

Zsolt Peter Nagy
Alex C. Varghese
Ashok Agarwal
Editors

Cryopreservation in Assisted Reproduction

A Practitioner's Guide to Methods,
Management and Organization

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Management and Organization

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Preface

Unlocking the Future: A Global Journey Through Cryopreservation

In the heart of life sciences lies cryopreservation, a revolutionary technique that freezes time, preserving gametes and embryos, safeguarding the potential for future generations. Our book embarks on a global exploration of this fascinating field, offering a comprehensive understanding of its history, its intricate workings, and its far-reaching potential.

We begin by setting the stage, exploring cryopreservation's global impact and the ethical considerations it raises. We then examine its rich history, tracing its evolution and celebrating the milestones that have paved the way for its success.

Next, we crack the code of cryopreservation, revealing the scientific principles that govern this seemingly magical process. Renowned authors contribute their expertise, offering a multifaceted view of the field, both foundational knowledge and glimpses into cutting-edge technologies shaping the future.

Navigating the crucial landscape of guidelines, regulations, and best practices, we ensure that you are equipped with the necessary knowledge to follow the highest standards of care and compliance.

Unleashing the power of innovation, the part Industrial Breakthroughs in Cryopreservation and Applications showcases remarkable advancements, particularly focusing on their industrial impact. Transformative technologies and methodologies are presented, highlighting their potential to revolutionize cryopreservation practices.

Finally, the Appendices serve as a practical guide, offering step-by-step procedures, standard operating procedures, and best practices. This invaluable resource ensures consistency, reliability, and adherence to regulatory standards, empowering you to achieve successful outcomes.

This book is not only a comprehensive resource for clinicians, researchers, and students, but also an invitation to explore and innovate. We aim to capture the current state of this art, best practices, and total quality management, all driven by a commitment to excellence and the advancement of reproductive science.

We extend our deepest gratitude to our most valued contributors for their invaluable insights and to you, our readers, who embark on this enlightening journey with us. As the field continues to evolve, we welcome your feedback and discussions, for they pave the way for future editions and a deeper understanding of cryopreservation's crucial role in shaping our future.

We are truly honored to present this book to you.

Atlanta, GA, USA
Auckland, New Zealand
Cleveland, OH, USA

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Alex C. Varghese
Ashok Agarwal

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Part I

Introduction

Cryopreservation in Global Perspectives: Editors' Thoughts

1

Alex C. Varghese, Zsolt Peter Nagy, and Ashok Agarwal

Key Points

- Cryopreservation strategies like social egg freezing, gamete/tissue freezing for fertility preservation, and freeze-all in IVF have become the standard practice of care in majority of countries where ART is practiced.
- Even though the vitrification technique has taken the field of cryopreservation by storm in the last one decade, the jury is still out on the efficacy of slow versus rapid cooling methods with regard to the molecular/epigenetic impact of them.
- Recent serious incidents in the fertility clinic cryostorage facilities have once again highlighted the need for better alertness and in particular improved risk management in relation to the cryopreservation of gametes/embryos/tissues.
- To address the need for risk reduction and standardization of cryopreservation across users and IVF clinics as well as a reduction in labor intensity, automated systems for cryopreservation, inventory management, and auditing hold much promise in ART lab.

1.1 Introduction

In the widely acclaimed first live birth of a “test tube baby,” Louise Brown, in England in 1978, the mother had a natural menstrual cycle and a single preovulatory oocyte taken from her ovary laparoscopically by the physicians; they fertilized it in the laboratory by conventional IVF and then transferred the resulting eight-cell embryo into her uterus. Today, IVF accounts for millions of births globally and 1–3% of all births every year in the USA and Europe [1].

The early IVF in women undergoing natural menstrual cycles yielded on average 0.7 oocytes per retrieval and a 6% per cycle pregnancy rate [2]. However, the initiation of controlled ovarian stimulation (COS) increased average oocyte yields to 2.1–2.6 and average pregnancy outcome to 23.5% per initiated cycle in 1982 and 30% in 1983 [3]. In the early phase of IVF where embryo cryopreservation was nonexistent, the success in IVF came with a huge price of multiple gestations due to transfer of more than one or two embryos. Even though Whittingham and colleagues in the early 1970s demonstrated that mammalian embryos could survive freezing to temperatures as low as -269°C and become normal fertile offspring after thawing and transfer to the uterus [4], the first human pregnancy following cryopreservation of an eight-cell embryo was reported only in 1983 by the Monash group [5] and the first live birth in 1984 by the Rotterdam group [6]. With the successful birth through the frozen embryos, the cryopreservation process slowly became the mainstay of assisted reproductive technology with its many facets being utilized by the fertility practitioners: (1) cryopreservation of sperm for donor banking/autoconservation for IVF/fertility preservation of male germ cells, (2) oocyte cryopreservation for fertility preservation/social reasons, (3) embryo cryopreservation for ART, etc. The increasing demand for fertility treatment worldwide drives intense research and development of technologies to optimize IVF regimens and success.

Gone are the days when we used to search the sections on cryobiology in popular fertility journals for new research

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papers, getting excited by bits of innovations/new technique improvisations in slow-freeze strategies so that improvisations could be introduced in each one of our cryopreservation protocols. How can we forget the pioneering works like (a) protocol utilizing a high sucrose concentration [7], (b) the effect of replacing sodium with choline in the freezing medium [8], (c) raising the seeding temperature to -4.50°C for oocyte slow freezing [9], and (d) innovations in sperm cryopreservation from Asymptote Ltd., UK [10].

One major development in the ART cryopreservation arena is the paradigm shift from the considerably less successful slow-freeze strategies to the highly successful vitrification methods across the laboratories. For nearly two decades, the freezing protocol employing 1.5 M propylene glycol (PROH) combined with 0.1 M sucrose as the cryoprotectants has been the most widely used [11] recipe. The vitrification, on the other hand, requires the use of high initial concentrations of cryoprotectants, low volumes, and ultrarapid cooling-warming rates. This approach was first introduced in human embryology for cleavage-stage embryos [12] and then for oocytes [13] and pronuclear-stage embryos [14]. The systematic review and meta-analysis by Rienzi et al. [15] support vitrification as being superior to slow freezing for cryopreservation of both human oocytes and embryos in clinical ART even though the quality of the evidence for clinical outcomes comparing the two freezing methods was mostly low and based on clinical pregnancy, rather than live birth, that for post-thaw cryosurvival of oocytes and embryos was moderate.

1.1.1 Global Infertility, ART, and Role of Cryopreservation

Worldwide data indicates that infertility affects both sexes. An accurate estimate on the burden of infertility is truly lacking on a global scale. Using survey data from 277 demographic and reproductive health surveys, a study [16] indicated differences in the prevalence of primary (high in North Africa and the Middle East) and secondary (high in Central and Eastern Europe and Central Asia) infertility between 1990 and 2010 in 190 countries and territories. As per a 2017 global study on the global burden of disease (GBD), the age-standardized prevalence rate of infertility increased by 0.370% per year for females and 0.291% per year for males from 1990 to 2017 [17]. These findings put emphasis on policymakers regarding the healthcare priority of infertility and implementing preventive and managerial interventions to address the growing burden of infertility in different regions. Increased maternal and paternal age has been associated with lower fertility and higher genetic/epigenetic risk for the offspring. Social sperm freezing could be developed in a way similar to social oocyte freezing because

postponing fatherhood has its consequences just like postponing motherhood [18]. If men can be screened (detailed andrology evaluation) at the postpubertal age, those falling within a potentially vulnerable group can be identified at an early stage. This strategy, in an era of male gamete survival at stake, opens up the option of cryostorage (“fertility insurance”) of samples from individuals with subnormal seminal parameters [19].

The International Federation of Fertility Societies’ (IFFS) Surveillance 2019 estimated the total number of ART facilities worldwide as 6201 [20]. This report corroborates to the dynamic, ongoing growth of the practice of assisted conception across the globe, the regional differences, and the continued international collaboration by various stakeholders that has characterized the field since its inception. It also reveals that progress has been made in developing registries, monitoring ART activities, and, in many countries, tracking the number of centers.

Sperm, oocytes, and embryos can now be frozen at various stages of development, making ART treatment potentially safer and more effective. There are considerable variations in the policies/popularity/type/technique of cryopreservation of reproductive cells/tissues across geographical regions. The 2018 IFFS Surveillance questionnaire did not uncover any countries that expressly prohibit cryopreservation of gametes or embryos for ART treatment or for fertility preservation performed for medical or other indications. Survey respondent in the range of 65–80% from the different countries noted the existence of laws, regulations, agency oversight, or professional guidelines that provided governance (Fig. 1.1).

The notable emergence of egg freezing in the last decade is not only life-changing as a means of “fertility preservation,” for the intended parents and carrier-savvy women, but it plays a major role for egg donation practices. Frozen donor egg banks and social egg banking programs are notably more popular in developed countries/regions like North America, Europe, and Australia (Fig. 1.2). Egg donation programs have emerged as a major treatment modality for infertile couples in many countries as part of the third-party reproduction. This entails that either intended parents travel to fertility clinics (even abroad) where donor eggs are located or the vitrified donor oocytes must be shipped to clinics where intended parents will do IVF and embryo transfer. With the help of a cryo-donor egg bank, frozen donor eggs can be shipped to receiving clinics for long distance/cross-border egg donation cases. In order to create transnational egg flows, egg banks rely on international courier companies that provide a well-established global infrastructure for moving frozen eggs through “cold chains.” Frozen egg trajectories are emerging between wealthy nations with egg shortages and popular donor egg IVF destinations with relatively unregulated egg procurement rules. An example is Ovobank

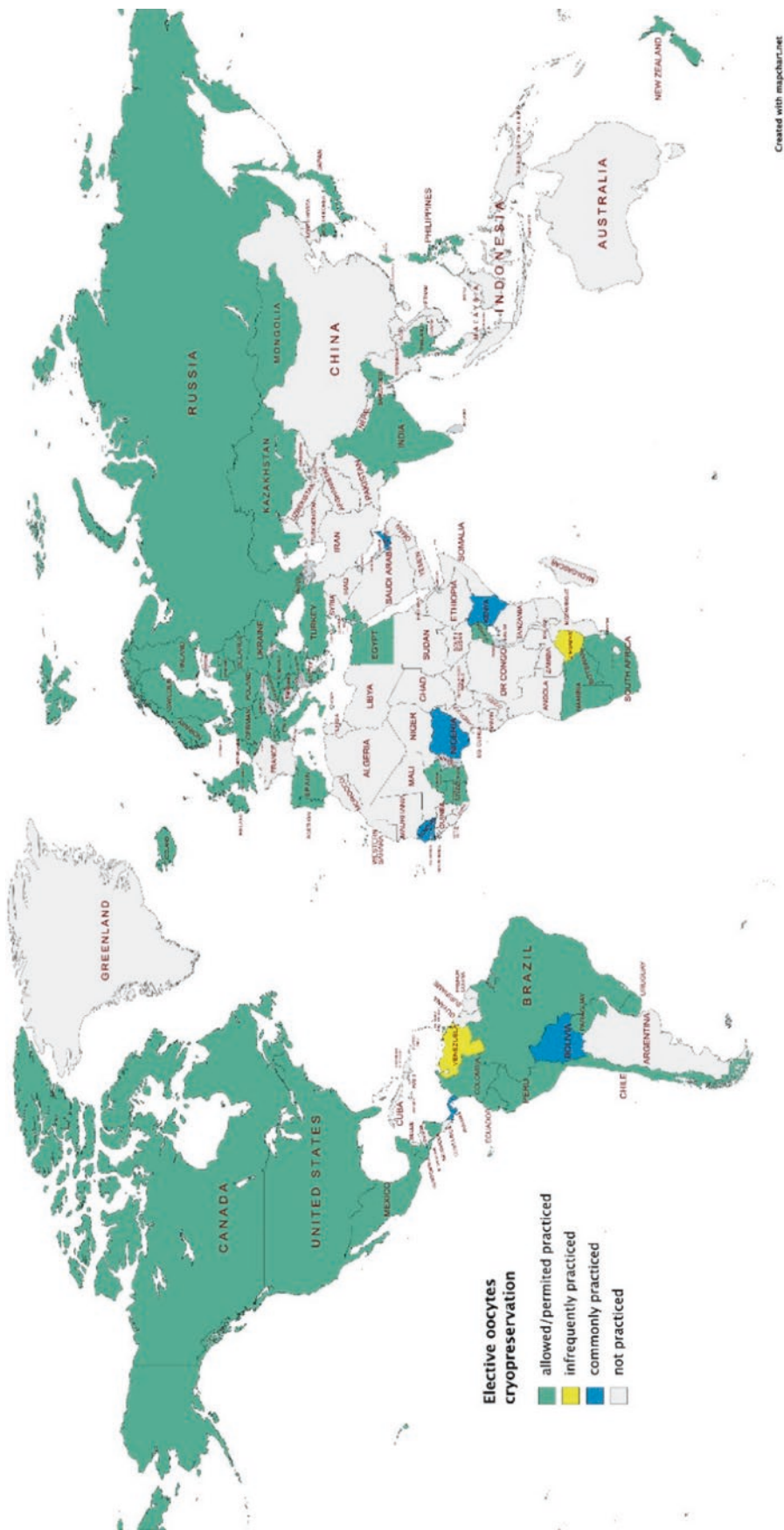


Fig. 1.1 Elective oocyte cryopreservation: worldwide map (copied with permission from Valentin Nicolae Varlas, author of the manuscript, doi: 10.3390/ijerph18158088)



Fig. 1.2 Geographical regions showing the prevalence of specific cryopreservation strategies and high research outputs

Spain, the first European egg bank shipping frozen eggs across national borders. Its location reflects the popularity of Spain as a destination for donor eggs for the third-party reproduction. Spain is said to be responsible for over half the donor-egg IVF cycles in Europe and is particularly popular among intended parents in the United Kingdom, for whom local shortage of female gamete is the top motivation for traveling to overseas clinics [21]. Similarly, some Eastern European countries, Romania, Cyprus, Ukraine, Russia, etc., are the favorite destinations for third-party reproduction, which leads to the establishment of multiple frozen egg banks in these countries.

Chang et al. [22] have proposed a strategy for the long-distance/cross-border egg donation cycles. This can be operated efficiently by a two-step process: (1) the transport of frozen husband/partner sperm to the donor egg bank for fertilization and (2) return shipment of resulting embryos to the recipient clinic for ET. Several embryology labs have reported compromised survival rate of frozen donor eggs from some banks. Since the embryos are created on-site at the donor egg bank in the abovementioned strategy, the quality and quantity of the resulting embryos can be assured before returning them to the recipient clinic for subsequent warming and embryo transfer. A “reverse traffic” like this has been reported in cross-border egg donation between Israel and Romania. The use of this language of “traffic” not only means the directionality of travel but

also hints to the reader that there may be an element of evading the laws in some European countries against egg donation by “importing” not oocytes but fertilized egg frozen at the two-pro-nucleate (PN) stage [23]. In a legal point of view, they are not oocytes, nor are they considered embryos yet.

Several methods are employed for ovarian tissue cryopreservation by various groups: (1) slow freezing (common in Europe, Australia), (2) vitrification (Japan, China, and some North American clinics), (3) stepped vitrification using liquidus tracking (UK), and (4) multi-thermal gradient freezing (Israel, USA), with the latter one being capable of freezing the whole ovary. The American Society for Reproductive Medicine [24] practice committee opinion that ovarian tissue cryopreservation (OTC) is no longer considered experimental and can be used in prepubertal patients or when there is no time for ovarian stimulation is a welcome move.

1.2 Vitrification for All: Does the Cells/Tissues Still Love Slow-Freeze Strategy

Majority of the IVF programs worldwide have embraced vitrification as the sole method for their cryopreservation need except for the sperm. Vitrification of sperm as practiced by few research groups and IVF clinics, by directly plunging the

sperm samples into LN2, is a fast, simple, and cost-effective method to cryopreserve human spermatozoa. Even though there are few commercially available devices for sperm vitrification (aimed at cryopreserving few sperms), the technique has not become popular yet, and manual/slow-freezing method is employed by the majority.

The slow-freezing strategy requires the use of a programmable freezing machine designed to provide accurate and consistent cooling parameters (various programs depending on the type of cells/tissues). Briefly, the sample is exposed in the beginning to a relatively rapid cooling rate of 2 °C/min to around −7 °C followed by a manual/auto-seeding to induce ice crystal formation in the solution. This is followed by a consistent slow cooling rate of 0.3–1.0 °C/min before the freezing device is plunged into liquid nitrogen after having reached temperatures of around −40 to −70 °C.

Ovarian tissue cryopreservation has been successfully performed using vitrification as well as slow freezing (also known as equilibrium freezing). Most widely reported pregnancy/live births from cryopreserved ovarian tissue have resulted from the use of the slow-freezing method [25]. However, two successful deliveries have been reported after vitrification [26] and two after VT followed by in vitro activation of the human ovarian cortex (IVA-OCTP) [27, 28].

A recent comparative study [29] however indicates that slow freezing for ovarian tissue cryopreservation is superior to vitrification process in terms of follicle survival and growth after xenotransplantation. Anti-Müllerian hormone (AMH) expression was low in transplanted samples subjected to both cryopreservation techniques. An increased primordial follicle deformation and cellular apoptosis were seen in the vitrification group as revealed by electron microscopy and Western blot analysis, respectively. However, another meta-analysis suggests that vitrification may be more effective than slow freezing, with less primordial follicular DNA strand breaks and better preservation of stromal cells. These advantages should lead to improved ovarian function after transplantation [30]. Interestingly, in a comparison study on the two techniques by Kutluk Oktay and Suzuki from Japan based on the intact primordial follicle survival, DNA damage, and apoptosis rates after thawing/warming, slow freezing vs. vitrification with either open or newly developed closed devices appear to be comparable [31].

We still cannot write off the clinical utility of slow-freezing strategies—the successful outcomes with ovarian tissue cryopreservation and transplantation will continue its place at least in some regions. More rigorous animal experiments and xenotransplantation studies are required to analyze the molecular intricacies of tissues subjected to either vitrification or slow-freezing methods.

1.3 How “Modern” Cryopreservation (Vitrification) has Changed the IVF World and IVF Treatment Paradigm

Due to the improved cryosurvival outcomes with vitrification, many laboratories worldwide have completely replaced slow freezing with vitrification. The optimized oocyte/cleavage-stage embryo/blastocyst cryosurvival rates and better clinical outcomes achieved with the use of vitrification have important clinical implications, which together allow a personalized approach in the fertility care. Since the introduction of vitrification, the number of frozen embryo cycles has increased tremendously across the fertility clinics, increasing the cumulative success rates of in vitro fertilization cycles without the fear of complications such as ovarian hyperstimulation syndrome (OHSS) and multiple pregnancies. Segmentation of IVF cycles became a reality with the introduction of vitrification program: 1) “freeze all” of all utilizable embryos in a fresh cycle and transfer in a subsequent frozen embryo cycle have led to the concept of OHSS-free clinics and 2) when one anticipates that pregnancy rates are compromised in fresh transfer, freeze-all embryo strategy is a viable option.

Preimplantation genetic diagnosis (PGT) programs are also reaping the benefit of the highly standardized embryo vitrification techniques. The biopsy results usually may take some time; freezing of all of the biopsied blastocysts with FET in a subsequent cycle is a preferred option now. The availability of reliable cryopreservation protocols like vitrification lets an extension of time available for embryo evaluation by indirect measures such as “-omic” profiling, metabolism, spent culture media analysis, and embryo quality, thereby affording a potential for implementation of new validated approaches for embryo selection.

Even though the first live birth from oocyte cryopreservation took place three decades ago, optimization and its widespread application in fertility clinics became possible only by the vitrification technology. In 2012, the American Society for Reproductive Medicine (ASRM) removed the experimental label for oocyte cryopreservation, and thereby more clinics started utilizing this technology. One of the most widely used applications of oocyte vitrification is for oocyte donation programs. The introduction of oocyte/egg banks offers myriad advantages: reduced waiting time for couples seeking third-party reproduction, no need for patient synchronization, improved safety to prevent disease transmission, and likelihood to increase donor pools. The increased availability of frozen donor eggs has also opened up implementation of new forms of collaboration between clinics—transborder fertility services. For example, an egg bank may cater to many needs of the IVF clinics: provide

donor evaluation and screening, ovarian stimulation, oocyte retrieval, and cryopreservation. These cryopreserved donor oocytes are then released for recipient use at associated IVF clinics [32]. Therefore, oocyte vitrification has provided important benefits for couples requiring donation, especially in those countries where there is a scarcity of donors.

Accumulating evidence of successful outcomes obtained with the vitrification technique employed for oocytes has greatly contributed to its application in the field of fertility preservation (FP). FP is now increasingly being used by women at a risk of losing their ovarian function because of either iatrogenic causes or natural depletion of their ovarian reserve. Hence, elective FP is offered widely by oncological patients and healthy women who wish to delay motherhood for various reasons.

Cryo-pooling of oocytes/embryos in poor responder patient is made possible by vitrification. In poor responders, especially those with advanced maternal age/low ovarian reserve, oocytes/embryos of 2–3 cycles are pooled to collect sufficient blastocysts that can be subjected to preimplantation genetic screening. Accumulation of embryos through natural modified (NM) IVF cycles before transfer has been shown to improve live birth rates and reduces the risk of lacking an embryo for transfer in poor responders aged ≥ 35 years [33].

1.4 Freeze-All Strategies: The Jury is Still Out

The policy of majority of IVF clinics was to do a fresh embryo transfer with the good-looking (best in morphological grade) embryos in an era of slow freezing/multiple embryo transfers and opt to freeze the rest good/moderate and even low-grade embryos. Embryologists and physicians were of similar opinion in this regard, based on the anticipated uncertainty of cryosurvival and unpredictable FET outcomes those days. A paradigm shift happened with the introduction of vitrification, enabling more confidence to IVF labs on the outcomes. The freeze-all (deferred embryo transfer) strategy is gaining popularity worldwide as an alternative to the conventional fresh embryo transfer. The objective of the “freeze-all” policy is the cryopreservation of the entire utilizable embryo cohort and replacement of the elective embryos in a more favorable intrauterine environment (segmentation of IVF cycles), without possible adverse effects of supraphysiologic hormone levels over the endometrial receptivity.

In a largest study to date examining trends in embryo cryopreservation among US ART cycles, Christianson et al. [34] used the SART Clinic Outcome Reporting System from 2004 to 2013. They observed that the number of freeze-all cycles increased from 7.9% in 2004 to 40.7% in 2013. The

authors observed that because of the increase in freeze-all cycles, the number of embryos cryopreserved per year increased from 158,383 in 2004 to 303,203 in 2013, and over the 10-year span, 1,954,548 embryos were cryopreserved with less than half, 717,345 embryos, transferred. The authors showed that over 1.2 million of the frozen embryos were potentially still in cryostorage. Due to the COVID-19 pandemic, some embryo transfers were delayed, leading to a higher number of cycles without a transfer during the pandemic period adding to the freeze-all cycles.

The results of several recent meta-analyses and randomized controlled trials (RCTs) on live birth rates (LBRs), either the LBR after the first embryo transfer or the cumulative LBR, are, however, controversial regarding freeze-all strategy. These reports show either no benefit of the freeze-all strategy compared with the fresh embryo transfer strategy or better outcomes [35–37]. The curious case report from a large dataset on the embryo storage time > 6 months is associated with reduced pregnancy rates that warrants further investigations [38].

In the recent Cochrane review [39] including 15 studies in the systematic review and 8 studies with a total of 4712 women, freeze-all strategy shows an overall evidence of moderate to low quality. The study reports a cumulative live birth rate between 57% and 63% following a freeze-all strategy compared to a 58% cumulative live birth rate following conventional strategy. The review also observed a low-quality evidence in less OHSS after the “freeze-all” strategy compared to the conventional IVF/ICSI strategy. The risk of pregnancy-related hypertension and of having a large-for-gestational-age baby and a higher birth weight of the children born may be increased following the “freeze-all” strategy. Hence, the question arises: When and for which clinical situations should a freeze-all strategy be used?

The recent study by Sargisian et al. [40] of almost eight million children in Nordic countries (Committee of Nordic ART and Safety—CoNARTaS group) raises the possibility that children born after FET had a higher risk of childhood neoplasm than children born after fresh embryo transfer and natural conception. Even though the results should be interpreted with caution based on the small number of children with cancer (48 cases), the findings raise concerns considering the increasing use of FET, in particular freeze-all strategies without clear medical indications. Even though the present study included both children born after slow freezing of cleavage-stage embryos and vitrification of blastocysts, which could be associated with different risks, another study from the CoNARTaS group comparing these techniques has not shown any major differences in perinatal outcome between these two techniques. Dimethyl sulfoxide (DMSO) and ethylene glycol (EG) are widely used as cryoprotectants in ART. However, DMSO is a known epimuta-

gen, and EG has been reported to be toxic in high concentrations. The measured residual DMSO and EG in ovarian tissue after vitrification and slow freezing indicate that concentrations of residual cryoprotectants were high after vitrification methods but almost zero with the slow-freezing method [41].

Even though data in the literature are not sufficiently robust to generally apply the deferred strategy to all patients requiring ART, an evidence-based medicine approach may be recommended for patients at OHSS risks, including cases of PCOS, and those undergoing PGT-A cycles. A thorough review on recommended deferred strategies has been discussed recently by Bourden et al. [42].

1.5 Risks, Challenges, and Solutions in the Organization of Cryo-Programs

We have come a long way in our ability to provide long-term storage of sperm, eggs, embryos, and reproductive tissues, and the field of reproductive cryotechnology is continually improving. This high level of achievement has also imposed a far greater responsibility on fertility clinics. The longer and longer we store the reproductive cells/tissues, the greater the risk and the bigger the need for more technical fail-safe attributes. As the keepers of the gametes and embryos, we, IVF lab professionals, reproductive endocrinologists, nurses, and administrative staff, have a different relationship with gametes and embryos and their stewardship than do our patients ([43], reference [43]). Recent serious incidents in the fertility clinic cryostorage facilities have once again highlighted the need for better alertness and in particular improved risk management in relation to the cryopreservation of gametes and embryos. Potential losses through litigation could be considerable for the IVF programs if the cryopreservation facility is found to be negligent and fails to provide the adequate resources and implement recognizable control measures.

It is estimated that nearly one-third of the cycles of assisted reproductive technology (ART) performed globally each year are categorized as frozen embryo transfers (FETs) [44]. Various studies show an association between FET and increased risk for hypertensive disorders of pregnancy, including preeclampsia [45–47]. Efforts to understand the reasons for this apparent risk may open up opportunities to better individualize the treatment modalities for the FET cycles. The ongoing randomized controlled trials will help us to understand whether the apparent risk is due to patient factors or the protocols being used [48].

A recent observation by Wang X [49] on the effect of chemically active compounds (SO₂, O₃) in the IVF laboratory environment on reduced FET live birth but not the con-

temporary fresh embryo transfers emphasizes the importance of active clean room implementation in ART laboratories. The differential effect of outdoor air pollution on fresh and FET cycles might relate to the stress caused by the cryotechnologies via the molecular and cellular perturbations getting augmented by the pre-freeze chemical exposure. ART laboratories must have updated protocols in place that address all the issues pertaining to a safe cryostorage system, and quality control must include checking tanks for both visual clues and monitoring evaporation rates of each storage tank. Schiewe MC et al. [50] have recently published a best practice guidelines for ART labs with regard to comprehensive assessment of cryogenic storage risk and quality management concerns. Several remote monitoring/alarm systems to detect tank failures have been introduced, and many hold promise for a better surveillance of stored cells/tissues. These include the recent innovative products such as multiple infrared cameras (Cryo Sentinel™, USA) aimed at the tanks, which can immediately sense temperature changes and sound an alarm if a tank is failing up to 20 h earlier than the other conventional temperature gauges. Weight-based cryomonitoring systems [51] like Boreas CryoScout (USA), WeighMed technology, ATRAX Medical (New Zealand), and DeTech (USA). The studies using weight-based monitoring systems demonstrate that a weight-based alarm system can detect tank failures prior to a temperature-based system.

Managing cryopreserved reproductive tissues in an ART laboratory requires skilled personnel, controlled processes, and specialized equipment. A number of sources of errors and failures, both human and mechanical, have been identified related to cryostorage in embryology and andrology laboratories. The recent practice committee document of ASRM has identified the major categories and common issues that have occurred in cryo-areas [52]. Many IVF units worldwide face the issue of space constraints with the increasing number of cryobanks each year. Efficient cryodewar models with stacking storage canisters and wider inner space have been introduced in recent years, which can accommodate more patient samples. A dedicated cryoroom is seen in majority of IVF units, but some units prefer to store their cryobanks in the IVF lab itself under the lab benches. There are also a lot of variability in the process of cryostorage (e.g., use of goblets, visotubes, cryocanes, cryotags), the witnessing steps, or the inventory management. Several incidents of floating goblets, straws, or losing the patient samples have been reported by many IVF laboratories, some of which leading to litigations too [53]. Even though a reliable sample management and inventory are being carried out as part of standard cryopreservation procedures including the verification and witness of all sample labels prior to storage, auditing of stored samples is a reassuring QC step in a com-

prehensive QM plan [50]. Live biological samples such as reproductive cells need to be kept below -135°C to avoid degradation. Uncontrolled temperature increase during inventory or retrieval of vials and straws is the biggest threat making gametes/embryos nonviable. Hence, the evaluation of the risk-benefits of performing complete or partial inventories should be assessed for each clinic based on the local regulations, manpower, and rate of incident reports. RFID tagging of cryopreserved samples (Cryogatt, UK) has been explored for inventory management, to identify and track movement of cryopreserved donor oocytes from cryostorage in one clinic, into and out of a transport dry shipper, and into cryostorage in a receiving sister clinic [54].

1.6 Saving the Private Krio: Frozen Gametes and Embryos in the War Zone

Fertility services could easily discontinue their elective ART treatments during the times of war. Though the patients find refuge, the safeguarding of all stored gametes and embryos can no longer be guaranteed in the war zones. Recent war on the Ukraine soil has affected the life of thousands of civilians, including the infertile couples from the country and abroad who were undergoing the IVF process. Tens of thousands of IVF couples and individuals that have pursued oncofertility cryopreservation have stored reproductive material in the numerous ART clinics in Ukraine. Such stored material is now at risk of demise due to lack of monitoring, security, and continuous provision of liquid nitrogen.

Ukraine is a preferred destination for infertile couples from Europe and the UK for third-party reproduction due to affordable IVF cost. The stories described by the IVF/egg bank owners unfold the ground realities of the grim situation and uncertainty of the ART clinics/profession in such scenario. The major problem the IVF clinics might face is the lack of liquid nitrogen supply, inadequate manpower, possibilities of destruction of the facility by shelling, etc. A particular egg bank was fortunate enough to shift their frozen cells across the border to safe destinations by adventurous to-and-fro trips by road carrying the cryocans filled with samples.

The narration by Birol Aydin and colleagues in Chap. 45 shows the kind of challenges faced and steps that helped them to transport the storage tanks from the war zone. A transborder collaboration with IVF clinics abroad and storage of patient/sample data in cloud are the essentials of risk management that we could learn from this unpleasant incident. This also opens up the importance of criteria that need to be set by IVF clinics in advance to save the thousands of eggs and embryos in their cryostorage.

Consortium of fertility service providers like societies and special interest groups should take initiative to form risk mitigation protocols/practice guidelines for saving frozen cells in adverse events like this. The corporate IVF groups with its spanning network of clinics across the borders have the added advantage of moving the reproductive cells/tissues without much regulatory hassles in times of conflict. The standalone private clinics are the ones that mostly get affected by such adverse situations unless there are tie-ups with foreign clinics.

1.7 Machines, Automation, and AI in Cryotechnology and Governance

It is conceivable that future automation enhancements in the ART laboratory may improve consistency and throughput and reduce the risk of human error associated with the performance of repetitive tasks. To address the need for standardization of vitrification across users and IVF clinics as well as a reduction in labor intensity, automated systems for vitrification hold much promise in ART lab. An ideal robotic/automated design should integrate vitrification—warming and storage of the samples in the same system without much human intervention. In a recent study, manual vitrification exhibited favorable total embryo survival rates and was more time efficient than automated vitrification [55]. A recent prospective, randomized, open non-inferiority trial conducted at three German IVF centers shows that semiautomated closed vitrification method (2PN oocytes) gives similar clinical results as compared to an exclusively manual, open system but higher procedure duration and less staff convenience [56]. This indicates that more efficient automated systems are needed for cryopreservation arena where the number of frozen cycles per clinics has been increased tremendously due to freeze-all strategies and elective single-embryo transfers. Microfluidic platforms are also being explored for automating the vitrification process ([57], reference [57]).

A proof-of-concept study using mouse embryos suggests that application of a robotic, liquid nitrogen vapor-based tank for storage of cryopreserved mammalian embryos is feasible [58]. The main features include sample management through robotic handling, digital inventory control, as well as continuous telemetric reporting of multiple tank parameters such as temperature, liquid nitrogen (LN2) level, and LN2 consumption rate with predictive analytics that can help avoid system failure. A detailed description of the system can be found in Chap. 47.

Artificial intelligence/deep neural networks have the potential to process complex datasets, to be better at identifying subtle but important patterns, and to be more objective than humans when evaluating embryos for scoring and mak-

ing disposition decisions for biopsy and cryopreservation. Many studies on oocyte cryopreservation show concern regarding fertilization and live pregnancy rates following an egg thaw, which, until recently, could only be predicted by age and number of eggs frozen. An AI system named Violet™ technology is being tried by one AI-based fertility clinic in Canada in determining egg quality for women undergoing oocyte cryopreservation. Such systems hold promise in patient counseling during social and oncofertility preservation.

1.8 Learning from Animal Models, Research, and Training

The history and evolution of the technical aspects of cryopreservation tell us the progress that we have made in our ability to provide long-term storage of sperm, eggs, and embryos. The development in cryobiology in animal breeding had revolutionized the field of reproductive medicine. The pioneering works by cryobiologists on different species of animal oocytes and embryos have thrown light on varied sensitivity of cold-shock injury by each, and the resultant knowledge helped immensely in the human ART cryopreservation. One major area is the fertility preservation research using animal models to standardize the best protocols.

The possible adverse effects of oocyte cryopreservation on epigenetic status of the resulting embryos have been studies extensively showing a reduction in the levels of DNA methylation and H3K9 acetylation in two-cell embryos in the vitrification group [59]. Development to the blastocyst stage partially adjusted the effects that oocyte vitrification had on the epigenetic status of embryos. The presence of antioxidants in the vitrification solutions has been shown by another study [60] to help in alleviating the negative effects of cryopreservation. The authors have also shown that the combination of antioxidants, acetyl-L-carnitine, N-acetyl-L-cysteine, and α -lipoic acid can ameliorate the reduction of acetylation of vitrified mouse blastocysts.

Slush liquid nitrogen, a supercooled form of nitrogen with a temperature from -207 to -210 °C, is another favorable candidate being tried by cryobiologists since the last one decade to improve the vitrification outcomes in ART with varied outcomes. Liquid nitrogen (LN) slush and sealed pulled straws (SPSs) have been studied to achieve a high cooling rate, enabling a reduction in cryoprotectant concentration [61].

A very recent randomized controlled trial analyzed whether the use of slush nitrogen (SN) can improve oocyte survival after vitrification and warming compared with conventional liquid nitrogen (LN) ([62], ref. [62]). There were no statistically significant differences noted in oocyte survival

rate, fertilization rate, rate of usable blastocyst formation, or rate of whole chromosome aneuploidy between the SN and LN arms, respectively. The vitrification of mammalian oocytes using closed carriers (CCs) has a low success rate. In a recent study, authors modified four key parameters, namely, thermodynamic characteristics (cooling and warming rates by slush LN), CPA exposure time, and CPA supplementation, to optimize the oocyte vitrification protocol using the CC. The results collectively indicated that increasing the cooling rate by applying slush LN2 to the CC system vitrification leads to improved survival and blastocyst formation rates of vitrified/warmed mouse oocytes ([63], ref. [63]). In another study using human ovarian tissue, it has been observed that the follicle quality, gene expression, viability, and progression are better preserved after SN vitrification [64].

An ultrafast blastocyst warming protocol eliminating the DS and WS steps is being trialed by many groups in recent times, and the initial studies indicate a favorable outcome [65]. Regarding embryology time savings, the total time for embryologists to warm was 1316 min and 106 min for the control and test group, respectively. The significantly quicker embryo warming technique also yielded comparable pregnancy and implantation rates while saving 13 min of tech time per warming. However, peer-reviewed studies in near future may authenticate these trials, and this protocol will allow for greater efficiency in the IVF lab too.

These data from the recent literature show that the cryobiologists and researchers in ART are still in the pursuit to optimize the vitrification technology to yield a superior outcome compared to the ones practiced routinely.

The shift from slow-cooling machines to faster vitrification has been based on the voluminous reports in literature and the superior outcomes achieved in the IVF laboratories. Vitrification is an important and routine procedure in the majority of IVF laboratories. Everybody will agree that the results from vitrification are quite dependent on the skill and experience of the technical staff doing it. Each laboratory should set out a rigorous training program for the new staff, and proficiency testing needs to be carried out from time to time as part of quality assurance.

1.9 Conclusion

Basic research and technological innovations hold promise for the advancement of cryopreservation in ART. Though vitrification has made a major impact on how the fertility practitioners manage the IVF cycles, the technology is still a hot subject for improvisation through modifications in various facets: cryodevices, vitrification/warming media, freezing/warming temperatures, additives like antifreeze proteins

or antioxidants, etc. More work on the molecular and epigenetic or epimutation level of the cryopreserved samples may shed light on the potential risks. A fully automated vitrification/warming approach and a secure cryostorage system with traceability and remote sensing capabilities are the need of the time. Artificial intelligence platforms may in near future assist physicians in assessing the viability ranking of eggs for fertility preservation—social and oncological cases.

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From Ice to Glass: Historical Cryobiological Events that Transformed the Application of Assisted Reproductive Technologies

2

Mitchel C. Schiewe and James E. Stachecki

2.1 Introduction

In the last century, assisted reproduction has evolved into the creation of thousands of healthy, high-tech miracle babies worldwide being produced every day. A critical piece of technology's advancement has been in the field of cryobiology, where freeze preservation of viable gametes, embryos, and reproductive tissues (ovarian and testicular) has been made possible. We recently celebrated the 50th anniversary of the first frozen mouse embryos producing live births after storage in liquid helium and nitrogen (-269 and -196 °C, respectively; [4]). In retrospect, it was a

unique scientific collaboration, where the Father of Modern Cryobiology, Dr. Peter Mazur, and his esteemed postdoc, Dr. Stanley Leibo, recruited a brilliant, young reproductive physiologist from England, Dr. David Whittingham, to join them in the USA (Oakridge Institute, TN). Instead of competing, together they celebrated history (Fig. 2.1). Indeed, their progress in applied cryopreservation methods, and those achieved today, is based on the scientific principles and discovery of how water molecules are manipulated in a frozen state by scientists in years and decades past. At the top of that pyramid was Peter Mazur for reasons that will be characterized further in the chapter.



Fig. 2.1 Dr. Mazur, shown here on the left in 1971 with his young postdoctoral fellow, Stanley Leibo, and guest scientist, David G. Whittingham, PhD from England, celebrating the birth and publica-

tion of the first frozen mouse embryo offspring in *Science* (photo courtesy of WF Rall approved by DG Whittingham)

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2.2 Early Cryobiological Discovery, Sperm Focused

In the 1930s, Walter Stiles first suggested that the protoplasm of plants may not extensively crystallize upon very rapid cooling, thus allowing botanical systems to maintain life [5]. Meanwhile, Professor John Hamond determined that rabbit sperm could be cooled to zero degrees and maintain useful viability, having the hindsight to speculate that in the new era of aviation, “the transportation of cooled sperm by airplane could allow for the worldwide distribution of genetic material” [6]. That truly historic observation has become a daily reality in the twenty-first century.

While studying syphilis, Jahnelt (1938) noted that human sperm survived for comparatively long periods when frozen on solid carbon dioxide (i.e., dry ice at -79°C), raising the possibility that the rate of freezing may be important. By 1940, Shettles confirmed Jahnelt’s findings by verifying sperm survival at temperatures reduced to -69°C [7] and introducing the idea of varying methods of thawing. That same year, Phillips and Lardy (1940) showed the beneficial effects of egg yolk to buffer membrane damage of the low-temperature preservation of bull sperm, which then aided effective on-farm artificial insemination of cooled extended ejaculates [8]. Hoagland and Pincus [9] reinitiated a discussion on feasibility of sperm banks by reporting a 67% survival of frozen thawed sperm. Sir Alan Parkes first determined that survival was volume dependent, thus confirming the importance of cooling rate (1945) [10]. Additionally, he agreed with the banking concept for prolonged storage and transport, claiming that there was no danger of genetic modification by freezing. A year later, Rostrand (1946) first used glycerol as an adjunct during cell freezing [3]. However, it was an agricultural doctoral candidate, Chris Polge, and physician, Audrey Smith, both mentored by Dr. Parkes, who are credited for accidentally discovering the protective role of glycerol to maintain post-thaw rooster sperm motility when studying the effect of various sugars as a cryoprotectant [11]. Glycerol-egg yolk diluents were then successfully applied to artificial insemination with frozen-thawed bull sperm [12, 13]. These early experiments were recently reviewed in detail [14].

Based on the bull model, frozen-thawed human sperm were proven capable of fertilization, inducing normal embryonic development and achieving pregnancy and live birth [15, 16]. These investigators concluded that lower temperatures, below -69°C , would be needed for effective freeze storage. Anticipating praise and professional glory, they instead found themselves the subject of criticism in Iowa and within the national medical community. Americans were skeptical of assisted conception, and even fertility specialists distanced themselves from the Iowa breakthrough [17]. It

would be decades before frozen sperm would be fully incorporated into the practice of reproductive medicine, with cryobanking of human sperm becoming a reality in the late 1960s and into the 1970s [2].

2.3 Forging Ahead in the Cryobiology of Eggs and Embryos

As early as 1947, Dr. Min-Cntjeh “MC” Chang demonstrated that rabbit oocytes could be cold stored for several days and still retain fertilizing capacity with corresponding normal development [18]. Subsequently, Audrey Smith demonstrated that a small percentage of rabbit embryos could experience cleavage divisions in culture after cooling to -79°C under glycerol cryoprotection [19]. Both Chang and Smith had chosen a challenging in vitro model, the rabbit, as its embryos are surrounded by a thick mucin coat which altered the permeability of early embryos to glycerol. Smith and her colleagues eventually achieved some post-thaw survival but no offspring. Alternatively, she conducted some fascinating whole lab animal body cooling studies [20], which Dr. Stanley Leibo described in his 2013 Cryobiology presentation (see Society for Cryobiology 2013, lectures). As a notable female cryobiologist of that era, Smith questioned the empirical observations of Father Basile Luyet regarding his concept of vitrification (solid freezing without ice formation; [21, 22]). Her first warning put the burden of proof onto Luyet to actually prove that vitrification had occurred by demonstrating the absence of ice crystals, not relying on a transparent state alone as proof. Her theory was later validated in experiments by Forsyth and MacFarlane [23], showing that when you rapidly cool various solutions, including lithium chloride or cryoprotectants, upon warming you can detect a glass transition point, devitrification event, or opaque crystallization event as temperatures elevate. Therefore, in relatively dilute solutions, devitrification and intracellular freezing may not be seen when warming rates are sufficiently high. The cautionary note being that even though a solution appears to vitrify by forming a transparent glass state, it does not mean that intracellular freezing does not still occur upon warming. The second cautionary note by Audrey Smith was issued toward Luyet’s belief that the survival of a cell after freezing and thawing was a key criterion proving that vitrification had occurred. She believed that survival could occur after ultrarapid freezing and rewarming even in the presence of intracellular ice.

Another pioneering cryobiologist of the 1950s was Smith and Polge’s colleague Dr. John E. Lovelock, who studied the colligative properties of various cryoprotective agents (CPA, e.g., glycols and sugars) and introduced dimethyl sulfoxide (DMSO) as an effective cryoprotectant [24, 25]. Up to that

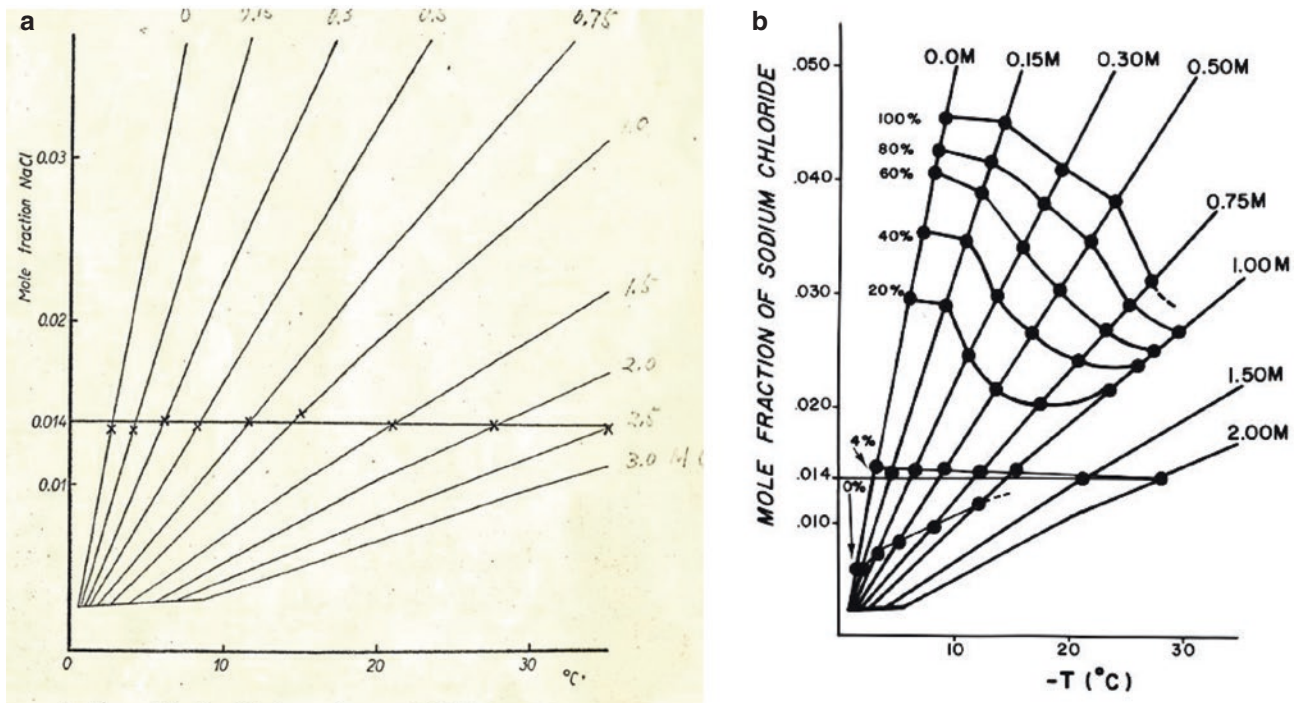


Fig. 2.2 Dr. Lovelock [24] is credited with providing us a modern-day understanding of how CPAs work. His experiments revealed that salt concentrations rise as a function of declining subzero temperatures and that the addition of increasing CPA concentrations depresses the salt concentration. He further determined that RBC injury occurred at the

mole fraction of salt, independent of glycerol concentration (see straight line, i.e., “Electrolyte Toxicity Theory of Cryobiology”). Fahy and Karrow [26] revalidated Lovelock’s curves and showed that CPA concentration did influence RBC injury relative to the salt concentration

point in history, the studies and successes with glycerol were empirical observations. Dr. Lovelock is credited for providing the scientific community with our modern-day understanding of how cryoprotectants work. His principle findings are shown in Fig. 2.2a, revealing the rise in salt concentration (Y-axis) as a function of temperature (X-axis) and that the addition of increasing CPA concentrations depresses the salt concentration. Lovelock reported that red blood cell (RBC) injury occurred at the mole fraction of salt, independent of the glycerol concentration, as indicated by the straight horizontal line. In turn, this gave rise to the electrolyte toxicity theory of cryobiology. Fahy and Karrow ([26]; Fig. 2.2b) reassessed Lovelock’s curves and pointed out if you assess survival at any other level (ranging from 4% to 20% to 100%), you find that RBC injury is not a function of salt concentration only and that there is an interaction with glycerol that makes them more susceptible to damage than Lovelock’s theory alone would indicate.

Further interpretation of Lovelock’s theories came from Harold Meryman, a pioneer in RBC cryobiology and the studying of ice growth. He is credited with questioning cell volume reduction being the critical factor in cell injury. Osmotically speaking, as the salt concentration goes up, the cell volume goes down. Under very slow cooling and slow

warming events, Meryman exhibited a correlation between % hemolysis and osmolal concentration of NaCl, regardless of the glycerol level present (Fig. 2.3; [27, 28]). This interpretation was a pivotal development in cryobiology, providing a more comprehensive understanding of freezing injury, publishing many articles on the topic of “solution effect” injury. Dr. Meryman became the Transplant Lab Director at the American Red Cross (ARC) in Bethesda, MD, and ultimately mentored the development of modern-day vitrification (reviewed by [29]). It is important to note that Dr. John Farrant [30] also played a pivotal role in understanding cell damage to cooling and warming events.

Our understanding and applied success with embryo freezing are credited to magna cum laude, Harvard graduate named Peter Mazur. In short, it was his thermo-kinetic theories of cellular water loss during subzero cooling being a function of temperature, cooling rate, cell membrane permeability, and cell surface-to-volume ratios that allowed for comparatively high levels of cellular and tissue survival post-thaw, including embryos [31–33]). His genius was perhaps first displayed in a 1964 American Cancer Society presentation where one quote stated, “It is possible therefore, if cells are cooled very rapidly the resulting cellular ice crystals will be so small as to be innocuous. In such a case the warming

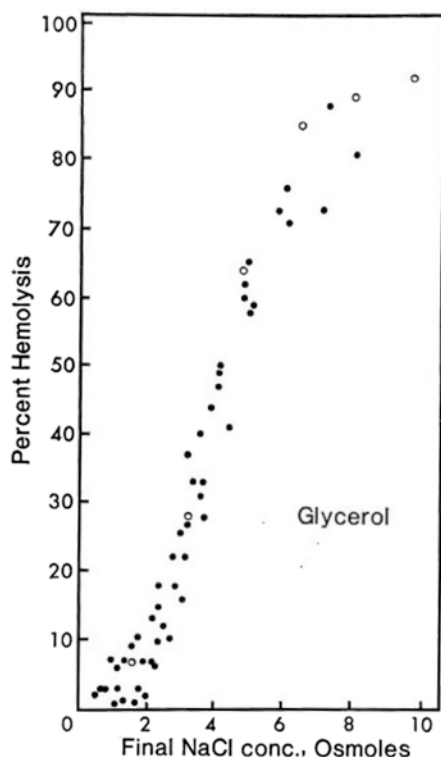


Fig. 2.3 Dr. Meryman [27] looked at RBC injury as a function of cell volume relative to salt concentration and the presence of a CPA. He found a strong correlation between % hemolysis and salt concentration independent of the glycerol level, even under very slow cooling and slow warming conditions

velocity could have a profound effect on survival. If the cells are warmed slowly, the unstable crystals could be converted to crystals of damaging size. But if the cells are warmed rapidly, the unstable crystals will melt before they have the chance to grow." A half century later, these insights are as valid today as they were then.

As Drs. Meryman and Farrant identified slow cooling injury, it was Mazur who characterized rapid cooling injury. His seminal publication in 1963 contained diagrams like those in Fig. 2.4, which revealed that the relative volume of cell water is influenced by the dehydrating effect of extracellular crystallizing water. Furthermore, that water loss from the cell (e.g., yeast cells in left panel) takes time and is indeed influenced by the rate of cooling. Increased cooling rates decrease the level of dehydration experienced by the cell, but the rate of water loss is cell dependent. In the case of yeast, very little water loss occurs when cooling at 1000 °C/min, while most all freezable water is lost when cooling at 1 °C/min. Conversely, the freezing of RBCs (Fig. 2.5) revealed that no freezable water remains in the cell whether cooling at 1000 °C/min or 10,000 °C/min. Both Drs. Meryman and Mazur conducted experiments assessing slow and rapid cooling injury, which allowed Dr. Mazur to formulate his "two-factor theory" of cell survival or injury. Factor 1 involved solution effects that can cause an osmotic stress injury, whereas Factor 2 focused on intracellular ice formation (IIF) injury associated with supercooling. In short, depending on the cryoprotective agent and water permeabil-

Fig. 2.4 Seminal experiments of Dr. Mazur identified a "two-factor theory." Not only was the relative cell volume influenced by the dehydrating effect of water possibly leading to "solution toxicity," but that water loss is inversely correlated to the rate of cooling. Increased cooling rates decrease the level of dehydration due to the formation of intracellular ice, which itself can be damaging to a cell

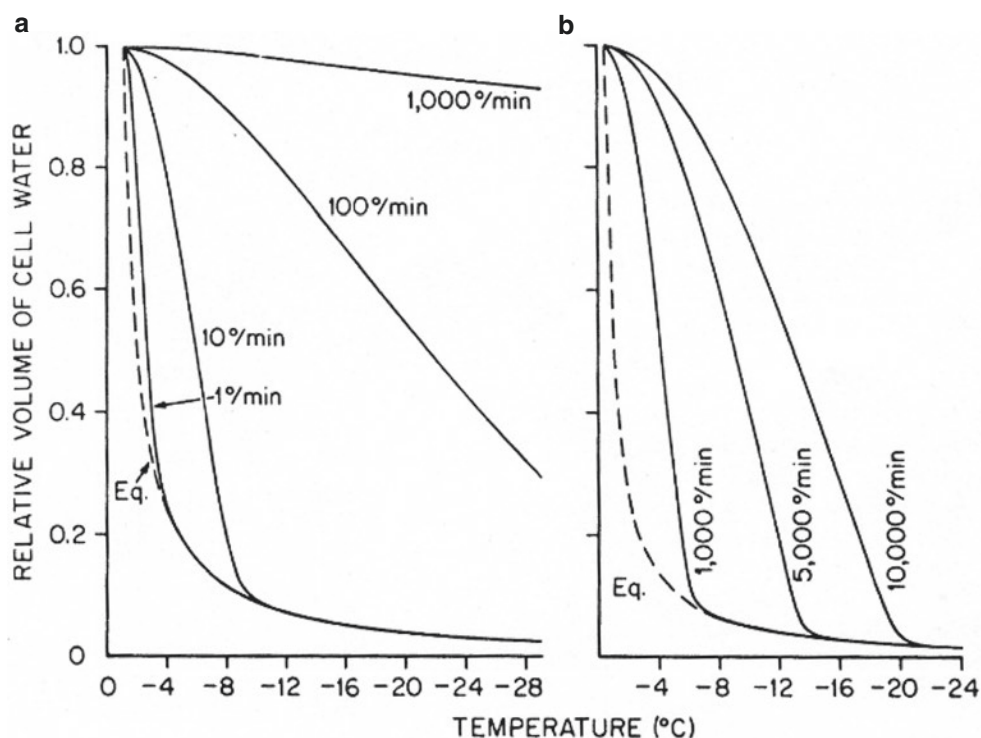
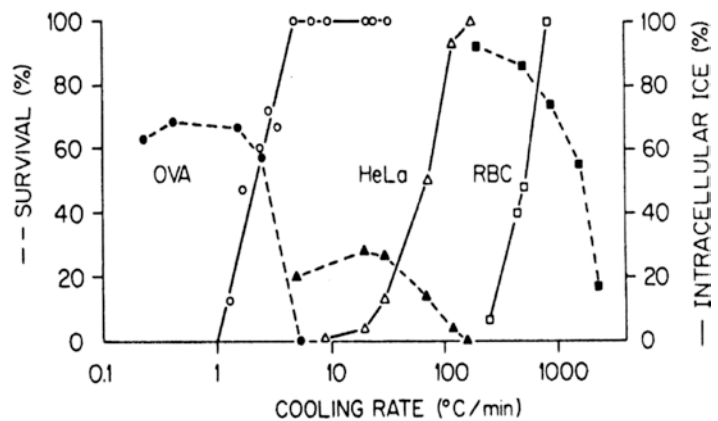


Fig. 2.5 Mazur's computer models were eventually applied to real cells. This graph shows that as the concentration of intracellular ice goes up (indicated by the open dots), a critical point occurs depending on the cell type, where cell survival is adversely correlated (black dots) to increasing cooling rates. The drop in viability at high cooling rates being caused by intracellular ice formation (IIF; 2nd Toxic factor; [31, 34])



ity of a given cell type, a delicate balance exists between cooling rates, water loss (i.e., cell volume), and IIF. If cooled too slowly, osmotic toxicity can occur, whereas if supercooled, then physical damage to the cell occurs due to IIF [31, 34, 35]. Mazur's computer models were eventually applied to real cells, where he did find correlations between cellular injury, or the lack of. Fig. 2.5 reveals a compilation of experiments performed by other investigators, which confirmed Mazur's two-factor theory [34], which gratefully permitted rapid progress in freeze-preserving more complicated multicellular, mammalian embryos.

2.4 Conversion of Scientific Principle to Applied Success

As highlighted in the introduction, successful embryo cryopreservation was borne in 1972, when Dr. Mazur extended a research collaboration to David Whittingham (from England) to join their cryobiology lab at a nuclear testing facility in Oak Ridge, TN. Together, they then applied Mazur's principles to David Whittingham's efforts to cryopreserve mouse embryos, developing the "slow-freeze/slow-warm" model that became successful using DMSO as the cryoprotectant. Later that same year, a young Scotsman at Cambridge University, Ian Wilmut, further validated their success [36]. Decades before being knighted for his pioneering contributions to the cloning of "Dolly" the sheep, Wilmut published his success the next year freezing bovine embryos [37]. Other notable firsts included Whittingham's efforts with rabbits [38] and rats [39], as well as the Australian efforts of Bilton and Moore [40] with goats.

The next major breakthrough, which made embryo freezing more practical, came in the mid- to late 1970s. Conventional slow-freezing wisdom was challenged by a young Danish veterinarian Steen Willadsen using a "rapid freeze-rapid thaw" technique with sheep embryos frozen in heat-pulled glass tubes [41–43]. In separate experiments,

Whittingham validated the latter model in the mouse (Fig. 2.6; [44]), showing that slow cooling (<0.5 °C/min) to -38 °C followed by a direct plunge into LN₂ (i.e., rapid freeze) was successful when combined with rapid warming directly in a 37 °C water bath (as opposed to previous ambient temperature warming). In 1982, sealed plastic straws were first introduced as a simplified container to freeze and store mouse embryos [45] and were readily adopted by the cattle ET industry. The comparative effectiveness of rapid freezing in straw containers was later proven beneficial in contrast to the slow-freeze/thaw method or the use of glass ampoules using a mouse model that identified genotypic variations in survival [46]. The latter studies also introduced the concept of thermal-electric cooling compared to forced liquid nitrogen vapor to achieve controlled-rate slow freezing [47]. In summary, rapid freeze/thawing in 0.25 ml plastic straws offered faster freezing and more efficient, high-capacity storage; thus, it was adopted worldwide as the conventional slow-freezing approach of the mid-1980s onward into the twenty-first century. Furthermore, programmable controlled-rate freezing units using LN₂ vapor (e.g., planar) or thermal-electric principles (e.g., freeze control) were commonly used in all IVF laboratories for embryo cryopreservation before essentially becoming obsolete after 2010, being supplanted by equipment-free vitrification technology.

Based on continued progress in animal models, the international community pioneered early success with human eight-cell embryos and expanded blastocysts using DMSO and glycerol solutions, respectively [48–51]. A few years earlier, Yusuke Yamamoto had confirmed the efficacy of glycerol to cryopreserve equine blastocysts and to produce live foals [52]. Meanwhile, his countryman Dr. Miyake Kasai was introducing effective alternative cryoprotective agents, the "glycols," in the mouse model [53]. This breakthrough led to the effective application of propylene glycol (i.e., 1,2-propanediol; PROH) producing live births with cow and sheep from slow-frozen blastocysts ([54, 55], respectively). The effective use of PROH for human cleavage

Fig. 2.6 A breakthrough in slow-freezing practices was validated in the mouse model [44], showing that high survival rates could be achieved when plunging embryos into LN2 before the intracellular freezing point (estimated -38°C ; i.e., “rapid freeze”) if they were also warmed rapidly ($>500^{\circ}\text{C}/\text{min}$). Conversely, conventional slow-cooling (plunge at -80°C) survival was optimized with slow warming ($20^{\circ}\text{C}/\text{min}$). {Figure approval by DG Whittingham}

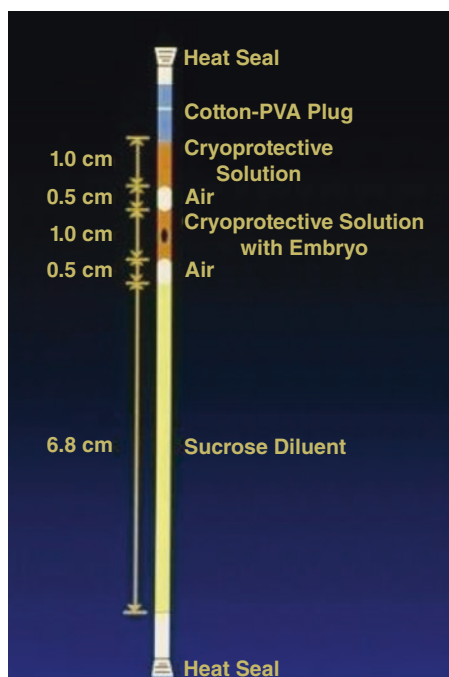
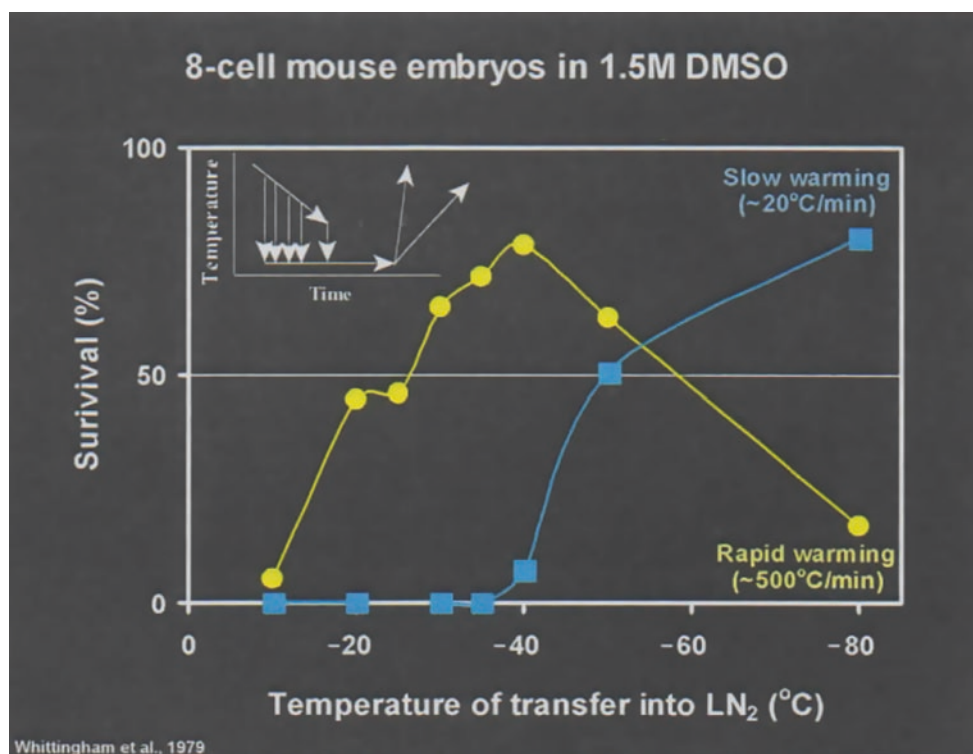


Fig. 2.7 The one-step straw schematic [60] illustrates how a 1 cm column of VS, with an embryo(s) separated by 0.5 cm bubbles, was associated with a 6.5 cm column of 0.75 M sucrose to facilitate the in situ dilution of vitrified embryos post-warming. This was the first approach used to vitrify mouse embryos [61] and sheep [60] blastocysts under ambient conditions in a 6.5 M VS3a glycerol solution and achieve healthy live births

embryos and zygotes was then proven safe and efficient [56, 57], becoming the standard slow-freeze technique in reproductive medicine for over two decades.

Historically, it is worth noting that with the adoption of slow-freezing embryos to -35°C in 0.25 ml plastic straws before plunging into LN₂, another interesting and practical concept was developed by Leibo [58, 59] for on-farm, direct embryo transfer. The one-step straw (Fig. 2.7) allowed for the in situ dilution of embryos with a column of 0.75–1.0 M sucrose solution in the straw, which was mixed upon thawing with a combined exposure to ambient air (30 s) and 37°C water bath (30 s–5 min). The one-step straw technique was ultimately used in the early vitrification success of Rall and coworkers with mouse [61] and sheep blastocysts ([60, 62]; Fig. 2.7), expediting the rapid elution of blastomeres post-warming directly in the straw.

Early slow freezing of human oocytes was first proven possible by Chen [63] and Al-Hasani et al. [64] with a second live birth reported by Van Uem et al. [65]. Yet, it was Deborah Gook of Australia who refined the standard PROH protocol for oocytes, validating its safety to the meiotic spindle and normal fertilization in mouse and human models [66, 67], as well as good fertilization and blastocyst development post-ICSI [68]. Following Gook’s mentorship, her approach to slow-freezing oocytes was first successfully adopted by Porcu et al. [69] and Tucker et al. [70], both achieving healthy live births. The slow freezing of eggs was used with variable suc-

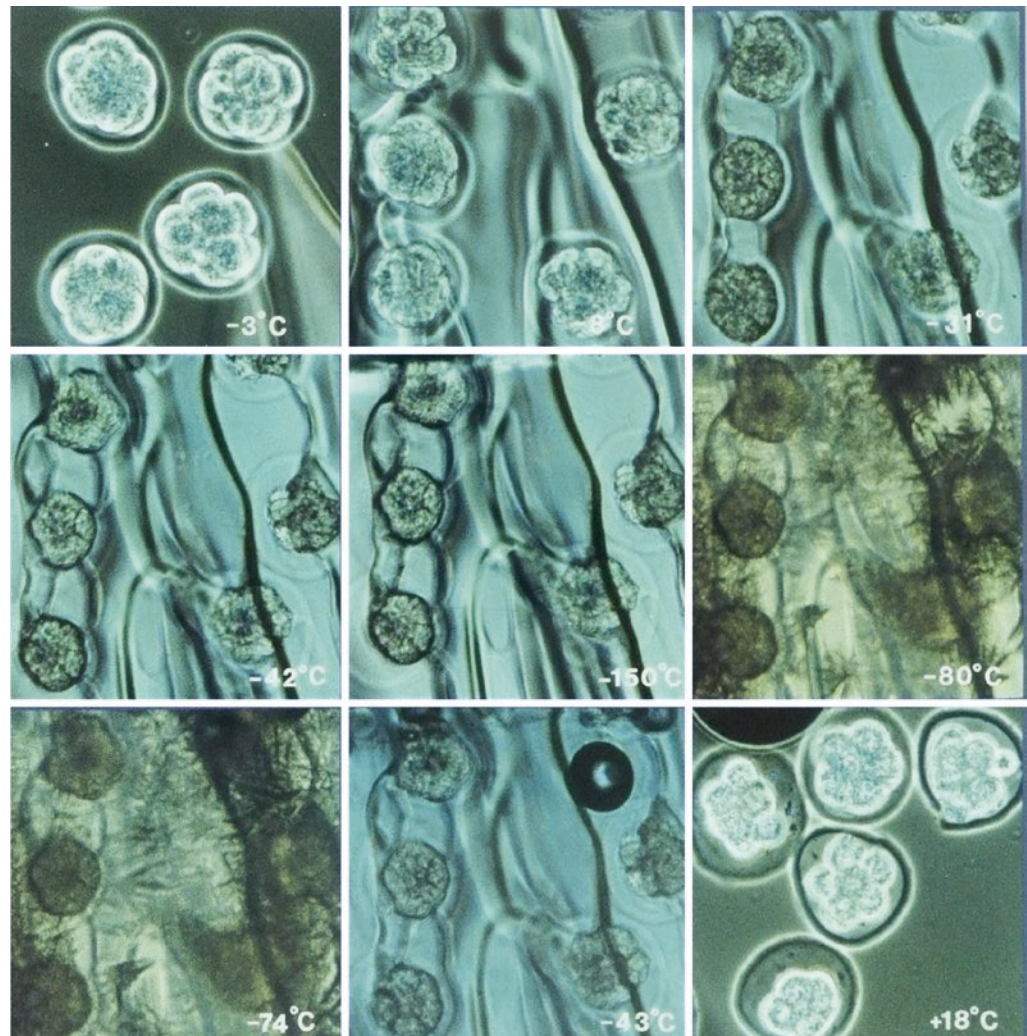
cess and efficiency for a decade. Interestingly, novel vitrification was applied successfully to mouse oocytes in 1989 [71] but was not duplicated on human oocytes for over a decade [72–74]. Vitrification technology ultimately proved to be significantly more reliable than conventional slow freezing [75], becoming the preferred procedure of egg banks and IVF clinics by 2010, as detailed in proceeding chapters.

2.5 The Path to Change in Cryobiology: Vitrification

In 1937, Father Basile J. Luyet, one of the founding members of the Society for Cryobiology and its first President in 1964, characterized the concept of vitrification working initially with frog sperm [22]. For reasons described elsewhere, Father Luyet had limited success developing a method to vitrify tissue successfully [76], but laid the foundation for the concept of vitrification to be pursued by those that fol-

lowed. Ultimately, it was the advent of cryomicroscopy in the late 1970s [77] and early 1980s that provided cryobiologists new insights into the physiochemical processes behind the underlying theoretical principles of cryobiology. Dr. William “Bill” Rall, mentored by Peter Mazur, fascinated by innocuous intracellular freezing, used the cryomicroscope to study fracture plane formations and IIF. During his postdoctoral fellowship with Chris Polge, he verified the concept of devitrification where the regrowth of ice crystals occurred in bovine and mouse embryos during slow warming (Fig. 2.8; [79, 80]). Years later, he determined that the sub -130°C metastable glass phase was susceptible to fracture plane formation if slow-frozen straws were warmed rapidly (i.e., directly warmed in a 37°C water bath), with damage to the zona of cattle blastocysts being reduced by slower initial warming at ambient temperature [81]. In total, these studies proved that changes in warming rates at critical points can dramatically alter physically damaging events.

Fig. 2.8 Cryomicroscopic observations enhanced our knowledge of physiochemical processes behind the cryophysics of slow embryo freezing ([78, 79]; (photo courtesy of William F. Rall)). Nonlethal fracture plane formations are revealed during freezing to -150°C , followed by non-harmful recrystallization events at -80°C to -74°C upon rapid warming, resulting in viable mouse embryos)



Rall et al. [78] determined that glycerol and DMSO depressed ice nucleation temperatures by substantially increasing the viscosity of intracellular regions, causing water diffusion to cease and the liquid cytoplasm to form a metastable glass (vitrify) upon rapid cooling (Fig. 2.8). Meanwhile, Fahy and others had assessed that when cryoprotectant concentrations are sufficiently high, crystallization of water molecules in the extracellular medium (i.e., aqueous solution) could be inhibited completely, forming a vitrified solid phase [82, 83]. As members of the Society for Cryobiology, Drs. Rall and Fahy had been in communication for years, developing a professional friendship before finally becoming colleagues at the ARC Laboratories in Bethesda, MD, in 1984. Together, they united their interests in vitrification and pioneered the technology for both whole-tissue preservation and embryo cryopreservation. Dr. Rall initiated a series of clandestine experiments under “cold-room” conditions (see review: [29]). The experiments were conducted at 5 °C to reduce the potential toxicity of acetamide in his initial VS1 solution. In their accomplishment of getting vitrified mouse eight-cell embryos to grow to the blastocyst stage, they emphasized the importance of warming rate relative to rapid cooling [84, 85].

Understanding the relevance of vitrification solution and cryo-toxicity [86], Rall and Fahy began working on solutions that could be safely applied to embryos at room temperature (1985–1986). Their initial attempts to use PPG/PROH (VS2) resulted in overt toxicity, while investigators in Belgium were reporting rapid freeze/vitrification success with PPG in combination with glycerol in the mouse and cow [87, 88]. Much like Audrey Smith’s challenge of Luyet’s concepts, Rall questioned whether Massip’s team was truly attaining a metastable vitrified state using the lower concentrated solutions (personal communication, 1986). The third generation of Dr. Rall’s vitrification solutions (VS3a, a 6.5 M glycerol solution) proved safe for use under ambient conditions, achieving comparable survival levels and pregnancy outcomes to conventional slow freezing of mouse embryos [61] and sheep blastocysts [60, 62], with four healthy lambs born in 1988. Shortly thereafter, embryo vitrification was successfully achieved in rabbits [89] and the goat model [90]. Perhaps more pertinent to this text was the pioneering efforts of Naomi Nakagata who successfully vitrified mouse oocytes and produced live young (1989). It is intriguing that these early proof-of-concept milestones did not find widespread application in the animal agriculture or human reproductive fields for more than a decade, as gradual progress and improvements with slow freezing occurred.

2.6 Advancing Embryo and Oocyte Vitrification into Clinical Practice

Still aspiring to cryopreserve whole organs, Fahy and coworkers worked diligently to define the physiochemical thermodynamics of potential ice growth in vitrification solutions, as it pertained to cellular viability under different cooling and warming conditions [91]. The importance of warming rates was again reconfirmed two decades later by Mazur and associates with respect to oocytes and embryos [92, 93]. With an understanding of how to control IIF, efforts were needed to reduce solution toxicity associated with the use of high molar cryoprotective agents. The advancement of low-toxicity vitrification solutions combining CPAs and other macromolecules was first reported by the Japanese teams of Kasai [94, 95] and Ishimori [96, 97]. Our understanding of different vitrification solutions and their relative toxicity to embryos was further enhanced by Drs. Jill Shaw [98, 99] and Jaffar Ali [100, 101]. Yet, it was not until the implementation of improved embryo culture systems supporting blastocyst growth [102] that vitrification was first incorporated into human reproductive medicine [103, 104]. Ultimately, clinical vitrification was linked to the formation of unique cryo-devices like the open pulled straw [105, 106] and cryoloops [107] that aimed to achieve ultrarapid cooling rates, despite the historical evidence characterizing the relative importance of warming rates to prevent damaging recrystallization events. As reviewed by others [108–112], proceeding chapters will discuss how various types of open (ultrarapid cooling; >5000 °C/min) and closed (rapid cooling; <2000 °C/min) device systems have been developed and effectively used for the vitrification of human gametes and embryos, supporting a growing industry promoting elective egg banking and “freeze-all” blastocyst cycles routinely implementing preimplantation genetic testing.

2.7 Conclusion

The history of cryobiology is based on understanding the properties of water molecules in a frozen state as they transition between liquid, ice, and glass phases. From early intrigue with semen in snow maintaining sperm viability to the cryophysics of thermodynamic principles, scientists have succeeded at freeze-preserving gametes and embryos for generations to come. For those truly interested in learning more about the historical stories behind the cryobiology of freeze preservation and vitrification, search the video library of the Society for Cryobiology’s 50th anniversary conference in 2013. These fascinating, informative, and sometimes

comical stories presented by current-day pioneers and mentors of the past are linked to the worldwide web via YouTube (search Cryo2013).

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Part II

Principles and Models

Cryopreservation Principles

3

James J. Stachecki

3.1 Introduction

The history of cryopreservation and those involved with making it a reliable technique used today in reproductive biology laboratories was discussed in the previous chapter. The review of these early works laid the foundation for modern cryobiology, as it applies to the storage of mammalian gametes and embryos. This chapter discusses the basic principles of cellular cryopreservation, whose understanding is important to fully appreciate the miracle of “vitrification” as it is used in the IVF lab today. Keeping in mind that gametes and embryos were not meant to be frozen, the fact that any survive and go on to make a viable offspring is truly remarkable. It is hoped that the concepts conveyed in this chapter will provide a useful knowledge base to help troubleshoot problems that may affect the efficacy of cellular preservation efforts through freezing. Overall, the collective works of Lovelock, Meryman, Mazur, Polge, Smith, Levitt, Farrant, Willadsen, Luyet, Fahy, Rall, and Leibo provided us an invaluable resource from which we can continue to learn about the effects of cryopreservation on cells.

3.2 Background: Intracellular Ice Formation (IIF) and Solution Effects (SEs)

Stable, long-term (practically infinite) preservation and storage of any perishable biological materials, particularly cells, are dependent on keeping them in the glassy (vitreous) state. This concept was apparent to Father Luyet when he titled his pioneering work “The vitrification of organic colloids and of protoplasm” [1]. It was Luyet’s work, which would make cryopreservation a science. From the outset, he recognized that ice damage must be avoided and that vitrification could be a method for long-term preservation of cell viability. Cells

are composed of >70% water, and thus, when they are cooled below a certain temperature, water begins to solidify into ice (T_m ; melting temperature). If ice forms intracellularly (IIF), the chances for survival upon rewarming are limited. However, if only minute amounts of ice form without causing particular damage to a cell, then that damage would likely not affect the cell’s ability to survive and continue development. Therefore, the avoidance of a “lethal” amount of IIF is necessary. This is outlined in Mazur’s two-factor theory of cell injury, whereby IIF and solution effects (SEs) make up the main reasons for cryopreservation injury [2–4]. The topic of SE is not new, but actually very old, especially if one simply considers the works of Luyet [1, 5]. The idea of SE or how the intracellular cellular environment as well as the extracellular environment will impact the cells during cooling comes into play. SE refers to those effects that can occur during cooling, whether it be in equilibrium or not. The intracellular response caused by solutes, penetrating and non-penetrating cryoprotectants, and the osmotic environment are collectively known as SE. The potential problem of SE involves the change(s) in a solution or cytoplasm that result from dehydration, increased solute concentration, pH changes, and precipitation of solutes [6, 7] that can be toxic or damaging to a cell. These effects can be described, if only in part, due to our incomplete understanding of exactly what happens during any form of cellular cooling and/or freezing. The importance of having a general understanding of IIF and solution effects is to enable us to understand how to store cells in such a way that they can sustain viability upon rewarming.

3.3 Principles of Ice Formation: Phase Diagrams

To avoid the deleterious effects of IIF, vitrification must occur [1]. Vitrification is the formation of an amorphous glass-like state. This simple definition is complete and accurate, despite excluding cooling rate, viscosity, thermodynamic changes, or rewarming events.

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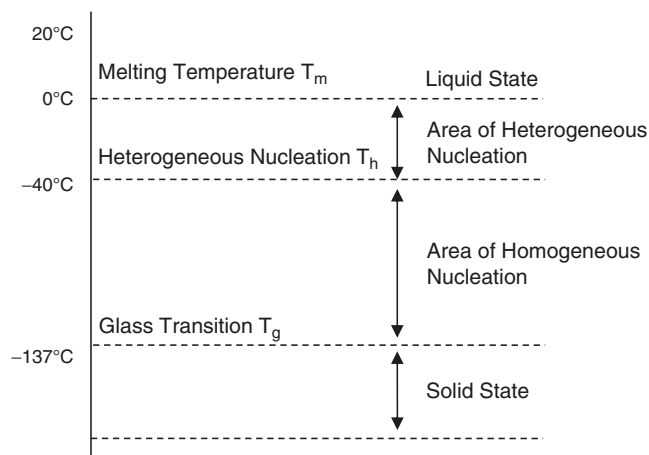


Fig. 3.1 Phases of pure water at different temperatures

In the diagram (Fig. 3.1), pure water is always in a liquid state above the melting temperature T_m . Falling below 0°C , heterogeneous ice nucleation can occur (T_h), and thus both states of liquid and solid may occur here. Water can form ice below 0°C , but needs a surface to nucleate on in order to form a crystal on which more ice can form and grow, similar to what happens with frost on a window pane. The glass, or dirt on the glass, acts as a surface whereby water can nucleate (crystallize and grow). At temperatures lower than -40°C , homogeneous ice nucleation can occur, where water does not need a surface to nucleate on and can spontaneously form ice (T_{homo}). At this point, both states of liquid and solid may still occur. As the temperature decreases below the glass transition temperature (T_g) of -137°C , molecules remain in the disordered pattern of a liquid; however, the physical properties become more similar to those of a rigid solid. Molecules become locked in place as though the liquid is frozen in time. The resulting “solid liquid” is called a glass or an amorphous glass-like state. The process of becoming a glass is called vitrification, derived from the Greek word “vitri” for glass. In order for water to become vitrified, without crystallization, it must be cooled at an extremely rapid rate exceeding $100,000^\circ\text{C}/\text{min}$ avoiding ice nucleation between T_m and T_g by a process referred to as kinetic vitrification [8–10].

In biological systems, the primary component is water, but not in a pure state inside cells containing solutes (salts, proteins, lipids, etc.), which must be taken into account when trying to cryopreserve the cell. The entire system is a collection of compartments filled with an aqueous solution. As aqueous solutions are cooled, the water forms a crystalline solid (ice), which has almost no solubility for the solutes that were in the aqueous solution. As pure ice forms, the solutes will be confined to the remaining liquid phase, becoming more concentrated. Since this lowers the freezing point of the aqueous liquid, the system can remain in equilibrium with a substantial unfrozen fraction. As cooling continues,

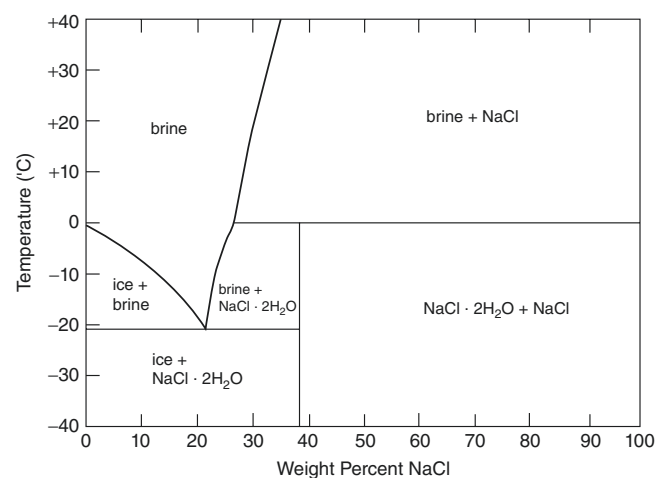


Fig. 3.2 Phase diagram for sodium chloride and water

the solubility limit of the solution will also be reached, leading to the precipitation of solutes. These events are succinctly described by a phase diagram. The simplest type of phase diagram is for binary systems, systems in which there are only two phases present (Fig. 3.2).

Starting at the left-hand side of the diagram, if the temperature of a solution with 0% salt is lowered, the freezing point occurs at 0°C . If the solution has salt dissolved in it (i.e., the concentration of salt is below the solubility limit), then the mixture will exist in the brine compartment. As the temperature is lowered, the weight percent of NaCl does not change until the thick line in Fig. 3.2 is reached. This line defines the freezing point of the solution. Further cooling will take the solution along the curve defined by the thick line until the eutectic point is reached at -21.2°C . At this point, the unfrozen compartment of the mixture is saturated with NaCl; any further cooling will cause salt to precipitate out of the mixture. For cryopreserving biological systems, this left side of the phase diagram is the most important as it describes the osmolality of the solution in which the cells exist. The osmolality will follow this curve down to -21.2°C , where it will hold constant until the temperature is raised once again.

In its most simplest form, a cell can be cooled at an extremely rapid rate to obtain a glassy state inside the cell, called kinetic vitrification [8–10]. Although kinetic vitrification is possible, it is far too costly and cumbersome to be used in IVF clinics today. One way to prevent IIF is to reduce the amount of water inside the cell by dehydration. This can be done by placing the cell in a hypertonic solution and draw the water out through osmosis. However, not all the water can be drawn out as some of it is bound to proteins and trapped in the intracellular milieu. Yet, removing too much water can, in itself, be damaging to the cell. Thus, some water is still in the cell and has the potential to nucleate and form ice crystals when cooled. This remaining water

can be “bound” with water-soluble chemicals, namely, cryoprotective agents (CPAs). These CPAs are typically alcohols that form hydrogen bonds with water, transforming its structure into a form that is more difficult to arrange into a crystalline structure when cooled. The higher the concentration of CPAs and/or the greater the cooling rate, the more likely the chance of vitrification. Although the binary phase diagram for sodium chloride and water is useful for understanding injury to cells, most cryopreservation protocols use at least one CPA to reduce the freeze-thaw injury. In such cases, there are three compounds that must be considered: water, NaCl, and the CPA. A system with three components is described by a ternary phase diagram. Diagrams have been published for the ternary systems DMSO-NaCl-H₂O and glycerol-NaCl-H₂O. From these diagrams, it is clear that the solubility and eutectic behavior of a single solute can be altered significantly by the amount and type of additional solutes introduced into the system (e.g., DMSO serves as a solvent for NaCl as well as a solute in water). It is also clear that equilibrium between solids and liquid becomes increasingly complex as the number of components is increased.

If we look at a phase diagram of a cryoprotectant solution in water, we will find several sections that are either liquid, solid, or a combination of both. Above T_m exists only liquid. Between T_m and T_h is the metastable region wherein ice can nucleate only by heterogeneous means. Below T_h , both heterogeneous and homogeneous nucleation exist. Once the temperature reaches the glass transition temperature, T_g , the remaining solution will vitrify. Simply stated, solidification of water above T_g can lead to IIF and cell demise if not properly cooled and warmed (Fig. 3.3).

The same graph below has been relabeled to show the phases as stable: no ice formation; metastable: ice nucleation

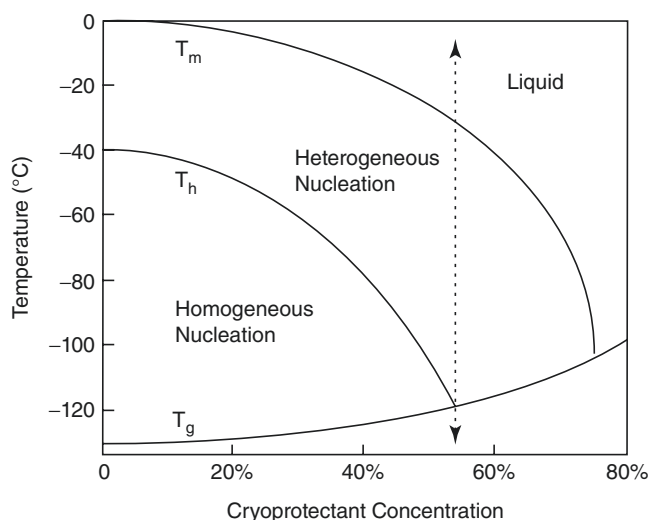


Fig. 3.3 Cryoprotectant solution in water phase diagram

by heterogeneous means only; and unstable or doubly unstable: ice nucleation by homo- and heterogeneous means (Fig. 3.4).

The graph in Fig. 4 can be separated into four sections representing the basic types of vitrification that can occur (Fig. 3.5). When cryopreserving a cell, one must cool it from above T_m to below T_g and then, upon rewarming, from below T_g raising above to T_m . On the far left and extending slightly to the right (the clear section), we have kinetic vitrification, wherein vitrification can only occur at extremely rapid cooling rates. Within the blue section, labeled “Unstable,” both types of ice nucleation can occur and cooling must occur at an ultrarapid rate (generally >5000 °C/min) so that the remaining water does not nucleate and grow during cooling. Furthermore, rewarming must occur at a rapid rate so that the ice that did nucleate during cooling will not grow (recrystallize) upon rewarming. During cooling in the metastable region, shown in yellow, nucleation occurs heterogeneously. This nucleation in and of itself is not hazardous to survival of

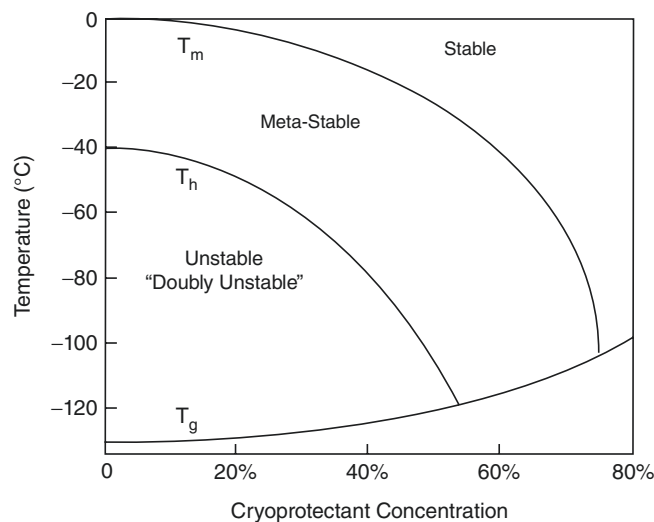


Fig. 3.4 Cryoprotectant solution in water phase diagram

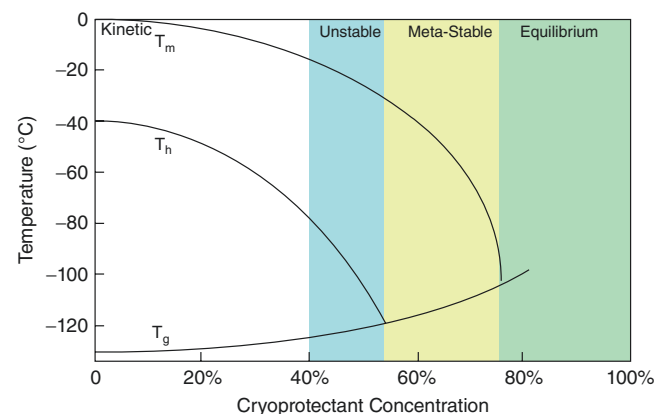


Fig. 3.5 Cryoprotectant solution in water phase diagram

the cell. However, it poses the opportunity for ice to recrystallize upon rewarming. Similar to the unstable region, if rewarming is fast enough, recrystallization can be avoided. The green section, “Stable,” represents a concentration of CPA high enough that the solution will vitrify at any cooling or rewarming rate with no chance for crystallization.

The graph in Fig. 3.5 visually conveys that increasing the CPA or solute concentration can shift our scenario towards the “safer” metastable area of the phase diagram. However, this increase [in CPAs] could eventually lead to cell death due to solution effects or cryoprotectant toxicity.

3.4 Cryopreservation of Cells

There are a number of methods available to achieve a vitrified state. To successfully cryopreserve a cell, specifically mammalian gametes and embryos, one must first dehydrate or remove some or most of the water. The next step involves addition of a CPA that will enter the cell and bind to the remaining water. Next, the cells are cooled until everything is solidified, usually below -80°C , and then stored in liquid nitrogen (-196°C) where metabolic activity ceases, and they can remain viable indefinitely. When it comes time to use the cells again, the opposite procedure occurs. The cells are rewarmed or thawed at which point they are rehydrated and the CPA is removed through a series of wash steps.

Storage of gametes and embryos was conventionally performed using slow cooling or equilibrium cooling (cells are cooled slowly to -30°C or below prior to storage in LN_2) for the past 50 years, and only in the last two decades has rapid, nonequilibrium cooling of cells treated at room temperature and plunged into LN_2 been routinely performed, referred to as vitrification. In actuality, both slow and rapid cooling are forms of vitrification. Slow cooling creates an intracellular vitrified portion, while the extracellular portion consists partially of ice. Conversely, rapid cooling consists mainly of vitrified portions. Rall and Fahy [11] demonstrated that mouse embryos could be vitrified and could survive by cooling them in a relatively concentrated solution and cooling at either $2500^{\circ}\text{C}/\text{min}$ or $20^{\circ}\text{C}/\text{min}$. The experiment exhibited that vitrification is not always rate dependent and, if solute conditions are right, is independent of cooling rate.

3.5 Slow Cooling (Conventional Slow Freeze)

The first theoretical basis for cryopreservation of cells was proposed by Mazur in 1965 and later applied to mouse oocytes [6, 12]. During slow cooling, embryos are exposed to relatively low concentrations of CPAs (1.5 M PROH and usually some sucrose, as a non-penetrating CPA, around

0.2 M) equilibrated for 10–25 min at room temperature, loaded into a straw or vial, sealed, and placed into a controlled-rate freezer. Ice formation is initially induced extracellularly by seeding at a temperature whereby ice can perpetuate (around -5.5°C or lower), and by briefly holding (5–10 min) at that temperature, we can minimize latent heat of fusion ice transformation and its potential detrimental side effects. As slow cooling continues ($0.3\text{--}1.0^{\circ}\text{C}/\text{min}$) and ice continues forming, a solute gradient is created and freezable water flows out of the cells directly via an osmotic gradient, minimizing the chance of IIF during cooling. As the temperature is gradually lowered, the concentration of cryoprotectant in the liquid phase, which includes the intracellular fluid, increases correspondingly until a level is reached (around 8 M CPA at -30°C) at which additional formation and growth of ice crystals, although possible, are unlikely, even if the temperature drops further [13]. Rather, the intracellular liquid phase turns into a glassy substance that solidifies without further crystal formation as the temperature continues to decrease (below -100°C). The unfrozen liquid phase remaining within the cells when they are plunged into LN_2 should ideally consist of this glassy substance with all the original cell solutes remaining in solution [13]. This suggests that when we slow-cool cells using a penetrating CPA such as PROH and standard slow-cooling protocols, we are actually vitrifying the cells by an innocuous, i.e., glass, conversion. Indeed, when we slow-cooled human embryos years ago, typical survival rates ranged between 60% and 100% depending on the developmental stage frozen. These survival rates would not be possible, at least according to Mazur and company, if IIF were occurring. Of course, this does not mean that IIF does not or cannot occur; it simply suggests that in slow-cooling protocols, IIF should not be a major source of cell damage. However, if water efflux is inhibited and does not occur in equilibrium, as the model suggests, and despite a slow cooling rate, IIF could pose a real problem along with SE [14].

Potential Problems:

The very successful slow-cooling protocols derived and modified from mouse and bovine embryo freezing work in the early 1970s do not work well for the storage of unfertilized oocytes, blastocysts, and other cells, at least using the conventional protocols. These cells, as well as gametes and embryos from other species, can be frozen effectively only if the stresses imposed on them are reduced or removed. For success, it would help to identify problem areas and then modify our approach to work around them. However, there are a number of unknowns. When a cell lyses after rewarming, the typical logic is that IIF and/or SE were responsible. This analysis is not based on experimentation, but on the seemingly logical, potential problems of IIF and SE. Hence, the freezing protocol is either modified or abandoned because “it does not work.” It is difficult to identify problems that

exist with simple morphological evaluation. Basic experimentation relies on visual observations. The cell is placed in the CPA solution and reacts by shrinking and sometimes re-expanding as equilibration occurs. It is then loaded into or onto a storage device, cooled, stored in LN₂, rewarmed, and rehydrated. We can only observe how the cell reacts to these treatments, without knowing anything about what is going on inside the cell. We do not know how the cell is affected by treatment with CPAs, by cooling, rewarming, and/or rehydration and removal of CPAs. It is a big black box. Where and when do problems arise? What are the problems?

Toner and coworkers [15, 16] specifically investigated IIF in mouse oocytes cooled in PBS without cryoprotectants and confirmed two distinct mechanisms of IIF: the first catalyzed by extracellular ice and the second heterogeneously by intracellular structures. Additionally, they discussed these mechanisms in the presence of cryoprotectants and conclude that both seeding (mechanism I) and heterogeneous nucleation (mechanism II) are rendered ineffective by the addition of cryoprotectants. In turn, it has been shown that mouse oocyte survival is inversely related to IIF [17–19] (Leibo et al. 1975, 1977, 1978).

The graph (Fig. 3.6) shows that the curve denoting IIF crosses the X axis and becomes zero (0% IIF) at and below a cooling rate of around 1 °C/min. This is substantially faster than the current protocol standard rate of 0.3 °C/min used for freezing mammalian embryos and oocytes, and accordingly IIF would not occur when a slower rate is used. In another study, Ashwood-Smith [20] looked at IIF in mouse oocytes using a cryomicroscope, reconfirming previous literature concluding that if IIF was present (occurred at –14 °C using a solution of PBS, 10% MeOH, and 10% dextran), the cells would lyse upon thaw due to a number of mechanical forces, including internal ice expansion rupturing membranes. They also say that cells could have been pierced by extracellular ice crystals, but earlier, Luyet and Gehenio [5] denied the existence of this mechanistic failure, stating that “experience

has shown that cells/tissues can tolerate the presence of extracellular ice and despite morphological appearances exert no mechanical pressure on the cells and certainly do not puncture them.” Ashwood-Smith [20] argued that mammalian eggs are much larger than the red blood cells used in Luyet’s paper, and physical forces could still play a significantly damaging role. But if these forces were lethal, it would not be possible to obtain near 100% survival of mouse embryos using the variety of cryoprotectants and slow-cooling protocols employed.

Indeed, early investigations showed that mechanical forces could contribute to the overall stress of freezing upon the cell, especially re-expansion or sheering forces upon rewarming (e.g., causing zona cracking for instance). The aforementioned studies collectively indicated that IIF is not necessarily a serious problem in modern protocols where penetrating cryoprotectants, slow cooling, and appropriate rewarming strategies are used.

The other potential problem of SE involves the change in a solution or corresponding cytoplasmic microenvironment that results from dehydration, increased solute concentration, pH changes, and precipitation of solutes [6, 7]. Half a century ago, Lovelock [21] showed that the increasing extracellular solute concentration that occurs during cooling when water freezes out as ice was responsible for red blood cell lysis (>0.8 M NaCl) and that the addition of a cryoprotectant (glycerol) reduced the amount of ice formed at any temperature, thereby effectively reducing the concentration of electrolytes produced. It has indeed been known for a long time that SE must be reduced to such a degree that cells can withstand cryopreservation. However, several more recent studies have reported higher tolerance levels to osmotic stress. Agca et al. [22] exposed bovine oocytes to PBS with increasing concentrations of sodium chloride increasing the osmolarity to supraphysiological concentrations (up to 4800 mOsm). They found that oocytes exposed to 2400 mOsm or lower developed to the blastocyst stage, albeit at slightly reduced rates, as compared to untreated controls. Van Os and Zeilmaker [23] exposed mouse zygotes to solutions up to 3100 mOsm (added NaCl) apparently without detriment to blastocyst formation. Others have exposed human or mouse oocytes and embryos to varying concentrations of cryoprotectants or sugars without cooling and found that cells have a considerable tolerance to heightened osmotic conditions [24–26]. When Toner et al. [27] cooled mouse zygotes to –40 °C in moderate to high concentrations of NaCl (300–2400 mOsm) without cryoprotectants, all the embryos lysed after rewarming. However, if the osmolarity of the PBS solution was increased (up to 2400 mOsm) using another salt, choline chloride, the majority of the cells remained intact despite the absence of cryoprotectants. Furthermore, when these zygotes were plunged directly into the highly concentrated solutions and not slowly dehydrated

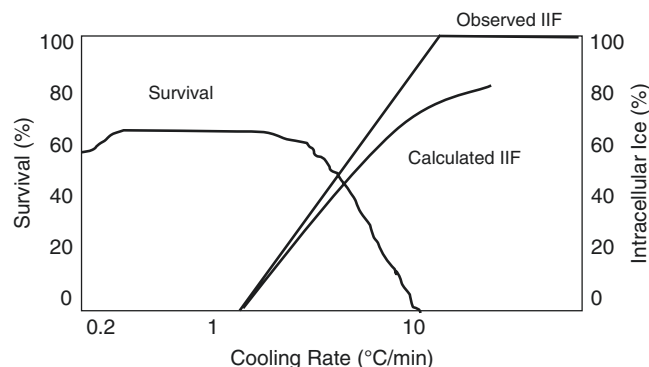


Fig. 3.6 Relationship between cell survival, cooling rate, and intracellular ice formation in a slow-cooling environment. Redrawn from Liebo et al., 1978 [19]

over a range of increasing osmotic concentrations, they still survived. This study showed that cellular demise from osmotic stress could be due to the type of stressor and not necessarily the ability of the cell to handle osmotic shock. Indeed, during the normal course of cryopreservation, cells (e.g., mouse embryos) that are exposed to very high osmolarities can still have $> 90\%$ survival. If osmotic stress were as lethal as originally suggested, the majority of the cells would die; yet this does not occur. Therefore, lethal IIF and SE may only play a small part in cellular demise during cryopreservation than generally thought, even though they can be lethal if the cells are not treated properly.

3.6 Rapid Cooling: Vitrification

The basic principles of vitrifying an embryo by slow cooling apply when rapid cooling is used. Initially, researchers tried many cooling techniques and cryoprotectants. They reasoned that if one could mimic, at room temperature, the intracellular cryoprotectant and water concentration when cooled slowly to $-30\text{ }^{\circ}\text{C}$ (where it can be vitrified), then the cell should survive storage from room temperature. All of these variables were calculated and tested in the cow, pig, sheep, rabbit, and mouse model, which led to a better understanding of what was possible [28–46]. Despite these successes, most of the solutions being used were more or less toxic to the cells, and although offspring could be produced, the technique overall did not work very well. The main reason was that for equilibrium vitrification to occur, the cells needed to be exposed to high CPA concentrations at a relatively low temperature (to reduce toxicity), and for a reasonable amount of time to dehydrate the cells and load the cell with cryoprotectant. Dimethyl sulfoxide (DMSO) was used as the primary or one of the primary CPAs because of its rapid permeation through cell membranes, despite its toxic potential [47–49]. The elevated cryoprotectant concentrations (6–8 Molar), temperature ($23\text{ }^{\circ}\text{C}$ to $39\text{ }^{\circ}\text{C}$), and duration (>5 min for adequate equilibration), all increase toxicity. Modifications in media composition, reduction of CPA concentration, and exposure led to some improvement in survival, but the big breakthrough came when very rapid cooling rates were used [38]. Vajta found that by using minute volumes of 1 μl or less along with direct submersion into LN₂, using an open container allowed for extremely rapid cooling rates on the order of $>10,000\text{ }^{\circ}\text{C}/\text{min}$. They found that despite using a high CPA concentration and relatively high temperatures of $23\text{ }^{\circ}\text{C}$ or higher, the extremely rapid cooling rate did not allow enough time for ice to form, even if it could. Longer exposure times and slower cooling rates proved detrimental to survival, most likely due to chemical toxicity [50]. By achieving an extremely rapid cooling rate, survival rates of over 90% and the establishment of live births have been since reported.

The basic principles and methodology outlined in Vajta's paper have not changed since it was published. All modern DMSO-based vitrification methods are basically slight modifications of what Vajta described. Despite posing a greater risk from the potential toxicity of the highly concentrated cryoprotectants and the relatively high exposure temperature, rapid cooling, in most instances, has met with greater success than slow-cooling procedures [24, 50]. The cryoprotectant solutions most often used consist of an equilibration solution of 7.5% DMSO and 7.5% ethylene glycol (for 10 or more minutes to allow for near-complete equilibration) and a final vitrification solution of twice that concentration [51]. To combat the potentially cytotoxic effects, exposure to the final vitrification solution is usually limited to around 45–90 s or less before plunging [52–57]. Also, in order to obtain a rapid cooling rate in the order of $>10,000\text{ }^{\circ}\text{C}/\text{min}$, it is necessary to use very small volumes of media, usually 1 μl or less, and direct exposure to LN₂. Alternative storage containers are used. These devices generally fall into one of the two categories: 1) micro-sized straws that could be heat-sealed or 2) a thin flat plastic blade with a handle (i.e., the Kuwayama model). With the cryo-top device, for example, the cell(s) are placed on the end of the device, and excess media is removed, leaving the cell(s) covered in a very thin film of media, before plunging directly into liquid nitrogen [51, 58]. This allows for ultrarapid cooling rates of over $20,000\text{ }^{\circ}\text{C}/\text{min}$ to be achieved. However, this is only one strategy for rapid cooling.

Understanding the principles of slow cooling, Stachecki developed a very different, yet traditional, vitrification approach using the standard 0.25 cc cryo-straw and much slower cooling rates than needed when DMSO is used (Stachecki et al. [59]). Other CPAs were less toxic than DMSO and had already been proven effective in slow-cooling procedures. Without DMSO, Stachecki avoided much of the toxicity issues and learning curve that Vajta's model required. Oocytes and embryos, including even fully hatched blastocysts, could be vitrified in practically any size container from a relatively large 0.5 cc sperm cryo-straw to the smallest open micro-volume container (e.g., cryo-top, cryo-tec, rapid-i). However, it should be noted that when vitrifying embryos without a zona pellucida (e.g., hatched blastocysts) on microvolume plastic surfaces, an additional physical stressor to cryopreservation is possible where membrane adherence can be damaging. The aforementioned S³ vitrification system [59] is now referred to as ICE and has been further improved and optimized [60] for the effective cryopreservation of human embryos and oocytes using DMSO-free CPAs. By using ethylene glycol and glycerol in lieu of DMSO, Stachecki found that the cells could be exposed to higher concentrations for longer periods of time (several minutes or longer), without chemical toxicity [59]. This meant that the cells were more dehydrated and had more intracellular loading of CPA than with Vajta's DMSO

system. The non-DMSO system allowed the cells to fall within the “metastable” portion of the latter phase diagrams (Figs. 3.2 and 3.3), whereby only heterogeneous nucleation could occur. Schiewe and coworkers [61, 62] further verified the low toxicity and the metastability of the ICE/non-DMSO system when they re-vitrified (rVTF) blastocysts up to five times with (discontinuous rVTF) or without (continuous rVTF) sucrose elution per event without a change in sustained blastocyst development 24 h post-thaw. By contrast, the DMSO system is “doubly unstable” and allows both homo- and hetero-nucleation to occur, which makes this system more susceptible to embryonic loss by repeated rVTF. Since we are shifted further right on the curve, to the metastable area, there is a smaller temperature range between T_m and T_g to traverse and thus less chance for nucleation to occur. This allows for slower cooling and rewarming rates, which in turn allows for a larger storage device, such as the conventional, sterile, heat-sealed 0.25 cc straw. The non-DMSO ICE system demonstrates that although vastly different approaches to vitrification exist, they still rely upon the basic principles of cellular cryopreservation.

3.7 Warming Rate: Key Importance to Vitrification Success

In addition to the above discussion on cooling, it would be amiss not to talk about rewarming after storage in LN_2 . It has been known for many years that warming rates are as important or arguably more so than cooling rates [13, 32, 63–65]. Cells properly cooled and stored in LN_2 may have 100% potential for survival, but with improper warming, cellular demise is inevitable. Upon rewarming, the frozen/vitrified solution must go through a temperature range, above T_g and below T_m (Fig. 3.3), wherein the solid phases will melt and/or devitrify, and the possibility of both liquid and solid phase will again exist. From the early 1970s, it was discovered that the rewarming rate affects the behavior of preexisting ice, the likelihood of additional ice formation and growth, and also the rehydration of the cell [13, 63, 65, 66]. Willadsen showed that embryos could survive plunging from temperatures of -30 to -36 °C provided that rapid thawing was used [32]. This opened the way for the development of shorter and simpler freezing and thawing procedures and helped optimize embryo cryopreservation.

During rewarming, not only can the water that has nucleated recrystallize and grow forming larger and potentially more damaging ice crystals, but there is also the potential for shearing forces to occur as these phase changes occur. This is similar to placing an ice cube in a glass of warm water and having it crack or shear apart. The stored potential energy in the ice (the larger the volume of ice, the larger the amount of potential energy stored) is released upon rewarming, and the rate of temperature increase will affect the

phase changes and energy released. Thus, zona cracking or embryo splitting is most often the result of too rapid a warming rate (or at least, the initial warming rate). The results of Stachecki and Willadsen [67] support this and were in general agreement with those of others in showing that the higher the equilibration/plunge temperature, the faster is the optimal thawing rate for cryopreserved cells. However, they also showed that even with a plunge temperature as high as 20 °C, excellent survival and development rates are obtainable and that contrary to what might have been expected, the most rapid thawing regime was not the one that allowed the most survival.

3.8 Conclusion

All of the rapid-cooling forms of vitrification procedures for human oocytes and embryos described in the recent literature are, in principle, the same. The high osmolarity of the vitrification solution rapidly dehydrates the cell, some of the CPA enters the cell and binds the remaining water, and submersion into liquid nitrogen quickly solidifies or vitrifies the cell so that any remaining intracellular water not bound by cryoprotectants does not have time to form ice crystals. The embryo is effectively vitrified without intracellular ice, similar to slow cooling. From these descriptions, both techniques (slow cooling and rapid cooling/vitrification), although seemingly very different, share the same outcome of vitrifying the cell. The principles outlined here should provide a basic understanding of cellular cryopreservation. It is with this knowledge that improvements can be made when trying to store other cells and multi-celled tissues.

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Animal Models for Human Oocyte, Embryo, and Ovarian Tissue Cryopreservation

4

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4.1 Introduction

Infertility is one of the most important health problems in all societies and ages and one of the most traumatic and delicate experiences that threaten the stability of the individual, marriage, family, and society [1]. The need to help these patients has propelled the development of efficient methods to preserve their precious gametes and embryos. Advances in cryobiology have allowed the cryopreservation of embryos, oocytes, and, more recently, ovarian tissue, revolutionizing reproductive medicine. These techniques are now a fundamental part of infertility treatments or fertility preservation strategies. They have allowed safeguarding the fertility of women diagnosed with pathologies and conditions that affect their reproductive potential. Recently, they have also been considered by healthy women who decide to delay child-bearing for personal or professional reasons.

The development and optimization of these approaches would not have been possible without studies applying animal models. The pioneering experiments on oocyte cryopreservation in rabbits in 1947, the first pregnancy using cryopreserved rabbit embryos in 1948, and mouse ovarian tissue in 1960 (Fig. 4.1) (Ludwig et al., 1999), among other studies in animal species, were crucial to understanding how cryoprotectants and low temperatures can affect embryos, oocytes, and preantral follicles. Indeed, the success of cryopreservation of human gametes and embryos is based on the achievements in different animal models. The viability of cryopreserved oocytes and embryos depends on the degree

of their cryodamage during freeze-warming. In this regard, it is imperative to develop freezing protocols that minimize the damages caused by the intra- and extracellular ice crystal formation, toxic effect of high cryoprotectant concentrations, and osmotic stresses. The effectiveness of cryopreservation of gametes and embryos is assessed based on morphological, functional, and genetic changes in the cells after warming.

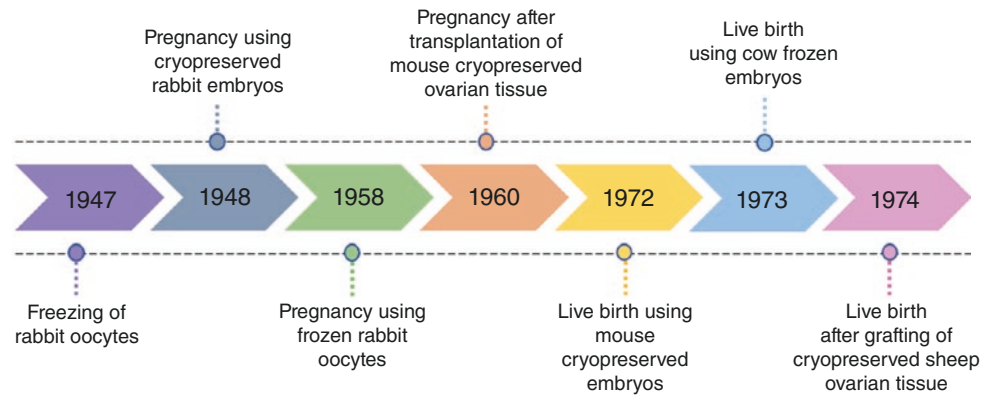
Recently, international health professional organizations have boosted the discussion about the importance of performing animal experimentation for reproductive disorders and fertility preservation research [2]. Nevertheless, special attention should be paid to the ethical issues of animal use in research, always pursuing the 3R principle: replacement, reduction, and refinement. Care should also be taken regarding assisted reproductive technology, including cryobiotech technologies, which in many countries remain open and in need of settlement. If animal experimentation is to be applied, choosing a model is a decision that depends on several aspects, including (1) the need for genetic variation control, (2) physiological/morphological similarities to humans, (3) intrinsic and (4) extrinsic factors that influence that species within the experiment, and (5) availability of an adequately adapted facility to keep animals.

In this chapter, in order to understand how cryopreservation protocols for human gametes and embryos have been established, we present a compilation of studies for cryopreserving embryos, oocytes, ovarian tissue, and isolated ovarian follicles in ruminants, rodents, and nonhuman primates. Moreover, we will discuss some ethical aspects related to the use of animal models in assisted reproductive technology.

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Fig. 4.1 Some pioneering studies using animal models for the cryopreservation of embryos, oocytes, and ovarian tissue



4.2 Cryopreservation Strategies

In the last decades, cryopreservation of embryos, oocytes, and ovarian tissue has been revolutionizing reproductive medicine, conserving cells, tissues, or organs by decreasing the temperature, aiming to reduce their metabolic activity. Although this allows storage for an unlimited amount of time without impairing cell or tissue viability, the decrease and subsequent increase in temperature during cooling and warming often result in cryoinjury [3, 4], especially in the intermediate temperature zone between 0 °C and – 15 °C [5]. During cooling and warming, cells are exposed to different forces (thermal, chemical, and mechanical), which may interfere with their normal function [3]. These forces vary according to the cryopreservation procedure applied, which generally falls into two categories: freezing or vitrification.

Freezing is the most applied method for cryopreservation of human ovarian tissue. For performing this method, the use of programmable freezers is essential, and throughout this process, ice crystals form first in the extracellular environment. Aiming to prevent intracellular ice crystal formation, which can potentially lead to cell damage, slow cooling rates are usually applied, allowing osmotic adjustments between intra- and extracellular fluids [5]. In most cases, slow cooling protocols apply a sole permeating cryoprotectant (e.g., dimethyl sulfoxide, ethylene glycol, propylene glycol), with concentrations around 1.5 M.

The vitrification method, which converts a supercooled liquid into a glass-like amorphous solid, prevents ice crystal formation through an ultrafast cooling rate associated with high concentrations of cryoprotectants [6]. This method is frequently applied for oocyte and embryo cryopreservation.

Still, the risk of ice crystal formation during warming or thawing is a factor to be considered, either with freezing or vitrifying protocols. For example, the formation of small ice nuclei during vitrification is inevitable. However, since warming takes a long time to be completed, further nucleation and crystal growth may occur, leading tissue to damage the structure [7].

4.3 Animal Models

While nonhuman primates are the animal models more similar to humans, their utilization in research has been drastically decreasing due to the current policies discouraging experimentation in these animals. Regarding domestic animals, ruminants, such as cows, ewes, and goats, are probably the best animal models for studies on developing or optimizing cryopreservation protocols. This is due to their similarities to humans: they have almost the same ovary size and stroma composition and similar follicle size and growth patterns (Fig. 4.2). Moreover, their ovaries are usually discarded in abattoirs, offering a large amount of material for research and avoiding ethical issues.

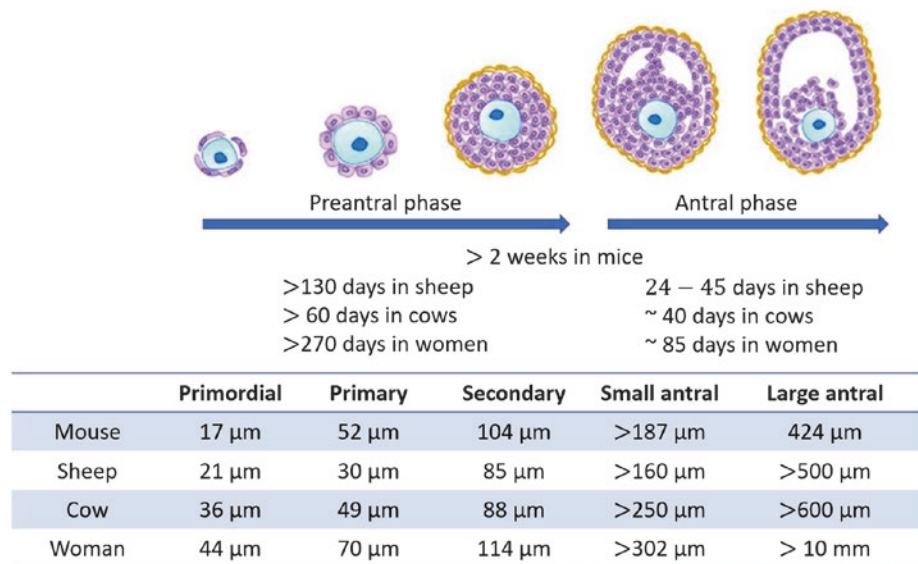
On the other hand, rodent ovaries are much smaller than their human counterparts, and they have a soft, spongelike structure with a low extracellular matrix content, which is very different from the stiffer, collagen-rich extracellular matrix of human ovaries. In addition, their follicles are much smaller too, and they take a shorter time to develop from the primordial to the preovulatory stage (Fig. 4.2). Nevertheless, the great majority of studies in assisted reproductive technologies are carried out using rodent ovaries, oocytes, and embryos.

4.3.1 Ruminants

4.3.1.1 Cryopreservation of Embryos

The demand for in vitro-produced bovine-sexed embryos has increased over the years to the point that more embryos have been produced in vitro than in vivo since 2016 [20]. Vitrification is currently the chosen method for embryo cryopreservation [21] due to its low cost, simplicity, and speed [22]. However, it requires high concentrations of cryoprotectants, which can cause cell toxicity. Moreover, there are many different vitrification methods, with a lack of a standard protocol, which has not yet offered a commercially viable solution [21]. Slow freezing has shown acceptable

Fig. 4.2 Follicle diameters calculated by Amorim et al. [8], Bartlewski et al. [9], Braw-Tal and Yossefi [10], Britt [11], Chen et al. [12], Driancourt et al. [13], Gougeon [14], Griffin et al. [15], Kacinskis et al. [16], Rosales-Torres et al. [17], and Verhoeven and Lambalk [18]. Figure adapted from Lee et al. [19]



pregnancy and livebirth rates in cattle, sheep, and goats, with a 10% gap between the results after fresh vs. frozen embryo transfer (reviewed by [21]). There are already some well-established protocols for embryo slow freezing in these species [23, 24]. One study compared embryo transfer of fresh, vitrified, or slow-frozen bovine embryos and reported that conception rates were similar between the different freezing techniques [25]. However, there is still no consensus on the slow-freezing efficiency for in vitro-produced bovine embryos [26].

In both cases, cryopreserved in vitro-produced embryos are less resistant to the freezing procedure [27], being influenced by the culture media [28] and also by their species [29]. In this sense, oocyte developmental competence is a way to improve in vitro-produced embryo cryotolerance, and this is influenced by a series of factors, such as oocyte origin, follicle environment, donor nutrition, calf history, stress level, health, and many others (reviewed by [30]). These factors influence oocyte morphology, transcriptome, and lipid composition, which, in turn, impact blastocyst yield [31].

Different amino acids and energy sources in culture and cryopreservation media impact embryo development. At around 2–3 days of culture, cattle embryos go through developmental arrest, called the “eight-cell block,” which can also be overcome with different culture processes, such as co-culture with different types of cells, addition of growth factors, culture under low oxygen concentrations, glucose deprivation, addition of reducing agents, cytokines, and conditioned medium [30]. The use of sera or the practice of co-culture may bring negative impacts, affecting cleavage, lipid accumulation, and gene expression. Moreover, the use of serum may inhibit initial cleavage but later stimulate the formation of blastocysts and cause asynchronous embryo age and uterine environment [30].

Since cryosurvival and gene expression can be altered by culture conditions [32], new protocols have been developed using microfluidic technology [33] and ultramicrodroplet volumes of up to 2 μL [34], which allow control of the fluid flow, automate the renewal of culture media, and maintain the concentration of embryotrophic factors, providing a monitored assessment of the culture [35]. Dynamic cultures with shaking, rotation, or vibration also benefit the cultures [36]. Moreover, nanotechnology with gold nanorods (GNRs) has been successfully applied in the vitrification of embryos [37, 38] and may provide important insight for future protocols on the case of ruminants and other species.

4.3.1.2 Cryopreservation of Oocytes

Oocytes are more sensitive to cryopreservation than embryos or even semen due to many cellular factors: their plasma membrane has a low permeability coefficient, making the movement of water and cryoprotectants slower [39, 40]; their cytoplasm has fewer actin filaments, which weakens their membrane [41]; they present a high lipidic content [40] being more susceptible to reactive oxygen species [42]; and their meiotic spindle is very vulnerable to chilling [43]. Moreover, cryopreservation promotes early cortical granule exocytosis, which leads to hardening of the zona pellucida and impairs sperm penetration, disorganizing the cytoskeleton and causing DNA abnormalities [44]. On the other hand, oocytes have a faster recovery after thawing/warming (especially after vitrification) [43]. There is an effect of the presence of cumulus cells, as well as oocyte maturation status and follicle size. Oocytes, as well as embryos, are impacted by in vitro culture and in vitro maturation systems that are applied before cryopreservation. Therefore, medium, oxygen pressure, pH value, and additives in media should be carefully chosen prior to vitrification to obtain an ideal blastocyst

yield after warming (reviewed by [45]). After fertilization, these conditions may change, making embryos easier to cryopreserve than oocytes (reviewed by [46, 47]).

Compared to embryos and sperm, cryopreservation of oocytes has a more successful history. In general, oocytes are frozen by vitrification, being immersed in an equilibration medium with lower concentrations (3–40%) of cryoprotectants than the freezing medium for 0.5–15 min [45]. Then, oocytes are immersed in a freezing medium inside a plastic straw and plunged into liquid nitrogen. The choice of cryoprotectants, their concentration, and exposure temperature and time affect oocyte survival and development. A study showed that 15% of matured oocytes developed into blastocysts after vitrification at an extremely rapid cooling rate in a solution containing 30% ethylene glycol and 1 M glucose, followed by in vitro culture [48]. Bovine oocytes exposed to an equilibrium solution containing 7.5% ethylene glycol and 7.5% DMSO at 38.5 °C for 2 min and 30 s prior to vitrification showed similar embryo development to fresh oocytes, showing spindle integrity and lower apoptosis rates than oocytes exposed at 25 °C [49].

Most vitrification protocols for MII oocytes have similar results in terms of blastocyst development and cleavage rates [47]. Cryopreservation of immature oocytes, in turn, is a challenging subject. Oocytes in the germinal vesicle state present homogenous lipid droplets that do not change after freezing, but intercellular coupling between cumulus cells and germinal vesicle-oocyte is very sensitive to osmotic stress [47, 50]. A study showed that only 8% of immature bovine oocytes developed into blastocysts after vitrification with ethylene glycol, Ficoll, and sucrose [51]. Protein-free media for vitrification of immature bovine oocytes provided high blastocyst quality with no noticeable toxic effects [52].

Storage of oocytes in liquid nitrogen requires high costs in terms of space and specialized equipment. In this sense, drying oocytes after vitrification is an alternative method for their preservation. One study compared different freezing methods for MII oocytes: slow freezing, rapid freezing, and vitrification followed by lyophilization with a trehalose-based solution. The third group presented 88% of oocyte recovery, against 70% and 71% in the other groups [46].

Similar to embryos, oocytes benefit from in vitro maturation before vitrification. Cytoplasmic lipid droplets can be partially removed from oocytes with centrifugation [53], reducing cryopreservation damage. Another impact of cryopreservation on bovine oocytes is the mitochondrial dysfunction and ATP loss [54, 55]. To stimulate ATP production and mitochondrial metabolism, L-carnitine can be used in the in vitro maturation medium [56, 57]. However, L-carnitine has shown an increase in aster formation, which may cause low developmental potential [58].

Glutathione can be used in the medium to reduce oxidative stress damages and regulate the syntheses of DNA and

proteins [59, 60]. Thiol compounds, such as β -mercaptoethanol and cysteamine, increase intracellular levels of glutathione and, thus, improve the development of bovine oocytes [61].

Post-vitrification treatments have also shown benefic effects on the developmental competence of bovine oocytes. The culture of vitrified-warmed oocytes with antiapoptotic drugs, such as Y-27632 [62], and antioxidant drugs, such as α -tocopherol and ascorbic acid [63], showed an improvement of bovine blastocyst yield.

Ultimately, standard approaches to cryopreservation of bovine oocytes rely on ultrarapid vitrification, using cryodevices [64] and equilibration with low concentrations of cryoprotectants prior to vitrification [65]. The most commonly used cryoprotectants are DMSO and ethylene glycol, in addition to sucrose. Improvements in the vitrification procedure to overcome the sensitivity of bovine oocytes to cryopreservation include chemical treatment of the oocytes, together with pre- and post-vitrification chemical treatment [45, 47].

4.3.1.3 Cryopreservation of Ovarian Tissue

Cryopreservation of ovarian tissue is an essential tool for preserving the genetic diversity of livestock [66]. Moreover, bovine ovaries are commonly used as models for human reproductive studies due to their physiological similarities [67], and many advances were made in human fertility preservation because of studies on bovine and small ruminants.

Immature oocytes present in cortical tissue are more resistant to the freezing process than isolated oocytes [68]. The optimal protocol for ovarian tissue cryopreservation, however, has not been well established yet. Although evidence shows better preservation of the ovary when slow freezing is performed [69], many studies have been performed to improve vitrification procedures. Vitrification has shown successful results in goats using ethylene glycol and DMSO [70] with live births after autotransplantation of cryopreserved ovarian tissue [71] and after in vitro culture of cryopreserved ovarian tissue [72]. Recent studies show the benefits of antifreeze proteins in the warming medium after vitrification, preventing ovarian tissue damage, reducing follicle apoptosis and tissue fibrosis, and improving follicle morphology and graft microvessel formation after transplantation [73]. The use of antifreeze proteins in the freezing medium, however, showed no significant difference in the quality of ovarian tissue and follicles [74]. Supplementation of the vitrification medium with *Aloe vera*, in turn, promoted follicle activation and development after in vitro culture of vitrified bovine ovarian tissue [75]. Injection of angiopoietin-2 or vascular endothelial growth factor (VEGF) in mice that were transplanted with bovine ovarian tissue enhanced angiogenesis and reduced apoptosis and tissue fibrosis [76]. Another study reported that oocyte vitrification was more

successful than ovarian tissue vitrification in preserving the developmental ability of cumulus-oocyte complexes [77].

Slow freezing, in turn, has shown to be most effective for bovine ovarian tissue using DMSO [78] and propanediol [79], preserving the structural integrity of follicles and stromal cells. DMSO was also efficient in preserving primordial follicles included in ovine [72, 80–82] ovarian tissue, while propylene glycol and glycerol did not maintain viable follicles in sheep ovarian tissue slow freezing [83]. Whole-sheep ovaries were successfully cryopreserved with DMSO, and the reproductive capacity and endocrine function of the sheep were well preserved 6 years after transplantation [84].

Follicles from sheep and goats respond differently to ovarian tissue cryopreservation [85], probably due to their morphological peculiarities. Goat follicles present different cytoplasmic components than other species [86]. Studies show that goat ovarian tissue can be frozen using ethylene glycol [87] as well as propylene glycol and DMSO [87]. The use of 20 IU/mL catalase in slow-freezing medium containing propylene glycol reduced lipid peroxidation of goat ovarian tissue [88]. Vitrification of goat ovarian tissue has also been well studied, showing benefits when melatonin was added to the freezing medium [89]. A vitrification device has been developed for vitrification of caprine ovarian tissue—the Ovarian Tissue Cryosystem [90, 91], opening a new possibility for commercial application of ovarian tissue vitrification and extrapolation for other species.

4.3.1.4 Cryopreservation of Isolated Preantral Follicles

While cryopreservation of mature oocytes promotes hardening of the zona pellucida and damage to the cytoskeleton, freezing oocytes contained in primordial follicles appears to be an interesting alternative. In this case, follicles are less differentiated, with few organelles, no zona pellucida, and no cortical granules. Thus, the oocyte is less prone to ischemia damages [91].

Cryopreservation of isolated primordial follicles can be performed using both vitrification and slow freezing in different species. The addition of 10 mmol/L α -tocopherol in the vitrification medium with DMSO increased follicle survival rates and preserved the morphology of cow preantral follicles [92]. Ovine primordial follicles have been successfully cryopreserved by slow freezing using ethylene glycol [93] and DMSO [80, 94].

4.3.2 Rodents

4.3.2.1 Cryopreservation of Embryos

Rodents are probably the most applied animal models in several research topics. In reproductive medicine and fertility preservation fields, for example, studies in embryo cryo-

preservation have been for a long time performed in rats [95] and mice [96]. Mouse is the most applied rodent for experiments with embryo cryopreservation. It is currently a meaningful model for the generation of embryonic stem cells. In this context, more effective methods for embryo cryopreservation have been proposed for the viability maintenance of blastomeres in this species [97]. Nowadays, a microfluidic system has been proposed as a method that contributes to the viability of mice embryos after cryopreservation, avoiding osmotic shock during cryoprotectant perfusion [98]. Such a system contributes to the perfusion of cryoprotectants during the dehydration process, reducing the time needed for cryoprotectant loading without compromising the outcomes. More frequently adopted methods for mice embryo cryopreservation include (1) slow freezing [99], (2) rapid cooling [100], and (3) vitrification [101]. For slow freezing, the use of propanediol and sucrose [102] allowed successful cryopreservation of embryos from the two-cell to the blastocyst stages, providing survival rates greater than 90%. For rapid cooling, ethylene glycol, Ficoll, and sucrose make an optimal solution (EFS40) for preserving morula-stage embryos, which must be initially placed in vapors and then submerged in liquid nitrogen before being stored at -196°C [103]. Vitrification of mouse embryos demands a preincubation in EFS20 (20% ethylene glycol) following incubation in an EFS40 (40% ethylene glycol) solution [101] before the cooling curve.

In rats, the first successful procedure of embryo cryopreservation was in 1975 [104], when two-cell and four-cell embryos were collected from mated females, frozen in a DMSO solution, and in vitro cultured after warming, reaching the blastocyst stage. The success of this experiment was confirmed by pregnancy; however, at that time, the authors recognized the limitation of the method performed in rats compared to the pregnancy rates observed in mouse experiments. Currently, one-cell [105], two-cell [106], and morula [107] stages are applied for rat embryo cryopreservation, with morula equilibrium freezing being the method of election for genome banking purposes [108]. In general, equilibrium [109, 110] and ultrarapid [111] cooling methodologies have been applied for rat embryos, allowing survival rates between 40% and 90%. It is worth mentioning that embryo survival rates after cryopreservation depend on the rat strain in question [110].

Experiments with cryopreservation of rabbit embryos started in 1974 when Bank and Maurer applied slow-freezing protocols with different DMSO solutions, providing a 65% viability rate [112]. Generally, the stage considered optimal for rabbit embryo cryopreservation by vitrification is morula [113], and solutions containing 20% ethylene glycol and 20% DMSO provide survival rates ranging from 45% to 65%. However, experiments with rabbit morulae revealed that ethylene glycol and Ficoll are less harmful when com-

pared to ethylene glycol combined with DMSO in the vitrification solution [114]. Also, intergenerational impacts of embryo cryopreservation were assessed in rabbits. Lavara et al. [115] confirmed that performing embryo vitrification and transfer impact the offspring in terms of live births, litter size, and postnatal survival rates in F1 and F2 generations [115].

In addition to the species mentioned above, other rodents, such as hamsters, have been applied for experimental embryo cryopreservation [116]. The first description of this species was in the 1980s, when Ridha and Dukelow froze preimplantation embryos in a DMSO solution, obtaining viable embryos as demonstrated by the trypan blue staining method [117]. For its recognized potential as a model for paternal behavior studies, the development of specific techniques for embryo cryopreservation and transfer in hamsters is also of great importance. In Campbell's hamster, the use of ethylene glycol and sucrose was more effective than ethylene glycol alone for slow cooling of eight-cell embryos [116]. In the guinea pig, although cryopreservation of embryos has been tried, attempts have not been successful, and authors attribute such failure to two challenges to be overcome: delipidation of the embryos and difficulties in developing embryo transfer techniques [118].

4.3.2.2 Cryopreservation of Oocytes

As for embryos, the first reports of oocyte cryopreservation, dating from the 1950s [119], were described in mice cells. These, however, were unsuccessful attempts described before the successful reports of Whittingham in the 1970s, applying mouse oocytes [120]. Matured mouse oocytes (MII) are usually cryopreserved by vitrification, which provides better rates of viability, fertilization, and spindle assembly [121]. Nowadays, such vitrification protocols usually apply commercially available vitrification media [122, 123].

In rats, the first reports of successful oocyte cryopreservation were also described in the 1970s [124, 125]. The studies performed for rat oocyte cryopreservation have focused on specific aspects of the process and its impacts on the cells. In this species, for example, a calcium-free medium for vitrification containing ethylene glycol inhibits cortical granule exocytosis [126]. Variable lipid content is present in oocytes from different species, and rats have been applied to analyze whether reactive oxygen species affect oocyte maturation [127], confirming that its moderate increase leads to oocyte meiotic resumption. The importance of lipid peroxidation and reactive oxygen species formation relies on the fact that cryoinjury usually damages cellular membranes, which are mainly composed of lipids.

Successful description of rabbit oocytes was described, and this model is commonly applied in experiments because the size and morphology of their oocytes are close to those of

humans [128]. A comparative study showed that propanediol provides a better viability rate than DMSO when a controlled cooling rate is applied [129]. Currently, the protocols described for rabbit cryopreservation apply slow freezing with propanediol [130] or vitrification with ethylene glycol [131].

Early in 1977, hamster and Mongolian gerbil oocytes were cooled to -75°C by Parkening and Chang [125], aiming to determine the best cooling rate for successfully reaching in vitro fertilization. The authors described a lower ability of gerbil's oocytes to survive all the cooling rates tested in comparison to the other rodent evaluated, as demonstrated by the number of blastocysts grown. In 1986, an experiment compared the impact of vitrification and slow-freezing methods for hamster oocyte cryopreservation, concluding that losses in viability within the freezing method are related to the exposure to cryoprotectant, while in the vitrification method, damages are more related to the cooling and warming procedures [132]. Costa-Borges et al. [133] described a protocol specific to hamster's oocyte cryopreservation, which consists of slow freezing the cells in a medium containing propylene glycol and sucrose [133].

4.3.2.3 Cryopreservation of Ovarian Tissue

Contrarily to male germ cells, which are isolated in seminal fluid, the ovarian follicles are contained in a specific structure, where the oocyte is surrounded by one or two types of cells: granulosa and theca. These are classified as preantral and antral, depending on their stage of development within the meiotic cycle. These small structures comprise the greater source of female genetic material [134], and oocytes within preantral follicles are more resistant to possible damage caused by cryopreservation procedures. This is due to the absence of zona pellucida or peripheral granules in the cytoplasm, which allows cryoprotectants to easily penetrate the cell. In addition, preantral follicles are constantly available in ovaries, contributing to efficient preservation of genetic resources [135].

The ovarian tissue cell composition is a challenge in terms of cryoprotectant perfusion and cooling rates. Not only cells but also the extracellular components must be preserved to provide an adequate reestablishment of tissue function when it is warmed [136]. Mouse ovarian tissue cryopreservation has been widely performed either by slow freezing [137, 138] or by vitrification [139, 140]. Some studies comparing both methods in mice did not find differences in their efficacy regarding follicle morphology or functionality [141, 142].

In rats, ovarian tissue has been more commonly cryopreserved by vitrification [143, 144], since in this species, experiments comparing both methods revealed that vitrification was superior to slow freezing [145].

Rabbits have also been applied as animal models for testing the efficacy of ovarian tissue cryopreservation methods. By slow freezing, the use of propanediol and trehalose allowed the maintenance of 85% morphologically normal follicles [146]. Slow freezing with DMSO led to the post-thawing reestablishment of ovarian function after autotransplantation, confirming the method's applicability [147].

Many other rodent species were applied as experimental models for ovarian tissue cryopreservation. In one experiment, in addition to rabbit and mouse, hamster and monkey ovarian tissue was also cryopreserved and transplanted to rat hosts [148]. The authors describe vitrification with DMSO as an adequate method for cryopreserving ovarian tissue from all the tested species. In the guinea pig, slow freezing with DMSO led to less cellular damage than vitrification with ethylene glycol and DMSO in the ovarian tissue [149]. In red-rumped agouti, vitrification performed with ethylene glycol and sucrose as cryoprotectants enabled good preservation of DNA integrity and follicle morphology [150].

4.3.2.4 Cryopreservation of Isolated Preantral Follicles

For cryopreserving isolated follicles, their excision from ovarian tissue is a mandatory procedure and must be tailored according to the species since differences have been described in the collagenous fibers of the ovary. In this aspect, most studies applying rodent models for developing cryopreservation methods to follicular structures perform ovarian tissue storage rather than isolating them before storage. However, according to an experiment performed on human ovarian tissue, there are no differences in the survival or in vitro growth rates of follicles cryopreserved before or after isolation [151]. On the other hand, experiments in mice revealed that vitrified isolated preantral follicles are more suitable for developing to mature oocytes than those isolated after tissue cryopreservation [152]. However, it is important to bear in mind that the cryopreservation of isolated follicles is challenging since these large structures are highly susceptible to plasma membrane disintegration, DNA damage, and cell death [153].

The first tests for preantral follicle isolation were performed in mice [154], applying collagenase and DNase associated with mechanical processing. In this experiment, follicles frozen in medium containing DMSO could successfully develop and have their oocytes in vitro fertilized [154]. The obtained embryos were transferred, yielding live births [154]. More recently, in addition to mice [155, 156], rats [157] were experimental models for developing better methods of isolated follicle cryopreservation. In mice, usually, vitrification is performed [155], while in rats, the results demonstrated that vitrification applying ethylene glycol and sucrose as cryoprotectants is better than slow freezing for cryopreserving follicles [157].

4.3.3 Nonhuman Primates

4.3.3.1 Cryopreservation of Embryos

The COVID-19 pandemic increased the necessity of applying primates as experimental models. Their physiological proximity to humans, especially within reproductive functions, made these models an ideal alternative to evaluate the impacts of this viral infection on reproductive cells [158, 159]. In such studies, the results indicate that performing fertility preservation protocols in COVID-19 patients is a safe procedure that does not implicate the possibility of offspring contamination.

Baboon (*Papio anubis*) has been recognized as a reproductive research model, especially for intrauterine studies, leading to significant advances in the biology of embryo implantation [160–162]. Their embryos have been cryopreserved since 1984 [163, 164]. For that, the authors modified a bovine cryopreservation method that resulted in two term pregnancies from the transfer of six embryos of eight-cell or blastocyst stage. Nowadays, studies performing cryopreservation of baboon embryos are frequently conducted in an African research center [165].

In the rhesus monkey model (*Macaca mulatta*), several assisted reproduction techniques have also been developed [166]. For embryo cryopreservation in this species, the first successful attempt was described in the 1980s [167], when early cleavage-stage embryos were frozen by a propanediol-based protocol, leading to pregnancy after transfer. The same group has successfully performed nuclear transfer in the rhesus model, applying cryopreserved embryos [168, 169]. Still, other technologies have been applied to this species for testing blastocyst storage: the cryoloop procedure with a glycerol + ethylene glycol vitrification solution showed to be effective [170]. More recently, blastocyst cryopreservation protocol has been described by applying commercial cryopreservation kits [171].

Cynomolgus monkey (*Macaca fascicularis*) embryo cryopreservation dates from 1986, when Balmaceda et al. froze four- to eight-cell embryos applying DMSO [172]. The viability rate was 70%, with 11 of the 39 frozen embryos cleaved at least once; lastly, three pregnancies from nine embryo transfers were described [172]. Recently, cynomolgus embryo freezing has been performed by applying bench medium and sucrose [173]. Other protocols were performed for marmoset monkey (*Callithrix jacchus*) embryos using slow freezing and a cryopreservation solution containing DMSO and glycerol [174], or vitrification [175] applying a commercial vitrification kit.

4.3.3.2 Cryopreservation of Oocytes

The number of experiments describing oocyte cryopreservation in primates is reduced, probably due to difficulties in manipulating these animals for superovulation protocols. In

these cases, primate oocytes have been mostly manipulated freshly, usually for performing in vitro fertilization with the aim of testing sperm cryopreservation methods, as described in the baboon [176], rhesus [177], and cynomolgus monkey [178], or for developing new superovulation protocols [179].

According to Younis et al. [180], the F-actin organization in the cortical oocyte region may be impacted by glycerol when freezing the rhesus monkey oocyte; in the cynomolgus monkey oocyte, these authors also describe irregular shrinkage in the germinal vesicle and MI, with the presence of hyperosmotic stress [180]. These are further indications of the difficulties in primate oocyte cryopreservation. Other experiments in rhesus monkeys compared DMSO, ethylene glycol, and propylene glycol regarding oocyte osmotic and permeability aspects [181], revealing that mature oocytes are most permeable to propylene glycol than to DMSO. Another experiment in this species revealed that germinal vesicle-stage oocytes are less susceptible to chilling injury or exposure to ethylene glycol, but the oocytes are damaged by freezing [182]. Later, studies from the same group confirmed that media containing propylene glycol and sucrose or ethylene glycol and DMSO induce disruption of microtubules from oocytes within cumulus cells, recognizing that such structures may be irreversibly damaged when subjected to cryostorage [183].

In the 1980s, the *Saimiri sciureus* model, also known as squirrel monkey, was applied for an experiment for cryopreservation of oocytes [184], where authors performed slow freezing with DMSO, describing a 50% fertilization rate.

4.3.3.3 Cryopreservation of Ovarian Tissue

Baboon ovarian tissue has been stored by vitrification by applying a solution containing ethylene glycol and DMSO [185]. After warming, the tissue was autografted and evaluated for 5 months, showing evident ovarian activity by the presence of corpora lutea and AMH and GDF-9 expression in follicles. Later, the same group confirmed that the transplanted ovarian tissue survives at least 18 months [186]. Another study showed that a similar protocol allows the maintenance of 67% of morphologically normal follicles [187]. Also applying a vitrification protocol, Lu et al. [188] suggested that the ideal tissue size for baboon ovary cryopreservation is 1.0x1.5x1.0 mm cortical pieces [188].

In the rhesus model, a comparison between slow freezing and vitrification revealed that the vitrification method is superior in terms of follicle and stroma morphology maintenance, allowing in vitro development up to the antral stage and production of steroid hormones [189]. Cryopreservation of ovarian tissue by applying synthetic polymers in a vitrification solution improved the in vitro viability and function of follicles [189]. On the other hand, Jin et al. [190] confirmed that follicle viability, as demonstrated by in vitro growth, is maintained after slow freezing of rhesus monkey

ovarian tissue as well, despite a decrease in follicle morphology [190].

The neonatal ovary of marmoset was vitrified by the Cryotop polypropylene strip method, applying a mixture of DMSO and ethylene glycol as cryoprotectants. No differences in morphologically normal follicles were present between fresh and vitrified tissue, confirming that such a method is ideal for preserving the rich genetic source available in the newborn ovary [191]. In the cynomolgus model, vitrified-warmed ovarian tissue led to the recovery of oocytes and resumption of hormonal cycles after autotransplantation [192]. In this species, ovarian tissue did not show different responses to open and closed vitrification methods [193]. A comparative study confirmed that vitrification is better than slow freezing to obtain functional autografted post-warming tissue in cynomolgus monkeys when associated with a technique that improves vascular bed during transplantation [194].

A prospective study applied cryopreserved ovarian tissue of rhesus and cynomolgus monkeys to evaluate if a co-culture system enhances follicle viability [195]. Applying a vitrification or slow-freezing approach, cryopreservation was performed using glycerol and ethylene glycol; then, after warming/thawing, tissue was co-cultured with a mouse fetal fibroblast monolayer. Results showed that co-culture increased the number of viable follicles recovered for both species and cryopreservation methods, suggesting a compensatory effect for a slightly lower viability rate found in the cryopreserved compared to the fresh tissue [195].

4.3.3.4 Cryopreservation of Isolated Preantral Follicles

The availability of experiments describing successful cryopreservation of primate isolated ovarian follicles is much lower than that described for ovarian tissue. The first description of this procedure was probably published in 2010 when isolated follicles from rhesus monkeys were cryopreserved [196]. In this work, the authors performed slow freezing of secondary follicles, applying a medium with ethylene glycol and sucrose. In vitro culture was performed post-thawing by 6-day incubation of follicles in an alginate matrix. The morphological and immunohistochemical evaluation showed that such follicles could survive cryopreservation, with early follicle growth and healthy somatic cell-oocyte connection [196].

4.4 Ethical Aspects

The increasing incidence of infertility is probably the main reason for the augmentation by 5–10% per year of the search for assisted reproductive technologies. Therefore, there is a pressing need to develop or optimize techniques. On the

other hand, there is a lack of rigorous preclinical studies on the safety and efficacy of these techniques as well as the follow-up information on patients [197]. Therefore, these novel/optimized approaches should be submitted to meticulous preclinical studies to prove their safety and efficacy [198–200]. Among the different options for preclinical studies, animal models are crucial in evaluating potential deleterious consequences of these new techniques for the patients and/or their offspring [197]. As we saw in this chapter, different animal models, ranging from rodents to nonhuman primates, have been applied to develop or optimize cryopreservation procedures for embryos, oocytes, ovarian tissue, and isolated follicles. While animal models offer information that the current *in vitro* approaches cannot provide yet, there are several ethical concerns regarding the research using animal models. Indeed, there is an increasing number of countries that have passed legislation limiting animal research and protecting animal rights, and the European Union plans to replace the use of animals in research entirely. However, research in medically assisted reproduction contemplates another delicate ethical issue: human embryos and gametes. As mentioned by Jans et al. [197], the “use of animals first” rule becomes a subsidiarity requirement that applies to human embryo research. As animal research is an indispensable step, it is necessary to balance its application with protecting its welfare. To this end, in 2010, the European Union passed a directive based on the principle of the “three Rs” (replacement, reduction, and refinement), which is a common ground for research workers who use animals and those who oppose their use [201]. If animal research is necessary, as few animals as possible should be used, and they should experience minimal pain or distress. The adoption of the 3Rs principle not only takes into consideration animal welfare but also has been shown to improve the quality of science [201]. Indeed, properly designed experiments that decrease variation, provide standardized optimal conditions of animal care, and minimize stress or pain often yield better, more reliable data [201].

While nonhuman primates are the best animal model for research in medically assisted reproduction, studies have demonstrated that these animals have complex social, emotional, and cognitive lives, and they suffer novice consequences after being used in invasive research [202]. For this reason, a growing number of researchers and bioethicists have been advocating against their use in research. Despite the utilitarian arguments such as the “least harm and the greatest good,” we have moral duties to nonhuman primates. Indeed, Carvalho et al. [203] defend that nonhuman primates should be used only if the research is carried out under the same deontological criteria that guide research with human beings.

Nonhuman primates can potentially be replaced by ruminants as these animals are a great model for humans regard-

ing the development or optimization of cryopreservation procedures for embryos, oocytes, ovarian tissue, and isolated follicles. One of the main advantages of using cows, sheep, and goats is that we can obtain ovaries for research in the slaughterhouse, which could significantly decrease the number of animals. Second, the studies would also benefit these species (e.g., safeguard of autochthonous breeds) or species from the same family (e.g., preservation of biodiversity).

Regardless of the chosen animal model, it is essential to implement the 3Rs principle, and, as outlined by Beauchamp and DeGrazia [204], we should also bear in mind the six moral principles that should govern animal research, where three are related to social benefit, and the other three are related to animal welfare:

1. The principle of no alternative method.
2. The principle of expected net benefit.
3. The principle of sufficient value to justify harm.
4. The principle of no unnecessary harm.
5. The principle of basic needs.
6. The principle of an upper limit to harm.

While caring for animals can present numerous challenges for scientists, a strong culture of care that supports the overall well-being of all the animals and professionals involved in biomedical discovery may drive research in extraordinary ways.

4.5 Conclusion

The great advances in reproductive medicine would not have been possible without the studies carried out in animal models. The studies on nonhuman primates, ruminants, and rodents have proved to be essential for our understanding of how cryopreservation can affect embryos, oocytes, and ovarian follicles and the development of assisted reproductive technologies. Since animal models are still necessary to evaluate the safety and efficacy of these procedures, it is important that the implemented research requirements should be met and research principles for animal research should be followed [205].

Authors' perspectives

Key Points

- The development and optimization of embryos, oocytes, and ovarian tissue cryopreservation for women would not have been possible without studies applying animal models.
- Ruminants, such as cows, ewes, and goats, are frequently applied in such studies due to the availability of their

reproductive tissue, which most of the time is discarded in abattoirs.

- Mice and rats make up the most currently applied rodent models. However, in addition to them, rabbits are usually used for human female germ cell cryopreservation methods due to their oocyte morphological similarity.
- Among all the experimental models, nonhuman primates provide the closest morphophysiological aspects to humans'. However, their maintenance in experimental environments demands more complex and expensive facilities.

Potential Areas of Research

While there are always ways to optimize or establish new procedures to increase fertility outcomes, a potential area of research could be developing in vitro and in silico strategies, such as organs-on-a-chip and computational approaches, to replace animals. Although this may not completely solve the problem of using animals in research on medically assisted reproduction, it may offer other invaluable information that animal models cannot.

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Considerations in Immature Oocyte Cryopreservation

5

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5.1 Introduction

Initial attempts to freeze oocytes employed the same slow-freezing methods that were considered the gold standard for embryo cryopreservation. However, the slow-freezing oocytes were met with very low survival and pregnancy rates [1–3], and this intervention was thus considered experimental [4]. With time, the efficacy of the slow freezing of oocytes has been improved by increasing the sucrose concentration in the freezing solution [5–8]. With the recent advancement of the vitrification technique, there has been a significant improvement in the efficacy of oocyte cryopreservation [9–18].

The outcomes are improving after the first birth resulting in oocyte vitrification being reported [19]. The guideline issued by the Practice Committees of the American Society for Reproductive Medicine (ASRM) and the Society for Assisted Reproductive Technology (SART) indicated that mature oocyte vitrification and warming should no longer be considered only an experimental procedure [20]: this technology is recommended in cases of gonadotoxic therapies when there is a lack of alternative options for fertility preservation. Several groups worldwide have reported high survival and pregnancy rates as well as high live birth rates using vitrified oocytes [21–28]. However, it appears that the majority of the live births were from egg donor programs [29] rather than from a clinic-specified population seeking to use this technology for fertility cryopreservation.

To date, for infertility treatment, the mature oocytes are derived from ovarian stimulation followed by in vitro fertilization (IVF) [30]. However, ovarian stimulation with gonadotropins may not be suitable for many women who are seeking fertility preservation, especially patients with breast cancer and other hormone-dependent cancers, and those who require immediate chemotherapy. There are different ways to obtain immature oocytes for clinical treatment and fertility preservation. Immature oocyte retrieval followed by in vitro maturation (IVM) from unstimulated or stimulated ovaries and then cryopreservation of those oocytes is indeed an attractive strategy for these women. Although it is possible to perform immature oocyte retrieval followed by cryopreservation of those immature oocytes immediately, the immature oocyte retrieval followed by IVM has proven more effective and resulted in pregnancies and many healthy live births [17, 18, 31].

With the development of cryopreservation technologies, it has been found that there is no difference in the survival rates between the oocytes cryopreserved at the immature GV stage and those cryopreserved at the mature MII stage [32]. However, poor maturation rate in vitro after immature oocyte cryopreservation is still the major problem associated with immature oocyte freezing [33, 34]. Although it seems that IVM rate can be improved after cryopreservation of the denuded immature oocytes [35], the efficiency needs to be confirmed.

5.2 Candidates for Immature Oocyte Cryopreservation

The effective oocyte cryopreservation technology provides a significant impact on the clinical practice of assisted reproduction. Oocyte cryopreservation benefits infertile couples with moral or religious objections to the cryopreservation of embryos. In fact, the cryopreservation of oocytes has become one of the leading options of female fertility cryopreservation in countries where the law forbids the cryopreservation

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of embryos. An efficient oocyte-freezing technology simplifies egg donation program and enables the establishment of egg banks by eliminating the logistics of coordinating egg donors with their recipients. The establishment of egg banks will then likely facilitate and improve the current shortage of egg donors. In addition to providing a fertility preservation option for young women requiring potentially sterilized (medical and surgical) treatments, cryobanking of oocytes will benefit a large population of women who wish to delay motherhood because of personal and professional reasons.

Considering cryopreservation of immature oocytes, they must have their own reasons. Oocyte cryopreservation provides an important alternative for women with cancers who require fertility preservation, and immature oocyte retrieval followed by IVM is an optimal option for those groups of patients [36]. Sometimes, mature oocytes and immature oocytes can be collected from the ovaries, if the oocyte retrieval is performed in the follicular phase after HCG priming, even though there has been no ovarian stimulation with FSH [37]. However, standard controlled ovarian hyperstimulation (COH) may not be feasible for some cancer patients, because there are serious concerns about the side effect of ovarian stimulation with hormones on the risk of cancer recurrence. Also, urgent gonadotoxic cancer treatment may not allow sufficient time for the patient to undergo COH. Particularly in women with breast cancer, fertility preservation has included retrieval of immature oocyte followed by IVM for fertility preservation with either oocytes or embryos [38]. This method provides a useful option for fertility preservation in breast cancer patients without ovarian stimulation and with no delay in cancer treatment [39], indicating that retrieval of immature oocytes followed by cryopreservation of oocytes and embryos is a feasible method for these groups of women with fertility preservation.

Another advantage of immature egg retrieval followed by IVM for fertility preservation is the possibility of retrieving oocytes regardless of the phase of the menstrual cycle [38]. It is possible to perform immature oocyte retrieval sequentially in either the follicular or the luteal phases of the menstrual cycle in breast cancer patients. Thus, in cancer patients under severe time constraints, immature oocyte retrieval in the luteal phase followed by IVM and cryopreservation can be considered before chemotherapy to maximize the possibility of fertility preservation, as suggested [40, 41].

Finally, immature oocyte retrieval without ovarian stimulation allows the avoidance of expensive medications and ovarian hyperstimulation syndrome (OHSS). The incidence of severe OHSS is 0.6–1.9% but may be as high as 6% in high-risk groups, such as young women with polycystic ovaries [42]. Although a number of strategies have been proposed to predict and prevent this potentially life-threatening complication, the only preventive strategy that is certain to

eliminate OHSS is the avoidance of ovarian stimulation altogether. Therefore, the option of immature oocyte retrieval followed by IVM and cryopreservation may be applied to young women who want to do fertility preservation with delaying motherhood without taking exogenous hormones for ovarian stimulation.

5.3 Immature Oocyte Retrieval

It must be mentioned here in advance that, firstly, it is common belief that the oocyte will be of full size in terms of diameter once the follicular antrum is formed in the ovary, and secondly, oocytes aspirated from the follicles without LH surge regardless of the size of follicles will be at immature stage. At the same time, the small follicles have no or less LH receptors; therefore, the aspirated oocytes may be at immature stage even after LH surge [43].

In a natural menstrual cycle, apart from the leading or dominant follicle developed, it is usual to have a subdominant or leading follicle developed together. Therefore, it is possible to retrieve mature oocytes from the dominant follicle and immature oocytes from the small size of follicles even after LH surge. In addition to the natural cycle treatment, the golden standard ovarian stimulation protocols may also result in retrieved oocytes being at immature stage. Thus, those immature oocytes can be used for infertility treatment or fertility preservation subsequently [44].

Oocytes retrieved from women with PCOS may be all at immature stage, because women with typical PCOS hardly produce the dominant follicle in the ovary. Therefore, all retrieved COCs may be at GV stage or metaphase I stage rather than mature metaphase II stage. For women with PCOS, there are different protocols before immature oocyte retrieval. One is given a few days of FSH to stimulate follicular development, and another is given both FSH and LH (HCG) before immature oocyte retrieval, or it is only given HCG before immature oocyte retrieval [44].

Transvaginal ultrasound-guided follicular aspiration has now become the preferred procedure for immature oocyte retrieval in IVM treatment cycles. The same principles applied to IVF oocyte retrieval are also valid for IVM procedure. However, a smaller gauge needle (19 G or 20 G) is preferable. Although IVM ovum pickup (OPU) requires more punctures per ovary, it is well tolerated and does not seem to be associated with a higher complication compared to conventional IVF OPU [45]. It is important to note that no report indicates increased bleeding or complications during immature oocyte retrieval compared to regular mature oocyte aspiration. This may be explained by the use of thinner needles and lower aspiration pressure for immature oocyte retrieval. Anesthesia is recommended for immature oocyte collection due to the length of retrieval time. Types of anes-

thesia used for transvaginal follicle aspiration for IVM include conscious sedation and local injection.

Because the intrafollicular pressure is already higher in small follicles, the aspiration vacuum pressure is reduced to 85 mmHg, which is approximately half of the conventional IVF aspiration pressure. A higher aspiration pressure provokes an increase in the number of denuded oocytes. Good ultrasonographic visualization is a vital element of successful immature oocyte retrieval. Follicular sizes vary, and certain follicles may be difficult to aspirate or, even if they are aspirated, no oocytes may be recovered, especially from the very-small-sized follicles (<4 mm in diameter).

Aspirates are collected in 10 mL culture tubes containing approximately 2 mL of heparinized warming medium (usually containing 2 units/mL of heparin). It is possible to use 0.9% saline containing 2 units/mL of heparin. It has been described that there are two ways to search and collect immature oocytes from follicular aspirates in the literature: (1) dish search: the follicular aspirates are poured directly into a petri dish and examined for immature oocytes under a stereomicroscope, and (2) cell strainer: the follicular aspirates are filled through a cell strainer (70 μ m nylon). After filtering, the collected aspirates can be rinsed with a prewarmed flushing medium and transferred to a petri dish to search for immature oocytes under a stereomicroscope. All handling procedures should be conducted on warming stages or plates at 37 °C.

Typical immature COC with compacting cumulus cells surrounds oocyte and is quite easy to identify with mature COC, because mature COC is with expanded cumulus cells surrounding the oocyte. To determine whether or not the oocyte is mature, a special observation technique called “sliding” can be employed. Briefly, the COC is allowed to slide slowly from one side to the other on the bottom of the dish while being observed under the microscope. During COC sliding, it is possible to observe clearly if the oocyte cytoplasm contains a GV and the oocyte has extruded the first polar body (1 PB) into perivitelline space (PVS). If neither GV is seen in the oocyte cytoplasm nor 1 PB is found in PVS, the oocyte is defined as germinal vesicle breakdown (GVBD) or metaphase I (MI) stage [46].

There is another source of immature oocytes from natural or ovarian stimulated cycles for intracytoplasmic sperm injection (ICSI) treatment cycles. Although most retrieved COCs are mature oocytes from stimulated cycles, it is often found that some of the collected COCs are immature oocytes after denuding from their cumulus cells before ICSI. This is a source of immature oocytes that may be used for cryopreservation with fertility preservation because it has been reported that the denuded oocytes can also be matured in vitro and produce live births [35].

Immature COCs may also be collected from the surgical materials when the patients are performing endoscopic gynecological

procedures or surgery [47, 48] or ovarian tissue banking for fertility preservation [49–51]. The procedure of aspiration of follicles from the surgical materials is similar to the procedure of transvaginal ultrasound-guided follicular aspiration. The difference is that the follicles are aspirated directly from the surgical materials using a syringe connected with 19–20 G needle or the follicles are punctured into culture dish.

5.4 In Vitro Maturation of Immature Oocytes

The oocyte is a unique cell in a woman’s body, owing to its special structure, function, and undergoing meiosis. Meiotic progression in the oocyte is defined as the oocyte maturation from reinitiation of the first meiotic division to the MII stage accompanied by cytoplasmic maturation to successfully prepare the oocyte for fertilization and early embryonic development [52]. In vivo meiotic resumption in the oocyte is initiated in response to the preovulatory surge of LH. The LH surge triggers oocyte maturation from the GV stage to MII. For infertility treatment with the use of IVF technology, the patients are given hCG to induce the completion of oocyte meiosis in the follicles to retrieve mature MII oocytes 36 h after hCG injection. Without hCG injection in IVF treatments, most of the retrieved oocytes would be at an immature GV stage. Human immature GV-stage oocytes can be matured spontaneously to MII in vitro when they are removed from the antral follicles and cultured in the proper culture media [31]. Time course of human oocyte maturation in culture is required for 36–48 h from GV to MII stage [52, 53]. It means that IVM technology involved in vitro culture human oocytes for 48 h in order to complete meiosis.

The techniques used for IVM of human immature oocytes differ in protocols, and the clinical definition of IVM treatment also differs from the biologic definition of oocyte IVM. Therefore, the definition of clinical IVM should be defined according to the origin of immature oocytes to clarify for the follow-up of outcomes derived from different sources of immature oocytes. We think that clinical IVM should be defined as IVM of any immature oocytes regardless of the stage from GV and MI to MII for clinical application, because it involves the procedure of IVM for immature oocytes [43, 44].

The clinical application with IVM of immature oocytes has come a long way since the first live birth reported in the early 1990s [54–57]. IVM is an effective treatment that has already achieved significant outcomes of acceptable pregnancy and implantation rates and has led to the births of several thousand healthy babies [58, 59]. The evidence from molecular analysis also indicated that no differences were

found between in vivo- and in vitro-matured human oocytes [60–62].

There is no unique IVM technology applied in the world. Human oocytes acquire a series of competencies during follicular development (oocyte growth and maturation) that play critical roles in fertilization and subsequent early embryonic development. Even if high rates of IVM of immature oocytes are obtained, the developmental competence of the in vitro-matured oocyte may be suboptimal as indicated by the relatively minimal development up to blastocyst stage and the poor viability to term after transfer. Therefore, oocyte maturation in vitro is profoundly affected by culture conditions [63].

Although numerous data have been accumulated from studies, the current rationale for choosing a specific medium for IVM of human immature oocytes appears to stem largely from adapting methods developed for culturing other cell types. All existing media for oocyte maturation in vitro have a base of complex culture media supplemented with different substances. There are commercially available IVM media, indicating that there was no difference in maturation rate or fertilization rate of oocytes matured in vitro in both media [64]. There is also no difference in the formation of good-quality blastocysts in the two groups. Therefore, in terms of human infertility treatment with IVM technology, it seems that no breakthrough has yet been made by improving the IVM medium itself.

It has been proposed that the nuclear maturation and cytoplasmic maturation were not synchronized during IVM of immature oocytes [65, 66], and ways to synchronize the nuclear and cytoplasmic maturations have been explored [67, 68]. In animal models of IVM of immature oocytes, some of these approaches resulted in high blastocyst formation and fetal production [69]. Although it has been reported that biphasic in vitro maturation was applied with human immature oocytes, its safety issue is still questioned even though it indicated that the method may improve the developmental capacity of oocytes from small antral follicles [70]. While the use of cell cycle-modulating agents or spindle formation inhibitors during oocyte IVM may be acceptable within the animal industry, the safety issues associated with the use of these agents for the clinical application of in vitro human oocyte maturation should be carefully investigated. The physical requirements of IVM system and culture platforms or devices both play a role in influencing immature maturation and subsequent embryonic development. Recently, several new systems have been introduced for culturing animal gametes and embryos as well as other cells [71–73], but the systems have not yet been used in the field of clinical practice for human IVM of immature oocytes.

Application of microfluidic technologies has been proposed in human ART, indicating that such technologies may be influential in the field of human gametes and preimplanta-

tion embryo biology [74]. Interestingly, a microfluidic system supports murine ovarian follicles to mimic the human 28-day menstrual cycle hormone profile, which controls human female reproductive tract and peripheral tissue dynamics in single-, dual-, and multiunit microfluidic platforms [75]. These systems simulate the in vivo female reproductive tract and the endocrine loops between organ modules for the ovary, fallopian tube, uterus, cervix, and liver, with a sustained circulating flow between all tissues. However, the practical application and the mechanical and biochemical characteristics of microfluidics still need to be studied properly before clinical implementation, especially for IVM of human immature oocytes.

In addition, an encapsulated three-dimensional (3-D) culture system was used for culturing mouse follicular growth [76]. The encapsulated 3-D culture system utilizes biomaterials to maintain cell-to-cell communication and supports follicle development to produce a mature oocyte that has been reported in nonhuman primate preantral follicles [77]. Although many reports indicated that the encapsulated 3-D culture system was efficient for follicle growth in vitro [78–81], it seems that there is still no report for human oocytes matured in vitro with encapsulated 3-D culture system.

Interestingly, it has been reported that liquid marbles (LMs) consist of a drop of liquid encapsulated by hydrophobic powder particles, in which 3-D bioreactor could form to support the growth of different types of cells [82, 83]. However, a recent report showed that the similar percentages of oocyte maturation were observed in LM and regular culture medium droplets (control) and that there were no differences in embryonic development (blastocyst formation rates) after IVF following IVM of sheep immature oocytes, indicating that LM microbioreactor may be a viable technique that provides a suitable microenvironment to induce oocyte IVM [84]. Nevertheless, the efficiency and the clinical outcomes of IVM with human immature oocytes by the encapsulated 3-D culture system must be further confirmed before clinical application.

As mentioned above, the immature oocytes can be obtained from different sources. Therefore, the results may be different due to immature oocytes derived from the different sources. Ideally, the immature oocytes should be from cumulus-oocyte complex (COC), because oocyte maturation, especially the cytoplasmic maturation of oocyte, is required for the structure of COC [85]. Cumulus cells are the somatic cells surrounding the oocyte, and they play an important role in the growth of the oocyte, meiotic maturation, ovulation, and fertilization in mammals [86].

Cumulus cells that are in contact with the oocyte form the COC. This complex allows communication between the oocyte and cumulus cells by directly affecting gene expression and protein synthesis, leading to differentiation and

expansion of cumulus cells as well as oocyte maturation [87, 88]. Cumulus cells connect to the cytoplasm of the oocyte and penetrate the zona pellucida (ZP) with gap junctions [89]. Following the development from the antral follicle to the preovulatory follicles, cumulus cells that undergo proliferation and the gap junctions are gradually released from the ooplasm with meiotic maturation of the oocyte. Normally, the function of cumulus cells is to support the growth of oocyte, produce hyaluronic acid (HA), and undergo cumulus cell expansion in response to FSH [90]. Following LH surge, the structure of the cumulus oophorus undergoes expansion by accumulating an extensive HA in the extracellular matrix (ECM) that helps ovulation and the subsequent fertilization in the ampulla of the oviducts [91].

For oocyte cryopreservation regardless of slow freezing or vitrification, the structure of COC will be destroyed in the high osmotic solution during the procedure of cryopreservation. Therefore, it should be considered carefully that the oocyte cryopreservation is performed before or after IVM of immature oocytes, because the damaged structure of COC must be affected negatively for the normal process of oocyte maturation, especially for cytoplasmic maturation of oocytes [92, 93]. Although it seems to supplement some components or factors into culture media and can improve the results of denuded immature oocyte IVM, its result needs to be confirmed subsequently.

5.5 Cooling and Warming Rates for Cryopreservation of Oocytes

The cryopreservation protocols for oocytes can be divided into (1) slow freezing/rapid thawing (cooling rates of 0.3–2 °C/min) and (2) rapid cooling/warming or vitrification (cooling rates at least >500 °C/min) [43, 46, 94]. Freezing induces precipitation of water into ice, leading to the separation of water from the dissolved substances. The presence of both intracellular ice crystals and a high concentration of solute can be lethal to the oocytes or embryos during the freezing procedure. Therefore, a slow cooling rate has been proposed in order to maintain the delicate balance between ice crystallization, osmotic, and chilling damages [95].

5.5.1 Slow Freezing/Rapid Thawing

The standard slow-freezing protocol is as follows: the cooling rate is of 1 °C/min from –5 to –9 °C, and ice crystallization is induced by the process known as “seeding,” which results in a heterogeneous ice nucleation that is more stable than a supercooling or homogenous nucleation. After seeding, the cooling rate is reduced to 0.3–0.5 °C/min until a lower temperature is reached (usually between –30

and –150 °C), and then the cells are stored in liquid nitrogen (LN₂). Thawing is a rapid procedure with temperature changes that can exceed >360 °C/min [95]. This procedure prevents the occurrence of recrystallization, a process where water enters the oocytes or embryos and transforms into a solid state around previously formed small ice crystals.

As mentioned above, the immature oocytes are arrested at the diplotene state of prophase I. The rationale of freezing immature GV-stage oocytes is to circumvent the risk of polyploidy and aneuploidies because the chromatin is diffuse and surrounded by GV nuclear membrane [94]. Although the immature oocyte survival rate seems improved when they were frozen at GV stage, the maturation and fertilization rates are poor, and embryonic development is stunted in immature oocyte freezing by the slow-freezing method [33, 95, 96]. Therefore, cryopreservation of mature oocytes (MII stage) using the slow-freezing method seems more efficient than immature oocyte freezing. Although using slow freezing/rapid thawing for immature GV oocytes resulted in immature oocytes capable of in vitro maturation, fertilization, and supporting viable births [1], cryopreservation of immature GV oocytes is inefficient for IVM after thawing and subsequent capacity of embryonic developmental competence [3]. Also the slow freezing/rapid thawing of immature GV oocytes met with low survival and pregnancy rates [97]. The modified slow-freezing/rapid-thawing methods have been introduced for cryopreservation of mature oocytes; particularly, increasing sucrose concentration [5–8, 98, 99] and Na⁺-free ion [100, 101] freezing solutions seem to be significantly improving oocyte survival and pregnancy rates.

5.5.2 Rapid Cooling/Warming or Vitrification

It is the concept that living cells can be successfully frozen if they are cooled so rapidly that ice crystallization does not occur [102]. Strictly speaking, a glass-like state also occurs when slow-freezing procedures are applied at the glass transition temperature (–130 °C). Rapid cooling/warming or vitrification is efficient for cryopreservation of oocytes and embryos. The definition of vitrification is a glass-like solidification of a solution at a low temperature. In other words, vitrification is the ice-free solidification of an aqueous solution. The mechanism of vitrification proposed is the use of a high concentration of cryoprotectant and an extremely rapid cooling/warming rate to pass through the glass transition temperature and avoid intra- and extracellular ice formation [103–107].1.

The majority in the field believe that the cooling rate is the most critical factor for oocyte vitrification. In order to accomplish this, a variety of devices have been used to obtain relatively high cooling rates. Initially, it has been considered using the open devices as the vitrified samples as they were

able to obtain $>20,000$ °C/min cooling rate through direct contact with LN₂ [108, 109].

Until recently, it was unknown as to what the optimal cooling and warming rates with a very small amount (<1.0 µl) of solution and samples should be [110]. Recent studies conducted with a mouse model indicated that the lethality of a slow-warming process is a consequence of the growth of small intracellular ice crystals by recrystallization [111, 112]. This means that the cooling rate is of less consequence than the warming rate during vitrification-warming procedures [113]. Interestingly, the authors of such studies have also demonstrated that a very high warming rate, as opposed to a high cooling rate, was the essential element to survival of the oocyte during vitrification-warming procedures [114]. The collected data also indicated that the oocytes can be vitrified successfully with approximately 500 °C/min cooling and 2000–2500 °C/min warming rates while having high survival and pregnancy rates [28, 115], suggesting that further understanding of the principle of vitrification is required in the field.

There is limited information on the oocyte survival, fertilization rates, and early embryonic development after vitrification at the immature GV stage. With the development of the vitrification method, there is no significant difference in the survival rate between oocytes vitrified at the immature GV stage and those vitrified at the mature MII stage [32]. From the few studies that have explored the question, the evidence suggested that oocytes should be vitrified at the mature MII stage following IVM rather than at the immature GV stage [116]. However, following insemination by ICSI, there were no significant differences in fertilization, cleavage, or blastocyst development rates between these two groups. To date, only a few live births resulting from the cryopreservation of oocytes at the immature GV stage [117, 118] or after IVM have been reported [17, 18]. These results suggest that vitrification of mature oocytes is more efficient than immature oocytes.

5.6 Cellular Damage During Oocyte Cryopreservation

With the special structure of immature oocytes with their GV compared to mature MII-stage oocytes, it has been considered that cryopreservation at the immature GV stage may reduce damage to the oocyte during the freezing procedure [33, 95, 96, 119].

5.6.1 Nucleus

The nucleus in the oocyte, also known as the GV stage, is composed of a nuclear envelope (NE), and nuclear material

such as chromatin, and the nucleolus. Once oocytes resume the first meiotic division, the nuclear envelope is disintegrated allowing mixing of the nuclear material into the cytoplasm [119]. Both slow freezing and vitrification could disrupt and/or distort the nuclear envelope. Theoretically, the use of immature GV-stage oocytes circumvents the risk of polyploidy and aneuploidy because the chromatins are diffuse in the diplotene state of prophase I and are surrounded by a nuclear membrane, which may avoid spindle depolymerization. It has been reported that reconstructed oocytes utilizing vitrified/warmed GV were able to mature up to MII [120]. However, their potential for further development was not optimistic.

5.6.2 Cytoplasm

The potential effects of cryodamage on the oocyte cytoplasmic function will be inherently related to the stage of the cell cycle, since the ultrastructural configuration in the cytoplasm is continually changing dependent upon the stage of meiosis. This is especially true with respect to cytoskeleton distribution and/or function, which is essential for normal segregation of mitochondria, chromosomes, spindle rotation, cytokinesis, and pronuclei/nuclei formation [121, 122].

5.6.3 Microfilaments

It has been reported that at 37 °C, 1,2-propanediol causes “blebbing” on oocytes, which were found to be lacking microfilaments. Cooling of oocytes has not been demonstrated to alter grossly microfilament polymerization [123]. However, there has been some suggestion that cooling effects on microtubule depolymerization in the spindle may also alter adjacent microfilaments. Moreover, DMSO and 1,2-propanediol have both been reported to disrupt polymerized cortical actin [124].

5.6.4 Mitochondria

Mitochondrial swelling has been observed after oocyte cryopreservation by both slow freezing [125] and vitrification [126]. It has been demonstrated that sublethal mitochondrial injury in mouse oocytes is heritable by resultant embryos and results in postimplantation pathologies similar to those reported for clinically subfertile women, including recurrent implantation failure or miscarriage and decreased live birth weight [127]. Supplementation of L-proline into vitrification solution could protect mitochondrial functions and improve the survival rate of vitrified oocytes [128].

5.6.5 Zona Pellucida

It has been reported that exposure of oocytes to DMSO [129, 130] and 1,2-propanediol [131] during the cooling process causes premature cortical granule release and zona hardening, resulting in compromising sperm penetration and fertilization. The cryoprotectants mentioned above, as well as ethylene glycol, cause transient calcium increases [132, 133], which were sufficient to cause zona hardening, presumably through triggering cortical granule exocytosis, which is a calcium-dependent event, indicating that removal of calcium from the vitrification solution facilitated fertilization following insemination and development to the two-cell stage at a rate approaching that of fresh oocytes [134].

5.7 Multiomic Analysis of Oocyte Cryopreservation

During oogenesis, the transcription is influenced intensely by epigenetic factors. The follicular growth phase is succeeded by a phase of resumed meiotic activity and transcriptional silencing [135], in which changes in gene expression depend on the translation and degradation of transcripts. This is crucial for assembling the molecular machinery, particularly the transcripts involved in epigenetic mechanisms, required for meiotic progression, fertilization, and embryo development [136, 137]. The transcript content shows that temporal changes are mostly regulated via epigenetic mechanisms and associated with oocyte competencies [138]. Therefore, both the slow-freezing and vitrification methods seem to differentially modify the epigenetic and gene expression profiles of human oocytes.

5.7.1 Methylation Changes

The impact of vitrification on human GV oocytes has no differences in epigenetic changes [139, 140]. However, a decrease in overall methylation has been reported after bovine oocyte vitrification [141, 142]. Studies from mouse oocytes demonstrated that enzymes related to methylation processes were differentially affected by vitrification as it decreases the gene expression of DNA methyltransferase 1o (Dnmt1o) [143]. At the same time, histone acetyltransferase 1 (Hat1) and deacetylase 1 (Hdac1) remain with no change in their expression levels after vitrification. Interestingly, none of these enzymes display alterations in their methylation patterns, suggesting that other mechanisms may contribute to the downregulation of Dnmt1o expression [144]. Bovine blastocysts derived from vitrified oocytes showed decreased levels of genome methylation. Finally, analysis of the methylation levels of pluripotency gene promoters

showed that oocyte vitrification procedure did have an effect.

5.7.2 Histone Modifications Changes

In mouse model, it has been reported that vitrification procedure induces changes in oocyte histone H4 acetylation and histone H3 lysine 9 methylation (h3K9) [145]; meanwhile, this process does not change acetylation levels in lysine 14 (H3K14ac). In contrast, there is also an increase in acetylation of histone 3 at lysine 5 (H3K5ac). In porcine oocytes, vitrification causes hyperacetylation of histone 4 2 h after thawing and displayed an increase in the methylation of histone 3 [146]. On the other hand, slow freezing/rapid thawing increases the trimethylation of histone 3 at lysine 27 (H3K27me3) and decreases the trimethylation of histone H3 at lysine 4 (H3K4me3), which could impair embryo development [147]. These results reveal a complex alteration of epigenetic marks of the DNA and histones that could change embryonic development and show a correlation between cryopreservation procedure and epigenetic alterations. Therefore, it seems that vitrification of human oocytes does not impair embryo development and does not generate methylation and hydroxymethylation at genomic scale [148].

5.7.3 MicroRNA Changes

The comparison of miRNA transcriptome in fresh and vitrified oocytes was performed in mouse [149]. Twenty-two miRNAs were differentially expressed between the two groups, and most of the target genes regulated by these miRNAs were identified as “metabolic pathway” regulators. Among them, miR-1345p, miR-210-5p, and miR-21-3p were significantly upregulated, whereas miR-465c-5p was downregulated. The expression of potential target PTEN, regulating cell apoptosis through oxidative stress, was reduced.

5.7.4 Transcriptomic and Genomic Changes

Recent evidence showed that oocytes submitted to either vitrification or slow-freezing procedures display transcriptomic alterations; these changes are characterized by the downregulation of several genes related to embryo development, energetic pathways, DNA integrity, and cell cycle. Interestingly, these changes are more conspicuous in oocytes submitted to slow-freezing procedures [150]. Following vitrification, oocytes are subjected to severe damage, and consequently their developmental potential is substantially reduced. The rate of in vitro maturation of vitrified-warmed

GV oocytes was significantly decreased in several animal models, such as bovine, equine, porcine, sheep, and human. It has been reported in mammals that oocyte vitrification could reduce the potential for embryonic development and decrease the oocyte developmental competencies [151–153]. In cryopreserved oocytes, genes related to oxidative phosphorylation, lysosome pathway, regulation of lipolysis in adipocytes, or AMPK signaling pathway were upregulated [154]. It has been found that 69 genes were differentially expressed after IVM-vitrified and fresh oocytes in murine model [155].

5.7.5 Proteomic Changes

With proteomic changes, most of the studies are focused on animal models. No differences were found in the protein profiles of vitrified mouse oocytes when compared with fresh oocytes [156]. However, after cryopreservation of porcine oocytes, proteomic analyses found a total of 59 and 94 proteins that were downregulated and upregulated, respectively. The differentially expressed proteins were related to several biological processes, such as metabolic processes, coagulation, heterochromatin organization, or immune responses [157]. These results highlight the importance of performing similar experiments in human oocytes to a better understanding of the biological processes mediated by proteins that could compromise the oocyte integrity after cryopreservation.

5.8 Duration of Oocyte Storage

Bacteria and fungi contaminations of stored semen and embryos that were in direct contact with LN₂ have been reported in early days [158–161] and further confirmed recently [162]. Interestingly, there was an idea that the vitrification procedure could be performed with a sterilized LN₂ [163] and that could store the vitrified oocytes with vapor LN₂ [164, 165] to prevent contaminations that occurred by the direct contact of LN₂ during the cryo-storage. However, it seems that storage in vapor phase of LN₂ also has the risk of contamination [166, 167]. The main issue remains to be whether or not it is absolutely necessary for the vitrified samples to be in direct contact with LN₂ in order to achieve the essential cooling rate for vitrification. If direct contact of LN₂ is not essential during vitrification procedure, this can be solved by using the closed devices for vitrification and storage.

Theoretically, the oocytes stored in LN₂ should be relatively safe without any obvious undesirable biological and metabolic changes in the ooplasm. It is generally believed that the biological activities of cells would be stopped com-

pletely when they are stored at the temperature of LN₂ (−196 °C). So far, most studies examining a prolonged period of storage have been focused on the quality of embryos cryo-stored at −196 °C, indicating that the quality of embryos is not influenced by the duration of cryo-storage [168–170]. Other studies have also indicated that a long-term cryo-storage of human embryos did not affect the pregnancy outcome [171, 172]. However, an early study has indicated that the pregnancy rate was higher when the embryos were cryo-stored in LN₂ for 1–2 months compared to the embryos that were cryo-stored for more than 2 months [173]. The recent report also indicated that the prolonged storage time of vitrified embryos negatively affected pregnancy outcomes, including biochemical pregnancy rate, clinical pregnancy, and live birth rate [174].

Regarding cryo-storage systems, especially those related to the vitrification method, there are two storage systems, i.e., “closed” and “open” systems to store oocytes or embryos in LN₂ after vitrification. The “closed” system refers to the oocytes that do not come into direct contact with LN₂ during storage, and the “open” system refers to the oocytes that come into direct contact with LN₂ during storage. Many studies have shown that an oocyte vitrification with the “open” system for storage does not have a detrimental effect on the subsequent fertilization, embryo development, and clinical pregnancy rates [10, 15–18, 21–24, 27, 175]. However, there is limited information available about the effects on the duration of the oocyte cryo-storage following an “open” system on the subsequent cryo-survival, fertilization, and embryonic development.

Apart from the concerns about the risk of contamination and infection from bacteria and fungi during the storage in direct contact with LN₂, it has been reported that the cryo-survival, fertilization rate, and embryonic development of the vitrified oocytes are affected significantly, in an adverse manner, by the cryo-storage duration in LN₂ [174, 176]. It is unclear whether or not this is due to the direct contact of the oocytes to LN₂ during storage. It is commonly believed that there are no thermally driven reactions that occur in cells in aqueous systems at −196 °C.

Nevertheless, it has been reported that many important photo-physical events or chemical syntheses occurred to produce some form of hazards in LN₂ [177]. With the result of photo-physical events in LN₂, it has been discovered that direct ionizations from background irradiation or cosmic rays are a source of damage at such low temperatures. This damage occurs as a consequence of the formation of free radicals and the production of breaks in macromolecules. Due to the rapid decomposition of ozone (O₃), ionizing radiation on oxygen dissolved in LN₂ causes the formation of free radicals [178]. Oxides of nitrogen are formed in LN₂, which enhances the yield of ozone by a catalytic effect. Ozone production may deleteriously damage DNA and cause

DNA breaks since an enzymatic repair cannot occur in LN₂ [179].

Many factors could affect the outcome of vitrified human oocytes, such as the age of the women, duration of infertility, and different stimulation protocols. As such, it would be premature to draw a solid conclusion that the cryo-storage duration affects the subsequent cryo-survival, fertilization, and embryonic development. As discussed previously, there are many potential risks attributed with the direct contact of vitrified oocytes into LN₂. Further study is required to confirm whether the cryo-storage duration could affect subsequent oocyte survival, fertilization, as well as pregnancy rates after warming.

5.9 Conclusions

Vitrification technology has made significant advancements and holds great promise, but many issues need to be addressed before it becomes the standard clinical laboratory procedure. The first issue is the use of a lower concentration of cryoprotectant without a compromise on the effects of the cryoprotective action. The second issue is to find an optimal cooling and warming rate with an appropriate vitrification device. The third question is to confirm whether or not the cryo-storage duration affects the subsequent cryo-survival. The fourth matter is to develop more effective culture medium and IVM procedure on human immature oocytes matured in vitro. The fifth matter is to know more information about molecular changes in the cryopreservation of immature oocytes. Lastly, the sixth matter is to further evaluate infants born from the vitrified immature oocytes in order to ensure safety of the vitrification technology.

Authors' Perspectives

- Key Points

The vitrification occurred when the cooling rate was not so tremendously high; therefore, maybe, direct-contact LN₂ is not required for cryopreservation of oocytes. The survival rates are same after warming when the immature and mature oocytes were vitrified by the vitrification method. However, maturation rate and oocyte quality are poor if vitrified at immature stage compared to mature stage, because the structure of gap junction between cumulus and oocyte was destroyed during the procedure of cryopreservation, in which the communication between cumulus and oocyte is essential for oocyte maturation in vitro. Thus, it is important to know that cryopreservation of oocytes should be performed after IVM rather than before IVM.

- Potential Areas of Research

For oocyte vitrification, relatively high concentration of cryoprotectants is used in order to obtain a good outcome. However, it is unclear whether or not the high concentration of cryoprotectants is necessary for vitrification of oocytes. Despite thousands of babies born after cryopreservation of oocytes, it is less known that if vitrification procedure damages the oocytes in gene levels. The infants born from cryopreserved oocytes should be monitored subsequently for their long-term health.

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Antioxidants and Antifreeze Proteins in Cryopreservation/Vitrification

6

David K. Gardner and Thi T. Truong

6.1 Introduction

While cryopreservation of gametes and embryos has always been an integral part of the human ART process, over the past two decades, we have witnessed an increase in the demand for such technology. The move to single blastocyst transfer as the standard of care in human ART and improvements in culture environment have increased the number of embryos available for cryopreservation. The advent of oocyte cryopreservation has seen an exponential increase in women wanting to preserve their fertility, and the increasing success of ovarian tissue cryopreservation is proving to be an invaluable option for oncology patients. Further, with the realization that sperm quality is significantly compromised with age, we may witness an increase in the number of younger men cryopreserving their sperm.

Cryopreservation of cells and tissues imparts significant trauma. Both vitrification and slow freezing induce oxidative stress [1], and therefore there is a need to combat the adverse effects of ROS in vitro which can exert long-term effects on gene expression [2, 3] metabolism [4], as well as the proteome [5] and epigenome [6, 7] of embryos. Here, we consider the roles and benefits of both antioxidants (AOX) and antifreeze proteins (AFP) in the cryopreservation of mammalian gametes, embryos, and ovarian tissue.

6.2 Antioxidants

In vivo, there exists a complex antioxidant system to provide a response to endogenous reactive oxygen species (ROS), formed through oxidative metabolism, which have adverse impacts leading to cellular dysfunction and dysregulation. Significantly, in vivo, low levels of endogenous ROS help facilitate cell signaling [8]. Consequently, a fine control of ROS levels is required to ensure normal cell functions. In vitro, such antioxidant systems are typically absent resulting in increased ROS levels and cumulative oxidative stress. Numerous adverse impacts of oxidative stress attributed to ROS have been associated with in vitro culture, particularly when culturing under atmospheric oxygen [9].

Oxidative stress during cryopreservation is attributed to many factors, including exposure of cells to atmospheric oxygen throughout the procedure. The mechanisms of cryopreservation itself cause osmotic stress through exposure to high cryoprotectant concentrations and extreme temperature shifts generating superoxide anions [1]. The associated increase in ROS, such as hydrogen peroxide, and related decrease in glutathione [10] can impact gamete and embryo physiology [11–15], with cytoskeleton fractures and DNA and cell membrane damage resulting in delayed development. Altered mitochondrial distribution and activity following cryopreservation can also disrupt pH regulation and mitochondrial transport systems crucial for energy production in the early embryo. Additionally, energy production is also affected as it is needed for adaptive rescue systems such as DNA repair, which in turn leads to an increase in the generation of ROS leading to increased oxidative stress [1].

Cryo-induced trauma to the embryo has downstream effects on gene expression [16–20] and epigenetic state [17, 21–24]. Studies show that fetuses and placentas from vitrified embryos not only exhibit lower weights [25] but also significantly altered gene expression [2]. In particular, reduced placental ENaC expression and placental transporter *Slc26a2*, gene associated with preeclampsia in human placentae, are affected, which therefore may contribute to pla-

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cental dysregulation and altered fetal programming [2]. Furthermore, vitrification may exacerbate the increased risk for preeclampsia and intrauterine growth restriction resulting from in vitro culture, thus demonstrating the cumulative oxidative stress of in vitro culture and cryopreservation particularly when in vitro culture is conducted at atmospheric oxygen [2]. This highlights how the damaging effects of an initial single oxidative stress (e.g., in vitro culture) can predispose embryos to a secondary stress (e.g., cryopreserva-

tion) [9, 26, 27]. Hence, an antioxidant intervention analogous to the system present in vivo may provide the mechanism for cellular function, repair, and oxidative redox balance to be achieved.

The inclusion of individual antioxidants during cryopreservation has been demonstrated to be effective in providing repair and protection against oxidative damage through their potent actions as free radical scavengers as well as activating the action of other antioxidants. In Table 6.1, those

Table 6.1 Antioxidant supplementation to cryopreservation media for mouse, sheep, cow, and human gametes, embryos, and ovarian tissue

Antioxidant	Effects	Species	Concentration	Tissue	Reference
Alpha-lipoic acid (ALA)	Increased follicular activation, development, and stroma cell proliferation while maintaining mitochondrial activity and normal follicular morphology	Sheep	100–150 μ M	Ovarian tissue	[28]
	Increased pre-antral follicle survival rates, formation, follicle growth, oocyte maturation, and MII oocyte and embryo development	Mouse	100 μ M	Pre-antral follicles	[29]
	Improved sperm motility and reduced DNA damage and lipid peroxidation (LPO)	Human	0.2 mM	Sperm	[30]
L-ascorbic acid	Reduced hydrogen peroxide levels and promoted inner cell mass development	Mouse	100 μ M	Embryo	[31]
	Reduced ROS levels with an associated decrease in DNA damage and apoptosis	Human	300 μ M	Sperm	[32]
L-carnitine	Improved sperm motility, viability, and membrane function and lowered LPO	Mice	5 mM	Sperm	[33]
	Reduced oxidative stress and apoptosis rate and increased cell numbers and acetylation levels	Mice	10 μ M	Embryo	[25]
	Improved spindle assembly, chromosomal alignment, and mitochondrial distribution	Mice	3.72 mM	MI oocyte	[34]
	Improved blastocyst development	Mice	0.6 mg/mL	GV oocyte	[35]
	Increased motility through a reduction in ROS and enhanced MMP	Human	5 mM	Sperm	[36]
	Enhanced motility, plasma membrane integrity (PMI), MMO and decreased ROS levels	Human	1 and 10 mM	Sperm	[37]
N-acetyl-cysteine	Reduced oxidative stress and apoptosis rate and increased cell numbers and acetylation levels	Mice	10 μ M	Embryo	[25]
	Reduced ROS levels, enhanced mitochondrial distribution patterns, and improved developmental competence	Mice	1.5 mM	GV oocyte	[38]
	Increased sperm motility, plasma membrane and functional integrity, and acrosome and DNA integrity	Sheep	1 mM	Sperm	[39]
	Reduced DNA and acrosome damage	Bovine	5 mM	Sperm	[40]
	Increased GPx antioxidant activity and concentration of malondialdehyde (MDA) and reduced chromatin and tail damage				
	Enhanced motility, plasma membrane integrity (PMI) and MMO and decrease ROS levels	Human	5 and 10 mM	Sperm	[37]
	Decreased ROS and DNA damage and increased motility, PMI, and MMP	Human	5 and 10 mM	Sperm	[41]
Catalase	Increased stromal cell density and reduced ROS levels and DNA damage	Sheep	10, 20, 40 IU/mL	Ovarian tissue	[42]
	Decreased ROS levels and increased viability, motility, and MMP while reducing DNA damage and apoptosis	Human	200 and 400 IU/mL	Sperm	[32]
Glutamine	Decreased ROS and DNA damage and increased motility, PMI, and MMP	Human	10 and 15 mM	Sperm	[41]
Glutathione (GSH)	Increased blastocyst development	Mice	1 mM	GV oocyte	[43]
	Enhanced sperm motility, viability, mitochondrial function, and membrane integrity	Bovine	0.5 mM	Sperm	[44]
	Preserved acrosome integrity	Sheep	2 mM	Sperm	[45]
	Increased viability and decreased ROS, DNA fragmentation, and LPO	Human	5 mM	Sperm	[46]
	Increased motility, viability and membrane protein sulfhydryl group while reducing ROS	Human	1 mM	Sperm	[47]
Superoxide dismutase	Maintains redox balance and scavenge ROS	Bovine	100 IU/mL	Sperm	[48]
	Preserved acrosome integrity	Sheep	100 IU/mL	Sperm	[49]

individual antioxidants which have been shown to have beneficial effects on gametes and embryos during cryopreservation over the past decade have been listed.

6.2.1 Synergies of Antioxidants Working in Functional Groups

Rather than an individual AOX, a combination of AOX better reflects the complexities of the antioxidant system present *in vivo*, whereby their interactions work in synergy to combat the multiple sources of oxidative stress. Consequently, groups of two or three antioxidants have been evaluated to help prevent oxidative damage.

Individually, both glutathione (GSH) and ascorbic acid improved boar sperm parameters when added to cryopreservation media. However, the benefits were greatest when they were added in combination, resulting in decreased intracellular peroxide levels and increased sperm motility, viability, and acrosome and nucleoprotein structure integrity [50]. A combination of lycopene and alpha-lipoic acid (ALA) added to cryopreservation media increased motility, acrosome, and membrane integrity in goat sperm, as well as led to increased mitochondrial activity and higher levels of antioxidant genes SOD, CAT, and GSH-Px resulting in higher pregnancy rates [51]. Melatonin and myoinositol added to the cryopreservation of goat sperm improved motility, viability, plasma membrane, and acrosome integrity. Levels of ROS, DNA damage, and lipid peroxidation (LPO) were also reduced, while blastocyst development rate was improved [52].

A combination of ALA with acetyl-carnitine (AC) and N-acetyl-cysteine (NAC) supplemented to cryopreservation media has been shown to successfully reduce oxidative stress during cryopreservation resulting in increased mouse blastocyst cell number, reduced apoptotic cells, and increased acetylation levels culminating in improved fetal development following transfer [25]. Furthermore, it was found that cryopreservation significantly perturbs subsequent fetal liver and placental gene expression compared to *in vivo*-derived blastocysts [2]. Combining ALA/LC/NAC in the vitrification solution reduced the number of altered genes and decreased overrepresentation in KEGG pathway gene expression related to preeclamptic and IUGR markers resulting in a more *in vivo*-like gene expression profile [2]. Thus, combined antioxidants have been successful in ameliorating oxidative stress during cryopreservation through different mechanisms, and their combined actions complement each other, with beneficial effects on embryo and fetal physiology, metabolism, and gene expression.

Although their use in clinical settings has yet to be fully established, a pilot clinical study using the combined antioxidants ALA/LC/NAC in culture and IVF media yielded positive clinical outcomes in patients 35–40 years of age

[53]. Therefore, combined antioxidants in cryopreservation media may represent a valuable strategy to help maintain the viability of human embryos.

6.3 Antifreeze Proteins

Antifreeze proteins (also known as ice-binding proteins or thermal hysteresis proteins) have the capacity to bind ice and consequently lower the freezing point below the melting point, thereby reducing or inhibiting ice crystal growth and conferring protection to the surrounding cells. These proteins, peptides, and glycoproteins all act extracellularly and were initially derived from cold-water fish that can live in sub-zero temperatures in polar regions, but have subsequently been discovered in several species including spiders, insects, plants, bacteria, and yeast [54]. Furthermore, and importantly from a regulatory perspective (as it is difficult to include animal products in human IVF media/solutions), some have recently become available in a recombinant form [55]. It is beyond the scope of this chapter to elucidate the mechanisms through which AFP acts (and there are several different types), but for those interested, such information can be found in reviews on their function [54, 56, 57].

Unlike the cryoprotectants commonly used in both slow-rate freezing and vitrification, such as ethylene glycol, propanediol, and dimethyl sulfoxide (DMSO), AFP have the advantage of being naturally occurring compounds and consequently exhibit lower cellular toxicity. This latter point is certainly worth considering given the known detrimental effects of DMSO on cell function and epigenetics.

AFP have been shown to have protective effects during vitrification of oocytes of the cow and mouse. Using AFP derived from several sources, it was determined that their inclusion in the vitrification and warming solutions was associated with improved oocyte survival, decreased DNA damage, improved spindle configuration, reduced ROS, and subsequently enhanced embryo development [58–61].

Sperm cryopreservation has also been shown to benefit from the inclusion of AFP in a number of mammalian species, including humans. Studies have demonstrated an increase in post-cryopreservation motility and viability, paralleled by a reduction in ROS and DNA fragmentation. Significantly, the effects are dependent upon both source and concentration of the AFP used [62–64].

As well as showing a beneficial effect on sperm, Nishijima and colleagues [64] have also determined that at 500 ng/mL, AFP type III has a beneficial effect on rabbit morula survival post-vitrification. Interestingly, at 1000 ng/mL, the AFP had an adverse effect. In a study on sheep blastocyst cryopreservation, involving recombinant AFP ApAF914, it was determined that at 10 µg/mL, AFP increased post-vitrification hatching rates of slow-growing sheep blastocysts [65].

Lee et al. investigated the effects of three different types of AFP on mouse ovarian cryopreservation and subsequent transplantation [66]. Supplementation with AFP, especially 10 mg/mL of LeIBP (from arctic yeast), improved follicle quality and decreased the apoptosis of follicles during the vitrification-warming procedures. Subsequent studies on the mouse have repeated such observations but using different types of AFP either alone or in combination [67]. Work on bovine ovarian xenotransplantation, which employed the AFP *Leucosporidium* ice-binding protein, determined that AFP reduced ovarian tissue damage and apoptosis and improved ovarian follicle morphology [68]. Similarly, in a recent study on the rabbit [69], it was determined that the addition of AFP type III improved follicle morphology, decreased apoptosis, and improved survival in both slow-frozen and vitrified ovarian tissues, with the beneficial effects most evident in the slow-frozen tissue.

It is evident that AFP can confer a benefit during the cryopreservation of gametes, embryo, and ovarian tissue, whether slow freezing or vitrification is used as the method for cryopreservation. However, much work remains to be done in this fascinating area, particularly regarding the types and concentrations used, as AFP appear to show toxicity at high levels. The majority of studies to date have utilized type III AFP, typically derived from fish. Given the availability of such proteins in recombinant form, much research should ensue to determine whether AFP can be of clinical benefit in ART.

6.4 Conclusion

It is evident that both AOX and AFP can confer significant benefits during the cryopreservation of gametes, embryos, and ovarian tissue. Further work now needs to be undertaken not only to establish which groups of antioxidants work best together, but also to determine whether there are synergies to be had by the inclusion of both AOX and AFP during cryopreservation. What is evident is that the addition of AOX and/or AFP clearly has the potential to confer significant benefit to human ART through improvements to cryopreservation by decreasing the damage and loss of gametes and embryos.

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Devitrification Thresholds and Warming Rates of Reproductive Cells

7

M. V. Santos and N. Zaritzky

Key Points Numerical prediction of warming and cooling rates in vitrified samples.

- Devitrification conditions in Dewar tanks correlated with lower viability of bovine reproductive cells.
- Temperature profile in cryodevices and nitrogen vapor inside liquid nitrogen storage tanks.

7.1 Introduction: Vitrification in Cryobiology

Matter in a liquid state submitted to a cooling process under ordinary conditions undergoes crystallization when the temperature reaches the melting point (T_m); these crystals form an orderly molecular arrangement as the freezing progresses with the release of the latent heat of melting. A heterogeneous freezing occurs when crystal nucleators are present; however, in the absence of nucleators, the sample may reach temperatures below T_m and still maintain a liquid state. This constitutes a nonequilibrium state called supercooled water, and the probability of ice crystal formation increases up to a value of 1 at the homogeneous freezing point (T_h) since crystallization is the thermodynamically favored state. In the case of pure water, the homogeneous nucleation temperature is near -40°C [1]. Between T_m and T_h , the solution is meta-

stable since ice formation can occur if there are particles that act as nucleators.

In solutions with a suitable concentration of cryoprotectants, the probability of ice formation can be very low and/or avoided below T_m when the viscosity of the liquid is high and there is a rapid cooling of the sample. Vitrification is a phenomenon that occurs when a supercooled liquid solidifies; it forms an amorphous crystal-free structure that retains its disorderly three-dimensional arrangement of molecules. The physical properties, such as specific heat, thermal expansion coefficient, and viscosity, are similar in value to a solid matter state usually called a glass. The glass transition temperature T_g is the temperature where the transition from a supercooled liquid into an amorphous glassy state occurs, in the absence of intracellular and extracellular ice crystals. During this phenomenon, there is no heat lost or released associated with this transition (no ice crystal formation), only a change of slope in the enthalpy vs. temperature curve due to the change of the specific heat capacity property between the liquid and solid glass. An amorphous crystal-free structure or glass is a non-thermodynamic equilibrium state. The minimum concentration of cryoprotectants where T_h crosses T_g is the lowest possible concentration to avoid ice crystal formation in order to achieve vitrification. Cryoprotectants during vitrification play a significant role since they help to inhibit the crystallization process. In vitrified cryoprotectant solutions, chemical changes depending on translational molecular diffusion are significantly slowed down to negligible rates. Once the sample is below T_g , there is only small local movements of molecules, diffusion is strongly inhibited, and therefore a restriction of chemical changes is observed when cells are stored vitrified at cryogenic temperatures [2].

When a vitrified solution is warmed above its T_g , ice crystal formation can occur since translational movement of molecules is enabled, and diffusion and nucleation of aggregates can cause a rapid crystallization of the solution. This phenomenon is called devitrification, and it occurs at the temperature defined as T_d . Devitrification during cryopreser-

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vation procedures must be avoided since ice formation both intracellular and extracellular has been associated with lethal damage to cells [3].

The critical cooling rates are the minimum rates that achieve vitrification, and the critical warming rates are the minimum rates to avoid ice formation during warming of a sample. Several authors suggest that in order to have a high survival of cells that have been vitrified during cooling, a high warming rate is critical [1, 4]. Specifically for several cryoprotectant solutions, the critical warming rates are two or more orders of magnitude higher than the critical cooling rates [1]. This important issue is often overlooked in the reproductive cryobiology during the manipulation of vitrified samples; usually, there is a strong focus on how the cooling procedure is carried out in order to reach the point below T_g , but not much attention is paid on the warming rate (the way up above the T_m).

There are several activities during the manipulation of vitrified samples that can pose a risk in terms of devitrification and ice formation during warming. For example, there is a risk of devitrification when cryodevices are uplifted for the correct identification of a patient since the sample is exposed to temperatures above its T_g ; this is especially critical when a double eye witness requirement must be fulfilled or when there are high-packed goblets (mushrooming) and several devices must be checked (uplifted) for their ID, exposing several cryodevices to higher temperatures. Also, devitrification can occur when longer times are needed during uplifting to better visualize the ID of cryodevices with handwriting, due to frost present at the surface. Another risk is when samples are transported in airplanes in dry shippers and the nitrogen vapors inside the tank reach temperatures above T_g , or when there is a low level of liquid nitrogen in a Dewar tank and several samples are exposed to a nitrogen vapor temperatures that are higher than the T_g of the sample. The latter is especially risky when there is subsequent lifting and lowering of canisters in semi-full Dewars and there is a disturbance of the nitrogen vapor layers [5]. In brief, every time a cryodevice that contains a vitrified sample is exposed to cryogenic temperatures above T_g , there is a high risk of suffering devitrification, even though low-temperature nitrogen vapor may give the sensation of working in a safe cool environment; this is not true if these temperatures are not below T_g of the sample.

Cryoprotective agents are important for vitrification purposes since the solute concentrations kinetically interfere with ice nucleation and crystal growth that is critical to achieve a “glassy state” at extremely low temperatures. There are many excellent reviews on the types of molecules and concentrations of cryoprotective agents in solutions that

have reached excellent results according to the type of cell or tissue [6, 7], but this information is beyond the scope of the current work, which is focused on the T_g of solutions with respect to the concentration of cryoprotective agents. The generally accepted value is that the onset glass transition T_g for vitreous pure water occurs at about 136 K (-137°C) for amorphous solid water and hyperquenched glassy water [8]. The glass transition temperature (T_g), which refers to a narrow temperature range in which the material undergoes a second-order phase transition (i.e., liquid to glass or vice versa), ultimately determines the appropriate biospecimen storage temperature. The T_g of solutions with cryoprotective agents and their change according to their concentration are relevant factors since experimental measurements using DSC show an increase in its value as the concentration of solutes increases [6, 9] with respect to the T_g of pure water.

The experimental measurements of the temperature versus time curve during cooling and warming in a specific protocol are usually very difficult due to the small volume of the solution mounted on the cryodevices for vitrification. Experimental observations of samples through modified cryostereoscopes for the visualization of crystallization and recrystallization are not always feasible for most laboratories.

The mathematical modeling of the most relevant mechanisms that govern the energy transfer during the cooling and warming stages of the cryopreservation protocol contributes to the knowledge of the key factors that affect the viability of cells.

In order for these models to be useful for cryobiologist, the correct parameters such as the surface heat transfer coefficients and thermophysical properties of the solution and plastic support as well as the actual geometry and dimensions should be considered.

Numerical simulations of the mathematical partial differential equations of the heat transfer process have been used in the field of cryobiology to analyze the performance of several vitrification devices for both cooling and warming process [10–12].

The objectives of this chapter are to mathematically model the complex thermal phenomena during the cooling and warming stages of a cryopreservation protocol; to predict time-temperature histories of vitrification devices using the finite element method considering surface heat transfer coefficients, thermophysical properties, and actual geometry of the system; and to determine devitrification thresholds and warming rates in OPS and Cryotop®, and how these may affect storage and handling conditions of the samples in Dewars, validating these results with *in vitro* embryo development using a bovine model and time-temperature measurements previously published in literature.

7.2 Predicting Time-Temperature Curves of Vitrification Devices Using the Finite Element Method

7.2.1 Mathematical Modeling

The heat transfer equation is a partial differential equation that describes the distribution of energy (heat) and how the temperature changes throughout a sample as time elapses:

$$\rho C_p \frac{\partial T}{\partial t} = \nabla \cdot (k \nabla T) \quad (7.1)$$

where ρ is the density, C_p is the specific heat, k is the thermal conductivity, T is the temperature, and t is the time. In some regularly shaped geometries that are homogenous in structure with constant and isotropic thermophysical properties (ρ , C_p , k), there may exist analytical and exact solutions to this equation; however, this is generally not the case for cryodevices. In the cryobiology field, these assumptions are generally not fulfilled, since devices and supports of biological samples involve two very different domains; the plastic support and the biological fluid, with distinctly different thermophysical properties. Additionally, the geometry of the devices used for vitrification processes is irregular. The heterogeneous structured systems, irregularly shaped objects, and temperature-dependent thermophysical properties force the mathematical partial differential equations to be solved using numerical techniques such as the finite element method, since exact analytical solutions are not available [13].

The finite element method is an established formulation for the solution of heat conduction problems such as cooling and freezing. It has the great advantage that it can deal better with problems where the object has an irregular geometry with defined regions (domains) of different compositions [14].

In order to correctly define the mathematical problem, the boundary conditions must be declared in the mathematical formulation [15]. The boundary conditions describe how the interface between the object (device with the biological solution) and the external cryogenic fluid works. The convective boundary condition (Eq. 7.2) at the interface involves the knowledge of the surface heat transfer coefficient (h):

$$q = -k \nabla(T) \cdot n = h(T - T_{ext}) \quad (7.2)$$

where T_{ext} is the external cryogenic fluid temperature, n is the unit outward normal vector at the interface, and h is the surface heat transfer coefficient. The h value used at the boundary condition characterizes the type of mechanism that governs heat transfer at the interface; it is strongly dependent on the geometry of the sample, the surface roughness, and

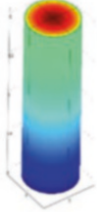
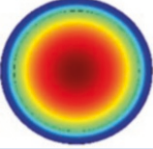
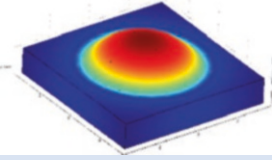
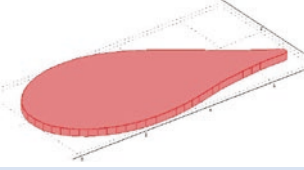
the cryogenic fluid and its movement or velocity field around the object. The h value provides information concerning whether natural or forced convection exists, and its value is strongly dependent on the type of external medium to which the sample is exposed such as air, nitrogen vapors, or liquid nitrogen and the velocity of the fluid in contact with the device.

By solving Eqs. 7.1 and 7.2 according to the geometry and structure of the system, the performance of vitrification devices during cooling and warming using engineering tools can be established.

7.2.2 Evaluation of the Cooling Rates

Table 7.1 shows the different cooling rates predicted by Sansinena et al. [10] using several devices for vitrification purposes. The tested vitrification devices were the following:

Table 7.1 Calculated cooling rates for three different h values in several vitrification devices reported by Sansinena et al. [10]

Device and geometry	Cooling rate, °C/min (from 20 to −130 °C)		
	External heat transfer coefficient, h (W/m ² K)		
	200	1000	2000
OPS (1 μ l) 	1694	5521	7826
Miniflex® 	1848	6164	8738
Cryotop® (100 μ m) 	10,465	37,500	60,000
Cryoloop® 	36,000	180,000	180,000

- (a) *The Cryoloop*[®] system (Hampton Research, CA, USA) is a device originally designed for crystallography used to mount, freeze, and secure the crystal during cryocrystallographic procedures and X-ray data collection. It consists of a mounted loop of 20 μm diameter nylon. These nylon loops are pre-staked to hollow, stainless steel MicroTubes[™]. Oocytes are loaded with a volume $< 1 \mu\text{l}$ onto the loop, maintained in the vitrification solution by surface tension and plunged into liquid nitrogen.
- (b) *The Cryotop*[®] (Kitazato Supply, Inc., JP) or *Cryoleaf*[®] (MedCult Inc., Jyllinge, Denmark) systems are thin polyethylene film strips of approximately 0.4 mm wide, 20 mm long, and 0.1 mm thick described by Kuwayama et al. [16] that allow for the oocytes to be vitrified when they are placed in an open drop of minimal volume and then plunged directly into liquid nitrogen.
- (c) *The Miniflex*[®] polyethylene tips (Sorenson Bioscience, Inc., UT, USA) have been used for vitrification and reported by Cremades et al. [17]. They were described to have 360 μm inner diameter and a 77 μm wall thickness.
- (d) *The Open Pulled Straw (OPS)* is normally manufactured in the laboratory from 0.25 mL polyethylene French straws (IMV, Orsay, France), typically by softening over a hot plate and pulling manually until the inner diameter decreases from 1700 to approximately 800 μm and wall thickness of the central part decreases from approximately 150 to 70 μm . The straws are air-cooled and cut at the narrowest point with a razor blade. Their commercial version, the Cryotip[®] (Irvine Scientific, CA, USA), is a specially designed drawn plastic straw with ultrafine tips and protective metal cover sleeves.

For each device, the cooling rate ($^{\circ}\text{C}/\text{min}$) was defined as the time needed to reduce initial core temperature (warmest point of the system) of the liquid from 20 $^{\circ}\text{C}$ to 130 $^{\circ}\text{C}$.

The abovementioned devices have been thoroughly studied and applied for the vitrification of oocytes since they are especially vulnerable to cooling processes and cryoinjuries. There are several reasons that are responsible for this sensitive behavior towards low temperatures: their large size and low surface-to-volume ratio (0.05 oocytes versus 4.5 for sperm), special intracellular content and structure, and high intracellular water content. There is robust evidence of the excellent results obtained using a vitrification method instead of slow-cooling protocols; it is considered nowadays the reference standard for oocyte cryopreservation [18].

Numerical results indicate that the nylon loop (Cryoloop[®]) is the most efficient heat transfer system analyzed, with a predicted cooling rate of 180,000 $^{\circ}\text{C}/\text{min}$ for an external heat transfer coefficient $h = 1000 \text{ W}/\text{m}^2 \text{ K}$ when cooling from 20 to -130°C . The performance of this device can be attributed to the absence of a solid support and its extremely small dimensions.

The OPS showed the lowest performance with a cooling rate of 5521 $^{\circ}\text{C}/\text{min}$ at the same h value.

In 2011, Mazur and Seki [19] achieved the experimental measurement of cooling and warming rates. In this study, the authors measured the time-temperature histories during cooling and warming of a sample mounted on Cryotop[®] using a 50 μm copper-constantan thermocouple and recorded data with a computer-oscilloscope. This experimental procedure allowed for the quantification of the warming rates achieved in a Cryotop[®] under several operating conditions. In the case of direct plunging of Cryotop into liquid nitrogen, the measured average cooling rate reported was 69,250 $^{\circ}\text{C}/\text{min}$. The numerical model by Sansinena et al. [10] showed a good agreement with experimental measurements predicting a cooling rate of 60,000 $^{\circ}\text{C}/\text{min}$ using as input a value of $h = 2000 \text{ W}/\text{m}^2 \text{ C}$ and glassy water thermophysical properties.

These results show the advantage and importance of developing numerical simulations of heat transfer phenomena since the analysis of the predicted thermal histories and cooling rates allows to determine the performance of the different oocyte cryopreservation devices under different laboratory conditions.

7.2.3 Evaluation of Warming Rates

Several works proposed that the warming rate of vitrified samples might be the most important factor that determines cell cryosurvival [3, 19]; therefore, the previously developed numerical models [10] were applied to the warming of Cryotop and OPS under different conditions in order to mathematically determine the time required to reach the T_g of a solution. If a vitrified sample reaches T_g , there exists a high probability of ice formation caused by devitrification.

Sansinena et al. (2014) determined the T_g of a vitrification solution using differential scanning calorimetry (DSC) (TA Instruments, New Castle, DE, USA) model Q100 with a cooling system of liquid nitrogen: the solution consisted of 2.8 M Me2SO (Sigma D2650) + 3.6 M EG (Sigma 102,466) and 0.65 M trehalose (Sigma T3663) in TCM199 (Invitrogen, USA, 12350–039) with 10% vol/vol fetal bovine serum (Invitrogen).

The thermogram obtained by DSC can be observed in Fig. 7.1; the midpoint temperature in the step curve was defined as $T_g = -125.49^{\circ}\text{C}$, and a detailed description of the experimental design can be found in the work of Sansinena et al. [20].

During the uplifting of a canister from the liquid nitrogen in a Dewar tank, the sample is no longer completely submerged in the cryogenic liquid that assures a constant temperature of -196°C ; lifting exposes the sample to nitrogen vapors which can be above the T_g of the sample. To determine the actual temperature profile inside a Dewar tank, which is the temperature versus distance above the level of liquid nitrogen, several temperature measurements at specific

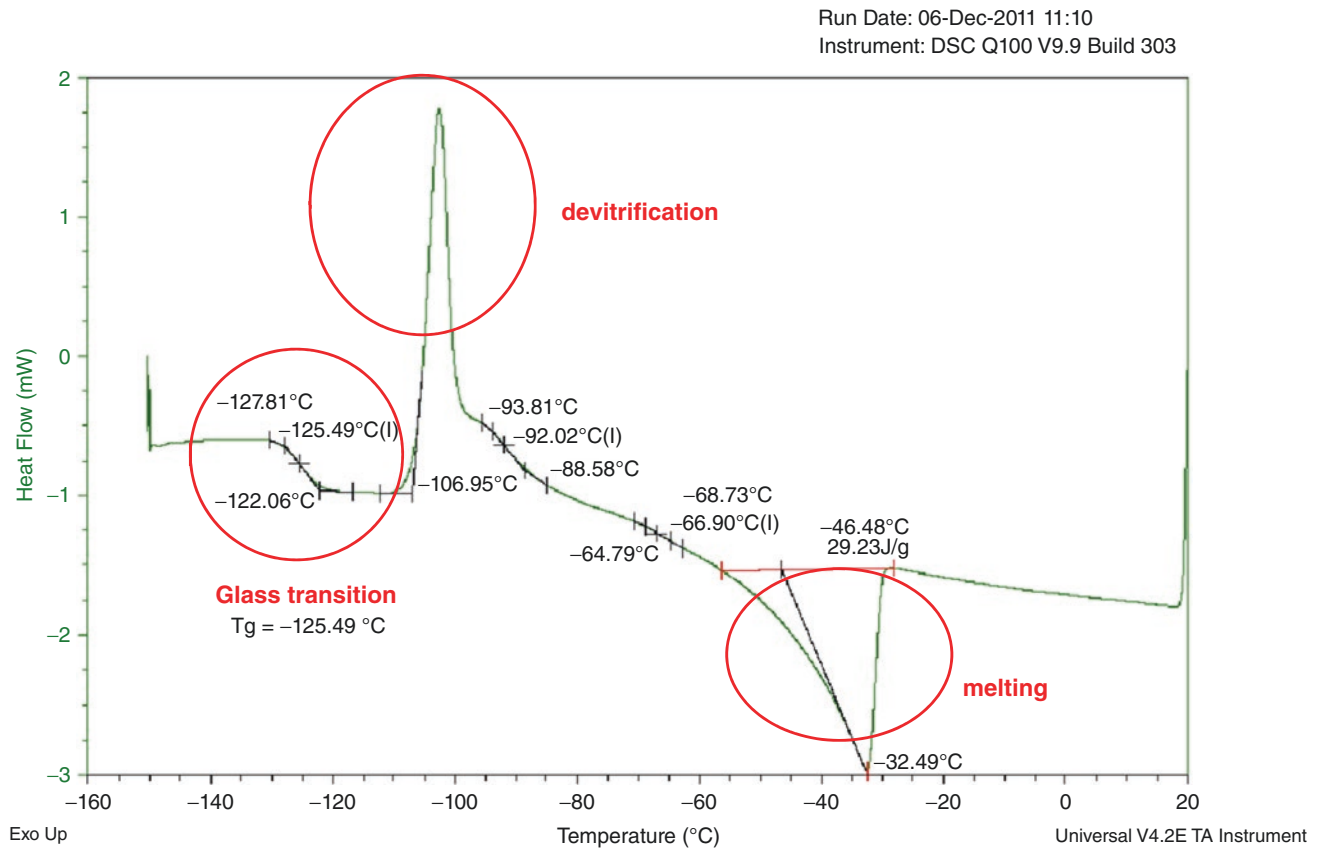


Fig. 7.1 Thermogram obtained by DSC and T_g of the vitrification solution, and the main thermal transitions are pointed in red circles in the curve

distances were carried out [20] considering a “full Dewar” (full with liquid nitrogen), “half-full Dewar,” and a usual laboratory practice where a canister is raised and then lowered when a half-full Dewar situation is encountered. The temperature measurements showed that under full Dewar conditions, a temperature of -97.87°C (higher than T_g) is achieved at 14 cm measured from the top of the Dewar, indicating that a rubbery state and molecular movement with a subsequent devitrification can occur at the neck of the Dewar tank if the sample is exposed to these nitrogen vapors because warming occurs at a very slow rate allowing to reach the most stable thermodynamic condition, which corresponds to ice crystals in the solution [19]. For a half-full Dewar, the high-risk zone of devitrification increased up to 16 cm (measured from the top of the Dewar) where the temperature of the nitrogen vapors reached -125.06°C ; only below this point (>16 cm) was the temperature lower than the T_g . For a half-full Dewar, after a common practice of raising and lowering a canister, the nitrogen vapor profile is disturbed and a new equilibrium condition is attained where the high-risk zone is now extended up to 26 cm; at this point, the temperature is -114.05°C . The different high-risk zones achieved with a full and half-full Dewar are shown in Fig. 7.2.

Since the effects of storage and handling of reproductive cells under rubbery conditions or above its T_g are

associated with a high risk of ice formation caused by devitrification, numerical simulations were carried out to quantify the critical time that elapses in a vitrification solution contained in an OPS for the warmest point to reach temperatures above its T_g [20], when the initial condition is a uniform temperature of -196°C (submerged in liquid nitrogen) and afterwards when the solution was exposed to a condition of being suspended in nitrogen vapors inside a Dewar tank.

In a Dewar tank, the top represents the hot surface and the liquid nitrogen interface (cold source) is at the bottom. Hot surfaces facing down produce a stratification phenomenon of the fluid (nitrogen vapor) since the density decreases with increasing temperature; therefore, less dense layers are located above denser, leading to a stable condition which does not cause any convection currents; heat transfer is produced by conduction. To quantify the critical time, the numerical modeling of the warming process was carried out using the finite element software COMSOL (2005). Heat transfer coefficients for this system were determined by Santos et al. (2013) and further corroborated with literature values (using Nusselt correlations); the h values selected for the simulations were 10 and $15\text{ W/m}^2\text{K}$ [13, 21]. Two different T_v values (70°C and -50°C) were considered for the calculation of the critical time needed for the system to reach the following final

Fig. 7.2 Different high-risk zones where devitrification may occur according to a full and half-full Dewar and after raising and lowering of a canister in a half-full Dewar

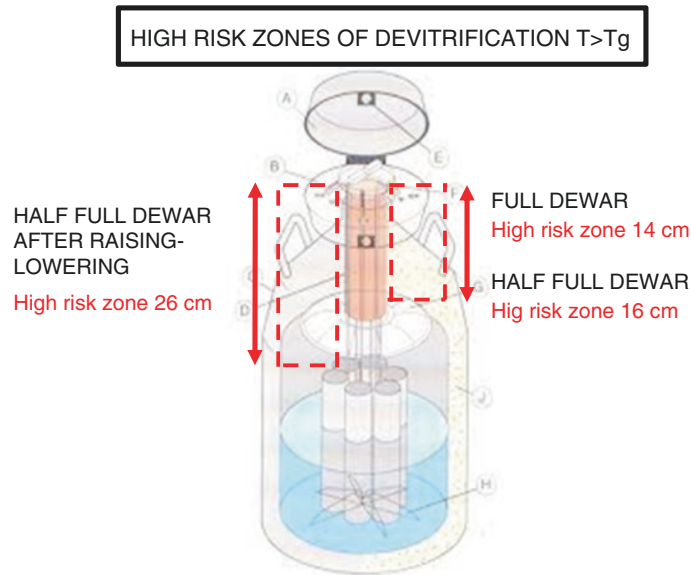
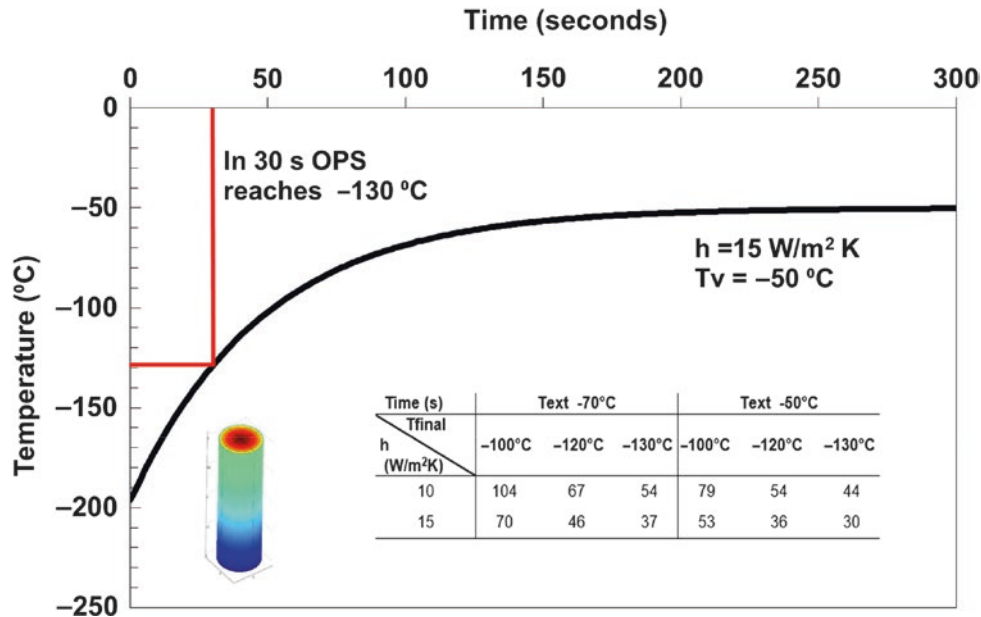


Fig. 7.3 Time-temperature prediction for the OPS when it is exposed to different T_v and using different values of h that correspond to stagnant nitrogen vapor conditions previously determined by Santos et al. [21]. The inserted table shows the numerical results indicating the critical time for the OPS to reach different final temperatures: -100 , -120 , and -130 °C



temperatures -100 , -120 , and -130 °C, which are values close to the T_g of the biological fluid in the OPS.

In the mathematical simulation, the initial condition was $T = -196$ °C; the thermal properties of the plastic were (k) 0.22 W/m K , (C_p) 1680 J/kg K , and (ρ) 900 kg/m^3 [10]; the thermal properties of glassy water introduced in the model were (k) 0.50 W/m K , (C_p) 4218 J/kg K , and (ρ) 983 kg/m^3 [8]. When the OPS is placed at a certain height over the liquid N_2 , it begins warming; as the height increases, the temperature of the N_2 vapor (T_v) also increases. If the OPS system reaches T_g , the vitreous biological solution has a greater risk of suffering damage

because it enables the transition into a rubbery state, which in turn allows the formation of ice crystals (devitrification). The devitrification condition is favored when the system is given enough time to equilibrate at temperatures above T_g and below T_m since ice formation is the most stable thermodynamic condition.

Figure 7.3 shows the mathematical simulation of the time-temperature history of the OPS when it is exposed to different T_v values, using two h values. The table inside the figure shows the results of the critical time for the OPS to reach temperatures of -100 , -120 , or -130 °C (values close to the T_g of the solutions).

7.3 Comparison of the Survival of Oocytes during Handling in Dewars and Under Different Warming Conditions

Sansinena et al. [5] analyzed the effect of storage and sample removal conditions on in vitro embryo development of parthenogenetically activated vitrified–warmed oocytes applying three different treatments that correspond to different situations during handling in Dewar tanks:

- Treatment 1: one-step removal of samples completely immersed in liquid nitrogen.
- Treatment 2: one-step removal of samples suspended in nitrogen vapors.
- Treatment 3: two-step removal of samples suspended in nitrogen vapors: sample lifting with 15-s pause at Dewar neck (8 cm from top, -17°C , simulating pause needed to read sample identification).

A full description of the experimental procedures and statistical analysis can be found in the work of Sansinena et al. [5]. In this chapter, the discussion is focused on the effect of handling conditions and possible devitrification when oocytes are warmed at low rates upon lifting in nitrogen vapor. Loading procedure of the OPS cryodevice was conducted as described by Vajta et al. [22], and vitrification was attained by directly plunging into liquid nitrogen.

As can be seen from the results presented in Table 7.2 and experimental observations, a significant reduction in morphological oocyte survival was observed with two-step removal from Dewar when compared with either one-step removal treatment (samples completely immersed in LN2 or N2-vapor storage) or non-vitrified control (50% versus 98.5%, 91%, and 100%, respectively; $P < 0.05$). Blastocyst rates in all vitrified–warmed groups were significantly lower

than non-vitrified control (17%, 17%, and 9% versus 33%; $P < 0.05$). Blastocyst development was significantly reduced in two-step removal treatment versus both one-step removal treatments (treatments 1 and 2) (9% versus 17% and 17%; $P < 0.05$). No significant difference was found between proportions of embryos reaching blastocyst stage between one-step removal treatments.

During storage of vitrified samples such as oocytes or embryos in liquid nitrogen, the tanks are filled completely, but there may exist loss of the cryogenic liquid due to evaporation or loss of vacuum in the Dewar tank, which is an important factor that allows the correct thermal insulation of the samples. This procedure, where samples can be suspended in nitrogen vapor, is not a common practice and should be avoided. However, technicians should also be aware that upon removal for ID or search of the correct cryodevice, the reproductive cells are exposed to a vertical temperature gradient that can surpass the T_g of the vitrification solution, and this warming may have lethal effects on cells. Several reports have indicated that, above the glass transition temperature of the cytoplasm, vitrified oocytes and embryos enter a liquid transition, promoting devitrification, followed by immediate ice nucleation and recrystallization [23, 24].

The cause can be attributed to the thermodynamic instability of vitreous samples to temperatures above the T_g in combination with a slow-warming rate since nitrogen vapors have a low h value that allow a sufficient time for molecules to rearrange and form ice crystals during warming (devitrification). No significant differences were observed in the in vitro development of chemically activated vitrified–warmed oocytes after one-step removal conditions, with samples either submerged in LN2 or stored in N2 vapor; these results are in agreement with recommendations previously proposed in numerical modeling studies that temperature of the sample must remain below the transition temperature of the cryoprotectant solution [24]. By contrast,

Table 7.2 Effect of three different treatments during storage and sample removal conditions on in vitro embryo development of parthenogenetically activated vitrified–warmed oocytes [5]

	Sample storage temperature T versus T_g^a	n vitrified/activated	Morphologically intact, n(%)	Blastocyst (day 7), ^b n(%)	Hatched (day 9), ^b n(%)
Treatment 1	$T < T_g$	135	133 (98.5) ^c	23 (17.3) ^c	4 (3.0) ^c
Treatment 2	$T < T_g$	140	127 (90.7) ^c	22 (17.3) ^c	3 (2.4) ^c
Treatment 3	$T > T_g$	135	67 (49.6) ^d	6 (9.0) ^d	0
Control	N/A	149	149 (100) ^c	49 (32.9) ^c	29 (19.5) ^d

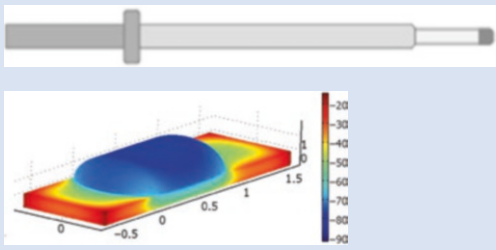
NA not applicable.

^aGlass transition (T_g) and devitrification thresholds reported by Sansinena et al. [20] from calorimetry measurements and finite element simulation

^bValues calculated from morphologically intact oocytes

^{c, d}Values with different superscripts within a column are statistically different; $P < 0.05$

Table 7.3 Geometry of the Cryotop®, convective heat transfer coefficients (h), and predicted warming rate values using numerical simulations of the system under two different protocols, droplet volume size 0.1 μL , experimental warming rates, and survival (%) of mouse oocytes in EAFS10/10 previously vitrified in Cryotop® with a cooling rate of 69,250 $^{\circ}\text{C}/\text{min}$

	Warming protocol Cryotop®	Heat transfer coefficient h ($\text{W}/\text{m}^2 \text{K}$)	Predicted warming rate using FEM ($^{\circ}\text{C}/\text{min}$) ^a	Experimental warming rate ($^{\circ}\text{C}/\text{min}$) ^b	Survival of mouse oocytes (%) ^b
<i>Cryotop – Open System</i> 	Open in air at 23 $^{\circ}\text{C}$	120	7845	7850 $\pm$ 415	15.4 $\pm$ 6.3
	Open in sucrose solution at 23 $^{\circ}\text{C}$	1850	96,000	117,500 $\pm$ 10,632	78.6 $\pm$ 8.7

^aSantos et al. [12]

^bMazur and Seki [19]

the two-step removal condition, which clearly surpasses the transition temperature of the vitrification solution, resulted in a significant reduction of morphologically intact vitrified-warmed oocytes, as well as reduced in vitro embryo development after activation. Under this condition, samples were exposed to temperatures shown to be in the range of rubbery and devitrification thresholds, which in turn resulted in decreased in vitro developmental competence. These results reinforce the importance of exercising extreme cautions while handling vitrified material during routine laboratory procedures such as identification confirmation and inventory keeping, owing to their inherent thermodynamic instability. A video webinar with full description of concepts and results can be found in [25].

Another important practice when reproductive cells are retrieved from long-term storage in liquid nitrogen is the warming rate protocol in order to achieve high survival rate. As explained and demonstrated by Santos et al. [12] and Mazur and Seki [19], vitrified samples cooled at high rates (69,250 $^{\circ}\text{C}/\text{min}$) and then exposed to low warming rates correlated with low oocyte survival and low values of h in the system.

Table 7.3 shows the effect of h values and warming rates in Cryotop under open protocols exposed to air or sucrose solution at 23 $^{\circ}\text{C}$ on mouse oocyte survival.

In the case of an open Cryotop® when it is raised from the liquid nitrogen and placed in contact with air, a high h value (120 $\text{W}/\text{m}^2 \text{K}$) was obtained when compared to typical values of h in stagnant air; this can be attributed to the fact that the liquid nitrogen film adhered to the Cryotop® device evaporates when it is exposed to the warm air. Nitrogen vapor released from the sample produced a higher convective flow that led to

higher h values. The individual contribution of radiation to the total rate of heat transfer was calculated resulting in less than 3%. The h values of the Cryotop® open system immersed immediately in sucrose solution at 23 $^{\circ}\text{C}$ achieved the highest survival according to Mazur and Seki [19], and the h value that best fitted experimental measurement for 0.1 μL was 1850 $\text{W}/\text{m}^2 \text{K}$. The h values calculated using numerical simulations correlated with warming rates. Therefore, these are useful parameters for cryobiologists when studying technologies associated with vitrification systems.

7.4 Conclusion

Storage of vitrified samples in liquid nitrogen vapor phase (as opposed to completely immersed in liquid nitrogen) constitutes a potential risk since devitrification can occur. Devitrification threshold should not be interpreted as a fixed value, as it is influenced by the T_g of the system, heat transfer efficiency of the vitrification support, volume of the sample, and other factors.

The mathematical modeling of heat transfer in cryodevices contributes to the understanding of different complex phenomena that govern the cooling and warming process of vitrified samples. Additionally, these models bring several advantages since they allow (1) prediction of temperature versus time curves under different laboratory conditions, (2) comparisons between protocols, (3) accurate determination of devitrification thresholds, and (4) evaluation of critical warming times, new storage designs, and inventory procedures prior to exposing real-life samples.

Author Perspective

(a) Key Points

It must be remembered that certain activities such as manipulations during ID, confirmation, or transport produce warming of vitrified samples, which pose a high risk of devitrification that could induce cell death. Mathematical heat transfer models help to assess these points and are reliable since they are independent of the technician, and thus no human factors are involved.

(b) Potential Areas of Research

Multidisciplinary approaches may help during the handling of cryodevices.

Recommendation: Think like an engineer when handling vitrified samples.

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Part III

Methodology and Management

Human Sperm Slow Freezing

8

Rakesh Sharma and Ashok Agarwal

8.1 Introduction

8.1.1 History of Sperm Cryopreservation

Sperm banking dates back to the 1660s when Lazzaro Spallanzani found that sperm could maintain the motility under cold conditions [1]. Sperm cryopreservation helped protect spermatozoa at low temperature, and the first reported human birth was in 1953 using sperm frozen on dry ice and thawed before insemination. However, the introduction of liquid nitrogen (LN₂) in the 1960s introduced the therapeutic sperm banks [2]. Since then, advances in freezing and storage techniques have resulted in the long-term storage of sperm for later use and serve as a fertility assurance for men to father a biological child.

8.1.2 Need for Sperm Cryopreservation in ART

There are a number of indications for freezing sperm. The most common medical and oncological indications in both adults and adolescents are diagnosed with cancer prior to the start of their treatment [3–6]. Cancer treatments are highly toxic, and chemotherapy can affect early stages of spermatogenesis especially the proliferating spermatogonia and lead to partial or complete azoospermia [7, 8]. Similarly, radiotherapy also affects spermatogenesis damaging Sertoli and Leydig cells, sperm DNA fragmentation, and obstruction of

ejaculatory ducts [9, 10]. Other medical but nononcological indications for cryopreservation include medical conditions such as lupus, multiple sclerosis, spinal cord injuries, and associated ejaculatory dysfunction [11, 12]. Sperm freezing is also done from autologous donors for future fertility or donor sperm where men serve as surrogate fathers to partners of couples suffering from refractory male infertility problems, or for single lesbian women planning for a child [13]. Sperm cryopreservation is also indicated in cases of azoospermia, both nonobstructive and obstructive, where sperm can be surgically retrieved from the testis or epididymis [14–17]. Spermatozoa can also be retrieved from men with ejaculatory dysfunction or spinal cord injury [11, 12] by using testicular sperm extraction or percutaneous epididymal sperm aspiration, thereby avoiding the need for repeated biopsies and aspirations [18]. In addition, sperm freezing is also indicated in prevasectomy patients [19]; before in patients with HIV, hepatitis C, or hepatitis B [20–24]; in posthumous use of sperm [25–28]; and in gender dysphoria or transsexual women [29–32]. Social freezing is done in men delaying fatherhood [33–35].

All samples that are cryopreserved must be screened for infectious diseases including HIV, herpes, and hepatitis before being transferred to long-term storage or being released for use [10, 36]. Cryopreservation of sperm is a fertility assurance when any of the situations, illness, or treatment threatens the fertility potential of an individual and limits the ability to father a biological child. Cryopreserved sperm can be used with assisted reproductive techniques (ARTs) including intrauterine insemination (IUI), in vitro fertilization (IVF), and intracytoplasmic sperm injection (ICSI) [37] depending on the total motile

Cryopreservation in Assisted Reproduction: A Practitioner's Guide to Methods, Management and Organization

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sperm available post-thaw. Patients are encouraged to bank multiple samples so that there is adequate number of sperm available for use with ART such as in case of IUI [38]. Fertilization and pregnancy rates are comparable with fresh and frozen surgically retrieved sperm when used with ICSI in case of both obstructive [39, 40] and nonobstructive azoospermia [41–43].

8.1.3 Cryoprotectives and Reagents

Freezing of sperm without cryoprotectants results in swelling of the plasma membrane, acrosomal leakage, and membrane damage because of freezing and expansion of water. Dramatic changes in the temperature, osmotic shock, and oxidative stress result in structural and functional damage to the sperm. Glycerol is the most common cryoprotectant used for freezing sperm. However, glycerol can alter the acrosomal membrane and alter mitochondrial membrane environment [44]. Cryoprotectants help reduce the water content and provide efficient dehydration, thereby minimizing the formation of intracellular ice formation during freezing. Extenders such as TES [N-Tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid], Tris [(hydroxymethyl) amino-methane] with sodium citrate, and egg yolk help maintain optimal sperm viability [45] and effective dehydration due to their zwitterion properties and their ability to bind free hydrogen and hydroxyl ions in the surrounding medium.

8.1.4 Non-Programmable and Programmable Slow Freezing

Slow freezing can be achieved without using a programmable freezer. Non-programmable freezing involves the gradual lowering of temperature from room temperature to -20°C and placing the semen sample in liquid nitrogen vapors at -80°C before plunging in LN_2 at -196°C . In rapid freezing, the semen samples are placed 15–20 cm above the LN_2 level for 15 min before coming in direct contact with LN_2 vapors. Programmable freezer allows the freezing to be controlled, and the cooling rate of $-5^{\circ}\text{C}/\text{min}$ to -80°C or -90°C is most frequently used, followed by immersion in LN_2 for storage.

8.1.5 Slow Freezing of Sperm

Conventional slow freezing is the most common method for freezing semen samples [37, 46]. In this method, the semen sample is mixed with the cryoprotectant such as glycerol and

an extender such as TEST-yolk buffer. The semen sample is mixed slowly in a dropwise manner with the cryoprotectant (1:1 vol:vol) at room temperature, placed in cryovials which are placed at -20°C for 8 min, and lowered in LN_2 vapor for 15 min before being plunged in LN_2 .

8.1.6 Cryopreservation of Sperm in Vapor and Liquid Phase of Liquid Nitrogen

Contamination of semen samples with infectious organisms such as hepatitis B which can survive in liquid nitrogen is a concern [47]. To prevent cross-contamination of semen samples, untested samples should be stored in separate containers. Also, straws or vials containing semen samples should be sealed and free from external contamination. Untested samples should be stored separate from samples that have been screened for infectious viruses and tested negative. One way to avoid the cross-contamination is the storage of samples in the vapor phase of liquid nitrogen. Studies have compared the effects of storage of semen samples from both normospermic and oligozoospermic as well as unwashed and washed semen samples in vapor phase and liquid nitrogen phase at -196°C . Greater decreases in motility were seen when samples were maintained at -70°C than those maintained at -196°C (47% vs. 39%) [48]. No differences were however seen in post-thaw sperm morphology at these temperatures. On the contrary, higher vitality and sperm motility recovery were reported in samples stored in vapor at -189°C [49]. This was attributed to lesser fluctuations in temperature between the vapor phase at -189°C and liquid phase at -196°C because of narrow intervals compared with -70°C and -196°C . This temperature of -186°C was maintained by keeping the samples at 15 cm height from the liquid nitrogen. Sperm can be stored for up to 3 months in vapor phase without significant effect on motility, viability, and morphology. DNA integrity and mitochondrial membrane potential are also similar for both vapor and LN_2 storage methods [50].

8.1.7 Advantages and Disadvantages of Slow Freezing

8.1.7.1 Advantages

Conventional or slow freezing is a standardized protocol that is approved by the Food and Drug Administration. It has been widely used in ART programs including infertility treatment and preservation of fertility in medical oncological and nononcological conditions. It is also cost-effective and does not involve expensive equipment, and as long as there

are viable spermatozoa available, ART can be used to achieve a pregnancy. Freezing of sperm also ensures the availability of sperm at the time of oocyte retrieval in ART avoiding the need for repeated surgical extraction procedures in azoospermic men.

8.1.7.2 Disadvantages

Slow freezing, either manual or automated, may cause extensive chemico-physical damage to the sperm. This is largely because of extensive ice crystallization and modification of the plasma, and mitochondrial membrane and modification in the mitochondrial membrane fluidity can result in peroxidative damage and DNA fragmentation [37, 51]. It is time consuming and has the risk of cross-contamination.

8.1.8 Strategies to Minimize Sperm Damage

Numerous studies show a decline in total motility, progressive motility, total motile count, total sperm count, viability, as well as morphology [52–55]. Due to increase in oxidative stress [56, 57], and reduced availability of antioxidants, sperm from infertile men [57] exhibit increased DNA damage after freezing [56]. Most of the damage occurs during the thawing process. Strategies aimed at reducing sperm damage include sperm preparation by swim-up before freezing helps improve sperm motility, viability, and number of non-apoptotic sperm [58]. Supplementation of the freezing medium with antioxidants is promising.

8.2 Conclusion

Authors' Perspectives

Slow cryopreservation has been successfully used for fertility preservation. It offers the ability for an infertile couple to have their own biological child. Although the sperm quality is poor after slow freezing, sperm frozen by slow freezing can be successfully used in ART.

Key Points

1. Cryopreservation of ejaculated spermatozoa can be successfully stored long-term using the slow-freezing technique.
2. Significant damage occurs as a result of the freezing process; good-quality spermatozoa can be used with ART especially with ICSI and result in successful pregnancies.
3. The methodology of freezing spermatozoa is simple and offers men the ability to father a biological child.

Potential Areas of Research

Newer techniques such as rapid freezing and vitrification are emerging as alternative methods that can freeze extremely low numbers of sperm. Improved protocol, methodology, and use of cryoprotectants that help minimize iatrogenic damage and result in higher sperm motility and membrane integrity are necessary to further improve the number of available sperm for achieving pregnancies.

Appendix I

CRYOPRESERVATION WORKSHEET

NAME: _____
MRN#: _____

FREEZE #: _____
COLOR: _____

DISPOSAL DATE: _____ SHIP DATE: _____
SERUM ACCESSION # _____ SERUM ALIQUOT LOCATION: _____

PRE-CRYOPRESERVATION

FILE # _____
SPECIMEN DATE: _____

SPECIMEN TYPE: _____
(i.e., fresh, epi aspiration, tissue)

COLLECTION TIME: _____
RECEIVED TIME: _____
ABSTINENCE TIME: _____
SPERM COUNT M/mL: _____
VOLUME mL: _____
TOTAL COUNT M: _____
PERCENT MOTILE: _____
VELOCITY μ /sec: _____
LINEARITY %: _____
ALH μ : _____

MOTILE SPERM M/mL: _____
CRYO MEDIA: _____ Lot # _____ Exp Date: _____
AMOUNT TYB ADDED mL: _____
NUMBER OF VALS ALIQUOTED: _____
VOLUME mL/VAL: _____
MOTILE SPERM M/VAL: _____
COMMENTS: _____

ROUND CELLS M/mL: _____
PRE-CYRO MOTILITY %: _____
PRE EN %: _____
ENDTZ M/mL: _____
TECH: _____

TIME: INTO VAPORS: _____ INTO LN₂: _____ LOCATION: _____

CRYOSURVIVAL

FILE # _____
THAW DATE: _____
THAW TIME: _____
% MOTILE: _____
VELOCITY μ /sec: _____
LINEARITY %: _____
ALH μ : _____
MOTILE SPERM M/mL: _____
MOTILE SPERM M/VAL: _____

% SURVIVAL: _____
TOTAL MOTILE M: _____
NO. OF INSEMS: _____
IVF ATTEMPTS: _____
COMMENTS: _____

POST EN %: _____
TECH: _____

VIAL LABEL CHECKED, TECH 1 : _____ DATE: _____
VIAL LABEL CHECKED, TECH 2 : _____ DATE: _____

Protocol for Human Sperm Slow Freezing

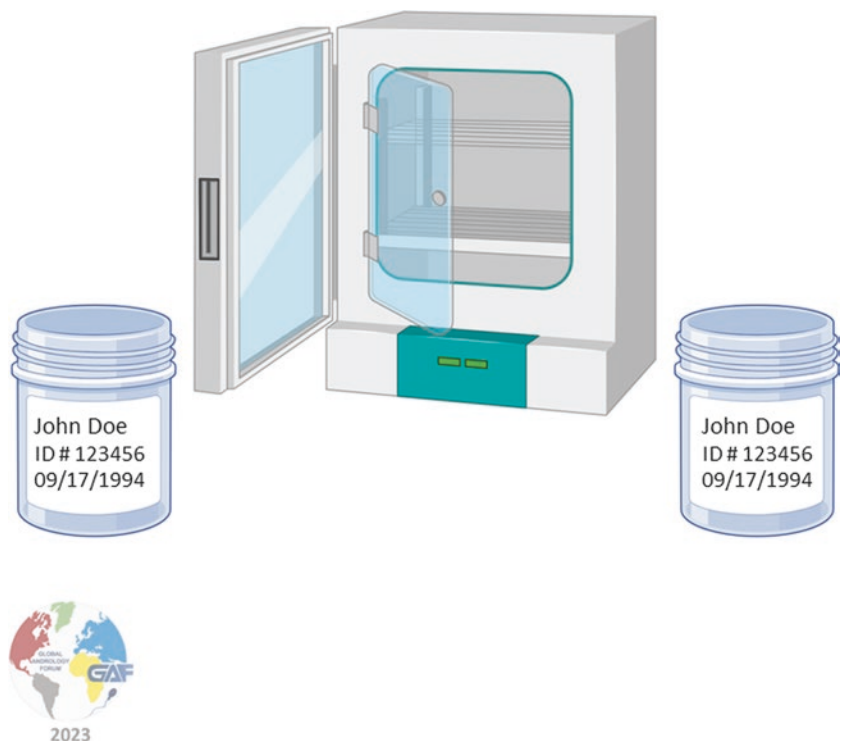
Step-by-Step Description on Performing Slow Freezing

Sterile technique should be used throughout specimen pro-

cessing. Gloves are mandatory for all procedures dealing with body fluids. Latex, however, can be toxic to sperm. Therefore, care should be taken to prevent contamination of the specimen with latex or talc, with preferably the latter being not used at all; instead, vinyl or nitrile gloves are recommended.

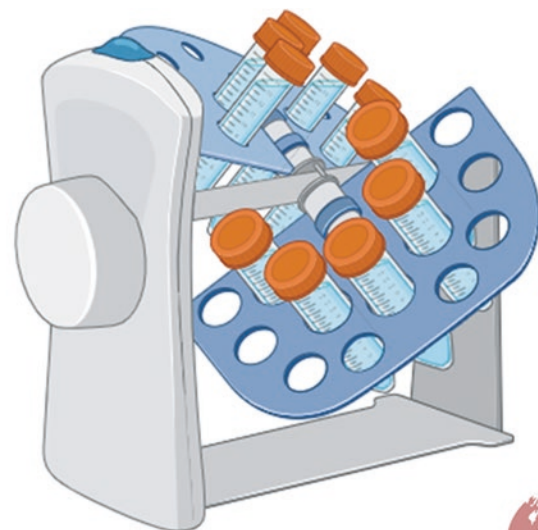
1. A bank file should be made for the client depositor. This consists of the following forms: (1). collection and storage agreement in duplicate and (2) sperm bank questionnaire and demographics consisting of patient information, date of birth, medical record number, age, address, and contact number. Person to be contacted and emergency contact number and contact information, diagnoses, reason for sperm storage, personal and sexual history, and any other document that is part of the legal contract for sperm banking are also required. The patient consult is done to explain the need for sperm cryopreservation and the process of sperm cryopreservation and utilization of the samples for later use with ART.
2. The patient/client depositor is then provided a sterile collection cup and asked to label the container with his name and collect by masturbation in the donor room adjacent to the laboratory.
3. After collection, the patient/client depositor is asked data about his abstinence time, method of collection, whether any specimen was lost during collection, and time of collection. The information should be recorded on the Cryopreservation Worksheet. The specimen is then accepted, labeled by the technologist, and placed in the 37 °C incubator for at least 20 min (Fig. 8.1). At this time, a bottle of TEST-yolk buffer with gentamicin is thawed in the 37 °C incubator for at least 20 min.
4. The patient/client depositor then signs the Semen ID Form in the presence of the technologist after checking the labeling of his vials. The lab technologist labeling the vials and witnessing the signature also signs this form. The cryovial label may not be altered, removed, or obscured after the patient checks the labeling of his vials. If the labeling is not acceptable to the patient, then the technologist should relabel the vials.
5. A positive identification is made by making a copy of the patient's driver's license.
6. Patient is provided instructions for a blood draw for testing of infectious disease, mainly HIV, Hep B, and Hep C. This is important for long-term storage of frozen samples once the report is confirmed to be negative for all the tests. If any of the results are positive, the samples are quarantined in a separate tank for 6 months. The physician is informed of the blood report as well.
7. After liquefaction, draw the semen specimen into a 2 mL sterile pipette, leaving at least 50 µL in the specimen container. Measure and record the volume, and note any unusual consistency on the patient's worksheet as well as the Cryopreservation Worksheet. Deliver the specimen into a labeled, sterile 15 mL conical tube that has been checked for cracks or defects. A moderate to highly viscous specimen can be further liquefied by pipetting up and down in the sterile pipette or adding viscosity treatment (VTS) when necessary.

Fig. 8.1 Collection container containing the semen sample is placed in an incubator set at 37 °C, which shows the collected semen sample for liquefaction



8. Analyze the remaining aliquot in the specimen container per the “Semen Analysis” protocol using the computer-assisted semen analyzer for concentration, motility, curvilinear velocity, linearity, and amplitude of lateral head movement and record on the patient’s worksheet. Presence of round cells in the specimen is quantified and an Endtz test conducted if ≥ 0.20 M/mL round cells are seen under wet preparation. At this point, any bacteria or foreign cells should be noted.
9. Within 1 h of specimen collection, add an aliquot of freezing medium equal to 25% of the original specimen volume to the centrifuge tube with a sterile pipette.
10. Gently rock the specimen with the freezing media for 5 min on a test tube rocker (Fig. 8.2).
11. Repeat steps 9 and 10 three times or until the volume of freezing media added is equal to the original specimen volume (Fig. 8.3).
12. During the mixing steps above, use appropriately colored cryomarkers to label the cryocanes (the labels should be labeled ahead of time so the patient may examine them).
13. Place preprinted labels such as Brady Labels on the cryovials. The label should contain last name, first name, medical record number, freeze number, and date of freezing, and label cells being frozen as “SPERM.”
14. Label the top of the cryocanes with client depositor’s last name and current freeze number with a cryomarker.
15. Label an additional 1.8 mL cryovial “Postthaw.” This cryovial will contain a small aliquot of 0.2 mL of the cryodiluted specimen to be assessed for cryosurvival 24 h after freezing. The top of the vial should be heavily marked with a black cryomarker to indicate that it is the post-thaw specimen vial.
16. A visual inspection should be performed for the cryodiluted specimen for motility. A manual motility should be done using a sperm counting chamber and phase microscope. The percent motility should be documented as “pre-cryo motility %.”
17. Evenly distribute the well-mixed, cryodiluted semen into the pre-labeled vials using a 2 mL sterile serological pipette (Fig. 8.4). Add at least 0.2 mL to the post-thaw cryovial.
18. Place labeled vials into the labeled cryocane(s). Place a maximum of two cryovials into top slots of canes while holding the canes upside-down (Fig. 8.5) (i.e., labeled portion of cryocanes should be facing the ground while placing the cryovials upright, with the orange top facing up). Cover cryocane(s) with cryosleeve(s), and place in the -20°C freezer for 8 min (Fig. 8.6). Do not open the freezer during this incubation. *Note:* Exposure to freezing conditions should occur within 1.5 h of specimen collection.
19. After the 8-min incubation, remove the cryocane(s) from the -20°C freezer. Place into an appropriate LN_2 vapor tank for a minimum of 2 h (Fig. 8.7). After 2 h, the vials are frozen by the liquid nitrogen vapors (-80°C) (Fig. 8.8). *Note:* Be sure that no less than 12 cm of LN_2 liquid fills the canister. Caution: Wear cryogloves and protective goggles.
20. After a minimum of 2-h incubation in the liquid nitrogen vapors (-80°C), turn the canes upside-down, immersing them into liquid nitrogen (-196°C).
21. After a minimum of 24 h in liquid nitrogen, thaw the aliquot in the 1.8 mL post-thaw cryovial as follows:

Fig. 8.2 Sample placed on a test tube rocker for 5 min after addition of TEST-yolk buffer



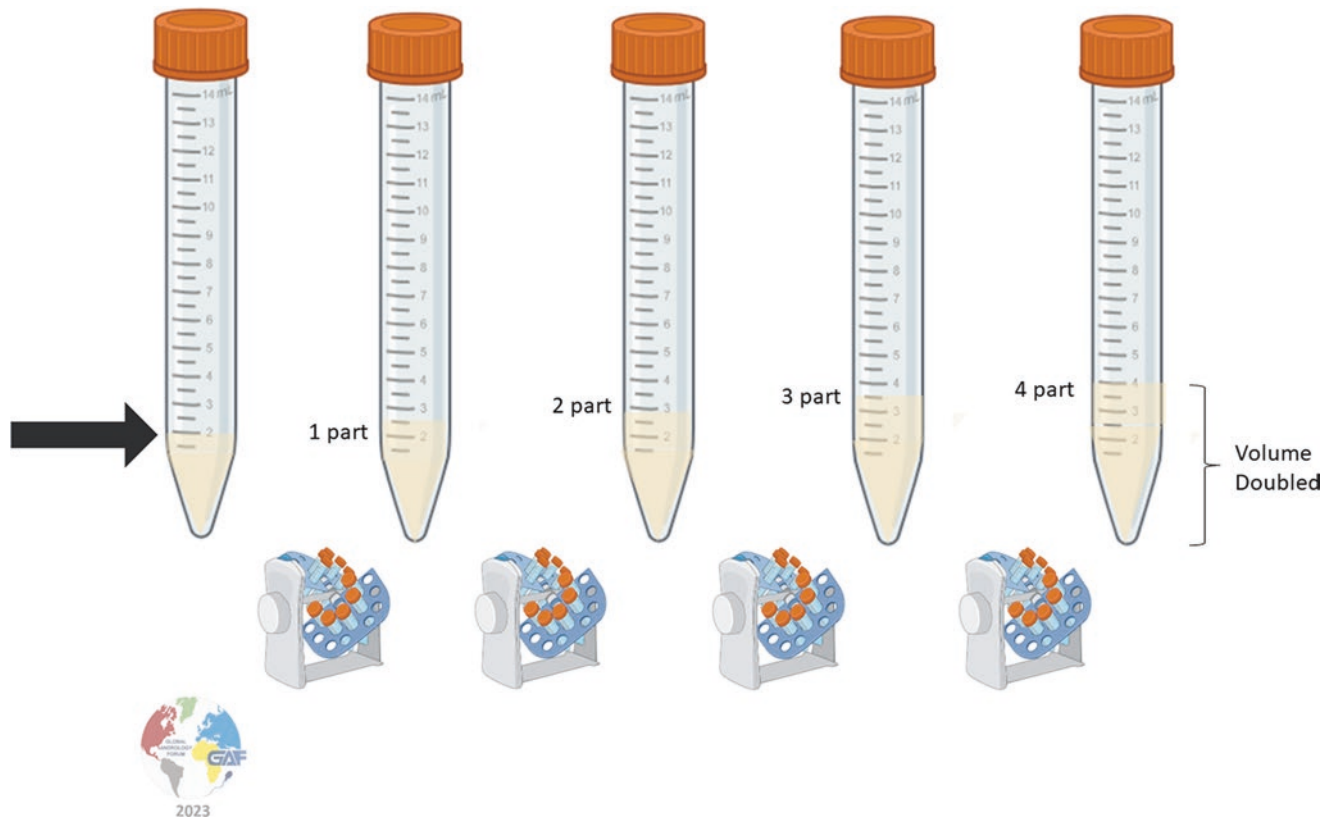


Fig. 8.3 Stepwise addition of TYB buffer to the semen sample. Volume of TYB equal to $\frac{1}{4}$ volume of patient sample is added four times, or until the total volume in the test tube has doubled

Fig. 8.4 Distribution of cryodiluted patient sample into cryovials using a sterile serological pipette





Fig. 8.5 Proper placement of cryovials into cryocanes

- Using cryogloves and protective goggles, remove cane containing the cryovial and snap it out. Loosen the cap and place in the 37 °C incubator for 20 min.
- Mix the vial well and analyze using the computer-assisted semen analyzer for count, motility, curvilinear velocity, linearity, and amplitude of lateral head movement.

22. Record results in the cryosurvival area of the Cryopreservation Worksheet. Assess cryosurvival using the following formula:

$$\frac{\% \text{motility of post-thaw specimen}}{\% \text{motility of pre-freeze specimen}}$$

23. Calculate the number of inseminations based on the following:
- 1 Insemination = 15–20 M.
 - 1/2 Insemination = 7.5–14.9 M.
 - 1/4 Insemination = 3.75–7.49 M.
 - 0 Insemination = <3.75 M.
24. Based on the number of available sperm, additional banks can be recommended.
25. The banked specimens are taken out of short-term storage, or quarantine, and transferred to the secured 17 K or 24 K freezer for long-term storage at –196 °C in liquid nitrogen.

Instruments/Disposables/Consumables/Components

- (a) Computer-assisted semen analyzer
- (b) Fixed cell sperm counting chamber
- (c) Automatic pipettor
- (d) Vortex
- (e) –20 °C Freezer.
- (f) LN₂ Dewar with 11" canisters
- (g) Sterile specimen container
- (h) Sterile 15 mL centrifuge tubes with screw caps
- (i) Sterile serological pipettes (2 mL capacity)
- (j) Sterile cryovials (1.8 mL capacity)
- (k) Colored cryomarkers
- (l) Test tube racks (for 15 mL test tubes)
- (m) Cryovial racks
- (n) Cryocanes
- (o) Plastic cryosleeves
- (p) Cryogloves
- (q) Protective goggles
- (r) Vinyl/nitrile gloves
- (s) 37 °C Incubator
- (t) LN₂
- (u) Viscosity treatment system (VTS)
- (v) Test tube rocker
- (w) Reagents

Freezing medium (TEST-yolk buffer with gentamicin sulfate; TYB). Each bottle is sterile and for one-time use.

Fig. 8.6 Cryovials are loaded on the cryocanes and inserted in a cryosleeve and placed upright in -20°C freezer for 8 min



Fig. 8.7 Loading cryocanes with cryovials into the cryotank canister





Fig. 8.8 Placing the canister with cryocanes in the liquid nitrogen vapor tank

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Vitrification of Human Spermatozoa: Effect of Cryoprotectants and Cooling Methods on Sperm Cryopreservation

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9.1 Introduction

Cryopreservation is a procedure to freeze several types of cells and tissues, documented since the early nineteenth century. Their use on the cryopreservation of human spermatozoa in the presence of cryoprotectants was first published in the late 1950s. Since those days, sperm cryopreservation became practical, widely used in assisted reproductive techniques (ARTs) and andrology laboratories. Their practical applications in humans include several possibilities such as infertility treatment, artificial insemination, in vitro fertilization, and use of frozen epididymal and testicular sperm samples with intracytoplasmic sperm injection (ICSI) treatments in obstructive and nonobstructive azoospermia patients.

Several cryopreservation protocols have been created looking for the best outcome to recover cells without cryo-damages and proper functional parameters such as sperm motility, viability, and DNA damage. Moreover, the conventional slow-freezing methods of human spermatozoa are widely used in ART, but the preferred expectations

have not been achieved. Overall, these methodologies promote dehydration of their intracellular water through cryoprotectants (CPA) to avoid ice formation and physical cell damage. However, these procedures imply an increased concentration of freezing media, triggering the possibility of osmotic shock and intracellular and extracellular ice formation, leading to mechanical damage and cell death.

Therefore, spermatozoa vitrification has been proposed as an alternative method, leading to the formation of a glass stage avoiding ice crystal formation instead of procedures that try to control a reduced ice formation as slow-freezing methods [1]. In our group, we confirm that permeable cryoprotectant-free vitrification preserves sperm quality better than slow freezing [2], and the vitrification method presented advantages in terms of the recovery rate of spermatozoa, acrosome intactness, and DNA fragmentation compared to slow freezing [1, 3, 4]. The vitrification technique is based on the ultrarapid cooling of human spermatozoa by directly into liquid nitrogen with or without the use of permeable CPA. In this sense, lethal intracellular ice crystal formation and harmful effects of high salt concentration are prevented, reducing the damage on spermatozoa functions [2, 5, 6], making this procedure much less labor-intensive, quicker, and simpler in application than conventional freezing. A systematic review and meta-analysis report compared the effectiveness of two techniques; they reviewed 2428 publications and 13 randomized controlled trials; 486 vitrified and conventional freezing human spermatozoa samples were included [7]. They found that vitrification was superior to conventional freezing regarding total and progressive motility in preserving spermatozoa [7]. Therefore, human spermatozoa vitrification demonstrated superior potential on sperm cryopreservation, but the procedure requires further optimization.

This chapter focuses on human spermatozoa vitrification, describing the current vitrification methodologies in use, advantages over other cryopreservation methods, and clinical application.

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9.2 Effect of Cryoprotectants on Sperm Cryopreservation

Cryoprotectants act by depressing the freezing point and binding intracellular water to prevent ice formation and reduce the cryo-damages [8]. Importantly, the cryoprotectant should be nontoxic to the cells during freezing and thawing. There are two main types of cryoprotectants (CPAs): permeable and non-permeable. Permeable CPAs include dimethyl sulfoxide, glycerol, ethylene glycol, and methanol; non-permeable CPAs include albumins and dextrans and egg yolk citrate, and sugars such as raffinose and sucrose [8].

9.2.1 Permeable Cryoprotectants and Non-permeable Cryoprotectants

Permeable CPAs reduce the concentrations of electrolytes present in the liquid phase of the sample and stabilize cell plasma membrane, popularly used including DMSO and glycerol; non-permeable cryoprotectants reduce intracellular crystallization, increasing the viscosity of the sample to protect the plasma membrane from extracellular damage [9]. Although CPA is important to prevent ice crystallization and cell damage, it would be toxic at high concentrations, and spermatozoa are vulnerable to osmotic changes.

The effectiveness of permeable CPAs for preventing intracellular ice formation during conventional slow freezing could only be achieved with a slow cooling rate, which might be damaged by itself [10]. The mechanical damages caused by the slow freezing result from intracellular or extracellular ice crystal formation and the osmotic damage, extensive cell shrinkage. The subsequent warming and thawing of the cells could further deteriorate their viability through possible excessive osmotic swelling and diminished antioxidant defense activity [10, 11]. Moreover, it would cause chemical and physical damage to sperm cell membranes due to alterations in lipid-phase transition or increased lipid peroxidation. The production of reactive oxygen species (ROS) leads to increased lipid peroxidation after freezing, which is also associated with a loss of sperm motility [12, 13]. All these negative effects of conventional freezing during the ice equilibrium process on cells could also lead to chromatin damage, and the chromatin abnormalities could damage sperm quality and male fertility status [14].

Therefore, the method of permeable cryoprotectant-free vitrification was developed. The breakthrough of human spermatozoa vitrification without permeable CPA was first reported by Nawroth et al. [15]. The combination of extremely high rates of cooling/warming and utilization of vitrification media containing proteins and polysaccharides made it feasible to avoid devitrification during warming without using the high-concentration CPAs.

Non-permeable cryoprotectants (carbohydrates like glucose, sucrose, and trehalose) could not pass through cell

membranes for the high molecular weight [16, 17]. Instead of the cytotoxicity of high concentrations of permeable CPAs, non-permeable CPAs could effectively promote rapid cell dehydration even with high freezing speed to avoid ice formation and stabilize proteins and cell membranes [2, 18]. Sucrose plays an important role in compensating the osmotic pressure and stabilizing the cell membrane during vitrification [19–21]. In our group, we first achieved CPA-free vitrification by human tubular fluid (HTF) medium supplemented with 1% human serum albumin (HSA) and 0.25 M sucrose as non-permeable cryoprotectants. It was shown that cryoprotectant-free vitrification of human spermatozoa with 0.25 M sucrose could efficiently cryopreserve the cells without significant loss of crucial physiological parameters we researched for the CPA-free vitrification technology of human spermatozoa [1]. A recent scientific report by Schulz et al. also reviewed some studies and concluded that vitrification techniques have demonstrated the preservation of selected spermatozoa without seminal plasma and with non-permeable cryoprotectants and protein [22].

Overall, the cryoprotectant-free vitrification technique avoids the additional negative impacts caused by the addition/removal of high-concentration permeable CPAs in conventional freezing.

9.2.2 The Vitrification of Human Spermatozoa: Permeable Cryoprotectant-Free Versus Cryoprotectant-Included Technologies

The effectiveness of the cryoprotectant-free vitrification process for human spermatozoa is well documented [1, 23–25]. These studies performed vitrification by direct plunging of small-volume spermatozoa into LN₂, from 1 to 10 μ L [23] diluted in 0.25 M sucrose medium, and from 20 to 30 μ L [1] by direct dropping into liquid nitrogen. In the absence of permeable CPA by decreasing the volume of the cryopreserved sample, increase the surface-to-volume ratio of the sample to achieve ultrarapid rates of vitrification [18, 26]. We also developed the aseptic technology of vitrification of human spermatozoa in a large volume (100 μ L) for intrauterine insemination [27] and capillaries for intracytoplasmic sperm injection [28]. After that, the birth of healthy children after ICSI and after intrauterine insemination confirms the ability of cryoprotectant-free vitrification to protect cells from lethal cryo-damage [29, 30].

Recently, human spermatozoa vitrification by direct plunging into liquid nitrogen in the presence of commercial cryoprotectant has been achieved. Berkovitz et al. [31] and Belenky et al. [32] have reported the study using Quinn's sperm freezing medium for spermatozoa vitrification. The studies reported that single motile spermatozoa from patients with nonobstructive azoospermia were transferred to 0.8 μ L

droplets of spermatozoa suspension that included sperm freezing media and plunged into LN₂; after thawing, high rates of recovery (96% and 99%, respectively), motility (33% and 37%, respectively), fertilization (59%), clinic pregnancies (55%), and delivery rate (32%) were established. More importantly, the freezing medium contains glycerol as the cryoprotectant. Glycerol is one of the four most popular permeable cryoprotectants for the cryopreservation of human spermatozoa. Additionally, the vitrification technique has also been successfully performed on animal spermatozoa in the presence of permeable CPAs [33–35].

However, Le et al. [36] and Agha-Rahimi et al. [37] used sperm freeze solution (Vitrolife) containing glycerol to play the protection role of permeable cryoprotectant during freezing, and no significant differences were found in post-thaw spermatozoa parameters between vitrification subgroups (with/without permeable cryoprotectant). Therefore, the presence or absence of permeable cryoprotectants on human spermatozoa vitrification remains controversial.

Specifically, we test the aseptic capillary vitrification technology using permeable cryoprotectant-included and cryoprotectant-free media to compare the cryoprotective efficacy of CPA for human spermatozoa preservation, and the results have been published [38]. In our study, one control (fresh spermatozoa) and three treatment groups were formed as follows: cryoprotectant-free (group 1), culture medium with 5% dextran serum supplement (DSS); cryoprotectant-free (group 2), culture medium with 5% DSS and 0.25 M sucrose; and cryoprotectant-included (group 3), culture medium with 5% DSS and 7.5% glycerol. Progressive motility, plasma membrane integrity, and total motility were determined after thawing. It was shown that progressive motility of spermatozoa in groups 1, 2, and 3 was $24.9 \pm 1.7\%$, $34.5 \pm 2.8\%$, and $34.0 \pm 1.4\%$, respectively ($P_{1-2,3} < 0.01$), and motility rate in all three treatment groups was lower than that in fresh spermatozoa ($68.8 \pm 5.8\%$, $P < 0.01$). The plasma membrane integrity rates of spermatozoa in groups 1, 2, and 3 were $33.3 \pm 2.1\%$, $48.4 \pm 2.9\%$, and $45.5 \pm 3.9\%$ ($P_{1-2,3} < 0.05$) with decrease in group 3 in comparison with group 2 ($P_{2-3} > 0.1$). After 24-h, 48-h, and 72-h in vitro culture, the total motility and viability of spermatozoa in group 1 were significantly lower than groups 2 and 3. The rates of apoptosis and mitochondrial membrane potential in groups 3 and 2 were all higher than those in group 1. However, there were no significant differences for these parameters between groups 3 and 2.

All those results support that glycerol and sucrose could preserve human spermatozoa from cryo-damages. Our data have shown that the rates of progressive motility, total motility, and plasma membrane integrity after vitrification in group 2 (with sucrose) were nonsignificantly higher than in the supplementation of the glycerol group. During the long-time in vitro culture, we have observed that the investigated physiological parameters of warmed spermatozoa in group 2 (with sucrose) have a tendency to be increased compared

with spermatozoa of group 3 (with glycerol). However, the toxic effect of cryoprotectant was observed during in vitro culture. We detected that the survival time of spermatozoa vitrified with the commercial freezing medium was significantly less than that in sucrose medium, which might be because of the lethal impact of osmotic shock and toxicity caused by permeable cryoprotectant.

Finally, we conclude that the supplementation of medium for the aseptic capillary technology for human spermatozoa cryoprotectant-free vitrification by permeable cryoprotectant glycerol does not improve the post-thawed spermatozoa quality. However, the main limitation of this study was the use of the commercial sperm freeze medium (FertiPro) instead of pure glycerol. And the presented results also supported a hypothesis: an adequate but tolerable (subtoxic) concentration of glycerol for human spermatozoa vitrification might be feasible.

9.3 Proteomic Profile Analysis Revealed Alterations in Key Proteins During Vitrification of Human Spermatozoa

Our research group and others have worked for several years on the implementation of human sperm vitrification without the use of permeable cryoprotectants. However, no one studied subcellular changes in spermatozoa after cryopreservation similar to conventional slow-freezing method. The mature spermatozoa mainly rely on posttranslational modifications as it is to be believed that spermatozoa are transcriptionally and translationally inactive. However, some recent literature indicates that mature spermatozoa are capable of using mitochondrial RNA transcripts for protein translation during the final maturation steps before fertilization [39, 40]. Another possible mechanism might be posttranslational modifications during the cryopreservation process. Moreover, it is widely reported that the conventional slow freezing adversely affects cytoskeleton proteins (actins, tubulins, outer dense fiber, and many more), heat-shock proteins, enzymes responsible for tyrosine/serine/threonine phosphorylation, ubiquitin-proteasomal system, and some recently discovered promising candidate proteins (like IZUMO, GLIPR1-like protein 1, ADAM, SPACA, and many more) [41–44].

Unfortunately, there is no such study conducted for vitrified sperm. Therefore, we do not know exactly what happened to the proteins responsible for sperm functions like motility, capacitation, and fertilization after sperm vitrification. Our previous study addressed the question by label-free quantitative proteomics analysis to reveal the key proteins altered during the vitrification process [45]. Additionally, our study also attempted to investigate the effectiveness of supplementation of the mitochondria-targeted antioxidant MitoQ and glycerol on ameliorating the spermatozoa vitrification process.

Our results showed that a total of 1759 proteins were detected in human spermatozoa, and from volcano plot results, we found that a total of 118 proteins were in low abundance and 21 proteins were in high abundance after vitrification. We classified identified proteins into the most important groups like cytoskeleton proteins (actin, tubulin, ODF) as well as proteins involved in phosphorylation and sperm-egg interaction. According to our study, the actin and actin-associated proteins influence a wide range of functions in the spermatozoa cell, including shape, movement, and interactions with extracellular matrices. We identified several actin and actin-binding proteins (actin, cytoplasmic actin 2, alpha-actinin-1, alpha-actinin-4, CAPZ family, and more) in the spermatozoa, but none of these is affected by the vitrification process, but slow conventional freezing processes resulted in the loss/damage of cytoskeleton actin in different species [45]. Tubulin is the protein that polymerizes into long chains or filaments and forms microtubules surrounding the axoneme of the spermatozoa tail. Several tubu-

lins were identified, tubulin alpha-1A chain, tubulin alpha-1B chain, tubulin alpha-1C chain, tubulin alpha-3E chain, tubulin alpha-4A chain, tubulin beta chain, tubulin beta-2A chain, tubulin beta-4B/4A chain, tubulin beta-8 chain, and TPPP2. Interestingly, none of these was affected during vitrification according to our results [45]. Phosphorylation/dephosphorylation is a dynamic process involving protein kinases and protein phosphatases. Several kinases and phosphatases have been identified in the spermatozoa, some of which are significantly altered during vitrification (Table 9.1) [45]. Several heat-shock proteins were also identified in human spermatozoa and were not affected during vitrification, except the DnaJ homolog (Table 9.2). One of the most important findings is that several candidate proteins (IZUMO proteins, GLIPR1-like protein, ADAM, Tektin, and more) involved in spermatozoa-egg interactions were also identified in the spermatozoa, but none of these was damaged during spermatozoa vitrification (Table 9.3), unlike the slow-freezing process [45]. Therefore, actin, tubulins, and HSPs were not

Table 9.1 List of identified proteins in sperm responsible for phosphorylation (kinases) and dephosphorylation (phosphatases) and showing differentially expressed proteins after vitrification

Protein IDs	Gene names	Protein names	Change in levels
Q14409	GK3P	Putative glycerol kinase 3	NS
O00764	PDXK	Pyridoxal kinase	NS
P14618	PKM	Pyruvate kinase PKM	NS
P54819	AK2	Adenylate kinase 2	↓MitoQ/Fresh
Q96M32	AK7	Adenylate kinase 7	NS
Q96MA6	AK8	Adenylate kinase 8	NS
P00568	AK1	Adenylate kinase isoenzyme 1	NS
Q86UN6	AKAP14	A-kinase anchor protein 14	NS
O75969	AKAP3	A-kinase anchor protein 3	NS
Q5JQC9	AKAP4	A-kinase anchor protein 4	NS
P17858	PFKL	ATP-dependent 6-phosphofructokinase, liver type	NS
P08237	PFKM	ATP-dependent 6-phosphofructokinase, muscle type	NS
Q01813	PFKP	ATP-dependent 6-phosphofructokinase, platelet type	NS
Q3LXA3	DAK	Bifunctional ATP-dependent dihydroxyacetone kinase/FAD-AMP lyase (cyclizing); ATP-dependent dihydroxyacetone kinase; FAD-AMP lyase (cyclizing)	NS
Q13557	CAMK2D	Calcium/calmodulin-dependent protein kinase type II subunit delta	NS
Q16566	CAMK4	Calcium/calmodulin-dependent protein kinase type IV	↓MitoQ-Gly/Fresh
O75952	CABYR	Calcium-binding tyrosine phosphorylation-regulated protein	NS
P17612	PRKACA	cAMP-dependent protein kinase catalytic subunit alpha	NS
P22612	PRKACG	cAMP-dependent protein kinase catalytic subunit gamma	NS
P10644	PRKAR1A	cAMP-dependent protein kinase type I-alpha regulatory subunit or N-terminally processed	NS
P13861	PRKAR2A	cAMP-dependent protein kinase type II-alpha regulatory subunit	NS
P48729	CSNK1A1	Casein kinase I isoform alpha	NS
P19784	CSNK2A2	Casein kinase II subunit alpha	NS
P68400	CSNK2A1	Casein kinase II subunit alpha; casein kinase II subunit alpha 3	NS
P67870	CSNK2B	Casein kinase II subunit beta	NS
Q9Y259	CHKB	Choline/ethanolamine kinase	↓MitoQ/Fresh ↓Glycerol/Fresh
O14578	CIT	Citron Rho-interacting kinase	↑Glycerol/Fresh

Table 9.1 (continued)

Protein IDs	Gene names	Protein names	Change in levels
P12277	CKB	Creatine kinase B-type	NS
Q16854	DGUOK	Deoxyguanosine kinase, mitochondrial	NS
P36507	MAP2K2	Dual-specificity mitogen-activated protein kinase kinase 2	NS
Q14410	GK2	Glycerol kinase 2	NS
P49840	GSK3A	Glycogen synthase kinase-3 alpha	NS
P19367	HK1	Hexokinase-1	NS
O75569	PRKRA	Interferon-inducible double-stranded RNA-dependent protein kinase activator A	NS
P29966	MARCKS	Myristoylated alanine-rich C-kinase substrate	NS
Q9UJ70	NAGK	N-acetyl-D-glucosamine kinase	NS
Q13232	NME3	Nucleoside diphosphate kinase 3	NS
Q9Y5B8	NME7	Nucleoside diphosphate kinase 7	NS
P15531	NME1	Nucleoside diphosphate kinase A	NS
P22392	NME2	Nucleoside diphosphate kinase B; putative nucleoside diphosphate kinase	↓Glycerol/Fresh
P56597	NME5	Nucleoside diphosphate kinase homolog	NS
Q8TBX8	PIP4K2C	Phosphatidylinositol 5-phosphate 4-kinase type-2 gamma	NS
P00558	PGK1	Phosphoglycerate kinase 1	NS
P07205	PGK2	Phosphoglycerate kinase 2	NS
Q93100	PHKB	Phosphorylase b kinase regulatory subunit beta	NS
P07311	ACYP1	Acylphosphatase-1	NS
Q9UKK9	NUDT5	ADP-sugar pyrophosphatase	NS
P34913	EPHX2	Bifunctional epoxide hydrolase 2; cytosolic epoxide hydrolase 2; lipid-phosphate phosphatase	NS
P50583	NUDT2	Bis(5-nucleosyl)-tetrakisphosphate [asymmetrical]	NS
Q9UHY7	ENOPH1	Enolase-phosphatase E1	NS
P09467	FBP1	Fructose-1,6-bisphosphatase 1	NS
Q15181	PPA1	Inorganic pyrophosphatase	NS
A0A024RBG1	NUDT4	Inositol monophosphatase 1	NS
P29218	IMPA1	Inositol monophosphatase 1	NS
P24666	ACP1	Low-molecular-weight phosphotyrosine protein phosphatase	NS
Q9UNW1	MINPP1	Multiple inositol polyphosphate phosphatase 1	NS
A6NDG6	PGP	Phosphoglycolate phosphatase	NS
Q9H008	LHPP	Phospholysine phosphohistidine inorganic pyrophosphate phosphatase	NS
P15309	ACPP	Prostatic acid phosphatase; PAPF39	NS
Q7Z5V6	PPP1R32	Protein phosphatase 1 regulatory subunit 32	NS
Q15435	PPP1R7	Protein phosphatase 1 regulatory subunit 7	NS
P35813	PPM1A	Protein phosphatase 1A	NS
Q9Y570	PPME1	Protein phosphatase methylesterase 1	NS
O14990	PPP1R2P9	Putative type-1 protein phosphatase inhibitor 4	NS
Q12913	PTPRJ	Receptor-type tyrosine-protein phosphatase eta	NS
P10586	PTPRF	Receptor-type tyrosine-protein phosphatase F	NS
Q13332	PTPRS	Receptor-type tyrosine-protein phosphatase S	NS
P30153	PPP2R1A	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	NS
Q15257	PPP2R4	Serine/threonine-protein phosphatase 2A activator	NS
P67775	PPP2CA	Serine/threonine-protein phosphatase 2A catalytic subunit alpha isoform	NS
Q08209	PPP3CA	Serine/threonine-protein phosphatase 2B catalytic subunit alpha isoform	NS
P48454	PPP3CC	Serine/threonine-protein phosphatase 2B catalytic subunit gamma isoform	NS
P60510	PPP4C	Serine/threonine-protein phosphatase 4 catalytic subunit	NS
Q8TF05	PPP4R1	Serine/threonine-protein phosphatase 4 regulatory subunit 1	NS
P53041	PPP5C	Serine/threonine-protein phosphatase 5	NS
O00743	PPP6C	Serine/threonine-protein phosphatase 6 catalytic subunit	NS
O15084	ANKRD28	Serine/threonine-protein phosphatase 6 regulatory ankyrin repeat subunit A	NS

(continued)

Table 9.1 (continued)

Protein IDs	Gene names	Protein names	Change in levels
Q9UPN7	PPP6R1	Serine/threonine-protein phosphatase 6 regulatory subunit 1	↓BM/Fresh ↓MitoQ/Fresh ↓Glycerol/Fresh ↓MitoQ-Gly/Fresh
O75170	PPP6R2	Serine/threonine-protein phosphatase 6 regulatory subunit 2	↓BM/Fresh
Q9BRF8	CPPED1	Serine/threonine-protein phosphatase CPPED1	NS
Q96HS1	PGAM5	Serine/threonine-protein phosphatase PGAM5, mitochondrial	NS
P62140	PPP1CB	Serine/threonine-protein phosphatase PP1-beta catalytic subunit	NS
P36873	PPP1CC	Serine/threonine-protein phosphatase PP1-gamma catalytic subunit	NS
O14829	PPEF1	Serine/threonine-protein phosphatase with EF-hands 1	NS
Q9Y3F4	STRAP	Serine-threonine kinase receptor-associated protein	NS
Q9BU02	THTPA	Thiamine-triphosphatase	NS
Q9NRX4	PHPT1	14 kDa phosphohistidine phosphatase	NS

NS Not significant, BM basal medium, Gly glycerol, ↓ Decreased in change levels, ↑ Increased

Table 9.2 List of heat-shock proteins identified in human sperm and showing differentially expressed proteins after vitrification

Protein IDs	Gene names	Protein names	Change in levels
P61604	HSPE1	10 kDa heat-shock protein, mitochondrial	NS
P10809	HSPD1	60 kDa heat-shock protein, mitochondrial	NS
O95433	AHSA1	Activator of 90 kDa heat-shock protein ATPase homolog 1	NS
P48723	HSPA13	Heat-shock 70 kDa protein 13	NS
P0DMV9	HSPA1B	Heat-shock 70 kDa protein 1B	NS
P34931	HSPA1L	Heat-shock 70 kDa protein 1-like	NS
P34932	HSPA4	Heat-shock 70 kDa protein 4	NS
O95757	HSPA4L	Heat-shock 70 kDa protein 4L	NS
P11142	HSPA8	Heat-shock cognate 71 kDa protein	NS
Q92598	HSPH1	Heat-shock protein 105 kDa	NS
Q12931	TRAP1	Heat-shock protein 75 kDa, mitochondrial	NS
P04792	HSPB1	Heat-shock protein beta-1	NS
P07900	HSP90AA1	Heat-shock protein HSP 90-alpha	NS
P08238	HSP90AB1	Heat-shock protein HSP 90-beta	NS
P54652	HSPA2	Heat-shock-related 70 kDa protein 2	NS
Q58FF8	HSP90AB2P	Putative heat-shock protein HSP 90-beta 2	↓NS
P31689	DNAJA1	DnaJ homolog subfamily A member 1	BM/Fresh
O60884	DNAJA2	DnaJ homolog subfamily A member 2	NS
Q8WW22	DNAJA4	DnaJ homolog subfamily A member 4	NS
P25685	DNAJB1	DnaJ homolog subfamily B member 1	NS
Q9UBS4	DNAJB11	DnaJ homolog subfamily B member 11	NS
P59910	DNAJB13	DnaJ homolog subfamily B member 13	NS
O75190	DNAJB6	DnaJ homolog subfamily B member 6	NS
Q8NHS0	DNAJB8	DnaJ homolog subfamily B member 8	NS
Q9UBS3	DNAJB9	DnaJ homolog subfamily B member 9	NS
Q13217	DNAJC3	DnaJ homolog subfamily C member 3	NS
Q9H3Z4	DNAJC5	DnaJ homolog subfamily C member 5	NS

NS Not significant, BM basal medium, ↓ Decreased in change levels

affected during the vitrification process. Only a few proteins responsible for phosphorylation were altered during vitrification. Similarly, several proteins involved in spermatozoa-egg fusion and fertilization were not affected during the vitrification process.

In our study, our results confirmed that MitoQ attenuates the vitrification-induced ultrastructural changes and alterations in the key proteins involved in spermatozoa functions

and fertilization, and we were able to explain a long-awaited answer to the question “why is glycerol not suitable for sperm vitrification.”

The results of investigations described in this work are reflected in a number of conclusions, the introduction of which to medical practice can improve the existing technology for cryoprotectant-free vitrification of human spermatozoa.

Table 9.3 List of candidate proteins identified responsible for sperm–egg fusion and fertilization but not affected by vitrification process

Protein IDs	Gene names	Protein names	Change in levels
Q8IYV9	IZUMO1	Izumo sperm–egg fusion protein 1	NS
Q6UXV1	IZUMO2	Izumo sperm–egg fusion protein 2	NS
Q1ZYL8	IZUMO4	Izumo sperm–egg fusion protein 4	NS
Q6UWM5	GLIPR1L1	GLIPR1-like protein 1	NS
P12830	CDH1	Cadherin-1; E-Cad/CTF1; E-Cad/CTF2; E-Cad/CTF3	NS
P07237	P4HB	Protein disulfide-isomerase	NS
P30101	PDIA3	Protein disulfide-isomerase A3	NS
P13667	PDIA4	Protein disulfide-isomerase A4	NS
Q15084	PDIA6	Protein disulfide-isomerase A6	NS
Q8N807	PDILT	Protein disulfide-isomerase-like protein of the testis	NS
P61626	LYZ	Lysozyme C	NS
Q6UWQ5	LYZL1	Lysozyme-like protein 1	NS
Q7Z4W2	LYZL2	Lysozyme-like protein 2	NS
Q96KX0	LYZL4	Lysozyme-like protein 4	NS
O75951	LYZL6	Lysozyme-like protein 6	NS
Q9UHI8	ADAMTS1	A disintegrin and metalloproteinase with thrombospondin motifs 1	NS
O14672	ADAM10	Disintegrin and metalloproteinase domain-containing protein 10	NS
Q8TC27	ADAM32	Disintegrin and metalloproteinase domain-containing protein 32	NS
Q9H2U9	ADAM7	Disintegrin and metalloproteinase domain-containing protein 7	NS
Q13443	ADAM9	Disintegrin and metalloproteinase domain-containing protein 9	NS
Q9UHI8	ADAMTS1	A disintegrin and metalloproteinase with thrombospondin motifs 1	NS
O14967	CLGN	Calmeglin	NS
P27797	CALR	Calreticulin	NS
P38567	SPAM1	Hyaluronidase PH-20	NS
Q969V4	TEKT1	Tektin-1	NS
Q9UIF3	TEKT2	Tektin-2	NS
Q9BXF9	TEKT3	Tektin-3	NS
Q8WW24	TEKT4	Tektin-4	NS
Q96M29	TEKT5	Tektin-5	NS
Q9BS86	ZPBP	Zona pellucida-binding protein 1	NS
Q6X784	ZPBP2	Zona pellucida-binding protein 2	NS
P00441	SOD1	Superoxide dismutase [Cu-Zn]	NS
P04179	SOD2	Superoxide dismutase [Mn], mitochondrial	NS
P04040	CAT	Catalase	NS

9.4 Aseptic Cooling Methods

Most of the proposed vitrification methods to obtain ultrarapid cooling rates are performed in open systems by plunging into liquid nitrogen, such as cryoloop, which would bring potential contamination risks as open systems expose the samples directly to LN₂. It is important to explore an aseptic cooling method to cryopreserve sperm cells. Therefore, our group carried out the aseptic vitrification technology and focused on microdrop methods and capillary-in-straw vitrification.

9.4.1 Microdrop Vitrification

The effectiveness of cryoprotectant-free vitrification was discovered by directly dropping small droplets of human spermatozoa suspension into liquid nitrogen [1]. In 2008, our group suggested the vitrification method that solved the

problem of tiny-volume sperm cryopreservation. In this method, 30 µL of sperm suspension was directly dropped in liquid nitrogen and collected in cryotubes for storing. However, one of the problems with this technique is that complete sterilization of commercially produced liquid nitrogen is impossible, and cryopreservation of non-isolated biological objects (with direct exposure to LN₂) would take potential risks of contamination by viruses, bacteria, fungi, and spores. Therefore, to avoid the potential risks of contamination during cryopreservation, a benchtop device for the production of sterile liquid air was developed. The CLAiR device was designed to prevent possible microbial contamination caused by commercially produced LN₂ for cell cryopreservation [46]. The device we used can produce clean liquid air (CLAiR, FertileSAFE, Ness Ziona, Israel) with a similar temperature (−195.7 °C) to LN₂. The temperature measurements, oxygen percentage measurements, and bioburden tests were performed on the liquid air, which indicated that the produced liquid air has the same properties as

LN₂ and could be adopted in the assisted treatment laboratory to eliminate potential contaminations. Arav et al. [46] and Isachenko et al. [47] reported using the device for the safety vitrification on human oocytes and mice embryos.

In addition, our group also achieved the vitrification of human spermatozoa by directly dropping human spermatozoa suspension into liquid air. According to our previous report [48], using a micro-pipettor at a distance of 10 cm from the respective liquid agent, 20 µL aliquots of spermatozoa suspension were dropped directly into this cooling agent. A sphere immediately formed and floated to the surface. After 10 s in liquid air, the sphere solidified and fell to the bottom of the strainer. After solidification, the spheres were collected, packaged into 1.8 mL cryotubes, and stored for at least 24 h in a tank with liquid nitrogen before warming. After warming, we found that the percentages of “progressive motility,” “viability,” and “apoptosis” of sperm suspension that dropped into LN₂ and liquid air were similar. These results suggested that cryoprotectant-free vitrification by direct dropping of human spermatozoa into a clean cooling agent (liquid air) is a good alternative to nonsterile liquid nitrogen, thereby minimizing the risk of microbial contamination.

9.4.2 Capillary-in-Straw Vitrification

The method was carried out in double straws packaged by a closed system. One filled sperm suspension straw inside another straw could protect against the cell suspension from bonding directly with the liquid nitrogen because it is wholly sealed, making it completely aseptic.

For the closed system, different kinds of packaging systems and devices were applied: the OPS [23], ICSI pipettes [49], nylon cryoloops [50], CryoTop [24], hollow-core agarose capsules [51], and micro-straw [52]. However, there were no standard tools with the uniform diameter and acceptable material presented in sperm vitrification. In 2012, Isachenko et al. [28] modified this method using commercial capillary in the packaging system for spermatozoa vitrification.

In 2021, we improved the aseptic capillary-in-straw method using the innovative manipulation microtool with a 600 µm diameter pipette (Vitromed GmbH, Jena, Germany), which can be the alternative capillary as reported by Isachenko et al. [28]. These capillaries are manufactured from hydrophobic plastic that prevents the capillary effect when immersed in a solution. That peculiarity of plastic prevents still-frozen samples inside the capillary from floating up to over the level of warming medium. The standard capillaries were supplied by industrial manufacturers. Commercial suppliers offer plastic capillaries of regular cylinder shape with stable (fixed) diameter for medical applications, which

made it possible to standardize the vitrification process using these standard capillaries.

The aseptic capillary vitrification procedure was performed as previously reported and illustrated in Fig. 9.1 [38]. Before the manipulation, the one end of all capillaries was labeled on top to mark the “cutoff” position (Fig. 9.1, solid arrows). In brief, 10 µL of spermatozoa suspension was aspirated with a micro-pipettor (Eppendorf, Hamburg, Germany) and dropped into a 35 × 10 mm Falcon petri dish (Life Sciences, Durham, USA). Next, the capillaries one by one were filled with 10 µL of spermatozoa suspension by IVF pipette controller (Medical Technology GmbH, Bruckberg, Germany), and the position was marked (Fig. 9.1, empty arrows). Next, the filled capillary was inserted into a 0.25 mL straw (Medical Technology GmbH, Bruckberg, Germany). One end of this straw was sealed in advance using a heat sealer (Cryo Bio System, Paris, France), and after sealing another end of the straw, the packaged capillary-in-straw system was directly plunged into liquid nitrogen (Fig. 9.1c). It was stored in liquid nitrogen for at least 24 h before warming. For warming, the upper part of the straw was cut off just above the mark position, without touching the end of the capillary (Fig. 9.1d). One capillary was transferred and immersed into 2 mL of pre-warmed HTF-HSA medium at 42 °C for 15 s (Fig. 9.1e). The sperm suspension was immediately expelled from the capillary for the next evaluation.

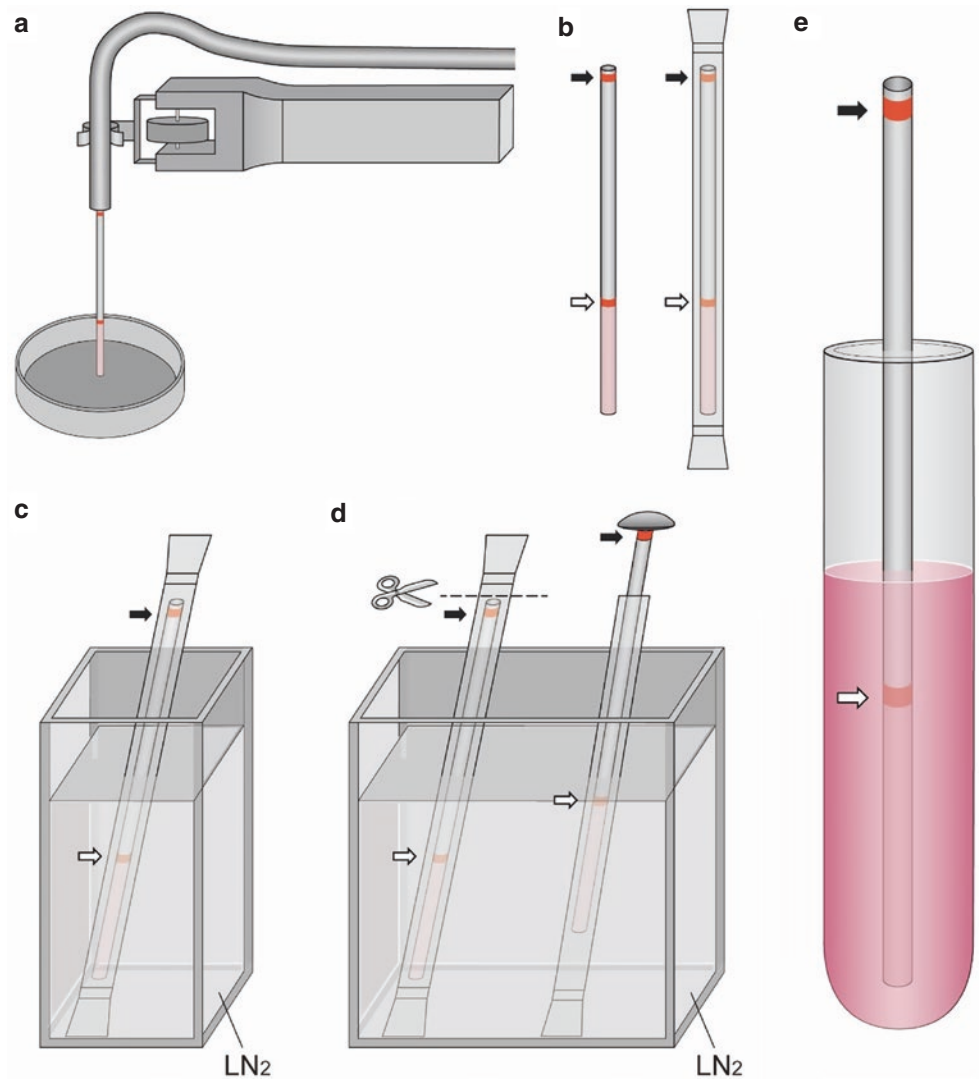
The study used the commercially supplied pipette of regular shape with stable (fixed) diameter for storing samples. Vitrification procedures can be standardized utilizing these standard microtools, and our data confirmed the safety and effectiveness of capillary with the 600 µm diameter for sperm vitrification, which unravels an excellent potential using the aseptic vitrification technique for sperm cryopreservation in ART.

9.5 Sperm Vitrification Applicability

Applications of sperm cryopreservation are multiplex. In animals, it could be a method of storage of gametes from animals with high genetic and the storage of gametes from endangered species to give some examples. In humans, these have an important role in preserving the fertility of cancer patients before chemotherapy or radiation treatments. Furthermore, sperm cryopreservation is also available to patients undergoing vasectomy and to donor semen samples. Regarding ART treatments, sperm cryopreservation is an even more important tool for the case of the male sterility, such as oligozoospermia, asthenozoospermia, obstructive azoospermia, and nonobstructive azoospermia, and the presence of pathologies as varicocele.

For the first time, we successfully established vitrification of human spermatozoa without permeable cryoprotectants

Fig. 9.1 Schematic illustration of spermatozoa vitrification using capillary. Arrows: Marked aspiration position (empty arrows) and end cutoff position (solid arrows) of capillary; (a) aspiration of 10 μ L spermatozoa suspension in the capillary; (b) capillary sealed in a 0.25 mL straw; (c) immersion of vitrification packaging system (0.25 mL straw) into liquid nitrogen (LN₂); (d) cutting of marked end of the packaging system and removing of the capillary from 0.25 mL straw; (e) warming procedure: immersion of capillary with sperm suspension into prewarmed 42 °C basic medium



[1, 15, 17]. We developed aseptic technology of vitrification of human spermatozoa in a large volume for intrauterine insemination [27] and in capillaries for intracytoplasmic sperm injection or in vitro fertilization [28]. We reported the birth of two babies after intracytoplasmic sperm injection using vitrified sperm [29] and a baby born after intrauterine insemination with vitrified sperm from an oligoasthenozoospermic man [30]. A study also reported the first birth of a healthy infant following intracytoplasmic sperm injection using a new permeable cryoprotectant-free sperm vitrification protocol [53]. The technology could also be used for the cryopreservation of epididymal sperm aspiration (MESA) and testicular sperm extraction (TESE) sperm. Our study demonstrated that the cryoprotectant-free vitrified MESA- and TESE-spermatozoa quality was higher than that of spermatozoa conventional slow freezing with permeable CPAs. This study reported two cases of babies born after ICSI using motile MESA and TESE spermatozoa vitrification without cryoprotectants [54].

9.6 Conclusion

The clinical application of vitrification was one of the most important achievements for human ART in the past decades. Nevertheless, despite many years of study, there are no standard optimal cryopreservation protocols to manage sperm samples from humans or animals. For instance, samples among different species, abnormal semen parameters, or even sperm retrieved from the epididymis and testicular biopsies can require unique cryopreservation conditions to obtain optimal outcomes.

Currently, conventional slow freezing is still the primary method used for spermatozoa cryopreservation, but spermatozoa vitrification has shown significant advantages. Through the continuous efforts made for the spermatozoa vitrification technique, our group has demonstrated the effectiveness of cryoprotectant-free vitrification for preserving human spermatozoa. Additionally, the efficacy of aseptic cooling methods by CLAiR device and capillary was confirmed. We found

that vitrification by directly dropping human spermatozoa suspension into clean liquid air produced by CLAiR is a good alternative to commercial liquid nitrogen, minimizing potential contamination. The supplementation of medium for the aseptic capillary technology for cryoprotectant-free vitrification of human spermatozoa by permeable cryoprotectant does not improve the quality of spermatozoa. Moreover, we also provide the first comprehensive description of the human vitrified spermatozoa proteome alterations after vitrification. Our work will also provide valuable information for future investigations involving spermatozoa vitrification.

Therefore, each cryopreservation method has its advantages and limitations. In-depth studies are required to detect the molecular function of cryoprotectants and methods of spermatozoa vitrification.

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Human Epididymal and Testicular Sperm Cryopreservation

10

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10.1 Introduction and Indications

Infertility is a common condition affecting 15% of couples, and the male factor is involved in approximately 50% of these cases [1]. Azoospermia occurs in 10–15% of infertile couples [2]. Since ICSI has been introduced in assisted reproduction techniques (ARTs) [3], assisting men with low sperm quality to conceive, surgical sperm extraction from epididymis and testis has been a breakthrough for men suffering from obstructive or nonobstructive azoospermia [4]. The freezing technique, which is widely used in ART, has a fundamental role in the preservation and storage of epididymal and testicular sperm.

Planning the sperm extraction in a prior day, before starting the ovarian stimulation, is a strategy adopted by many IVF clinics, in order to verify whether there are spermatozoa available for fertilization. In these cases, cryopreservation is a very useful and necessary tool, as thawing can be done on the day of the oocyte retrieval. This option offers the advantage to avoid unnecessary ovarian stimulation and oocyte retrieval in case of failure to obtain sperm while giving more time to the couple to decide their following steps.

Moreover, when both the oocyte and sperm retrieval are scheduled on the same day, cryopreservation of the remaining sample of sperm after ICSI is recommended. The cryopreserved sample can be thawed and used in the future,

without the need for multiple biopsies that carry a considerable risk of temporary or permanent hypogonadism [5].

Cancer patients also benefit from surgical extraction of sperm and cryopreservation if the collection of ejaculated sperm is not possible or in case of tumor-induced azoospermia [6]. Onco-TESE and cryopreservation are effective methods to preserve fertility in cases of severe sperm alteration (SSA) and sperm collection failure (SCF) [7].

10.2 History and Success Rates

In 1985, the first pregnancy using percutaneous epididymal sperm aspiration (PESA) and IVF was achieved, while in 1993, surgically extracted testicular sperm was employed, by Sherman Silber and his co-workers, offering a solution to many cases of azoospermia [8]. In 1996, the first successful pregnancies employing ICSI with cryopreserved testicular sperm were reported by Manuel Gil-Salom and his co-workers [8]. During the following years, the freezing method of epididymal and testicular sperm has been routinely used in IVF clinics, and many pregnancies have been achieved from couples suffering from azoospermia, a condition that has been considered untreatable.

Obstructive azoospermia (OA) is the absence of sperm in the ejaculate due to acquired or congenital conditions that block the passage of sperm from the testicle through the reproductive tract and sperm. Testicular sperm from men with OA is associated with better clinical performance and higher fertilization rates, compared to sperm retrieved from men with nonobstructive azoospermia (NOA) [5]. According to the AUA/ASRM guidelines, in cases of OA, either cryopreserved or fresh sperm may be used as fertilization, and pregnancy rates are comparable [9].

On the contrary, NOA is a condition usually characterized by impaired spermatogenesis, and the sperm obtained surgically may be limited and of low quality [10]. After cryopreservation, sperm quality may be further impaired in terms

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of presence, motility, and morphology of testicular spermatozoa [11]. As a consequence, many centers choose to schedule simultaneous sperm extraction and oocyte retrieval to avoid a negative impact of sperm cryopreservation on fertilization and embryo development. Nevertheless, according to a recent systematic review and meta-analysis by Liu et al., the majority of studies in the literature showed that there are no differences in fertilization, clinical pregnancy rates (CPRs), or live birth rates (LBRs) between fresh or frozen sperm in men with NOA [12].

10.3 Epididymal and Testicular Retrieval Techniques

Azoospermia is diagnosed when the absence of sperm is observed in two semen samples after 600 ×g centrifugation and screening at 400× magnification using an inverted microscope, according to the World Health Organization guidelines [13].

Several techniques have been developed to retrieve sperm from epididymis or testis, such as percutaneous epididymal sperm aspiration (PESA), microsurgical epididymal sperm aspiration (MESA), testicular sperm aspiration (TESA), and testicular sperm extraction (TESE) [14]. PESA or TESA is commonly used in men with obstructive azoospermia.

During PESA, the surgeon uses a fine needle to pass through the skin of the scrotum and aspirate fluid from the head of the epididymis, while in MESA, the epididymis is being dissected and the fluid of a single tubule is aspirated.

In cases that motile spermatozoa are not detected after MESA, TESA or TESE can be performed at the same operative time. TESA is a technique in which the needle is inserted through the scrotal skin into the testis with negative pressure and the fluid is drawn out from the tissue [14].

Conventional TESE is an open biopsy in which the surgeon extracts a small fragment of the testicular tissue containing a large quantity of seminiferous tubules. This can be achieved with a single incision in the tunica albuginea or through multiple biopsies via many small incisions [15].

The goal of surgical sperm recovery is to retrieve an adequate number of sperm for ICSI. In men with nonobstructive azoospermia, surgical sperm retrieval is performed by means of either single or multiple conventional TESE or by micro-TESE. Under operating microscope, it is possible to identify and selectively extract larger seminiferous tubules, which have higher probability of harboring spermatozoa [16].

Microsurgical testicular sperm extraction (micro-TESE) is a biopsy under 6–8× magnification, where seminiferous tubules are extracted from superficial and deep testicular regions, avoiding the vascular regions [17].

10.4 Epididymal and Testicular Sperm Processing

The reagents, supplies, and equipment used for the epididymal and testicular sperm processing are listed in Table 10.1. The procedure is performed at 37 °C laminar flow cabinet surface.

After epididymal aspiration, the seminiferous fluid is transferred into the tube and mixed with sperm medium to avoid agglutination. Then the fluid is spread in a petri dish with a pipette and is observed under the inverted microscope. If there are motile spermatozoa in the sample, 1–2 mL of the sperm washing medium is added to the conical tube containing the epididymal fluid and centrifuged at 400 ×g for 10 min. The supernatant is discarded, and the pellet is washed with 1–2 mL of the sperm washing medium by centrifuging the tube at 400 ×g for 10 min. The pellet is resuspended in 1.0 mL of the sperm washing medium, and the sample can be frozen.

During TESE, the specimens from testicular tissue are placed in the petri dish, which contains sperm washing medium; thus, it is important to keep it moist. The petri dish is placed under the stereomicroscope, and the seminiferous tubules in the tissue are being observed. The tissue is minced with iris scissors, and the tubules are being torn apart with a fine needle. Another method is to spread the tissue with forceps, dissect the tubules into short lengths (1–2 cm) with a

Table 10.1 Laboratory setup for sperm retrieval and freezing

Media	Consumables	Equipment
HEPES-based sperm washing medium with HSA	Sterile iris scissors and forceps	Laminar flow cabinet
	Pasteur pipettes 3 mL	Stereomicroscope
RBC lysis buffer	1 mL 25 G syringe with needle	Inverted microscope
	Petri dish 60 × 15	Benchtop clinical centrifuge
IVF sperm gradient	14 mL sterile centrifuge polystyrene tubes with caps	Incubator
	CBS straws 0.3 mL	Planer controlled-rate freezer
Sperm freezing medium (20% TEST yolk buffer, 12% glycerol with gentamycin sulfate)	Aluminum canes	Liquid nitrogen Dewar
	Plastic cryosleeves	Sealer
	LN ₂	

fine needle, and then squeeze them out. The sample is examined under the inverted microscope at 200 $\times$ or 400 $\times$. The presence of seminiferous tubules, immotile spermatozoa, motile spermatozoa, Sertoli cells, and round blood cells is noted (Fig. 10.1). Usually, the spermatozoa in testicular sperm are immotile or show twitching movements. As soon as several motile spermatozoa are detected, the surgical procedure is terminated. After the examination of all the received specimens, they are transferred into a conical tube and incubated for at least 30 min at 37 °C with 2 mL of sperm washing medium. Before incubation, it is needed to pipet the tissue pieces many times, so that the spermatozoa can be released from the tubules out to the sperm washing medium. In order to induce the motility, pentoxifylline or recombinant FSH can be added as stimulating agents [18].

If many RBCs are present, there is the option of using erythrocyte lysing buffer (ELB) in order to facilitate the detection of sperm. After tissue shredding, the suspension is centrifuged at 750 $\times g$ for 5 min. The supernatant is aspirated and discarded, and the pellet is resuspended in 1–2 mL of ELB. After the incubation of the mixture at room temperature for 5 min, the tube is filled up with buffered medium to dilute the ELB and is centrifuged at 750 $\times g$ for 5 min. The supernatant is completely aspirated and discarded, and the pellet is resuspended in 0.5–1 mL buffered medium [19]. However, this option is controversial, as ELB may damage sperm cells, by means of decreasing motility and viability and increasing DNA fragmentation. Therefore, the use of ELB in testicular semen handling is not recommended except for cases of sperm recovery failure [20].

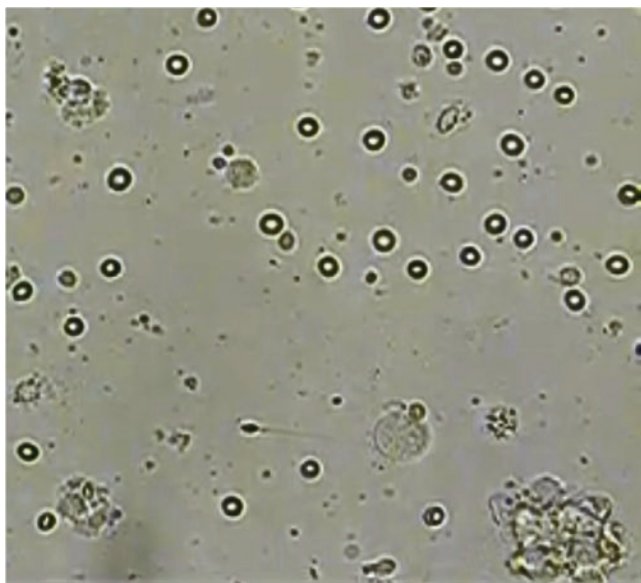


Fig. 10.1 Testicular sperm after micro-TESE. A mature spermatozoon is observed under inverted microscope

10.5 Principles of Sperm Freezing and Thawing

Cryopreservation affects sperm motility, viability, chromatin stability, and membrane integrity and causes significant morphological alterations. This loss in sperm quality is especially significant in patients with poor-quality sperm parameters [21] and may result in lower fertilization rates after the freezing and thawing procedure [22].

During freezing, various factors such as sudden temperature changes, intracellular ice formation, and hyperosmotic conditions have been suggested as the cause of irreversible membrane damage. The cryoprotectants protect spermatozoa by decreasing the freezing point and reducing the intracellular ice formation, but at the same time may exert toxic effects on the cells, the extent of which depends on factors such as type, concentration, incubation time, and freezing method. To minimize ice damage, it is important to optimize the parameters of cryopreserved conditions, including the concentrations of CPA, cooling rates, cooling temperature, and warming rates.

In conventional slow freezing, more commonly used permeable cryoprotectants include dimethyl sulfoxide, glycerol, glycol, ethylene, and methanol, while albumin, dextran, and egg yolk citrate are often used as nonpermeable cryoprotectants [23]. In slow freezing, the cryoprotectants cause dehydration of the cells, while the temperature is gradually decreased by using a controlled-rate freezer. Controlling the osmolarity during cryopreservation is crucial. Using permeable cryoprotectants in the cryopreservation media increases the osmolarity. Glycerol is used in the media as the main cryoprotectant agent for the mature spermatozoa [24].

The cytotoxic effect of cryoprotectants may be avoided by employing the cryoprotectant-free vitrification method, which results in higher mitochondrial membrane potential and motility than conventional slow freezing [25]. The vitrification method is an ultrarapid freezing procedure, during which the sample passes from a liquid to a solid glass-like state, thus preventing ice crystal formation. According to a meta-analysis by Li et al., several studies have reported the successful cryopreservation of epididymal and testicular spermatozoa by vitrification and have demonstrated the superiority of this method over conventional slow freezing [26]. However, this method has several drawbacks, including the limitation to cryopreserving low volumes of sperm and the concerns over contamination in the liquid nitrogen.

The warming process may cause damage to the cells due to the growth of intracellular ice crystals regardless of the method used for freezing. Rapid thawing of semen decreases the harmful effects of recrystallization processes and hydra-

tion, preventing damage to sperm membrane and cytoplasm. In this case, the ice crystals do not have time to be formed [27].

Mostly, all cryopreservation protocols use 37 °C as an optimal temperature of thawing. Thawing of cryopreserved human sperm at 40 °C results in a statistically significant increase in motility recovery compared with thawing at 37 °C. The procedure of epididymal and testicular sperm retrieval and freezing-thawing of human sperm at 40 °C for 3 min may become beneficial for the recovery of functional human spermatozoa after cryopreservation [28].

After thawing, the simple in vitro incubation of retrieved sperm may aid to obtain motile and functionally normal spermatozoa and avoid using immotile sperm for ICSI [14].

10.6 Epididymal and Testicular Sperm Freezing

Spermatozoa from epididymal aspiration or testis biopsy can be cryopreserved using common protocols that are used for ejaculated sperm. In cases with no motile spermatozoa in testicular sperm, it is not recommended to freeze the sample.

10.6.1 Conventional Slow Freezing

In slow freezing, the freezing medium (test yolk) is initially warmed at room temperature, and subsequently, it is mixed dropwise with the dilution of the sperm medium with extracted spermatozoa, in a ratio of 1:1. A very good mixing is important. Then the sample is left at room temperature or 10 min. The sample is loaded in 0.3 mL straws, which are tagged with the name or the code of the patient. It is beneficial for the patient to divide the sample to as many aliquots as possible, in order to maximize the number of fertilization attempts. Vials may be used instead of straws for storing the sample in LN₂. After loading, the straws must be sealed at both ends.

The straws are placed vertically at the controlled-rate freezer. The program used for slow freezing is described in Table 10.2. Once the program is finished, the samples must be plunged into the LN₂.

Table 10.2 Controlled-rate freezer program

	Steps	Cooling rate
1	Cooling from +16 to −7 °C	2 °C/min
2	Hold at −7 °C for 10 min (manual seeding)	
3	Cooling from −7 to −30 °C	0.3 °C/min
4	Cooling from −30 to −150 °C	50 °C/min

10.6.2 LN₂ Vapor Freezing

In vapor freezing, the straws are placed 10 cm above the LN₂ surface, in an open Dewar, in the vapor for 30 min (Fig. 10.2). The straws must be placed in horizontal position, to minimize the heat difference between the two ends during freezing [29]. Subsequently, the straws are plunged into the LN₂.

10.6.3 Vitrification

Although the traditional method for sperm cryopreservation is slow freezing, vitrification has been a quickly growing alternative method of rapid freezing [30].

In vitrification, sperm samples are plunged directly into LN₂. Permeable cryoprotectant-free vitrification presented a higher percentage of live spermatozoa, better preservation of acrosomes, and lower DNA fragmentation [31]. Two different nonpermeable cryoprotectants 0.25 mol/L sucrose and 0.1 mol/L trehalose are used in vitrification protocols, preserving sperm membrane integrity after thawing [32].

A simple method of vitrification is by mixing the sperm solution with sucrose solution (ratio of 1:1) and dropping aliquots of 30 µL of the mix into LN₂, which contains a stainless tea strainer. The aliquots are solidified on contact with the LN₂ and formulate spheres, which are immediately packed into pre-cooled cryovials for storage. Thawing is preferred to be performed at 42 °C [33].

Comparing cryoprotectant-free vitrification protocol with slow freezing (5% glycerol) at retrieval sperm from MESA and TESE, it has been demonstrated that the quality of sperm motility, capacitation-like changes, acrosome reaction, and mitochondrial membrane was higher after vitrification.

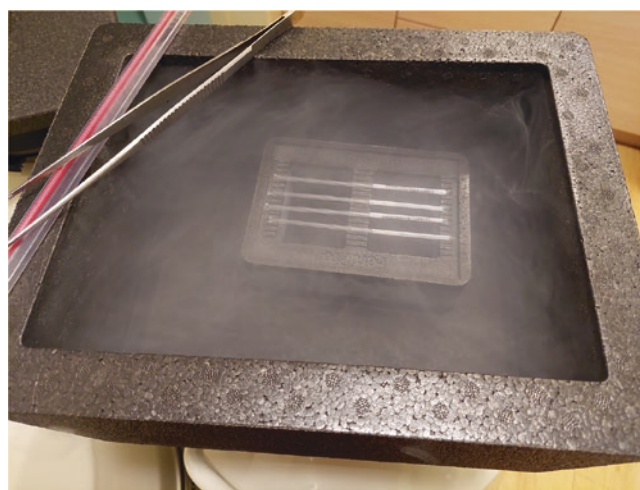


Fig. 10.2 Freezing of four straws of an individual patient in the LN₂ vapor

Three healthy babies from two women following ICSI with motile MESA- and TESE-spermatozoa were vitrified without cryoprotectants [25].

10.6.4 Single Sperm Freezing

Men with azoospermia usually have a very limited number of motile sperm in the epididymal or testicular sample. In 1997, Cohen et al. first reported the use of empty zona pellucida (ZP) for freezing of individual spermatozoa [34]. Since then, human, mice, hamster, and gold hamsters have been used as carriers for sperm freezing. The main advantage of ZP is that they are easy to locate and manipulate after thawing, facilitating the recovery of thawed spermatozoa. However, this method has also several drawbacks such as the limited availability of ZP and the bioethical issues that arise. Moreover, the New FDA and European Tissue Directive regulations impose limitations on the exposure of human gametes to animal products [35]. Based on the concept of the empty ZP, several carriers have been used for single sperm freezing such as the Volvox globator sphere, agarose capsules, and hollow hyaluronan-phenolic hydroxyl microcapsules. In addition, several vitrification devices have been employed such as cryoloops [36], culture dishes [37], cell sleepers [38], cryotops [39], and novel sperm vitrification devices.

The sperm vitrification device (sperm VD) is a multiwell mini plate that has been designed to freeze individual or limited numbers of spermatozoa. The sperm VD contains 0.8–1 μL microdroplets of sperm washing medium mixed with sperm freezing medium in a ratio of 1:1 (Fig. 10.3) in which the spermatozoa are transferred under oil (Fig. 10.4). Subsequently, the sperm VD is stored in cryovials in LN_2 as shown in Fig. 10.5. After thawing, the sperm vitrification device may be placed inside the ICSI plate, facilitating the transfer of spermatozoa to the ICSI droplets without harming the spermatozoa (Fig. 10.3). Furthermore, this device offers the advantages of high sperm recovery rate and reduced post-thaw search time of sperm [40].

Another device, developed to cryopreserve individual sperm or small sperm numbers from patients with severe oligozoospermia or NOA, is the novel cryopiece. Spermatozoa with normal morphologies and high motility were added to droplets on the cryopiece. The freezing tubes were placed into LN_2 vapor for 15 min and subsequently plunged in LN_2 . After thawing, 83% of the spermatozoa completely recov-

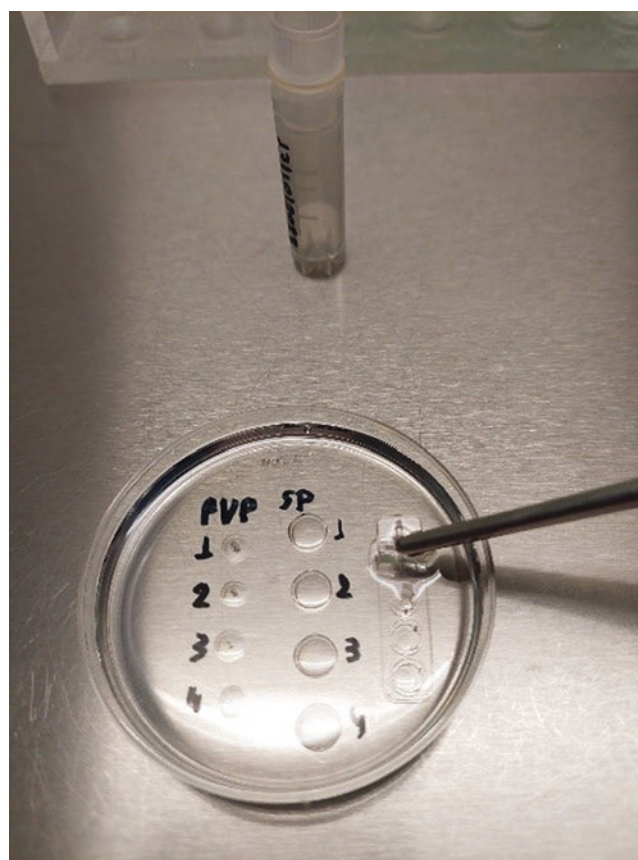


Fig. 10.3 Sperm VD preparation

ered with movement rate of 48%. Following ICSI, fertilization rate was 73%, and 19 (86%) zygote cleavage events were observed. Four healthy babies were born at term [41].

Recently, sperm from PESA, TESE, or micro-TESE has been vitrified using micro-straws made by cutting a 0.25 mL straw into small pieces. These micro-straws were placed on a dish, and then 10 mL of liquid containing 5 mL sperm dilution and 5 mL of cryoprotectant were transferred on the micro-straw's groove. The micro-straws were exposed to LN_2 vapor for 10 min and then placed in a 1.8 mL cryotube and transferred to LN_2 for storage. Micro-straws are easy to manufacture, economical, and easy to use, with a survival rate of $73 \pm 8.3\%$ and no difference in normal sperm morphology, normal *acrosome* integrity, or *DNA fragmentation* index between the micro-straw and cryotubes. An additional advantage is the fact that contamination is avoided because there is no direct contact of the micro-straw with the liquid nitrogen [42].

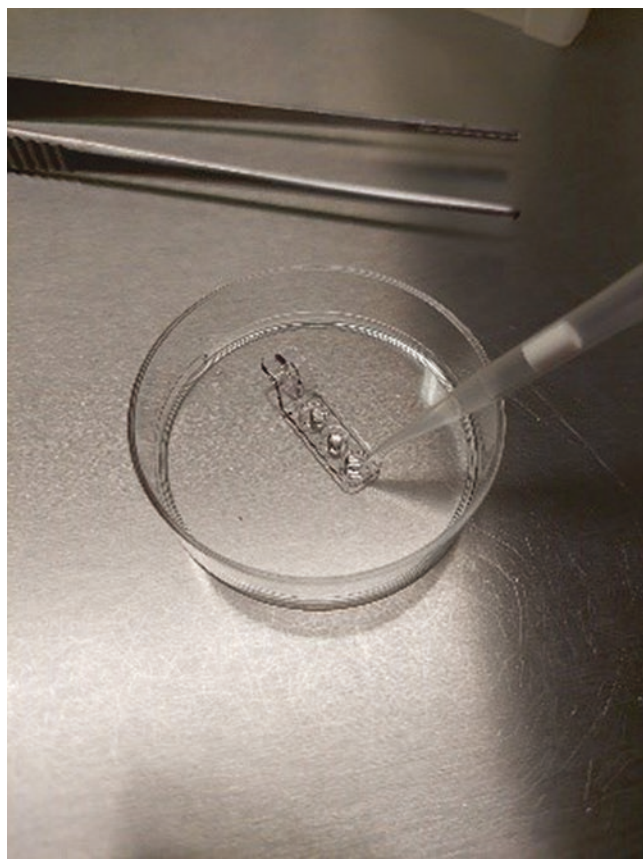


Fig. 10.4 Loading retrieved spermatozoa on sperm VD under oil

Single sperm freezing methods result in improved sperm recovery rates after thawing. However, these methods have not been widely employed mostly due to increased cost, technical requirements, and need for highly skilled operators. Furthermore, there exist risks of contamination because most of the carriers have direct contact with the liquid nitrogen [35].

10.6.5 Cryopreservation in a -80°C Freezer

Finally, a novel simplified method, similar to vapor rapid freezing, is freezing by directly moving the samples to a -80°C freezer for 24 h and then plunging into LN_2 . The freezing of testicular sperm in a -80°C freezer in a freezer tube ensures that all spermatozoa in the testicular tissue are captured; it only takes a short time and simplifies the process, while several samples can be processed simultaneously. Short-term storage of sperm at -80°C freezer could be a viable alternative to liquid nitrogen vapor freezing at -196°C due to their comparable post-thaw results and lower ROS and is necessary to confirm the clinical value of freezing testicular sperm [43].

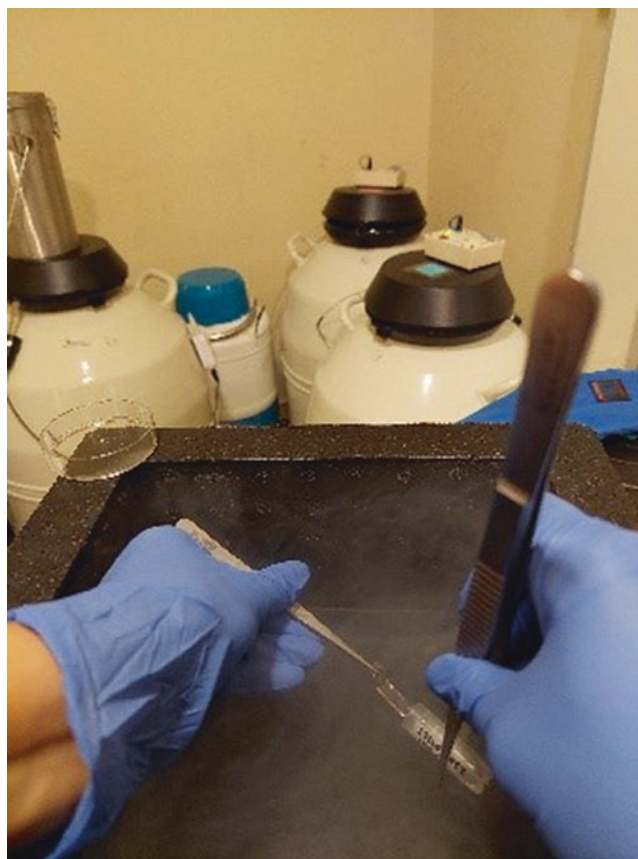


Fig. 10.5 Placing sperm VD into the cooled cryovial

10.7 Epididymal and Testicular Sperm Thawing

On the day of the oocyte retrieval, at least one aliquot of the cryopreserved sperm is thawed. In case that not enough spermatozoa for ICSI are detected after thawing, more cryopreserved aliquots can be thawed.

The first step of the procedure is to locate the straw in the LN_2 Dewar and identify it. Then the straw is removed from the LN_2 and soaked rapidly in a water bath at 37°C for 30 s. If the sperm has been cryopreserved in vials, it needs 10 min to thaw. The next step is to first cut the bottom end of the straw and secondly the top end allowing the sample to drop into the conical tube. The sample is diluted with 2 mL sperm washing medium and centrifuged at $400 \times g$ for 10 min. The supernatant is gently aspirated and discarded, in order to wash off the freezing medium.

In case of retrieval sperm with motile spermatozoa, preparation with centrifugation above density gradient is done, unless this step has been completed before freezing. There is an ongoing debate in the literature about the proper timing of sperm preparation techniques such as density gradient cen-

trifugation. Post-thaw semen analyses demonstrated significantly improved sperm counts when density gradient centrifugation was performed before freezing [44]. However, no consensus has been reached yet about the optimal timing of sperm preparation: before or after freezing.

For the density gradient centrifugation, a two-layer gradient is prepared using solutions of 80% and 40% PureSperm. Media is warmed to room temperature. The thawed semen sample (up to 0.5 mL) is placed on top of the upper layer into the conical tube. The tube is centrifuged at 300 $\times g$ for 20 min, and the supernatant is removed. The pellet must be suspended in a volume of 1 mL sperm washing medium, and a second centrifugation is done at 500 $\times g$ for 10 min. The pellet is resuspended in a volume of 0.5 mL medium, and then the final sample is spread in the ICSI petri dish to be examined for spermatozoa.

In case of testicular sperm that has only immotile spermatozoa or spermatozoa with twitching movements, after the first washing, the pellet is resuspended in 1–2 mL of the sperm washing medium, and the tube is centrifuged again at 400 $\times g$ for 10 min. The supernatant is gently aspirated, and the sperm pellet is resuspended in 0.5 mL of the sperm washing medium. Then the sample is incubated to allow the spermatozoa to induce their motility. Finally, the sample is spread in the ICSI petri dish and checked for motile spermatozoa under the inverted microscope.

Several techniques in the past have been developed in order to use viable immotile spermatozoa for fertilization. In some cases, where only immotile morphologically normal sperm are observed, the mechanical touch technique may be employed. A lateral pressing against the upper third of the immotile spermatozoon tail is applied by using the ICSI micropipette. If the tail is flexible and recovers its original position, it is considered viable. In both fresh and frozen TESE-ICSI cycles, similar fertilization rates were obtained with immotile and motile testicular sperm [45]. The hypo-osmotic swelling test evaluates the functional integrity of the sperm membrane and facilitates the selection of viable spermatozoa. A modified HOS test has also been successfully applied to select immotile testicular sperm for ICSI [46]. In addition, when frozen-thawed testicular samples were treated with pentoxifylline, significantly higher motility was observed. The use of pentoxifylline for the selection of viable nonmotile testicular sperm seemed more effective than the use of the HOS test [47].

10.8 Considerations and Conclusion

There is a worldwide current trend of decline in human sperm quality [48]. Although several explanations have been suggested, such as environmental factors and lifestyle decisions, reasons are unknown till now. As a consequence, an

increased number of patients will be in need of ART techniques, surgical sperm extraction, and cryopreservation in the future, and an advanced, safe, and reliable method to cryopreserve epididymal and testicular sperm will become a necessity.

A common consideration concerning men with NOA azoospermia is the likelihood of the presence of genetic defects that may adversely affect the offspring. NOA men are at a higher risk of being carriers of karyotype abnormalities and Y-chromosome microdeletions compared to the general population of oligo-normospermic men [49]. Besides, the damage caused to spermatozoa due to cryopreservation is more probable in cases of low sperm quality. The freeze and thaw procedure and the use of cryoprotectants lead to increased production of reactive oxygen species. As a result, even viable, motile spermatozoa after thawing may contain structural and functional damages due to oxidative stress that may affect fertilization rates and subsequent embryo development [50].

Recent developments in sperm cryopreservation resulted in the design of new vitrification devices with promising results. The optimization of the vitrification technique with the modification of cryoprotectants and the use of antioxidants may improve sperm survival rates and laboratory outcomes.

To conclude, further studies and research are needed to improve the already existing single sperm freezing devices in order to maximize the chances of recovery of viable sperm after thawing, especially in NOA patients, and increase fertilization and pregnancy rates with frozen epididymal of testicular sperm. Moreover, although the efficiency of the cryopreservation technique has been documented, in case of novel technologies that have been used only for a small number of cases, further follow-up studies are recommended to ensure the safety and health of the offspring. Finally, it is crucial to highlight the importance of providing detailed information to the patients about the risks and benefits of cryopreservation of surgically extracted sperm and counseling the couple about the chances of success depending on their background, diagnosis, and treatment that is followed.

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Key Points

- Oocyte cryopreservation allows preservation of female reproductive potential, circumventing the undesired implications of embryo cryopreservation.
- Human mature oocytes have biophysical features that make their cryopreservation arduous. Oocyte cryopreservation is challenging and requires control of the two main causes of cell death: ice formation and lethal increase in solute concentrations.
- The correct extension of the intervals between (a) oocyte recovery and freezing and (b) thawing and insemination remains a matter of discussion, but ideally their overall duration should not exceed the normal time span of oocyte culture in a fresh cycle.
- Evidence on the safety of oocyte cryopreservation is so far reassuring.

11.1 Introduction

Oocyte cryopreservation allows preservation of female reproductive potential [1–3], circumventing the undesired implications of embryo cryopreservation. Oocyte cryopreservation was initially implemented as a technical extension of embryo cryopreservation. Slow-freezing protocols originally developed for embryo cryopreservation were successfully adapted for oocyte cryopreservation. This technique may be used to rescue cycles complicated by ovarian hyperstimulation syndrome and failure to obtain sperm on the day of oocyte pickup, in oocyte donation programs, in the treatment of congenital infertility disorders or premature ovarian failure (POF), as an alternative to embryo freezing, or, for fertile women, to electively delay childbearing.

In general, cryopreservation makes it possible to freeze cells and tissues at very low temperatures and to keep the biological specimen genetically stable and metabolically inert in order to preserve it for future use [3]. The oocyte cryopreservation procedure involves initial exposure to cryoprotectants, cooling to subzero temperatures, and final storage in liquid nitrogen. At this storage condition (−196 °C), biological reactions do not take place, and in principle, storage can be prolonged indefinitely.

Human mature oocytes have the biophysical features (a diameter of 120 µm, overall high sensitivity to low temperature, high water content, low surface-to-volume ratio, relatively low plasma membrane permeability) that make their cryopreservation very difficult to achieve.

Oocyte cryopreservation, while preserving its physical and functional integrity, is therefore challenging. To achieve this, it is necessary to eliminate the two main causes of cell death associated with cryopreservation: ice formation [4] and lethal increase in solute concentrations [5] while maintaining the functional capacity of the intracellular organelles. Devising procedures that take biological samples from a physiological temperature (37 °C) to a cryogenic temperature (−196 °C) is an extremely complex task. Changes have been made to cryopreservation methods in order to improve results in terms of biological and clinical outcomes.

The first human births from frozen oocytes were reported in the 1980s by Chen [6] and Al-Hasani [7].

Slow-cooling cryopreservation methods were improved by testing different combinations and compositions of cryoprotectant solutions [8, 9–11], to avoid damage to chromosomes and the meiotic spindle, cytoskeleton, and cortical granules [12, 13].

The first protocol used was that devised by Lasalle, based on 1.5 M PROH and 0.1 M sucrose, originally applied to embryo freezing [14]; however, this protocol did not ensure adequate dehydration, with consequent formation of intracellular ice and cell death. It was therefore necessary to increase the concentration of sucrose to obtain better dehydration and lesser ice formation. Fabbri et al. [15] therefore

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increased sucrose concentrations from 0.1 to 0.3 M, which resulted in a significant increase in the survival and fertilization rate, without, however, achieving good clinical results in terms of implantation and pregnancy rate. Therefore, on the basis of these results, Bianchi et al. [8] further modified the oocyte cryopreservation protocol to include a freezing solution containing 1.5 M PROH plus 0.2 M sucrose, in order to obtain lesser shrinkage during cooling and a thawing solution with a higher sucrose concentration (0.3 M). This change made it possible to achieve biological results comparable with those of previous studies and significantly better clinical results.

11.2 Biophysical Principles of Controlled Rate Cooling and Methodology

To stabilize biological materials at cryogenic temperatures, the slow-freezing procedures take into account a number of important aspects.

The high water content (75–85%), related to the large cell size, can convert into ice crystals at low temperatures, generating significant and irreversible damage to the cellular ultrastructure. In addition to this, the cytoplasmic oocyte is also characterized by a high sensitivity of the cytoskeleton to low temperatures [16], elements that give way to relevant difficulties in the successful application of the technique. The cryopreservation process involves replacing some of the water inside the cells with other compounds that will not form large ice crystals when frozen.

11.2.1 Cryoprotectants

All freezing methods rely on the presence of one or more cryoprotectants at molar concentrations. One feature that is common to these compounds is their ability to interact with water via *hydrogen bonds* and, prior to slow cooling, to decrease the freezing point of the solution and ultimately to produce a large effect of cell dehydration without excessive shrinkage [17]. Cryoprotectants can be divided into two groups according to their molecular weight and, therefore, their ability to pass through the cell membrane:

- *Permeating agents* chemically characterized as having a relatively low molecular weight and that are able to penetrate the cell's lipid bilayer: They also osmotically replace intracellular water, decrease the formation of intracellular ice crystals, lower the freezing point of the solution, and support enhanced cellular dehydration during freezing.
- *Non-permeating agents* are unable to cross the cell membrane due to their molecular size. They generate an

osmotic gradient that causes cell dehydration and decreases the intracellular water content; consequently, the risk of intracellular ice crystal formation as the temperature drops is reduced.

In all freezing techniques, permeating and non-permeating agents are usually used in association; their capability to replace water, which is so important at low temperatures, also means that they are characterized by an innate toxicity if they are used on cells at physiological temperatures. Toxicity is proportional to exposure time and concentration. During the procedure, they are added and removed gradually, in order to reduce excessive osmotic gradients, which may arise during the process. It is also essential to balance the benefit of the slow addition of the cryoprotectants with the detrimental effects caused by consequent increase in exposure time.

11.2.2 Slow Freezing

- **Control of the cooling speed:** Thanks to the automated machine, the decrease in temperature is finely regulated and recorded; indeed, it is achieved using a computerized freezing system consisting of a computer and freezing chamber connected by a pump to a cylinder from which the liquid nitrogen is extracted in gaseous form. The temperature-reduction curve to be achieved is saved on the computer; according to the temperature detected by the temperature sensor, the computer opens and closes the solenoid valve and regulates the introduction of gaseous nitrogen into the freezing chamber, thus permitting controlled temperature lowering. Cryofreezers are equipped with printing devices that produce graphical representation of temperature changes occurring during freezing. This output is an essential documentation of the thermal condition at which the cryopreservation process occurs, especially for quality management purposes. Technical failure of cryofreezers is rare, but not impossible. This imposes that IVF laboratories are provided with two cryofreezers for backup purposes. Access to two cryofreezers also allows parallel execution of cryopreservation cycles, if needed.
- **Dehydration step:** In slow-freezing protocols, two-step dehydration is normally carried out before cooling the cell to subzero temperatures.
 - **First phase of dehydration:** Concentrations of permeating cryoprotectants (1.0–1.5 mol/L) are utilized to remove water from the cell preventing ice formation. The most widely used cryoprotectants in this phase are PROH, DMSO, or EG. The presence of permeating cryoprotectant in the extracellular

media creates an osmotic gradient, which draws water out of the cell. The oocyte, once dehydrated and shrunk, returns to near the original volume as the cryoprotectant enters the cell replacing the water. This causes a double flux across the membrane (the water exits the cell while the cryoprotectants enter) that influences both the intracellular solute concentration and the cell volume. The extreme extent of shrinkage and swelling can cause damage or even cell death because of the osmotic stress acting on the oocyte membrane.

Second phase: A non-permeating cryoprotectant is usually added to minimize the phenomenon of shrinkage. The most common agents successfully applied are sucrose and trehalose [17–19]. This agent creates a second-phase dehydration in which the shrinkage rate is faster with a higher reduction of the cell volume before freezing. This phenomenon decreases, further on, the likelihood of intracellular ice crystal formation.

- **Seeding:** When the slow-cooling method is used to cryopreserve oocytes or embryos, the freezing solution is usually manually “seeded” at a temperature of -7°C to induce ice formation. This is usually achieved by touching the wall of each straw with cold forceps (cooled in liquid nitrogen). The aim of this procedure is to induce the first ice crystal [20] in the freezing solution some distance away from the oocytes and to prevent the whole solution from supercooling.

11.2.3 Rapid Thawing

During thawing procedures, cryoprotectants are removed by stepwise dilution with final return to a physiological environment, and rapid temperature transition is preferred to prevent the recrystallization of water with the potential for ice crystal damage. Here, caution must be taken to avoid osmotic shock from the permeating cryoprotectant, which now is at a very high concentration in the intracellular space. Therefore, an additional non-permeating cryoprotectant is used. As the permeating cryoprotectant gradually diffuses out of the oocyte, the concentration of the non-permeating cryoprotectant gradually decreases, until the oocyte is returned to standard culture medium [17].

11.3 Management and Organization

Oocyte cryopreservation is challenging from a technical and organization standpoint. Several technical (protocols, materials, and devices) and processing (pre- and post-freezing oocyte culture, oocyte manipulation, and expertise of the

operator) factors require efficient management to preserve the viability of the stored material.

11.3.1 Selection of Freezable Oocytes

The cryopreservation of female gamete has many difficulties due to morphological features such as its size and its physiology: the human female gamete is the largest cell in human body, with a low surface/volume ratio and with a high cytoplasmic water content (75–85%). The selection of freezable eggs is certainly a difficult choice for embryologists. Just after the retrieval, the oocyte is surrounded by two types of somatic granulosa cells: cumulus oophorus cells that are more dispersed and corona radiata cells that are in contact with zona pellucida.

Choosing the suitable oocytes for cryopreservation is important to make an assessment of oocyte maturity and quality, so the complete denudation of cumulus–corona complex using hyaluronidase enzyme and mechanical pipetting before freezing is recommended, since the presence of the cumulus cells does not allow to effectively evaluate the nuclear maturity and the cytoplasm characteristics.

It is generally accepted that a “morphologically normal” human mature oocyte should have a round, clear zona pellucida, a reduced perivitelline space containing a single and nonfragmented first polar body, and a uniform or moderately granular cytoplasm, with no inclusions. There are several different cytoplasmic and extracytoplasmic abnormalities; in fact, after ovarian hyperstimulation, some of the retrieved oocytes exhibit one or more morphological cytoplasmic or extracytoplasmic abnormalities [21–23].

The cytoplasm can result to be dark, homogeneously granulous, or located in the middle; it can have vacuoles, various formations which are constituted by endoplasmic reticulum. It is also possible to find oocytes with a larger diameter called “giant” that are generally discarded because of their abnormal chromosomes [24]. Also, the extracytoplasmic abnormalities seem to be associated with oocyte function and embryo development. Abnormalities of zona pellucida, perivitelline space, and first polar body are part of this category. It is preferable to cryopreserve only “morphologically normal” oocytes since the cytoplasmic and extracytoplasmic abnormalities seem to affect oocyte competence. Unfortunately, methods to assess the quality of the oocytes are usually invasive and thus nonconservative.

11.3.2 Cryopreservation Timing

In the mainstream IVF process, oocytes are cultured for several hours following the retrieval and before insemination. This interval time amounts to approximately 40 h after

human chorionic gonadotropin (hCG) administration; this is the time believed to be optimal to achieve oocyte nuclear and cytoplasmic maturation.

With oocyte cryopreservation, one of the concerns is the risk to extend excessively the period of culture between recovery and fertilization. The correct extension of the intervals between (a) oocyte recovery and freezing and (b) thawing and insemination remains a matter of discussion, but ideally their overall duration should not exceed the normal time span of oocyte culture in a fresh cycle.

11.3.3 Survival and Insemination of Cryopreserved Oocyte

A relevant indicator of the efficiency of oocyte cryopreservation process is the percentage of the survival rate. The most evident cryodamage is the lysis of the cell membrane as a result of the intracellular ice. Over the years, the improvement of protocols for human oocyte cryopreservation has led to an increase in the survival rate. Initial oocyte cryopreservation with DMSO was associated with survival rates ranging between 20 and 30% [7, 25–27]. Substituting the permeating cryoprotectant with PROH and adding 0.1 M sucrose have doubled the survival rate. The evolution of the protocols has also led to increase in the sucrose concentration from 0.1 to 0.2 or 0.3 M during the dehydration, increasing the survival rate of up to 60–80% [28–32]. These changes have led to obtaining a survival rate of around 70% when the protocol of 0.2 M sucrose was performed [33–35] and slightly higher (74%) using 0.3 M sucrose [28–30, 36].

Morphologically, a mature human egg survived after cryopreservation should look like fresh, although survival does not mean ability to be fertilized. In order to identify the right time for the microinjection after thawing, it is important to balance carefully the recovery time that oocytes need in order to restore the meiotic spindle with oocyte aging, which must be avoided.

The insemination of surviving oocytes takes place anyway not earlier than 1 or maximum 2 h after the end of the thawing procedure in order to avoid exceeding a total culture time and to recover meiotic spindle after thawing.

11.3.4 Cryodamage

Cryostorage should achieve preservation of oocyte developmental competence, not only post-thawing survival. Post-thaw lethal oolemma damage occurs relatively frequently, with a rate of 20–30%. This outcome makes slow freezing less competitive compared with vitrification, whose survival rates range between 80 and 90%. Oocyte death occurs mostly during thawing or immediately afterwards, but also

during the preparation for ICSI: Survival after thawing is certainly crucial, but does not necessarily coincide with cell integrity. It has been widely demonstrated that there are several structures that must be preserved in order to consider the oocyte functional and suitable to progress towards the generation, because low temperatures may induce sublethal damage or alteration to different organelles and structures: zona pellucida, cortical granules, mitochondria, and meiotic spindle.

Zona pellucida: There is evidence that cryopreservation, irrespective of the adopted method, causes the hardening of zona pellucida through premature cortical-granule release, which then acts as a barrier to fertilization [37–39]. If the standard insemination technique were used, a reduction of normal fertilization rates [40] would occur. After the introduction of the intracytoplasmic sperm injection (ICSI) by Palermo [40] in 1992, it was possible to bypass the hardened zona, leading to improved fertilization and live birth rates [41].

Meiotic spindle: This is composed of microtubules and is important for chromosome segregation. The meiotic spindle apparatus is a dynamic structure, and it has been demonstrated to depolymerize when exposed to low temperatures [42]. Nevertheless, the return to normal conditions shows that the spindle is able to significantly reassemble [26, 43].

During slow-freezing procedure, the oocyte undergoes a drastic change in temperature that can be detrimental for the spindle; on the other hand, CPAs play an important role in protecting the spindle.

Mitochondria: It was displayed (with electron microscopy TEM) long since a deformation and subsequent damage of these structures in response to a lowering of the temperature up to 0 °C in the presence or absence of DMSO [44]. Nottola revealed the presence of mitochondrial damage by comparing fresh with frozen oocytes using PROH and different concentrations of sucrose (0.1 M and 0.3 M), while areas of microvacuolation have been described in the presence of high concentrations of sucrose (0.3 M). These areas can be correlated with a reduced potential of embryonic development after thawing [45].

In fresh samples, mitochondria had a regular shape with few short cristae, whereas in the frozen-thawed group, a high percentage of oocytes (72%) showed a variable and, in some cases, a very high fraction of mitochondria with decreased electron density of the matrix or ruptures of the outer and inner membranes. Moreover, in those oocytes, the mitochondrial damage was associated with SER swelling.

11.4 Safety of Oocyte Cryopreservation

Safety is another important aspect of oocyte cryopreservation.

Oocyte cryopreservation has generated concerns especially because cell damage may occur sublethally, with possible developmental consequences.

The first report addressing this issue was published by Porcu in 2000 [46]. The paper reported on obstetric complications, perinatal outcomes, and children's follow-up of 17 pregnancies from cryopreserved oocytes. Results were reassuring, with no malformations and normal physical and cognitive development observed in the follow-up all of newborns. Noyes in 2009 [47] published a large study including 963 babies born from oocytes cryopreserved by slow freezing or vitrification between 1986 and 2008. The rate (1.3%) of birth anomalies—ventricular septal cardiac defects, choanal atresia, biliary atresia, Rubinstein–Taybi syndrome, clubfoot, and skin hemangioma—was comparable with that of spontaneous pregnancies. The birth anomalies observed were as follows: Experience on the duration of storage [48] shows no differences between oocytes cryopreserved with slow freezing and thawed after up to 48 months compared to earlier thaws, in terms of survival, fertilization, cleavage, embryo quality, implantation, and live birth rates. More recent data of the health of children born from frozen-thawed oocyte is missing, because slow freezing has been almost entirely replaced by vitrification.

11.5 Conclusion

Slow-freezing techniques originally developed for embryo cryopreservation have made oocyte cryopreservation possible. This has required development of protocols more suitable to manipulate an extremely fragile cell, such as the human oocyte. Data on babies born from this cryopreservation approach have denied dogmatic concerns on possible health risks, opening oocyte cryopreservation to a range of applications.

Safety concerns have not been confirmed, although further and more rigorous studies are needed. Future development of oocyte cryopreservation slow freezing will depend on the application of novel theoretical models, which can predict better cryopreservation conditions and thereby improve oocyte quality after freezing-thawing.

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12.1 Background

The field of cryopreservation has expanded steadily since the first official announcement of a baby born from a frozen embryo that was stored in liquid nitrogen [1]. Although this was reported in 1984 by Zeilmaker, it is now generally accepted that the first birth from frozen human embryos actually occurred almost 6 years earlier, but since it was published in a low-impact journal and the parents were unwilling to have their names made public, the event went largely unnoticed [2]. Nonetheless, the success gave rise to an entire new industry, opening doors for delayed childbearing and preservation of fertility before fertility-altering medical treatments, and gave fertility providers the ability to wait until the perfect lining and avoid the risk of pregnancy following ovarian hyperstimulation syndrome before transferring embryos into their patients. Even for IVF cycles during which fresh embryo transfer is performed, the vast majority have extra embryos that can be frozen for subsequent cycles regardless of the success of the fresh transfer.

It is impossible to know exactly how many frozen human embryos are in existence today due to the enormous proportion of them being abandoned (especially the ones from many years ago before cryostorage management became more rigid), constant addition and removal of specimens, lack of a unified global reporting system for frozen inventory, and continuous growth in the industry as the demand increases. Just in the USA, it was estimated that the number of embryos created between 2004 and 2013 was almost two million, of which less than half were transferred [3]. The authors also noted that potentially over 1.2 million were still in storage. If we look at the growth trends in the industry, we can say with relative certainty that the current number of frozen embryos in the USA alone must be pushing around five million. Although slow freezing of embryos has been phased out due to the vast majority of programs switching over to

vitrification, a large proportion of the specimens in storage across the globe were slow frozen, which necessitates that reproductive biologists maintain an understanding of the principles of slow freezing and especially of thawing of frozen embryos.

12.2 History of Cryopreservation

Over 300 years ago, an alchemist called Robert Boyle was studying the effect of temperature on the volume of gases. His discoveries are largely accepted as the foundation for the work by Jacobus van't Hoff, who won the first Nobel Prize in chemistry for describing the laws of chemical dynamics and osmotic pressures in solutions [4]. One of the fundamental principles in cryobiology even today is demonstrated by the Boyle-van't Hoff plot, which describes the effect of osmotic pressure on cell volume. Today, we know that dehydration is an essential step in the preparation for freezing of cells, and this even now these fathers of the field are contributing with their basic research.

The first demonstration of preservation of mammalian reproductive cells was when Italian military physician P. Mantegazza noted in 1866 that spermatozoa became non-motile after cooling in snow. This observation sparked the idea that potentially a fallen soldier could have children after death by preserving sperm through cooling [4].

In the early 1900s, there were several prominent scientists with a deep interest in life at low temperatures and the effects of cold on various biological systems. Most notable was Basile J. Luyet, who is considered the father of cryobiology. He was a Roman Catholic priest who immigrated to the USA in 1929 to study "Life and Death at Low Temperatures." He established the faculty at St. Louis University, founded the journal *Biodynamica*, and was the first president of the Society for Cryobiology [5].

Others, such as John Hammond and Arthur Walton, were also studying the effect of low temperature on cells, specifically in ejaculated and non-ejaculated sperm, and Hammond

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predicted cross-continental transportation of semen samples by airplane after cooling.

Then in 1938, Luyet and Hadopp reported survival of spermatozoa following vitrification in liquid air using sucrose as a cryoprotectant.

Although encouraging, success rates remained low until the accidental discovery of the cryoprotective properties of glycerol by Jean Rostand in frog sperm [4] and Polge and Smith in fowl sperm in the late 1940s [6]. Subsequently, three pregnancies following artificial inseminations using frozen-thawed human sperm were reported [7].

While successful cryopreservation in sperm seemed to be quite straightforward, it was soon discovered that eggs and embryos brought other challenges, mostly due to their large cell sizes and small surface-to-volume ratio. In addition, oocytes have intricate cell machinery very susceptible to temperature fluctuations and cryodamage [8]. A detailed description of the work on cooling of zygotes and embryos at different stages by M.C. Chang's group can be found in the study of Leibo [4]. But it was Sherman and Lin who reported the first births following transfer of frozen-thawed eggs in the mouse model [4]. This was followed by the first live birth from mammalian embryos [9] and eventually human embryos [10].

It is remarkable and fortunate that the first success in humans coincided so closely with the first successful human IVF. This combination led to the birth of a new, highly prolific industry where infertility clinics have the ability to create supernumerary embryos and freeze them for patients. We have referred to the large collection of cryopreserved embryos in existence above. Although the technology has evolved, most notably a transition from slow freezing to vitrification, we should not forget the challenges that these pioneers have had to overcome to give providers and patients the gift of cryostorage as we know it today.

12.3 Principles of Cryopreservation and Slow Freezing/Thawing

The purpose of cryopreservation, by definition, is to store specimens at cold temperatures while preserving its viability and preventing irreversible cryodamage. To understand how we can achieve this preservation, we need to appreciate the mechanisms of cryoinjury, a series of often irreversible changes that can lead to cell death. Although there are so-called second-order effects that can contribute to cell damage and death, there are two primary factors that are most often the culprits of the insult that causes the irreversible damage. These are damage by osmotic effects and injury due to ice crystal formation inside and outside of the cell. Both of these occur as a result of the movement and

behavior of water as cells are cooled. Therefore, the fundamental principle of cryobiology is the control of water inside and outside of cells during the freezing and thawing process [11].

Mammalian cells are made up of up to 85% water, which needs to be managed during the freezing or vitrification process. We know that around 10% of water inside cells is closely associated with cell structures and is unable to freeze, but the remaining "free" portion is capable of moving across the membrane via osmoses, can form ice crystals, and acts as a solvent, all of which poses risks to cell survival through various mechanisms [11].

Our two biggest defenses against freeze damage are:

1. Removing water from the cell
2. Cryoprotective agents that replace the water that is removed from the cell

Below we will describe the various modes of cell damage and the methods that we can use to compensate or prevent the damaging effects by utilizing the abovementioned defense mechanisms.

12.3.1 Damage Via Osmotic Effects

When cells are exposed to temperatures below the freezing point of water during slow cooling ($<0.5^{\circ}\text{C}$ for intracellular water), water in the solution surrounding the cells undergoes ice crystal formation, which means that water is progressively removed from the extracellular solution and solutes concentrate in the remaining fluid as the crystals exclude them from the forming lattices. This causes concentration of solutes in the remaining, unfrozen water portions surrounding the cells. Therefore, intracellular water is removed from the cell cytoplasm due to extracellular ice crystal formation, by osmoses through the semipermeable cell membrane due to the increase in osmolarity outside of the cell. Inside the cell, the increase in solutes, especially salt concentrations, could damage proteins and other particles and affect cryosurvival.

Remedy: Addition of permeable cryoprotectants to cryopreservation media allows for replacement of water as it leaves the cell. These agents interact with water through hydrogen bonding and decrease the freezing temperature of water, thereby reducing the amount of free water that is available to interact with one another and crystalize into ice. Therefore, the increase in osmolarity inside the cell is depressed, and lethal levels of solutes are prevented. As cooling progresses, the remaining liquid phase of the cytoplasm becomes supercooled and eventually vitrifies, thereby preventing intracellular ice crystal formation.

12.3.2 Damage by Ice Crystals

The most likely cause of cryodamage during freezing is puncture of the cell membrane or organelles due to intracellular ice crystal formation. During rapid cooling, intracellular free water will crystalize and grow significantly and eventually damage cell membranes and organelles. During slow freezing, however, the extracellular free water crystalizes, essentially removing water from the solution and leaving supercooled, hyperosmotic intracellular cytoplasm content. This causes increase in the solute concentration and movement of free water from the cytoplasm out of the cell to maintain chemical equilibrium via osmosis. As the cytoplasm becomes even more supercooled, the chance of nucleation and crystal formation increases. Therefore, slow-freezing protocols protect cells from intracellular ice crystal formation via cell dehydration. However, extracellular ice crystals can also be damaging by cell puncture as well as placing physical stress of the cells that end up in hypertonic channels of unfrozen solution between the free water ice crystals. In addition, when extracellular ice crystals grow, they can form narrow channels in which cells get trapped, further causing stress on cell membranes as they are squeezed into unusual shapes between crystals, even though they may not be punctured by sharp crystals.

Remedy: The most effective way to prevent ice crystal formation is to minimize the amount of water available to form nucleation sites. By using non-permeable, hyperosmotic freezing solutions, we can pull water out of the cell by osmosis and minimize extracellular ice crystal formation. To further reduce the risk of ice intracellular crystal formation, we can add permeable cryoprotective agents that prevent water crystallization by virtue of its chemical interaction with water molecules. Examples of such agents are glycerol, dimethyl sulfoxide (DMSO), ethylene glycol (EG), and propylene glycol (PE). Used in combination with non-permeating cryoprotectants that dehydrate cells as described above, these agents act as a water substitute inside of the cells and prevent continued increase in solute concentration as well as ice crystal formation.

12.3.3 Damage Via Physical Volume Dynamics

A direct effect of dehydration during the freezing process is cell shrinkage. This can cause cell damage by shear stress on the cell membrane as it shrinks. Furthermore, should intracellular ice crystals be present, excessive shrinkage will cause the membrane to close in on sharp ice crystals, which can puncture the membrane. There is also evidence that cells can tolerate a maximum amount of shrinkage, so should this level be exceeded, irreversible

cell damage occurs. Conversely, when cells are thawed and water moves back into the cell, too rapid re-expansion can cause cells to rupture due to the large osmotic pressure into the cell, which can result in cells bursting like an over-filled balloon.

Remedy: Damage by too rapid shrinkage and expansion is prevented by slowing down the osmosis of water across the cell membrane and allowing replacement of the lost volume when cells are dehydrated. This is achieved by gradual, usually stepwise equilibration that allows maintenance of chemical equilibrium inside and outside of the cells. Addition of permeating cryoprotectants to freezing solutions ensures restoration of the osmotic equilibrium and cell volume as the removed water is replaced. It is critical to reverse this process during the thawing process by gradually reducing osmolarity in the washing solutions and slowly rehydrating cells so as not to supersede the rate at which the cryoprotectants can be removed from the cell, which will cause the cells to overexpand and burst.

12.4 Protecting Cells During Freezing/Thawing

12.4.1 Addition of Cryoprotectants

As the name indicates, cryoprotectants are additives to freezing media that will protect the cells against cryodamage by facilitating the remedies described above. Their modes of action depend on what type of molecules they are, although all are water soluble at low temperatures as they help control or counteract the dynamics of intra- and extracellular water and must be relatively nontoxic. Basically, these agents can be divided into two broad categories, namely, permeating and non-permeating, which are typically used in combination during cryopreservation procedures.

12.4.1.1 Permeating Cryoprotectants

There exists a collection of permeating cryoprotectants that have been shown over the years to be effective at protecting while being tolerable to most cells, depending on the cell type and species. These molecules must be able to diffuse into cells, necessitating them to be small and highly water soluble. Some of these do exhibit toxicity at higher temperatures and prolonged exposure, which is why most cryopreservation protocols are performed at room temperature to negate potential harm, and exposure is kept to a minimum. During freezing of embryos, the choice of permeating cryoprotectants depends mainly on the stage of the embryos to be frozen. Their protective properties act both inside and outside of the cells, although they are slightly different.

The main purpose of permeable cryoprotectants is to enter the cell as water leaves the cell, replace the water in order to restore the lost volume, and prevent excessive and prolonged increase in solute concentration [12] while preventing ice crystal formation through hydrogen bonding with water molecule, thereby preventing water molecules to bind to one another to form nucleation sites and resultant ice crystals. It is also thought that some of these agents, such as DMSO, may increase membrane permeability to further allow crossing of water as well as cryoprotectants through the cell membrane to facilitate more effective equilibration [13–15].

Extracellularly, permeating cryoprotectants mainly prevent crystallization through the same action and allow cells to be encapsulated inside the crystals rather than being excluded into narrow channels which can be damaging. The most commonly used permeating cryoprotectants for slow freezing of human embryos are 2,1-propanediol and dimethyl sulfoxide (DMSO) for all stages except blastocyst, for which glycerol is typically used [8].

12.4.1.2 Non-permeating Cryoprotectants

As the name suggests, these agents do not enter the cell due to their large size and chemical composition. Therefore, their role in cryopreservation of cells is purely extracellular, to aid in dehydration (osmoses of water out of the cell) and to prevent lethal crystal formation in the surrounding solution. Its mode of action to protect cells is similar to that of permeating cryoprotectants, by inducing vitrification instead of freezing and preventing puncture or deformation of cells by ice crystal formation. The most commonly used non-permeating cryoprotectant is sucrose, due to its tolerability, high solubility in water, availability, and low price point [8].

12.4.2 Controlling the Cooling and Warming Rates

The above-discussed mechanisms through which cryoprotectants prevent cryoinjury will mostly be circumvented, and the procedures will fail, despite addition of protectant agents, unless the cooling and warming rates are carefully controlled. In fact, Mazur demonstrated that the likelihood of ice crystal formation and survivability is dependent on the cooling rate [16]. The reason is that, as mentioned above, the principles of cryobiology heavily depend on the movement and behavior of water in and around the cells. For example, non-permeable cryoprotectants aid in dehydration of cells. But unless the protocol allows for some time of water to move out of the cell, the procedure will fail due to intracellular crystallization of water that did not have time to evacuate the cells. Furthermore, if we consider the action of permeable agents, these are large molecules that take time to

enter the cell and replace the water that was lost. Unless we provide time for equilibration of the solutions inside and outside of the cells, the system will fail and cells will not be protected from intracellular hyper-isotonic conditions and ice crystal formation [11].

During slow cooling, since the cryoprotectants are thought to be toxic at higher temperatures, part of the equilibration process is performed during the initial cooling process. Therefore, the cooling process is typically slow at first (0.5–2.0 °C/min depending on the protocol), followed by a hold period for manual seeing [17]. This provides ample time for water to be removed from the cell and be replaced by permeating cryoprotectants. Then, once a critical subzero temperature is reached (typically at around –7 °C), due to the supercooled state of the solutions in and around the cells, a high risk of crystallization exists as the temperature further decreases. Thus, after seeing, the cooling rates beyond the supercooled state are increased to a rapid –10 °C, which does not allow time for large ice crystals to form. Finally, once –30 °C is reached, the cryodevices containing the now frozen specimens can be rapidly cooled (30–50 °C/min) before being submerged in liquid nitrogen.

Similarly, when slow-frozen embryos are thawed, we have to pay close attention to the warming rates as ideal warming rates have been shown to be closely correlated to the cooling rates used during the freezing process [18]. The mechanisms behind these phenomena are beyond the scope of this chapter, but are summarized in the work of Mazur [11].

Regardless of the exact cooling and warming curve called for, the desired cooling rate can be achieved either by hanging or suspending the straw of vial containing the embryos to be frozen in a homemade liquid nitrogen vapor environment with carefully calibrated distances from the liquid nitrogen or by using a programmable freezer. Regardless of the cooling technique used, thawing is typically achieved either by exposing to ambient air or by submersion in a water bath at a specific temperature, typically between 30 and 37 °C until the entire volume of freeze solution is thawed. Although several freezing and thawing methods exist, the thawing protocol should always correlate to the freezing protocol.

12.4.3 Slow-Freezing Carrier Devices

Several carrier devices have been used for slow freezing. The most common are cryovials, similar to those used for freezing of cell suspensions and tissue, and 0.25 or 0.5 mL straws. Occasionally, there may still be some frozen from historical freezing procedures in glass ampules. Cryovials and straws are still used for slow freezing of animal embryos in agriculture and research purposes, but due to the switch to vitrifica-

tion, these are only seen in clinical labs from embryos frozen years ago, and for frozen sperm, which they are still routinely used for.

The biggest difference between slow-freezing devices and those used for vitrification is the volume of cryopreservation media that is used. For slow freezing, the volume ranges between 0.25 and 1 mL, while vitrification devices aim to minimize the volume to less than a microliter to achieve the highest cooling rate. In addition, these devices usually are open systems that expose the cryomedia containing the embryos directly to liquid nitrogen, while slow-freezing devices are all closed systems. There is a theoretical risk of cross infections using these open devices, which is something that is never a concern with slow-freezing devices and considered a major advantage of slow freezing. However, the overall benefit of vitrification still outweighs this one advantage, and some companies now manufacture closed-system vitrification devices to circumvent this concern.

12.5 Ancillary Procedures

12.5.1 Lysed Cell Removal

When embryos are frozen at the cleavage stages (usually day 3 and between 6 and 12 cells), individual blastomeres may be damaged during the freeze/thaw process. At the cleavage stage, individual blastomeres are easily identified, and lysed cells are very apparent. Since the acceptable survival rate for transfer in embryos at these stages is around 50%, often embryos sustain cryodamage but still fall into the acceptable range for embryo transfer. It has been shown that lysed blastomeres remaining in the embryo may negatively affect the undamaged viable cells, prevent cell-cell communication, and could reduce success rates dramatically. Therefore, it is recommended that damaged cells are readily removed from thawed embryos by a process called lysed cell removal (LCR) before transfer, to maximize the outcome potential [19–21]. In fact, one study showed that the presence of necrotic cells reduced the success rates three times, suggesting removal could improve rates accordingly [22]. One limitation of these studies is that due to the nature of the LCR procedure, assisted hatching (discussed below) was used in conjunction with LCR, which makes it difficult to assess how much of the improved outcomes were due to assisted hatching and how much was due to the LCR procedure. Nonetheless, the procedure is relatively simple and easy to perform with demonstrated benefits. A small hole is made in the zona pellucida (either chemically or mechanically), and the necrotic cells are aspirated or squeezed out of the zone to leave only non-lysed cells. The remaining embryo is then transferred as usual.

12.5.2 Assisted Hatching

For an embryo to successfully implant in the uterus, it is imperative that the embryo properly (trophectoderm and inner cellular mass) breach and exit from inside of the zona pellucida, to put the blastomeres in direct contact with the uterine lining. During natural conception, the embryo secretes factors that facilitates thinning of the zona as the blastocyst expands, and eventually a hole forms through which the embryo escapes the zona. There are, however, instances where the embryo fails to hatch due to abnormal zona properties or failure of the embryo to expand and “dissolve” a gap in the zona. In such cases, implantation cannot occur, and no pregnancy can be established.

Data exist that indicate that we can assist the embryo in this process by making an artificial hole in the zona prior to embryo transfer, which can be achieved either by chemical drilling (e.g., acid tyrodes) or via mechanical slicing (fine needle) or drilling (laser) through the zona. It is generally believed that cryopreservation could affect zona properties, causing it to harden during the freeze-thaw process. Therefore, most programs now perform assisted hatching as standard procedure for all embryos that were cryopreserved before transfer. The only exception would be if the embryo was already hatching at the time of freezing, or if the embryo was biopsied beforehand, leaving a hole in the zone. More recently the value of assisted hatching prior to embryo transfer has been challenged, and the routine use of this procedure is not longer recommended [23].

12.6 Why Slow Freezing Is Being Phased Out

Today, IVF clinics around the globe have switched to vitrification as their standard cryopreservation method. In fact, most manufacturers have stopped making their slow-freezing solutions, and a few are now selling only the thaw solutions for those clinics that have a remaining collection of slow-frozen embryos that occasionally need to be thawed. This transition was driven by two factors: Firstly, data suggest that survival rates are superior using vitrification vs. slow freezing [24–27]. However, some groups have failed to find significant differences between the two methods [28, 29]. Secondly, the technique for vitrification is significantly faster, more practical, and more cost effective compared to slow-freezing techniques, so even if convincing data about survival superiority exists, the ease and swiftness of the procedure were enough justification to replace slow freezing in most labs. Slow cooling typically takes between 1.5 and 2 h to perform, whereas a vitrification procedure can be completed in 20–30 min. It is thus understandable why most labs have switched to the faster and cheaper technique and why slow freezing in clinical embryology labs will be completely phased out soon.

12.7 Storage of Slow-Frozen Embryos

Since slow freezing has mostly been phased out, one would naturally assume that the vast majority of slow-frozen embryos in existence are several years old. This brings up issues that have become the liability nightmare for many clinics and storage facilities worldwide. The challenges surrounding long-term cryobanks will be described in more detail in other chapters of this text, but it is important to mention that slow-frozen embryos are subject to the same challenges with storage liability, space constraints, alarms, and abandonment. For a recent review, see Pomeroy et al. [30].

One important point to mention is that slow-frozen embryos are arguably less risky to handle as they are frozen in larger volumes than vitrified embryos. With the latter, there is almost no margin of safety when exposed to ambient temperatures, even for a second or two, whereas slow-frozen embryos, especially those stored in cryovials and larger volume straws, will take longer to warm to detrimental temperatures outside of a planned thaw procedure. Therefore, thaw procedures for slow-frozen embryos can be considered less technically challenging and could be performed by less experienced embryologists, whereas warming of vitrified embryos should only be performed by experienced lab staff.

12.8 The Future of Slow Freezing of Human Embryos

While writing this chapter, a quick poll was done on social media to inquire how many clinics still performed slow freezing of embryos. From over 50 embryologists from all over the world, not a single person said that they are still performing slow freezing of human embryos in their labs. The only use of slow freezing of embryos currently is for agriculture (especially bovine) and commercial mouse embryo freezing. Several laboratories are still using slow freezing for ovarian tissue cryopreservation, but many are switching over to vitrification as well. A lot of embryologists said that they are still occasionally performing slow thawing in their jobs, although many said that they have not done it in such a long time that they would not feel comfortable doing it anymore, or they never had to do the procedure in their careers. It is clear that slow freezing has reached the end of its role in clinical embryology. In contrast, slow freezing of embryos is still used routinely in research, agriculture, and animal breeding programs. The technique has a vast amount of data supporting its effectiveness in a wide array of species. Moreover, slow freezing is used for cryopreservation of sperm, tissues, and other biological materials. Therefore despite having been apparently completely

phased out for clinical human embryo cryopreservation, it should remain a valuable technique for other applications. Also, as mentioned above, a large collection of frozen embryos are still in existence, so the thawing part of the technology should be around for the foreseeable future, and it is advisable that every IVF laboratory should have the ability to thaw slow-frozen embryos. It is thus imperative that clinical lab staff have the knowledge and technical expertise to perform thaws of embryos previously cryopreserved using slow freezing.

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Human Oocyte Vitrification

13

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13.1 Introduction

The first live human birth from a cryopreserved oocyte was reported in 1986, following an automated slow-rate freezing (“slow-freeze”) protocol. Despite the much efforts to improve oocyte slow-freeze protocols over the past three decades, the available evidence suggests that embryos derived from oocytes cryopreserved by “slow freeze” have compromised development and implantation relative to fresh controls. Significantly reduced pregnancy and implantation rates have been shown in embryos derived from slow-freeze versus fresh oocytes. On the other hand, there is now a substantial body of evidence suggesting that vitrified oocytes are functionally equivalent to their fresh counterparts in terms of fertilization, development, and implantation potential. In contrast to slow freezing, vitrification is a cryopreservation method that allows solidification of the cell(s) and extracellular milieu into a glass-like state without the formation of ice. To achieve vitrification within a cell, both a reduction in water content and a highly viscous cytoplasm are necessary. The most widely adopted vitrification method requires the use of high initial concentrations of cryoprotectants, low volumes of loading solution, and ultrarapid cooling-warming rates. The first successful clinical application of human oocyte vitrification involved the use of a very high concentration of a single permeating (7.1 M EG) and single non-permeating (0.6 M sucrose) cryoprotectant resulting in a single live birth [1]. A modification of this approach using 5.5 M EG and 1.0 M sucrose proved to be successful in other small studies [2]. Later, a method based on a mixture of permeating cryoprotectants (2.7 M EG and 2.1 M DMSO) and 0.5 M sucrose (non-permeating cryoprotectant) was introduced, and it rapidly became the most widely adopted method [3, 4]. In the past two decades, several oocyte vitrifi-

cation protocols, which differ from one another in the type of cryoprotectants (EG and/or DMSO and/or propylene glycol (PROH) and sucrose and/or Ficoll and/or trehalose), equilibration and dilution parameters, carrier tools, and cooling, storage, and warming methods, have been proposed [5].

Though the effects of cryopreservation on oocyte quality (structure and functionality) can be minimized through vitrification, there are still factors that can negatively impact oocyte quality translating to diminished embryo developmental potential or unfavorable subsequent clinical outcomes. To date, there are very limited evaluations of vitrification that assess the procedure’s potential negative effects and the consequence of these effects on outcomes. In this chapter, we attempt to assess the potential laboratory factors that can impact the overall efficacy and efficiency of oocyte vitrification. Even so, more studies remain necessary to explore the future directions to better clinical oocyte vitrification.

13.2 Factors Affecting Oocyte Vitrification

13.2.1 Osmotic Shock

From the evidence of the mouse model, the oocyte has specialized mechanisms of regulating cell volume [6, 7]. To date, one of the most utilized protocols for human oocyte vitrification involves the combination of 15% DMSO (2.1 M), 15% EG (2.7 M), and 0.5 M sucrose (or trehalose) in a minimum volume (≤ 1 μ L) cooling method [8]. The extreme osmolality changes encountered during exposure and removal of CPAs used in vitrification and warming processes can be anticipated. Using the most commonly adopted protocol as an example, the osmotic pressure changes can be shifting from 280 (the osmolality of culture media) to 2700 mOsM (the osmolality of equilibration solution) during the equilibration stage. Osmolality significantly increases to 5600 mOsM of vitrification solution before the oocyte is plunged into liquid nitrogen on any type of several carriers

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[9]. In the warming process, the osmotic alterations are abruptly turned around with rapid dilution from 5600 to 1280 mOsM in the warming solution followed by a couple of rehydration steps with 780 mOsM and subsequently 280 mOsM [9]. As one can imagine, the cell membrane of the oocytes must have enough strength in order to stand the water abruptly flowing in and out of the cell membrane during the process of pre- and post-vitrification. The vitrification of oocytes is possible in large part because the oolemma is able to withstand intense and rapid fluid shifts without collapsing. Although stepwise CPA exchange concept, which was originally from the bovine vitrification model, has been broadly utilized for human oocyte vitrification, the exact procedure differs from lab to lab contributing to the variability in cryopreservation success rates. The stepwise CPA exchange can be operated with the use of manual pipetting and microfluidic devices [10, 11] to create a microenvironment with gradual exposure to CPAs and rehydration processes to alleviate osmotic shocks.

13.2.2 The Use of Cryoprotectant Agents (CPAs): CPA Type, Concentration, and Toxicity

13.2.2.1 Permeable CPAs

All successful oocyte cryopreservation methods have one common objective: to avoid ice crystal formation. Though the first successful mammalian oocyte cryopreservation was performed using dimethyl sulfoxide (DMSO) as a cryoprotectant, several other agents such as ethylene glycol (EG) and propylene glycol (PROH) have also been widely applied to oocyte cryopreservation since then. These permeable cryoprotectants consist of small molecules that can penetrate cell membranes to form hydrogen bonds with water molecules, thereby preventing formation of ice crystals. Therefore, successful vitrification requires a combination of high cryoprotectant concentrations and rapid cooling and warming rates. However, exposure of oocytes to high concentrations of cryoprotectants can also be detrimental to the subsequent developmental potential because of osmotic and toxic effects.

Ethylene glycol (EG) is an effective cryoprotectant for oocyte vitrification without any known compromise to morphology and development. Thus, in recent years, ethylene glycol became a universal cryoprotectant for human oocyte vitrification. The mixture of permeable cryoprotectants (EG and DMSO) provided advantages over solutions containing one permeable cryoprotectant in the studies of animal model [12, 13], since the mixture allowed the use of lower concentrations of each individual cryoprotectant to achieve the same level of protection. Thus, the permeable cryoprotectant mixtures (i.e., EG + DMSO or EG + PROH) have often been

chosen as a vitrification formula for vitrifying human oocytes in recent years. However, the advantage of the vitrification solution with a mixture of permeable cryoprotectant has not been evaluated systematically for human oocyte vitrification yet. As such, it is still unknown what affects the outcomes of oocyte vitrification with solution containing single permeable cryoprotectant (EG or DMSO or PROH) versus permeable cryoprotectant mixtures (EG + DMSO, EG + PROH, and DMSO + PROH).

13.2.2.2 Non-permeable CPAs

A wide variety of organisms in nature (including arctic frogs, tardigrades, insects, bacteria and fungi) are able to survive extreme temperatures by accumulating large amounts of intracellular sugars such as sucrose, trehalose, and glucose. The extracellular sugar draws free water from within the cell, since mammalian cell membranes are not permeable to large-molecule sugars. In combination with permeating cryoprotectants, non-permeable CPAs (extracellular sugars) could further prevent intracellular ice crystal formation. The most commonly used extracellular sugar for oocyte vitrification is sucrose with 0.5 or 0.6 molar concentration. However, other types of sugars, such as trehalose, have also been used for oocyte cryopreservation. In a mouse model, it has been demonstrated that extracellular trehalose permitted lower concentrations of permeating cryoprotectants, which reduces the toxicity of the cryo-medium during oocyte cryopreservation.

13.2.2.3 CPA Toxicity

Despite success with oocyte vitrification, there is still much work to be completed in terms of suppressing sublethal oocyte damage. Although most successful oocyte vitrification methods require the use of high concentrations of CPAs, some of these CPA compounds can themselves cause possible toxic effects, such as DNA and cellular damage [14–16].

The exposure to CPAs alone can cause genotoxicity, even without cryopreservation [17, 18]. Three commonly used vitrification CPAs, EG, DMSO, and PROH, have been assessed for genotoxic effects in somatic cells and oocytes [14, 17, 18]. Using the comet assay, it has been proven that high concentration of PROH (7.5% and 15%) induced significant DNA damage in mouse oocytes, regardless of the duration of CPA exposure [14]. Studies also show that oocyte vitrification using PROH caused significantly more DNA damage compared with EG and/or DMSO vitrification protocols [14, 19].

Moreover, it has been indicated that DMSO can induce cytotoxicity by triggering mitochondria-dependent apoptosis and consequently decrease the implantation and full-term delivery rate in the mouse model [16]. DMSO can also induce extreme changes in miRNA and create alterations in

the epigenetic landscape during cell culture [20]. Genome-wide hypermethylation induced by global deregulation of methylation mechanisms, especially when it affects genes crucial to development, may trigger negative consequences immediately, or possibly in subsequent generations. Overall, the use of DMSO should be prudent, and the effects that DMSO may have on epigenetic alterations should be considered. The concentration of DMSO should be kept as low as possible in the vitrification solution. The exact mechanisms by which the genotoxicity and cytotoxicity of cryoprotective agents arise are still being identified; hence, we should choose CPAs for human oocyte vitrification with caution until more studies and long-term evaluations on CPA toxicity are available. In addition to individual CPA's toxicity evaluation, little information is available regarding the assessment of toxicity when a combination of two or multiple CPAs is used for vitrification.

13.2.2.4 CPA Concentrations, Temperature, and Time of Exposure

Regarding CPA exposure time and concentration, it is necessary to determine the minimum time and concentration at a certain temperature required for effective CPA exposure regimes. Progress has mostly been made through research that can only be tested by trial-and-error experiments. The conditions of CPA exposure can impact cell function and survival. The condition factors like concentrations, temperature of exposure, and time of exposure are crucial variables that are just as important of a factor as the type of CPA used. Higher temperatures increase the energy and therefore the movement of the molecules, increasing the rate of diffusion. Since the loading and unloading of permeable CPAs totally depend on the rate of diffusion, the higher the temperature used for oocyte vitrification, the lesser the time required to load and unload the CPAs. Based on most protocols devised for oocyte vitrification, it is very common to use room temperature (22–25 °C) as a baseline temperature for loading and unloading the CPAs through oocyte membrane due to its simplicity. However, it is clear that not all CPAs function equally well with oocytes at a certain temperature. This likely stems from a balance between the CPA's permeability in conjunction with the size and unique permeability of the oocyte membrane, as well as the CPA's toxicity. In order to eliminate the negative effects from the CPAs, several approaches have been implemented into oocyte vitrification protocols. For example, a high concentration of a single permeating cryoprotectant may in itself be lethal or may result in impaired development in mouse and hamster models. Multiple cryoprotectants have been used at lower concentrations in combination to ameliorate individual cryoprotectant toxicity while achieving a highly viscous solution. Currently, the most used human oocyte vitrification method is a combi-

nation of EG and DMSO in order to achieve the lowest concentrations required from each CPA. Currently, the exposure time of most protocols can be reduced to 1 min in the final CPA exchange in order to minimize the impact of the toxic, high osmotic conditions.

13.2.3 pH Changes and Buffer Systems

It is well accepted that to maintain a stable pH in the culture media and handling system, it is critical to optimize IVF outcomes. The external pH of the culture media (pH_e) can impact intracellular pH (pH_i) and cellular resources expended in maintaining intracellular homeostasis. Maintaining a stable pH_i in cells is accomplished primarily by various membrane regulatory transporters and exporters that keep pH_i within a specific range (human embryo 6.98 and MII oocyte 7.03) [21]. If pH_e deviates too far outside the specified range, the pH_i value can change more significantly. Denuded mature oocytes have diminished ability to regulate pH_i and are therefore dependent upon pH_e . Thus, in procedures such as oocyte vitrification, where the protective cumulus cells are purposefully removed, oocytes are extremely susceptible to perturbations in pH_e during the whole pre- and posttreatments of vitrification.

The zwitterionic buffers, such as 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 3-(N-morpholino)propanesulfonic acid (MOPS)-buffered media, are commonly used to stabilize the pH_e for the embryo and gamete-handling media at room atmosphere. The ideal pH for handling media (pH_e) is usually targeted at 7.2–7.4. On the other hand, temperature can affect the pH of zwitterionic buffered media. For example, HEPES pKa is 7.30 at 37 °C and 7.48 at 25 °C, while MOPS pKa is 7.02 at 37 °C and 7.20 at 25 °C. Therefore, it is imperative to select the suitable buffer system for the appropriate working temperature. In the most adopted oocyte vitrification and warming protocols, temperature dramatically changes during procedures for pre- and posttreatment of oocyte vitrification (addition and removal of CPA) (pre-vitrification: from room temperature 25 to –196 °C; post-vitrification: from –196 to 37 °C, and then to 25 °C). Hence, two novel modifications can be considered: (1) using combined buffer systems with different pKa values (HEPES combined with MOPS) in the media for pre- and post-vitrification treatments and (2) selecting the exact pKa value of buffer system that is suitable for each specific step of pre- and post-vitrification treatments (HEPES for 37 °C and MOPS for 25 °C). Nevertheless, those two potential approaches have not been examined for oocyte vitrification. It is necessary to have more validated outcomes from future studies of oocyte vitrification with adjusted buffer systems.

13.2.4 Cryo-Device Design

Human oocytes can be efficiently cryopreserved only when an extremely high cooling and warming rate ($\sim >20,000$ °C/min) is applied to oocytes with a limited amount of surrounding solution. This protocol is also known as minimum volume cooling (MVC) vitrification. Various types of cryo-devices have been developed; these cryo-devices can be separated into three main categories: tubing, surface, and a combination of tubing and surface types. These cryo-devices mainly serve to improve cooling and warming rate [22]. There are some representative tubing devices that have been used for oocyte vitrification such as open pulled straw (OPS cooling rate: $>20,000$ °C/min), closed pulled straw, gel-loading tip, Flexipet denuding pipette, and CryoTip (FujiFilm Irvine Scientific). Secondly, surface-type devices have been gaining more attention due to its high efficiency to heat conduction for cooling and warming; representative devices have been applied to oocyte vitrification such as Cryotop (the cooling and warming rate: 69,000 and 118,000 °C/min, respectively) [23], Cryoloop, Cryoleaf, Cryolock, and Cryotec. The last category is the combination type, which functions by loading the oocytes on the surface device and then enclosing the loaded device into hermetically protective tubing in order to avoid potential cross contamination in the liquid nitrogen. Although the representative devices for this category are also commercially available such as high-security vitrification (HSV), Rapid-I, and Cryotop CL, the actual cooling and warming rate still needs to be assessed. It is interesting to note that the occurrence of vacuolization seemed to be cryo-device related, since a lesser degree was detected in human MII-stage oocytes vitrified by open carrier devices (vitrified oocytes directly contact liquid nitrogen) compared to closed ones [24]. Since temperature conductivity can be largely affected by different materials and designs (close or open system) of the cryo-devices, it indirectly indicates that the occurrence of vacuolization is highly correlated to cryo-injury during vitrification and warming process.

13.2.5 Human Factor

Vitrification involves equilibration of a specimen in a cocktail of CPAs before being plunged in liquid nitrogen. It normally requires high molar concentrations of CPAs and a very rapid rate of cooling to reach below the glass transition temperature (T_g). An ultrarapid rate of warming is also required to avoid ice nucleation. A major drawback of the technology is toxicity from the high solute concentrations, which can have severe biochemical and osmotic effects. In most of the clinical oocyte vitrification and warming procedures, all the

CPAs' loading and unloading are still operated by human hands. Vitrification is one of the most time-consuming, manual, and operator skill-dependent procedures performed in the ART laboratory, because success rates are still highly operator dependent due to the narrow limits of tolerance for CPA loading, cooling, and warming when executing vitrification [25].

13.3 Strategies to Improve Oocyte Vitrification

Vitrification relies on high concentrations of CPAs and a rapid cooling to well below the glass transition temperature (T_g). The result is that the sample is out of thermodynamic equilibrium; hence, the liquid in the sample forms glass. In order to achieve this glass state, a sample will have to traverse a metastable region, in which ice crystals can possibly nucleate and grow (between freezing point and T_g during cooling). In order to avoid any ice crystal formation during cooling, the threshold cooling rate has to be obtained. The cooling rate during the actual vitrification process has been suggested to be the key factor to the success of oocyte vitrification. Likewise, if a sample is heated above T_g after successful vitrification, the metastable region is entered again allowing ice crystals to nucleate and grow (between devitrification temperature and melting point during warming). Therefore, high warming rate is also widely accepted to be critical to cell viability.

In order to traverse this metastable region without forming ice crystals, a couple of principles can be followed: (1) minimizing this metastable region by adjusting the CPA compositions and (2) increasing the cooling and warming rates. A few strategies can be considered to facilitate a vitrified state: (1) using small sample volume, (2) increasing heat conductivity of the cryo-device, (3) using high concentrations of CPAs that reduces the cooling rate that is required to reach the glass state, (4) choosing a good glass former as CPAs, and (5) increasing the difference in the temperatures between the sample and its surroundings (using slush liquid nitrogen with -207 °C instead of liquid nitrogen with -196 °C). On the other hand, not all CPAs perform as well in forming glass, and unfortunately, the best ones tend to be more toxic. Since a successful vitrification normally requires high concentrations of CPAs, the major drawbacks of the technology, typically toxicity, stem from the high solute concentrations. There are various strategies for mitigating the toxic effects of CPAs: (1) choosing a combination of CPAs to reduce an individual CPA's toxicity (1 CPA vs. 2 CPA combination vs. 3 CPA combination), (2) modifying CPA loading and unloading protocols to mitigate the biochemical and osmotic impact (fine-tune the ambient temperature and treat-

ment duration, and gradually increasing/decreasing CPA concentrations), and (3) using agents that can improve the oocyte resistance to the impact.

13.4 Clinical Applications and Outcomes of Oocyte Vitrification

The superior clinical efficiencies and increased live birth rates associated with vitrification allow for meaningful fertility preservation and have led to a significant increase in the use of oocyte cryopreservation. Oocyte cryo-storage permits fertility preservation for a variety of individuals, including those experiencing fertility-threatening disease or treatments, those who wish to delay reproduction, those who are unable to co-locate during IVF treatment (such as failure to obtain sperm on the day of a fresh oocyte retrieval), as well as those who are unable to preserve preimplantation embryos for social, medical, ethical, or legal reasons. Furthermore, oocyte cryopreservation provides logistical alternatives for human IVF cycle management and facilitates emerging services such as donor oocyte banking. Donor oocyte banking offers advantages to all involved parties (donor, recipient, and clinic); such benefits include reduced waiting time, patient synchronization, improved safety measures to prevent infectious disease transmission, and extended donor pool accessibility. The increased availability of vitrified donor oocytes has allowed the widespread application of oocyte donation and the implementation of new forms of collaboration between centers (even across international borders) [11, 26, 27].

During the rapid “adoption and validation” phase of human oocyte vitrification, oocyte donation programs provided most of the data regarding efficacy and efficiency with vitrified oocytes. Early papers on the subject used a variety of approaches to compare vitrified oocytes to fresh oocytes. The donor oocyte model provides adequate evidence for the equivalence of fresh and vitrified oocytes. Using donor oocyte model, most of the oocyte vitrification studies lead us to conclude that the developmental capability of embryos obtained from vitrified oocytes is not affected by the vitrification procedure, as fertilization, embryo cleavage, embryo quality, and clinical results were similar to those achieved with fresh oocytes [27–31]. Moreover, it has been concluded that vitrification had no detectable effect on the *in vitro* developmental competence using autologous sibling oocytes as a model. The results show that fertilization and early embryo development (defined by day-3 good-quality embryo rates and blastocyst formation rates) were not statistically significantly different in the vitrified oocytes compared with the fresh sibling oocytes, and these results strongly imply that the potential negative impact of vitrification on oocytes can be minimized [32, 33].

Like many prior revolutions in IVF laboratory technique, vitrification gained wide acceptance before any safety data was available for children born from the process. Vitrification has been a “care standard” for less than a decade, and there are no long-term follow-up studies on childhood health. Similarly, the technique is far too new to have any data on development through to adulthood. Analysis of perinatal outcome, as well as the long-term development of the children born, is necessary to rule out any adverse effect on offspring. Cobo et al. reported a study with a large group of children born after the use of vitrified oocytes (1027 babies from 804 pregnancies) and fresh oocytes (1224 babies from 996 pregnancies). According to the large series of data, it is very encouraging to note that there is no increase in adverse obstetric and perinatal outcomes in children conceived with vitrified oocytes when compared to fresh oocytes [34]. Long-term follow-up of children born from vitrified oocytes remains necessary to establish the overall safety of the process. Two recent studies for the follow-up report of children born from vitrified-warmed oocytes look very promising. It indicated not only that oocyte vitrification does not adversely affect the growth and health of offspring beyond the neonatal period [35], but also that the mental and physical development of children was comparable to the data of reference group in the 6-year follow-up study [36].

13.5 Future Directions for Human Oocyte Vitrification

13.5.1 Automation

In the ART field, addressing the need for standardization of oocyte vitrification across users and clinics has always been of paramount importance. The semiautomated vitrification system was first tested with limited number of human blastocysts, and the *in vitro* outcome was equivalent to the manual vitrification control [37]. A prospective randomized multicenter trial was conducted with human 2PN embryos to compare the outcome between the semiautomated vitrification system and manual vitrification; it was indicated that the primary outcome (survival, top-quality embryo, and pregnancy rate) was comparable between the semiautomated and manual vitrification [38]. Interestingly enough, in the same study, the operators assessed for user experience that was in favor of not only manual vitrification, but also reduced procedure time spent for vitrification in manual vitrification [38]. The first successful live birth using the semiautomated system to vitrify human oocytes was reported from a case study [39]. The first step of the automation for the purpose of oocyte vitrification has been achieved successfully, and the future is promising for the standardization of oocyte vitrification. Nevertheless, it seems safe to assume that the future will

develop more automated technologies, enabling a welcome reduction in the operator factor while generating more uniform results on oocyte vitrification.

13.5.2 Artificial Intelligence (AI)

In order to prevent water from forming intercellular and intracellular ice crystals, the oocyte has to be dehydrated with the use of highly concentrated cryoprotectants during vitrification. During the warming procedures, the water is directed into the oocytes for rehydration. One can imagine that the cell membrane of oocyte needs to withstand intense and rapid fluid shifts without damages during vitrification and warming. However, biological material is by its nature variable in size and composition, so not every oocyte has the same size and composition. If every oocyte only has a narrow margin for optimum performance in vitrification, conventional protocols have to compromise by focusing on the central tendency, implying that some outlying oocytes fall out of range. Therefore, one vitrification protocol may not be the best protocol to fit all oocytes. AI, or more specifically deep learning, has recently been introduced to the field of ART to automate embryo grading and eliminate the subjectivity of manual techniques. The potential advantages of using AI for embryo selection include increased standardization and the ability to identify subtle features that are not obvious to human eyes [40]. The rapid development of AI in recent years has made possible the ability to objectively process and interpret vast quantities of data to potentially improve embryo selection. Likewise, AI can be applied to enhance protocols according to minuscule cell membrane response to the osmolarity changes and water movement during vitrification and warming procedures. As a result, the CPA exposure and removal conditions can be adjusted to be optimal for each individual oocyte. In the near future, it can be foreseen that individual tailored vitrification protocol for each oocyte can be achieved with the assistance from AI and automation.

13.6 Conclusion

Based on previous invaluable research and intensive validation of human oocyte vitrification studies thus far, the ability to vitrify human oocytes efficiently has already provided tremendous advantages to the field of human ART. Bonds continue to be made in knowledge regarding important factors involved for oocyte vitrification (such as the effects of osmotic effects, CPAs, buffer systems, cryo-device design, and human factors). Even though oocyte vitrification has been adopted broadly by human ART, we should make every attempt to improve its consistency, efficiency, and safety.

Using the principles of vitrification, strategies are attainable to upgrade the current oocyte vitrification procedures. Further improvement of clinical oocyte vitrification can be made by embracing novel technologies, such as automation and AI.

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14.1 Introduction

Since the announcement in 1972 of the survival of mouse embryos after cryopreservation at -196°C (1), the impact of cryopreservation on the growth and improved efficiency of assisted reproduction in humans has become increasingly appreciated. Moreover, cryopreservation has also been shown to increase overall pregnancy rates while allowing for further selection of embryos. Indeed, it is possible to achieve implantation and pregnancy rates with frozen-thawed embryos as high as those achieved with fresh embryos. Routine in vitro blastocyst culture and cryopreservation have been shown to increase pregnancy rates while allowing for better selection of embryos. With more reliable cryopreservation techniques, lower numbers of embryos are now being transferred, resulting in less high-order multiple pregnancies, as well as increased healthy implantations.

14.2 Principles and Purposes of Cryopreservation of Human Embryos

The fundamental objectives for successful cryostorage of cells in liquid nitrogen at -196°C can be summarized as follows: (1) arresting the metabolism reversibly; (2) maintaining structural and genetic integrity; (3) achieving acceptable survival rates after thawing; (4) maintenance of developmental competency post-thaw; and (5) the technique must be reliable and repeatable.

14.3 The Relevance of Human Embryo Cryopreservation

Blastocysts have been shown to increase pregnancy rates while allowing for further selection of embryos. Lower numbers of embryos are being transferred resulting in less high-order multiple pregnancies and increased implantations. In addition, decreased numbers of embryos are transferred increasing the potential for more embryos to be placed in frozen storage and thereby exposing the patient to fewer fresh cycles.

14.4 The Vitrification Component and Cryoprotectant Agents

The major disadvantage of using cryostorage is that it can lead to crystallization of water and thereby can create new and unwanted physical and chemical events that may injure the cells that are being preserved. As early as 1985, ice-free cryopreservation of mouse embryos at -196°C by vitrification was reported in an attempted alternative approach to cryostorage. Since then, vitrification techniques have entered more and more the mainstream of animal and human reproduction as an alternative cryopreservation method to traditional slow-cooling/rapid-thaw protocols. In addition, the last few years have seen a significant resurgence of interest in the potential benefits of vitrification protocols and techniques in human-assisted reproductive technologies. The radical strategy of vitrification simplifies and improves cryopreservation resulting in the total elimination of ice crystal formation, both within the cells being vitrified (intracellular) and in the surrounding solution (extracellular). Vitrification, however, avoids ice formation altogether during the cooling process by establishing a glassy or vitreous state, wherein molecular translational motions are arrested without structural reorganization of the liquid in which the reproductive cells are suspended. The physical definition of vitrification is the solidification of a solution at low temperature, not by ice

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crystallization, but by extreme elevation in viscosity during cooling (2, 3). In general, vitrification solutions are aqueous cryoprotectant solutions that do not freeze when cooled at high rates to cryogenic temperatures. In addition, the rate of cooling/warming and the concentration of the cryoprotectant required to achieve vitrification are inversely related. In addition, recent publications have shown the relatively greater importance of warming rate over cooling rates regarding the survival of oocytes subjected to a vitrification procedure (4–6).

The earliest attempts using vitrification as an ice-free cryopreservation method for embryos were first reported in 1985 (7). In 1993, successful vitrification of mouse embryos was demonstrated (8). Furthermore, bovine oocytes and cleavage-stage embryos were vitrified and successfully warmed a few years later (9). In 1999 and 2000, successful pregnancies and deliveries after vitrification and warming of human oocytes were reported (10, 11). Since that time, and because it seems to be that both entities appear to be especially chill-sensitive cells in ART, blastocysts appear to have received a significant boost in survival rates by avoiding ice crystallization through the use of vitrification (12).

Vitrification is very simple, requires no expensive programmable freezing equipment, and relies principally on the placement of the embryo in a very small volume of vitrification medium that must be cooled at extreme rates not obtainable in traditional enclosed cryostorage devices such as straws and vials.

It is important to mention that cryopreservation interferes without doubt with the intracellular and extracellular homeostasis and therefore poses a risk for inducing spindle abnormalities, chromatid nondisjunction, fertilization errors, and abnormal mitotic divisions during embryonic development. In addition, specific subcellular compartments containing accumulated transcripts and proteins necessary for spindle formation, epigenetic control, and chromosome alignment are also mandatory for proper spindle functionality. Although it is far too early to draw conclusions about the risk of imprinting mutations and birth defects after vitrification of oocytes and embryos, the current literature is reassuring. An increased incidence of imprinting diseases in live births after cryopreservation of oocytes and preimplantation embryos has not been identified (13).

One “drawback” of vitrification considered by embryologists not familiar with the vitrification technique is the use of high concentrations of cryoprotectants, which does potentially mean that vitrification solutions are potentially more toxic than their counterpart solutions used for conventional slow freezing. This is necessitated by the practical limit for the rate of cooling, and the biological limit of tolerance of the cells for the concentration of toxic cryoprotectants being used to achieve the cryopreserved state. It is important to

Table 14.1 Summary of vitrification and warming solutions (Irvine Vit-Kit “Freeze” and “Warm”)

	Composition		
	Ethylene glycol (%)	DMSO (%)	Sucrose (M)
Equilibration solution (ES)	7.5	7.5	0
Vitrification solution (VS)	15	15	0.5
Warming solution (TS)	0	0	1.0
Diluent solution (DS)	0	0	0.5
Wash solution (WS)	0	0	0

EG ethylene glycol, DMSO dimethyl sulfoxide, M-199 + 20% SSS wash solution—WS

note that recently published papers (14–17) have shown that the use of relatively high concentration of cryoprotectants such as 15% (v/v) ethylene glycol (EG) used in an equimolar mixture with dimethyl sulfoxide (DMSO) had no negative effect on the perinatal outcomes from blastocyst transfers following vitrification when compared with those from fresh blastocyst transfers. Overall, more than 20 years of vitrifying cells have discovered that the problem of cryoprotectant toxicity can be eliminated or controlled by careful attention to the technique. Cryoprotective agents (CPAs) are essential for the cryopreservation of cells. Basically, two groups of cryoprotectants exist: (1) permeating (e.g., glycerol, EG, DMSO) and (2) non-permeating (e.g., disaccharides, proteins, and polymers) agents (Table 14.1). The key component of a vitrification solution is the permeating agent. These compounds are hydrophilic nonelectrolytes with a strong dehydrating effect. Furthermore, these CPAs are able to depress the “freezing point” of the solution. Additionally, these highly permeating cryoprotectants are also more likely to diffuse rapidly out of the cells, so that the cells quickly regain their original volume upon warming, thus preventing osmotic injury. The second component of a vitrification solution is the non-permeating CPAs such as disaccharides, for example sucrose, which does not penetrate the cell membrane, but helps to draw out more water from cells by osmosis, and therefore lessens the exposure time of the cells to the toxic effects of the permeating CPAs. The non-permeating sucrose also acts as an osmotic buffer to reduce the osmotic shock that might otherwise result from the dilution of the cryoprotectant after cryostorage. Furthermore, permeating agents are able to bind with intracellular water, and therefore water is very slowly removed from the cell. During warming, using a high extracellular concentration of sucrose (e.g., 1.0 M) counterbalances the high concentration of the cryoprotectant agents in the cell, as it reduces the difference in osmolarity between the intra- and extracellular compartments. The high sucrose concentration cannot totally prevent the cell from swelling, but it can reduce the speed and magnitude of swelling (18–20).

14.5 Artificial Collapsing and Assisted Hatching

Most importantly, residual fluid in the blastocoel can reduce post-warming survival of embryos. Vanderzwalmen et al. (21) showed that survival rates in cryopreserved, expanded blastocysts could be improved by artificial reduction of the blastocoel cavity, and others consider that blastocoel collapse is necessary pre-vitrification on whichever day the blastocyst forms (21, 22) and the most effective strategy for promoting both permeation of cryoprotectant and dehydration (23).

Although one study has suggested that expanded blastocysts show no significant differences in viability, implantation potential, or pregnancy outcome when frozen on day 5 versus day 6 (24), our “body of data” (15–17) refutes the comparable implantation rates for blastocysts cryopreserved on day 5 or 6. And while there are several possibilities why day-6 blastocysts perform less well than day-5 blastocysts, one explanation could be that the older blastocysts are often more expanded with a significantly larger blastocoel impacting water loss and CPA absorption. Being mindful of previously published research on artificial collapse (AC) of blastocysts prior to cryopreservation, we looked for an opportunity that could potentially help us improve the outcome for day-6 blastocysts using AC of the blastocoel prior to vitrification. Because the concept showed improvement for the day-6 blastocysts, we applied to day-5 blastocysts that had well-expanded blastocoels.

AC of the blastocoel can be performed using different techniques, such as microneedles, sucrose solutions, or lasers (22, 25–29). In 2003 and 2004, two groups independently reported a beneficial effect by applying AC to blastocysts prior to vitrification. Son et al. (25) observed an improved clinical pregnancy rate of 48% and an implantation rate of 29% with the use of AC. Hiraoka et al. (26) collapsed day-5 and day-6 blastocysts by manually pipetting embryos until they collapsed and achieved a clinical pregnancy rate of 50%, with an implantation rate of 33% after warming. Moreover, Mukaida et al. (22, 27) found that the survival rate of vitrified blastocysts was negatively correlated with the size of the blastocoel. They speculated that a large blastocoel may disturb the efficacy of vitrification. They collapsed the blastocoel by puncturing it with a microneedle or by making a hole between two trophectoderm cells with a laser pulse. After applying AC, the survival improved from 86 to 97.2%. Moreover, their pregnancy rate went up from 34.1 to 60.2%, with an implantation rate of 46.7%. Iwayama et al. (28) used a laser pulse or osmotic shock resulting from exposure of the whole embryo to sucrose, and the implantation rate was significantly higher in both groups compared to the control group without AC (59.7% and 49.3% vs. 34.2%). Furthermore, Hur et al. (29) looked at the effect of AC,

achieved using a 29-gauge needle or laser pulse, on clinical outcomes in fresh transfers, and they observed a significant increase in the clinical pregnancy in the study group compared to the control group (58.8% vs. 39.0%). All publications mentioned, including Liebermann and Conaghan (30) and Liebermann (31), conclude that AC has a beneficial effect both on frozen blastocyst transfers and for overall cumulative pregnancy and implantation rates. A recent study by Kazemi et al. (32) assessed the effect of pre-vitrification blastocoel fluid reduction on the survival, hatching rate, and expression of genes related to apoptosis (*Tp53*), pluripotency (*Pou5f1* , *Nanog*), and differentiation (*Cdx2* , *Eomes* , *Gata6*) in mouse blastocysts. The survival rate of treatment groups was similar to the control group, whereas the hatching rate of artificial collapse/vitrified blastocysts was significantly higher than vitrified blastocysts. Quantitative reverse-transcription PCR analysis revealed a considerable reduction in the expression of *Cdx2* , *Eomes* , *Gata6* , *Grb2* , and *Tp53* transcripts following artificial collapse/vitrification in comparison to the vitrification-alone group; the abundance of *Pou5f1* and *Nanog* , however, did not change. The authors concluded that artificial collapse of the blastocoel cavity before vitrification leads to relatively normal expression of apoptosis and development-related genes plus higher hatching rates.

Because of the post-thaw phenomenon called “zona hardening” (33, 34) and based on recently published data (35), all thawed embryos underwent assisted hatching using a laser by removing about a third of the zona pellucida.

Warming of blastocysts occurred 1–2 h prior to transfer. Both natural and hormone replacement cycles were used to increase the receptivity of the endometrium. Progesterone was supplemented on day 15 of the cycle, and blastocysts were warmed on day 6 of progesterone supplementation.

14.6 Successful Application of Blastocyst Vitrification

Between 2004 and 2021, the Fertility Centers of Illinois “IVF Laboratory River North” (Chicago) has vitrified 56,770 blastocysts from 14,143 patients (Table 14.2). Most blastocysts were vitrified on day 5 (52.0%), 47.0% on day 6, with a minority on day 7 (1.0%).

Table 14.2 Retrospective data from 14,143 patients (average age 35.1 ± 4.7) with blastocyst cryopreservation by vitrification between 2004 and 2021

	Day of development			Total
	Day 5	Day 6	Day 7	
Number of blastocysts vitrified	29,388 (52.0%)	26,660 (47.0%)	722 (1.0%)	56,770 (100%)

The perinatal outcome (live births) from 9323 FETs **between 2004 and 2018** using an open (Cryotop) as well as closed (HSV) system with an average number of 1.5 embryos transferred (total of 14,394 embryos being transferred) is available and is as follows: number of babies from 3605 deliveries equals 4220 (2154 girls and 2066 boys). Over 80% of all deliveries were singletons with around 16% twin deliveries (Table 14.3). No abnormalities were recorded.

The outcome with regard to the day of development and age of the patient including donor-recipient cycles, between 2004 and October 2021, is summarized in Tables 14.4 and 14.5. In good prognosis patients under 35 years old, when transferring day-5 blastocysts, an ongoing pregnancy and implantation of 52.6% and 51.0% were noted (Table 14.4). In contrast, transferring day-6 blastocysts in patients younger than 35 years of age, an ongoing pregnancy and implantation of 39.1% and 37.0% were recorded (Table 14.5). Despite 96 transfers using day-7 blastocysts only, an ongoing pregnancy and implantation of 18.8% and 18.5% were observed (Table 14.6).

Table 14.3 Perinatal outcome of vitrified blastocysts after 9323 transfers between 2004 and 2018

	Day of development			
	Day 5 + day 6 + day 7	Day 5	Day 6	Day 7
Deliveries (total)	3605	2553	1040	12
Babies born (total)	4220	2990	1217	13
Female	2154 (51.0%)	1545	604	5
Male	2066 (49.0%)	1445	613	8
Singletons/deliveries	3005 (83.5)	2126 (83.0)	868 (83.5)	11 (92.0)
Twins/deliveries	585 (16.0)	417 (16.5)	167 (16.0)	1 (8.0)
Triplets/deliveries	15 (0.5)	10 (0.5)	5 (0.5)	–

Values are numbers unless otherwise described; percentages are indicated between brackets

In October 2007, the Fertility Centers of Illinois “IVF Laboratory River North” moved forward from the use of an open carrier system (*Cryotop—embryos are in direct contact with liquid nitrogen*) to a closed system (*embryos are sealed before contact with liquid nitrogen*). Using a closed carrier (high-security vitrification (HSV) kit) for aseptic vitrification, the following data from day-5, day-6, and day-7 blastocysts were observed and are summarized in Table 14.7: (a) clinical pregnancy rate (*cPR*): 57.6% vs. 47.0% vs. 26.3%; (b) ongoing pregnancy rate (*oPR*): 49.7% vs. 38.6% vs. 20.8%; and (c) implantation rate (*IR*): 49.4% vs. 38.2% vs. 21.5% (Table 14.7). As shown in Table 14.7, *oPR*, *cPR*, and *IR* occurring in the day-5 blastocyst group were significantly higher than transferring day-6 or even day-7 blastocysts.

Between 2007 and October 2021, the Fertility Centers of Illinois “IVF Laboratory River North” (Chicago) performed 2870 vitrified blastocyst transfers (VBTs) without collapsing prior to vitrification, with a mean patient age of 35.8 ± 4.9 years (group A), whereas in 9272 VBT (group B) with a mean patient age of 35.7 ± 4.5 years, artificial collapse was performed prior to vitrification (Table 14.8). On average, 1.6 vs. 1.3 embryos were transferred in groups A vs. B, which means 40–70% of all VBTs were single-embryo transfers. Survival in group A versus group B was not significantly different (98.6% vs. 99.5%). However, there was a significant improvement in group B compared to group A for the following: (a) clinical pregnancy rate (*cPR*): 56.9% vs. 43.3%; (b) ongoing pregnancy rate (*oPR*): 49.1% vs. 34.2%; and (c) implantation rate (*IR*): 50.0% vs. 33.2% (Table 14.8).

When the vitrified-warmed blastocysts were divided into day 5 and day 6 + 7 groups, the following data were observed (Table 14.9): in 1662 VBT transferring day-5 blastocysts from group A (mean age of 35.6 ± 5.0), the *IR*, *cPR*, and *oPR* were 38.3%, 48.5%, and 39.7%, compared to 53.2%, 60.1%, and 52.4% of day-5 blastocysts from group B ($n = 6140$; mean age of 35.4 ± 4.5). As shown in Table 14.9, *IR*, *cPR*,

Table 14.4 Retrospective outcome data (2004–2021) at the Fertility Centers of Illinois, Chicago, from vitrified day-5 blastocysts (untested and genetically tested normal) regarding the patient’s age

	Patient’s age (y)				Donor
	<35	35–37	38–40	>40	
Average age	31.4 ± 2.3	35.9 ± 0.8	38.9 ± 0.8	42.5 ± 1.9	42.9 ± 5.0
Cycles	3781	2040	1321	666	606
Transfers	3778	2039	1319	663	604
Blastocysts survived (%)	99.0	99.0	99.4	98.4	98.5
Blastocysts transferred (mean)	1.4	1.3	1.4	1.4	1.5
Positive pregnancy/VET (%)	68.3	67.0	63.0	59.6	65.4
Clinical pregnancy/VET (%)	59.9	55.9	53.7	49.5	55.1
Ongoing pregnancies/VET (%)	52.6	48.3	44.2	40.6	45.9
Implantations	2724	1306	813	372	400
Implantation rate (%)	51.0	48.3	43.7	38.8	45.2

Values are numbers, unless otherwise described

Table 14.5 Retrospective outcome data (2004–2021) at the *Fertility Centers of Illinois, Chicago*, from vitrified day-6 blastocysts (untested and genetically tested normal) regarding the patient's age

	Patient's age (y)				Donor
	<35	35–37	38–40	>40	
Average age	31.5 ± 2.2	36.1 ± 0.8	38.9 ± 0.8	42.5 ± 1.7	43.8 ± 5.0
Cycles	1795	1191	999	636	262
Transfers	1786	1186	995	631	261
Blastocysts survived (%)	98.4	99.0	98.6	98.6	99.5
Blastocysts transferred (mean)	1.5	1.4	1.4	1.4	1.5
Positive pregnancy/VET (%)	58.6	56.3	56.8	49.4	49.4
Clinical pregnancy/VET (%)	46.9	47.0	46.9	39.6	40.2
Ongoing pregnancies/VET (%)	39.1	39.0	38.7	29.8	30.7
Implantations	1007	644	527	269	121
Implantation rate (%)	37.0	38.1	37.2	30.6	30.6

Values are numbers, unless otherwise described

Table 14.6 Retrospective outcome data (2004–2021) at the *Fertility Centers of Illinois, Chicago*, from vitrified day-7 blastocysts (untested and genetically tested normal) regarding the patient's age

	Day 7
Patient's age (y)	35.8 ± 4.2
Cycles	98
Transfers	96
Blastocysts survived (%)	93.8
Blastocysts transferred (mean)	1.4
Positive pregnancy/VET (%)	35.4
Clinical pregnancy/VET (%)	22.4
Ongoing pregnancies/VET (%)	18.8
Implantations	25
Implantation rate (%)	18.5

Values are numbers, unless otherwise described

and *oPR* occurring from the day-5 blastocysts in group B were significantly higher than from the day-5 blastocyst in group A (χ^2 ; $P < 0.001$ respectively).

If we compare day 6 + 7 in group A ($n = 1208$; mean age of 36.1 ± 4.8) with day 6 + 7 outcome in group B ($n = 3132$; mean age of 36.4 ± 4.3), the following data in terms of *IR*, *cPR*, and *oPR* were observed: 26.6%, 36.2%, and 26.7% vs. 43.7%, 50.8%, and 42.8%, respectively (Table 14.9). As shown in Table 14.9, *IR*, *cPR*, and *oPR* occurring in the day-6 blastocysts of group B were significantly higher than transferring day-6 blastocysts from group A (χ^2 ; $P < 0.001$ for any comparison, respectively).

In Table 14.10, the results for patients under 35 years in group A (**no assisted collapsing**) and B (**assisted collapsing**) are summarized. Comparing day 5 from group A ($n = 767$; mean age 31.4 ± 2.3) with day 5 from group B ($n = 2756$; mean age 31.6 ± 2.2), we found the following for *IR*, *cPR*, and *oPR*: 41.3% vs. 57.2%; 51.6% vs. 63.6%; and

Table 14.7 A comparison of retrospective data from the cryopreservation program (*Fertility Centers of Illinois, Chicago*) of vitrified untested and genetically tested normal day-5, day-6, and day-7 blastocysts using aseptic vitrification technology between October 2007 and 2021

	Day 5 + day 6 + day 7	Day 5	Day 6	Day 7
Patient's age (y)	35.7 ± 4.6	35.4 ± 4.6	36.4 ± 4.5	36.7 ± 4.2
Transfers	12,142	7802	4268	72
Blastocysts warmed	17,067	10,812	6150	105
Blastocysts survived	16,942 (99.3)	10,745 (99.3)	6095 (99.1)	102 (97.1)
Blastocysts transferred	16,713	10,605	6006	102
Blastocysts transferred (mean)	1.4	1.3	1.4	1.4
Implantations	7563 (45.2)	5241 (49.4) ^a	2300 (38.2) ^a	22 (21.5) ^a
Positive pregnancy/VET	7741 (63.8)	5238 (67.1) ^b	2473 (57.9) ^b	30 (41.7) ^b
Clinical pregnancy/VET	6523 (53.7)	4496 (57.6) ^c	2008 (47.0) ^c	19 (26.3) ^c
Ongoing pregnancies/VET	5542 (45.6)	3878 (49.7) ^d	1649 (38.6) ^d	15 (20.8) ^d
Deliveries (total)	3344	2435	900	9
Live births (n until 2018)	3843	2783	1050	10

Values are numbers unless otherwise described; percentages are indicated between brackets

VET vitrified embryo transfer

^{a,b,c,d} $P < 0.001$

44.7% vs. 55.1%. Looking at day-6 outcomes for group A versus group B, we observed the following for *IR*, *cPR*, and *oPR*: 30.2% vs. 45.0%; 40.6% vs. 51.9%; and 32.1% vs. 44.5% (see Table 14.10).

Table 14.8 A comparison of retrospective data from the cryopreservation program (*Fertility Centers of Illinois, Chicago*) of vitrified day-5, day-6, and day-7 blastocysts without artificial collapsing (group A) and with artificial collapsing day-5, day-6, and day-7 blastocysts (group B; starting in 2011) using aseptic vitrification technology between 2007 and October 2021

	Technique	
	Group A	Group B
Patient's age (y)	35.8 ± 4.9	35.7 ± 4.5
Transfers	2870	9272
Blastocysts warmed	4898	12,169
Blastocysts survived	4829 (98.6)	12,113 (99.5)
Blastocysts transferred	4707	12,006
Blastocysts transferred (mean)	1.6	1.3
Implantations	1564 (33.2) ^a	5999 (50.0) ^a
Positive pregnancy/VET	1469 (51.2) ^b	6272 (67.6) ^b
Clinical pregnancy/VET	1243 (43.3) ^c	5280 (56.9) ^c
Ongoing pregnancies/VET	983 (34.2) ^d	4559 (49.1) ^d
Deliveries (total)	946	2398
Live births (n) until 2018	1142	2701

Values are numbers unless otherwise described; percentages are indicated between brackets

VET vitrified embryo transfer

^a $P < 0.01$; ^{b,c,d} $P < 0.001$

Table 14.9 A comparison of retrospective data from the cryopreservation program (*Fertility Centers of Illinois, Chicago*) of vitrified day-5, day-6, and day-7 blastocysts without artificial collapsing (group A) and with artificial collapsing (group B; starting in 2011) using aseptic vitrification technology between 2007 and 2021

	Technique			
	Group A		Group B	
	Day 5	Day 6 + 7	Day 5	Day 6 + 7
Patient's age (y)	35.6 ± 5.0	36.1 ± 4.8	35.4 ± 4.5	36.4 ± 4.3
Transfers	1662	1208	6140	3132
Blastocysts warmed	2795	2103	8017	4152
Blastocysts survived	2760 (98.7)	2069 (98.4)	7985 (99.6)	4128 (99.4)
Blastocysts transferred	2682	2025	7923	4083
Blastocysts transferred (mean)	1.6	1.7	1.3	1.3
Implantations	1026 (38.3) ^a	538 (26.6) ^{aa}	4215 (53.2) ^a	1784 (43.7) ^{aa}
Positive pregnancy/VET	945 (56.8) ^b	524 (43.4) ^{bb}	4293 (69.9) ^b	1979 (63.2) ^b
Clinical pregnancy/VET	806 (48.5) ^c	437 (36.2) ^{cc}	3690 (60.1) ^c	1590 (50.8) ^{cc}
Ongoing pregnancies/VET	660 (39.7) ^d	323 (26.7) ^{dd}	3218 (52.4) ^d	1341 (42.8) ^{dd}
Deliveries (total)	640	306	1795	603
Live births (n) until 2018	772	370	2011	690

Values are numbers unless otherwise described; percentages are indicated between brackets

VET vitrified embryo transfer

Day 5: ^a $P < 0.01$; ^{b,c,d} $P < 0.001$; Day 6: ^{aa,bb,cc,dd} $P < 0.001$

Table 14.10 A comparison of retrospective data from the cryopreservation program (*Fertility Centers of Illinois, Chicago*) of vitrified day-5, day-6, and day-7 blastocysts without artificial collapsing (group A) and with artificial collapsing (group B; starting in 2011) using aseptic vitrification technology in patients younger than 35 years old between 2007 and 2021

	Technique			
	Group A		Group B	
	Less than 35 years old		Less than 35 years old	
	Day 5	Day 6 + 7	Day 5	Day 6 + 7
Patient's age (y)	31.4 ± 2.3	31.7 ± 2.1	31.6 ± 2.2	31.7 ± 2.2
Transfers	767	495	2756	1055
Blastocysts warmed	1313	885	3585	1435
Blastocysts survived	1290 (98.2)	864 (97.6)	3574 (99.7)	1430 (99.7)
Blastocysts transferred	1255	842	3552	1415
Blastocysts transferred (mean)	1.6	1.7	1.3	1.3
Implantations	519 (41.3) ^a	254 (30.2) ^{aa}	2031 (57.2) ^a	637 (45.0) ^{aa}
Positive pregnancy/VET	445 (58.0) ^b	237 (47.9) ^{bb}	1997 (72.5) ^b	713 (67.6) ^{bb}
Clinical pregnancy/VET	396 (51.6) ^c	201 (40.6) ^{cc}	1752 (63.6) ^c	548 (51.9) ^{cc}
Ongoing pregnancies/VET	343 (44.7) ^d	159 (32.1) ^{dd}	1547 (55.1) ^d	469 (44.5) ^{dd}
Deliveries (total)	338	151	926	232
Live births (n) until 2018	417	188	1051	271

Values are numbers unless otherwise described; percentages are indicated between brackets

VET vitrified embryo transfer

Day 5: ^{a,b,c,d} $P < 0.001$; day 6: ^{aa,bb,cc,dd} $P < 0.001$

14.7 The Continuing Evolution and Practical Implications of Blastocyst Vitrification

Data presented in this chapter have shown that freezing at the blastocyst stage provides excellent survival, implantation, and clinical pregnancy rates. To achieve these outcomes, consider these points: (a) Without a successful blastocyst vitrification storage program, extended culture should never be attempted. (b) The blastocyst is composed of more cells and is therefore better able to compensate for cryoinjury. (c) The cells are smaller, which makes cryoprotectant penetration faster. On average, fewer embryos per patient are cryostored, but each one has a greater potential for implantation when thawed.

14.8 Improving Current Embryo Cryopreservation Protocols

Regardless of the success of vitrification as a fast and reliable cryopreservation technique, there is always space for improving current vitrification protocols. This could include (a) the choice of cryoprotective, i.e., (1) the use of one or a mix of two cryoprotectants; (2) one which is DMSO free; (3) replacement of sucrose by trehalose; and (4) containing macromolecules also called polymers such as polyvinylpyrrolidone (PVP), Ficoll, or dextran. In addition, simplifying protocols by exposing cells for a precious duration of time in the different solutions, and even increasing the drop size of the solution for equilibration, and finally vitrification will have a supporting impact for a positive outcome regarding survival and potential to implant.

14.9 Conclusion and Future Directions

Vitrification has itself established as a standard technique to cryopreserve human eggs, zygotes, cleavage-stage embryos, and embryos at the blastocyst stage. It has replaced slow-freezing protocols entirely. This is due to its consistency in clinical track records and reliability and reproducibility of data and results independently from the user after a quite short introduction and training phase. A standardized vitrification protocol applicable to all stages of the preimplantation embryo is not realistic because of (a) different surface-to-volume ratios, (b) rather more stage-specific pattern of the movement of water and cell-permeating cryoprotectants is, (c) differing cooling rate requirements between oocytes, zygotes, cleavage-stage embryos, and blastocysts, and (d) variable chill sensitivity between these different developmental stages. Currently, however, the most widely used protocol applied to any embryo stage is the two-step equilibration in an equimolar combination of the cryoprotectants ethylene glycol and DMSO, at a concentration of 15% each (v/v) supplemented with 0.5 mol/L sucrose. For the adoption of vitrification in ART, as with all new technologies, there has been initial resistance, but as clinical data has been accrued, this technology has become commonly adopted as the standard procedure in many IVF programs worldwide. The increased use in human assisted reproduction as well as in commercial animal production such as porcine and bovine will be pushing forward its development to higher levels of clinical efficiency, utilization, and universal acceptance.

14.10 Establishing and Troubleshooting Clinical Storage: Tips and Tricks for the Clinical Embryologist

1. Special care must be given to the selection of the vitrification carrier type. It is necessary to use types of carrier or vessel material with rapid heat transfer that also supports the process of uniform heat exchange to achieve higher cooling rates.
2. In addition, although no reports of contamination in human IVF following cryopreservation exist, the user should be encouraged to choose a closed carrier system, which in our experience works for blastocysts without any problems.
3. To minimize the toxicity of the cryoprotectant, a step-wise exposure of cells to pre-cooled concentrated solutions (approximate room temperature of 24 °C) is recommended.
4. Utilizing higher concentrations of cryoprotectant allows shorter exposure times to the cryoprotectant, but be careful because the potential toxicity of the cryoprotectant increases at higher concentrations. As almost all cryoprotectants are toxic to some extent, it is important to carefully monitor the duration of exposure to the final cryoprotectant before plunging into liquid nitrogen.
5. To facilitate vitrification by higher cooling rates, it is also necessary to minimize the volume of the vitrification solution (VS) as much as practical (preferably less than 1 μ L). From this point of view, it is very important to use a small pulled pipette. Furthermore, by collecting the blastocysts in one place and loading no more than two blastocysts at the same time in the pipette, it is possible to keep the volume small. However, if the load of media is too large, it can still be reduced before plunging in LN₂ by “drawing down” the droplet to flatten the blastocysts slightly while removing all surplus vitrification solution.
6. To make sure that the blastocysts are loaded on the carrier, perform the loading process under a stereomicroscope. Always confirm the number of loaded blastocysts.
7. After sealing the carrier, submerge the carrier loaded with the blastocysts directly in liquid nitrogen by passing rapidly through the vapor phase (nitrogen gas).
8. Store the cryo-cane in a pre-chilled PVC cryo-sleeve sitting in the goblet in the Dewar. It is essential to always maintain exposure of the HSV to LN₂ to eliminate the risk of warming and devitrification.

9. Before moving the carrier quickly from the liquid nitrogen into the warming solution, have a stripper tip (micropipette) ready. Fill the pipette with a small amount of the first warming solution (TS). When using the HSV as the vitrification carrier, rinse the open edge of the straw after placing into the pre-warmed (37 °C) 1.0 M sucrose; because the droplet is so small, it warms immediately, and it is essential to pull/stir the blastocysts off the carrier surface as soon as possible to avoid any toxic effect of the VS. A stirring motion is recommended when plunging into the warm TS to agitate the cell off the carrier surface without the need to remove it actively.
10. When switching the cells between different concentrations of warming solutions, fill up the pipette with the next lower concentration of warming solution, before picking up the blastocysts to move into the next concentration (dilution effect).
11. In general, during vitrification and warming, the LN₂ Styrofoam box needs to be as close as possible to the working area to minimize any lag in cooling and warming rates.
12. Be aware of the expiration dates of the vitrification and warming media; once opened, shelf life is 6 weeks (according to the recommendation of Irvine Scientific).

7. Styrofoam container.
8. Visotubes 10 mm (IMV 5561).
9. Cryo-canes aluminum (ThermoScientific 5015-0001).
10. Cooper wire cutter (GB; GS-360; 0.80–2.6 mm).

Reagents

1. Sage Protein Supplement (SPS) (Sage).
2. Modified Human Tubal Fluid (HTF-HEPES) from Sage.
3. Vit-Kit, Freeze (Irvine Scientific #90133DSOC).
4. Vit-Kit, Thaw (Irvine Scientific # 90137DSOC).

Equipment

1. Dissecting stereomicroscope (Olympus SZX-12, Bausch Lomb or Leica) with warming stage.
2. Laminar flow hood (Origio).
3. Inverted microscope (Olympus 1X-71).
4. Infrared 1.48 µm diode laser (Hamilton Thorne, Zilos or Lykos laser, Hamilton Thorne Research, Beverly, MA).

Stepwise Blastocyst Vitrification Procedure

Vitrification of blastocysts needs to be undertaken utilizing a “closed system” (HSV: High Security Vitrification Kit; CryoBioSystem, L’Aigle, France; FDA 510(k) clearance for cleavage-stage embryos in blastocysts) after a two-step loading with cryoprotectant agents at 24 °C. If assisted collapsing is done prior to vitrification, then the blastocyst needs to be put on an inverted microscope equipped with a laser system (ZILOS-tk, or Lykos, Hamilton Thorne), the junction of two trophoctoderm cells in each blastocyst needs to be located, and one shot (100% power, 500 µs pulse length) was applied. Then the blastocysts can be moved back in the incubators for 5–10 min. Briefly, blastocysts must be placed in equilibration solution, which is the base medium (M199 with 20% serum supplement substitution (SSS) containing 7.5% (v/v) ethylene glycol (EG) and 7.5% (v/v) dimethyl sulfoxide (DMSO)). After 8 min, the blastocysts need to be washed quickly in vitrification solution, which is the base medium containing 15% (v/v) DMSO, 15% (v/v) EG, and 0.5 M sucrose, for 60 s and transferred onto the HSV using a micropipette. Immediately after the loading of one or not more than two blastocysts in a 1 µL drop on the HSV, the straws can be heat sealed, then plunged in LN₂, and secondarily stored inside 5 mL liquid nitrogen-prefilled canes (Visotube Rond, IMV, France). Each single step is described in detail below. The timeline for vitrifying blastocysts requires a total of 9 min (Fig. 14.1).

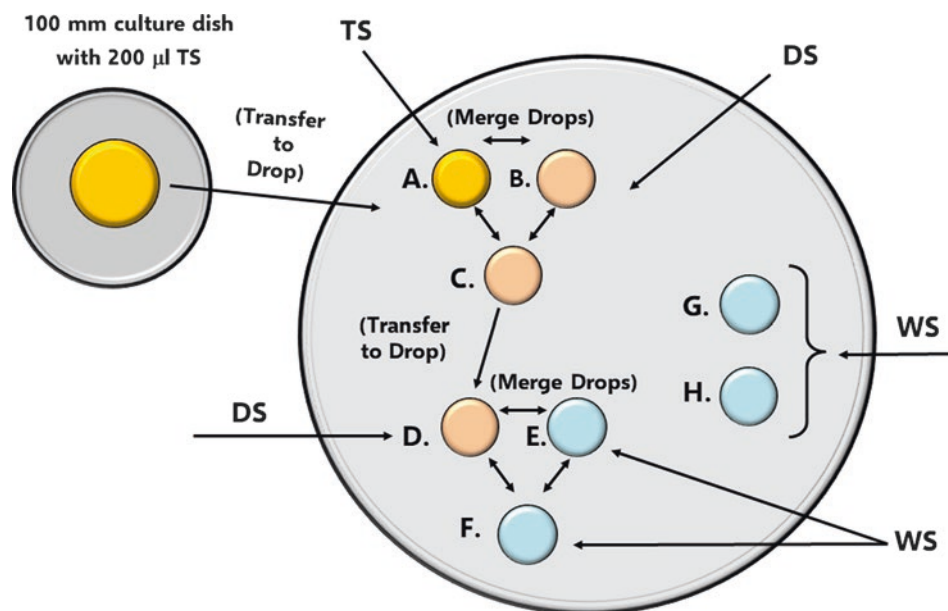
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Addendum. Stepwise Vitrification and Warming Protocol (Standard Operation Procedure) at Fertility Centers of Illinois (Chicago, IL, USA)

Materials

1. HSV (High Security Vitrification Kit (Catalog # 022137; CryoBioSystem)).
2. Heat sealer (CryoBioSystem).
3. Polycarbonate micropipettes, 170 and 140 µm end hole (MidAtlantic Diagnostics# KFPIP-1170-10BS).
4. Brady labeler (BMP 61).
5. Brady labels (PTL-19-427).
6. 90 × 15 mm Petri dish (Nunclon # 150362).

Fig. 14.2 Setup for the warming procedure on a plain 100 mm Petri dish bottom surface. *TS* thawing solution, *DS* diluent solution, *WS* washing solution



a 90 mm dish lid surface. Blastocysts can be picked up directly from the HSV and placed in a fresh 50 µL drop of 1.0 M sucrose at 24 °C, and immediately connected with a 50 µL drop of 0.5 M sucrose. After 3 min, connect with another 50 µL drop of 0.5 M sucrose for 2 min. Then blastocysts can be transferred to a 50 µL drop of 0.5 M sucrose solution and connected with a 50 µL drop of wash solution for 3 min. Then connect to another 50 µL drop of wash solution for additional 2 min. Thereafter, transfer the blastocysts through the 2 × 50 µL of wash solution and return into the transfer dish containing the culture medium (SAGE Blastocyst Medium, Trumbull, CT, USA) until transfer. Each single step is described in detail below. The timeline for warming blastocysts requires a total of 10–11 min (Fig. 14.2).

1. Take reagents from the refrigerator, and allow them to warm to room temperature. All cryoprotectants are removed at 25 °C.
2. Place a 200 µL drop of TS on a dish lid surface, and place on a warming plate.
3. Label a petri dish (Nunc) with the patient's name under the lid as follows: TS, DS, and WS. Prepare 1 × 50 µL of TS, 3 × 50 µL of DS, and 4 × 50 µL of WS (see Fig. 14.2).
4. Before warming, use a Stripper tip with 200 µm end hole for removing the blastocysts from the carrier.
5. Fill Styrofoam container with LN₂.
6. Confirm location and identification with a second embryologist before warming any HSV kit. Warm one kit at a time.
7. Each sample that is warmed will be done in a separate hood and verified by a second embryologist before proceeding.

8. With the HSV kit under LN₂, open the kit by cutting the outer straw using a wire cutter. Use the blue handle to remove the inner stick if needed.
9. Submerge HSV kit directly in the pre-warmed drop containing TS, which should be as close as possible to the LN₂ Styrofoam container. As soon as the HSV kit contents liquefy (within 1 min), try to locate the blastocysts before removing them with a stripper tip. After locating all the blastocysts, remove them from the tip, place them in the droplet of TS (drop A), and connect immediately with the first droplet of DS (drop B) for 3 min.
10. Then connect with the second droplet of DS (drop C) for another 2 min.
11. Then transfer the blastocysts to a fresh drop of 0.5 M sucrose (drop D), and connect immediately with the first drop of WS (drop E) for 3 min. Then connect with the second drop of WS (drop F) for another 2 min.
12. Finally dilute and rinse through a series of two wash drops of WS (G to H).
13. Place the blastocysts into a transfer culture dish, and put it in the incubator for subsequent culture.
14. Record the survival and appearance of all blastocysts. Update log with warm data, and notify the physician of the result.

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15.1 Introduction and Indications for Ovarian Tissue Cryopreservation by Slow Freezing

In most female cancer patients, aggressive chemotherapy and/or pelvic irradiation result in ovarian failure [1], which is why human fertility preservation must be considered a legitimate complement to cancer treatments. Indeed, thanks to considerable improvements in treatments and prognoses, ovarian tissue cryopreservation is a technique that has been gaining ground, not only to safeguard fertility, but also to enhance quality of life by restoring hormone levels [1, 2].

Ovarian tissue cryopreservation and transplantation procedures have so far been almost exclusively limited to avascular cortical fragments, and for now, this is the only procedure that has yielded live births in humans after autologous transplantation [2]. To date, more than 200 babies have been born after grafting of frozen-thawed ovarian tissue [3]. The technique should be recognized as a routine approach in prepubertal patients who require gonadotoxic chemotherapy or in adult patients who need to preserve their fertility without postponing chemotherapy [2, 4].

15.2 History of Ovarian Tissue Slow Freezing

The concept of storing primordial follicles was born in an attempt to give women who cannot undergo controlled ovarian stimulation for oocyte retrieval and freezing a chance to

have their fertility restored. Primordial follicles, being less metabolically active, were expected to be more tolerant to the freezing procedure. However, successful cryopreservation of follicles is a challenge, since they are multicellular and heterogeneous in size. Their water component needs to be replaced with a cryoprotective agent in order to protect them from cryoinjury [5].

The first evidence of the feasibility of frozen-thawed ovarian tissue transplantation was in the 1960s, with a study by Parrot that demonstrated successful pregnancies in mice after ovarian tissue cryopreservation at -79°C using glycerol as a cryoprotectant prior to further transplantation [6]. In 1993, Carroll and Gosden confirmed these earlier reports by successfully cryopreserving previously isolated murine primordial follicles using dimethyl sulfoxide (DMSO) as a cryoprotectant, subsequently proving their fertility potential after thawing [7]. In those first studies, rodents were used as models, and their intact ovaries were preserved and grafted after thawing [8, 9]. However, unlike in mice where it is possible to cryopreserve the entire ovary [10], ovaries from large mammals like sheep, cows, and humans present a greater challenge due to their size and the difficulty of distributing cryoprotectants throughout the whole organ. In these species, ovarian cortex needs to be cut into small fragments, precluding the possibility of direct anastomosis [11, 12].

Sheep were chosen by Gosden et al. as an experimental model to devise a protocol for ovarian tissue cryopreservation using a slow-freezing procedure [12]. Cortical slices were prepared from ovaries of young ewes and autotransplanted to the ovarian pedicle without vascular anastomosis. The experiment proved that primordial follicles can survive cryopreservation after ovarian tissue grafting and go on to produce fertile oocytes and ovulate. Hormone profiles showed that estrous cycles started 3–4 months after transplantation consistent with the folliculogenesis time frame to develop from the primordial stage. Indeed, growing follicles within cryopreserved ovarian tissue do not survive after transplantation, but primordial follicles were shown to remain normal and viable for further development. Fertility

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restoration was also demonstrated, as one ewe conceived and gave birth to a healthy lamb after transplantation [12]. Ovarian cyclicity for up to 2 years postgrafting was evidenced in ewes in subsequent experiments by the same group [13].

Over the following years, a number of reports emerged in the literature about human ovarian tissue autotransplantation for endocrine resumption [14–17]. Then came the first live birth in a woman who had had her ovarian tissue cryopreserved 7 years earlier, having been diagnosed with stage IV Hodgkin's lymphoma [18]. The second live birth followed soon after in a woman with non-Hodgkin's lymphoma who had had her tissue frozen before treatment with high-dose chemotherapy [19].

From 2004 onwards, ovarian tissue transplantation became much more widespread, using cortical strips cryopreserved by slow-freezing approach obtained from thousands of children and young women who had undergone cryopreservation before gonadotoxic treatments all over the world [4]. Indeed, live births have increased exponentially over the years, numbering more than 200 live births to date [3].

Based on increasing evidence of the safety of cryopreservation and further transplantation of ovarian cortical tissue as a fertility preservation and restoration strategy, the American Society for Reproductive Medicine (ASRM) concluded in 2019 that the technique should no longer be considered experimental [20].

15.3 Principles and Challenges of Slow Freezing

During the process of ovarian tissue cryopreservation with a slow-freezing protocol, the tissue can suffer a great deal of chemical and physical damage. Formation of ice crystals is

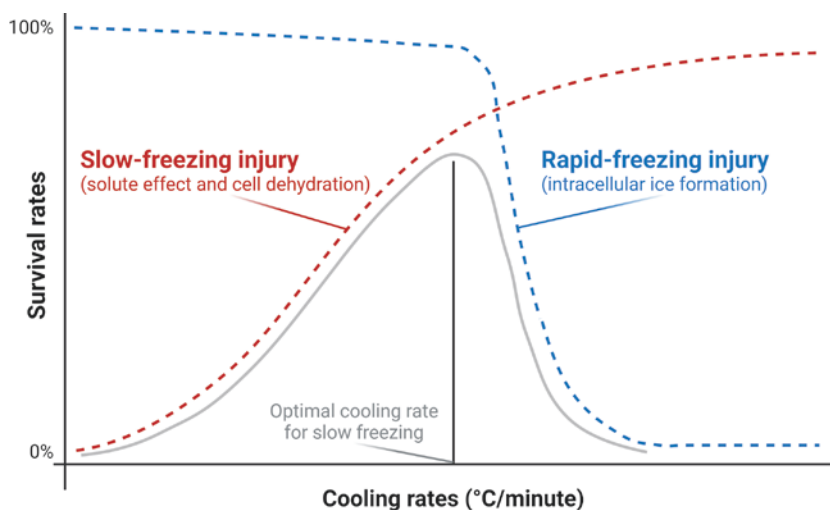
one of the key challenges during the process because, during cooling, there is an inevitable water-to-ice transition phase, potentially causing mechanical damage to cells. As ice crystals form both inside and outside cells, ice-induced injury can be divided into extracellular and intracellular during the freeze-thaw cycle process. Localization of potential ice crystal formation depends on cooling rates and the water permeability of cells with respect to each specific tissue. Fast-cooling rates prevent water from leaving cells, resulting in intracellular ice crystal formation. Slow-cooling rates allow osmotic adjustments, with an increased risk of ice crystal formation in the extracellular space. Moreover, the latter may lead to excessive cell dehydration and shrinkage [21, 22] (Fig. 15.1).

Success of the cryopreservation procedure depends on the setup of all technical steps, including cooling and warming rates and cryoprotectant concentrations, which may be optimal for some cell types, but not ideal for other cell components of ovarian tissue. In this case, the best setup aims to preserve oocytes and granulosa cells of primordial follicles, rather than growing follicles, stromal cells, and vasculature [24].

15.3.1 Use of Cryoprotectants

Addition of cryoprotective agents to the freezing medium helps to protect cells against injury caused by both crystal formation and hypertonicity during the cryopreservation procedure. However, they also have some unwanted effects, as their presence in the solution can cause cell dehydration and shrinkage, before returning to their original volume as the cryoprotectants enter in the intracellular space. Change in volume can lead to cell damage and death, subject to the magnitude and speed of the process [22]. Moreover, cryoprotectants may have some chemical toxicity impact on cells,

Fig. 15.1 Inverted U-curve of cell survival as a function of cooling rate. During slow freezing with low cooling rates, solute impact and cell dehydration are the main determinants of reduced cell survival. Increased cooling rates, on the other hand, cause greater intracellular ice formation, which is also detrimental. The best survival rates are obtained using an optimal tissue-dependent protocol that balances the two damaging components. (Created with Biorender. Adapted from [23])



depending on their concentration [25]. Nevertheless, optimal exposure to cryoprotectants is able to minimize osmotic stress and avoid chemical toxicity while allowing sufficient permeation and dehydration to achieve protection from freezing injury [26].

Cryoprotective agents are further divided into (1) permeating and (2) non-permeating agents. Slow-freezing cryopreservation protocols for ovarian tissue usually involve the use of both. Permeating agents include glycerol, DMSO, ethylene glycol (EG), and 1,2-propanediol (PROH). They are characterized by low molecular weight and the ability to pass through the lipid layer of cell membranes, albeit more slowly than water. Investigations into human ovarian tissue slow freezing, thawing, and xenografting evidenced no significant differences in primordial follicle survival with DMSO, EG, or PROH (survival range: 84–96%), but primordial follicle protection was significantly lower in glycerol (survival rate: 35%) [27]. For this reason, glycerol saw a downturn in the use for slow freezing of ovarian tissue, while the other permeating cryoprotectants are all still utilized in different protocols worldwide. Non-permeating agents include sugars (sucrose, trehalose, raffinose) and macromolecules (Ficoll and polyvinylpyrrolidone), protein, and lipoproteins. They are extracellular solutions and promote controlled dehydration.

15.3.2 Equilibration Time

Since cryoprotectants are potentially toxic to tissues, especially at room temperature and at higher concentrations, the equilibration time before cooling has to strike a compromise between toxicity and sufficient penetration [27]. Equilibration needs to be optimized in terms of time and temperature for each cryoprotective agent [24]. Data on human ovarian fragments evidenced tissue penetration of at least 70% after 30 min at 4 °C [27] compared to just 5 min at 20 °C in murine models [28]. This difference is due to tissue density, which is much higher in humans. Cutting human ovarian cortex into thin strips of similar size favors more homogeneous penetration of cryoprotectants inside the tissue. This minimizes the risk of insufficient or excessive cryoprotectant exposure in the presence of different cell types, a rigid extracellular matrix, and reduced vasculature.

15.3.3 Ice Nucleation

Formation of ice in pure water becomes energetically favorable when water at atmospheric pressure is cooled to below 0 °C. A first nucleus of water molecules fuses into a stable crystal of ice. Additional molecules then attach as the crystal grows, as long as the temperature and pressure are maintained. Initially, ice crystals form randomly because of the

reduced motion of liquid molecules, but become more and more apparent as the temperature drops [23]. In a liquid, like the cryoprotective solution in which ovarian tissue is immersed for cryopreservation, the presence of non-water molecules can guide ice formation to occur within a given temperature range (usually up to −18 °C), hence favoring formation of organized ice crystals outside cells first [29]. In order to do so, ice is seeded in a controlled manner in extracellular solution at a given temperature by manual seeding [23].

15.3.4 Cooling Rates

When ice forms, very small amounts of solutes are incorporated within, hence increasing their concentrations in the remaining water. This leads to (1) a decrease in the freezing point of the remaining solution and (2) an increase in the osmotic pressure gradient across the cellular membrane. For this reason, cooling rates after seeding need to fall progressively until they reach the temperature at which the remaining liquid solidifies through “glass transition,” like in the vitrification process. At this point, the system can be further cooled in liquid nitrogen without any significant change in cells [30]. Cooling rates need to be customized according to cell permeability, upon which dehydration and shrinkage depend, and cell tolerance to toxicity due to increasing concentrations of electrolytes in the unfrozen fraction of the solution [31].

The best results obtained for organ cryopreservation involve a slow-cooling rate of <1 °C/min [21]. Cell damage incurred during faster freezing is likely to be due to intra- and/or extracellular ice formation. Indeed, fast-cooling rates, like those used for vitrification, were shown to significantly increase the proportion of abnormal granulosa cells and oocytes in frozen-thawed ovarian tissue, with more cytoplasmic vacuoles and organelle abnormalities [24].

15.4 Slow-Freezing Procedure

15.4.1 Ovarian Tissue Removal and Processing

Depending on the age of the patient, ovary size, and risk of gonadotoxicity, different surgical approaches can be applied, including (1) uni- or bilateral ovarian biopsy looking to retrieve around one-third of the total cortex, (2) uni- or bilateral hemiovariectomy, or (3) unilateral ovariectomy [32]. Ovarian tissue is surgically removed, avoiding the use of diathermy, and then placed in enriched medium for transportation to the laboratory. It can be processed right away if surgery is done in a large hospital with a dedicated biobank or transported to the closest facility thanks to the organiza-

tion of tissue transportation networks [33–35]. Various media are used for transportation, such as minimum essential medium alpha, IVF medium, Leibovitz-15, and HEPES-MEM. Temperatures range from 0 to 39 °C, the most common being on ice (4 °C) or room temperature (20 °C). Although there is no standard procedure, the best conditions appear to involve transport at 4 °C to minimize ischemic damage and use of a medium able to sustain tissue metabolic activity [36]. Upon arrival at the biobank laboratory, ovarian biopsies are placed beneath laminar flow hoods and processed. Slivers of cortex are shaved from the surface with a scalpel blade and/or scissors to a depth of around 1 mm and then further cut into small strips of around 15–45 mm² (Fig. 15.2).

15.4.2 Preparation of Freezing Medium

Ovarian cortical strips are transferred to cryogenic vials containing 1 mL freezing solution, which differs according to protocol. The slow-freezing protocol developed by Gosden et al. involves transfer of cortical strips to Leibovitz-15 medium supplemented with 10% donor calf serum and 1.5 mol/L DMSO, followed by 15 min of equilibration at 4 °C [12]. This protocol is among the most widely used, including in countries like the UK, Belgium, France, Germany, Spain, Sweden, and the USA [26]. One of the most common variations to this protocol is addition of 0.1 mol/L

sucrose as a non-permeable cryoprotectant to the freezing solution containing DMSO.

Ovarian fragments can also be frozen in a solution containing 0.1 mol/L sucrose and 1.5 mol/L EG in phosphate-buffered saline after 30 min of equilibration at 4 °C, according to the protocol used in Denmark [17]. A third protocol, published by Gook et al., involves transfer of ovarian tissue to a solution of HEPES-buffered human tubal fluid containing human serum albumin at 37 °C and enriched with 1.5 mol/L PROH and 0.1 mol/L sucrose, followed by 30 min of equilibration [24].

15.4.3 Cooling Rates and Seeding

Cryovials are placed inside a programmable freezer and cooled progressively at a cooling rate of 2 °C/min [12, 17, 24]. Cooling is then paused to perform manual seeding for ice nucleation at an optimal temperature that depends on the cryoprotectant characteristics in the freezing solution and can vary between –7 and –9 °C [12, 17, 24]. Manual seeding is achieved by touching each cryovial with an instrument (a spatula cooled or a cotton swab) cooled to cryogenic temperatures (Fig. 15.3). Ovarian tissue cooling continues at a slow rate of 0.3 °C/min to –40 °C and thereafter at a much faster rate of 10 °C/min to –140 °C [12, 17] or 50 °C/min to –150 °C [24] (Fig. 15.4). The cryovials are subsequently removed and stored in liquid nitrogen at –196 °C.

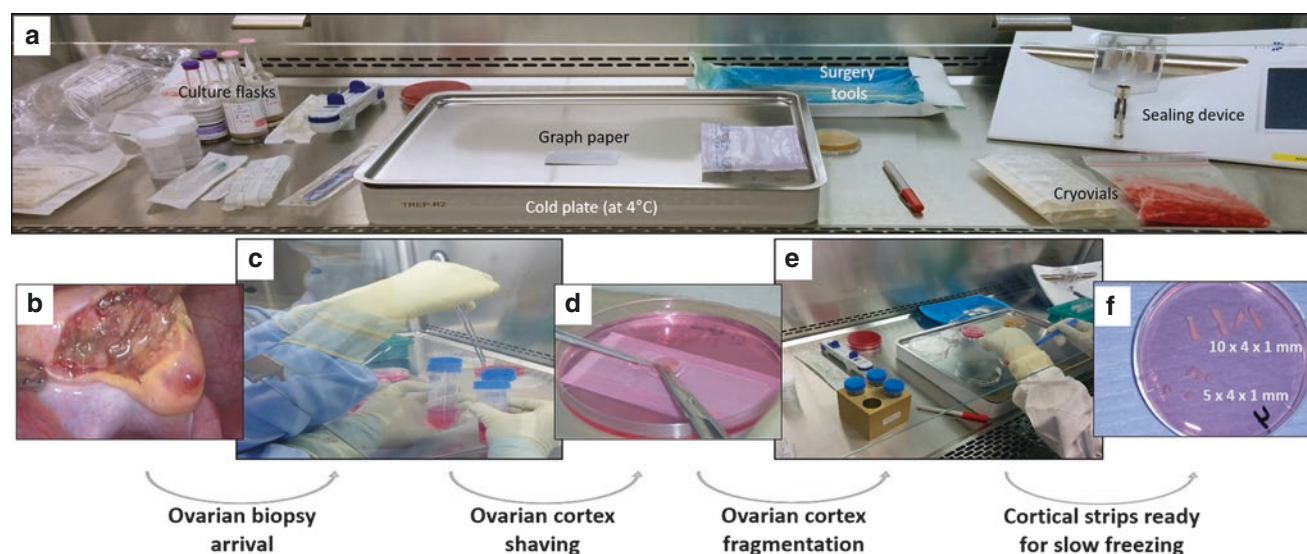


Fig. 15.2 (a) Workstation at the tissue biobank for ovarian tissue slow freezing, including (from the left) culture flasks for quality control of discarded medium, a cold plate at 4 °C with graph paper to prepare ovarian fragments, a sterile surgery kit, cryovials, and a sealing device. (b) Large ovarian biopsies of ovarian cortex are retrieved from the oper-

ating room. (c) The biopsies are transported inside enriched medium like Leibovitz-15 to the tissue biobank. (d) Slivers of ovarian cortex are shaved from the remaining medullary tissue with a scalpel blade and scissors on the cold plate and (e) divided into small fragments of different sizes (f) (10 × 4 × 1 mm or 5 × 4 × 1 mm)

Fig. 15.3 Cooling from 0 °C is paused for manual seeding, done with either a spatula (a) or a cotton swab (b) cooled to cryogenic temperatures in liquid nitrogen, the optimal temperature ranging from –7 to –9 °C

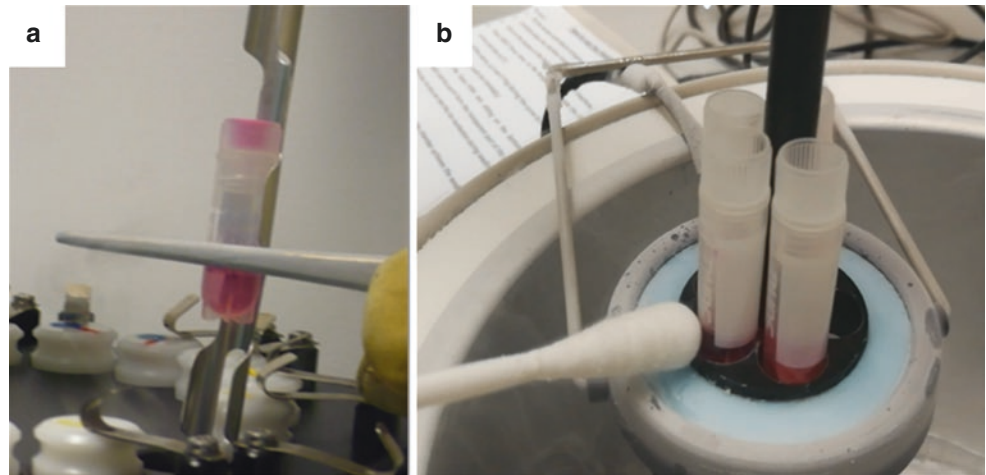
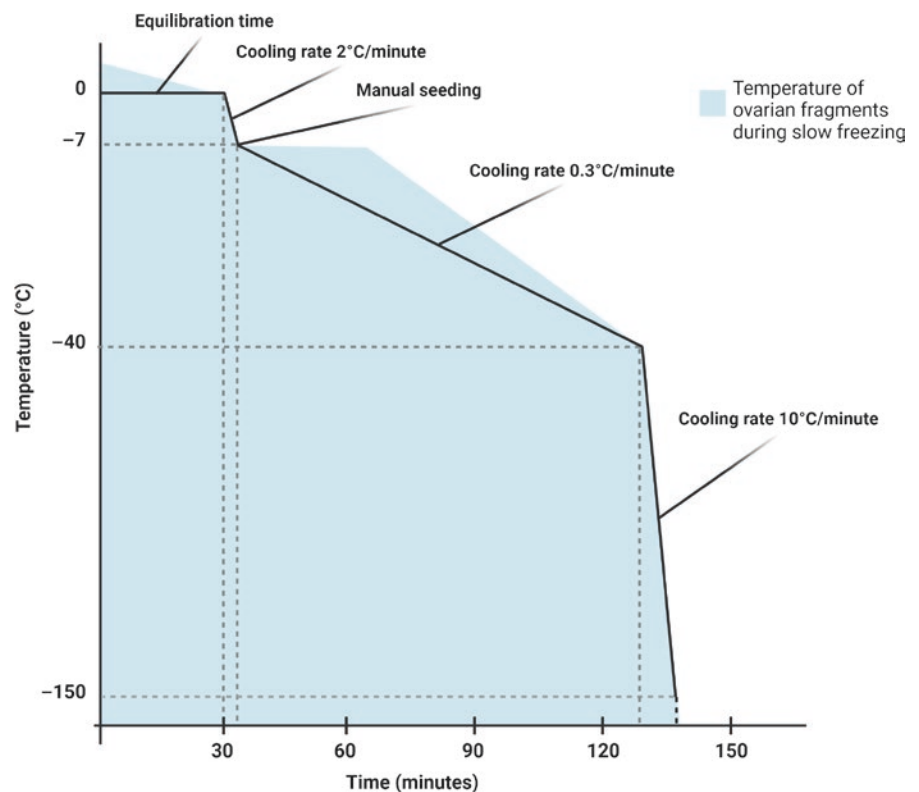


Fig. 15.4 Curve of cooling rates used in the slow-freezing procedure, according to Gosden et al. [12]



15.5 Principle and Challenges of Thawing

During thawing, there is a risk of tissue damage due to ice formation and an osmotic imbalance caused by water uptake by cells, making them swell. The risk of compromising tissue viability by either of these two threats means that the thawing procedure is just as precarious as slow freezing itself [23]. The best strategy to prevent tissue thawing-related damage is slow and progressive removal of cryoprotectants,

associated with addition of proteins like human serum albumin or sugars like sucrose to control the process [23, 32].

Since cryoprotective agents may be toxic at higher concentrations, a number of research groups have raised concerns about their residual presence after thawing and possible detrimental effect on ovarian tissue. Nakamura et al. found evidence of residual DMSO and EG cryoprotectants immediately after thawing [37]. However, cryopreserved ovarian tissue is rarely transplanted straight after thawing,

and additional washing may decrease cryoprotectant concentrations. Indeed, gas chromatography showed cryoprotectant concentrations to be minimal after 30 min and negligible after 60 min, irrespective of the type of cryoprotectants used and with no evidence of DNA damage to follicles after thawing [38].

15.6 Thawing Procedure

Thawing protocols may vary in terms of time and thawing solutions, but all aim to ensure optimal clearing of cryoprotectants from tissue [39]. The most frequently used is a short protocol based on several baths in Leibovitz-15 without cryoprotectants [12, 27, 40]. Frozen slices of ovarian cortex are first thawed for a short time in ambient air and then in a water bath at room temperature to speed up the process. The tissue slices are then removed from the cryovials with sterile forceps before being washed three times in fresh medium in order to gradually remove all traces of the cryoprotectant [12]. Another protocol applied in a clinical setting involves slow thawing and several baths in decreasing cryoprotectant concentrations using PROH [41, 42] or DMSO [43, 44] and sucrose. Thawed ovarian fragments are then transported to the operating room in fresh medium for ovarian tissue transplantation back to the pelvic cavity.

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Cryopreservation, In Vitro Maturation, and Transplantation of Ovary Tissue

16

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16.1 Introduction

The purpose of this chapter is to update the results of the author's study in the USA and include what has been learned from in vitro gametogenesis about IVM, i.e., obtaining competent metaphase II (MII) oocytes at the same time as cryopreservation of the ovarian tissue. Unique to this report are (1) results from vitrification as well as slow freezing, (2) birth of six healthy infants from women with leukemia, (3) relative simplicity within the series of IVM from ovary tissue, and (4) success rate from just one center using the same techniques [1–6]. Here, the authors report the latest update of a single large series of cryopreserved transplants from one center in the USA, carried out with the same technique assessed uniformly over long-term follow-up and studied carefully to elucidate the mechanisms of ovarian longevity and failure. The authors also wished more recently to see if mature oocytes could be obtained easily in vitro from the removed ovarian tissue, prior to freezing, and thus avoid the need for hormonal stimulation.

Over a period from 1997 to 2020 (24 years), 119 female patients (out of 142 who were initially consulted) between age 1 and 42 years underwent ovary tissue freezing for fertility preservation by either slow freezing or vitrification, and the 13 most recent also underwent IVM with vitrification of the resultant MII oocytes. Of these 119 cases, 85 were related to cancer, 8 were for threatened premature ovarian failure, 13

were for social reasons, and 13 were for a variety of conditions including Turner's syndrome, multiple sclerosis, endometriosis, aplastic anemia, a daughter born with no ovary, or massive bilateral ovarian teratoma. All patients underwent slow freezing prior to September 2007, and all subsequent participants underwent vitrification of their ovarian tissue.

16.2 Cryopreservation and Transplant Surgery

The technique for slow freeze and thaw of ovarian tissue has not changed since the original description by Gosden and colleagues in 1994 [7, 8]. Slow freezing was the approach initially used when this program began in 1997, and it has been previously described in detail [9].

16.3 Freezing Technique: Slow Freeze

The cortex is removed from the medulla; divided into multiple strips measuring $1.0 \times 1.5 \times 0.5$ cm each; incubated in 1.5 mol/L 1,2-propanediol ethylene glycol or DMSO, 0.1 mol/L sucrose with 10% SSS at 37 °C for 30 min, and then 0.2 mol/L sucrose for 5 min; and finally transferred to cryovials and cooled by computerized lowering of temperature [11]. Cooling is first done at 2 °C/min to –9 °C and then seeded. Thereafter, the cooling rate is –0.3 °C/min down to –40 °C and then –10 °C/min down to –140 °C, and then it is plunged into liquid nitrogen. Thawing is performed rapidly (37 °C/min) in a warm water bath, and the tissue is trimmed under an operating microscope before transplantation [7, 10, 11, 13]. This slow-freeze method is well established and has resulted in most of the reported pregnancies and live births to date.

The thaw for slow freezing has, however, not been well described in the literature. (Thawing is performed rapidly (100 °C/min) in a warm bath after first holding the cryovial in air for 30 seconds. Serial transfer to the thawing solu-

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tions of 1.0 M cryoprotectants, 0.5 M cryoprotectant, 0.2 M sucrose, and then standard media is undertaken for 5 min for each step.) As there is usually redundant tissue under the cortex after thawing with slow-freeze cases, the tissue is trimmed under an operating microscope before transplantation, as previously described in great detail [7, 8, 10–12]. The purpose of this trimming is to avoid interference with revascularization, or diffusion of nutrients to the transplanted cortex, and thus prevent ischemic damage.

16.4 Freezing Technique: Vitrification

Since 2007, the authors have exclusively used vitrification, because in vitro viability analysis studies have shown no oocyte loss with vitrification, but a 40% oocyte loss with slow freezing [14–16]. Nonetheless, slow freezing seems equally robust for ovarian tissue cryopreservation because the loss of half of a huge number of eggs (200,000) would not be likely to affect the clinical result. The authors' practice exclusively adopted vitrification in 2007 because it was simpler and more feasible for the series. However, in vitro viability analysis studies show that vitrification may be preferred due to its negligible oocyte loss rate as compared to the 50% oocyte loss with slow freezing [14–16]. For vitrification, the ovarian cortex is cut into slices measuring $10 \times 10 \times 1$ mm. The tissue slices are initially equilibrated in 7.5% ethylene glycol (EG) and 7.5% dimethyl sulfoxide (DMSO) in HEPES-buffered handling media supplemented with 20% synthetic serum substitute (SSS) for 25 min. Then, a second equilibration is performed in 20% EG and 20% DMSO with 0.5 M sucrose for 15 min or until the slices descend to the bottom of a 50 mL centrifuge tube indicating complete absorption of the cryoprotectants. The tissues are then placed on a thin metal strip (Kitazato BioPharma, Japan), which are then plunged directly into sterile liquid nitrogen and inserted into a "closed" tube which contains liquid nitrogen for storage.

The pieces of thawed cortex are first quilted together with 9-0 monofilament nylon under an operating microscope and continuous pulsatile irrigation with ice-cold media. This quilted ovarian cortex is then transplanted onto the residual ovary medulla after the oocyte-depleted cortex has been resected. Any hemorrhages on the medulla are controlled with micro-bipolar forceps and pressure stitches of 9-0 nylon to avoid micro-hematoma formation under the graft. The graft was placed orthotopically to allow natural conception from ovary retrieval by the fallopian tube.

For thawing, the metal strip is immersed swiftly into 40 mL of 37 °C HEPES-buffered handling media supplemented with 1.0 mol sucrose for 1 min and then 40 mL of 0.5 mol sucrose for 5 min at room temperature and washed



Fig. 16.1 Thin slices of ovarian cortical tissue preserve the resting follicle pool



Fig. 16.2 A thin (1.5 mm) slice of ovarian cortex



Fig. 16.3 Histology of thinly sliced ovarian cortex containing primordial follicles

twice for 10 min in standard handling media. Standard H + E histology and dye exclusion are performed to check for viability and presence of cancer cells (Figs. 16.1, 16.2, 16.3, 16.4, 16.5, and 16.6).



Fig. 16.4 (Previously published) Ovary Transplant Tissue Quilting (Silber SJ, Goldsmith S, Castleman L, Hurlbut K, Fan Y, Melnick J, Hayashi K. In-vitro maturation and transplantation of cryopreserved ovary tissue: understanding ovarian longevity. *Reprod Biomed Online*. 2022 Mar;44(3):504–514)

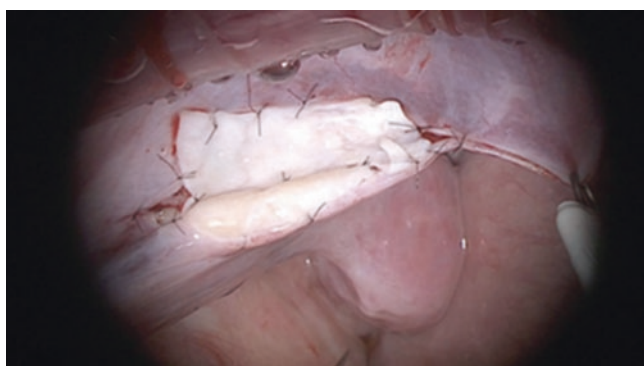


Fig. 16.5 (Previously published) Ovary Transplant (Silber SJ, Goldsmith S, Castleman L, Hurlbut K, Fan Y, Melnick J, Hayashi K. In-vitro maturation and transplantation of cryopreserved ovary tissue: understanding ovarian longevity. *Reprod Biomed Online*. 2022 Mar;44(3):504–514)

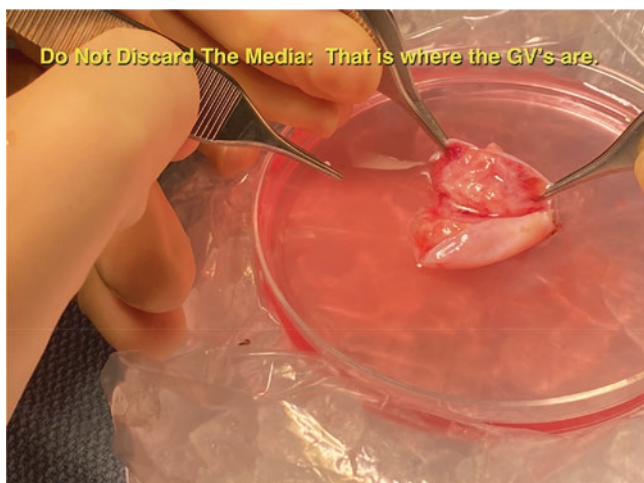


Fig. 16.6 (Previously published) Dissection of ovarian cortex from the medulla. (Silber SJ, Goldsmith S, Castleman L, Hurlbut K, Fan Y, Melnick J, Hayashi K. In-vitro maturation and transplantation of cryopreserved ovary tissue: understanding ovarian longevity. *Reprod Biomed Online*. 2022 Mar;44(3):504–514)

16.5 IVM Culture

For IVM culture, a variety of concentrations of FSH and HCG (LH) were employed. Those concentrations were (1) 75 mIU/mL FSH with only 10 mIU/mL HCG with ordinary cleavage media; (2) 150 mIU/mL FSH with only 20 mIU/mL HCG with ordinary cleavage media; (3) 75 mIU/mL FSH with 1000 mIU/mL HCG in cleavage media; (4) 75 mIU/mL FSH with 1000 mIU/mL HCG in Sage IVM media, USA; and (5) 75 mIU/mL HCG in Sage IVM media. This variety of ordinary medium was used because of previous results with in vitro gametogenesis demonstrating the stages of in vitro development (IVD), in vitro gametogenesis (IVG), and IVM, postulating that many of these germinal vesicles would already have completed the full phase of IVD and IVG in vivo in the ovary. The authors suspected that no special IVM media would be needed and that almost any concentration of HCG would suffice.

16.6 Results

Thirteen transplants resulted in spontaneous pregnancy and the delivery of at least one live healthy infant (76%). In one case, five singletons so far have resulted from just one participant from one transplant, two singletons from another woman, and in a third case, two singletons and one spontaneous set of twins from just one patient. There have thus been a total of 19 live, healthy infants without IVF in these 17 cases. There have been a total of 22 pregnancies, 3 of which miscarried before 3 months (14%). Ovarian function is still continuing in 3 of the 6 cases involving vitrified tissue and 5 of the 11 slow-freeze cases.

Three of the transplants were for leukemia, for which the oncologists involved gave approval. The basis for approval was that the ovary had been removed and frozen after the patient was in her first remission, before the bone marrow transplant, and that stains for residual cancer cells were negative [2]. Two of these three resulted in spontaneous pregnancy, with the delivery of a total of six healthy infants. There has been no recurrence of the leukemia, and in fact no recurrence in any of the cases involving cancer. All three women who had leukemia still have ovarian function. These were among the first cases of success in terms of leukemia patients having babies from transplanting their frozen tissue, although the first such case was published in 2017 [9]. One of the participants who had had leukemia now had five live births from her transplanted ovary tissue.

The most recent 13 cases involved IVM of the cumulus complexes recovered in the media after cortico-medullary dissection. The number recovered varied widely—between 3 and 37 cumulus complexes depending on the age of the women and whether there had been any prior chemotherapy.

The rate of maturation to an MII oocyte varied from a low of 19% to a high of 56% (average 35%). The average values for other centers publishing on IVM have varied between 30% and 39% [17–25]. Maturation of germinal vesicle to MII oocytes was detected between 24 and 48 h of exposure to the HCG-containing media. For most participants, the number of mature oocytes was what would be obtained from ovarian hyperstimulation. Surprisingly, the success of IVM was not related to the media or to the concentration of gonadotrophin in the media. A variety of media and concentrations were intentionally used in light of now-established mechanisms of in vitro oogenesis, to see if this understanding could be used for a simplification of IVM [18–21, 26, 27].

16.7 Discussion

The unique features of this report include (1) the robustness of results with this technique; (2) the inclusion of both slow freezing and vitrification; (3) some of the first successful results with three leukemia patients, who delivered six healthy infants; (4) a well-studied series from just one center (the only such center in the USA); and (5) very recent addition of IVM from ovarian tissue to freeze MII oocytes at the same time as cortical tissue cryopreservation. The advantages of ovary cryopreservation over oocyte vitrification for cancer patients include no delay of cancer treatment, avoidance of ovarian stimulation, and a resumption of endocrine function. As these results (and those reported by other groups) with IVM demonstrate, one might consider the option to dispense with ovarian stimulation, and go right to oophorectomy, with no delay in cancer treatment. Surprisingly, there might therefore possibly be no need for stimulation to obtain mature oocytes [22, 25, 28–30]. Mature oocytes may possibly be directly obtained from the excised ovarian tissue. Other groups have preceded these current efforts at IVM from excised ovarian tissue and achieved pregnancy with healthy infants [25, 28–32].

Perhaps the most striking scientific postulate about ovarian function resulting from this series is the high rate of maturation of germinal vesicle oocytes from ovary tissue to MII in less than 2 days, with no ovarian stimulation. The success of IVM did not correlate in this series with the specifics of the media used or the concentration of gonadotrophin. This might have been expected from the early work of Edwards and of Cha and colleagues, as well as Hayashi's group [18, 19, 27, 33, 34]. The success of IVM appears to be intrinsic to the cumulus, in which the germinal vesicle oocyte has already in vivo developed meiotic competence, and because the germinal vesicle oocytes have already achieved meiotic competence, by in vivo exposure to the eight “core genes,” and in vivo FSH [26].

It is easy to collect many germinal vesicle oocytes from these tiny follicles when you have the ovary in hand instead of using a needle, and their intrinsic 30–40% meiotic competence is universal. So why do we even need ovarian stimulation, or even the normal ovulatory cycle? The normal ovulation cycle is not needed for meiotic competence. Ovarian stimulation for IVF and even for the normal ovulatory cycle is only required for easy oocyte retrieval or just to allow oocytes to exit the ovary.

Without the ovarian cortex, which induces the formation of primordial follicles, fetal oocytes would continue in meiosis and be completely depleted by birth [1, 2, 12, 18–20, 27, 35–38]. Nagamatsu and colleagues demonstrated that dense cortical tissue pressure caused oocyte nuclei to rotate, and this held the primordial follicles in arrest [21]. As they encountered less tissue pressure internally, the rotation stopped, and the primordial follicles were then recruited [21].

The high success rates here with pregnancy and healthy babies after frozen ovary tissue transplantation are most likely aided by having tissue only from younger women with no prior history of infertility. Nonetheless, the live baby rate for these otherwise sterile cancer survivors and the obvious effectiveness of standard slow freezing (despite a previously demonstrated high oocyte loss compared with vitrification) for ovarian tissue cryopreservation testify to its robustness and simplicity [12, 18, 19, 27, 38–45]. One may be able to obtain as many mature oocytes from IVM using this tissue as one would from ovarian stimulation. Furthermore, following the lead of Greve and co-workers, this approach could even be used for patients with leukemia [2]. Most leukemia patients present with severe illness and require initial chemotherapy to go temporarily into remission before having their bone marrow transplant. This is likely to reduce the risk of transmission of leukemia cells back to the woman after thawed ovarian tissue is transplanted back. The already recruited, developing follicles will be destroyed by this initial chemotherapy. However, the primordial follicles will be resistant [3]. Thus, the only option for leukemia patients may be ovarian freezing and transplant.

Ovarian tissue cryopreservation thus appears (24 years later) to be a robust method for preserving fertility, without any need to delay cancer or other treatment because of the time required for ovarian stimulation. Simple cortical tissue pressure (associated with primordial follicle nuclear rotation) has been found to be a key regulator of primordial follicle arrest, recruitment, and ovarian longevity in humans, similar to mice [21]. However, eight “core genes” are also necessary to allow the primordial follicles to escape arrest and develop over 4 months to be meiotically competent germinal vesicle oocytes. The ability of ovarian tissue germinal vesicle oocytes to undergo normal IVM indicates that the

normal ovulatory cycle and ovarian stimulation are not necessary for oocyte maturation. Their only purpose may be just to grow a follicle for mono-ovulation or for egg retrieval, i.e., to get the mature oocyte out of the ovary.

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Vitrification: Implementation and Training

17

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17.1 Introduction

Vitrification represented a game changer in IVF. It is a fast, safe, and efficient protocol to cryopreserve oocytes and embryos [1–4], and it is more effective than the slow-freezing technique [2–4].

The introduction of vitrification has allowed to implement several clinical strategies:

- The systematic application of *elective single-embryo transfer* policy, thus reducing the risk of multiple pregnancies and increasing the safety of treatments.
- *Deferred embryo transfer*. By performing cycle segmentation, it is possible to avoid transferring embryos in a stimulated endometrium, possibly improving the chance of implantation and decreasing the risk of ovarian hyperstimulation syndrome.
- *Preimplantation genetic testing* as it is currently conducted. The improvements in cryo-survival rates achieved via vitrification of biopsied blastocysts boosted the widespread implementation of PGT.
- *Female fertility preservation* for both medical and non-medical reasons. In 2013 and 2018, the ASRM and the ASCO, respectively, stated that oocyte vitrification would no longer be considered experimental. In fact, it was reported as the gold standard technique for fertility preservation, thanks to its excellent results and the reproductive autonomy its application offers to women [5–9].

- *Egg banking for donation*; the consistent survival rates achieved by vitrification [10–12] laid foundations for the implementation of effective egg donation programs through vitrified oocytes, thereby increasing safety and improving management.

The access to these high-standard clinical strategies is possible thanks to vitrification and its impressive effectiveness.

For a proper implementation of vitrification in IVF, some key aspects must be considered. A proper laboratory design should enable appropriate staff and equipment layout for an optimal workflow as well as proper equipment and consumable selection. As current vitrification protocols demand major hand-operated steps, the effectiveness of this technique may be highly variable and dependent on embryologists' experience. Therefore, adequate staff training programs are mandatory to perform it successfully. Lastly, a robust and reliable quality control system is required; to evaluate and monitor staff performance and equipment, to identify gametes and embryos correct handling during cryopreservation, and to ensure traceability and witnessing during vitrification, storage, and warming processes.

17.2 IVF Laboratory Design

17.2.1 General Safety Requirements

In terms of resources and time, cryopreservation covers an important portion of the IVF laboratory activity. Therefore, a specific working area should be dedicated to the manipulation and storage of cryopreserved material.

Laboratory design is critical: it should ensure both, good laboratory practice, minimizing any damaging or stressful effects upon reproductive cells, and a safe working environment for the operators, reducing the risk of errors or accidents. Hereafter, some general requirements are described:

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- *Construction materials*, painting, flooring, and furniture should be appropriate for clean room standards, minimizing volatile organic compound (VOC) release and embryo toxicity.
- The *design and layout* of the laboratory and its equipment shall ensure optimal workflow by minimizing distances and avoiding the presence of obstacles while handling reproductive cells.
- Only *authorized personnel* should have access to the laboratory. A system for clean access of personnel and materials to the laboratory should be defined. Changing rooms, hand-washing facilities, administrative work offices, and cleaning and sterilization areas should be separated and outside the laboratory.
- The *air quality* is one of the most critical issues in the IVF lab. To optimize environmental conditions, the laboratory air should be subjected to high-efficiency particulate air (HEPA) filtration and VOC control. Positive pressure is also mandatory to minimize air contamination. According to the European Union Tissues and Cells Directive (EUTCD), tissue and cell processing must be performed in a Good Manufacturing Practice (GMP) Grade A environment with a background of at least GMP Grade D (revised guidelines for good practice in IVF laboratories of the European Society of Human Reproduction and Embryology [13]). To accomplish it, the air quality should be regularly monitored.
- IVF and vitrification laboratories should be *controlled conditions facilities*. They shall count on a highly accurate and reliable heating/air conditioning equipment that ensures the control of the humidity and temperature inside the facility. A temperature near 25 °C seems a reasonable meeting point between the tolerance zone of embryologists and that of the embryos ([14] book).
- Some of the *occupational health and safety requirements* include bench height, adjustable chairs, adequate workspace per person, microscope eye height, efficient use of space and surfaces, and sufficient environmental lighting.
- *Gas cylinders* should be located outside the laboratory, with the latter being equipped with an automatic change-over system and sufficient cylinders stocked for immediate replacement. High-purity gas and inline HEPA and VOC filters are highly recommended.
- It is mandatory to count with an automatic *emergency backup power system* for all critical equipment present in the lab in case of power cut (revised guidelines for good practice in IVF laboratories of the European Society of Human Reproduction and Embryology [13]).
- Regarding the *equipment*, the laboratory should contain all essential items required for IVF. All of them should be in a number appropriate to the workload, conveniently distributed, properly disinfected, validated, calibrated, and performance verified and with updated maintenance service (equipment should preferably be CE-marked). Incubators, workstations, heating devices, stereomicro-

scopes, inverted microscopes, cryostorage tanks, and N₂L reservoir tanks, among others, are part of the list.

17.2.2 Cryostorage Facilities

All the above are general conditions that rule all IVF laboratories which must also be addressed in the area of the laboratory designated for cryopreservation. Regarding specifically with **cryostorage facilities**, there are some others to note.

About the **location**, this area should be outside but adjacent to the main lab space, with visible access to the interior (e.g., via a window, camera).

For **security** reasons due to N₂L manipulation,

- Adequate ventilation, air changes, and low oxygen alarms should be installed to prevent accumulation of nitrogen in its atmosphere.
- This sector should be equipped with a system for O₂-level monitoring.
- Protection devices (e.g., glasses, face shield, cryogloves, apron, footwear) should be available for use during N₂L handling.
- Members of the laboratory staff should be instructed on how to handle liquid nitrogen properly.
- Cryostorage units should be continuously monitored and equipped with alarm systems, detecting any out-of-range temperature and/or levels of liquid nitrogen (N₂L).
- The lab must count with an emergency plan with specific procedures in case of an exceptional failure of infrastructure and facilities to protect all cryopreserved human material (revised guidelines for good practice in IVF laboratories of the European Society of Human Reproduction and Embryology [13]).

The **equipment** needed in the cryopreservation area include:

- Laminar flow hood
- Stereomicroscope
- Tri-gas incubator
- Stripper holder or similar device for holding a pipette tip
- Automatic pipettes
- Liquid nitrogen container
- Cryostorage tank
- Tweezers
- Hot plate
- Digital timer

All the equipment should be properly validated, calibrated, and regularly monitored with a registry of all measured parameters. If measurements are out of range, corrections should be made and their effectiveness verified (revised guidelines for good practice in IVF laboratories of

the European Society of Human Reproduction and Embryology [13, 15]).

All maintenance services and repairs must be documented, and data recorded.

About **consumables**, the following products are required:

- Vitricification and warming kit
- Cryodevice to load the material
- Micropipette tips 20–200 µL
- 6-Well dish
- Pipette flexipets
- Storage items (metal rod, goblet)
- Oocytes/blastocysts culture media supplemented with human serum albumin (HSA)
- Culture dish
- Mineral oil for oocyte/embryo culture

All the consumables must be specific for IVF and certified for such use. This means that plastic supplies have been appropriately mouse embryo tested (mouse embryo assay (MEA) tested) and regulatory approved for contact with human gametes and embryos, and thus are nontoxic and safe. It also means that they can be used for media and oil aliquoting and containing, without the risk of toxic molecules from the material entering the medium or oil. In addition, packaging integrity and appropriate delivery conditions should be checked. Documentation of quality control testing must be supplied for any commercial media, and this must correspond with the delivered batch.

Plastic that is deemed safe in IVF is usually marketed as such, although if in doubt, it is worth checking with your supplier that the plastic is IVF-grade. It is useful to rely on an appropriate stock management system for media and consumables, including the batch number, date of entry, date of opening, and expiration date (revised guidelines for good practice in IVF laboratories of the European Society of Human Reproduction and Embryology [13, 15]).

17.2.3 Workflow of IVF Operators and Reproductive Cells

Vitricification is about stopping and restarting cell metabolism. To achieve it successfully, it is essential, as embryologists, to be aware that our working material is dynamic, subject to aging and evolution. To perform vitricification/warming in a timely manner and considering the biology of the oocytes and embryos are one of the key points to ensure not only viability but also developmental competence of the reproductive material. That is why there exist intervals, configurations, and technical arrangements that should be considered to optimize an effective workflow for vitricification/warming performance.

The oocyte is a very special cell, with lots of functions and features that make it intrinsically sensitive to vitricifica-

tion. Zona pellucida hardening, membrane permeability alteration, cytoplasmic and cytoskeleton damage, meiotic spindle depolymerization, and aging are some of the most important processes to which the oocyte is subjected when it is vitricified; for this reason, the fine-tuning process for an efficient oocyte cryopreservation technique was a long journey. To address all these events and minimize damage caused by vitricification, there are certain time intervals to consider:

- Vitricify the oocyte preferably within 38 h of hCG trigger. If the aspiration is scheduled at 36 h post-hCG, vitricification should be performed within 2 h to preserve oocyte viability and minimize postovulatory aging [16, 17].
- Denude the oocytes immediately before vitricifying them. It is well known the essential role of the cumulus oocyte complex (COC) for a correct oocyte maturation, metabolism, etc. [18, 19].
- Oocyte vitricification and warming inevitably cause meiotic spindle disassembly. Performing ICSI 2 h after warming has been demonstrated to be compatible with spindle recovery [10–12, 20].
- When many oocytes are vitricified/warmed, two or more available operators may be required to comply with the mentioned timing requirements.

The human blastocyst expansion process requires the Na⁺, K⁺ pump and aquaporin channel activity, which demands large amounts of energy. During the vitricification protocol, the blastocyst undergoes shrinkage and re-expansion as the exchange occurs and equilibrium is reached. Many studies showed that blastocyst artificial collapse improves the diffusion of cryoprotectant agents inside the cavity, reducing the formation of ice crystals and increasing the survival rate after warming (reviewed in [21]). When trophectoderm cell biopsy is performed, unavoidably the blastocyst collapses. It has been demonstrated that the longer the time from biopsy to vitricification, the higher the no re-expansion rate after warming. Therefore, cryopreservation should take place as soon as possible after biopsy, ideally within 30 min and no later than 90 min [22]. In case the number of embryos to be biopsied exceed the capacity of a single operator to vitricify them in a timely manner, it is useful to count with an additional operator to avoid delaying vitricification.

The vitricification/warming protocol is subject to time intervals, configurations, and technical requirements that must be kept under control to ensure success:

- Approximately 30 min before vitricification, bring the equilibration solution (ES) and vitricification solution (VS) to room temperature (RT) (25 °C). All the vitricification steps must be performed at RT since cells are more resistant to cryoprotectant agents (CPAs) toxic effects.
- Time in VS must not exceed 1 min. Approximately 10 s before the end of the minute of incubation, place the cryo-

device under the microscope, adjust the focus on the black mark (i.e., the tip of the device), and place the material with a minimum volume of VS.

- At least 1 h before the thawing, bring the thawing solution (TS), dilution solution (DS), and washing solution (WS) out of the fridge. DS and WS should be at RT, in contrast, TS must achieve 37°C. Make sure that the TS reaches this temperature before starting the warming process. To guarantee an extreme warming rate is one of the critical steps (if not the most) for embryo/oocyte survival.

At last, it is essential to set up an efficient working area. This means making sure that all the necessary equipment is in the right place at the right moment. For each step to be accomplished, it is necessary to consider certain indispensable components to avoid unnecessary delays. Organize the layout to limit distances from the workstation and facilitate the workflow. Have at hand the necessary number of pipettes of the right diameter, timers, cryodevices, medium at the right temperature, tips, dishes previously warmed and/or pre-equilibrated, etc. Labeling all plastics and cryogenic devices before starting the procedure is both safe and time saving. Likewise, before the end of the vitrifications' equilibrium stage, make sure that you have a full container of liquid nitrogen for a successful final step.

17.3 Training of Operators

17.3.1 Learning Process: Achievement of Proficiency

Vitrification involves multiple steps of stringently timed pick-and-place operations for processing oocyte/embryo/blastocyst. It requires precise washing sequences and timings in each cryoprotectant medium since high concentrations are used and there are risks of toxicity if overexposed. The technique demands the handling and control of minimal volumes, and as the media presents different densities, it means a challenging training for visualizing oocyte/embryo/blastocyst in a few seconds.

Unlike slow freezing, vitrification does not need a freezing machine to provide specific cooling parameters, and it is performed totally manually. For this reason, vitrification effectiveness is highly operator dependent, being very variable and depending on the *laboratory protocols, training programs, and technician proficiency*.

Laboratory protocols should be detailed in a standard operating procedure (SOP) manual. It includes all the validated, updated, and authorized written instructions for each process that takes place in the lab. The vitrification/warming technique shall have an exclusive section in the SOP. This manual should contain clear and detailed infor-

mation for both, trainees who need to learn the technique and experience operators that need to keep a high performance level.

Troubleshooting and tips and trick sections should be included in the SOP. This could be very useful to anticipate the embryologist-in-training about what may occur during the procedure and how to proceed.

The *training program* includes both a theoretical and practical approach with the technique. For the theoretical aspect, the trainee must be aware of the fundamentals and basics of the technique and the reasoning behind each aspect of the protocol: the timing, the media composition, and the critical details. Inside the clinical lab, the new staff should follow a structured training program supervised by experienced clinical embryologists. It is mandatory to mention that adequate training must include all the procedures to ensure that traceability of each individual specimens' identification and patients is guaranteed during all the process.

When a new person is in training, it is necessary to have an in-house quality control procedure to assess the learning process and the trainee *technician proficiency*. There exist a few approaches to define when the operator competency level is reached. Confirming a recommended number of procedures under supervision is the standard practice, but also statistical tools designed to indicate when a process has reached a pre-defined level of performance can be used as well. Dessolle et al. [23] proposed a graphical representation and recording of the learning curve, which might be useful for tutors and teachers responsible for certification. However, they also stated that the learning curve for vitrification procedure should not be so long even for junior embryologists as the achievement of proficiency is mainly limited by manipulation challenges [23].

Although it is important to accomplish and complete the learning curve before applying the technique with viable oocytes/embryos, in expert hands, vitrification tends to suffer from shifts perhaps because of the repetitive actions or the relatively long time intervals, which tend to reduce concentration on adherence to the protocol timeframe and may lead to attempts to speed up the procedure, thereby threatening standardization. For that reason, it is important to continuously monitor each individual operator's performances. Key performance indicators (KPIs), which will be discussed later, are essential for accurately and regularly assess the maintenance of competency (Alpha consensus [5]). Cimadomo and colleagues published a study outlining the use of KPIs in clinical practice and how to monitor the operator performances in terms of cryo-survival outcomes after warming; they proved that by eliminating all patient confounders that can impact the outcome, all the operators were able to perform vitrification with similar outcomes [22, 24]. These approaches could be used to monitor the performance of not only the junior staff but also senior operators with the aim of achieving standardization and prompt action in case of a shift in laboratory outcomes.

17.3.2 Critical Steps in the Vitrification Protocol and Troubleshooting

As the body temperature of mammals is strictly regulated, cooling of human oocytes and embryos to subzero temperatures is a situation they will never meet under physiological conditions. Consequently, the cell tolerance to survive such conditions is limited, and therefore, cells may suffer irreparable damages ([14] book). Vitrification is a pseudo-second-order phase transition (IUPAC Compendium of Chemical Terminology) that induces a glass-like solidification inside the cells preventing ice crystal nucleation and growth, the main causative factor of cell injury during chilling [15]. To achieve this specific phase state, vitrification/warming techniques deal with many factors and variables that must be strictly controlled to preserve not only viability but also developmental competence of the cells. Hereafter, we list some of the crucial points, together with a troubleshooting perspective, in order to get over them and achieve a successful vitrification:

- *Volumes* are critical. Evaporation of the drops during equilibration and vitrification should be avoided. RT and air hood affect the evaporation and consequently the consistency of the environment surrounding the gamete or embryo (Swain et al. RBM online [25]).

For troubleshooting: Prepare the droplets as quickly as possible and immediately before use; consider large volumes of droplets (<30 μL is not recommended); pay attention to the opening date of the vial of medium as osmolality can change with multiple openings. Especially when drops are of small size, it is important not to carry medium between drops during the protocol. To that end, rinse the pipette when passing from one medium to another and load the oocyte/embryo almost at the tip of the pipette. Using a 6-well plate can be useful to standardized volumes and reduce evaporation, also sterile water or PBS can be added to the adjacent reservoir to limit evaporation.

- Extremely high *cooling and warming rates* are another important topic. To achieve the glass-like solidification state, a cooling rate > 20,000 $^{\circ}\text{C}/\text{min}$ is necessary. This is accomplished by both minimizing loading volumes (<2 μL) and direct and quick exposure of the samples to liquid nitrogen. On the other hand, the formation of ice nuclei or ice crystals must be avoided while the oocyte/embryo is being warmed. It occurs when the cryopreserved material temperature rises above -130°C . To prevent it, the difference in temperature between the two states and the speed of this switch must be maximized [26].

For troubleshooting: Volume and temperature of the TS are the main factors to control. It should be not minor to 0.5 mL and properly warmed to 37°C (at least 1 h before the warming). At first glance, it seems an easy task, but it

deserves many points to be taken into account to ensure its compliance: a heated stage set at 37°C does not necessarily imply that the working temperature is 37°C ; It must be considered that the temperature in the medium may be lower than that set in the heating plate as it is influenced by heat loss the design of the dish, the medium volumes, among others. All necessary tests should be made to ensure the correct temperature of the thawing medium.

Additionally, the external device for temperature measurements must be appropriate and properly used: the Pt100 thermocouple could be a suitable option to measure media temperature in dishes.

Other points to achieve the necessary cooling and warming rates include the following: the liquid nitrogen rack should be filled to the edge of its capacity and placed as close as possible to the stereomicroscope; the operator should be as fast as possible while transferring the material to the nitrogen during vitrification or from it to TS during warming; and finally, during storage and handling of cryopreserved material, the operator must keep the cryodevice submerged in N_2L to avoid temperature increases and crystallization.

- *Timings* are crucial in vitrification, being particularly sensitive to the 1-min moves: the VS loading step during vitrification and TS step during warming.

For the VS- loading step, it is important to mention that VS is the most CPA concentrated media of the kit, thus cell contact with it must not exceed 1 min, and this minute shall almost include the loading on the cryodevice. Oocytes/embryos/blastocysts must be picked up in a very small volume of vitrification solution to support an appropriate cooling rate (Fig. 17.1) [10–12, 27]. On the other hand, each media is made of different cryoprotectants, which confer them a particular density and viscosity. Due to this condition, the identification and manipulation

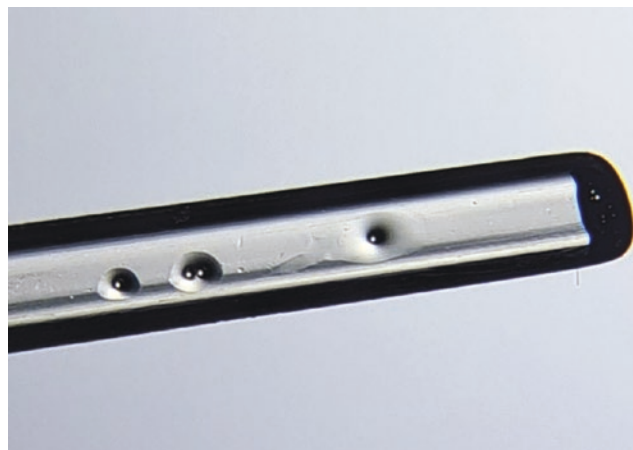


Fig. 17.1 Example of a correct loading volume

of the cells could be a visual challenge that may lead to delays in the protocol.

For troubleshooting: For timing accomplishment, it is essential to maintain the focus during all the process and avoid other activities during vitrification/warming procedures. To choose the right diameter for a proper loading of the oocyte/embryo/blastocyst will be helpful when the loading time comes (from 170 μm for oocytes up to 300 μm for a biopsied blastocyst) [15, 28]. To accomplish the VS minute, start the loading procedure in the last 10 s before the timer starts to beep.

To comply with all the abovementioned critical points, the development of skills is essential, and for that there is no magic: “practice makes perfect.”

17.3.3 Monitoring of Competences: Key Performance Indicators and Benchmarks

Performance indicators are required to quantify specific accomplishments and monitoring and constantly improve the results of a given procedure (revised guidelines for good practice in IVF laboratories of the European Society of Human Reproduction and Embryology [13, 29]). In an IVF laboratory, the tracking of *key performance indicators* (KPIs) is essential for monitoring all techniques and constantly improve outcomes [24].

KPIs are essential for:

- Evaluating the introduction of a technique or process, as minimum standards for proficiency
- Monitoring ongoing performance as part of a quality management system
- Benchmarking and quality improvement

They can generally refer to three main areas: processes, structures, and outcomes [30, 31]. *Structural KPIs* measure the quality of the IVF laboratory by outlining the characteristics of physical and human resources. An example of a structural KPI related to the facility in a cryopreservation program might be the number of cryotanks relative to the total number of ART procedures requiring cryopreservation conducted in a given time period or the number of cryotanks relative to the total square meters of the cryostorage room.

Process KPIs measure how well the IVF laboratory works. Examples of process KPIs are the percentage of laboratory staff injuries while handling liquid nitrogen per number of IVF procedures per year, percentage of gametes/embryos lost/damaged during vitrification/warming procedures, and audits per number of procedures per year.

Finally, *outcome KPIs* measure the effectiveness of the IVF laboratory through competency and benchmark values that shall be defined by international societies. Vitrification values have been defined by the Alpha Consensus of 2012. About oocyte vitrification, they include post-warming oocyte survival, rates of fertilization, embryo development, and implantation. For embryo vitrification, they defined different KPIs for cleavage- or blastocyst-stage embryos. Morphological survival, post-thaw development, transfer, and implantation rate are the KPIs suggested (Alpha Consensus [5]). The competence and benchmark values are summarized in Table 17.1.

Table 17.1 Key performance indicator values for vitrification technique (Alpha Consensus 2012)

KPI	Competence	Benchmark
Oocyte key performance indicator values		
O1	Morphological survival	70% 75% 85% (95% for donors <30 years)
O2	Fertilization rate	No more than 10% (absolute; i.e. 10 percentage points) lower than that for the comparable population of fresh oocytes at the centre
O3	Embryo development rate	The same as for the comparable population of fresh embryos at the centre
O4	Implantation rate	No more than 10–30% (relative) lower than that for the comparable population of fresh embryos at the centre
Zygote key performance indicator values		
Z1	Morphological survival	70% 85%
Z2	Cleavage rate	The same as for the comparable population of fresh embryos at the centre
Z3	Embryo development rate	The same as for the comparable population of fresh embryos at the centre
Z4	Implantation rate	No more than 10–30% (relative) lower than that for the comparable population of fresh embryos at the centre
Embryo key performance indicator values		
E1	Morphological survival: fully intact	70% 85%
E2	Morphological survival: 50% intact	85% 95%
E3	Post-thaw development (including implantation rate) for fully intact embryos	≤10% (relative) lower than that for the comparable population of fresh embryos at the centre The same as for the comparable population of fresh embryos at the centre
Blastocyst key performance indicator values		
B1	Survival rate	80% 95%
B2	Transfer rate	80% 95%
B3	Implantation rate	≤10% (relative) lower than that for the comparable population of fresh embryos at the centre The same as for the comparable population of fresh embryos at the centre

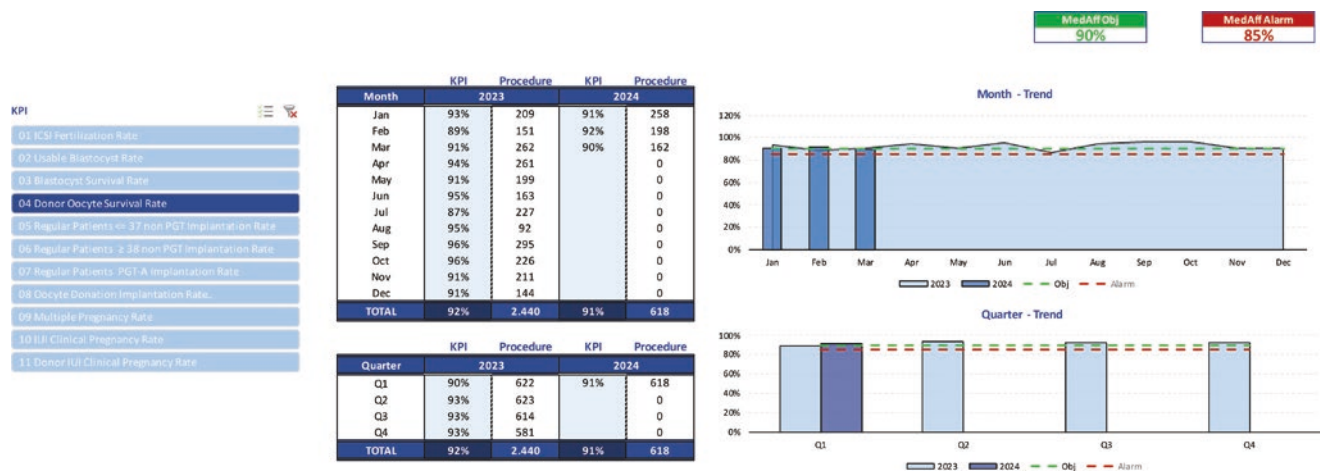


Fig. 17.2 Example of a control chart for KPI monitoring

KPI control charts are the most common tool for constantly monitoring them. The Shewhart chart or the Levey-Jennings quality control chart is the most frequently used [24]. Briefly, a control chart should report a “control mean” over the preceding 6 months, upper and lower “warning limits” set at 2 standard deviation, and upper and lower “control limits” set at 3 standard deviations (Fig. 17.2). Ideally, an indicator should always remain within the control limits oscillating between the lower and upper control as per physiological fluctuation due to patient variability. If the indicator crosses the limits or shows some systematic tendency, an action is required to determine the reason. If lower limits are crossed, the cause should be determined and corrected. If the high control limits are crossed and the improvement is real and sustained, the control limits must be redefined. Of note, KPIs can be based on a reference group of good prognosis patients, as in the Vienna consensus (women younger than 40 years using own fresh oocytes and ejaculated fresh/frozen sperm during non-PGT cycles) (ESHRE Vienna Consensus [32]), or on the whole patient population (revised guidelines for good practice in IVF laboratories of the European Society of Human Reproduction and Embryology [13]).

As vitrification/warming is a 100% manually performed technique, KPIs become a useful and necessary tool to monitor the overall efficiency of the technique as well as the proficiency of each of the operators who execute it.

17.3.4 How to Improve Standardization?

As in many biotechnology fields, it is mandatory to ensure a standard, consistent, and reproducible vitrification technique avoiding operators subjectivity and deviations. Automation has been demonstrated to be a successful strategy for this purpose.

A semiautomated vitrification device has been introduced on the market, developed by Roy, Hajek, and colleagues. They have developed “Gavi,” a system that automatically performs

equilibration steps of the vitrification process. The Gavi allows precise and reproducible control of all vitrification variables including temperature, time, volume, media concentration and fluidics speed. It also reduced embryo handling, minimizing potential mechanical stress or loss of the embryo. Outcomes for embryos automatically vitrified with the Gavi system were similar with those treated with the manual Cryotop method and with fresh (non-vitrified) controls [33, 34]. However, a recent randomized control trial conducted by Hajek and colleagues has shown that the Gavi system, at least for now, is a more time-consuming procedure with less staff convenience than the classic manual process [33].

Automation is a widespread trend in all fields of biotechnology. While at the moment it does not seem to be able to compete with the manual method, its refinement will surely do so.

In the future, automated systems should not be perceived as a threat for the embryologist role, but rather as an opportunity to devote more time to other tasks that require creativity, scientific thinking and reasoning.

17.4 Traceability During the Vitrification and Warming Process

In the IVF lab, traceability refers to the capacity to ensure the chain of custody of patients’ (or donors) tissues, cells and embryos. Each IVF laboratory must have a comprehensive, effective, and accurate system to uniquely identify, trace, and locate reproductive cells during each procedural step, not just for avoiding mix-ups but also to track every detail of the processes to which they were subject. Traceability is thus essential for the success of a cryopreservation program as well as the whole treatment.

Guarantee traceability is a matter of critical details and simple but key steps. Even though each IVF unit should have its own protocol, some general points are listed below:

- *Proper training in traceability* procedures for all laboratory staff is mandatory. All members of the laboratory should be aware of the traceability importance and that its compliance is one of the embryologist's duties.
- *Labeling of the cryodevice* with patient details such as full name, date of freezing, and unique identification number is crucial for the traceability of the sample. Additional information due to the specific needs of the clinic or national guidelines is also applicable. It must be clearly and permanently labeled.
- *Cryostorage* systems should be organized to ensure easy access and identification of the biological materials therein. Special attention should be paid to documentation of samples in storage for easy identification. A periodic inventory of the contents of the cryobank is recommended, including cross-referencing contents with storage records.
- *Technical data of each manipulation* step is also important. The date, time, and identity of all operators and witnesses must be documented throughout the treatment. At warming, documentation on biological material should also include thawing method and post-thawing sample quality. All the consumables and media used must be traceable.
- A *double-check and witnessing* protocol must be implemented in every IVF unit to ensure a clear process of patient and cell identification during both vitrification/warming procedures and the entire IVF steps.

Regarding this last point, although mismatches in IVF are very rare but unfortunately possible, *witnessing* protocols are essential to avoid them. Each IVF laboratory must have an effective and accurate witnessing system; this refers to a double-check system that ensures the proper patient identity traceability during all the manipulation and decision-making steps (revised guidelines for good practice in IVF laboratories of the European Society of Human Reproduction and Embryology [13]). It becomes particularly important in those critical and error-prone steps of IVF treatment. Rienzi and colleagues conducted a multicenter failure mode and effects analysis (FMEA) to identify potential failures in the traceability process and develop strategies to mitigate the risk of mismatches. In this study, they highlighted that all process phases of an IVF are potentially vulnerable to errors and that cryopreservation is one of the most [3, 4]. The authors showed that correct labeling is a high-risk point of the procedure and that not only cells must be correctly identified but also the location in the cryostorage container. In this context, double checks by a second person (witness) and/or an electronic identification system are strongly advised [35]. However, human witnessing is also considered prone to error, being electronic witnessing systems (EWSs) a better choice for continuous traceability. By using tags and

barcodes, this systems are mainly automatic and do not require additional steps for the operator nor interruptions for the witness. Although EWS has been demonstrated to be more efficient [36], both witnessing methods improve procedure traceability, minimize the risk of mismatch, help embryologists to work more confidently and deliver a better and safer service to the patients.

17.5 Conclusion

Vitrification has been demonstrated to be the best protocol for cryopreservation at all stages of development, from oocytes to blastocysts. An efficient cryopreservation program allows access to high-standard technologies (single-embryo transfer, freeze all, fertility preservation, biopsied embryo cryopreservation, donor egg banking, etc.). Vitrification versatility and efficiency have given much more flexibility in the laboratory practice and clinical decision-making. It has also offered new opportunities for embryo evaluation, for instance through genetic analysis. In the future, combining vitrification with many other novel technologies, more time and effort could be dedicated to fine-tune embryo selection strategies.

Vitrification is a unique mixture of a manual procedure—resembling 100- to 150-year-old techniques—fine-tuned to obtain twenty-first-century significant achievements. This technique has the powerful capacity of making the impossible possible—to stop and restart the un-restartable: life ([14] book). As embryologists, we must be aware of the incredible opportunities vitrification offers, aimed for the highest standards during its' performance and consider all the points addressed in this chapter for its' proper implementation and training.

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Establishing a Donor Oocyte Cryobank Network: US Experience

18

Kathryn J. Go, Jay C. Patel, and Daniel B. Shapiro

Key Points

- Vitrication has enabled the reliable cryopreservation of human oocytes.
- The availability of cryopreserved donor oocytes has catalyzed access for patients requiring donor eggs for family building.
- A network model for the cryobanking of donor oocytes provides standardization of laboratory and clinical protocols for best practices, safety, and optimized outcomes.

18.1 Introduction

In vitro fertilization (IVF) was revolutionary in its breadth and range for surmounting challenges to fertility and providing solutions to family building. The application of donated, or donor, oocytes to women who had lost their ovaries or ovarian function was realized early in the era of assisted reproductive technology (ART) [1], but the earliest cases of donor oocyte IVF presented potential challenges of inconvenience, minimal selection of donors, and high cost [2].

For women who did not have an oocyte donor readily identified, e.g., a sister, other relative, or friend, finding a suitable donor required either independent research and management of recruitment, logistics, development of an

agreement, and possible remuneration or the reliance upon an independent agency or the infertility clinic to fulfill these activities. The cost of such a resource-intensive treatment could be significant and was, undoubtedly, prohibitive for many [2].

Anonymous oocyte donor selection could be limited. For the agencies and infertility clinics, establishing a “catalogue” of appropriate donors entailed advertising and recruitment, screening by an array of processes—clinical, genetic, social, and psychological—and then, following acceptance of the candidates, presenting them to clients/patients. When a donor was selected, scheduling of the treatment cycle required the coordination and synchronization of donor and recipient menstrual cycles. This could be a hurdle when their personal schedules could not be matched, and for the recipient, the potential delays could be extremely stressful.

There could be logistic considerations as well to preserve the donor’s anonymity and privacy. Efforts were often taken at the infertility clinic during the donor’s oocyte retrieval to ensure that she was not encountered by, for instance, the recipient’s husband or partner or by the recipient herself.

This chapter outlines the array of elements and considerations that underpin the establishment of a network of clinics producing cryopreserved donor oocytes—a substantive solution to address the continuously increasing demand for this resource for family building [3–5]. Once a validated, consistent technology for cryopreserving oocytes is established in the laboratory, the planning and organization that follow must include standardized protocols for the (a) training on technology and authorization of its users; (b) complete management of the oocyte inventory, i.e., storage and transport, documentation, and recording and tracking of data for each oocyte; (c) recruitment, screening, acceptance, stimulation, and post-retrieval care of the oocyte donors; (d) compliance with all regulatory standards; and (e) conduct of business and financial activities to operate and develop the network.

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18.2 The Revolution Through Vitrification

The intensity of effort for patients seeking family building with donor oocytes was alleviated by the introduction of vitrification to the continuously expanding repertoire of assisted reproductive technologies. Early reports suggested that, despite the oocyte's large diameter and high intracellular water content—a hurdle to effect dehydration—the slow-cooling methodologies that had worked well with zygotes and embryos might be adapted [6–8]. But it was vitrification, first reported in 1985 for mouse embryos [9], that was pivotal in achieving reproducible oocyte cryopreservation.

Applied to blastocysts, vitrification yielded high post-warming and implantation rates [10–12] and was successfully adapted to oocytes for fertility preservation for women who were meeting the challenges of gonadotoxic therapy or who wanted to stave off age-related decline in oocyte number and quality [13–15]. Its application to provision of donor oocytes was immediately recognized and catalyzed the creation of cryobanks [16–19]. In addition to the relief from cycle synchronization with the donor required in fresh donation models, cryobanks offer more cost efficiency and access to a wider array of potential donors, i.e., more phenotypes for physical matching, more ethnicities, and ethnic blends.

The establishment of a donor oocyte bank entails extensive and careful planning and organization as it is a multidisciplinary endeavor requiring an array of expertise. This comprises the embryologists in the reproductive technology laboratory, a shipping department trained in best practices of cryohandling for cold-chain maintenance, reproductive endocrinologists and nurses to manage and care for the patients undergoing ovarian stimulation and retrieval of the oocytes, a mental health professional to evaluate the donor applicants, administrative specialists to organize the cryobank's records, information technology experts to manage the cryobank's databases, business officers to structure the financial and commercial parts of the bank and the network, marketing and social media specialists, and replication of all of these elements in each of the network member clinics.

With this chapter, the authors set forth a framework for establishing a cryopreserved donor oocyte bank network. By addressing a catalogue of elements central to network development, the authors aspire to offer guidance and a template for planning.

There are five general categories of activity for consideration: technical, clinical, administrative, financial/commercial, and support/development of the cryobank network. Table 18.1 lists the components in each category. In develop-

Table 18.1 Operations within a cryopreserved donor oocyte bank

Laboratory	Clinical	Administrative	Financial/commercial	Network support and development
Technical expertise in cryobiology	Donor medical evaluation and vetting	Donor recruitment and vetting	Oversight of fiduciary infrastructure	Multidisciplinary approach to obtaining new partners for the cryobank
Training and certification of all personnel	Ovarian stimulation and management of cycles	Maintenance of donor records	Provision and allocation of resources to operate the cryobank	Regularly scheduled meetings among partners to exchange information on clinical outcomes, any problems requiring adjustment of protocols and policies, suggestions for enhanced operations
Maintenance and security of cryostorage	Oocyte retrieval	Development of donor catalogue and website	Development of donor remuneration	Maintenance of all donor catalogues and information for patients
All shipping and receiving activities	Post-retrieval care and monitoring	Assurance of compliance with all regulatory standards for donor cells and tissues and biobanking	Client services and support through guarantee-structured programs	Maintenance of network-wide database of inventory and clinical outcomes from warmed oocytes
Maintenance of inventory and outcome database	With laboratory colleagues, decision-making regarding continued inclusion of donors based on outcome data	Interface with potential clients	Development of new business	Standardized procedures for shipping and receiving and document management
Technical innovations for continuous improvement	Consistent review of outcomes for each oocyte donor cycle for building knowledge about optimal stimulation strategies and recipient preparation	Client services and support	Provision of resources for advertising, marketing, consumer relationships, market presence, and acquisition of greater market share	Collegial cooperation to develop the network and continuously improve performance and client experience
Assurance of compliance with best laboratory and cryostorage practices and all related regulatory standards		Oocyte donor support and follow-up		
With clinical colleagues, decision-making regarding continued inclusion of donors based on outcome data		Provision of marketing and website expertise		
Consistent review of outcomes for each oocyte donor and cycle for building knowledge for quality assurance				

ing the network and cultivating new partners, some events and phases may proceed simultaneously, while others may need to be sequential, requiring the completion of a step or phase to set a foundation. These will be addressed in this chapter to suggest a checklist approach for organization and planning.

18.3 First, the Vision

Before there is a “network,” there is, likely, a single visionary clinic and laboratory with the ambition to establish a donor oocyte cryobank and then disseminate that vision to partner laboratories to build a matrix of centers for breadth and depth of donors. The benefits of this approach are the establishment of a broad geographical footprint, standardization of donor oocyte treatment, broad consensus on ethical standards in egg donation, expanding access to donor oocytes [20], and ability to provide an expanded range of third-party reproductive services (such as embryo creation or gestational surrogacy) through coordination of multiple network centers for the benefit of a single patient.

Oocyte banking networks use multiple criteria to determine which centers they will ask to join. Primary among these is the ability of an IVF laboratory to vitrify and warm oocytes consistently, with equally consistent pregnancy rates. Clinics with a high volume of donor oocyte cycles are likely to both embrace being a partner and have competence as one. The transition from coordinated fresh donor cycles to ovarian stimulation cycles destined for oocyte vitrification is seamless and often a relief to laboratory workflows.

Partner centers must also make a commitment to donor recruitment and screening, which requires a dedicated marketing team and aggressive investment in advertising in social media outlets and more traditional media as well. A dedicated nurse manager and donor nursing team are also a typical requirement. Centers joining a network must build an adequate donor catalogue or list, integrate the vitrification and warming methodologies into their laboratory workflow, maintain the donor oocyte inventory, provide time and additional resources for shipping of oocytes and embryos, and provide Internet and/or telephone access and support to patients, donors, and recipients in need of assistance.

Although this chapter is embryology-centric with focus on the technical and handling aspects of being a cryodonor egg bank member, administrative, financial, marketing, information technology, and regulatory expertise are required to achieve the operational objectives and consistent clinical outcomes. A successful oocyte banking network fulfills its clinical and business goals through partnership with professional marketers and client satisfaction assessors. The

administrators of a functional oocyte bank will put emphasis on coordinating the clinical side of operations with the messaging to donors and recipients and with what recipients and donors say they prefer in their care. Oocyte bank administrators, including the clinical manager, the laboratory and medical directors, and the business officers, must also find the right balance between maintaining best practices throughout their network while simultaneously allowing each partner clinic autonomy in its own clinical and business management.

It is also our opinion that each network should have a mission statement that acknowledges the autonomy and dignity of the donors. Oocyte banking networks, typically, will have regular clinical, laboratory, and business committee meetings to resolve issues for individual recipients and donors, problem-solve when laboratory outcomes fall below standard, and ensure that services are advertised under the appropriate guidelines of the American Society for Reproductive Medicine (ASRM) and the Society for Assisted Reproductive Technology (SART). Business committees usually meet to monitor and compare donor compensation, pricing for services, and reimbursement to member clinics for services they provide to other partner centers in the network.

18.4 Operations

The prerequisites of establishing a donor oocyte cryobank are (a) a reliable, reproducible, and effective vitrification method underpinned by validation data, a training program, and defined materials, supplies, cryopreservation, and warming solutions; (b) an organizational hierarchy with defined roles and responsibilities; (c) dedicated administrative and clinical laboratory space, instruments, and resources; and (d) a well-designed, enforceable quality management program to ensure optimized operations. As well as uniformity in the technical procedures, there must be consistency in all aspects of managing the cryobank’s inventory. Protocols for labeling of the cryodevices, formatting of the inventory for listing and tracking, provision of documents to accompany the shipped cryo-oocytes, and collection of outcome data for quality analysis must be established.

18.4.1 The Laboratory and Mastery of the Cryobiological Procedures

There can be no cryobank without a proven vitrification methodology. There must be a way to generate the products offered by the cryobank. The validation phase of the cryopreservation program for oocytes must comprise the vitrification and warming of a sufficient cohort of oocytes to

demonstrate, with data, that a protocol consistently yields results that meet or surpass appropriate thresholds and that multiple operators can produce these results. A strong, centralized, and uniform training program with a troubleshooting component to help trainees recognize the markers of effective freezing and warming is fundamental to obtaining best results in a network model where oocytes will be vitrified in an array of laboratories.

The standardization of methods and materials is one of the strongest efforts towards achievement of uniformity in practice and consistency when multiple partners in a network produce an entity for patient care. Here, cryopreserved oocytes and their outcomes—high-quality embryos for embryo transfers leading to live births—are the goal as well as the foundation for the model. Prescribed protocols are not confined to the cellular aspects of banking, but must also address instructions for handling of the oocytes at all phases of acquisition and processing, storage, movement among containers, and downloading at the destination laboratory to preserve integrity, i.e., “cold chain.”

Achievement of highest quality is attained through (a) optimal and consistent performance of all technical steps—preparation of reagents, vitrification, cryodevice loading, storage, handling to maintain cold chain, shipping and downloading, warming, and placement into culture; (b) application of best materials, such as chemicals, supplies, and equipment; and (c) collection, analysis, and appropriate follow-up of data, including # of mature oocytes per total number of oocytes per donation cycle, # of intact oocytes per total number of oocytes warmed, # of fertilized oocytes per total # of oocytes receiving intracytoplasmic sperm injection (ICSI), # of high-quality embryos per total number of oocytes warmed and total number of fertilized oocytes, # of embryos transferred and # of embryos cryopreserved, and # of clinical pregnancies per embryo transferred.

18.5 Training Program

A standard operating protocol for vitrification and warming—cryopreservative and warming solutions, equilibration times, and number of oocytes on the cryodevice—must be available for distribution to the partner laboratories. An ideal training program will be held at a single site and with dedicated trainers to ensure a consistent experience in this critical activity. A list of reagents to comprise the vitrification and warming solutions as well as all disposables and equipment contributes to the desired consistency that will yield the same high quality of oocytes and their outcomes in multiple hands in multiple laboratories: high post-warming survival, fertilization and high-quality embryo rates translating to high implantation, and healthy live born rates.

18.6 Labeling

Accurate, stringent, and consistent labeling of the cryo-oocytes among the member laboratories is essential. A standardized label and its content must be specified by protocol, reflecting, for instance, the name of the cryobank, oocyte donor's unique identification number, number of oocytes on the cryodevice, cryopreservation date, and possibly serial numbers of the oocytes. This model will ensure the ability to track the origin and disposition of each oocyte and cryodevice by the members of the network and contribute to best practice in understanding the outcomes from each donor's oocytes and the donor's retrieval cycles in which the oocytes were obtained.

18.7 Cryostorage Security and Safety

A laboratory embarking on building inventory for a cryodonor oocyte bank must anticipate the requirements for expansion of its storage facility, i.e., the number or capacity of its storage Dewars, liquid nitrogen consumption rate, strength of oversight and maintenance protocols, and provisions for emergency response.

Current treatment paradigms in ART are already leading to a burgeoning of cryoinventory in many laboratories from more frequent cryo-all cycles, preimplantation genetic testing, autologous oocyte cryobanking, and a greater volume of embryos for cryopreservation from single-embryo transfer (SET) [21]. The addition of cryodonor oocytes is likely to require additional storage tanks and the resources to support them. As expanding cryoinventory is already challenging for some laboratories, preparing for the increment of the donor oocytes may involve evaluation of existing storage space to apply any engineering solutions needed to support the cumulative tanks and achieve adequate ventilation and access to liquid nitrogen with incorporation of the tanks into the monitoring and alarm systems for liquid nitrogen and temperature as well as emergent response [22–24].

18.7.1 Inventory Control and Management

A standardized format for the listing and tracking of inventory in each partner center and shared access to this inventory provides consistency of information throughout the network on the availability and outcomes for each donor cohort of oocytes. The ability to query and perform analytics from this database will be invaluable to understanding the individual metrics and outcomes for each of the oocyte donor's cohorts, the performance of both the clinics producing the cryoinventory and using the cryoinventory, the status

and disposition of all oocytes, and the general status of the volume and activity in each partner center.

18.8 Handling of Inventory and Shipping

A bedrock principle of cryostorage is the maintenance of cold chain: the avoidance of any transient warming that might expose the specimen to temperatures above the glass transition temperature. These brief excursions out of the safe temperature zone can cause sufficient damage to reduce or preclude the post-warming recovery, viability, or developmental potential of the cryopreserved cell or tissue. While moving samples between containers, and in particular during shipping, oocytes must be confirmed for identity and number and then transferred to the dry shipping container, or at receipt, when the converse activities are performed, i.e., confirmation that the specimens match the accompanying documents.

In addition to vitrification, warming, labeling, inventory management, and troubleshooting, the cryobank training record should also include a refresher course or instruction in safe handling—safe for the embryologist and safe for the specimens—to avoid cryoinjury and transient deviations from cold chain, respectively [25]. This will ensure conservation of oocyte integrity and realize the goals of high post-warming outcomes of survival, fertilization, and embryo development.

18.8.1 Recruiting Clinical and Laboratory Partners

The prospective clinic and laboratory partners who will participate in a network must, first and foremost, have the infrastructure to support the activities of a donor oocyte cryobank. Their partnership will likely entail a significant increase in the scheduled events and tasks in their laboratories and may require augmenting or installation of new resources to support their production role. The partner clinic and laboratory must accommodate the additional retrievals and the time required for processing and vitrification of the oocytes, the addition of inventory to their cryostorage, and the record-keeping, shipping, and receiving of the samples.

In addition to clinical and laboratory factors, consideration must be given to the geographic location of the prospective partner: is the clinic located in an area that will be advantageous for donor recruitment to achieve desired goals of volume and diversity in donor traits such as phenotype and ethnicity and allow accessibility to donors making the process convenient. The aspiration is to make the donation process safe, attractive, and manageable for high compliance and sustained motivation [26].

As the cryobank is a multidisciplinary endeavor, a global organizational meeting within the clinic will consider the required elements and develop a plan to emplace them. Guidance from the originator of the network will ensure advantageous standardization to eliminate duplication of effort and the achievement of consistency in practices.

18.8.2 Clinical Protocols and Oocyte Donor Care

The clinic may wish to assign a single physician or a group of dedicated physicians to oversee the ovarian stimulation cycles of the donors and may, similarly, have a nurse specialize in the care of the donors as they progress through their cycles for continuity. Methodologies for the screening and review of the donor applicants [27, 28], acceptance and rejection criteria, regimens for ovarian stimulation [29, 30], post-cycle follow-up, and maximal number of cycles per donor must be addressed in standardized operating protocols for consistency [31].

Protocols can anticipate management of information obtained during donor screening, e.g., genetic carrier status, medical conditions, or mental health issues, that may preclude the donor's participation in the cryobank.

Considerations of anonymous versus open donation must also be delineated to ensure that the donor's rights and expectations are fulfilled. In support of the oocyte donor, the cryobank may wish to offer the individual the opportunity to vitrify her oocytes for her own possible use as an adjunct to her remuneration.

Clinical protocols for clinics using the cryobanks' oocytes will address preparation of the recipient for embryo transfer [32, 33].

18.8.3 Administrative Protocols

Although every clinic is an autonomous entity with its unique protocols, policies, and operations, standardization of the cryobank materials and resources for both its donors and client-users will catalyze cooperativity, communication, and shared problem-solving when needed.

Administering a network of cryobanks will include systems to achieve oversight and coordination of all operational, clerical, and financial activities. Just as the laboratory and clinical teams will, ideally, meet regularly to review outcomes and discuss any problems, the administrative, financial, and business specialists from the partner clinics can benefit from routine meetings for exchanging perspectives and exploring new directions. These may address donor recruitment, cryobank's marketing and representation in social media, website design, and all points of the network

partners' interaction with potential purchasers and clients of the cryobank.

Provisions of the cryobank for warranties or guarantees and any new programs that may be attractive to prospective clients can be continuously evaluated. The number and nature of any cases failing to meet a guarantee can be reviewed to determine what appropriate follow-up action must be taken for remediation.

The administrators in each partner clinic will oversee the review of all regulatory and legal standards pertinent to the cryobank's operations, as the latter will vary by state. Compliance with the regulations or practice guidelines of all relevant agencies and entities, e.g., the American Society of Reproductive Medicine, the Society for Assisted Reproductive Technology, the Food and Drug Administration, and the American Association of Tissue Banks, as well as the avoidance of any legal issues that have arisen with respect to oocyte donors, e.g., antitrust activity, can be achieved through their diligence and communication with the individual departments within the clinic.

18.9 Quality Management

Each partner laboratory and clinic is anticipated to have an effective program in place to assure quality and achieve quality control in its practices and best outcomes and safety of its patients [34].

A shared quality control and assurance program among the cryobank network partners will regularly review operations and performance metrics and serve the network well in supporting attainment of best results from the cryo-oocytes. The program will prescribe protocols for consistent performance of all laboratory procedures, labeling, listing of oocytes in the inventory, tracking and management of inventory, recording of clinical outcomes from warmed oocytes, handling of cryodevices to preserve cold chain integrity, and shipping and receiving donor oocytes that must be written, validated, and regularly reviewed and updated as needed. A single source of training, e.g., the headquarters laboratory of the donor bank network, will ensure consistency among operators and in the expectations for the outcomes from the collectively vitrified oocytes.

There must be regularly scheduled conferences among the various departments of each partner clinic: the embryologists who oversee creation of the inventory, physicians who administer the ovarian stimulation cycles, retrieval of the oocytes and transfer of the embryos from warming cycles, and business directors who are responsible for the fiduciary operations of the network. These meetings ensure that poor outcomes receive adequate attention and that the network

fulfills any warranties and/or guarantees with respect to expectations from individual egg cohorts and their warming cycles.

Provision of a shared global outcome database will allow comparisons among users of oocyte lots and identify factors benefitting from attention in the technical or clinical aspects. The metrics comprising a cycle review include donor ID, donor cycle ID or retrieval date, # of oocytes warmed, # of oocytes survived, # of oocytes fertilized after intracytoplasmic sperm injection (ICSI), # of high-quality embryos and # of developmental stage embryos transferred, and # of embryos cryopreserved. An advantage of the network is that comparison of outcomes from a single cohort of donor oocytes among one or more clinics is possible, making troubleshooting more focused. Having data showing that a "lot" of donor oocytes (typically, six oocytes) performed well in one center suggests that review of laboratory or clinical factors may be indicated—of handling/warming or embryo transfer protocols, for instance.

A quality manager who can review both laboratory and clinical factors can be available for site visits and provide the assurance of an objective appraisal of methodology, materials, training, and operations. The achievement of best outcomes is integral to the network's mission and success [35].

18.10 Conclusion

Vitrification was the catalyst to the establishment of donor oocyte banks by providing the consistent, reproducible, and reliable method for cryopreserving human oocytes. Availability, lower cost, greater array of donor phenotypes and other desirable factors, increased convenience through elimination of synchronization of donor and recipient cycles, and reduced time to pregnancy and live birth are all attributes of cryopreserved donor oocytes. Network creation benefits from a single source of cryopreservation and warming technology, training and certification of trainees, materials, standard operating protocols for all facets and phases of specimen handling, labeling, shipping and receipt, recordkeeping, inventory and control, and accompanying documents. These comprise the tenets of the network's quality control program and set the goal for a consistently high-performing oocyte cohort as measured by the key indicators that the members will emplace: live birth per warmed cohort and number of high-quality embryos available for transfer or for cryopreservation. Regularly scheduled meetings among the members of the network drive communication for opportunities to improve methodology, methods, and/or operations and provide a forum for troubleshooting or addressing problems or indicators for positive change.

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Establishing a Donor Oocyte Cryobank Network: European Experience

19

Jose Remohi Gimenez and Pilar Alama Faubel

19.1 Introduction

The first reported pregnancy after the use of vitrified donor oocytes was made in 1999. Vitrification constitutes today a method of safe storage with impressive efficacy and high rates of successful thawing [1]. Since January 2013, cryopreservation of oocytes is no longer considered an experimental technique [2]. In the last 5–7 years, there has been a proliferation of biobanks that preserve human gametes, mainly for the purpose of providing gametes for heterologous fertilization pathways (establishment that stores oocytes from donors that makes them available to other parties) or preserving their own gametes for future use, alongside the need to establish standard institution and conservation procedures [3]. The increasing use of donor egg banks (*heterologous bank*) would reduce the waste of surplus embryos, the waiting time for recipients, and the cost to patients [4].

The oocyte cryobank in the field of medicine-assisted reproduction is regulated under the EU Tissues and Cells Directive 2004/23/EC, as it is considered a tissue bank, a term commonly used to describe an establishment that collects and stores human cells or tissues for medical research or human application (guide to the quality and safety of tissues and cells for human application is published by the European Directorate for the Quality of Medicines & HealthCare of the Council of Europe (EDQM) [5]).

Biobanks perform a public function, although they are not necessarily established in public institutions.

There are wide variations in the techniques available and permitted by law in each country and the types of reproductive cell that can be donated. Whatever the situation, national

laws must be respected and donors recruited accordingly. Gamete donation can be classified as partner or non-partner donation depending on the link with the donor.

It is essential that all gamete donors should be recorded in national registers, and all centers should participate in the collection of national and international data. Medical assisted reproductive (MAR) centers and health authorities should collect data on a systematic basis to follow up the donors, including the collection of data on serious adverse events or adverse reactions in donors, recipients, and the resulting offspring.

In addition, there should be a limit to the number of times a woman may donate.

19.2 Donor Recruitment

The scarcity of donor gametes has stimulated discussion about acceptable systems of recruitment, especially since it has become evident that different clinics and countries are using different approaches to recruit donors, with various modes of compensation for donors. Donor recruitment is an active process and requires awareness of the general population by educating society, at local and national levels, about the benefits of the clinical applications. It would be useful to work for the spread of an authentic culture of altruistic and gratuitous donation in favor of other people.

However, the donor recruitment activity, whether performed by a public health system or by private centers (where allowed by national legislation), must be authorized by the health authority. In some countries, publicizing or advertising the need of donors is forbidden and regulated by law.

It is important to emphasize that the donation of reproductive material should strictly follow the same principles of being voluntary and unpaid as for other donations of tissues or cells.

National regulations will need to pay special attention to the existence of advertising and false or misleading promotion. In addition, the activities to donation should have a non-

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profit character, which means that only the actual costs of the additional services (those required to allow the donation to be performed) should be charged.

The Directive 2004/23/EC on Tissues and Cells states that donors may receive compensation, but this is strictly limited to making good the expenses and inconveniences related to the donation procedure. Financial compensation should reflect the time, inconvenience, and physical and emotional demands and risks associated with oocyte donation and should be at a level that minimizes the possibility of undue inducement of donors and the suggestion that payment is for oocytes themselves [6]. In this case, member states define the conditions under which the compensation may be granted.

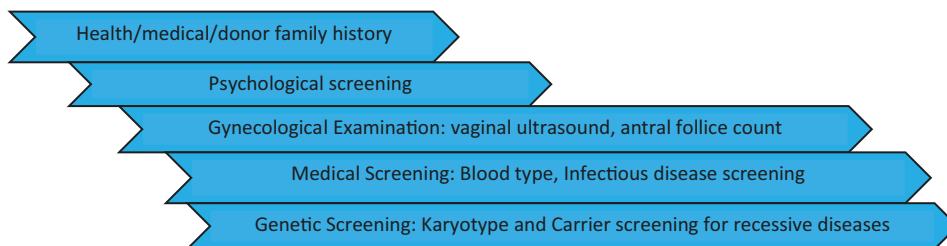
Many studies suggest that a mix of altruism and financial interest leads men and women to donate gametes [7]. Payment is often considered inappropriate, while compensation for expenses is acceptable. Motivation and views on compensation seem to depend on the type of donation and the culture and values of the country in which the donors are recruited.

19.3 Donor Suitability Assessment

The purpose of evaluation donors is to ensure that donors whose gametes may cause a health risk in the recipient or to the offspring (e.g., infectious disease, genetic disease) can be excluded, in addition to ensuring that the donation process does not cause harm to the health of the donor (see Fig. 19.1). According to the EU Tissues and Cells Directive 2004/23/EC, to donate the gametes, the potential donor must be submitted to:

- Consultation and counseling with a healthcare professional.
- Completion of a health/medical/donor family history questionnaire.
- Psychological screening: psychological evaluation and counseling by a qualified mental health professional. The donor will be asked to speak with a psychologist to ensure that she fully understands the benefits and risks of egg donation and is properly motivated to become a donor.
- Gynecological evaluation of the donor's menstrual cycles and a vaginal ultrasound are made to examine ovaries, to count antral follicles, and to ensure that there is no pathology in her ovaries.
- Medical evaluation and body mass index.
- Laboratory testing (including screening for infectious diseases. The following biological tests must be carried out: anti-HIV-1 and anti-HIV-2, HBsAg and anti-HBc, anti-HCV, and syphilis (a treponemal specific test or a nonspecific treponemal test can be used). In some cases, further tests may be required, for example, testing for HTLV-1/2.
- Blood group type.
- Genetic testing as indicated by family history and prevalence of carrier status in specific populations; karyotype testing is strongly recommended, and other extensive genetic screening for common recessive genetic mutations is now available and should be considered in order to reduce the risk of transmitting genetic disease to the child.
- Informed consent before any procedure. Potential donors must beforehand be given appropriate information as to the type of cells to be procured, the purpose and nature of the intervention, its consequences, possible side effects and risks, and whether the oocytes can be processed and stored. Potential donors should be informed that tests will be performed to assess the possible existence of any communicable diseases that may be a risk to the recipient, and the measures that will be taken in the event of a positive result. Donors should be informed that they may withdraw their consent at any time (Fig. 19.1).

Fig. 19.1 Donor oocyte requirements



19.4 Recurrent Suitability Evaluation

Once donors are accepted as a donor, the following biological tests must be carried out for each donation: anti-HIV-1 and anti-HIV-2, HBsAg and anti-HBc, anti-HCV, and syphilis (a treponemal specific test or a nonspecific treponemal test), and in some countries, testing for HTLV-1/2 is mandatory. The guidelines recommend for increase in the safety of the procedure and NAT of HIV, HCV, and HBC. Oocyte donation could be considered as starting on the first day of stimulation, with the sample for testing to be taken at that time (guide to the quality and safety of tissues and cells for human application is published by the [5]).

19.5 Donation Process

Processing, preservation, storage, and distribution conditions must comply with European regulations on human tissue and cell donation.

Here, we will describe some of the technical features that an oocyte bank has to meet to fulfill the European regulations:

Storage: The material with which the oocytes are handled must have been tested for cytotoxicity (pipettes, culture plates, vitrification devices) and if possible be CE marked. Storage temperature is appropriate when $<-132^{\circ}\text{C}$ and is detrimental to the survival and quality of frozen material if $>-132^{\circ}\text{C}$. Storage tanks should have protocols for monitoring temperature and/or nitrogen levels. Oocyte storage devices can contain one or more oocytes and can be open or closed. There is no scientific evidence that oocytes, if kept in proper storage conditions, deteriorate over time; hence, they can be stored for long periods [8, 9]. Cross-contamination between environmentally derived pathogens and stored reproductive material has also not been demonstrated.

19.5.1 Distribution and Transport

The transport of reproductive cells and tissues requires the identification of the distributing, importing, and exporting institutions, as well as the identification of the biological material and its conformity for clinical use. A collaboration

agreement should be signed for inter-institutional transfers that reflects the obligations of the sending and receiving institutions. At both institutions, the accompanying documentation and the identification of the sample in the storage device should be verified to ensure that it corresponds to the donor records. Transport between centers should be by the most appropriate means of ground or air transport and through systems capable of maintaining cell viability and functionality. These systems should be specified in documented procedures according to the type of cell or tissue to be transferred. The transfer of oocytes from or to countries outside the European Union (import/export) requires prior administrative authorization.

19.5.2 Quality Assurance and Traceability

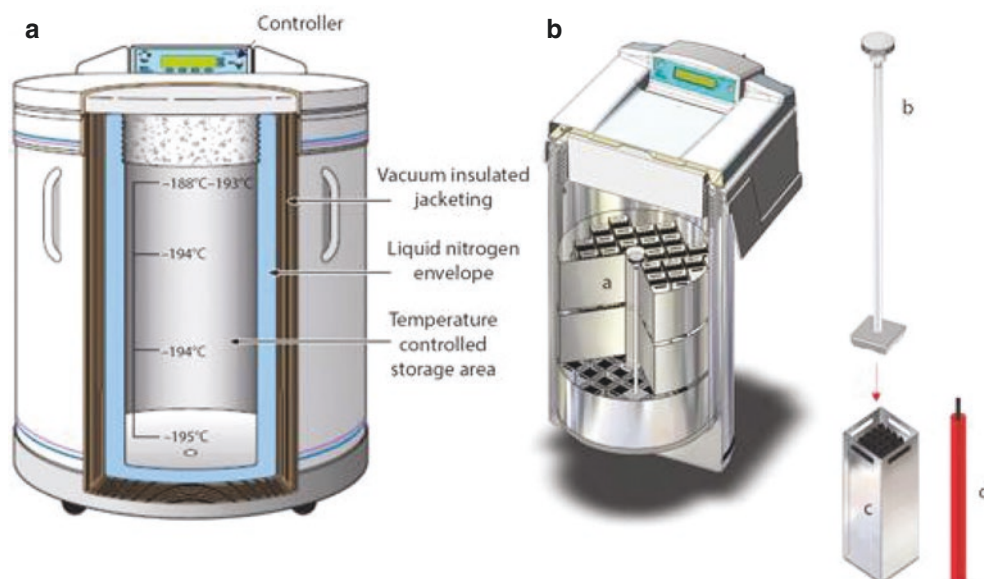
All devices containing donated gametes should be clearly and permanently labeled with the unique identification of the donor. Biological material from different donors should not be processed in the same work area at the same time. During specimen handling, traceability should be verified by double-checking the identity by a second person and/or by using an electronic identification system.

Products and materials used with biological materials should be traceable. The date of each handling and the identity of all responsible persons and witnesses should be documented throughout the treatment. These records must be kept for a specific period of time according to current European legislation.

Figure 19.2 illustrates the interior of a nitrogen tank. Some experts suggest calculating the space based on a linear increment within a 10-year plan basis or to have an off-site storage room in case of urgent need for extra space [10].

Donated gametes require a specific coding, called SEC code ("single European code") (Fig. 19.3), for those countries that are regulated according to European Commission directives, specifically Directive 2015/565 which amends Directive 2006/86/EC. The "Single European Code" or "SEC" is a unique identifier that consists of two elements, a donation identification sequence, essentially indicating the origin of the tissue or cells, and a product identification sequence, essentially classifying the type of tissue or cells.

Fig. 19.2 (A) Diagram to illustrate the inside of the tank, showing the jacket with liquid nitrogen and the vapor area for storage. (B) (a) Three storage levels assembled on a rotating carousel. (b) Retrieval tool to place and retrieve the canister (c). (d) Goblet containing the samples that are placed into the canisters. Source from: BookTextbook of Assisted Reproductive Techniques. Managing an oocyte bank Edition fifth Edition. Imprint CRC Press. ISBN 9781351228220



THE STRUCTURE OF THE SINGLE EUROPEAN CODE

DONATION IDENTIFICATION SEQUENCE			PRODUCT IDENTIFICATION SEQUENCE			
EU TISSUE ESTABLISHMENT CODE		UNIQUE DONATION NUMBER	PRODUCT CODE		SPLIT NUMBER	EXPIRY DATE (YYYYMMDD)
ISO country code	Tissue establishment number		Product Coding System identifier	Product number		
2 alphabetic characters	6 alpha-numeric characters	13 alpha-numeric characters	1 alphabetic character	7 alpha-numeric characters	3 alpha-numeric characters	8 numeric characters

Fig. 19.3 The structure of the single European code

19.6 Legislation and Regulation in European Countries

A recent survey was performed by the European IVF Monitoring Consortium of the European Society of Human Reproduction and Embryology (ESHRE) on the legislation in the EU about ART, sperm, and egg donation [11]. Egg donation is not allowed in Germany, Norway, and Switzerland.

19.6.1 Egg Donor Details

Egg donors must be over 18 years old in about half of the countries where the donation is performed (Belgium, Bulgaria, the Czech Republic, Spain, Portugal, Russia, etc.). A maximum age for donors is established in 25 countries, ranging from 34 years in Serbia to 38 years in France, with many countries setting the limit at 35 years. Bulgaria

and Denmark are less restrictive about the donor's maximum age if the donors/relatives are known to one another. In Belarus, Bulgaria, Hungary, and Ukraine, egg donors must have at least one child. This condition is considered desirable but not mandatory in the selection of egg donors in Romania and Sweden. In Romania, women must be married [11].

19.6.2 Number of Egg Donation Cycles

The maximum number of donations is specified in 10 countries—from 1 (2 in rare exceptions) in Slovenia to 20 in Belarus; most common numbers are between 4 and 6. These data are similar to those established by the ASRM, where the maximum number of stimulations to be performed by a donor should be up to 6 [12].

19.6.3 Children from the Same Donor

A maximum number of infants originating from the same donor is defined in 25 countries, although in the Czech Republic and Romania, it is just a recommendation (and not a legal requirement). This value ranges from 1 in Cyprus and 2 in Montenegro and Macedonia to 10 in France, Greece, Poland, Kazakhstan, and Italy. In 6 out of the 25 countries (Belgium, Finland, Serbia, Slovenia, Sweden, and the UK), there is a maximal number of families/women that may have children resulting from the same egg donor (ranging from 1 for Serbia to 10 for the UK) [11].

19.6.4 Anonymity

The biggest recent change moving towards homogeneity in Europe is in the anonymity of egg and sperm donors. The map of Europe shows the differences within European countries with respect to anonymity in donations (Fig. 19.4). As regards gamete donation, four different scenarios were identified:

- *Strict anonymity* remains the law in 18 countries, although in 5 of these countries, disclosure of donors' identity is possible in cases of severe health conditions of the child born. A particular situation is seen in Lithuania where a donor's identity can be known for other (non-specified) important reasons, after a court decision. In some countries (Estonia, Poland, and Russia), general information about the donors (nationality, age, weight, height, education) is available for recipients and children born.
- *Anonymity applies to recipients*, but the born children can have access to donor identity when above a defined age (Austria, Croatia, Finland, Malta, Portugal, the UK).
- *A mixed system* (anonymous and non-anonymous) as was described in 13 countries, but in each country with a very different regulation.
- Finally, *non-anonymity* is reported as the rule for gamete donations in Georgia and the Netherlands.

A registry of donors exists in 16 countries (in France for egg donors only) and is mandatory in all of them except Iceland. It is organized by a competent authority in six countries, the Ministry of Health in four (including Spain), another governmental body in two, a professional association in Sweden, and a committed volunteer in Iceland; information was missing for three countries (Fig. 19.4).



Fig. 19.4 Europe map adapted according to anonymity. Author: own elaboration

19.7 Follow-Up Children and Donors

Donors should be informed about unanticipated results of medical (and where applicable genetic) screening and, where required, offered a follow-up consultation to explain the results and potential implications.

Oocyte donors should be informed of all health risks associated with hormonal stimulation and oocyte retrieval. A follow-up consultation with oocyte donors shortly after donation is recommended. It is also important to talk about the possible short- and long-term emotional consequences of their donation [13].

19.8 Donor-Recipient Match

Donor-recipient matching is the process by which a donor is selected for a recipient couple based on **phenotypic criteria** (physical characteristics, such as ethnicity, eye color, hair color, height and physique, blood type), **biometrics** (facial resemblance study), and carrier genetic test. Some argue that the family history of disease should also be considered in the matching process [14].

The final aim of carrier genetic screening studies on gamete donors and recipients is to reduce the risk of having offspring who are affected by the serious hereditary diseases studied in the recipient.

According to the recommendation of the consensus reached by a work group consisting of representatives from the Spanish Fertility Society and other scientific societies, there are two types of genetic screening: a basic mandatory screening of all donors and an extended genetic screening for donor-recipient genetic matching. For their prevalence, penetrance, and severity, this basic genetic screening should include the analysis of pathogenic variants related to cystic fibrosis (CFTR), spinal muscular atrophy (SMN1), non-syndromic sensorineural deafness (GJB2), and the most prevalent hemoglobinopathies (HBA1/HBA2, HBB; alpha- and beta-thalassemia, sickle cell anemia). In addition, in oocyte donors, the molecular study of the FMR1 gene related to fragile X syndrome should be done. All gamete banks should carry out basic genetic screening on their donors.

The other recommended strategy would be that all patients who provide gametes for assisted reproduction techniques should be informed about carrying out expanded genetic carrier screening. If donor gametes are used in these techniques, donors who do not share pathogenic variants in the same gene as the recipient will be selected. This is called genetic matching. Each gamete bank can decide which diseases to include in these expanded genetic carrier screening studies, considering that these panels should be limited to serious diseases with defined phenotypes and variants of high penetrance, with a high frequency of carriers and a causal relationship between the pathogenic variant and the phenotype, avoiding identifying variants of unknown biological significance [15].

In addition to such screening tests in the context of reproductive treatment, direct-to-consumer genetic testing and other diagnostic tools are now proliferating on the Internet that make it feasible to access the wealth of medical and personal data or information on genetic relatives without going through “official” channels that require navigating gatekeepers and regulatory structures [16].

Donors should be informed about the implications of direct to-consumer genetic testing in combination with social media and online information. They need to be fully aware that their genetic identity could be revealed at any point through DNA testing by themselves or one of their relatives, even if they were granted anonymity by the legislation of their home/donating country.

19.9 Conclusion

The main advantages of an oocyte cryobank include the fact that donor and recipient no longer must be coordinated in time and space, with a reduced risk of cycle cancellation. The ability to use one batch of donor oocytes for more than one recipient also helps to improve access, reduce recipient

wait times and costs, and decrease excess embryo storage and wastage [4]. The biggest recent change moving towards homogeneity in Europe is in the anonymity of egg and sperm donors.

Authors' Perspectives

The organization of egg banks is carried out from a commercial point of view, generating ethical doubts (to whom do the eggs belong?) and legal challenges, since there are differences between the legislation of each country of the European Union regarding egg donation. The change in the anonymity policy in European countries can be a setback in the management of oocyte bank samples from a commercial point of view:

1. Differences in regulation.
2. Differences in eligibility criteria with more restrictions.
3. Differences in how competent authorities approach situations.
4. Changes policies in a very short period of time.

On the other hand, there is a lack of international guidance on what information should be provided to prospective donors, recipients, and donor-conceived offspring.

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Managing a Sperm Bank: European Experience

20

Sarah B. Morgan, Henriette Roed Nielsen,
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20.1 Introduction

Within the European Union (EU), companies working with tissues and cells must be licensed for the specific activities they perform. The European Tissues and Cells Directive [1] poses a series of minimum quality and safety requirements for gamete donation that are implemented in national legislation in member states.

All tissue establishments (TEs) must have a person responsible (PR), who ensures that licensed activities are conducted in accordance with EU and national requirements. Additional personal roles and key competences can be required by national legislation, e.g., a clinical geneticist, a quality manager, or competent laboratory staff.

European Sperm Bank (ESB) was founded in 2004 and is currently located in multiple EU member states. Consequently, the organisation comprises several licensed TEs authorised by the competent national authority in the country in which they reside. Each TE is headed by a local PR. To determine the overarching medical guidelines and requirements employed, a medical director has been appointed. Further, it is advisable that quality management is controlled centrally so employees, regardless of the location, have access to the same documentation and are subjected to the same training requirements.

This chapter outlines the donor suitability assessment and sample processing used at ESB. Further, the special consideration relating to distributing gametes within the EU will be presented.

20.2 Donor Suitability Assessment

Gamete donation has lifelong implications for the donor, recipients, and the children potentially conceived. Therefore, it is important to ensure that a person is both physically and mentally suited to be a gamete donor. The requirements in the Danish Tissue Act [2] provide a comprehensive implementation of the EU Directive [1], which forms the basis of the ESB donor suitability assessment.

All males between the ages of 18 and 45, regardless of ethnicity, religion, sexual preferences, educational level, and parental or marital status, can apply to become a sperm donor with ESB and partake in the donor suitability assessment.

As outlined in Fig. 20.1, the suitability of a potential donor is typically assessed through six steps.

The purpose of the suitability assessment is to exclude individuals with a high-risk profile from becoming donors. A risk profile includes a person's hereditary predispositions to physical and mental conditions and risk behaviour related to acquiring sexually transmitted infections. High-risk behaviour is determined in national legislation and varies within countries, but in general, it includes a high number of different sexual partners, injecting pharmaceuticals for non-medical purposes, traveling to countries with endemic outbreaks, using human-derived products, or having sexual relations with a partner that practices high-risk behaviour.

20.2.1 Assessment of Sperm Quality

The first step in the donor suitability assessment is an evaluation of sperm quality. To ensure correct identification of donation, procurement should be performed on site under controlled conditions. To protect the privacy of each donor, samples are produced in private, soundproof donor rooms, with cleaning facilities and adult entertainment available.

For the initial assessment, ESB primarily evaluates the motility of the sample, whereas morphology and DNA fragmentation are of less concern for samples from normozoo-

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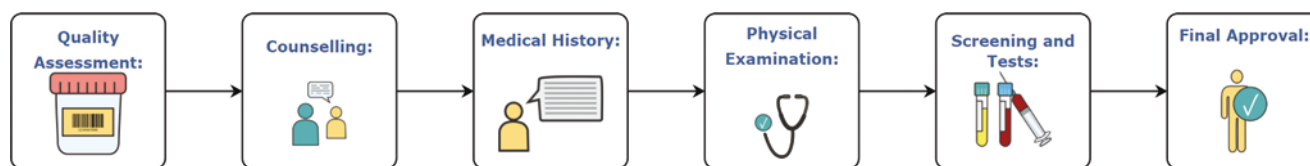


Fig. 20.1 The donor suitability is assessed through six different steps: (1) assessment of the sperm quality, (2) counselling and information provision, (3) assessment of the medical history of the donor and his

family, (4) a physical examination performed by a general practitioner, (5) screening and test on blood and urine, and (6) final combined assessment and approval

spermic and fertile men, as opposed to men with subfertility [3–7]. At ESB, more than half of the potential donors are rejected for not meeting our internal sperm quality requirements. The potential donors that have a sperm quality above the established threshold are invited to proceed in the suitability assessment.

20.2.2 Counselling and Information Provision

Choosing to be a gamete donor is a significant commitment, not only because of the children that will be born, but also due to the information that will be uncovered about a potential donor himself during suitability assessment. Hence, it is of great importance to ensure that all potential donors are adequately informed and have been offered applicable counselling, prior to committing to become a donor [8]. This information includes the scope of the assessment, the implications of being a gamete donor, and the data protection rights that do and do not apply to gamete donors.

Specifically for genetic screening, the sperm bank should carefully consider a policy for genetic counselling both prior to genetic screening and when the result is available, as the results of genetic screening can have consequences for the donor himself and his close relatives.

20.2.3 Medical History of Donor and Family

The medical assessment of a potential donor aims to identify and exclude people whose donation could present a health risk to the recipient, the donor child, or both. This includes signs of physical or mental disease, malformations, evidence of disqualifying infections, and significant risk behaviour. The medical information is typically self-reported; therefore, it is advisable to establish a procedure that increases the accuracy and ensures consistency, e.g., different collection methods over a span of time.

A three-generation pedigree with medical information on family members is created for the potential donor to account for hereditary conditions and predispositions to disease. The pedigree is extended if the collected information gives rise to suspicion of predisposition to late-onset diseases. A potential

donor cannot be qualified if the use of his gametes poses a significantly increased risk of severe disease or predisposition to severe disease in his offspring.

20.2.4 Physical Examination

To diminish the risk of approving a donor with a predisposition to serious hereditary condition and/or an infectious disease, a physical examination is performed by a licensed general practitioner. The examination covers the donor's general physical health, his mental well-being, physical evidence of sexually transmitted infection, evidence of skin manifestations of hereditary conditions, malformations, and/or tumours. To assist the assessment of the potential donor's health biochemical screening is included in the evaluation.

20.2.5 Screening for Infectious Diseases and Hereditary Conditions

An indispensable part of donor suitability assessment is screening for infectious diseases. The screening is strictly regulated within the EU and must include screening for hepatitis virus B and C, HIV1/2, and syphilis. Individuals who are or have been infected with these diseases cannot be accepted as gamete donors.

According to the EU directive, genetic screening for autosomal recessive conditions that are known to be prevalent in the donor's ancestry or geographic origin must be performed [9]. At ESB, all donors are karyotyped and screened for a carrier status of cystic fibrosis, spinal muscular atrophy, and hemoglobinopathies. The screening is expanded if the donor has Jewish, Faroese, or French-Canadian ancestry or is from other populations with a known founder effect and high carrier frequency of specific conditions.

Until recently, it was believed that only a limited number in a population carried a genetic condition. Consequently, the prevalent approach is to disqualify potential donors who are known carriers. There are several drawbacks to this practice although the purpose of avoiding a serious condition in a child is clear. Further, the risk of an affected child depends on both the maternal and paternal carrier status.

Hence, by excluding donors who are carriers of a recessive condition without knowing the carrier status of the recipient, a substantial number of potential donors will be disqualified on an irrelevant basis. This does not only have a negative effect on cost and access to donated gametes, but also poses a significant risk of harm to the donor who is told that he is unsuitable for reproduction. An alternative approach is to approve donors who are known carriers and subsequently make information about their carrier status available to recipients. This allows the recipient to decide, on an informed basis, whether a donor is suitable for her or not. Though this provides the recipient with reproductive autonomy, publication of a donor's carrier status can have a negative impact on the privacy of the donor and his right to remain unaware of certain information, e.g., his own carrier status [10].

A more relevant method is to offer a matching solution where the genetic information of the recipient and the donor is matched to ensure that they are not carriers of the same recessive condition, thereby reducing the risk of an autosomal recessive inherited condition in a child without harming the donor by either disqualification or excessive disclosure of his carrier status. Likewise, the recipient may remain unaware of her carrier status.

20.3 Recurring Suitability Assessment

When the donor is assessed as suitable, donations can be obtained, processed, and stored for the purpose of being distributed. The EU Directives require verification that the

donor is still suitable for donation prior to release of obtained donations [9]. Recurrent donor suitability assessment is conducted periodically, whilst the donor is actively donating, to identify any factors which could impact the safety and quality of the gametes donated since the initial assessment or the latest approved recurrent assessment.

A recurrent assessment can include triage questionnaires used to identify new medical information about the donor and his family members, travel history, changes in the donor's risk profile, or other findings with potential impact on the safety of the gametes. Even though the donor has stopped donating, he will still be obligated to report any new medical information concerning himself and his family to the sperm bank.

Further, physical examinations and infectious disease screening should be repeated as objective indicators of continued suitability. The frequency of screening and the number of infectious diseases included in the screening are regulated by the Directive [9]. Unfortunately, this has not been implemented unanimously across all member states, which is problematic for the cross-border exchange of gametes. Most member states accept what is known as the 'Danish Protocol' based on nucleic acid testing (NAT) every third month. This method was described and evaluated by the European Centre for Disease Control and Prevention [11]; however, other countries only rely on serological testing to verify recurrent suitability. If a sperm bank seeks to distribute gametes throughout the EU, several different release methods may be applied to comply with the regulations in the recipient country. The basics of release are outlined in Fig. 20.2.

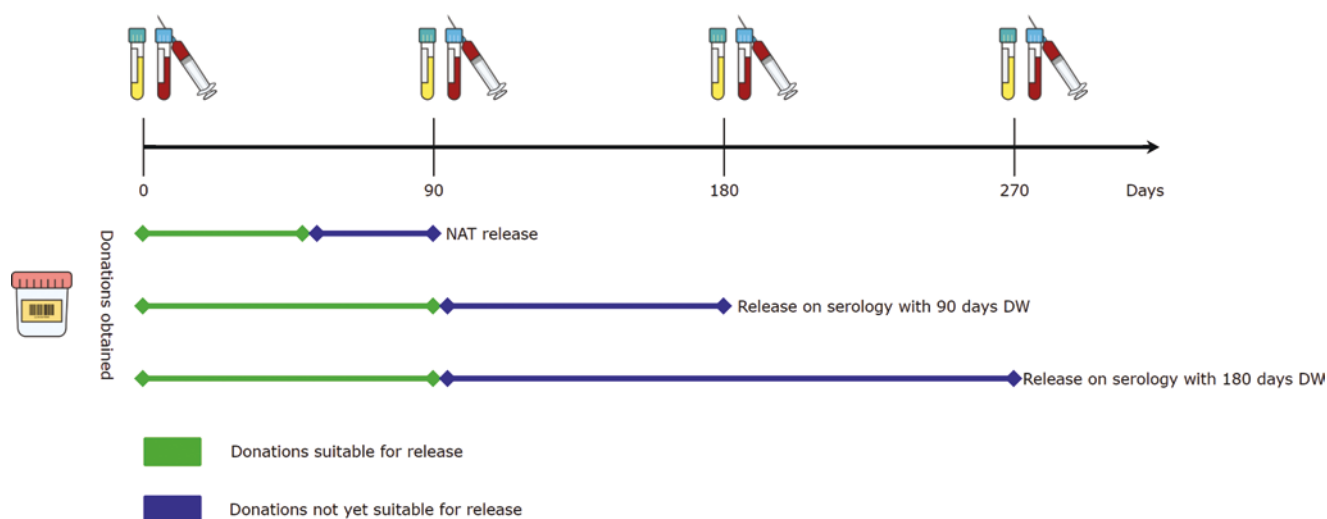


Fig. 20.2 The release standard that can be used to assess when a donation is safe for use depends on the method of analyses and the length of the associated diagnostic window (DW) period. The screening on blood and urine should include HIV1/2, hepatitis B and C, syphilis, and chla-

mydia, as well as additional testing based on the travel history of the donor and requirements in the recipient country. NAT: nucleic acid testing

20.4 Donor Recruitment

Prior to assessing any donors, they must first be recruited and apply to be part of a donor programme. Donor recruitment rests on a basis of marketing campaigns aimed towards destigmatising society's view on sperm donors, as well as explaining the difference a sperm donor makes to families in need. These campaigns can be physical or online and are often combined with targeted campaigns on social media inviting men to apply to become a sperm donor.

National regulation differs on what can be included in the marketing campaigns and what is allowed in terms of compensation for the donor. Most countries do not allow sperm donors to be paid due to the principle of unpaid and voluntary donation, but compensation for loss of earnings and transportation cost is generally accepted. The allowed amount varies but ranges from 40 to 80€.

20.5 Donation Process

20.5.1 Sample Process Flow

The physical arrangement of the laboratory must account for the sample process flow, i.e. workstations placed in a meaningful order, and separation of samples at the workstations should be possible to avoid mix-ups. To further prevent mix-ups, double witnessing can be implemented either as an electronic system or by manual process with two employees witnessing the identity of the sample at each critical stage, i.e. where the sample changes container (as outlined in Fig. 20.3). Specific requirements for laboratory facilities are specified in the EU and national legislation, e.g. air quality in processing areas.

The sample process should be simple and standardised to ensure high and reproducible quality as well as traceability. It is essential that samples are not mixed, contaminated, or lost during the processing; hence, the process should be well-described, and staff properly trained.

Semen samples can be frozen directly after liquefaction or subjected to processing before freezing. The predominant way of processing semen samples is by the swim-up tech-

nique or density gradient centrifugation. Before freezing in liquid nitrogen, the samples are filled into sperm straws and sealed to physically separate samples from each other. The process of freezing depends on the media used and the laboratory experience.

Analysis of sperm quality should be done in accordance with the WHO manual [12], but as already mentioned, some sperm parameters are more relevant for fertile men in the setting of the sperm bank. It can be a help for the laboratory staff to employ a computer-assisted sperm analysis (CASA) system. A CASA system can also minimise the potential counting bias between laboratory staff.

Regular assessment of staff counting competences both internally and via participation in external quality programmes, i.e. ESHRE or other national programmes [13–17], is recommendable.

Sperm straws are stored in liquid nitrogen, and no maximum storage time is defined in the EU directives. As sperm is a biological product, a minor degree of deterioration over time must be expected. Additionally, some countries have national regulations, specifying the maximum age of sperm straws in storage before they are used in a treatment.

Sperm cells are sensitive to temperature, and under storage and transport of frozen samples, it is vital for the quality of the gametes that the temperature is kept as constant as possible. To accomplish this, frozen straws should not be exposed to ambient temperatures. Transport of frozen straws can, however, be done in nitrogen vapour shippers without affecting the quality of the gametes [12].

20.5.2 Donation Frequency and Limits

There is no legislation controlling a donor's donation frequency; however, as recommended by the WHO, a minimum of 48 h between ejaculations along with regular ejaculations is ideal to achieve high sperm quality [12]. At ESB, we constantly review our donors' donation frequency, their number of straws produced per donation, and the market demand and market limits of the donor. We strive to collect and store an adequate number of sperm straws to meet the demand from our markets, also considering the client's wish for siblings

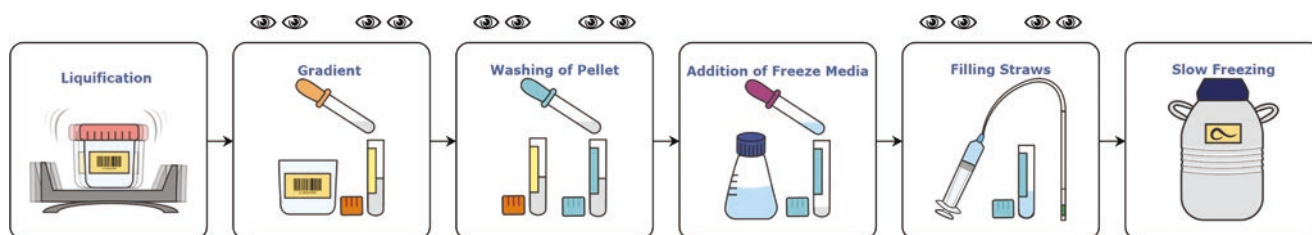


Fig. 20.3 Generic flow of samples at ESB including the three main steps in which a donation changes container (marked with two sets of eyes)

from the same donor. The demand is, however, not without limit, as there is an increasing tendency among member states towards regulating the use of donated gametes.

20.6 Regulating Pregnancies from Donor Insemination in Europe

In the current EU Directives regulating artificial insemination with donor sperm (donor insemination), no limit on the number of children born from a single donor is described; nevertheless, several member states have implemented national limits [18].

The main reasons to impose such regulations are to limit excessive spread of genetic conditions and protect the genetic variation of the population [19, 20]. However, other considerations are often debated, e.g. the emotional implications of the parties involved and the risk of unintentional consanguineous unions between donor-conceived individuals. Current research shows that the actual risk of consanguinity is not greater for donor-conceived individuals than it is for the general population [21]; hence, this should not be a main consideration when establishing reproductive limits. The greatest risk mitigation that can be employed to limit the risk of consanguineous unions is transparency from parents towards their children. Informing a child that they are donor conceived, and educating the child of what that entails, enables the child to make informed decisions later in life.

The psychological implications of gamete donation on a larger scale have not yet been thoroughly reviewed due to the young nature of the field. As a result, there is great variation between national approaches.

One of such differences is whether regulations aim to limit the total number of children born from a specific donor or the number of families using the gametes of a donor (Table 20.1). By regulating the total number of children associated with a donor, a family might not be able to have full siblings, if the donor they have previously used has subsequently reached the reproductive limit. This can cause great distress, hence, there is a tendency of changing the limit of children per donor to the number of families per donor, thereby avoiding regulations limiting the growth within a family.

Distribution of donor sperm to clinics around the world requires close monitoring of national regulations to ensure continued adherence to these. It is critical to ensure traceability through the distribution chain and, subsequently, to

monitor the outcome of treatment, i.e. if the treatment has resulted in a live birth. In general, there are two main ways to control the number of pregnancies associated with a single donor:

- Pregnancy reporting is a practice in which the responsibility relies on the treating/fertility clinic reporting a clinically verified pregnancy to the associated sperm bank. Unfortunately, this method often results in underreporting and therefore requires continuous oversight from the sperm bank.
- Pregnancy slot is a method used by sperm or egg banks to control the right to use a specific donor's gametes either for one or for multiple children. The potential problem with a pregnancy slot is that in the unfortunate case where no child is born, the donor's gametes are not available for treatment of new recipients, even though the actual pregnancy limit has not been reached. To avoid unused pregnancy slots, a fee can be charged to acquire a pregnancy slot, which in turn is fully refundable if no child is conceived, thereby allowing another recipient to purchase the pregnancy slot.

20.6.1 Donor Registers

Today, several member states have identified the importance of registering and overseeing donor conception. As a result, national registers have been created where donors and associated pregnancies are reported, which allows competent authorities to monitor adherence to reproductive limits. Sperm banks are in many cases not allowed access directly to these registers and cannot use them to monitor the pregnancies of their respective donors. Additionally, these registers are autonomously regulated by each member state resulting in divergent approaches to data protection and access.

An alternative would be the establishment of a common European gamete donor register, in which data could be securely stored and used in a manner aligned across the EU. A common register can further ensure that a donor's relationship with a TE is recognised and traceable, ensuring that a donor does not donate to more than one TE. This, in turn, provides higher confidence in the adherence to reproductive limits in all member states and enables traceability when handling adverse outcome as described later.

Table 20.1 Examples of reproductive limits within the EU

Member state	Reproductive limits per donor
Belgium	6 families
Denmark	12 families
Netherlands	25 children to 12 families
Sweden	12 children to 6 families

20.7 Balancing Privacy and the Right to Know

The identity of a donor must not be disclosed to the recipients according to the current EU directive. However, depending on which donor type the recipients have chosen, there

might be an option for the donor-conceived individual to get the information about the donor's identity upon reaching legal age.

The donor type allowed for treatment is exclusively determined by each member state; however, as donated gametes are distributed across member states, confusion exists between donor, recipients, and donor children regarding what a specific donor commits to or what the donor child can expect.

At ESB, we distinguish between two types of donors, ID release donors and no ID release donors, where the only noticeable difference is whether or not the donor has consented to having his ID disclosed to a donor child, if requested by the child when it reaches legal age. The specific details that can be disclosed about a donor likewise differ among member states, but often include disclosure of the donor's full name, year of birth, and nationality. There are no general regulations for the subsequent use a donor child can make of the disclosed data.

When establishing internal procedures relating to disclosure of a donor's data and potential contact between donors and donor-conceived individuals, the right to know your heritage must be balanced against the donor's right to privacy. No donors have committed to having contact with potential donor children, which is important for the parents to communicate to their children to avoid unrealistic expectations or hopes. To accommodate the need for information, sperm banks may offer access to a non-identifying donor profile. This profile consists of extensive information about the genetic heritage, family medical history, and personal traits of the donor, which can be of great importance to a donor child and their identity.

20.8 Follow-Up on Donor-Conceived Children

Although the suitability assessment and screening of the potential donor reduce the incidence of genetic anomalies passed on from a donor to donor children to below general population levels, it is not possible to exclude all risks. The donor can develop a hereditary condition years after the suitability assessment was made, or he could be a healthy carrier of a genetic condition, as penetrance and expression of a genetic condition may vary within individuals. This means that the donor may appear healthy but is harbouring an autosomal dominant inherited condition with a 50% risk of passing the condition to children. When it is suspected that a donor has passed a condition to a donor child, it is known as an adverse outcome. As defined in the EU directives, a 'serious adverse reaction' means an unintended response, including a communicable disease, in the donor child, the recipient, or the donor that is fatal, life-threatening, disabling, or inca-

pacitating or which results in, or prolongs, hospitalisation or morbidity [1].

It is of highest importance that a suspicion of an adverse outcome of donor insemination is reported to the sperm bank, which in turn must assess if the use of the donor's gametes poses a risk. If the suspicion is confirmed, the gametes from the associated donor should be blocked and not used for further treatment to limit the disease risk. The sperm bank must ensure that there is a system in place to report, investigate, register, and transmit information about serious adverse reactions, which may influence the quality and safety of the donor gametes, and member states in the EU should communicate the information across borders [1]. Further, all relevant clinics must likewise be informed to enable them to stop treatment with gametes already in their possession, counsel the relevant recipients, and ensure diagnosing and/or surveillance of potentially affected individuals.

20.9 Closing Remarks

The field of donor conception is still relatively new; therefore, the information on how donors and donor-conceived individuals respond emotionally throughout their life is not adequately covered. As a sperm bank, we follow our donors, recipients, and donor-conceived individuals and try to accommodate the knowledge, feedback, and regulatory changes that will emerge over the coming years.

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Sperm Bank Management Best Practices: US Experience

21

Jaime Shamonki

Key Points

- A best practices approach to sperm bank management goes beyond donor screening and considers numerous medical, ethical, legal, and cultural issues involved in donor conception.
- Optimizing outcomes for donors, recipients, and donor-conceived persons should include screening for sperm quality, infectious disease, heritable disease through robust practices established by genetic professionals, psychological wellness with the direction of mental health professionals, and an assessment of donors' commitment, maturity, orientation toward disclosure, and altruism.
- Genetic screening and risk mitigation involve collecting and processing complex health information and have both prospective and retrospective components. Banks should be prepared to receive, evaluate, and manage updated health information in perpetuity.
- It is important to survey and consider the attitudes of donor-conceived individuals to create sperm bank practices that are in line with the best interests of the people who have the least power and decision-making ability in this process.

21.1 Introduction

From the time Dr. William Pancoast of Jefferson Medical College facilitated the first anonymous therapeutic sperm donor insemination resulting in a live birth in 1884 [1], sperm donation has been the subject of enormous interest and occasional controversy. Today's modern sperm banks are both contributors to and a consequence of much progress in science and society. Sperm bank practices continue to evolve, as do the families who grow every year as a direct benefit of sperm dona-

tion from the 200,000 annual donor inseminations in the United States alone [2]. It is interesting to consider that the mere existence of modern sperm banks is the result of numerous interdependent factors, including advancements in cryobiology, evolving societal norms around parentage and the definition of family, and even the emergence of the AIDS epidemic.

Today, a best practices approach to sperm bank management must consider the complex medical, ethical, legal, and cultural issues [3] implied in donor screening and compensation, in facilitating donor conception, and in navigating the network of biological relationships that exist outside of nuclear family and kinship. This chapter is intended to serve as a "practitioner's guide" to optimizing outcomes for all parties involved in the donor insemination process. The best practices put forth in this chapter rely on learnings from management of one of the oldest and largest US donor sperm banks, incorporate feedback from adult donor-conceived persons, and consider decades of multidisciplinary academic studies in third-party donation.

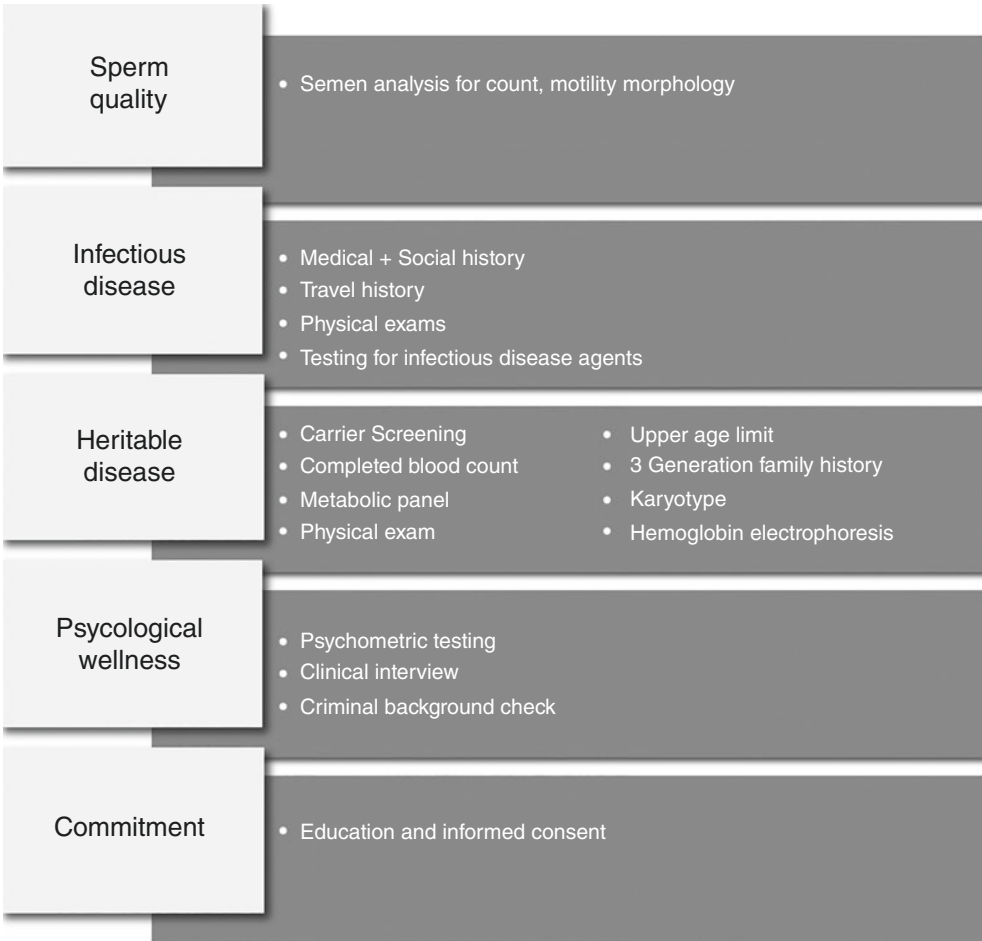
While important consideration is given to current regulatory requirements and accrediting body standards, this chapter is not meant to serve as a comprehensive review of regulatory guidance documents or as a replacement for first-hand review of applicable regulatory requirements. It should also be clarified that this chapter is written from the experience of managing a US sperm bank, so the regulatory considerations predominantly reference those of the FDA or a US state health department requirement.

21.2 Defining the Primary Objectives of the Sperm Bank

A recurring theme of this chapter is that best practices for facilitating donor insemination continue to evolve. However, the primary objectives of the sperm bank have always been to find healthy, preferably "desirable" sperm donors, freeze vials that have the quality and the quantity of sperm required to induce pregnancy, and mitigate risks where possible to

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Fig. 21.1 Sperm bank donor screening should encompass these five areas to optimize the health and safety of all parties involved in what we call the “donor ecosystem”: the donor, the insemination recipient, and the donor-conceived person. Several of these screening steps are considered industry best practice even if they are not required by regulatory bodies



optimize the health of an unborn child. On examination, the straightforwardness of these objectives seems deceptively simple, particularly when viewed in the context of our current definitions for a “healthy” sperm donor and a “healthy” child. For example, a donor-conceived person may assert that their health is as much dependent on the ability to know their donor’s identity as it is on their donor being thoroughly screened for heritable disease.

The sperm bank has the duty to adopt practices that serve to protect the health and safety of all parties involved in what we call the “donor ecosystem”: the donor, the insemination recipient, and the donor-conceived person. These parties are best served when the sperm bank screens the sperm donor for the five categories as depicted in Fig. 21.1.

One may wonder how extensive a role regulatory bodies play in defining these screening practices and whether the sperm bank is obliged to screen beyond what is legally prescribed by the regulator. The section of the FDA that pertains to donated human cells and tissue exists for the chief purpose of mitigating the spread of infectious diseases from donors to recipients. The FDA guidelines effectively reduce the risk of transmitting certain communicable diseases; however, they do not address many of the other elements of health for the donor, donation recipient, and donor-conceived person.

Practices such as limiting the number of vials distributed, establishing a policy on knowableness of a donor’s identity, and establishing donor compensation that is commensurate with altruistic donations, further strengthen the sperm bank’s ability to protect the vulnerable recipients and donor-conceived people who are dependent on this gift. This chapter provides an overview of the practices and methods that can be deployed to conduct high-quality screening and some of the policies that can be adopted to achieve the primary objectives of the sperm bank more confidently.

21.3 Donor Recruitment and Compensation

Historically, when screening was less rigorous and men were promised anonymity, sperm donation was a quick and easy way to make extra money. Today, that is not the case. In one large US sperm bank, the average donor spends almost 40% of his time with the sperm bank in a rigorous screening program moving through the qualification process [4]. Even after donors leave the program, they are expected to engage with the sperm bank indefinitely, such as when the bank inquires about developments in his or his

family's health. The time commitment, and the additional fact that fewer than 1% of applicants ever qualify as donors [4], means that there is nothing quick or easy about making money as a sperm donor.

If screening is oriented to altruistic motivation, then applicants who are interested in sperm donation solely for financial reasons should not pass the screening steps or should self-select out of the process. Sperm bank recruitment messages that primarily emphasize the compensation for donating sperm create a flood of applicants who are not particularly good investments. A savvy marketing department in charge of donor recruitment will figure out that attracting donors solely through promises of quick cash is a foolish strategy.

Sperm banks should be recruiting donors with messaging that is consistent with their expectations that donors be altruistically motivated to help people of all gender identities, sexual orientations, and family compositions to have children. Donors should be committed to undergoing a rigorous screening process, making healthy choices while donating, and be available well into the future for communication that serves in the best interest of the donor-conceived person. Donors should appreciate that their gift is not purely transactional; therefore, the marketing messages that seek to recruit these men should not suggest that it is.

Some compensation is certainly expected, and arguably justified, for the degree of work sperm banks demand from these men. (It is important to point out that remuneration is only for service, never for the gametes.) Although a paycheck should not be the primary motivation to donate, even the most altruistically motivated donor applicants typically expect some compensation for their time and effort.

So how much should sperm donors be compensated for their service? The answer must carefully weigh the benefits of maintaining a supply of willing donors, with the potential safety, coercion, and commodification concerns that arise when payment for services is "too" compelling [5]. Consider the fact that the blood bank industry stopped compensating donors many years ago when remuneration for blood donations correlated with higher rates of infectious diseases in donated blood [6]. Strictly limiting compensation to mitigate for coercion or conflict of interest donations can have unintended negative consequences as well. After years of litigation, the class of human-egg donors who challenged the American Society for Reproductive Medicine (ASRM) for suggesting a price cap on the amount of compensation donors can receive (a suggestion to, among other things, help protect donors from exploitation and coercion) reached a settlement in which the ASRM agreed to remove the guidance for limiting egg donor compensation. The class effectively argued that the ASRM was illegally price fixing and limiting egg donors' opportunity to benefit from consumer demand in a free market [7].

Donor recruitment should also take human diversity into consideration. A widely diverse group of people are looking for a donor on sperm bank catalogs, and their ideas of a perfect donor are as diverse as humans themselves. Families who rely on sperm donors, particularly non-white donors, often have far fewer choices because of chronic underrepresentation on donor catalogs and high and growing client demand for minority donors [8]. Sperm banks cannot solve this complex challenge in a vacuum. Creating inclusive recruitment campaigns and ensuring donor screening criteria are not discriminatory toward race, and engaging mindfully with the recipients and potential donors who comprise these minority communities may help improve diversity on sperm donor catalogs.

21.4 Donor Screening

Reproduction is never going to be a 100% risk-free endeavor, although this does not typically assuage the parent of a donor-conceived child who is born with a rare disabling genetic condition. This is an unfortunate reality of this area of clinical practice and a sobering reminder that the primary goal of donor screening is risk mitigation, not risk prevention. It is noteworthy that a US sperm bank can be legally compliant with the regulatory requirements to distribute reproductive tissue without following many of the five screening categories identified above. The sperm bank business and its customers are best served when the bank follows industry best practices and adopts evidence-based, data-driven tools and well-trained professionals to screen donors. The challenge is that industry standard practices are continually evolving. New technology, regulatory responses to emerging infectious diseases, refreshed society guidelines, and industry adaption to the voice of donors, recipients, and donor-conceived people all drive changing definitions of best practices.

A caveat: Sperm banks tout tongue-in-cheek facts that it is harder to get accepted as a donor than it is to get into Harvard. The rigorous potential donor screening steps should be performed with empathy for the candidate donor, recognizing the potential consequences of rejection from the program. Sperm banks should have a policy and supportive training for employees to manage the applicant disqualification conversation. A donor who is refused for sperm quality may worry about his own reproductive health. Likewise, a donor who is suspected to carry a familial cancer syndrome or be at risk for a psychiatric condition should be properly counseled on the findings, with appropriate health care professional referral, if necessary. It is important that men who do not qualify as donors are not inadvertently concluding that they are "unfit for reproduction" or being cast aside as "rejects" or commodities.

21.4.1 Screening for Infectious Diseases

The key elements of donor infectious disease screening are prescribed by the FDA and are necessary to meet the FDA criteria for donor eligibility. Screening components comprise repeated laboratory testing, physical examinations, and a thorough review of medical and social history of risk factors for, and clinical evidence of, relevant communicable disease agents and diseases to determine FDA eligibility [9].

The FDA-required screening focuses on “relevant communicable diseases” or RCDs, as not every communicable disease can or should be screened for in the donor population. The FDA’s *Guidance for Industry Eligibility Determination for Donors of Human Cells, Tissues, and Cellular and Tissue-Based Products (HCT/Ps)* specifies the following:

The FDA considers (1) risk of transmission, (2) severity of effect, and (3) availability of appropriate screening measures or tests, in accordance with § 1271.3(r)(2), as factors in determining whether a communicable disease or disease agent, not listed under §1271.3(r)(1), is **relevant**. The importance of these factors in determining relevance may be based on the clinical significance of the disease agent or disease. For example, *Ureaplasma urealyticum*, although highly prevalent and transmissible, is not considered a relevant communicable disease agent because its pathogenicity to reproductive cell and tissue recipients has low clinical significance.

The FDA regulations require testing for human immunodeficiency virus (HIV) types 1 and 2, hepatitis B virus (HBV), hepatitis C virus (HCV), *Treponema pallidum* (i.e., syphilis), *Chlamydia trachomatis*, and *Neisseria gonorrhea*. Sperm donors must also be tested for human T-lymphotropic virus (HTLV) types I and II and cytomegalovirus (CMV) given the leukocyte-rich nature of semen [9].

CMV is a ubiquitous virus that has infected between 50% and 70% of the adult population [10, 11], depending on the geographic location. It is not considered an RCD; however, given the severe consequences of congenital CMV if a primary infection is acquired during pregnancy, the FDA requires sperm banks to develop a testing algorithm to ensure that donors are not acutely infected with CMV at the time of donation. Typically, a total antibody screening test with a reflex to specific IgG and IgM antibodies allows for the distinction between remote and acute infection; however, this test is fraught with challenging laboratory results, such as false-positive total antibody screens and persistent production of IgM well into the latent phase of infection. A more detailed discussion of CMV and recipient risk factors is out of scope of this chapter; however, the authors advise expert consultation when designing a CMV donor testing algorithm and donor summary of records reporting procedure.

Certain emerging infectious diseases, such as West Nile virus, among others, have been added to the list of required laboratory screening tests in the last several years [12].

Emerging diseases can be caused by newly identified pathogens, mutated/variants of an existing pathogen, pathogens with recent transfer to human host, or pathogens with changes in geographic distribution [13].

While most disease agents can be screened for through direct testing, certain agents require indirect screening by a targeted medical and social history and physical examination. An example is Zika virus, which is spread by mosquitos and is still endemic in many parts of the world. The more rapid clearance of Zika from plasma than from the genitourinary tract [14] and lack of a validated test for the virus in semen mean that screening must rely on proxies for exposure, such as travel history. Sperm donor travel to a Zika-endemic region results in a 6-month deferral for that donor to mitigate the risk that the virus could be transmitted through his sperm [15].

A donor can also be deferred for nonspecific high-risk behavior, such as illicit drug use or recent history of unknown sexual partners. These indirect screening tools allow the sperm bank to mitigate for the RCDs above, as well as other potentially adverse and often sexually transmitted infections, such as human papillomavirus (HPV) or herpes simplex I or II (HSV I/II).

A frequent query to sperm banks is why do they not screen directly for common sexually transmitted agents, like HPV and HSV I/II viruses, when clinical assays for their detection exist. Recall that to qualify as an RCD, the infectious agent must be both clinically significant to the recipient *and* have an appropriate screening test available for donor specimens. There is no evidence that an asymptomatic donor poses any meaningful clinical risk for these viruses to a recipient, nor are appropriate tests available for screening the sperm of asymptomatic donors. The tests that exist are not intended or validated for use on sperm and do not have any demonstrated clinical value in their application for sperm banking.

Sperm banks may forgo direct testing for these types of viruses; however, it is still prudent to question donors for a prior history of or current infection with these common agents. Policies and procedures designed to mitigate the risk of transmission should be put into place. For example, donors who have a reported history, or have been with a partner with a history of active or recurrent genital herpes should be excluded from donation, even if the risk of active viral shedding and transmission is extraordinarily low.

The introduction of nucleic acid testing (NAT) has significantly narrowed the window between infection and detectability [16]; thus, the FDA-required 6-month quarantine period between semen collection and release is scientifically more conservative than needed to mitigate RCD spread. The same is arguably true of social history requirements that render men with male sexual partners ineligible. While there does not appear to be an immediate plan for the FDA to

revisit these criteria, it should be noted that recipients may always consider the use of ineligible *directed* sperm donors. Sperm banks should be ready to offer reduced quarantine periods and facilitate donations from men who do not meet social history requirements. In this case, it is prudent to ensure that the inseminating physician, the insemination recipient, and the intended parent(s) are all aware of the findings that render a donor ineligible and that proper informed consent has been obtained and documented. The Practice Committees of the ASRM and the Society for Assisted Reproductive Technology (SART) recently updated the guidance regarding gamete and embryo donation [17] with a recommendation that directed donations should be quarantined for greater than 35 days and then followed by repeat infectious disease testing.

21.4.2 Semen Analysis for Count, Motility, and Morphology

Semen analysis using basic light microscopy is still the best proxy for male fertility. Tools such as the WHO laboratory manual [18] for the examination and processing of human semen, now in its sixth edition, help examiners use standardized techniques for the assessment of semen health and diagnosis of male infertility based on several criteria, including count, motility, and morphology. These established industry criteria alone have limited value in identifying a suitable sperm donor applicant because the proxy threshold for fertility based on semen analysis is substantially lower than that which is typically required to produce a single vial of intra-uterine insemination (IUI)-washed sperm. Sperm banks targeting at least ten million total motile sperm after thaw will need to aim to bank at least double that number in post-wash, motile sperm, before freezing. A donor who cannot produce at least one IUI vial per donation, even if he meets the WHO criteria for healthy sperm, may be a poor candidate for the sperm bank.

Defining protocols and criteria for morphology assessment is a source of consternation for sperm bank laboratory directors. Various criteria have been introduced over the years. A significant change was introduced by the WHO fifth edition [19], which introduced the “strict criteria” Kruger method. Many sperm banks have considered aligning with andrology laboratories which are end users of the sperm bank product. However, data suggest that this “strict” level of scrutiny applied to sperm morphology criteria offers limited predictive value for pregnancy; therefore, the additional cost and effort of training, implementing, and performing strict morphologic assessments may not be the best approach for the sperm bank [20].

As new molecular technologies emerge, there will undoubtedly be new tools, such as the epigenetic profile of

semen, to assess a potential donor’s suitability. These assays promise to offer a depth of analysis more meaningful than light microscopy and evaluation of social history; however, they must still meet the primary sperm bank goals and demonstrate practical value beyond an academic exercise.

21.4.3 Screening for Heritable Risk Factors and Management of Genetic Information

An abundance of genetic-based information flows through a modern sperm bank. Mitigating heritable risks to donor-conceived children requires establishment of robust protocols and, ideally, trained genetic professionals to execute these protocols. Sperm banks must also collect, evaluate, and manage relevant health information that develops in donors, their families, and donor-conceived children long after donors have left the program.

Prospective genetic screening and heritable risk mitigation comprise the following steps:

1. Carrier screening for monogenic, recessively inherited diseases.
2. Complete blood count and metabolic panel.
3. Physical exam.
4. Upper age limit for donation.
5. Genetic counselor-conducted three-generation family history.
6. Karyotyping (optional).
7. Hemoglobin electrophoresis.

ASRM and SART practice committee guidance [17], FDA requirements [15], and New York State law [21] all include provisions for genetic screening and management of adverse outcomes reported, though stated objectives are relatively high level and not prescriptive enough to form the basis of a thorough genetic risk mitigation strategy. Much of the interpretation of information collected from donors requires interpretation and clinical judgment; thus, it is strongly recommended to rely on professionals with reproductive genetic training to implement and execute a genetic screening program.

Carrier screening is currently performed using next-generation sequencing technology, which allows low-cost, high-throughput identification of sequence variants across many genes simultaneously [22]. In consideration of the improvement in technology and ubiquitous access to large carrier screening panels, the American College of Medical Genetics (ACMG) updated their recommended screening for autosomal recessive and X-linked conditions in 2021 [23]. A primary review of this publication is highly recommended to

ensure that the testing panel used on sperm donors meets society criteria for analytical and clinical validity.

With the nearly ubiquitous adoption of pan-ethnic expanded carrier screening (ECS) panels screening sperm donors for hundreds of monogenic disorders, a few meaningful changes in sperm bank practice are worth noting. First, these panels are run on all potential donors regardless of ancestry and ethnicity, a departure from prior risk-based testing criteria that helps overcome the shortcomings of inaccurate ethnic heritage self-reporting. The second change involves a movement from exclusion testing [24], where all carriers of certain conditions like cystic fibrosis were disqualified from donation, toward acceptance of heterozygous carriers for recessive conditions. In theory, if the person contributing the egg screens negative for a condition the sperm donor is a carrier for, there is no meaningful risk to offspring to inherit this condition. In truth, there is always measurable, albeit very small, residual risk for inheritance given limitations in analytical sensitivity; however, the risk is orders of magnitude lower than procured by historical small carrier screening panels (or no such panel at all). Donors who carry mutations for certain genes, such as ataxia telangiectasia mutation (ATM), fumarate hydratase (FH) deficiency, and Nijmegen breakage syndrome (NBS), may be considered for excluded from donation as these genes have been associated with risks for certain manifesting conditions even in heterozygotes, thus posing risk to 50% of offspring [25]. It should be noted that the list of specific genes associated with these so-called expressing carrier states, and our understanding of their actual risk, is evolving, therefore recommended management may be updated since the time of this publication.

In circumstances where the egg contributor does not or cannot receive complementary testing to their donor, the intended parent can still be advised on risks of inheritance by multiplying incidence of the condition in the untested person by the 25% likelihood of homozygous inheritance of the disease in question to make an informed decision on the risk for inheritance. Reference labs should provide the population incidence for every condition on their carrier screening panel to inform this calculation.

ECS is not a replacement for a thorough, three-generation family history review, ideally conducted by a trained genetic counselor. Genetic professionals are trained to construct a pedigree and elicit a more detailed history than can be gathered by a family health questionnaire. Understanding patterns of inheritance, variable expressivity and penetrance, and genetic variants are key to interpretation of risk to offspring [26]. The family history review is useful to identify major Mendelian disorders that may be passed on to offspring, such as von Willebrand's disease or Ehlers-Danlos syndrome. The review also identifies risk factors for multifactorial/polygenic disorders such as learning disabilities,

mental health disorders, or autism spectrum disorder. Family history review may reveal increased risk for heritable risks like familial hyperlipidemia or heritable forms of cardiovascular diseases.

Metabolic panels and complete blood count are performed as proxies for general health but are also useful tools for identification of potential hereditary conditions such as familial hyperlipidemia or hemoglobinopathy. Critical values should be flagged for review by the medical director and ultimately triaged under their clinical supervision for follow-up and management.

Current guidelines recommend hemoglobinopathy carrier screening by complete blood count, with follow-up hemoglobin electrophoresis or fractionation based on abnormal result or ethnicity; however, advances in molecular genetic testing are thought to increase sensitivity of carrier detection. A retrospective analysis of a multiethnic population of men who underwent screening for hemoglobin disorders via complete blood count, hemoglobin fractionation, and molecular testing found that 68% of carriers identified on molecular testing were missed by hemoglobin electrophoresis and currently recommended methods [27]. No identified carriers were detected by recommended methods but missed by molecular testing.

The physical exam is an FDA requirement for the evaluation of evidence for high-risk behavior or infectious disease; however, it can also be a useful adjuvant screening tool for certain inherited disorders. Example signs of genetic disease identifiable by physical exam include pectus excavatum or hyper-elasticity suggestive of connective tissue disorder, café au lait macules suggestive of neurofibromatosis type 1 (NF1), and depigmentation suggestive of vitiligo.

The use of sperm from donors who are above the age of 40 years has been correlated with increased incidence of genetic diseases such as autism spectrum disorder [28]; therefore, an upper age limit for sperm donors is recommended by many groups, including the ASRM [17].

Balanced structural chromosome abnormalities are estimated to occur in 0.2–0.5% of healthy individuals in the general population and are associated with an increased rate of miscarriage, and structural and developmental birth defects in offspring [29]. There are limitations of karyotype screening when read on the average 550–600 band level, as clinically meaningful cryptic chromosomal abnormalities may be read as normal. ASRM guidelines indicate karyotypic analysis as an optional screening tool [17]. Sperm banks must weigh the cost-benefit ratio for testing, knowing that karyotyping will identify up to 0.5% of men with structural abnormalities, theoretically preventing some adverse outcomes in pregnancies achieved with their sperm, with a high cost of testing which is only performed by a limited number of reference laboratories.

Genetic risk assessments must be performed on sperm donors on an ongoing basis, even after men have stopped donating and have left the program. Sperm banks should pro-

vide a receptive and efficient mechanism for the reporting of new medical issues arising in offspring, donors, or their family members. If new information suggests a significant risk for previously unidentified medical issues in donor-conceived offspring, distribution of that donor's vials should be restricted and relevant client populations notified. Investigation of the clinical significance of reported information can be a time-intensive process, involving the cooperation and consent of the informing party and typically requiring review of special genetic testing and medical records.

The genetic origin of Mendelian disorders in donor-conceived offspring may be readily identified through evaluation of medical records and genetic testing. Though notification to donor-conceived families may not be a pleasant task, the clinical risk assessment and management of information are typically straightforward. Greater advancement in molecular testing available in the workup of children with clinical findings brings greater complexity for sperm banks' adverse outcome management. Sperm banks should be prepared to receive information such as whole-exome sequencing reports performed on symptomatic children with only variants of undetermined significance detected. One case study [30] perfectly exemplifies the complexity and unique aspects of sperm banking, describing the discovery of NF1, an autosomal dominantly inherited condition, arising in two donor-conceived offspring related only by their sperm donor. Conventional understanding of Mendelian inheritance suggests that this condition would have been clinically detectable in either biological parent or would have arisen as a *de novo* mutation in the embryo. These cases were ultimately attributable to gonosomal mosaicism (Fig. 21.2) in the sperm donor, detected in 20% of sperm cells only after

vials were distributed to families. Without the unique constitution of a sperm donor-conceived "family tree," there would be no reason to suspect mosaicism; however, this phenomenon clearly has high relevance to the management of genetic information that sperm banks collect.

21.4.4 Psychological Assessment

Today, third-party reproduction is a thriving subspecialty area within the mental health profession. A vast body of academic literature has helped shape and improve clinical practice and sperm banks' approach to third-party conception. Evidence resoundingly supports the finding that donor-conceived children bond equally well with non-biologic parents, thereby advocating for disclosure of donor conception to both children and community and documenting the importance of donor-conceived people to know their biological origins [31]. This professional group has informed the modern approach to psychological screening for sperm donors, which includes assessment for mental health and genetic psychiatric risk factors and serves as a key opportunity to optimize long-term outcomes for donors and donor-conceived families through donor education and informed consent. The psychological assessment is an educational session, proactively assessing potential donors' understanding of the long-term implications of donation, their comfort with identity disclosure, and alignment with the sperm bank's expectations for altruistic motivation, commitment, and maturity.

The psychological screening should be performed by trained mental health professionals, ideally those who receive continuing education from industry special interest

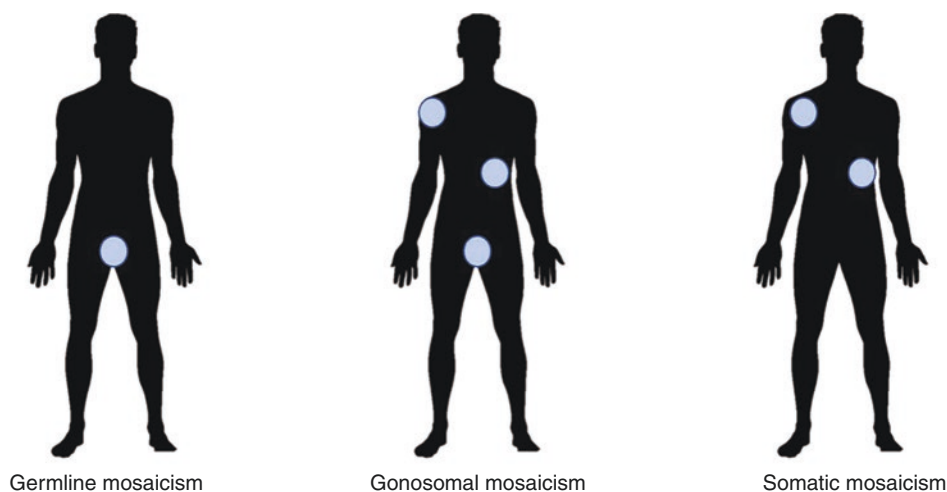


Fig. 21.2 Inherited genetic mutations that first present in donor-conceived offspring may be attributed to mosaicism in the sperm donor. Genetic evaluation for mosaicism must take into consideration the three variations pictured here. Donors may be gonadal mosaic, in which a percentage of sperm are affected by the mutation but will not be

detected in the somatic cells, gonosomal mosaic, in which the mutation affects a subset of both germline and somatic cells, or somatic mosaic, in which the mutation affects a subset of somatic cells in a mosaic pattern. Number of children affected is typically proportional to the rate of mosaicism

groups, such as the ASRM Mental Health Professional Group [32]. Thorough psychological screening comprises two components and should endeavor to standardize as many elements as possible, particularly when multiple locations and professionals are participating in a donor screening program.

One element of psychological screening is assessment with a standardized psychometric test, such as the Minnesota Multiphasic Personality Inventory (MMPI) or the Personality Assessment Inventory (PAI), designed to assess respondents' [personality](#) and [psychopathology](#) on multiple clinical scales, expressed as a function of standard deviations from large measured populations. Criteria for suitability should include testing within established ranges of standard deviations from the mean. These tests should only be administered and scored by professionals who have received specialized training in their use and interpretation, as the findings must be interpreted in the context of validity indices as well as those gathered from the second element of psychological screening, the clinical interview.

The clinical interview should feel like a natural conversation between applicant and interviewer, rather than follow a regimented set of questions. However, a standardized clinical interview guide can be a very useful tool to ensure that key elements of screening are covered. Suggested areas to cover through this conversation include [33] openness to family about donation and reactions of the applicant's support network, feelings about donor compensation, romantic relationship history and current partner's perceptions of donation, history of personal or family mental health challenges, chemical dependency, and feelings toward identity disclosure to the donor-conceived child. This is by no means an exhaustive list but meant to illustrate this interview as a key tool that sperm banks can use to select donors who are psychologically healthy and committed for the long-term implications of gamete donation.

When performed correctly, the psychological screening step should be expected to disqualify several candidates either based on a recommendation by the mental health professional or due to donor self-deselection [33]. Given the challenges of donor recruitment, it may seem "bad for business" to introduce a screening step which is not a regulatory requirement and leads to disqualification of several applicants; however, the short-term frustrations of losing potential donors are ultimately outweighed by long-term benefits to families and donors and ultimately the sperm bank.

21.5 Donor Identity Disclosure

Advancements in technology and increased access to donor sperm have facilitated significant social progress in family building and shaped societal norms around the definition of

family. Reciprocally, the increasing use of sperm and egg banks to build families has stimulated further evolution of the approach to operating sperm banks. Perhaps the greatest example of this interconnected progress has been in the shift from anonymous donors toward "knowable" donors or "identity disclosure" donors.

The first documented use of sperm donation that resulted in a live birth occurred in the late nineteenth century in Philadelphia and was facilitated by Dr. William Pancoast [1]. When a couple sought medical assistance after failing to conceive naturally, the doctor determined that the issue was azoospermia in the husband. The most attractive medical student was selected as an anonymous donor, and an insemination was performed while the wife was under anesthesia. The husband was told about the use of the donor later and was reported to have been pleased that his wife was pregnant, but he asked that the wife not be told about the use of a donor. While such an act today would be seen as egregiously unethical, it is important to consider the motivations of the physician and husband within the appropriate historical context.

Society and the law have long considered genetics to be central to kinship and therefore central to the determination of parenthood [1]. Sperm donor-conceived children were ostensibly being protected from illegitimacy through the secrecy of their conception and anonymity of their donor.

The prevailing belief that sharing sperm donor origins with a child could threaten the bond between the child and the non-biological male parent persisted well into the 1990s. Sperm banks promised anonymity to donors because the entire social contract was predicated on the fact that donor conception disclosure was highly discouraged by medical professionals and society for decades. In the traditional medical model, the inseminating doctor was often the sole decision-maker, frequently selecting the sperm donor for an infertile couple, who were passive participants in the building of their families through gamete donation. People were told to go home and forget about how they achieved pregnancy.

As the structure of the family has changed over the past few decades and options for family building have grown, the role of the intended parents has evolved from passive patients to informed consumers [1]. Academic study of donor-conceived kids' mental wellness and concurrent increasing LGBTQ (lesbian, gay, bisexual, transgender, queer) and single-mother use of donor sperm has provided insight into the belief that donor-conceived people are socially well-adjusted members of society, who deserve not only to know that they are donor conceived, but also to learn as much about their biological origins as those who are raised in a two-biological parent family [31, 34].

The online space *WeAreDonorConceived.com* conducted a survey in 2020, in which 481 donor-conceived people ranging in age from 13 to 74 participated [35]. Most participants

were between 20 and 40 years old. 89% of respondents indicated that the donor used in their conception was anonymous, with no identity disclosure agreement. When asked about the method of their conception, 70% of participants strongly agreed or agreed with the statement “the method of my conception sometimes causes me to feel distressed, angry, or sad” [35]. Of note, nearly 80% of respondents were raised primarily by heterosexual parents, in stark contrast to the nearly 75% LGBTQ or single-parent families who use sperm banks today (Fig. 21.3).

Regardless of the timing of discovery that they were donor conceived, findings among donor-conceived individuals are similar as to feelings about donor anonymity and the importance of knowing the donor and their donor siblings. With respect to identity, one study by Harrigan et al. [36] found that those who expressed a desire to contact their donor were strongly driven by the quest for identity. Ninety-two percent of those surveyed agree with the statement “The ART (Assisted Reproduction Technology) industry has a responsibility to act in the best interest of the people it helps to create.”

Insight that the promise of anonymity is unrealistic has been growing for at least a decade; however, sperm banks have been concerned that they would not be able to recruit enough donors to meet consumer demand if the option was removed [37]. A 2016 study by a US sperm bank reported that 29% of current anonymous sperm donors would refuse to donate if the law changed such that they were required to put their names in a registry available to donor-conceived children at age 18 [38].

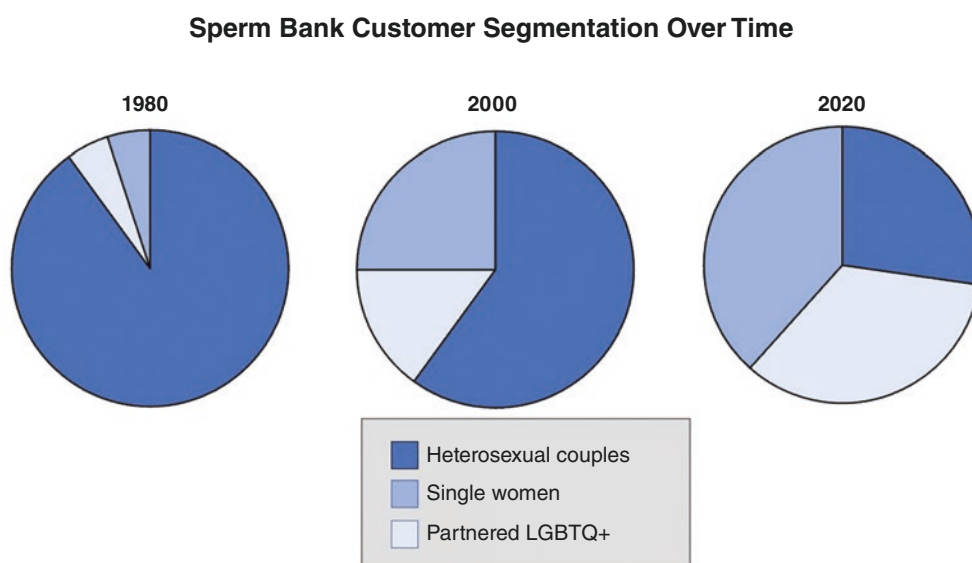
Within 3 years of that study’s publication, a Consumer Reports survey reported [39] that one in five American had

taken a direct-to-consumer (DTC) genetic test. Many of these tests led to unexpected discoveries of biological parenthood, including donor conception. When asked about their discovery of their donor origin, a third of donor-conceived people indicated that they learned the truth as the result of taking a commercial DNA test. A large proportion of individuals who knew they were donor-conceived have taken a DNA test intentionally to learn about their biological origins [35]. The age of the Internet made finding sperm donors possible; however, DNA testing has effectively ended any illusion of donor anonymity. A new era of understanding of what sperm banks can truthfully represent to donors and donor-conceived families regarding anonymity has arrived.

In 2016, California Cryobank changed its policy to require all new sperm donors to agree to ID disclosure status, meaning that their offspring would have access to their donor’s identity when they turn 18 years of age [4]. Other sperm banks have followed suit. After counseling about this requirement, 14% of candidates self-selected out of the California Cryobank program, which is substantial, but not nearly as significant as the 29% predicted by earlier studies [38]. Perhaps it should not be surprising that these men, who identify as Millennials or Gen Z, who grew up with the Internet and social media are less concerned about their identity being discoverable online.

A key message for all parties—the donors, insemination recipients, donor families, and donor-conceived people—is that respect for privacy is essential to optimize the outcomes for the many people who are connected through the donor ecosystem. Professionals like the mental health team help support all parties through education and reinforcement of these values.

Fig. 21.3 The profile of a major US sperm bank client has changed significantly over the last 30 years. As fewer heterosexual couples require sperm donation to conceive, there has been a significant concurrent increase in the number of single parents by choice or people who openly identify as LGBTQ+ seeking sperm donor services. As of 2020, approximately 75% of clients comprised the latter two groups, with the share growing year over year. *Source: California Cryobank internal marketing data*



21.6 Family Unit Management

People tend to agree that it is a good idea to limit the number of offspring conceived from one sperm donor, whether to prevent accidental consanguinity or, far more practically, to mitigate the consequences of a previously undetected genetic condition affecting too many children.

Often, heritable disease cannot be detected prior to reproduction. Conditions can manifest for the first time in offspring, at a rate determined by several genetic factors (e.g., autosomal dominant, recessive, or polygenic inheritance, penetrance, mosaicism) multiplied by the number of children conceived. Sperm banks should not only limit numbers of family units, but they must also serve as the repository of information and expertise to ensure that a genetic finding in one child does not warrant notification to other families or, rarely, suggest that nobody should use this sperm again given the implicit risks.

US sperm banks have shared policies that typically target a maximum number of 20-30 family units from each donor. In practice, they report an average of around 18 family units per donor [4]. These targets have not changed for decades, although the actual number of vials that can be shipped to remain compliant with policies continues to decline, thanks to advances in IVF technology and the improved efficiency of achieving pregnancy.

The UK and Australia are examples of countries where the law specifies the maximum number of offspring that can result from a single sperm donor [40, 41]. US sperm banks who export to these countries must be compliant with these laws, which include having a system to manage the outcome of insemination data and limit vial distribution. Compliance is facilitated by the infertility practices in the UK and Australia who are registered with their government and licensed to facilitate donor insemination.

One can debate the merits and practicality of aligning US regulations with the more conservative governments like the UK and Australia; however, such a shift in requirements would require an arguably insurmountable change in how American clinics and donor recipients currently practice and would likely lead to adverse unintended consequences. A full discussion of these regulatory considerations is beyond the scope of this chapter; however, a discussion on this subject can be found following [here](#) [42].

Practically, all sperm banks should be highly incentivized to limit the number of children who result from one man's donations. As discussed in the section on genetic screening, awareness of a mosaic donor resulting in a subset of children who inherit NF1 should illustrate the point.

21.7 Long-Term Management of the "Donor Ecosystem"

Stating the obvious, sperm banks exist to help create babies and grow families. Unlike the doctors and other professionals who participate in this baby-making, the sperm bank has a relationship with the donor and the donor-conceived family that endures for generations. When things go well, which thankfully most of the time they do, people are immensely grateful to the sperm bank and their donor. However, as illustrated in the genetic screening section, occasionally adverse outcomes occur and must be quickly and responsibly managed.

Donor-conceived babies grow into adults, and even the most well-adjusted ones may want to learn more about their donor. Most of the time, when facilitated by the sperm bank, donors are happy to engage on some level to satisfy the biological queries of their donor-conceived offspring. Sperm banks should go to great lengths to support all parties involved in this communication. However, donors do not always respond the way we hope they will. We predict that this will improve as more psychologically screened and properly prepared donors' offspring come of age; however, it is still heart-wrenching to deliver the news that a donor does not want to respond to the well-intentioned inquiry of a person who shares half their DNA.

The take-home lesson here is that knowing adverse outcomes are inevitable, a sperm bank must establish practices that meet or exceed the standard of care and endeavor to reduce risk through rigorous and standardized processes. The other important lesson is that sperm banks cannot solely rest on the achievement of standard of care. The author would argue that sperm banks must be deeply connected to the human element that is the center of their reason to exist. Building families requires empathy for all the humans involved in this process. Management of a truly successful sperm bank means fostering an environment where employees are connected to the mission and will help the organization continually grow and evolve, along with the thousands of families it serves.

21.8 Conclusion

Like any business, the sperm bank must anticipate and meet their customers' evolving needs to survive. To thrive, the modern sperm bank must do more. Today's sperm bank is many things: a consumer-driven business, a health care service, and a regulated tissue bank. Sperm banks may additionally serve as facilitators and advocates for human rights

issues such as family equality, privacy, and the right to know one's biological origins. While it may not be possible to please every customer, donor, and donor-conceived person, being mindful of these three parties' unique perspectives, interests, and needs is an excellent guiding principle. Establishing best practices for sperm bank management should be balanced and always serve to protect the health and safety of all parties involved in the donor ecosystem.

Authors' Perspectives

Key Points

- Fertility clinics and sperm banks hold a tremendous amount of responsibility in facilitating family building through sperm donor conception. This does not mean that sperm banks and clinicians can promise families a risk-free procedure nor be considered liable in the case of every adverse consequence of human reproduction. Quite the contrary, the industry should take great care not to sell families and donors an impossible promise. The key message is that these institutions are at the center of the "donor ecosystem," with the power to recruit, screen, and inform donors through transparent, clinically robust, and ethical practices. These institutions cannot be expected to predict every way that further evolution in society and science will further change sperm donor conception; however, the abundant information available today should inform socially, clinically, and ethically responsible choices that serve to optimize outcomes for all parties involved in donor conception.

Potential Areas of Research

- Predictive molecular markers that improve assessment of sperm health beyond light microscopy semen analysis.
- Value of familial cancer screening panels in a low-risk sperm donor population.
- A follow-up with psychologically screened "ID disclosure" donor's attitudes toward donor-conceived person communication when offspring reach majority age.

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Maintenance and Management of Cryopreserved Samples and Cryotanks in an IVF Laboratory

22

Kimball O. Pomeroy and David E. Battaglia

22.1 Introduction

Storage of cryopreserved human reproductive tissues began decades ago with the freezing of sperm samples for patients or sperm donors. This was often performed in outpatient clinics or research laboratories where there was little oversight or management of storage conditions. With the advent of effective cryopreservation of human embryos in the mid-1980s, in vitro fertilization clinics began to routinely store these embryos for patients wanting to save their extra embryos for later embryo transfer. At this time, accreditation standards for IVF labs were being developed which advocated for the need for oversight and effective quality control procedures for maintaining safe storage.

The introduction of highly effective vitrification methods for “freezing” these embryos resulted in a dramatic increase in the demand for embryo cryopreservation. Patients increased the use of this service for their extra embryos since survival rates for thawed embryos were vastly improved. Expanded use of embryo biopsy with cryopreservation for preimplantation genetics and fertility preservation also resulted in greater need for cryopreservation. Vitrification also offered a very effective way to preserve oocytes for later use. Thus, the amount of cryopreserved reproductive tissues has increased dramatically in clinics worldwide. The management of safe and effective storage has become paramount, and IVF laboratories take these responsibilities very seriously.

Effective storage of cryopreserved tissues requires an ultralow temperature environment and specialized equipment. The required storage temperature is achieved using liquid nitrogen (LN2), which exists at a temperature of -196°C . The devices containing the tissues (embryos,

oocytes, or sperm) are best stored immersed in LN2. The most common storage method utilizes LN2 dewars (akin to large-vacuum thermos) that are filled with LN2. Most laboratories have multiple dewars which are each capable of storing hundreds of samples. Due to evaporation of the nitrogen, the optimal LN2 levels must be restored manually when they approach the lower level for effective storage. Very large storage systems are capable of handling thousands of samples. These systems utilize electronic methods for monitoring LN2 level and automated refilling.

Clearly, the importance of vigilant oversight and management is essential to meet the expectations of the patients who are extremely vested in safe storage, both emotionally and financially. While no system of cryostorage management is 100% fail-safe, many methods of oversight and quality control have reduced the risks to extremely low levels. In 2018, two separate incidents occurred in unrelated clinics where proper cryostorage conditions failed and hundreds to possibly thousands of embryos were destroyed [1]. These events have raised awareness within the IVF community and the public about the consequences of errors in cryostorage oversight. In both incidents, large cryostorage systems were in use, but technical malfunctions and human error were involved that jeopardized the safety of the stored embryos and oocytes.

22.2 Risks and Managing Risks with Off-Site Storage

Clinical IVF laboratories have an enormous liability in storing gametes and embryos. This has been vividly pointed out in the recent court case of five plaintiffs against Pacific Fertility Center, Prelude, their parent company, and Chart Industries, Inc., the manufacturer of the tank. On June 11, 2021, a jury awarded the plaintiffs almost \$15 million when a tank’s vacuum seal failed at a fertility clinic. Chart Industries Inc., the manufacturer of the tanks, was found 90% responsible, and Pacific Fertility Center was found 10%

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responsible for the egg and embryo losses (“Jury Awards \$14.975 Million in IVF Tank Failure Trial,” 2021). Three women in the California case were each awarded between \$2 million and \$3 million, and a couple was awarded \$7.2 million. Hundreds of other patients also have claims pending against Chart and Pacific Fertility Center, and additional trials are scheduled. Publicized cases though are just the tip of the iceberg. What about those that never reach the public?

A study by Letterie and Fox searched past court dockets in the United States to find any suits related to cryopreservation accidents [2]. The authors examined dockets from January 1, 2009, to July 1, 2019, almost 10 years. They found 133 cases—122 in state court and 11 in federal court. These cases were sorted into one of these categories—lost or misplaced in laboratory, lost or misplaced in transit, damaged or destroyed through mishandling, damaged or destroyed through miscommunication, and damaged or destroyed through storage tank failure.

Tank failure made up the largest number of cases. Most of the 111 cases originated from two separate incidents that occurred in early March of 2018, one in California and the other in Ohio. In both situations, the nitrogen level in a storage tank dropped, causing the frozen embryos and ova to possibly warm and lose viability.

When one examines all of these cases, most claims included breach of contract, bailment (improper property transfer), and negligence (failure to meet the standard of care). Just two other claims appeared in a substantial minority of cases: breach of fiduciary duty (37.1%) and conversion of personal property (28.6%). Of these 133 lawsuits, 90 are closed and all but two were settled out of court (97.8%).

The authors mention that “medical malpractice claims require showing that patients were harmed in physical or economic ways. These showings are hard to make in claims for embryo loss. In terms of liability risks, practitioners may do better to invest and insure against contract and property claims associated with storage malfunctions.”

One of the claims filed against University Hospitals in Cleveland warrants special mention: in addition to their negligence claim, the plaintiff couple sought a legal declaration that their lost embryos should be given legal standing as persons, sufficient to let them sue for wrongful death [3]. The Cuyahoga County Court of Common Pleas dismissed their case without giving a reason or explanation about whether any settlement was reached. The couple said that they would appeal their claim to Ohio’s Supreme Court. This means that if the ruling is overturned, defendants could be found guilty of manslaughter.

These recent high-profile cases show that juries will return high judgment amounts against IVF clinics that do not take care of their gametes and embryos. Clinics and labora-

tories should rethink their systems and spend new effort and capital in safeguarding these precious tissues.

Because of the liability in tissue storage, some IVF clinics have taken advantage of commercial, off-site tissue repositories. This may provide some protection from liability, but it does not remove all liability and there are some additional issues with using an off-site storage facility. For off-site storage, the IVF clinic will usually provide short-term storage of patient tissue for 6 months to a year, during the active part of the patient’s treatment cycles. After a fixed amount of time or once a pregnancy has occurred, the tissue will automatically be shipped to the commercial tissue repository. The patients sign a contract with the repository, but the clinic may still have some liability during the short-term storage, during the multiple shipments back and forth from the repository to the clinic, and during the storage at the clinic prior to transfer.

Two major problems with this method are that (1) the tissue undergoes more shipping back and forth than were it to remain at the laboratory and (2) there is an increase in workload for the laboratory due to the multiple shipping events to and from the clinic to maintain proper inventory records and in adding and removing tissue from inventory. Some of this workload is offset by time savings in tank monitoring as well as carrying out final dispositions. IVF clinics need to weigh the benefits of on-site and off-site storage against the risks of each.

22.3 Location of Tissue Repository

The location of cryopreserved specimens in the IVF laboratory can vary greatly depending on the size of the lab and its budget. The storage system can also vary from a few LN2 dewars situated within the IVF lab itself to much larger scenarios with a multitude of LN2 dewars or large-scale automated systems.

If a few dewars are present in the working footprint of the IVF lab, they should be located in an area with minimal foot traffic so as not to be inadvertently bumped into. If room allows, the LN2 supply tanks for the dewars should not be kept in the lab due to continuous outgassing of pressurized LN2 and the danger this presents of asphyxiation. They should be located in a space adjacent to the lab and fit with oxygen monitors and alarms. The dewars should each be equipped with an electronic alarm (see Sect. 22.5) for continuous monitoring of liquid nitrogen levels. In this situation, the refilling of the dewars with LN2 when levels are low can be problematic. Either the dewars need to be moved to the supply tank to be topped off, which poses risks by possibly damaging the dewars, or the dewars can be topped off in the lab by bringing in insulated pitchers filled with LN2 from the supply tank.

A more ideal situation is having storage dewars or large automated systems located in an adjacent room. This offers a convenient location for placing new specimens into the tanks or for retrieving specimens to be warmed. This location also offers close proximity to lab staff for monitoring LN2 levels and the integrity of the storage tanks. This room should ideally have separate ventilation and a monitor/alarm for oxygen levels. The LN2 supply tank(s) should be located in an adjacent room with an insulated pipe and valve assembly that permits LN2 to be dispensed through a wall and directly into the repository space. This separate space for LN2 supply tanks prevents their release of nitrogen vapors into the working lab space and provides a convenient location for delivery of full tanks. Such spaces can also be used to house CO₂ tanks for IVF incubators and O₂ tanks to supply the surgical suites in the clinic. Such a location for the repository also offers space to manage multiple alarm cables (if the alarm system is not wireless) and alleviates inadvertent jostling of the dewars. A separate space like this also offers greater flexibility for the addition of more dewars to expand capacity than housing them in the IVF lab itself.

22.4 Tanks

Two types of tanks are used in reproductive medicine—tanks for shipping tissue (which meet the International Civil Aviation Organization technical instructions for safety when shipping in a plane) and storage tanks. Storage tanks can be divided into both small- and large-capacity storage tanks and then into liquid nitrogen or nitrogen vapor tanks.

Shippers are relatively small tanks that can be filled with 1–5 L of liquid nitrogen. Prior to shipping though, the tanks must be inverted to remove all free-liquid nitrogen. An absorbent material inside of the shipper will sequester some of the liquid nitrogen so that should the tank fall over or even be turned upside down, the liquid nitrogen will not spill out of the tank. The most common shippers in the United States are the MVE SC 2/1V and SC 4/2V (Chart Industries, manufacturer). The SC 2/1V holds about 1.5 L, and the SC 4/2V holds about 3.5 L of liquid nitrogen. Once the tank has been filled with liquid nitrogen, it is left to be absorbed by a hydrophobic material. These tanks are one of the most common to fail, and when they fail, there is little time for recovery. If the tank fails during shipping, there is usually no opportunity for recovery as the tanks are usually not accessible for real-time monitoring or refilling during shipping. One study indicates that the liquid nitrogen evaporation rate of an intact SC 4/2V goes from 0.15 L/day to 11.3 L/day if the vacuum of the tank has been compromised [4]. This means the static holding time for the tank goes from 22.5 days to about 4–6 h.

There are two major types of standard storage tanks, small-capacity tanks which contain less than 70 L of liquid

nitrogen and large-capacity tanks which often contain hundreds of liters of liquid nitrogen. Large-capacity tanks can be further divided into liquid nitrogen or nitrogen vapor tanks. Large-capacity tanks are constructed of steel, while small-capacity tanks are constructed mainly of aluminum. Storage tanks contain a tank-within-a-tank design where the inner tank is where the liquid nitrogen and the actual tissue are stored, and the outer tank provides an insulative layer between the inner tank and the outer environment (see Fig. 22.1). In smaller tanks, these two tanks are connected by a fiberglass-like neck, which is glued to the inner tank and the outer tank (see Fig. 22.2). It is this gluing of two different types of materials, the neck to the aluminum, that is the weak point of the tank and is often the source of failure. When a crack develops in this glued area, the insulating vacuum that decreases evaporation of liquid nitrogen is lost and evaporation rates increase up to 100-fold [4]. Another weakness of these small-capacity tanks is that the entire weight of the inner tank (which can be as much as 100 pounds due to liquid nitrogen and inventory racks) is suspended from this neck. When the tank is tilted or jostled, it can put enormous strain on the weld and result in the failure of the vacuum. Large-capacity tanks on the other hand are welded steel to steel, and they are so large that they are difficult to move during routine use.

In the liquid nitrogen version of the large-capacity tank, the specimens are stored in the liquid portion of the liquid nitrogen. Often, there is an automatic refill system to maintain the liquid nitrogen level of the tanks so that it never drops below a certain level. These automatic refills can fail though in either the open or the closed position and are the biggest weakness of this system. The automatic refill system can also make it difficult to monitor liquid nitrogen evaporation rates, which are crucial to determining the integrity of

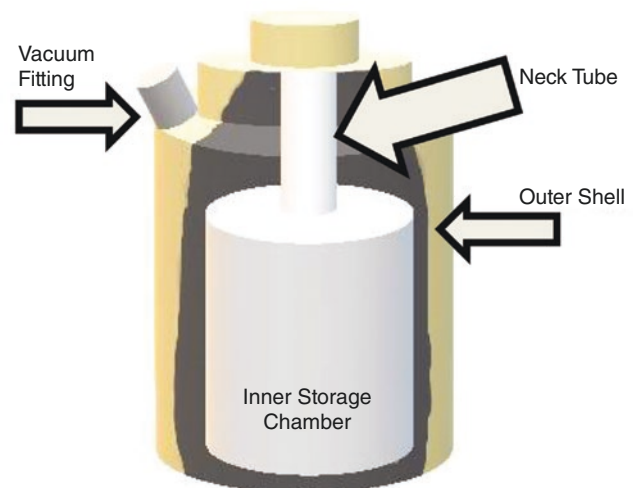
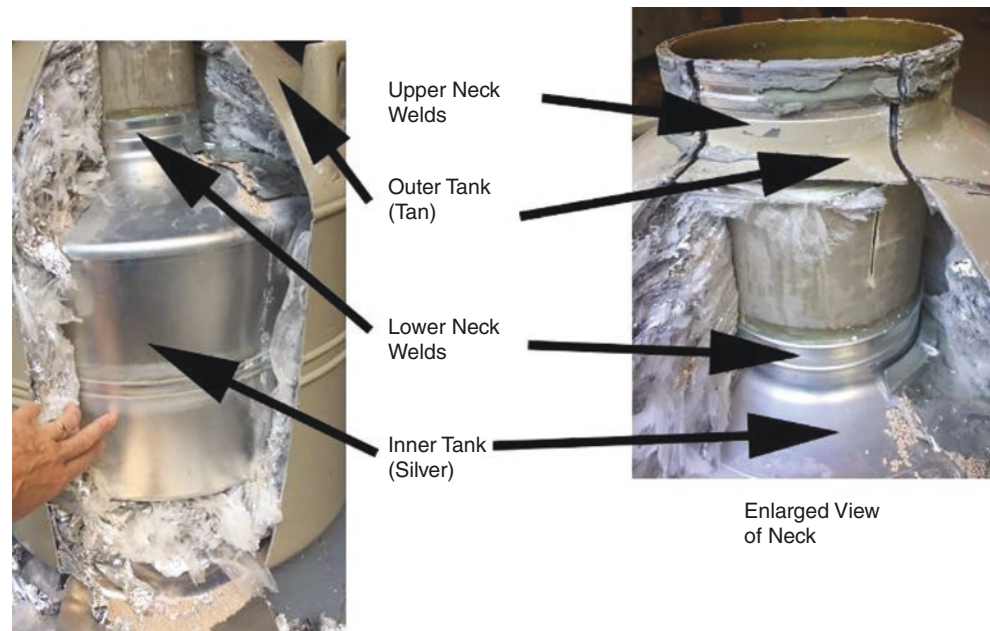


Fig. 22.1 Diagram of typical small-capacity tank showing the vacuum port, the tube neck, and the tank within the tank

Fig. 22.2 Cutaway view of a small-capacity tank (MVE 47/11). The lower and upper neck welds are the most common sites of vacuum failure. (Photo courtesy Dawn Kelk)



the tank. Vapor tanks possess a small area where liquid nitrogen is stored, but the specimens are stored in a separate area where the tank is cooled down by conduction and the specimens are never in direct contact with the liquid nitrogen. It is felt by some that vapor tanks will keep the specimens safer from the potential of cross-contamination of microbes in the tank, while others feel that avoiding direct contact of the specimen devices and liquid nitrogen makes them inherently less safe. There is little evidence of cross-contamination risk though from the scientific literature [5].

Almost all tanks used today have been developed mainly for the cattle industry for use in storing semen and embryos. Not until recently has a tank been developed specifically for the human reproduction industry (Abeyance storage tanks).

22.4.1 Validation of a New Tank

Validation of a new tank is a simple but time-consuming process that involves four steps: (1) visual inspection of the tank, (2) recording of base measurements, (3) determining the tank's liquid nitrogen evaporation rate, and (4) determining the tank's time from alarm to it reaching either a critical temperature or liquid nitrogen level. Although weight-based methods are described below, one can use a calibrated dipstick to perform the same measurements. The weight-based methods will be more accurate.

22.4.1.1 Visual Inspection

Check the entire tank for imperfections, cracks, or discoloration. Pay particular attention to the area around the vacuum port.

Use a pen light to examine the interior of the tank. Pay particular attention to the neck welds.

Examine the top of the tank for a secure fit and tight attachment of the cork to the lid.

22.4.1.2 Base Measurements

Record the tank's serial number and date of manufacture.

Write on the tank the date it was put into use.

Weigh the tank with its inventory rack and lid. Record this on the side of the tank as its empty weight with the date.

Record the weight of the lid by itself.

Fill the tank with liquid nitrogen just so that when the lid is placed, the nitrogen reaches the base of the cork but does not spill out. Record this as the tank's full weight with the date. For a shipper, fill as indicated for preparing for a shipment. After pouring off the liquid nitrogen, record this as its full weight. Just prior to shipping, the tank's full weight should be compared to this number to ensure that it is completely full.

22.4.1.3 Liquid Nitrogen Evaporation Rate (Liters Per Day)

1. Place an inventory rack into a tank, and fill it with liquid nitrogen.
2. Allow it to sit overnight.
3. Refill the tank the next morning, but not to overflowing, and place the full tank on a scale.
4. With the lid on, record the time and the weight of the tank.
5. Record the weight of the tank and the hours since the initial weight every 24 h for 2–4 weeks.

6. Calculate the mass evaporation rate by dividing the number of days into the loss of weight for this time period. This is the base “mass” evaporation rate for that tank and should be recorded.
7. Convert this weight to the “volume” evaporation rate by converting the mass of liquid nitrogen to the volume of liquid nitrogen. This is accomplished by dividing this number by the density of liquid nitrogen (0.8064 g/ml). This number should be close to the static evaporation rate stated by the manufacturer.
8. This evaporation rate is the “static” evaporation rate, and the actual rate will be slightly higher due to the inventory rack and increased evaporation from accessing the tank periodically.
9. One should compare this static evaporation rate to its working rate by measuring either its weight or the height of the liquid nitrogen weekly while it is being used.
10. During these initial measurement periods, the tank should be observed twice daily for any frosting of vapor on its side, hissing, or other strange noises.
11. As a test for the integrity of a small-capacity tank’s welds, the tank can be raised about 6 in. off the ground and dropped. Another evaporation rate should be measured to see if there is a difference after the drop test, indicating a potential vacuum leak. For obvious reasons, there are some that feel drop testing may be damaging to the tank and should be avoided.

22.4.1.4 Measuring Response Time (Hours)

1. Set a liquid nitrogen level probe at the top of a cane (or any other height that is determined to be a critical height below which you do not want the nitrogen level to fall), and place it in the nitrogen tank.
2. Add liquid nitrogen to the tank so that it is just above the level at which a trigger would occur.
3. When the tank alarms, measure the time from the initial alarm until when the nitrogen level alarm goes off. This is your response time.

An alternative way to set up the alarm is so that the temperature does not go above a critical temperature, -130°C , at a particular point in the tank (say the top of a cane). One would then substitute the nitrogen level probe for a temperature probe. It should be placed at the top of a cane, and the time should be measured from alarm to when the temperature probe records -130°C . For a 47/11 tank, it has been shown that the temperature at this point is not reached until the tank is nearly empty of liquid nitrogen [4].

22.4.2 Retiring Tanks

Most tanks come with a warranty of about 10 years. This guarantee though does not indicate the useful life of the tank. For example, a microscope that is warranted for 1 year is not expected to last just one year, but the one year is the risk the manufacturer accepts for free repairs or replacement. Especially in the current situation where there are questions regarding the quality of some newer tanks, as evidenced by manufacturer recalls [6] and failures of almost new tanks (Pomeroy et al., unpublished), decommissioning a 10-year-old tank that is currently working for a new tank may be a gamble. A tank should be retired based on its performance, not on its age.

22.5 Alarms

It makes intuitive sense that the function of LN2 storage tanks should be routinely monitored for tank integrity and LN2 levels. The viability of the stored tissues depends on optimal conditions which are entirely dependent on optimal LN2 levels. For accreditation purposes, there are also requirements for this (e.g., College of American Pathologists reproductive laboratory accreditation program). While manual inspection of cryotanks can provide a fair level of security for storage, this, coupled with automated alarm systems, can enhance the level of confidence in optimal storage conditions.

Automated alarm systems for monitoring LN2 levels in dewars can come in many forms and with varying levels of expense. In basic concept, the alarm consists of either a temperature probe capable of sensing temperature of at least -196°C or a nitrogen level probe that is attached to a wireless transmitter or hard wired to a monitoring system. The monitor receives the signal from the probe and can dial out to a list of recipients to alert them when an alarm condition exists (low LN2 level or high temperature). Typically, the probe is passed through a hole in the dewar’s removable top and positioned into the LN2 reservoir a few centimeters above the critical LN2 level. In this way, the probe will continuously monitor the temperature and will sense a rise in temperature if the LN2 level drops significantly below the optimal level. An alarm condition would exist if the temperature climbs to a preprogrammed level, typically -140°C or -150°C , in a small-capacity tank.

The alarm monitor has the capability of receiving signals from the probe and can be programmed to call a list of individuals if an alarm condition exists. Having such monitoring 24/7 does offer a sense of security, but labs must realize that

it is not foolproof. There are pitfalls to any automated system. First, the laboratory must ensure that when an alarm call is made and received by someone on the list, there is an appropriate response time for an individual to make it to the lab and remedy the situation. Second, placement of temperature probes must be done carefully so that it will sense a drop in LN2 level well before the LN2 reservoir is empty. If the probe is placed too deeply into the dewar, it will not sense a temperature rise until there is very little LN2 left in the tank, thus reducing an effective response time. Third, any automated system can malfunction at any level. The system should be routinely tested for its ability to sense a temperature rise and can effectively call out to all people on the list.

For the above reasons, total reliance on an automated system for monitoring storage conditions is a mistake. It does not take the place of routine manual QC. The condition of the tank should be assessed morning and evening by someone actually touching the side of the tank and looking for signs of imminent failure (frost on the tank). If the tank feels cold and condensation is felt on its surface, then the vacuum seal inside the walls of the tank has become compromised. In this condition, the tank will lose LN2 at an accelerated rate, and it would be prudent to move the specimens to an intact backup tank. LN2 probes do not sense external conditions on the dewar and are therefore only useful several hours after tank failure has occurred. In addition, the level of LN2 in the tank should be measured at least 3 times/week. This level should be recorded and charted so the lab can determine if the rate of LN2 loss is changing (i.e., increasing). Increasing loss rates are indicative of an impending tank failure. Measurement of LN2 levels is also the best means of determining if the LN2 needs to be topped off to return it to optimal depth. Large, automated storage systems with internal temperature probes and the ability to refill LN2 automatically are not fail-safe either. Manual QC monitoring activity needs to be developed for them too and could vary greatly depending on the manufacturer.

There are relatively new systems for assessing LN2 levels in a dewar that are beginning to appear commercially. They are based on continuously monitoring the weight of the dewar and its contents. Excessive loss of LN2 is accurately picked up by these systems, which are linked to a monitoring system like those for LN2 temperature probes. The scale needs to be carefully calibrated so that routine movement of specimens in and out of the tank does not set off false alarms. Here too, manual QC activity like that described above is also necessary to back up the activity of the automated balance system. Scales are not feasible for a large steel tank that can weigh a ton when filled with liquid nitrogen; however, measuring the liquid nitrogen loss with a dipstick can be another method of monitoring rapid vacuum loss for both small- and large-capacity tanks.

A new infrared video camera system (CryoSentinel) may be able to continuously monitor a room of tanks for tank failure due to loss of vacuum. This system detects decreases in temperature on the outside of tanks. Often, when a tank fails, the outside of the tank begins to frost up, sweat, and/or increase its temperature. The user sets a trigger point below the ambient temperature of the tank's shell and how much time this temperature must be measured for an alarm to be sent. One test of this system indicated that it reported a failed tank almost 23 h ahead of an internal temperature probe [7]. This type of system will not be able to detect failure to fill a tank though.

22.6 Validation of the Entire System

An alarm system should be set up so that it can detect (1) failure to refill a tank, (2) partial failure due to loss of integrity, and (3) catastrophic failure due to loss of integrity. Ideally, the system should be able to detect these events with an alarm that provides adequate time to acknowledge the alarm, drive to the laboratory, and repair or move specimens to a backup tank before the specimens are exposed to devitrification temperatures of around -130°C . An important part of any tank failure plan is to have adequate backup tanks that are ready to receive specimens from failing or failed tanks. A key to any alarm system is that the entire alarm system, for each tank, must be evaluated and constantly tested. Any time a new probe, alarm, or tank is added to the system, it should be evaluated to failure.

The most important piece of information that must be determined for each tank is the response time. This is the time from alarm until the liquid nitrogen alarm reaches a critical level *for each tank*. This critical level can be either a certain height in the tank where liquid nitrogen will be kept above or a location where a certain critical temperature is not allowed to be reached during evaporation of nitrogen. In the first case, this could be the top of the canister or the area just above where the tissue is located. For the latter case, it might be where the temperature will not be allowed to go above -130°C . One must also decide if the alarm and probe system will be set up to detect catastrophic and slow failure (as well as failure to refill), or will it be set up to just detect slow failure (and failure to fill).

To set up an alarm system to detect catastrophic failure (say, loss of vacuum), one must know the evaporation rate after vacuum loss in a specific model of tank. To set up the alarm to detect slow failure, one needs to know the average rate of nitrogen evaporation for the specific tank model. This average rate (static evaporation rate) has been measured and is reported by the manufacturer. For the most common small-capacity storage tank, the MVE 47/11, it is 0.39 L/day. For

these calculations though, it is easier to use the measurements in liters/hour. This would be 0.01625 L/h for the MVE 47/11.

If one will be setting up for measuring the response time for a catastrophic failure event, the evaporation rate after vacuum failure must be known. The rate for an MVE 47/11 is 35 L per day or 1.458 L/h [7]. In other words, for a slow failure, 0.39 L of liquid nitrogen will give 24 h of response time. For catastrophic failure, each 1.458 L of liquid nitrogen represents 1 h of response time. To convert this volume to the height within a specific tank, we can use the formula to calculate the volume of a cylinder given its radius and height. $V = \pi r^2 h$. Volume is in milliliters, and r and h are in centimeters (see Fig. 22.3). Using these figures, one can estimate where an alarm probe should be placed in the tank, and then after placement, the probe should be tested to see if it responds as calculated.

Example

Goal: Have 6 h of response time from the alarm to the top of the cane.

Measurements: Cane height (h) is about 30 cm; MVE 47/11 radius is about 20 cm.

From the alarm point, one needs $0.01625 \text{ L/h} \times 6 \text{ h} = 0.0975 \text{ L}$.

Calculate the height of the liquid nitrogen in a tank of 20 cm radius that represents about 97.5 ml:

$$V / \pi r^2 = h = 97.5 / (3.1416 \times 20 \times 20) = 0.078 \text{ cm}$$

Now let us say that we have the alarm set 6 inches above the cane. How much response time from the alarm do we have? 6-in. (15.2 cm) height of a cylinder represents $\pi r^2 h = 3.1416 \times 20 \times 20 \times 15.2 = 19,101 \text{ mL} = 19.1 \text{ L}$.

For a catastrophic event, this represents $(19.1/1.458 = 13.1 \text{ h est})$.

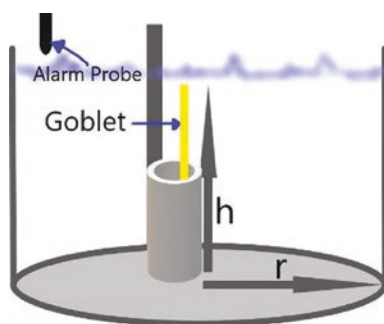


Fig. 22.3 Diagram of the inside of a liquid nitrogen storage tank. Goblet (or cane in yellow). Canister holds several goblets. The liquid nitrogen in the tank can be described as a cylinder that contains the goblet with radius (r) and height to the top of liquid nitrogen (h)

For non-catastrophic event $(19.1 \text{ L}/0.01625 = 1175 \text{ h or } 49 \text{ days})$.

These numbers are only estimates. One must check these estimations for each tank.

22.7 Protocols for Maintenance and Training

As explained in previous sections of this chapter, the monitoring and maintenance procedures attendant to cryostorage systems are paramount for providing safe and effective storage of patients' reproductive tissues. These processes are fortified by the presence of detailed operating procedures that all lab staff must become fully acquainted with. Such standard operating procedures (SOPs) are important for uniform activities by all of the appropriate staff.

The methods for maintenance of a cryostorage system are intimately tied to the handling of LN2 since effective storage requires its ultralow temperature of -196°C . While LN2 can be safely contained within the storage dewars, it cannot be overemphasized that its handling, if not done correctly, can be an extremely dangerous situation. Optimal levels of LN2 within the laboratory's tanks must be maintained through manual transport of LN2 from supply tanks to the dewars themselves, thus making lab staff undergo acute exposure to such a low-temperature fluid. Dangers of this exposure are many. First, direct contact with LN2 by skin, eyes, or any other bodily surface can cause immediate frostbite and associated damage. When exposed to room-temperature air or lab surfaces, LN2 immediately boils with resultant release of nitrogen gas. Nitrogen gas displaces oxygen in the air and can therefore create hypoxic conditions that can be life-threatening.

To manage the handling of LN2, it is important that SOPs include the proper techniques for its handling and methods for training of lab staff. Initial emphasis should include the use of proper personal protective equipment. This should include insulated gloves that are rated to withstand short-term exposure to LN2, eye protection, and face protection provided by a face shield. Personnel need to be trained in the handling of appropriate insulated canisters for transport of LN2 from storage tanks to dewars. Included also should be procedures for handling an LN2 spill in the lab. Spills certainly will result in large quantities of nitrogen gas being released, but even normal handling of LN2 will release this gas. The SOPs should cover what to do in the event that a dangerously low oxygen level is detected by the oxygen sensor in the lab.

As described in previous sections, effective monitoring of LN2 levels and cryotank integrity are essential for safe and effective operation. The SOPs need to cover, given a lab's type of equipment, how to measure LN2 and what to look for

with signs of impending cryotank failure. Also, in this regard, automated alarm systems are certainly an essential safety feature of effective monitoring of LN₂ storage. All lab staff need to be trained on how electronic alarms are programmed, who is on the emergency call list in the alarm system, and what to do in the event that they are contacted by the system with an alarm condition.

22.8 Regulatory Items

There are several agencies that regulate the management of IVF laboratories, and many include items that regulate the maintenance and management of cryostorage. These agencies may include the American Society for Reproductive Medicine, the College of American Pathologists (CAP), the Joint Commission, the European Society of Human Reproduction and Embryology, and individual states/provinces. Many new regulations were implemented after the terrible disasters of failed tanks in 2018 and are designed to reduce the risks of these events occurring again.

CAP has about 15 checklist items in their regulations related to cryostorage management and safety [8]. These checklist items address the monitoring of tanks, functioning of alarm systems, keeping of records of alarms and responses, and safeguards of inventory records. Checklists also describe necessary paperwork to convey to patients the length of storage, informed consents, and long-term disposition of cryopreserved gametes or embryos. Finally, they include items to make the storage areas safe for employees by describing necessarily oxygen monitors and training. Each clinic must know, understand, and implement the proper regulations regarding cryostorage according to the agencies they are responsible to.

22.9 Disaster Preparedness

Another requirement of most regulatory agencies is to have disaster preparedness plans. These are not just plans of what to do when disaster hits. They are plans in how to prepare for a disaster and how to minimize the effects of a disaster. All laboratories are subject to some type of disaster whether it be power outages, hurricanes, tornados, earthquakes, terrorist activities, or pandemics. One should consider not only the safety of frozen specimens, but also the safety of records. Assistance on writing a disaster plan can be found at <https://www.readyrating.org/How-It-Works> [9].

A disaster preparedness plan should address such items as follows: Is the current location and storage of the tissues ideal in case of the disaster of interest? Under what situations, where, and when should tanks be moved? Who will move tanks? How will one keep an adequate supply of liquid

nitrogen for tanks during the emergency? Is there adequate storage room when potential fresh cycles must be converted to “freeze alls” until the danger passes? Is a copy of the entire storage database kept off-site and easy to access? Are there security risks that might occur should a long-term emergency arise and how will those risks be handled?

A disaster plan must be written with input from physicians, nurses, and laboratory personnel. As a group, everyone should write down a list of all the potential risks that might occur in a particular disaster. Taking a photo of the involved area may be an important start in helping to understand the location of the tanks and how that location might be affected by a disaster. Roles should be assigned to each person. A way of communicating should be prearranged in case the phone systems are overloaded or down.

The recent Covid pandemic has shown us how ill prepared we were despite recent infectious disease disasters with Zika and HIV. Preparation for a disaster should occur well before the disaster hits. The time to find a flashlight is before a disaster hits, not while wandering aimlessly in the laboratory trying to use the dim light of an iPhone to only find a flashlight whose batteries are out. One must devise plans on how to minimize infection of staff and patients. What will be the requirements for shutting down cycles? If treatments are halted, how will the staff employment be handled? How will these cancelled cycles be taken care of? How will the laboratory obtain important laboratory materials with stoppages in work and shortages of supplies? What items can be stored to avoid a shortage in important medications, supplies, or equipment? How will the laboratory and clinic be staffed to decrease contact among staff and patients?

Ideally, once these plans are written, the plan should be tested to see how well it works and whether everyone knows their job. Then the plan should be modified and reexamined at least yearly. The use of checklists can be a crucial part of ensuring that the plan is carried out correctly [10].

22.9.1 Inventory

The importance of a comprehensive and effective system for tracking the inventory of cryopreserved oocytes and embryos is obvious and attends to many aspects of safely storing these tissues. Safety, as referred to in this chapter, has focused on the storage environment. But an inventory system goes beyond that to address (1) how many embryos or oocytes does a patient have, (2) who has custodial control over the use of these tissues, and (3) where are the tissues located. Each of these will be briefly addressed in this section. A comprehensive inventory is also essential for billing for storage, but this aspect will not be addressed here.

Inventories for stored reproductive tissues have been created pretty much since the beginning of cryopreservation.

The early systems were entirely paper-based, either in log-books, index cards, or data sheets in patients' medical charts. Paper-based systems can be very useful if accurately maintained and kept in duplicate in separate locations. But as cryostorage demands have increased, many programs have been compelled to move to electronic recordkeeping. This can be comprised of simple spreadsheets (e.g., Excel), relational databases (e.g., Access), electronic medical records (EMRs like EPIC), or complex databases that have been designed for reproductive medicine (e.g., eIVF, BabySentry).

Regardless of the format of the inventory, be it kept on paper or in electronic form, there are key aspects that all of them must manage. The first is who did these tissues come from and who has custodial control over their use? Perhaps the most common example is a patient couple who each supplied oocytes and sperm to create the embryos. By consent, it is commonly understood that both partners have control over their use and must be in agreement as to what will be done with them. This seems simple enough to state in the inventory records. However, what happens when the couple is no longer together? Has either of them been given sole control, either by contract or by another legal instrument? What if one former partner is now with another? Do they both have control? Inventory records must be updated with such information so that the laboratory knows who has authority over the tissues and does not inappropriately warm an embryo(s) for a partner who no longer has such authority. While cumbersome, such changes in custodial control can be managed in paper records, but care must be exercised that every part of the records is kept together and intact. Electronic records can also address such issues, and if the programming of databases is done carefully, the process can be efficient and accurate. But users must be aware that there are pitfalls even in sophisticated databases. EMRs often have difficulty linking two patients (who are partners) together in a cryopreservation record. One clear example of this is that EPIC, which is probably the most widely used EMR in the United States, has difficulty with this task. Careful programming can partly overcome this, but such a feature is not something that you can "buy off the shelf." Even relational databases like eIVF struggle with this, and careful auditing is needed to ensure accuracy. The example here of patients changing partners is just one example of a wide range of complex relationships of patients with their cryopreserved specimens. Circumstances involving donor gametes (anonymous or directed), donor embryos, same-sex partners, deceased partners, etc. all require an adaptable yet powerful inventory system to keep track of them. And again, oversight and audits are essential.

With regard to the number of oocytes and/or embryos that a patient(s) has in storage and the locations of the specimens, inventory systems must be plastic. The laboratory needs to be

able to accurately and easily update the inventory with what specimen was used, how was it used, and how many specimens remain? We have all been asked many times by the clinic and/or patient as to how many embryos or oocytes remain for the patient or couple. An efficient inventory system should easily provide that answer. But the consequences of getting the answer wrong can be devastating to the patient (not to mention making everyone in the program look bad). Thus, whatever inventory system is in use, be it electronic or paper, it must be frequently audited for accuracy. For a more detailed look at cryostorage inventory, see Pomeroy and Marcon [11].

22.10 The Future

The field of reproductive medicine and the public at large are becoming increasingly aware of the consequences of a failure in a cryostorage facility. Considering the hundreds of thousands of reproductive tissues that are in storage, the incidence of a failure is, fortunately, extremely rare. However, for the patient(s) affected by an accident, this is a devastating situation and one that we must endeavor to avoid with great vigilance.

As described here, the systems and methods employed to cryopreserve specimens and maintain safe and effective storage range from purely manual to increasingly automated approaches. For those who use any given approach, they can spell out the advantages and potential pitfalls of all of them. The common denominator for a safe and effective approach is reliance on a multitude of methods aimed at cross-checking functions and validating the system. Interest in increased sophistication of automated equipment for specimen safety and inventory management is spreading throughout our field. An extreme example of this is the recent introduction of systems that not only automatically maintain an optimal LN2 environment and alarm surveillance, but also automatically update inventories when specimens are introduced or removed. In addition, some of these systems robotically locate and retrieve specimens for use. One example is the TMRW robotic cryostorage tank (TMRW Life Sciences, New York, New York). It is tempting to think that these systems represent our future and will improve the safety of cryostorage of reproductive tissues. This may be true, and future development will be of interest to all. However, as has been described here, automation can fail and cannot completely substitute for expert human oversight to prevent potential catastrophe. No matter how much work advanced automated systems can take off the hands of embryologists, they need to be open for human oversight and complacency cannot be allowed.

The growth and high effectiveness of the cryostorage of reproductive tissues are an amazing story of biology, tech-

nology, and medicine. Countless fertility patients have benefitted from this option in their treatment regimen. Expertise in this has grown around the world, and we have all benefited from advancements that have provided very safe and effective results. With greater awareness and knowledgeable oversight, what is already a highly successful addition to reproductive medicine will only improve.

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Part IV

Advances in Cryotechnology, Research, and Animal Models

Artificial Intelligence and Oocyte/ Embryo Assessment in Cryopreservation Cycles

23

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Key Points

- AI has been shown to play a promising role in the future of reproductive medicine, from its ability to evaluate sperm, oocytes, and embryos to its ability to predict treatment success rates.
- In evaluating oocytes, AI can assist in identifying competent oocytes at the time of retrieval, classification of nuclear maturity, and detection of dysmorphisms. It can also be used to predict the likelihood of an oocyte becoming a usable blastocyst as well as the likelihood of live birth.
- AI has been utilized to evaluate embryos at each stage prior to transfer and assist in embryo ranking, tracking, and ploidy assessment.
- AI technology is limited by the quality of the data used to train it; ideally, it should be trained on data that accurately represents the population this technology will serve. Adequate validation studies performed in a prospective manner with a diverse dataset in independent settings are necessary before routine integration in the IVF lab.

23.1 Introduction to Artificial Intelligence

Artificial intelligence (AI) is a field that lies at the intersection of computer and data science. It leverages data and algorithms to mimic the problem-solving capabilities of the human mind. The field of machine learning (ML) and the subfield of deep learning (DL) are included in the overarching term of AI. Through ML, mathematical models allow

computers to detect patterns within large datasets and use these patterns to make future predictions. These algorithms can learn from data without requiring explicit programming. These algorithms are trainable and can modify output without requiring explicit changes to programming. DL, a subset of ML facilitated by artificial neural networks, requires less ongoing human intervention. A classic example of DL's power and rapid evolution is how programs such as Deep Blue or Alpha Go can now consistently beat world champions at chess and Go, respectively. Even more impressive is the ability to create such programs by teaching the algorithm the set of rules for each game and allowing the program to train itself to run simulation games. The application of DL to image, video, or unstructured data analysis represents an exciting new frontier in healthcare, particularly in reproductive medicine.

Narrow AI, sometimes called “weak AI,” refers to an AI program trained to perform a specific, well-defined function to replicate or surpass human ability in that task. Narrow AI has been integrated into our daily lives; examples of this technology include facial recognition systems, virtual intelligent chatbots, and even self-driving vehicles. Although simpler to create, narrow AI systems are limited by specific tasks and can only make decisions based on their training set. Thus, they have inherent biases, and their decisions are generally not explainable to the user.

The cornerstones of a successful AI application include delineating a well-defined problem; using a large, high-quality dataset on which the algorithm can be trained; and appropriate validation studies. Thus, it is critical to understand the intended clinical application of a narrow AI system and how it was designed, trained, and validated.

The growing interest in the application of AI within healthcare stems from its numerous potential benefits, including the possibility of lowering the cost and time needed to complete tasks, scalability of its technology, and improved accuracy due to its ability to be less subjective, decrease human error, and unlock new insights not possible by a trained professional. An additional advantage is that, in the-

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ory, its accuracy should continue to improve as more data and better DL techniques are integrated.

The integration of AI with medicine seems inevitable. Indeed, medicine has already been impacted by the AI revolution. The ability of AI-enabled technology to gather insights from robust biologic datasets has led to its use to assist with clinical decision-making, diagnosing, managing, and treating clinical cases. There are already numerous FDA-approved AI-based medical devices and algorithms, the majority of which are in cardiology and radiology [1]. As the AI healthcare sector matures, so will the regulatory bodies that evaluate, approve, and monitor the efficacy and safety of AI technologies. Currently, AI technologies are considered “software as medical devices” (SaMDs).

23.2 AI Application in Reproductive Medicine

AI technologies have also begun to play an essential role in enhancing several aspects of care in reproductive medicine, with an initial focus on improving pregnancy rates per treatment cycle. Simply put, a successful pregnancy is dependent on a viable embryo and a receptive endometrium. A viable embryo is the downstream product of a high-quality oocyte and sperm that fertilizes, cleaves, blastulates, and ultimately hatches and implants in the endometrium. Although there is ample opportunity for narrow AI to be applied to every step of this process, this chapter focuses on oocytes and embryos in cryopreservation cycles. It is important to highlight that with the rapid growth of AI capabilities and the ongoing interest and efforts in AI by academia and industry, the current state of AI in reproductive medicine will change and progress quickly.

The applications of AI to oocytes and embryos can be broken down into potential and current uses.

23.3 Potential Uses

A list of potential uses for narrow AI is extensive, and future applications are continuously being proposed. This chapter is focused on oocytes and embryos in cryopreservation cycles. It, therefore, will not cover pre-IVF lab applications such as clinical decision support tools that may include optimal IVF protocol selection, personalized gonadotropin dosing, ideal day of trigger, and predictive calculators for IVF success rates, to mention just a few [2, 3]. Also, post-IVF lab applications, beyond the embryo transfer, such as endometrial receptivity assessment or miscarriage risk prediction of an established pregnancy, will not be discussed. The role of AI concerning oocytes and embryos will be focused on within the IVF lab.

One of the pillars of narrow AI within the IVF lab is to define a specific task that a trained embryologist may or may not currently be able to complete. A deep working knowledge of the IVF lab is required to determine a clinically relevant exercise. It is also important to highlight the primary objective of such an application, which could include improving outcomes, delivering more cost-effective care, or decreasing lab errors. Below are some proposed applications:

23.3.1 Oocytes

Identification:

- Identify cumulus-oocyte complexes (COCs) at the time of retrieval.
- Identify oocytes and link them with unique patient identifiers (oocyte tracking).

Evaluation and automation:

- Determine which COCs are more likely to be immature or mature prior to denudation (COC scoring).
- Identify nuclear maturity (presence of a polar body and absence of a nucleus).
- Identify oocyte dysmorphism (intra- and extra-cytoplasmic).
- Analysis of cumulus cells or culture media “omics” (proteomics, genomics, transcriptomics, etc.).

Prediction:

- Identify oocytes more likely to successfully fertilize and develop into cleavage- or blastocyst-stage embryos (oocyte scoring).
- Identify oocytes more likely to develop into high-quality blastocysts or blastocysts determined to be euploid by preimplantation genetic testing for aneuploidy (PGT-A) (oocyte scoring).

23.3.2 Embryos

The stage of development can subdivide embryos into a zygote, cleavage stage, and blastocyst stage. Each stage can also include witnessing checks (embryo tracking) and culture media assessments. Unique to embryos, AI can be applied to either single-image analysis (two-dimensional) or video analysis of development up to a certain evaluation stage (time-lapse).

Zygote:

- Identify fertilization (the presence of two pronuclei).

- Automate zygote scoring.
- Predict outcomes including blastocyst development, PGT-A results, and sex identification.

Cleavage:

- Identify the number of blastomeres.
- Automate cleavage-stage scoring.
- Predict outcomes including blastocyst development, PGT-A results, and sex identification.

Blastocyst:

- Automate blastocyst-stage scoring.
- Predict outcomes, including PGT-A results and sex identification.
- Embryo ranking/selection.
- Predict successful cryopreservation and post-thawing survival.

23.4 AI and Oocyte Evaluation

Oocyte morphology is one of the first physical indicators of an assisted reproductive technology (ART) cycle's chances of success, often defined as fertilization competency, ability to produce high-quality blastocysts with high pregnancy potential, and ability to survive a subsequent freeze/thaw cycle. At the time of oocyte harvest, gross abnormalities in COCs are observable, and the embryologist can estimate the number of mature oocytes available. After denuding and at the time of intracytoplasmic sperm injection (ICSI) or cryopreservation, each oocyte's morphology can be assessed in detail. Currently, no standardized oocyte scoring system is used in clinical practice [4].

In practice, morphologic assessment of oocytes is often done in passing, without too much weight given to small abnormalities; because of the effort involved in their procurement, even poorly graded oocytes will be used. In contrast, in sperm selection assessment, morphologically poor sperm are generally deselected and discarded when there are higher quality sperm in the sample to use. Additionally, in its current state, morphologic assessment of oocytes is highly subjective, and there is still no concrete quantitative measure of its impact on ART success [5]. Abnormalities make it difficult for an embryologist to objectively quantify an oocyte's competency, especially without significantly slowing down the lab's workflow. An AI tool should be able to rapidly assess several oocyte dysmorphisms to give an objective and reproducible assessment of each oocyte's competence.

Oocyte morphological assessment traditionally focuses on abnormalities. There are no known features associated with increased competency or higher quality. Although some

dysmorphisms have been correlated with negative lab outcomes, these are not consistently demonstrated. Currently, no visual oocyte scoring system has been integrated into routine practice [6].

The assumption that abnormalities at the molecular level will manifest as an observable phenotypic change at some point in development (from oocyte to blastocyst) must be challenged. A morphologically normal oocyte, as viewed after the removal of cumulus cells with a light microscope at around 100× magnification, is traditionally classified as one with a rounded cell with a uniform textured appearance and a light-colored cytoplasm. The zona pellucida is a single continuous membrane of even thickness that is similarly light-colored and uniformly textured. A small perivitelline space (PVS) with a single bean-shaped polar body is a sign of maturity in oocytes that have completed meiosis II. In other words, it is assumed that a lack of visible dysmorphisms classifies an oocyte as high quality.

Deviations from this normal profile are common and do not necessitate an oocyte's exclusion from a patient's cohort for clinical use. Nuclear maturity (metaphase II [MII]-stage oocytes) is relatively easy to classify based on the presence of a polar body and the lack of a nucleus within an oocyte. Cytoplasmic maturity is a vague term and a complex entity to study. Given that it is subjectively classified, it frequently encompasses oocytes with multiple concurrent abnormalities. It is difficult to determine the impact of any individual dysmorphism. Oocyte dysmorphisms are generally classified based on their location: cytoplasmic (ooplasm pattern and the presence of smooth endoplasmic reticulum (SER) or inclusions/vacuoles) or extra-cytoplasmic (general oocyte diameter and shape, polar body morphology, as well as PVS and zona pellucida characteristics).

AI can help standardize the classification of oocyte dysmorphisms so that their impact on oocyte quality can be better studied. Moreover, by using image analysis, AI can unlock further insights into oocyte quality, identifying impossible features for humans to detect and develop a clinically useful oocyte scoring system. Unlike sperm (WHO 2020, <https://www.who.int/publications/i/item/9789240030787>) or embryos [4], there is no established visual grading system for oocytes. Developing an automated, noninvasive, objective, and accurate oocyte evaluation tool would greatly benefit ART. It would have clinical relevance in oocyte cryopreservation, oocyte donation, and oocyte utilization (IVF or ICSI) cases.

23.5 Clinical Uses

23.5.1 AI and Oocytes

As mentioned above, AI technology applied to oocytes has the potential to assist in the identification, evaluation, automation, and prediction of ART outcomes.

In vitro maturation: In [7], Cavallera et al. utilized time-lapse analysis for training an artificial neural network to identify competent versus incompetent, immature mouse oocytes. The team recorded mouse oocytes during in vitro maturation from the germinal vesicle (GV) to the MII stage and looked specifically at cytoplasmic movement velocities during this time period. This analysis went on to inform a feed-forward artificial neural network (FANN) to predict the oocyte's competency. Overall, this algorithm was 91.0% accurate when classifying immature oocytes as competent or incompetent to mature in vitro.

COCs: AI can also play a role in improving the identification of mature oocytes within COCs, which can help minimize the chance of immature oocytes being identified after denudation, preventing them from potentially reaching full maturation after the loss of surrounding cumulus cells. Studies have proposed using molecular analysis of COCs as a tool to help identify MII-stage oocytes at the time of egg retrieval. Sun et al. [8] suggested performing a noninvasive biopsy of COCs at the time of egg retrieval to assess oocyte maturity by evaluating messenger RNA, microRNA, and protein biomarkers of MII-stage oocytes present in cumulus cells. AI can be applied to the analysis of COC biomarkers to improve the accuracy of predicting mature oocytes. However, it seems clinically impractical to have a biomarker analysis of COCs for this specific task, as the results would be required quickly, since the decision to denude COCs or not is required within hours of retrieval. However, as Sun et al. pointed out, this concept can be applied to identify better quality oocytes that subsequently had better outcomes in the IVF lab. It is plausible that embryo selection might be improved by considering upstream evaluations, such as COC biomarker analysis.

Polar body identification: Researchers have also been able to use deep convolutional neural networks (CNNs) to locate extruded polar bodies to distinguish MII oocytes from metaphase I and GV oocytes and identify the correct location for sperm injection for ICSI [9]. This is a typical example of a defined task, identifying and locating a polar body, an embryologist can and consistently perform that, when automated by an AI tool, can improve standardization, efficiency, and accuracy, as well as serve as a safety check to ensure that embryologists and the software program are in agreement.

Dysmorphisms: A meta-analysis in 2011 determined that a large first polar body, large PVS, and inclusion of refractile bodies or vacuoles were associated with decreased oocyte fertilization [10]. Building on this research, subsequent work has been done to train AI models to classify various oocyte morphologic features with high accuracy. These models offer the ability to evaluate the morphologic characteristics of oocytes in an objective, time-efficient, accurate, and reproducible fashion while simultaneously helping eliminate the potential for subjectivity and human error. For example,

these models, which have been trained to identify oocytes with morphologic abnormalities associated with poor fertilization, can be used to directly assist in selecting oocytes from a cohort better suited to IVF instead of ICSI.

Outcome predictions: Currently, there is no standardized oocyte scoring system, which diminishes the ability to select better oocytes or predict their outcomes in the IVF lab. Having an oocyte scoring tool would be particularly beneficial in places where the creation of supernumerary embryos is limited (e.g., due to legal restrictions based on the jurisdiction), as practitioners could identify and target the oocytes most likely to succeed while cryopreserving surplus oocytes. In addition, all patients with cryopreserved oocytes (medically indicated or for elective reasons) would benefit from more personalized feedback on the quality of their oocytes and the potential for a future successful pregnancy. Such feedback could also help patients and clinicians better understand the oocytes' contribution to failed IVF cycles, which are still the most common outcomes of treatment cycles.

Researchers have used AI technology to evaluate images of MII oocytes (two-dimensional) and use these features to predict outcomes for a given cycle. A 2020 study looked at the ability of a trained CNN to predict fertilization potential based on oocyte images [11]. The proposed model accurately identified oocytes with the highest fertilization potential 86% of the time.

AI technology has also been employed to predict an oocyte's potential to become a usable blastocyst, thus creating a visual oocyte scoring system [12]. Violet™, an AI software program, is a noninvasive tool that uses light microscopy to capture images of mature oocytes. When tested against senior embryologists, Violet™ was better able to predict blastocyst development (62.8% vs. 52.1% ± 3.4%; $P < 0.001$). Further, when repeated 2–3 months later, the program's reproducibility was 100% compared with intra-observer repeatability of 81.4% for the same embryologists. The software is used in clinical settings to provide personalized feedback on oocyte quality to patients freezing their oocytes. The current standard of care consists of a general prediction for patients based solely on female age and the number of MII vitrified oocytes. Such an AI-enabled oocyte quality tool could also be used by donor oocyte programs to help with top oocyte selection or to more evenly distribute oocytes across intended parents so that the best quality oocytes are equally allocated instead of randomly sorted.

23.5.2 AI and Embryos

As highlighted above, the potential uses of AI at the zygote, cleavage, or blastocyst stage are numerous. They include automating developmental milestones (2PN, cleavage, and blastocyst development), enabling a grading/scoring system

(zygote, cleavage, and blastocyst), and predicting outcomes ranging from blastocyst development to ploidy status and toward successful implantation and clinical pregnancy.

Unique to embryos, AI can be applied to single-image analysis (two-dimensional) or video analysis on development up to a certain evaluation stage (time-lapse).

23.6 Zygote

CNN has been developed to distinguish zygotes with two pronuclei from those without with a high degree of accuracy [13]. AI technology has also been applied to the grading system used for zygotes in clinical practice to improve the accuracy and speed with which this task can be performed. Zhao et al. [14] explored the use of a CNN to evaluate pronuclear-stage embryos by examining the morphokinetics of the zygote cytoplasm, zona pellucida, and pronuclei. They found that their algorithm could accurately analyze these features of a day-1 embryo. The evaluation of cytoplasmic kinetics is predictive of blastocyst development. When compared with human evaluation, AI analysis of cytoplasm movement and velocity was found to be more accurate in predicting blastocyst development [15]. As such, the advent of AI technology in this space allows for grading to be done in an accurate and time-efficient manner and enables grading systems to expand to consider parameters undetectable by the human eye.

23.7 Cleavage

Concerning the assessment of cleavage-stage embryos, researchers have trained algorithms to make predictions about outcomes from day-3 embryos. One of the earliest software tools that tackled cleavage-stage embryos was the Early Embryo Viability Assessment (EEVA) test that aimed to better select day-3 embryos for transfer, a practice that is no longer considered the standard of care in the IVF lab. Cleavage-stage prediction algorithms continue to be updated to allow earlier assessment times during embryo development [16]. When the performance of these AI models was compared with that of embryologists, the neural network's predictions were found to be more accurate at identifying embryos that will develop into blastocysts.

23.8 Blastocyst

AI models have also been applied to the grading of blastocysts. Similar to earlier models trained for embryo evaluation, CNNs have been trained to evaluate images of blastocysts. Bormann et al. [17] trained their model to evaluate embryos 113 hrs after insemination. They found that their

algorithm could accurately differentiate between blastocysts and non-blastocysts in 90.97% of cases, indicating a high degree of concordance between AI evaluations and those of expert embryologists.

23.9 Time-Lapse Incubators

An innovative approach to embryo evaluation, at least in terms of available data, is time-lapse incubators that gather development videos from fertilization to the blastocyst stage. Although time-lapse systems hold great potential, their clinical efficacy in selecting better embryos or correlating them with ploidy status remains marginal. A recent systematic review and meta-analysis on the subject that included 58 studies analyzing more than 40,000 embryos identified ten morphokinetic variables significantly delayed in aneuploid embryos. However, a robust model to replace PGT-A is not yet available [18].

23.10 Embryo Selection

Embryo evaluation plays an important role in optimizing the success of an IVF cycle. In current practice, noninvasive examination methods are used to evaluate an embryo's morphology and its development to the blastocyst stage. This process is generally undertaken by embryologists, who can select the embryos best suited for transfer. This process has high inter-operator variability in and of itself [19]. The selection of embryos best suited for transfer improves the likelihood of success in an IVF cycle and helps limit the number of embryos transferred in a cycle. This, in turn, leads to fewer multi-gestational pregnancies, thus decreasing the risks of serious complications associated with these pregnancies, including preeclampsia, preterm delivery, and postpartum hemorrhage. Several classification systems have been created to streamline embryo evaluation. Nevertheless, in its current form, this process is inherently subjective and, as such, ripe for AI innovation.

Time-lapse incubator companies such as VitroLife have continued developing and implementing AI into their software programs. The EmbryoScope™ uses guided annotation to assist embryologists in reviewing time-lapse videos and document important milestones such as fertilization and time intervals between early cell division and blastocyst grading. Guided annotation aims to make embryo evaluation more objective and less time-consuming for embryologists. The KIDScore5 was developed as a decision support tool based on morphokinetic data inputs to rank embryos based on their implantation potential [20]. The evolution of the KIDScore5 is the iDAScore, which automatically evaluates time-lapse sequences to rank embryos to improve the selection process

while decreasing time commitment from the embryology team [21].

Other groups have focused their narrow AI efforts on embryo selection, some with static single two-dimensional images [22, 23], while others have used the time-lapse video approach.

Bormann et al. [17] also trained CNNs to determine the highest quality embryos within a cohort. In this model, the algorithm selected the highest quality embryo in 90% of cases. When the CNN was trained to assess the implantation potential of an embryo, the algorithm outperformed embryologists in predicting the implantation outcome of euploid embryos. Similarly, other researchers have used deep neural networks (DNNs) trained on time-lapse images of human embryos to predict blastocyst quality accurately. This information was then combined with patient age to determine better pregnancy likelihood [24].

There is reflexive fear that a validated AI algorithm might fully replace the evaluation or decision-making of an embryologist. Practically speaking, at least initially, most AI tools will likely be used with trained clinicians. Fitz et al. [25] demonstrated that embryologists could improve their ability to select the best quality embryo when aided by an AI-trained algorithm.

23.11 PGT-A

AI models have also been applied to identify embryos with chromosomal abnormalities. Several DL models have been trained to recognize noninvasive indicators of ploidy. Although some AI models have been trained on static blastocyst images, others have used time-lapse images [26–28]. These AI models may prove to be an important step in the embryo evaluation process, as they may reach a certain level of accuracy that allows them to determine whether embryos should undergo PGT-A biopsy (inconclusive prediction) or not (strong positive or negative prediction). Currently, PGT-A is a costly, labor-intensive procedure that can only be performed by experienced embryologists and is unavailable to all patients. Given its limitations on accessibility to patients and ongoing debates on its clinical benefits in all patient populations, its routine use continues to raise controversy [29]. It is important to highlight that PGT-A might be a surrogate marker for a successful IVF cycle but certainly not the gold standard of an established clinical pregnancy.

23.12 Embryo Witnessing

Ensuring that gametes and embryos are accurately tracked to patients is critical; this must be a zero-error task. IVF labs have strict protocols to ensure that no mistakes are made

during embryo handling, including ensuring that two embryologists must witness each process. Nevertheless, the opportunity for misidentifying embryos still exists. In rare cases, it has led to the births of children from transferred embryos that do not belong to the parents. AI has been used to target this mix-up problem. CNN has been trained to identify embryos and link them with unique patient identifiers. When put to the test, a recently reported model could correctly match 100% of embryo cohorts with the patient identifiers they belonged to [27]. Using AI to identify the unique morphologic characteristics specific to an embryo and associate it accurately with the correct patient is an important potential application of AI in the IVF lab. These models can be utilized with existing security protocols in labs as an added safety measure.

23.13 Limitations

Within the field of reproductive medicine, AI technologies have the potential to assist embryologists and reproductive endocrinologists in evaluating sperm, oocytes, and embryos in a fast, accurate, and reproducible manner that minimizes the potential for subjectivity and human error. Impressively, the potential uses for narrow AI continue to expand, as does the development of clinical use cases.

However, the use of AI is not without limitations. Inherently, an AI model is only as valuable as the proposed clinical question and the data it was developed on. For example, many important variables beyond embryo quality determine successful implantation, such as a receptive endometrium and evaluation of the uterine cavity [19, 27]. AI can play an important role in embryo selection, but it also has the potential to be utilized to assess uterine anomalies and endometrial defects better. However, at the current time, few AI technologies can incorporate an evaluation of this full clinical picture.

Another example of the dependence of the underlying dataset is that it is critical to determine if an embryo selection AI tool was developed based on embryo transfers only in surrogates (assumed to have a receptive endometrium based on their clinical histories) or on the other spectrum, in patients with failed multiple embryo transfers in the past (presumably due in part to an underlying uterine factor). The accuracy and generalizability would be dramatically different, even with the same embryo images and AI network capacity, further illustrating the importance of the data quality used to train AI models.

Small training datasets have increased the potential for bias, which in turn can lead to wrong outputs, and this inappropriate conclusions or decisions being carried out. As such, to optimize this technology, we must ensure that large, high-quality datasets are used to train algorithms. Procuring

datasets that meet these requirements can be a challenge in and of itself [30].

To adopt widespread use of AI technologies, models must be generalizable to populations beyond those on which it is trained. Data collected in specific contexts, such as at select clinics or IVF labs or on certain population demographics, may train models specific to these scenarios and thus not generalizable to the wider population. As such, it is important that before these technologies are employed on a large scale, they have been reevaluated in different settings to ensure that the data they have been trained in adequately represents the population they will be serving. Ideally, AI technologies will be trained and validated on large, diverse, and realistic datasets that appropriately represent the clinical context in which these technologies will be employed [31].

Like all scientific research, validation studies are necessary to continue to develop AI tools. The first tier of evidence usually stems from a retrospective dataset, with a test experiment performed on a blinded subset of the data never before seen by the AI algorithm. Next, comparing the AI technology with a clinically relevant control group can highlight the benefit of a narrow AI application over standard care. For example, an AI tool that aims to predict blastocyst ploidy can be compared with PGT-A results from the same embryo. The evolution of validation studies includes confirming similar results in diverse settings (different clinics, IVF labs, and patient populations) and a real clinical setting (prospective studies). Prospective studies have the additional benefits of shedding light on the impact of AI on workflow and clinical productivity. Of course, RCTs should be pursued if they are practical to the specific AI application.

AI faces the challenge of being translated into the workplace. Physicians and embryologists employing this technology are not classically trained in AI principles, so interpreting active research is less familiar to them. The term “black box” AI refers to the unknown nature of how the software program reached its conclusion. This is inherently risky, as the end user (clinician or patient) cannot easily determine how the system reached its conclusion or if the program is biased [31]. There is a push to develop “interpretable” AI tools that attempt to highlight what variables the algorithm is relying on to make its analysis. For example, it is important to ensure that an embryo selection algorithm is not analyzing the image of an adjacent holding pipette as part of the analysis (information outside the embryo of interest), as that likely represents a bias where data from a specific IVF lab that routinely uses a pipette to capture images might skew results in a certain direction.

Unfamiliarity with this field poses a potential barrier to AI’s widespread implementation. Nevertheless, as an increasing number of advances in medicine incorporate AI, clinical practitioners will need to develop the background

and skills to utilize these technologies to benefit their patients.

The implementation of AI technology raises the concern that algorithms will be used to replace human decision-making. There is also the associated ethical concern of relying partly or fully on a computer to make important clinical decisions. Patients and practitioners may fear that the advent of these technologies can lead to further dehumanization of IVF, a process that is mentally and physically taxing on patients. In this regard, it will be important to ensure that when AI technologies are implemented in the workplace, they are done in a way that bolsters, instead of replacing, the role of practitioners and embryologists in clinical decision-making.

Ultimately, there are many steps between the current use of AI in reproductive medicine and its eventual routine integration into the IVF lab. These include streamlining the various models that currently exist in order to identify those best suited for clinical practice, testing them to ensure their clinical utility, and training them to be accurately applied in various clinical settings. Once ready for implementation in the IVF lab, AI technologies need to be integrated into current clinical practice stepwise and responsibly. Decisions made by humans and machines will comprise the future IVF practice.

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Vitrification: A Reliable Method for Cryopreservation of Animal Embryos

24

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Key Points

- Vitrification is a reliable method for cryopreservation of animal embryos.
- Vitrification is the best alternative for cryopreservation of very cryosensitive embryos such as porcine embryos and embryos produced in vitro.
- Progress in vitrification of animal embryos requires standardization of methodology, resolution of biosafety issues, and expansion of knowledge about animal embryology, especially in wildlife species.

24.1 Introduction

The cryopreservation of animal embryos is becoming increasingly interesting due to its numerous applications [1–3]. A key benefit of this technology for livestock is the development of embryo transfer (ET), facilitating the transport of genetic resources with very low cost and health risks compared to the transport of animals [3]. Furthermore, the development of efficient methods for the cryopreservation of animal embryos is essential for cryobanking, which contributes to the conservation of endangered species and breeds [4] or other highly valuable genetic resources, such as genetically modified embryos, for biomedical research [5]. In 1985, Rall and Fahy [6] described, for the first time, the successful cryopreservation of mouse embryos without ice crys-

tal formation; this was the first report of vitrification of a mammalian embryo. This “glass-like” state is achieved in vitrification by the extreme viscosity of the solution and very high cooling rates [7].

Vitrification has several advantages over slow-freezing systems. With regard to the impact on embryos, vitrification reduces cold damage and prevents the formation of ice crystals [8]. Moreover, vitrification is also faster and simpler than slow freezing, and the necessary equipment is cheaper [8, 9]. Since the investigations of Rall and Fahy [6], vitrification has been widely applied for the cryopreservation of human oocytes [10] and embryos [11]. Additionally, vitrification has been used to cryopreserve embryos from a variety of domestic and laboratory animals [5, 9, 12], as well as other mammalian embryos. Currently, vitrification is considered to be the best alternative for the cryopreservation of highly cryosensitive embryos such as in vitro-produced (IVP) and cloned embryos [13, 14]. For most livestock species, vitrification has been developed as an efficient alternative to slow-freezing systems, which give satisfactory results only with bovine and ovine morulae and blastocysts [9]. In this chapter, we present the embryo vitrification state of the art in livestock animals, paying special attention to practical considerations such as vitrification solutions and devices and to the results that we can expect in each species.

24.2 Vitrification and Warming Solutions

Vitrification requires a very high concentration of cryoprotectants (CPs) to provide the medium with the necessary viscosity and prevent the formation of ice crystals. Media include permeable and nonpermeable CPs. Ethylene glycol (EG), dimethyl sulfoxide (DMSO), and glycerol are the most used permeable low-molecular-weight CPs for animal embryos [15]. These CPs can easily penetrate the cytoplasmic membrane and replace the water in cells, quickly reaching the concentration required for vitrification. In contrast, nonpermeable cryoprotectants, such as trehalose,

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sucrose, or Ficoll, cannot penetrate the cytoplasm but have an important role in regulating osmotic pressure inside and outside cells, stabilizing membranes and promoting vitrification [16]. One of the main concerns of vitrification has always been the use of extremely high concentrations of CPs. However, the final concentration of CPs is higher in traditional freezing than in vitrification because of the growth of ice during slow cooling [17]. This observation suggests that vitrification may not have greater long-term toxicity than traditional freezing.

Vitrification is performed in a viscous solution containing a phosphate- or HEPES-buffered medium supplemented with CPs, macromolecules, and saccharides. Vitrification media have been improved using less toxic and more permeable chemicals, combining CPs to reduce toxicity, and increasing cooling and warming rates, resulting in a reduction in the amount of CPs required for vitrification [18]. Traditionally, vitrification media contain serum or serum components. However, the trend is to develop chemically defined media that allow better traceability of samples and greater homogeneity and avoid potential contamination risks. Therefore, chemically defined media for vitrification and warming have already been developed with excellent results [19, 20].

The most commonly used combination of CPs is EG and DMSO due to the low toxicity and permeability of EG and the good glass-forming property of DMSO [15]. Animal embryos are usually vitrified using a two-step equilibration protocol. First, embryos are equilibrated for 1–5 min with a lower concentration of permeable CPs (total concentration of 15–20%, v/v) and then for 20 s to 1 min in vitrification medium with a high CP concentration (0.25–0.5 M nonpermeable CP and a total concentration of 30–40% (v/v) of permeable CPs). Then, the samples are immersed in liquid nitrogen.

The basal media for warming embryos are the same as those for vitrification. Classic warming protocols include step-wise equilibrations in decreasing concentrations of sucrose, starting with 0.4–0.5 M concentrations [15]. The problem with these warming protocols is that, although effective, they require manipulation of the embryos and, therefore, do not allow direct transfer to recipients in farms. To solve this technique aspect, several direct warming systems have been developed that allow direct transfer of vitrified embryos under field conditions. Direct warming methods have been successfully used in cattle [21], pigs [22], horses [23], donkeys [24], sheep [25], and goats [26]. Currently, direct warming is the method of choice for the practical application of ET with vitrified embryos under field conditions in livestock species.

24.3 Vitrification Devices Used for the Cryopreservation of Animal Embryos

Vitrification depends on the rate of cooling and viscosity, which are related by an exponential factor [27]. Thus, the higher the cooling and warming rates, the lower the required CP concentration [9]. Accordingly, the best strategy for successful vitrification is to increase the cooling rate, which makes it possible to reduce the concentration of CP and thus reduce the osmotic hazardous effect and the toxicity of the vitrification medium and minimize the damage caused by low temperatures [9]. Fast-cooling rates are achieved by directly immersing the sample in liquid nitrogen and by developing vitrification systems that allow the sample (as little as few microliters) to be vitrified. Many devices have been developed to reduce the volume of vitrification solution, commonly referred to as “minimum volume cooling (MVC) methods” [7]. The MVC methods available for the vitrification of human and animal embryos have been described in detail by Saragusty and Arav [28]. The vitrification methods used for animal embryos (Tables 24.1, 24.2, 24.3, and 24.4; Fig. 24.1) are generally the same as those developed for human embryos and are classified as “open” or “closed” systems, depending on whether the sample is in direct contact with liquid nitrogen during cooling [29]. For both animal and human embryos, the results of closed vitrification systems are commonly considered worse than those of open systems [29]. For this reason, currently, most human and animal oocytes and embryos worldwide are vitrified using open systems [29]. The main criticism of these systems is the potential risk of contamination of the sample with pathogens present in the liquid nitrogen. However, experience has shown that the risk of pathogen transmission through liquid nitrogen in assisted reproduction is extremely low (less than 1:250,000 or much lower), because an estimated 1,000,000 transfers have been performed with embryos vitrified with open systems with no reports of disease transmission [29, 30].

To achieve high viability rates, the MVC concept requires that oocytes/embryos are vitrified either individually or in a very small number. This limitation is a problem in the vitrification of embryos from polytocous animals. Commercial pig ET, for example, requires a method that can preserve a large number of embryos at one time (preferably between 20 and 40 embryos, which is the number of

Table 24.1 Comparative pregnancy rates after the transfer of cryopreserved (slow freezing or vitrification) and fresh in vitro-produced embryos in cattle over the last 10 years (table adapted from Ferré et al. (2020) [47])

Cryopreservation	Direct ET	Number of ETs	Pregnancy rate (%)	Reference
IVP				
Fresh	+	20	40	Inaba et al. (2011) [43]
VIT-Cryotop	+	9	44.4	
Fresh	+	12	41.6	Caamaño et al. (2015) [21]
VIT-Cryologic	+	12	41.6	
VIT-Cryotop	—	3392	36.6	Marinho et al. (2015) [42]
Fresh	+	259	51.3 ^b	Sanches et al. (2016) [14]
Slow freezing	+	311	40.2 ^b	
VIT ^{1,a}	—	234	35.9 ^b	
Fresh	+	109	41.3	Do et al. (2018) [44]
VIT-Cryotop	+	85	40.0	
Fresh	+	30	63.3	Gómez et al. (2020) [46]
Fresh	+	17	64.7	
Slow freezing	+	40	55.0	
Slow freezing	+	14	64.3	
VIT-Cryologic	—	47	61.7	
VIT-Cryologic	—	11	45.4	
Fresh	+	58	51.7	
Slow freezing	+	80	50.0	Gómez et al. (2020) [46]
Fresh	+	44	43	Oliveira et al. (2020) [48]
VIT-Cryologic	+	25	40	
IVD				
Fresh	+	55	49.1	Inaba et al. (2011) [43]
Slow freezing	+	62	45.2	
VIT-Cryotop	+	30	46.7	
Fresh	+	51	58.8	Zhang et al. (2015) [45]
VIT-0.25 mL straw	+	46	56.5	
Fresh	+	34	47.1	An et al. (2016) [49]
Slow freezing	+	71	35.2	
VIT-Microdroplet	—	78	39.7	

ET Embryo transfer, VIT Vitrification, IVP In vitro-produced, IVD In vivo-derived

¹The vitrification device is not specified

^{a,b}Significant differences $p < 0.05$

Table 24.2 Embryo transfer of vitrified sheep morulae and blastocysts (data are from publications of the last decade)

Cryopreservation system	No. of ETs ^a	No. of transferred embryos	Pregnancy rate (%)	Lambing rate (%) ^b	Reference
IVP					
VIT-OPS	75	149	–	45.0	Mara et al. (2013) [52]
IVP + IVD					
Slow-freezing	73	136	38.3	18.4	Santos-Neto et al. (2017) [50]
VIT-Cryotop	94	175	66.0	30.9	
VIT-spatula	76	126	32.9	16.7	
IVD					
VIT-micropipette	127	127	38.6	38.6	Gibbons et al. (2019) [54]
Slow freezing	17	35	~50 ^c	37.1	Khunmanee et al. (2020) [53]
VIT-0.25 mL straw	18	36	~60 ^c	36.1	

VIT Vitrification, ETs Embryo transfers, IVP In vitro-produced, IVD In vivo-derived

^aEmbryo transfers were performed by laparotomy [52–54] or laparoscopy [50]

^bPercentage of lambs born with respect to the number of embryos transferred

^cApproximate values according to the graph in the publication

Table 24.3 Embryo transfer of vitrified porcine embryos (data are from publications of the last decade)

Vitrification system	Stage	No. of ETs ^a	No. of embryos/ET	Pregnancy rate (%)	Farrowing rate (%) /litter size	Reference
IVP						
Fresh control	Morula	3	28–29	100	100/9	Maehara et al. (2012) [72]
Hollow fiber		3	28–29	100	100/5.6	
Hollow fiber	Blastocyst	3	21–25	100	100/9.7	Matsunari (2012) [31]
Cryotop	Blastocyst	3	30	100	100/4.7	Mito et al. (2015) [19]
Cryotop		3	30	33.3	33.3/4	
Cryotop	Zygote	8	114	25	25/7.5	Kamoshita et al. (2017) [32]
IVD						
SOPS	Blastocyst	10	35	50	50/10.4	Gomis et al. (2012) [73]
Cryotop	Blastocyst	5	66	40	40/4.5	Misumi et al. (2013) [69]
SOPS	Morula and blastocyst	40	30	75	75/9.6	Martínez et al. (2015) [65]
SOPS+NS-ET		36	30	38.9	38.9/5.7	
SOPS+NS-ET		33	40	77.8	72.7/9.5	
SOPS	Blastocysts	14	25	78.6	71.4/9.8	Cuello et al. (2016) [20]
MVAC	Morula and blastocyst	2	29	100	100/6	Sakagami et al. (2016) [74]
Metal mesh	Morula	5	20	60	20/1	Nakamura et al. (2017) [75]
OPS	Blastocyst	7	20–23	85.7	57.1/5.5	Tajima et al. (2020) [76]
MVAC+NS-ET	Blastocyst	7	110	42.9	42.9/2.3	Tajima et al. (2020) [66]

ETs Embryo transfers, IVP In vitro-produced, IVD In vivo-derived, MVAC Microvolume air cooling, NS-ET Nonsurgical embryo transfer

^aAll embryo transfers were performed surgically unless otherwise stated

Table 24.4 Embryo transfer of vitrified equine blastocysts (data are from publications of the last decade)

Vitrification system	Blastocyst size (treatment)	No. of ETs ^a	Pregnancy rate (%)	Reference
IVP				
Fresh control	<300 µm	47	74	Choi et al. (2017) [80]
Micropipette tips		29	59	
Cryolock	<300 µm	40	50	Canesin et al. (2020) [82]
IVD				
Cryolock	821 µm (PA)	6	83.3	Diaz et al. (2016) [84]
Micropipette tips	<300 µm	17	76	Choi et al. (2017) [80]
	>300 µm (PA)	11	55	
Cryolock	<550 µm			Wilsher et al. (2019) [86]
	(P)	12	75	
	(PA)	10	80	
	>550 µm			
Cryolock	(P)	10	10	Wilsher et al. (2021) [85]
	(PA)	18	72	
	<300 µm	10	80	
	300–560 µm			
	(P)	10	20	
	(None)	17	82	
	>560 µm			
	(P)	20	5	

ETs Embryo transfers, IVP In vitro-produced, IVD In vivo-derived, VIT Vitrification, P Punction of the blastocoel, PA Punction and aspiration of the blastocoel liquid

^aOne blastocyst was transferred to each recipient

embryos required for a potentially successful ET). A large volume of vitrification solution is needed to vitrify these many embryos in one unit, making it difficult to comply with the concept of MVC. Some attempts have been made to develop or adapt devices for the vitrification of large numbers of embryos while maintaining the MVC concept. In this sense, hollow fiber (HF) can hold up to 40 mouse or 20 pig embryos in less than 0.1 µL of solution [31]. Cryotop has also enabled the successful simultaneous vitrification of 20 pig embryos at different stages of development ([32]; González-Plaza unpublished data) in small microdroplets while maintaining the MVC principle. Even though vitrification was originally considered a cost-effective alternative to slow freezing, the situation has changed with the introduction of commercial vitrification kits and devices, which are very expensive [9]. In addition, it is important to consider that the cost–benefit ratio in animal breeding is not the same as that in human-assisted reproduction clinics. Therefore, one of the major challenges for vitrification of animal embryos is to develop cost-effective devices and devices that allow vitrification of large numbers of embryos in polytocous species while maintaining the MVC principle. Efforts are being made in this direction. For example, a low-cost vitrification system that allows simultaneous cryopreservation of 20–30 embryos has recently been developed and is being tested in different polytocous species (Marco-Jimenez et al. personal communication).



Fig. 24.1 Images of Cryotop (a, a'), open pulled straw (b, b') and Cryologic (c, c') containing a rabbit morula (a', b', and c'). Photos were kindly provided by Dr. Francisco Marco-Jimenez and Dr. WenBin Chen

24.4 Lipid Content and Cryotolerance

There is evidence that cellular lipids play an important role in the cryopreservation of embryos. This issue is particularly important in the cryopreservation of animal embryos because many species, such as Carnivora and Artiodactyla, have embryos that are rich in lipids, making cryopreservation challenging [33]. Both the amount and composition of lipids are important because they correlate with embryo survival after cryopreservation [33]. They are species-specific and depend on the developmental stage and origin of the embryo. Among livestock species, pig embryos have the highest proportion of lipids [33]. In relation to the embryonic stage, it has been shown in pigs that the content of triglycerides,

phospholipids, and cholesterol in lipid droplets is stable from zygote to morula but decreases at the blastocyst stage [34], which may lead to better cryopreservation ability. The origin of the embryo also determines the amount and composition of lipid droplets, which is higher in embryos produced in vitro [34]. In this sense, the composition of the culture media also influences the lipid profile and thus the cryotolerance of the embryos [35].

The mechanisms of chilling injury in lipid-rich embryos are still poorly understood. It has been suggested that when cooling is below the optimal temperature and above the freezing point, lipid-phase transition is the main cause of embryo damage [33]. The high cooling rates used in vitrification partially mitigate this problem. Therefore, vitrification has been

shown to be the only efficient method for cryopreservation of embryos with very high levels of lipids, such as porcine embryos or embryos produced *in vitro*. In addition to high cooling rates, various strategies have been tried to improve the vitrificability of lipid-rich embryos. In pigs, delipidation by micromanipulation after centrifugation increased the survival of vitrified porcine four-cell embryos and morulae generated by *in vitro* fertilization [36], and the combination of mechanical delipidation of four- to eight-cell-stage embryos and vitrification allowed the birth of first piglets after the transfer of IVP porcine embryos [37]. Additionally, delipidation and vitrification of morulae produced by somatic cell nuclear transfer (SCNT) transferred at the blastocyst stage were able to produce piglets [13]. Despite the efficacy of mechanical delipidation, this treatment should be avoided because it leads to disruption of the zona pellucida, which can damage embryos and poses a risk for disease transmission, and is also expensive and time-consuming. Furthermore, complete delipidation may not be advisable because the lipid droplets in the embryos are more than just energy stores. They are active organelles that can perform a range of functions [38] and are involved in embryo development and embryo-maternal interactions [33]. A more conservative alternative to mechanical delipidation is partial delipidation with solutions of high osmolarity and trypsin, which has been used to obtain piglets from vitrified IVP porcine embryos [39], or chemical induction of lipolysis [40], which has been shown to increase the vitrificability of IVP porcine blastocysts. These possibilities need to be explored, and further research on the cryobiological behavior of cellular lipids, i.e., cellular membranes and lipid droplets, is required to improve the vitrification of lipid-rich embryos, such as those generated *in vitro* or by SCNT.

24.5 Vitrification of Bovine Embryos

Considering the long reproductive cycle, small litter size, and high beef or milk mass per embryo, cattle are the most important livestock species for embryo cryopreservation [9]. Direct transfer of frozen embryos has become a routine technology for reproductive management in beef and dairy herds [41]. Conventional freezing is an effective method for preserving bovine morulae and blastocysts produced *in vivo* and *in vitro* [41, 42]. Vitrification was developed as an inexpensive, quick, and easy alternative to slow freezing [7]. Although vitrification is widely used for cryopreservation of human embryos and oocytes and is the method of choice for embryo cryopreservation in some livestock species, such as pigs, it has had limited commercial use in cattle. There are few controlled studies comparing the success rate of vitrification with that of slow freezing, but vitrification appears to be the most suitable method for cryopreservation of IVP embryos, which are much more cryosensitive, mainly due to their higher lipid content [9].

In the last decade, there have been few reports on the vitrification of bovine embryos produced *in vivo*, but the best and most consistent results have been obtained with the Cryotop system [42–44]. For embryos produced *in vivo*, similar pregnancy rates (~40–50%) have been achieved after the transfer of fresh and vitrified blastocysts [43, 45]. For IVP embryos, some studies reported transfers with blastocysts vitrified with Cryotop [43, 44], 0.25 mL straws [45], or Cryologic [21, 46], which have shown similar pregnancy rates (~40–50%) as transfers performed using their fresh counterparts. Interestingly, similar reproductive performance is achieved in cattle after the transfer of vitrified, *in vivo*-derived, or IVP blastocysts. Table 24.1 shows comparative results of transfers performed in recent years with fresh, frozen, and vitrified IVP embryos in cattle.

One of the major obstacles to the commercial use of vitrification in cattle has been traditional warming systems, which require the gradual removal of cryoprotectants, making direct transfer under field conditions difficult. However, in recent years, several protocols have been developed for warming *in-straw* and direct transfer of vitrified *in vivo* [43, 45] and in IVP embryos [21, 43]. All abovementioned studies showed similar pregnancy rates regardless of the method used for warming. The results of the direct warming method *in straw* allow the direct transfer of vitrified embryos and promote the application of vitrification under field conditions in cattle. In addition to investigation on the technical aspects of the warming and ET methods, research is also underway on how to improve the quality of IVP embryos and their cryopreservation resistance by modifying and adapting the *in vitro* culture conditions [47].

24.6 Vitrification of Small Ruminant Embryos

In small ruminants, as in cattle, slow freezing is still the most commonly used cryopreservation method for *in vivo*- and IVP embryos [50]. In recent years, most reports on the transfer of vitrified embryos from small ruminants relate to ovine species. Vitrification is as effective in sheep as conventional slow-freezing methods, and pregnancy and lambing rates (~45–50%) following transfer of vitrified embryos (Table 24.2) are comparable to those of embryos cryopreserved by slow freezing [51]. Embryo survival rates after vitrification are similar in both sheep and goats [52], but there are differences between species related to the optimal vitrification medium [51].

OPS vitrification has provided promising results following the transfer of *in vivo*-derived embryos [25], with pregnancy rates of ~70% and lambing rates of ~60%. *In vivo*-derived ovine embryos have also been successfully vitrified in mini-straws [53]. In this study, similar results were obtained after the transfer of ovine 2–8 cells or morulae-

blastocysts cryopreserved either by slow freezing or vitrification with pregnancy rates between 50% and 60%. Micropipette tips have also been used for vitrification of sheep embryos [54] with an overall pregnancy rate of 47.5% for transfers performed under extensive field conditions. Santos-Neto et al. [50] evaluated the efficiency of cryopreservation of sheep morulae and blastocysts produced in vivo and in vitro and cryopreserved either by slow freezing or by using MVC methods (Cryotop and spatula). These authors reported a better overall pregnancy rate after the transfer of in vivo-derived embryos (68.9% vs. 29.9%). The best results were obtained with the Cryotop method, with survival rates of 67.1% and 38.7% for in vivo-derived and IVP embryos, respectively, showing the superiority of vitrification with Cryotop compared to slow freezing and vitrification with spatulas [50]. Good results have also been reported in sheep after the transfer of IVP embryos vitrified with SOPS [52]. In this study, the influence of different culture conditions on reproductive outcomes after the transfer of IVP vitrified embryos was investigated. Pregnancy rates of ~45% were achieved in the best groups [52]. The development of vitrification of goat embryos is still at an early stage. In the last decade, the number of studies on the vitrification of goat embryos has been very limited and based on in vitro experiments. In goats, the same vitrification devices, such as OPS [55], Cryotop [56], and micropipette tips [57], have been used for the vitrification of both in vivo-derived and IVP embryos. The best results were reported after vitrification with Cryotop, with survival rates of 88.3% [56] after vitrification and warming of IVP embryos and 66.3–90% for SCNT blastocysts [58].

24.7 Vitrification of Porcine Embryos

From the early 2000s until today, vitrification has been developed as the only efficient alternative for cryopreservation of embryos in pigs [3, 59, 60]. Table 24.3 shows the results obtained after transfer of vitrified porcine embryos published in the last 10 years.

Progress in cryopreservation of porcine embryos has been limited due to their low cryotolerance, related to their high lipid content, and the technical difficulties of embryo collection and transfer in this species [3, 16]. In the last decade, considerable advances have been made in vitrification protocols, and numerous studies have investigated which factors are crucial for improving the survival of vitrified porcine embryos. Today, successful vitrification and transfer of porcine embryos are limited to in vivo-derived morulae and blastocysts, which are the most appropriate embryonic stages for commercial applications [3]. The best results have been obtained with blastocysts, which have the highest cryotolerance, probably due to their higher permeability to cryoprotectants [61] and lower lipid content [34]. SOPS is one of the most commonly used vitrification devices in pigs and has shown excellent in vitro viability with in vivo-derived morulae (>80%; [20, 62]) and blastocysts (>90%; [20, 63]) without any treatment prior to vitrification. Recently, excellent in vitro results have also been obtained with intact morulae and blastocysts vitrified with the Cryotop device (Cuello et al., unpublished data; Fig. 24.2). The SOPS vitrification process has been continuously improved through the development of chemically defined conditions [20] and the adaptation of the device to nonsurgical ET under field

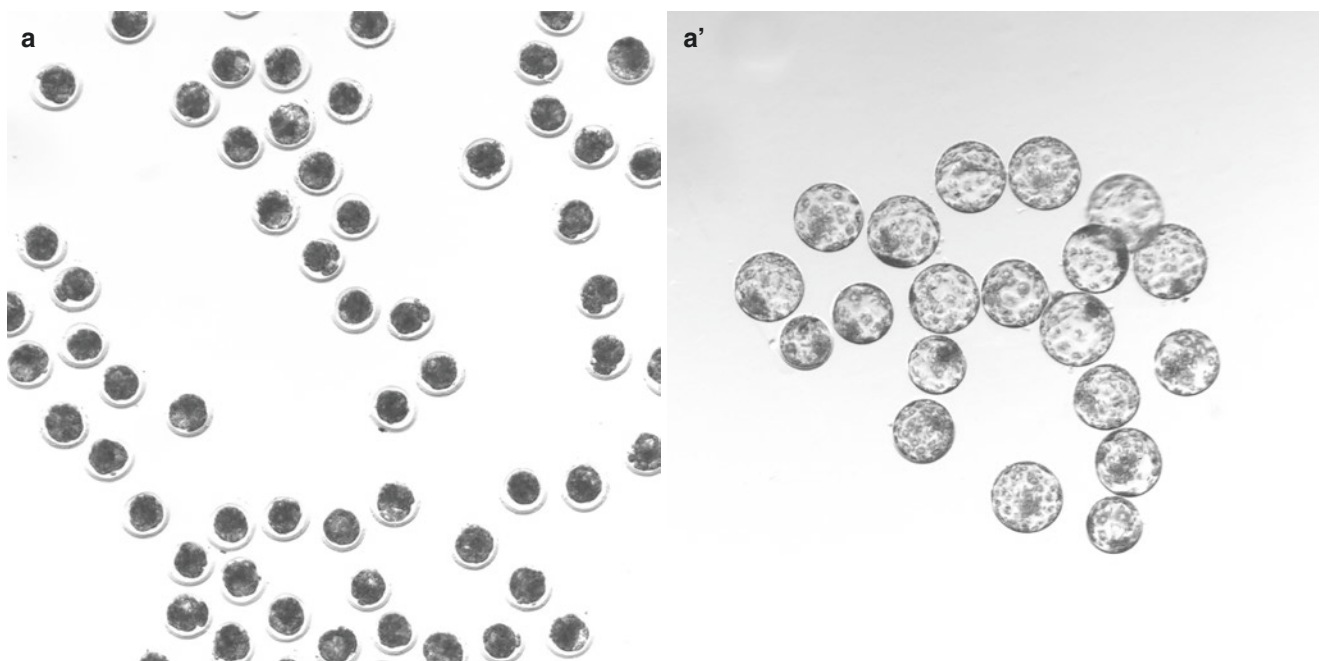


Fig. 24.2 Porcine fresh morulae (a) and blastocysts from morulae vitrified with Cryotop and cultured in vitro for 24 h after warming (a')

conditions [64], which represents a breakthrough for the practical application of this technology. SOPS vitrification has resulted in piglets born after the transfer of vitrified-warmed morulae and blastocysts, with acceptable fertility (~70%) and prolificacy (~9.5 piglets born) after surgical or nonsurgical transfers [20, 65]. Additionally, piglets have been born after nonsurgical transfers of in vivo-derived blastocysts vitrified by the microvolume air cooling (MVAC) method with farrowing rates between 33.3% [66] and 66.7% [67]. Literature on transfers of in vivo-derived porcine embryos vitrified with other systems is scarce, and only a small number of surgical transfers have been reported. Matsunari et al. [31] performed three ETs using morulae and early blastocysts vitrified with a hollow fiber (HF) device. The three recipients became pregnant and farrowed an average of 9.6 piglets. Despite these good results, there are no further studies, probably because the HF system is difficult to handle and presents technical storage difficulties due to its fragility, which limits its practical application. Piglets have also been obtained after surgical transfer of blastocysts vitrified with the MMV method (10 ETs: 70% pregnancy rate and an average of 5.3 piglets; [68]). Additionally, some attempts have been made to use closed systems that avoid direct contact of the samples with the liquid nitrogen, with worse results than for open systems. In this context, farrowing rates less than 20% were reported after surgical transfer of blastocysts vitrified using MVAC and cryogenic methods [69, 70].

Due to the high polyspermy rate and low embryo quality in vitro, pigs are not as efficient as cattle in in vitro embryo production [71]. In addition, vitrification of IVP embryos is much more complicated than that of embryos produced in vivo due to their higher cryosensitivity [34]. Although piglets have been obtained after the transfer of blastocysts derived from intact IVP morulae vitrified with the HF system [72], several studies suggest that some treatments, such as mechanical removal of lipids, chemical delipidation, lipid polarization, or induced collapse of the blastocoel, may be necessary to improve the vitrifiability of IVP embryos [16].

On the other hand, pigs have considerable importance as animal models or as potential organ donors for regenerative medicine and xenotransplants in humans, mainly since the development of nuclease zygote genome editing technology. For this application, the use of in vivo-derived zygotes is commonly preferred because the quality of zygotes is essential for their success. The development of an efficient vitrification method for these zygotes would be of undoubted value because it would facilitate the creation of a stock and its use when necessary. Although there are very few studies, in vivo-derived zygotes have been successfully vitrified, without special treatments, using the SOPS method, with a blastocyst formation rate of 43% after warming [77]. Studies on the vitrification of IVP zygotes using MVC or microdrop

systems or a novel CP (carboxylated ϵ -poly-L-lysine) have reported low (~20%) postwarming blastocyst formation rates [32, 78] and piglet production efficiency (number of piglets born to the number of transferred embryos) as low as 1.5% [32]. It is clear that more studies are necessary to improve the postwarming survival of this type of embryo.

24.8 Vitrification of Equine Embryos

Embryo transfer is commonly used in the equine industry to achieve pregnancies from subfertile mares or from competing mares and to obtain more than one foal from a mare in one breeding season. Even though ET is a common technology in equine breeding, only 2–5% of collected embryos are cryopreserved. Data from commercial embryo transfer stations reported a 50–60% pregnancy rate with vitrified-warmed equine embryos (Table 24.4). Although these pregnancy rates are similar to those achieved in cattle, there are some obstacles to the development of ET and embryo cryopreservation in this species. A major hurdle is that there is no efficient superovulation protocol. Therefore, the collection of embryos is based on only one ovulation; thus, no additional embryos are collected for cryopreservation [79]. In addition, the most important factor determining the ability of the equine embryo to survive cryopreservation is its size [79]. While there are difficulties in cryopreserving blastocysts larger than 300 μm in diameter, embryos smaller than 300 μm can be successfully cryopreserved by slow freezing or vitrification, resulting in pregnancy rates of 45–71% [80, 81]. Moreover, IVP blastocysts, which are usually approximately 300 μm , can also be vitrified, with pregnancy rates similar to those of their fresh counterparts (~45%; [80]). These pregnancy rates have been recently improved (~80%) after the transfer of IVP blastocysts using a semiclosed vitrification system, commercial media, and warming under field conditions [82]. Although vitrification of embryos with a diameter of less than 300 μm is preferable, their recovery is subject to some technical limitations. Equine embryos reach the uterus on days 5.5–6 of gestation with a diameter of 150–220 μm . These embryos increase in diameter (>300 μm) within 0.5–1 days and transform into blastocysts with a large blastocoele and an acellular capsule [79]. Therefore, the collection of morulae and early blastocyst-stage embryos (<300 μm) requires several ultrasound examinations during the mare's estrus to determine the exact time of ovulation. Strategies such as administration of hCG and collection of embryos 8 days later have also resulted in a high proportion of small blastocysts at collection [23]. The large volume of blastocysts with a diameter of more than 300 μm and the acellular glycoprotein capsule, whose thickness correlates with freezability, make them poorly permeable CPs, which conditions their cryoresistance [79].

Among the attempts made to improve the vitrification ability of large blastocysts, the most promising method has been to collapse the blastocoel and aspirate blastocoel fluid. The best results were achieved when more than 70% of the blastocoel fluid was removed [79]. With this approach, pregnancy rates of up to 71% (5/7; [83]) and 83% (5/6; [84]) were obtained after the transfer of day-6–8 vitrified expanded blastocysts. To enable vitrification of expanded blastocysts (>300 μm) in standard veterinary practice, there is a need for a simple device that can safely collapse the blastocoel without requiring a micromanipulator. In this sense, manual collapse of large blastocysts was reported by Wilsher et al. [85], who achieved an 82% (14/17) pregnancy rate after vitrification and transfer of large blastocysts less than 560 μm in diameter. These results show that this is a promising tool that must be explored.

24.9 Vitrification from Other Animal Species

Among the other species where vitrification has been applied, the murine and rabbit stand out because of their importance as experimental models in different areas of research, including embryo technologies [87, 88]. Mice and rabbits have also been used to test vitrification devices for their subsequent application in humans [12, 87].

Another animal family in which vitrification is of great interest is camelids. ET is a technology that is routinely used in breeding programs for dromedaries in Middle Eastern countries [89] and alpacas and llamas in Australia and South America [89, 90] to improve their reproductive output [91]. However, frozen embryos are not used commercially due to their low cryoresistance [89]. Vitrification could be an alternative method to vitrify camelid embryos, which are highly cryosensitive due to their high lipid content [91]. Despite the low pregnancy rates (10–50%), live births have been achieved after the transfer of dromedary, llama, and alpaca embryos. Although vitrification has also been suggested as an important tool for the conservation and multiplication of genetic resources of wildlife and endangered species, the possibilities of vitrification are still limited due to the lack of embryology background in this species [92]. Strategies for gene resource banking for rare and endangered animal species will be addressed in a forthcoming chapter.

24.10 Conclusion

Vitrification of animal embryos has made considerable progress and is constantly being improved as an important tool for the cryopreservation of animal embryos. Currently, vitrification is proposed as the most suitable method for cryo-

preservation of highly cryosensitive embryos such as transgenic, IVP, or SCNT embryos, porcine embryos, or those in early stages of development. In all livestock species, vitrification gives similar or better results than slow freezing. However, we believe that in the near future, it is imperative to do some work to overcome the shortcomings of vitrification that limit its commercial application.

24.11 Author Perspectives

Some possible areas of research to increase the efficiency and applicability of vitrification include standardization of protocols, equipment, and storage systems. Automation of vitrification should also be implemented, because currently the skills of the person performing the process have a major impact on the results. For the practical application of vitrification, the optimization of procedures for the direct transfer of vitrified embryos under field conditions is an important issue. The development of devices that allow simultaneous vitrification of large numbers of embryos is also important for polytocous species. Biosafety aspects of open vitrification devices and storage should be addressed. Several treatments to increase the vitrificability of embryos, including the treatment of large equine blastocysts, are also being investigated. In addition, the susceptibility of IVP embryos to low temperatures is reduced by exploring in vitro culture conditions. We believe that the use of vitrified embryos will be a routine alternative for breeding programs in most livestock species in the near future.

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Cryopreservation Effect on Genetic/Epigenetic Function: Neonatal Outcomes

25

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Key Points

- Neonates conceived by frozen embryo transfers are more likely to have higher birth weights and experience complications related to maternal preeclampsia and pregnancy-related hypertensive diseases compared to fresh embryo transfers.
- The incidence of imprinting disorders is higher in ART, and it has been demonstrated that cryopreservation induces epigenetic changes in the oocyte, sperm, and embryo. However, it is suggested that the diagnosis of infertility and not cryopreservation nor ART increases this risk.
- Some data is suggestive of possible adverse neonatal impact from cryopreservation including increased risk of infection, as well as respiratory and neurologic disorders. No increase in chromosomal nor non-chromosomal defects has been shown.

25.1 Introduction

Over 8 million children have been born by assisted reproductive technology (ART), and many of them were conceived after a frozen embryo transfer (FET) [1]. The transfer of cryopreserved embryos has been associated with an improvement of live birth rate in specific populations, such as high responders to ovarian stimulation compared to fresh embryo

transfers [2]. FETs also significantly decrease risk of ovarian hyperstimulation syndrome, a serious complication of ART [3]. Beyond live birth rates and hyperstimulation, there are differences noted in both maternal and neonatal outcomes between fresh and frozen embryo transfers. Singletons conceived by FET had a higher risk of being large for gestational age, while children conceived by fresh embryo transfers were at risk for low birth or very low birth weight [4–6]. Additionally, women who underwent a programmed FET with artificial preparation of the endometrium with estrogen and progesterone supplementation without an ovulatory cycle were at increased risk for preeclampsia during pregnancy compared to fresh transfers [7–11].

Likewise, when comparing differences between IVF and naturally conceived pregnancies, IVF pregnancies have a higher incidence of preeclampsia, preterm delivery, and term low birth weight [8, 12–14]. Although these findings are associated with multiple gestation, a known risk of ART, these adverse events remained even in singleton pregnancies. It is reassuring that although children born after ART have a lower birthweight, data have suggested no difference in height, weight, or BMI by adolescence compared to those who were not born of ART [15]. This finding however does not negate the possible long-term impact of neonatal morbidities such as low birth weight, preterm birth, and preeclampsia on adulthood health, which include an increased risk for metabolic and cardiovascular diseases. This leads to the hypothesis that not only can IVF impact neonatal and pregnancy outcomes, but that specifically the process of cryopreserving embryos may play role in these observed changes. Therefore, despite the advantages of FETs, cryopreservation has its own set of consequences and may contribute to the adverse neonatal outcomes observed in ART.

Success of IVF has relied on the improvements in laboratory techniques, with significant improvement in embryo culture and cryopreservation resulting in the transition to blastocyst instead of cleavage stage transfers and the increasing usage of trophectoderm biopsy for pre-implantation genetic testing (PGT). The modern process of cryopreserva-

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tion, including the transition from slow freezing to vitrification, has played a critical role in this change in ART practice. Evidence has shown that vitrification is more efficient and can produce more consistent results in embryo survival and viability after thawing. Although an optimized slow freezing processes can still produce excellent results, vitrification has now become standard practice in most ART labs [16]. The principle behind successful embryo freezing and survival is the balance between the cooling rate, the toxicity of cryoprotectants, and the reduction of intracellular ice formation [17–21]. The introduction of higher concentrations of cryoprotectants to replace water content in the vitrification process allows for sufficiently lower temperatures that intracellular ice does not form. This has improved embryo survival rates [22]. Due to the need for higher concentrations of cryoprotectants, the use of multiple solutes was introduced to reduce the cellular toxicity of any single cryoprotectant [23, 24]. Embryo survival has also improved with the adaptation of rapid freezing through direct contact with liquid nitrogen [25]. This effective cryopreservation process has improved embryo viability allowing for long-term storage for multiple clinical purposes, including fertility preservation, decreasing the need for multiple IVF treatments, as well as the use of embryo biopsy for PGT.

One of the consequences of multiple embryo manipulations include epigenetic and genetic alterations. One hypothesis suggests that vitrification and embryo biopsy disrupt DNA methylation and histone posttranslational modification leading to adverse pregnancy and neonatal outcomes as previously discussed above. Indeed, epigenetic changes have been observed in children conceived by ART [26]. Some data have linked epigenetic changes not to the diagnosis of infertility but to the ART procedures themselves [27].

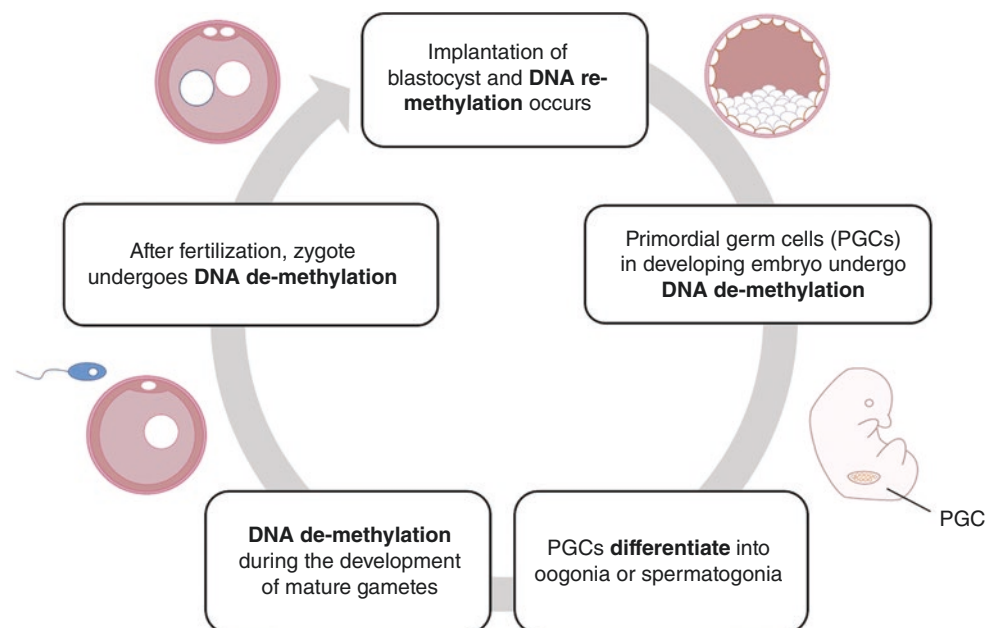
Additionally, there have been historical concerns of the possible increased risk for birth defects and chromosomal abnormalities after embryo manipulation. In this chapter, we will address the impact of embryo manipulation and cryopreservation on both epigenetic and genetic function and how these changes can affect neonatal outcomes. At this time, current evidence for a long-term impact of epigenetic changes due to cryopreservation is unclear, but it remains important to understand due to its importance for public health now and in generations to come.

25.2 Embryo Manipulation and Epigenetics (1000)

Normal epigenetic programming occurs in two distinct phases. Prior to differentiating into a gamete, primordial germ cells have similar genetic expression as somatic cell lines due to epigenetic programming. In the first phase during gametogenesis, DNA methylation patterns are erased and then re-methylation occurs into the appropriate imprinting pattern based on parental origin of the chromosomes (during oogenesis or spermatogenesis). After the parental origin of the gametes is established, the second important phase of epigenetic reprogramming occurs after fertilization in which an embryo then has its unique epigenetic profile established by both demethylation and re-methylation [28] (Fig. 25.1).

Ovarian stimulation, in addition to the additional processes, such as in vitro fertilization or intracytoplasmic sperm injection (ICSI), embryo culture, and blastomere or trophectoderm biopsy, performed prior to cryopreserving an embryo, are all manipulations that may trigger an embryo's stress response. These adaptive changes can include epigen-

Fig. 25.1 DNA methylation programming



etic alterations, which may manifest through abnormal development and susceptibility toward particular diseases in adulthood [29]. Although this chapter will focus on the cryopreservation effect on genetic and epigenetic function and the neonate, the current literature has not yet completely extricated the effect of cryopreservation on epigenetics from other laboratory manipulations that are inherent in the ART process and may also play a key role.

Common epigenetic mechanisms include DNA methylation, histone posttranslational modifications, and microRNA (miRNA) regulation [30]. DNA methylation and demethylation, or the addition or removal of a methyl group to a nucleobase, regulate gene expression and are vital in the programmed development and implantation of embryos. In mammals, DNA methylation events typically occur on cytosines in CpG islands and direct tissue-specific gene expression, genomic imprinting, and X chromosome inactivation [30, 31]. DNA methylation affects how tightly histones are bound to DNA affecting gene expression and repression. Other histone modifications include acetylation, ubiquitination, and phosphorylation, which all affect transcription in a similar manner. Furthermore, DNA methylation can regulate the expression of miRNA. miRNA regulation of gene expression is an epigenetic mechanism that utilizes non-coding RNA to bind to target messenger RNA causing either RNA cleavage and degradation or direct inhibition of RNA translation. These three mechanisms are also interdependent regulating each other's activities [30]. It is not surprising that aberrations in programmed DNA methylation, histone posttranslational modifications, and miRNA activity can result in a disruption in normal development. There have been associations of alterations in DNA methylation with genomic instability leading to malignancy and increased risk for imprinting defects such as those seen in Beckwith-Wiedemann syndrome and Angelman syndrome [32]. Whether these disease processes are due to ART itself or to the fact that ART permits the growth of embryos that would otherwise not progress to successful implantation is not fully understood. Regardless of these hypotheses and concerns, we do not fully understand how these epigenetic alterations manifest themselves phenotypically and it is uncertain if they play any significant role at all in the ART population. Much of the data is limited and inconclusive.

For example, when studying common DNA methylation regions (DMRs) in both placenta and cord blood of infants conceived by either ovulation induction or in vitro fertilization, there were small but significant differences in DNA methylation common to Beckwith-Wiedemann syndrome. However, it did not change the transcriptional levels of the genes next to the DMRs suggesting that with proper gene expression the risk of imprinting disorders does not increase [33]. Other large epidemiologic studies also support this negative conclusion [34–36], yet others do not [37–39]. This leads some to believe that the disturbances in genomic

imprinting seen in ART are associated with a subset of children who are more susceptible to these changes [40].

25.3 Controlled Ovarian Stimulation

Supraphysiologic hormonal milieu observed in controlled ovarian stimulation may contribute to the epigenetic changes seen in oocytes and embryos. These epigenetic changes have been observed in superovulated oocytes in mouse embryos [41–45], and similar alterations were observed in human oocytes [42, 46, 47]. Even in this, the data are mixed; one study did not find these methylation changes and suggested that these alterations occur after fertilization [48]. The preponderance of evidence, however, suggests that superovulation itself plays a significant, if separate, role in epigenetic alterations in ART compared to cryopreservation.

25.4 Intracytoplasmic Sperm Injection

Fertilization is performed in IVF either through conventional in vitro insemination or intracytoplasmic sperm injection (ICSI). ICSI is commonly performed in IVF centers worldwide accounting for nearly 65% of all cycles [1]. ICSI differs from conventional methodology in which a single viable sperm is injected directly into the mature oocyte for fertilization. Some previous observational case series have suggested that the use of ICSI is associated with increased risk for epigenetic changes, especially through maternal DNA methylation, causing imprinting errors in children conceived by ART [49, 50]. However, when studied retrospectively, it appeared that perhaps it was the oocyte environmental changes during superovulation that triggers the imprinting defects [51, 52]. Regardless of conventional insemination or ICSI, there did not appear to be a difference in the amount of DNA methylation errors. The manipulation of the embryo via ICSI itself compared to conventional in vitro insemination does not increase DNA methylation and epigenetic changes [53]. However, long-term follow-up of offspring in future studies is recommended.

25.5 Assisted Hatching and Embryo Biopsy

Assisted hatching (AH) creates an artificial opening in the zona pellucida in effort to improve implantation of the embryo. It is a technique that is also used to obtain trophoctoderm cells for embryo biopsy for pregenetic testing. It is hypothesized that the environmental stress that embryos undergo during AH and biopsy can induce epigenetic changes especially if thermal laser energy is utilized. Evidence is limited regarding the impact of AH in human embryos but current data in mouse embryos are inconsistent. Laser disruption of the zona pellucida is the most common technique for

AH. One study did not show a significant change in the epigenetic signature of the mouse embryos with laser AH [54]. When laser drilling (complete breach) was compared to laser thinning of the zona in mouse embryos, manipulation by either method induced changes in the DNA methylation compared to the control group in the blastocysts, placenta, and the mouse offspring [55]. There are no known studies regarding epigenetic changes with other AH techniques such as mechanical drilling or chemical AH [56, 57].

25.6 Embryo Culture

During embryo development and implantation, programmed epigenetic changes occur in multiple phases for parental-specific gene activation, cell differentiation, and metabolism. Therefore, embryo culture and the environment can affect its epigenetic programming. Tight regulation in culture composition and oxygen concentration is important for embryo growth and metabolism [58]. In animal models, suboptimal in vitro culture induced epigenetic changes as well as phenotypic changes in the offspring and the placenta. Phenotypic changes observed in these animal studies include glucose intolerance, subfertility, behavioral and neurodevelopmental deficiencies [58]. However, despite these concerns, ART in humans compared to animals is not associated with a significant increase in imprinting disorders. The low absolute risk for imprinting defects in humans conceived by ART may be due to the robustness of human embryos and optimization of embryo culture over the years [59].

25.7 Vitricification and Thaw

The process of vitricification can be used to store both oocytes and embryos. The manipulations involved in the freeze/thaw cycle have been shown in superovulated mouse metaphase II oocytes to decrease methylation levels; specifically, *H19*, *Peg3*, and *Snrpn* differentially methylated regions (DMRs) were hypomethylated in mouse blastocysts after oocyte vitricification compared to blastocysts resulting from control oocytes [60]. Additionally, a separate study found that with increasing manipulations of the mouse blastocyst, whether it is a single vitricification/thaw or double vitricification/thaw, with or without biopsy, there was greater disruption of gene expression, as assessed by microarray. This study specifically found disruption of genes related to innate immunity, apoptosis, and mitochondrial function related to additional vitricification and biopsy, in addition to disruption in cytoskeletal function as assessed by immunohistochemical staining [61]. However, it is difficult to isolate the effects of embryo freezing/thawing process from superovulation. One unique study utilized a novel mouse model to examine these effects independently. The authors found that superovulation itself

and not vitricification led to decreased fetal weight and differences in placental vasculogenesis. They also found that gene expression did not differ in term placentae resulting from blastocysts transferred fresh or following vitricification [62]. This model is difficult to replicate in humans due to the nature of in vitro fertilization. In a human study, where Day 3 cleavage embryos were embryos biopsied, vitricified, and thawed, there were no differences between CpG methylated levels in the *H19/IGF2* DMR between vitricified and non-vitricified blastocyst [63].

25.8 Epigenetic Changes After Cryopreservation

Although superovulation itself is suggested to induce epigenetic changes, there is evidence that the additional manipulation after superovulation continues to induce more defects in the embryo as well. With the cumulative changes, it is unclear how this is clinically significant in the newborn and during pregnancy. In a study examining DNA methylation profiles in bloodspots of twins, those conceived by ART and IUI were significantly altered compared to naturally conceived newborns, particular at the *H19* DMR [64]. Specifically, methylation changes in newborns conceived by cryopreserved embryos that underwent ICSI or IUI were similar suggesting that both the diagnosis of infertility (as measured by IUI) and manipulation of cryopreservation/ICSI have long-term effects on the epigenetics of a child [64]. Despite this finding, it is interesting that in a study of global methylation patterns from human placentae utilizing the LUMA and LINE-1 assays, that placentae from deliveries resulting from frozen embryos did not have significant global methylation difference from placentae formed from natural conception, while those from fresh transferred were significantly different [65]. It is important to note that freezing technology may differ in these human studies as ART labs shifted between slow freezing and vitricification. In a mouse model, cryopreservation via slow freezing induced the most significant changes in gene expression of the embryos compared to vitricification and fresh embryos [66].

It is generally accepted that there are global changes in DNA methylation with ART. However, to understand the relevance of these changes, focusing on changes observed in unique epigenes has been important. Targeted epigenes for maternal hypomethylation and global DNA methylation levels studied in animals and human embryo studies are outlined in Table 25.1.

One of these epigenes include *GRB10* (growth factor receptor-bound protein 10) which is a maternally inherited negative regulator of insulin-like growth factors. With normal function, this allele limits the placental volume and fetal growth [71]. Therefore, when disrupted in a mouse model,

Table 25.1 Targeted epigenes affected by vitrification

Epigene	Description	Associated phenotype	Location [67]	Methylated allele of origin
Grb10	Growth factor receptor bond protein 10	Placental overgrowth and fetal LGA (Silver-Russel Syndrome or Beckwith-Wiedemann)	7p11.2-p12	Maternal
H19/IGF2	H19 imprinted maternally expressed transcript /insulin-like growth factor 2	Large offspring syndrome in sheep [68] Potential difference in birth weight with IVF [69]	11p15.5	Paternal/maternal
PEG1/MEST	Paternally expressed gene 1/mesoderm specific transcript	IUGR and abnormal maternal behavior toward offspring [70]	7q32.2	Maternal
GNAS	Guanine nucleotide binding protein, alpha stimulating	Pseudohypoparathyroidism	20q13.2	Maternal

the lack of inhibition led to pups being born 30% larger than wildtype [72]. When the mouse blastocysts were vitrified, there was an aberration in the expression of *Grb10*, which was significantly lower in in vitro blastocysts compared to fresh blastocysts and in vivo blastocysts [73]. This correlates with findings in humans, where singletons conceived by FETs had a higher risk for being large for gestational age [4, 5]. Deletion of *GRB10* has been associated with Beckwith-Wiedemann syndrome phenotype, characterized by overgrowth with macrosomia, macroglossia, hemihyperplasia, and organomegaly [74]. Duplication or maternal uniparental disomy of *GRB10* has been associated with Silver-Russell syndrome's undergrowth, including decreased weight at birth that is prevalent throughout adulthood and abnormal cognitive development [75]. However, findings for methylation changes for *Grb10* have been inconsistent.

Another important epigenetic region that has been under investigation is chromosome 11p15.5 imprinted region. This imprinted region has two domains that is controlled by imprinting center 2 or KvDMR1. Normally this region is methylated only on the maternally inherited chromosome. Domain 1 involves *IGF2* (insulin-growth factor 2) and *H19*, a gene for non-coding RNA, and is distal from domain 2, which includes *CDKN1C* (cyclin dependent kinase inhibitor 1C), *KCNQ1* (potassium voltage-gated channel subfamily Q member 1), and *KCNQ1OT1* (*KCNQ1* overlapping transcript 1), a gene within *KCNQ1* for non-coding RNA. A normally imprinted region would express paternal *IGF2* and maternal *H19*. However, abnormal imprinting that leads to Beckwith-Wiedemann syndrome includes the methylation of *KCNQ1* locus that leads to the expression of *IGF2* in both maternal and parental alleles, instead of only paternal *IGF2*. Another mechanism that occurs in 50% of patients with Beckwith-Wiedemann syndrome includes the loss of methylation within this region that leads to decreased expression of *CDKN1C* [76]. Vitrification of mouse embryos or oocytes have resulted in notably abnormal methylation at *KvDMR1* upstream from *H19* and *IGF2* [60, 77, 78]. Similar findings have been observed in bovine studies as well [79, 80]. However, in humans, frozen embryo transfers did not appear to be associated with methylation changes in *H19/IGF2* nor *KCNQ1OT1* in placenta compared to fresh embryo transfers.

And, regardless of how the pregnancy was conceived, the neonatal cord blood did not show any differences in these imprinted areas as well [81]. Even when the human embryos obtained by IVF were vitrified on day 3, there were no changes in methylation in *H19/IGF2* compared to controls [63]. These findings suggest that although there is an increased risk of Beckwith-Wiedemann syndrome by 10-fold with ART [82], it may not be due to aberrant methylation but rather by a separate mechanism.

Other epigenetic changes including histone modifications and miRNA expression may also be affected by cryopreservation. Studies regarding histone modifications after vitrification were performed in oocytes and none in embryos to our knowledge. Vitrification of mature mouse oocytes showed changes in histone 3 lysine 9 (H3K9) methylation, a marker of condensed DNA that is transcriptionally silent, and H4K5 acetylation, a modification implicated in active transcription and mitosis [83]. However additional studies are needed to further elucidate if these changes have any clinical relevance in ART population. Studies conducted in a mouse model found that there is a difference in miRNA expression in vitrified and fresh mouse embryos that regulate implantation and embryo development [84–86]. In a study in human blastocysts, miRNA expression of miR-let-7a, which regulates implantation of the embryo, and miR-16, a regulator of programmed cell death and proliferation, were significantly reduced with vitrification and even further reduced with re-vitrification compared to fresh blastocysts [87]. This adds to the evidence that additional manipulations to embryos result in increased epigenetic disturbances. Whether these changes persist and are the mechanism for the clinical difference seen in neonatal outcomes following frozen and fresh embryo transfer is unclear.

25.9 Gamete Cryopreservation

Epigenetic reprogramming occurs in two phases during the life of a gamete. The first is during the gametogenesis phase, and the second is after fertilization [88]. Therefore, the possibility of cryopreservation causing epigenetic disturbances in the gamete needs to be considered.

25.9.1 Sperm Cryopreservation

With the effects of cryopreservation on the embryo, it is important to consider the effects of cryopreservation on gametes as well. Men with normal semen analysis had high levels of global DNA methylation, while men with infertility and abnormal semen analysis were more likely to have hypomethylated DMRs, including *H19* and *IGF2* [89]. When abnormal methylation was observed in the sperm of infertile men, there was an increase in poor pregnancy outcomes utilizing the sperm, [90] with lower embryo quality and birth weight following ART [91]. However, it is not uncommon in IVF cycles to have sperm frozen prior to oocyte retrieval, especially in male factor infertility, in the event fresh sperm cannot be retrieved on the day of the procedure. In addition, sperm cryopreservation is indicated for fertility preservation in patients undergoing chemotherapy or pelvic radiation. Overall, the data is limited regarding the effect of cryopreservation on sperm. Post-thaw sperm is generally lower quality than fresh sperm with loss of motile viable sperm. Abnormal methylation levels were observed to be significant in animal models including cryopreserved boar [92] and murine semen [93]. Additionally, fertilization was significantly decreased with lower fertilization rates and decreased blastulation rates with frozen then fresh murine sperm [93]. In humans, it has been shown that cryopreservation of human sperm can affect the methylation and gene expression of sperm associated with male fertility [94]. This may raise concerns regarding the safety of cryopreserving sperm. When nine important spermatozoa genes were analyzed, including maternally and paternally imprinted genes associated with abnormal semen parameters, there were no changes in methylation whether the sperm was fresh or frozen for up to 4 weeks [95] suggesting that there is no clear clinical significance of routinely freezing sperm.

25.9.2 Oocyte Cryopreservation

Oocyte cryopreservation is commonly used for fertility preservation in patients who are undergoing gonadotoxic treatment or who are not yet ready to build a family but desire to protect themselves from age-related decline in egg quality. Similar to the data surrounding embryo vitrification, the evidence regarding the effect of cryopreservation of oocytes on epigenetics is mixed. In one animal study, there was overall reduction in global methylation in both the mature vitrified mouse oocytes and the resulting embryos [96]. More specifically, the resultant blastocysts had decreased methylation of *H19*, *Peg3* (a gene involved in cell apoptosis and tumor suppressor activity), and *Snrpn* (a gene suspected to be associated with Prader-Willi and Angelman syndrome) compared to blastocysts that resulted from fresh oocytes [60]. When stud-

ied in human oocytes, global DNA methylation was not different in embryos derived from vitrified or fresh oocytes demonstrating the safety of oocyte vitrification [97]. Methylation profiles of *H19* and *KCNQ1OT1* did not change even after in vitro maturation when vitrified in the germinal vesical stage and thawed [98]. Other than DNA methylation, there were also changes in histone modifications and miRNA after vitrification of mouse oocytes, although studies in human oocytes are limited [83, 99]. Whether these epigenetic changes have any clinical impact is still under investigation.

25.10 Ovarian Tissue Cryopreservation and In Vitro Maturation

Ovarian tissue cryopreservation is no longer an experimental procedure and has growing availability to oncologic patients. Often, oncologic patients do not have time to delay life-saving treatment to undergo ovarian stimulation. Therefore, ovarian tissue cryopreservation has become an option for fertility preservation. However, mouse offspring conceived from oocytes of transplanted cryopreserved ovarian tissue has noted to have increased changes in methylation compared to transplanted fresh ovarian tissue or naturally conception [100]. Whether the tissue was frozen via vitrification or slow freezing method, *IGF2R*, *H19*, and *PLAGL1* had significant changes in methylation compared to control groups. However, despite these epigenetic differences, no phenotypic differences defined as birth defects, weight gain, fatigue, and exercise capacity were observed in the mouse offspring [100].

Another potential option for fertility preservation is in vitro maturation which requires little to no exogenous gonadotropin stimulation and can be completed in any phase of the menstrual cycle. The method of oocyte maturation does not appear to affect DNA methylation profile [101]. In a single study, whether the oocytes were matured in vivo, in vitro without vitrification, or in vitro after vitrification and thaw at the germinal vesicle stage, there was no difference in global DNA methylation. Despite no epigenetic differences, there were higher abnormalities in the spindle fibers and chromosomes in the third group [101].

25.11 Neonatal and Long-Term Childhood Outcomes After Cryopreservation

Although many studies have focused on the clinical pregnancy and live birth outcomes with ART, there has been growing concern regarding the increased risk of adverse neonatal and long-term childhood outcomes with ART. ART is known to increase the risk for poor neonatal outcomes such as prematurity and term low birth weight in singletons [102].

However, long-term outcomes such as malignancy, chronic disease, and behavior and learning disorders are inconclusive but remain areas of investigation [103]. One of the concerns is whether ART-induced methylation changes are the cause for the differences in these outcomes. Although altered methylation has been demonstrated in children born after ART at birth and during childhood [104, 105], it has not been shown that these methylation changes are the cause for the differences in neonatal outcomes. In fact, studies have shown that although aberrant methylation has been noted at birth, these changes for the most part do not persist later in life [105, 106]. One study compared the methylation profile in serum at birth versus 9 years old [105] and another at birth versus adulthood from ages 22 to 35 years [106]. Although there are a multitude of epigenes that are involved in development, there is a paucity of data connecting subclinical epigenetic changes to the long-term health and development of children born after ART.

One particular topic of interest is whether cryopreservation itself is the culprit for the differences in neonatal outcomes. Although there are known differences in outcomes between frozen and fresh embryo transfers, these differences have not been attributed to cryopreservation-induced methylation changes. There are multiple other factors that can affect these outcomes including endometrial preparation and hormonal environment of the endometrium after ovarian stimulation that are confounding factors. A large retrospective study found that the differences observed in newborn weight are due to ovarian stimulation rather than vitrification by studying fresh and frozen embryo transfers in autologous and donor recipients [107]. Specifically, recent studies have suggested that programmed FET cycles, compared to FET cycles with natural ovulatory or stimulated ovulatory preparations, are associated with higher risks of perinatal morbidity including preeclampsia as well as neonatal outcomes including macrosomia, suggesting that the hormonal environment, rather than cryopreservation, may play a larger role [10].

Additionally, methylation differences have been observed in placentae from IVF-conceived pregnancies compared to placentas from spontaneously conceived pregnancies, including at *H19*, *MEST*, *KCNQ1OT1*, and *LINE1* methylated regions [65, 81, 108]. Even though the placentae differ between IVF-conceived and spontaneously conceived, no differences were observed in placentae of newborns conceived by frozen embryo transfer compared to those conceived spontaneously in multiple studies [65, 81]. Interestingly, cord blood methylation profiles of neonates born from frozen embryo transfers did not differ from IUI-conceived [64] nor spontaneously conceived pregnancies [81]. If cryopreservation affects neonatal outcomes, it is likely due to its effect of the placenta on fetal development rather than directly on the fetus itself. In fact, there is a significant increased risk of pregnancy-induced hypertension

and placenta accreta in frozen embryo transfers than fresh transfers [109].

There is, however, a noted increase in large for gestational age (LGA) infants born following frozen embryo transfer compared to fresh embryo transfer, which has been observed in multiple studies [5, 6, 110–113]. The etiology of this has not been elucidated. Animal studies have long noted an overgrowth syndrome following IVF and embryo culture in species such as cattle, sheep, and, less commonly, mice [114, 115]; however, in humans, LGA has been specifically observed following cryopreservation. Whether this is an effect of cryopreservation or the hormonal environment is yet to be determined; and if an effect of cryopreservation, the potential epigenetic mechanism has yet to be elucidated [110].

In addition to neonatal outcomes, long-term effects from frozen embryo transfers have also been studied. From a cardiovascular standpoint, fetal echocardiograms completed on IVF pregnancies suggest that there are early cardiac changes including thicker myocardial wall and lower systolic and diastolic function in both frozen and fresh embryo transfer [116]. However, in a study examining children ages 8–9 years, they did not have significant differences in cardiovascular health whether conceived spontaneously or by fresh or frozen embryo transfer [117]. Also, with the risk of pregnancy-induced hypertension with frozen transfers, children born from women with pregnancy-induced hypertension have higher blood pressures observed as early as 19 months and as late as 5 years old [118]. Offspring risk for hypertension and chronic kidney disease is elevated [119].

Additionally, children born from frozen embryo transfers may also have a slight increased risk of malignancy. There is a small but increased incidence of leukemia in children born after frozen embryo transfers versus spontaneously [120]. Lastly, there was no difference in the risk of congenital defects between fresh and frozen embryo transfers [121, 122] but there are small increased odds of respiratory, neurological, and infectious diseases among children born from frozen embryo transfers [122].

25.12 Genetics and Cryopreservation

Although still controversial, cryopreservation has the potential to increase cytotoxicity by inducing DNA breaks. In human ovarian tissues, a systematic review and meta-analysis demonstrated that the protocol utilized matters, and that overall, vitrification had significantly lower likelihood of DNA damage than slow freezing [123]. It can also result in damage in embryos as seen in animal studies leading to decreased embryo development [124, 125]. Similarly, compromises in DNA integrity can also occur in human sperm [126, 127] as well as changes in gene expression in human

oocytes [128] after cryopreservation. Additionally, there are concerns that cryopreservation may affect the meiotic spindle and normal cell division. Although the majority of vitrified and thawed embryos develop normally, vitrified embryos were more likely to have abnormal spindles than fresh embryos [129].

Although there is an overall small but significant increase in risk in birth defects in children conceived by ART [130], current evidence does not show an increased risk for chromosomal abnormalities with cryopreservation. In a large study of 14,491 neonates comparing those who were conceived by frozen versus fresh embryo transfer, there was no increased odds for either chromosomal or non-chromosomal birth defects [122]. Multiple other studies support this finding and the safety of cryopreservation [131, 132]. Similarly, in terms of cryopreserved oocytes, it has been demonstrated that there was no significant difference in aneuploidy rates or pregnancy rates from human embryos derived from vitrified or fresh oocytes [133]. The American Society for Reproductive Medicine supports oocyte cryopreservation and current research does not support a concern for increased risk of genetic defects in children conceived from frozen oocytes [134].

25.13 Potential Areas of Research

As alluded to throughout this chapter, additional research is needed to fully understand the clinical implications of cryopreservation-induced epigenetic changes, including neonatal outcomes and long-term adult health issues such as malignancy and cardiovascular or metabolic diseases. This can help us understand further the role of particular epigenes on the development and implantation of the embryo as well as long-term health. Other issues that need additional investigation include the role of maternal and paternal age, the diagnosis of infertility, and specific laboratory manipulations of the gametes and embryos on the risk of epigenetic disturbances. Although there is evidence that epigenetics changes do occur outside of normal programming with ART, whether they persist and are clinically relevant remains an important question.

25.14 Conclusion

Overall, there is research suggesting that cryopreservation of gametes and embryos does result in epigenetic changes, including alternations in methylation. However, it is unknown if and how these epigenetic changes manifest themselves clinically. It is also difficult to separate the epigenetic effects of cryopreservation from those of other ART manipulations,

as well as the non-epigenetic impact of ART manipulations on neonatal outcomes. Current evidence suggests that epigenetic alterations appear to be attenuated at birth and through early childhood, and the adverse long-term health outcomes of children born from cryopreserved embryos are limited. Although current data suggest that the absolute risk overall is low and cryopreservation is a safe practice in IVF, more research is needed to further our understanding of the impact of cryopreservation on epigenetic alternations and long-term health.

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Recent Advances in Fertility Preservation and Genome Resource Banking for Rare and Endangered Animal Species

26

Pierre Comizzoli and William V. Holt

Key Points

- Fertility preservation and germplasm cryo-banking efforts in wildlife are still limited to few species (mainly mammals) and cell types (spermatozoa) because of the lack of fundamental knowledge in wildlife reproductive biology and cryobiology, as well as the limited access to individuals for research and development.
- While slow freezing of semen is the most common approach, ultra-rapid freezing and optimized reanimation of tissues will soon enhance genome resource banking efforts in wildlife.
- Fertility preservation and genome resource banking are highly relevant to wildlife conservation if properly integrated into the management of animal populations in breeding centers and in the wild.
- The use of new biotechnologies to produce germplasms and embryos from frozen somatic cells of wild animal species should be carefully analyzed while considering important ethical and welfare aspects.

26.1 Why Do We Need Fertility Preservation and Genome Resource Banking in Wildlife Conservation?

Studies and maintenance of biological and genetic diversity are critical to ensure survival of animal species in their natural environment. Unfortunately, habitat degradation, overexploitation of natural resources, pollution, emerging diseases, invasive alien animals and plants, and climate change have led to a considerable loss of biodiversity [1]. In terms of con-

servation actions, the protection of large-size natural habitats is an important strategy to preserve genetically diverse species or populations, but it is not sufficient. Too often, wildlife populations have become too small to be self-sustainable and are highly vulnerable to inbreeding depression as well as environmental catastrophes and epidemics. Based on the characterization of wild species biology, the critical set of conservation actions is to propagate small animal populations in their natural habitats (in situ) or in conservation breeding centers (ex situ) for eventual reintroduction in the wild. In that area, there are specific advantages to preserve and extend fertility, largely for animals that have not yet produced enough descendants to ensure the passing on of their genes to the next generations. Specific targets include individuals that (i) are dying before puberty, (ii) are living but fail to natural reproduce, (iii) unexpectedly die, or (iv) are nearing reproductive senescence, because there is value in rescuing and re-infusing genes from these individuals into the current populations [2].

So far, studies characterizing reproduction in wild animal species have revealed an extensive diversity in traits and physiological mechanisms. This diversity also translates into differences in conditions required for the handling, cryopreservation, and in vitro culture of germplasms (gametes, embryos, and gonadal tissues) [3]. Thus, development of assisted reproductive techniques, fertility preservation strategies, and germplasm freezing for long term (genome resource banking) must take into account that diversity to successfully produce enough individuals for subsequent relocation or reintroduction in their habitat [3, 4].

Regarding genome resource banking, the value of maintaining data-rich biological samples (including somatic cells, blood products, and DNA samples in addition to germplasms) has long been recognized for human medicine. It is also a fundamental component of most basic scientific research as well as population management tool for livestock species [5]. Reproductive biologists studying wildlife have greatly benefited from advances in the human infertility and livestock production fields. However, the

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overall goals of these programs are substantially different—overcoming infertility (humans) versus more efficient/higher quality food production (livestock) versus retention of all gene diversity (wildlife). Nowadays, wildlife genome resource banks can be found on all continents [5, 6]. All biobanks contain samples from multiple species that are often collected opportunistically during routine health examinations, captures/releases, or necropsies. The first goal is to create banks of biomaterials that can be used to study and understand the biology of species. This is also a convenient way to provide open access to rare and valuable biomaterials to qualified researchers who have no access to wild species or normally work outside the conventional mainstream of wildlife conservation biology. The second goal is to actively use the genome resource banks to preserve the fertility of genetically valuable individuals through assisted reproductive techniques, such as artificial insemination, in vitro fertilization, and embryo transfer. With the help of fertility preservation and biobanks, there is also the possibility to only move germplasm and embryos instead of live animals between conservation sites (in situ or ex situ) to maintain the same levels of heterozygosity [5].

Currently, there are real-life illustrations of using biobanks for conservation breeding. The iconic giant panda is routinely managed in ex situ collections and on a large-scale in China using artificial insemination with fresh and frozen-thawed spermatozoa [7]. The black-footed ferret, once the most endangered species in North America, has also been recovered and restored by a combination of natural mating and artificial insemination, including with sperm that had been frozen and stored for up to two decades [8]. There also are many examples of “milestone” births using frozen-thawed spermatozoa or even embryos with the incidence of success completely dependent on having an excellent understanding of the target species’ reproductive physiology [9, 10].

The objective of this chapter is to review recent advances in wildlife fertility preservation and biobanking of germplasm. Note that embryo freezing is not covered in this chapter as it does not represent a primary biobanking effort for maintaining genetic diversity in wild animal populations.

26.2 Recent Advances in Slow Freezing and Vitrification for Fertility Preservation in Wildlife

26.2.1 Semen Can Be Successfully Frozen from Corals to Elephants

Sperm biology studies and sperm cryobiology (ejaculates or epididymal flushing) represent most of the germplasm preservation efforts in wildlife species. A better understanding of

sperm biology in multiple species over the past decades has helped to develop optimal handling of sperm cells, survival after cryopreservation, and successful fertilization and embryo development (after artificial insemination or in vitro fertilization) [11].

Slow freezing (around $-40^{\circ}\text{C}/\text{min}$) has been and remains the most common method. Even though successful sperm freezing has been reported in a very broad spectrum of species (from corals to elephants), progress is still limited to a very small proportion of animal species [6, 12]. Offspring have been produced through artificial insemination or in vitro fertilization using frozen semen in >50 mammalian species, but only few of those species are managed through the extensive use of sperm cryopreservation and assisted reproductive techniques (namely the black-footed ferret and giant panda) [3]. Most of the successes have been reported in mammals because semen cryopreservation is still highly challenging in other groups like birds [13]. Semen cryopreservation and artificial fertilization are now being reported in amphibian species as well, but a very small number of species have been studied so far [14]. Sperm freezing in wild fish species is rarely reported but few pioneering studies have paved the road for more successes in the future [15]. Interestingly, recent studies in fish (studying the trout model) or amphibians have demonstrated that sperm cryopreservation may reduce the growth of resulting offspring [16, 17].

In addition to slow freezing procedures, directional freezing has been explored to cryopreserve large semen volume from elephants or rhinoceroses [18]. The technique controls ice crystal growth by orienting the ice formation in one direction, which creates more space between the damaging ice crystals for cells to survive the freezing process. Effectiveness of ultra-rapid freezing (over $-2000^{\circ}\text{C}/\text{min}$) as an alternative to slow freezing has also been explored in different species [19], including in fish [20, 21], and showed no differences with conventional freezing.

26.2.2 Oocyte Freezing Has Not Been Reported Yet

Besides laboratory species, livestock, or companion animals, successful oocyte freezing leading to viable embryos after in vitro fertilization has not been reported in any wild species. In addition to the usual challenges to freeze oocytes (large size and fragile structures [2]), main limiting factors of success in wildlife are the access to enough oocytes to understand the cryobiology and optimize the protocols, which considerably slows down the progress. Importantly, freezing fully grown oocytes is impossible in species like fishes, amphibians, reptiles, or birds because of the very large size, the yolk content, and the cellular membranes that are not permeable to cryoprotectants [22].

26.2.3 Testicular Tissue Cryopreservation Is a Powerful Tool for Male Fertility Preservation

Preservation of testicular tissues (post-mortem or after orchiectomy for medical reasons) is the best option when semen cannot be collected when prepubertal individuals or adult males that are outside of their breeding season die unexpectedly. The age of the animal is still the most representative factor for the successful preservation of testicular fragments, with tissues from young individuals being more homogeneous and more resilient to freezing temperatures than from sexually mature animals [11]. Testicular biopsies on live animals are not performed in wild species because of possible damages to the gonads and the risk of infections after the procedure. Testicular tissues contain a vast number of germ cells, including at early stages (spermatogonia and spermatocytes), that can potentially develop into viable spermatozoa after grafting or culture *in vitro*. Resulting sperm cells then can be used for assisted reproductive techniques. After tissue dissection, testicular fragments can be cryopreserved using slow or ultra-rapid freezing, or even vitrification methods. The composition of cryopreservation media can vary according to species-specific characteristics, especially regarding the cryoprotectant type and concentration. Species-specific cryopreservation protocols have been explored in rodents, felids, cervids, tayassuids, and bovids [23, 24].

26.2.4 Ovarian Tissue Freezing for Female Fertility Preservation

In addition to the lack of precise knowledge about reproductive physiology in many wild species, there are still few comparative studies on follicular development. Studies related to the recovery of primordial and primary follicles have been conducted only in few wild species,

but improved culture systems and the possibility promote development of these follicles after thawing are promising [25]. Regarding cryopreservation, protocols developed to date are efficient for storing preantral follicles in some wild animal species; however, it is necessary to refine protocols for a more optimal storage through systematic approaches [25]. In non-mammalian species, the development of germ cell transplantation techniques for propagating fish species, most commonly farmed species such as salmonids, has been gaining interest among conservation scientists as a means of regenerating endangered species [26]. Cryo-banking of primordial germ cells might be the only options, not only for fishes, but also for wild birds and amphibians to preserve female fertility [3].

26.2.5 Cryopreservation of Somatic Cells and Other Sources of Germplasms

Freezing somatic cells for nuclear transfer (cloning) in wild animal species has been supported by the successful application of that advanced technology for several domestic mammals, including horses, rabbits, mice, and dogs [27]. Somatic cell cryopreservation also appears less challenging than for germplasms. Although some births have been reported in wild species several years ago [28, 29], the cloning procedure itself has a low success rate and cloned mammals frequently suffer from developmental abnormalities [30].

Stem cell technologies have often been recognized as promising methods for producing gametes from embryonic stem cells, spermatogonial progenitors, or from differentiated cells [22]. However, recent studies reporting the production of induced pluripotent stem cells (iPS) from northern white rhinoceroses or wild felids show that they remain challenging for gamete production [31, 32]. More research efforts are still needed to cryo-bank those cells, derive gametes from it, and consistently produce embryos.

26.3 Recent Advances in Ancillary and Alternate Approaches Relevant to Wildlife Fertility Preservation and Biobanking

26.3.1 Customized Thawing or Warming Processes Followed by Optimized Use of Germplasms

Mitigation solutions have been developed mainly in mammals to improve sperm recovery and reanimation post-thawing. Despite the advancements in germplasm cryopreservation, more research has to be conducted to improve outcomes and reduce variability, especially post-vitrification. Cryopreserved sample recovery depends on rapid heat penetration during warming to avoid ice recrystallization. For instance, rapid warming of testicular tissues has been explored in the domestic cat model [33, 34]. Recent development of ultra-rapid warming using lasers has improved survival of large frozen specimens preloaded with wavelength-absorbing materials (India ink or nanoparticles). Immediately upon removal from liquid nitrogen, the sample can be exposed to a laser beam which evenly warms the tissue via the dispersed nanoparticles. This approach has resulted in improved viability of zebrafish embryos [35] and coral larvae [36]. Recent success in laser warming also has been reported on cat reproductive cells and tissues [37].

Regarding fertility preservation, strategies used to ensure human reproductive health have inspired efforts in conserva-

tion biology [2]. Rescuing gonadal tissues in vertebrate species implies the availability of efficient *in vitro* tissue culture to produce mature gametes, a technique that is still in its infancy, even in laboratory and domestic species [24, 25]. Species-specificities mentioned for the preservation of samples also apply to the manipulation and culture of tissues and gametes as well as the many complexities related to *in vitro* fertilization, selecting/managing recipients, and conducting embryo transfer. Despite the possibilities of using xenografts for sperm or follicle development, *in vitro* culture has become a more attractive tool since it may help to better understand and monitor the complete spermatogenesis or folliculogenesis in adult individuals. Although *in vitro* conditions have to be optimized, the approach is less constraining than xenografts in immunodeficient rodents [2, 24, 25].

26.3.2 Alternate Approaches for Germplasm Preservation

Germplasm structures and functions are currently stabilized by freezing temperatures. This state of “suspended animation” ensures long-term longevity and quality of cellular and molecular components. Before, during, and after cryopreservation, samples are exposed to stresses related to toxic cryoprotectant(s), cooling/freezing, detrimental ice formation, and thawing/rewarming. As mentioned earlier, resilience of tissues, cells, organelles, and DNA depends on protocols that have to be adapted for each type of tissue and each species [22]. Moreover, liquid nitrogen tanks have to be permanently filled and maintained, electrical freezers operating at -80°C require a lot of energy, and staff are needed for security, preparing and keeping track of samples, and maintaining databases. Recently, some attention has been directed to alternate approaches for storing any type of germplasms for long term at ambient temperatures. The research has been driven by examples of small invertebrate organisms (nematodes, tardigrades, insects, and brine shrimp) that can survive extreme cellular water loss for extended periods of time through a process called “anhydrobiosis,” by down-regulating their metabolism and synthesizing intracellular key protectants, including trehalose [38]. In these cases, cellular and molecular activities can be suspended above freezing temperatures. Knowledge from anhydrobiotic invertebrates has been translated and adapted to mammalian cells over the last decades [39]. Currently, sample drying in the presence of trehalose is mainly achieved by freeze-drying (lyophilization), which is the sublimation of ice at low atmospheric pressure. Embryo production or live births following intra-cytoplasmic sperm injection with frozen-dried sperm in the mouse or livestock species has been reported [40]. More recently, microwave-assisted desiccation (without preliminary freezing) in the

presence of trehalose has been explored using the domestic cat model. Dried sperm was stored in sealed pockets at temperature above freezing for several weeks and proved led to successful embryo development after rehydration and injection into oocytes [41]. Partial dehydration of cumulus-oocyte complexes can also preserve cellular functions in the domestic cat model [42]. Desiccating and storing germplasms at ambient temperatures will offer many advantages (simple, flexible, and cost-effective options). However, despite promising progress, more research is still required to develop optimal conditions of non-cryogenic storage in wild species.

26.4 Status of the Integration of Fertility Preservation and Genome Resource Banking into Wildlife Conservation Efforts

The integration of gamete and embryo cryopreservation activities into wildlife conservation is relatively rare, albeit increasing. The giant panda and black-footed ferret breeding programs cited above are routinely including sperm cryopreservation as part of their efforts to lose as little genetic diversity as possible and keep it available for the future. Recent models in the marsupial species have demonstrated the same benefits [43]. Efforts to impede the loss of threatened amphibian species via gamete preservation and biobanking are being particularly active at present, especially in Australia, the Americas, and Russia [44]. There is still a need to develop strategic, yet species-specific, approaches to the collection of amphibian sperm via the administration of reproductive hormones for the control of gamete release, and also the use of ultrasound for ovulation detection [45]. These are very basic yet important issues, without which it would be impossible to develop a widely applicable set of fertility preservation strategies.

Large biobanks containing germinal and somatic cells can be found worldwide (Table 26.1). As previously mentioned, the practical drawbacks associated with the establishment and maintenance of biobanks that aim to support conservation breeding programs are usually centered on the financial burdens involved in their management. However, it is noteworthy that current research based on economic modelling has shown that it is financially beneficial to integrate the use of frozen and banked spermatozoa into captive breeding programs as a conservation strategy [46]. When the financial model was applied to the captive breeding program for the Oregon spotted frog (*Rana pretiosa*), it revealed that significant cost savings would be achieved by combining genome storage and the planned use of stored spermatozoa in the breeding program.

The integration of cryo-banking programs with conservation activities can be considered from two distinct perspec-

Table 26.1 Examples of major biobanking efforts for germinal and somatic cells from wild animal species

Institution/Consortium	Location
Smithsonian Institution https://www.si.edu/psci	USA and Panama
San Diego Frozen Zoo® https://science.sandiegozoo.org/resources/frozen-zoo%C2%AE	USA
Kunming Wild Animal Cell Bank http://english.kiz.cas.cn/gre/skl_Facility/	China
'Time Capsule' at the National Institute for Environmental Studies https://www.nies.go.jp/timecaps1/indexE.htm	Japan
Conservation Genome Resource Bank for Korean Wildlife http://www.cgrb.org/	Korea
FAUNA Research Alliance https://faunaresearchalliance.com/fauna/projects/faunabank/	Australia
The Frozen Ark https://www.frozenark.org/	Consortium based in the United Kingdom
CryoArks https://www.cryoarks.org/	Consortium based in the United Kingdom
Biobank of the European Association of Zoos and Aquaria https://www.eaza.net/conservation/research/eaza-biobank/	European consortium

tives: (a) are the programs focused on providing genetic support for conservation breeding in zoos and wildlife parks? or (b) whether the aim is to support wild, but dwindling populations in their natural environment. When the aim is to provide genetic support for captive bred animals, it is likely that the populations are healthy and well managed. Zoos and wildlife parks have a long history of involvement in genetic management programs and often hold detailed information in studbooks and databases about the breeding histories of the animals in their care. Modern software for pedigree and demographic analysis allows studbook keepers to make informed decisions aimed at minimizing inbreeding depression and often involves the exchange and transport of animals between different organizations [1]. Within this scenario, cryopreserved gametes, embryos, and other genetic materials are ideally incorporated into the breeding plans and treated as if they were whole animals. Indeed, if the genetic materials are intact and have been well maintained, the animals in question may have died decades previously. One of the most important considerations in relation to this scenario is that the preserved materials are not infected by unwanted microorganisms that could lead to disease transmission [47]. This can be a major problem if cryopreserved materials are to be transferred between locations, especially if international transport is required. Veterinary authorities that govern the international transport of semen and embryos from domestic species such as cattle, pigs, and sheep, have

well-defined protocols for the avoidance of disease transmission (manual of the International Embryo Technology Society <https://www.iets.org/Publications/IETS-Manual>), but these are much less well defined for wild species. Regulatory authorities will demand evidence that cells and tissues are not infected, especially if there are any risks to their local agriculturally important species. The requisite evidence may therefore be very difficult to provide without extensive veterinary intervention and testing. In addition, protection of genetic resources against the unfair exploitation of intellectual property rights has to be taken into account [48]. The Nagoya protocol obliges researchers, managers, and industrial companies to follow strict rules for the international transport of frozen gametes, embryos and stem cells, and other animal components (there needs to be binding agreements between the sending and receiving countries). The use of genome resource banks for “within-country” animal breeding programs is therefore more practical and easier than programs involving import and export. In such scenarios, there is less need to emphasize precautions about infectious disease transmission between localities, although it obviously remains important.

26.5 Caveats and Ethical/Welfare Considerations

As biotechnology advances, it opens new and exciting opportunities for species conservation. In the 1960s, shortly after the development of sperm cryopreservation methods, artificial insemination with frozen semen from threatened species was widely regarded as a solution to captive breeding problems in zoos and wildlife parks. The development of embryo transfer technologies for domestic animals followed shortly afterwards, and the revolutionary production of the cloned sheep “Dolly” cemented the popular belief that reproductive technologies would be widely used for genetic management and the avoidance of inbreeding depression. More recently, it has been possible to clone a few wild species, in some cases using cryopreserved and easily obtained somatic cells such as fibroblasts. Advanced culture methods have enabled the production of sperm and oocytes from stem cells created by the genetic manipulation of somatic cells; however, there is a growing sense that such techniques should be treated with some caution and widely evaluated before being widely adopted for animals and even for humans [49].

Advocates of advanced biotechnologies for animal conservation have frequently stimulated lively ethical debates about how to judge both the feasibility and desirability of applying their methods to projects involving the restoration of lost genetic diversity within small and vulnerable populations, and

Critical Information to Consider before Using Frozen Somatic Cells for Wild Animal Breeding	
✓	Is the species identification reliable?
✓	What are the available metadata (e.g., donor age, sex, collection date and location, tissue type, live biopsy, postmortem samples)?
✓	What are the characteristics of the donor cells?
✓	Do established cell lines exist?
✓	Can the cells replicate well in culture?
✓	Are the chromosomes normal and is the epi/genome intact?
✓	Are the cells free of contamination?

Fig. 26.1 Main practical issues to be considered when planning to exploit cryopreserved somatic cells for de novo production of gametes, embryos, and induced pluripotent stem cells

Key Ethical Questions that Need Answers when Planning a Conservation Breeding Program Including Advanced Biotechnologies	
✓	What is the scientific merit of the project?
✓	Is it related to the ecological benefit to the target species?
✓	What is the ecological value to the wild population if successful?
✓	Does the project only benefit zoo populations?
✓	What is the social value of the project?
✓	Does it benefit local communities?
✓	Does the project involve live animal procedures?
✓	Does the project involve invasive procedures (if yes, is there a harm/benefit analysis?)

Fig. 26.2 Main ethical questions to be considered before commencing an animal breeding project that involves advanced assisted reproductive technologies

even the potential recovery of extinct species [29, 50]. However, there are factors to consider when planning to exploit frozen somatic cells for cloning or stem cell technologies (Fig. 26.1).

Recently, Sandler et al. [51] proposed a detailed set of criteria for evaluating the ethical desirability of undertaking animal breeding projects that involve advanced technologies such as nuclear transfer and the exploitation of stem cells. They suggested that the initial ethical review should involve three assessment stages: (a) assessment of the goals of the project; (b) are the means available to achieve those goals? and (c) what is the ecological benefit if the goals are achieved? As the technologies are usually rather invasive the next phase of the assessment should involve a rigorous harm/benefit analysis (Fig. 26.2). In countries such as the UK this step is extremely crucial; if the project proponents cannot convince the ethical review board that the project is worthwhile, they would be denied permission to take it forward. The analysis from Sandler

et al. [51] includes many other factors, including public acceptability of the project and whether there would be any scientific benefits.

Importantly, systematic biobanking of germplasms remains the highest priority to address short-term needs in fertility preservation and rescue of rare species. The return on investment in terms of producing more individuals and building faster sustainable population remains higher with germplasms than with somatic cells.

26.6 Conclusions and Perspectives

Semen cryopreservation still represents the vast majority of efforts in germplasm preservation research in wild animal species. There are few reports on frozen oocytes but advances in gonadal tissue preservation are significant. It is now possible to preserve almost any germplasm from a genetically valuable animal of any age or reproductive state using reasonably simple, affordable techniques. However, there are still vast needs in basic cryobiological studies in many species. Progress can only be made if we continue to explore basic gamete and gonad biology to identify appropriate quality criteria before and after freezing. In some cases, progress has been made by relying on data already available in domesticated counterparts (e.g., cattle for antelope, dog for wild canids, or cat for wild felids). Interestingly, inter-species (mammals vs. non-mammals, carnivores vs. ungulates) as well as inter-individual variations or sometimes commonalities also can be remarkable. The recovery of species as diverse as the whooping crane, black-footed ferret, and giant panda owe at least partial credit to modern reproductive science and tools in fertility preservation and genome resource banking.

While there is no doubt that slow freezing protocols will continue to be broadly used, it is expected that more field-friendly vitrification or ultra-rapid freezing approaches for wildlife germplasms will be developed in the coming decade. Novel and likely more cost-effective strategies, like desiccation, will be explored and developed. Lastly, research priorities should also include exploring the developmental potential of early gamete stages within gonadal tissues, developing in vitro culture systems to secure more mature stages, and determining how stem cells can be converted into gametes that can produce viable embryos.

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The End of the Ice Age and the Start of the Space Age: The Freeze-Drying of Gametes

27

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27.1 Introduction

Genetic resources are assets for the future of humanity. Although all genetic traits are not required for survival, these traits must be preserved to ensure the species' survival in the event of the spread of unknown diseases or environmental changes, such as global warming [1]. The routine storage of spermatozoa in liquid nitrogen (LN2) for extended periods requires a constant supply of LN2. Additionally, LN2 may not be easily accessible in certain regions of the world. Therefore, it would be ideal if spermatozoa could be stored indefinitely at ambient temperature, and numerous attempts have been made to achieve this.

Polge et al. first attempted to freeze-dry spermatozoa by combining fowl sperm with glycerol, followed by freeze-drying while removing 90% of the water content [2]. Up to 50% of spermatozoa regained motility after being rehydrated, but their fertility was never determined. However, many crucial technical passages were omitted from the

paper; additionally, glycerol cannot dry, making it a substance that remains liquid at room temperature and is, more importantly, extremely toxic. Subsequent attempts to freeze-dry viable human and bull spermatozoa failed, with a few exceptions [3, 4]. Then, Yushchenko reported the birth of 12 normal bunny rabbit offspring using freeze-dried spermatozoa [5], and Meryman [6, 7] reported that cows became pregnant after artificial insemination with freeze-dried spermatozoa, but these results have not been confirmed by other groups [8]. In stark contrast to the largely successful long-term storage of viruses, bacteria, and yeasts by freeze-drying, these results indicate that this method is not suitable for long-term storage of mammalian spermatozoa.

Therefore, we attempted to completely dehydrate mouse spermatozoa in glass ampoules (Fig. 27.1a) and store them at room temperature or in the refrigerator without glycerol for several months. Due to the lack of a cryoprotectant, the cell membranes of the FD sperm ruptured, and all spermatozoa died during freezing or shortly after the addition of water [9]. However, since the development of mouse intracytoplasmic sperm injection (ICSI) techniques in 1995, it has become possible to produce offspring using ICSI, even from spermatozoa that have died [10, 11]. Therefore, we attempted ICSI on mouse oocytes with FD sperm. Surprisingly, even though all spermatozoa died after freeze-drying, their DNA remained intact, and healthy offspring were produced when spermatozoa were injected into oocytes [9]. This was the first time that mammalian spermatozoa preserved at room temperature for more than 1 month successfully produced offspring. In addition to rats, hamsters, rabbits, and horses [12–15], this technique has been used in other species. The ability to store FD sperm at room temperature would allow us to reduce costs and increase safety. Even in developing nations, it is now possible to preserve their genetic resources.

Thus, freeze-drying technology of mammalian germ cells will be essential for our future, but it is not yet widely used. This section will discuss the current issues and benefits of FD sperm.

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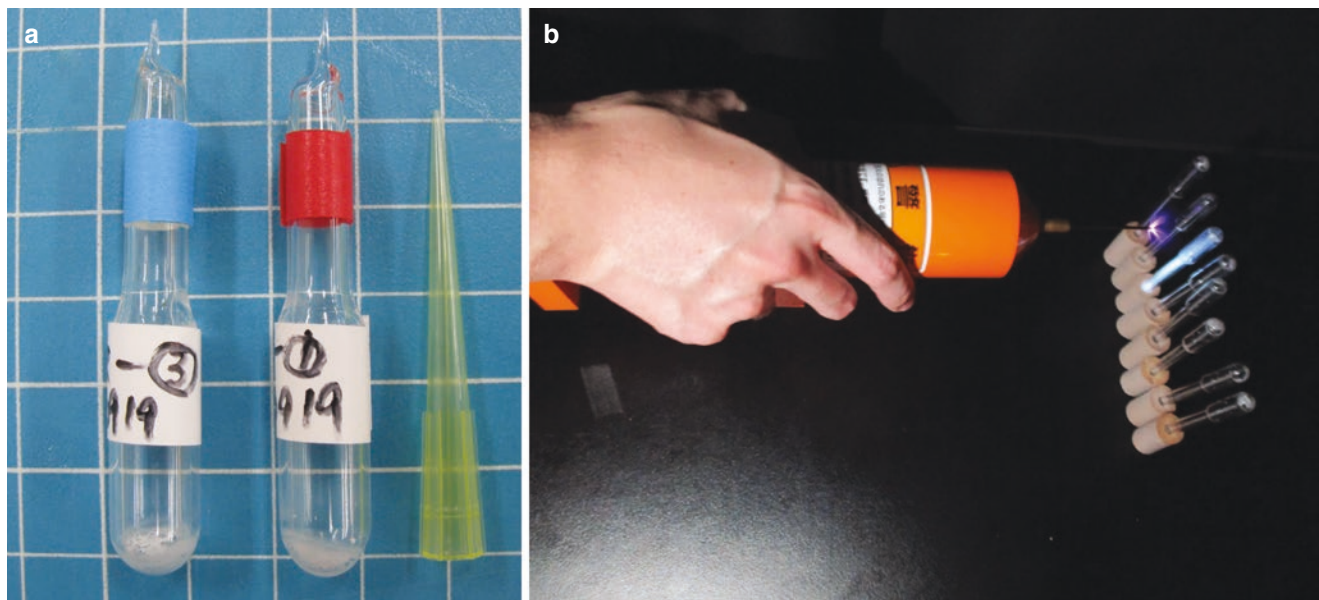


Fig. 27.1 Ampoules of FD sperm and detection of trapped air by non-destructive assay. (a) Comparison of the size of glass ampoule. (b) Tesla coil leak detector and ampoules of positive. If the ampoule

contains only a small amount of residual air, its ionization produces a spark inside the ampoule. Photo credit: Daiyu Ito, University of Yamanashi

27.2 Problems with Freeze-Drying of Spermatozoa

Studies have outlined several reasons for the poor implementation of germ cell preservation technology. Here we have outlined the three major issues with FD sperm that must be resolved for its global dissemination.

27.2.1 Freeze-Drying Sperm Death Following Freeze-Drying Treatment

The biggest problem with FD treatment of spermatozoa is that after FD treatment, all spermatozoa die. Cryoprotectants are required to freeze all cells, including spermatozoa, but standard cryoprotectants, such as ethylene glycol and EDTA, cannot be used as they cannot be dried. Therefore, in the current FD treatment, all spermatozoa are killed by freezing. When FD-treated sperm were observed under an electron microscope, the membranes of the sperm cells were destroyed [9]. Consequently, ICSI is required to produce offspring using FD sperm when used to conserve genetic resources. Moreover, the birth rate of embryos fertilized with FD sperm is substantially reduced due to the abundance of abnormal spermatozoa [16].

As such, apart from FD, other methods, including convective drying, evaporative drying, microwave drying, and heat drying, have been attempted. However, none of these methods have been able to increase the birth rate [17, 18]. Recently, we have succeeded in producing healthy offspring

using the vacuum drying (VD) method in which the spermatozoa were not frozen before the drying process [19]. This experiment revealed that the low birth rate of embryos derived from VD sperm is a result of the drying process instead of freezing. Additionally, it has become evident that rapid drying causes more DNA damage to the sperm. Thus, we must determine where and what damage occurs during the drying process and then find ways to enhance it.

27.2.2 Preservation of Freeze-Drying Sperm for Longer Periods at Room Temperature

If FD sperm are to be preserved as a genetic resource, they must reliably produce offspring when necessary. Conventional cryopreservation techniques using LN_2 are reliable because germ cells can be preserved indefinitely as long as LN_2 is continually replenished. It is currently unknown how long FD sperm can be stored at room temperature or in the refrigerator. Kawase et al. applied the theory of accelerated degradation kinetics to FD sperm and estimated that the maximum storage time at room temperature at only 1 year [20]. However, although these data were merely obtained from simulation results, the current FD method has vastly improved. For example, spermatozoa preserved by evaporative drying in a LiCl sorption jar with trehalose as a cryoprotectant was used to produce healthy offspring after 2 years of storage at ambient temperatures for 2 years [21].

Our data also demonstrated that maintaining a vacuum in ampoules is essential for preserving the viability of FD spermatozoa, as the stability of spermatozoa DNA increased when imperfectly vacuumed ampoules were detected using a non-destructive test and discarded (Fig. 27.1b) [22]. Although it should be possible to extend the period of preservation at room temperature with future technological advancements, such as the addition of an appropriate cryoprotectant [23], even if the mouse strain is weak. Recently, we have been able to produce offspring from FD sperm stored at room temperature for 6 years (unpublished data). However, long-term preservation experiments can only be facilitated by long-term preservation, so we must continue until obtain the reliability.

27.2.3 Storage Container of Freeze-Drying Sperm

Additionally, the integrity of the sperm storage container must be considered. The current method requires the use of fragile, expensive, and cumbersome glass ampoules that are difficult to store or transport. In fact, when we examined glass ampoules containing FD sperm after several months of storage, some ampoules were broken, and the FD sperm contained in these broken ampoules was no longer usable [22]. Subsequently, we devised a technique for preserving FD sperm in inexpensive commercially available PCR tubes [24] or thin plastic sheets (Fig. 27.2a–c) to address this issue. Its DNA integrity was comparable to that of spermatozoa stored in glass ampoules, and healthy off-

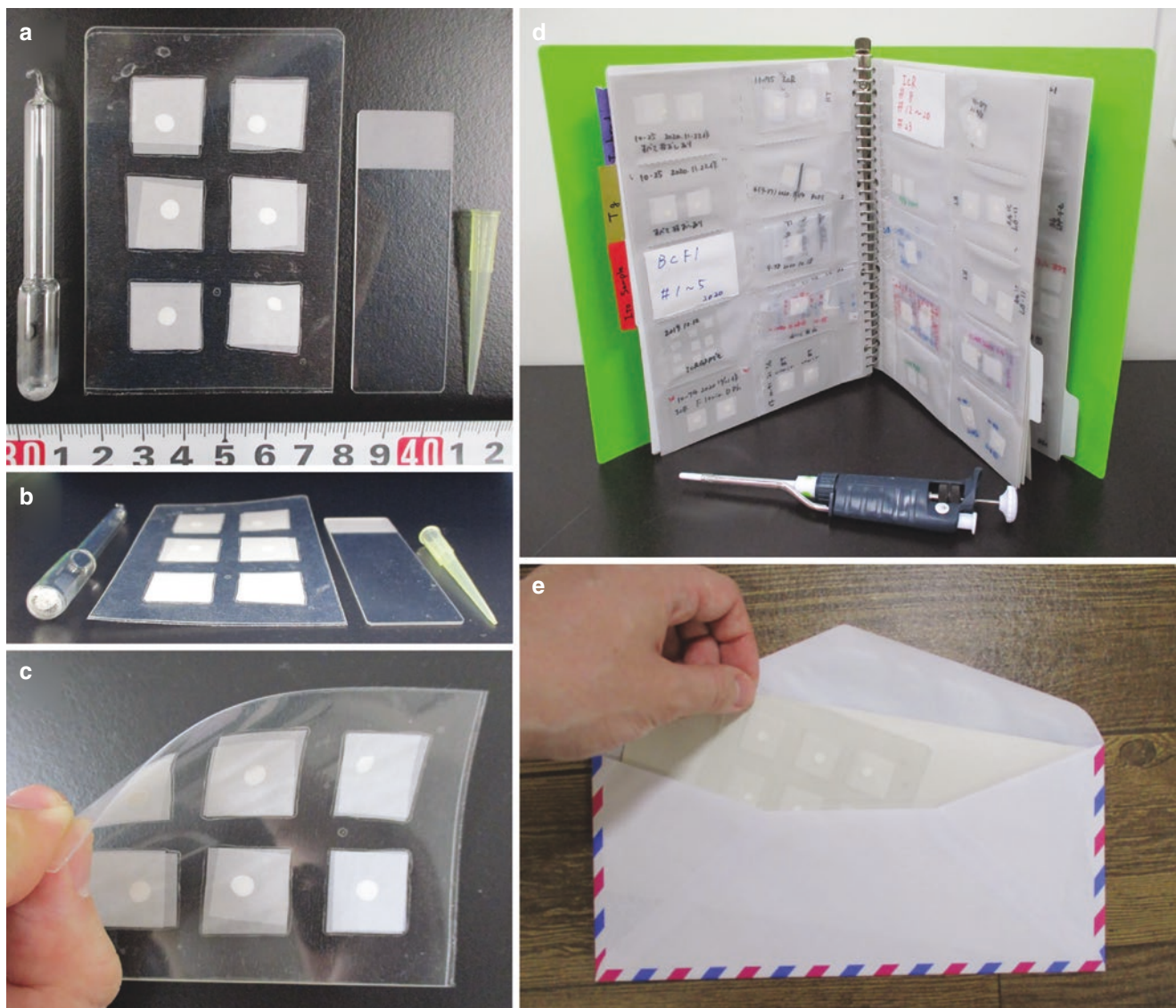


Fig. 27.2 Preservation of FD sperm by plastic sheet. (a, b) Comparison of the size of plastic sheet. (c) The sheet is soft and does not break even when bent. (d) FD sperm in the plastic sheet were placed

into the cardholder of a book. (e) Plastic sheet of FD sperm was put in the envelope and dropped directly into the mailbox without any protection. Photo credit: Daiyu Ito, University of Yamanashi

spring were obtained after storage at -30°C for more than 3 months [25, 26]. This method will facilitate the storage of thousands of mouse strains in a single book or cardholder (Fig. 27.2d).

27.3 Advantages of Freeze-Drying Sperm

Initially, we began developing a method of FD sperm to preserve any genetic resources at room temperature. However, when this becomes possible, there will be numerous additional benefits in addition to storage.

27.3.1 Simplifying the Transportation of Mammalian Spermatozoa

Conventional cryopreservation using LN2 necessitates the use of a dry-shipper for sperm transport, and the cost of transporting even one cryotube or straw containing frozen spermatozoa is extremely high. After the success of preserving FD sperm at room temperature, spermatozoa can now be transported without temperature control. Professor Yanagimachi at the University of Hawaii inserted several FD sperm ampoules into his pocket and traveled to Japan. Approximately 1 week later, he returned to Hawaii, and we were subsequently able to produce numerous offspring via ICSI using FD sperm [9]. This is the first time it has been reported that international sperm transport was successful without additional protection or cost. Although it is difficult to preserve FD sperm at room temperature for an extended period using the current method, the delivery of FD sperm worldwide prior to fertility loss is possible using air transport. If genetically modified mice could be shipped around the world as FD sperm, shipping costs would be very low; therefore, international collaborative research would be facilitated.

However, glass ampoules are at risk of shattering during the transport of FD sperm, which is a concern. As previously mentioned, FD sperm could be preserved in a thin plastic sheet instead of a glass ampoule. Therefore, we attempted to attach the sheet containing FD sperm to a postcard or envelope (Fig. 27.2e) and send it unprotected from another lab to my lab. When the postcard arrived, we were able to produce offspring from the sperm attached to it; the cost of transporting the spermatozoa was less than 1/100 of what it would have been using dry-shipper or LN2 [25].

27.3.2 Extreme Tolerance of Freeze-Drying Sperm

ICSI is required to fertilize oocytes due to the inability of FD sperm to be revived following rehydration. However, it has been discovered that FD sperm have an advantage over fresh sperma-

tozoa. Even after repeated treatment with conditions ranging from LN₂ to room temperature up to 10 times, it was possible to produce offspring from FD sperm nuclei. Conversely, when FD sperm samples were heated at 95°C , although the birth rate decreased with increasing heating duration, FD spermatozoa heated for 2 h still produced offspring. This period was increased to 6 h when trehalose was substituted for glucose in the freeze-drying medium. The resistance temperature was increased to 150°C for brief treatment [27].

Similarly, the radiation resistance of FD sperm increased compared with that of fresh spermatozoa. When spermatozoa were gamma-ray irradiated before and after FD treatment, those irradiated before FD treatment showed chromosomal aberrations at 4 Gy exposure, whereas those irradiated after FD treatment showed no aberrations even when exposed to 8 Gy radiation [28]. We also attempted to obtain offspring from these irradiated spermatozoa after ICSI using X-ray irradiation of fresh or FD sperm. Our findings revealed that FD sperm could produce offspring even after 30 Gy of irradiation, whereas fresh spermatozoa were incapable of doing so after 5 Gy. Although the maximum level of radiation tolerance varied among mouse strains, it was evident that FD treatment [29] increased the radiation tolerance of mouse spermatozoa [29].

When the space age arrives, we will travel to the moon and Mars via deep space, where we will be exposed to deep-space radiation, which consists of various heavy particle rays. In other words, X-ray and gamma-ray experiments alone are insufficient to predict the effects of deep-space radiation. As a result, we have been experimenting with irradiating FD sperm with heavy-ion beams and developing a method of protection against such powerful radiation. Using proper protection, we have been able to produce healthy offspring from FD sperm irradiated with 50 Gy (unpublished observation).

Although they may be somewhat unrelated, our findings may partially support or extend the Panspermia hypothesis [30, 31], which states that interplanetary objects could have seeded life on Earth. The most significant criticism of this hypothesis is that living organisms cannot withstand prolonged exposure to space radiation or high temperatures [32]. Furthermore, the hypothesis only proposes the transmission of “lower” species, such as microorganisms; therefore, it cannot explain the evolution of contemporary life forms. Nevertheless, our research suggests that the genetic information of spermatozoa may be preserved even after exposure to such extreme environments and that it is possible to convey the DNA.

27.4 Toward the Space Age

NASA and several organizations are currently planning human missions to Mars. In the future, humans will likely inhabit other space habitats or planets with animals such as

dogs, cats, and livestock for many generations. Thus, if only a small number of livestock and pets were carried in that situation, inbreeding degeneration would occur. To resist unknown diseases, it is also necessary to preserve as many genetic resources as possible and maintain the planet's diversity. Even if artificial hibernation technology was developed, transporting numerous living animals by spacecraft would be prohibitively expensive. Therefore, to preserve diversity, germ cells rather than individuals will be transported to the spacecraft, and for reasons of safety and cost, they may be transported in a freeze-dried state as opposed to LN₂ cryopreservation.

In contrast, sustaining life beyond Earth on space stations or other planets will require a thorough understanding of how the space environment affects critical phases of mammalian reproduction, such as embryo development in microgravity (μ G). However, to date, very little research has been conducted on the ISS using mammals [33]. This is because it is difficult to care for mice or rats on the ISS, frozen spermatozoa or embryos cannot be transported to the ISS due to a lack of LN₂, and astronauts who have never practiced embryo handling cannot conduct experiments with spermatozoa or oocytes/embryos in space.

27.4.1 Effects of Microgravity on Fertilization and Embryo Development in Space

First, because we did not know how to obtain permission to conduct experiments on the ISS, we used Clinostat, which mimics nullifying the gravitational vector through continuous averaging of G forces using three-dimensional (3D) rotation, to study the *in vitro* fertilization of mouse oocytes and their development into blastocysts under μ G [34]. *In vitro* fertilization occurred normally under μ G. Despite obtaining many offspring from μ G-fertilized and cultured embryos after transfer to recipient females, the birth rate was significantly lower than among the 1G controls. Immunostaining of embryos revealed that *in vitro* culture under μ G resulted in slower development and fewer trophoblast cells than 1G controls but had no effect on blastocyst polarization. These findings indicate that normal fertilization can occur under μ G, but normal preimplantation embryo development may require 1G. Other groups have published similar findings [35, 36]. However, these results were obtained through simulations and not actual space experiments.

In order to conduct real experiments on the ISS, untrained astronauts must be able to thaw and culture-frozen embryos on the ISS while subjected to μ G. Therefore, we developed a

new device that would allow astronauts who had never thawed and cultured frozen embryos to conduct experiments on the ISS [37]. The frozen 2-cell stage embryos [38] are placed in a small bag, which is then covered with a mesh sheet to prevent the embryos from flowing out of the bag, and a syringe is used to inject thawing solution and culture medium into the bag so that even untrained individuals, such as astronauts, will be able to perform this experiment anywhere, including the ISS. Using this apparatus, we conducted an experiment on the ISS to determine whether mammalian embryos can develop in real μ G, whether differentiation into ICM and TE is possible in the absence of gravity, and whether embryonic gene expression is normal. These bags have been returned to the ground and are presently undergoing analysis [39].

27.4.2 Effect of Space Radiation on Freeze-Drying Sperm Stored on the International Space Station for up to 6 Years

Deep-space exploration may cause DNA damage to spermatozoa, raising concerns about the transmission of mutations to future generations. Since FD sperm does not require LN₂ for long-term preservation on the ISS, we could launch FD sperm to the ISS to examine the biological effects of space radiation and the reproductive capacity of mouse spermatozoa. The Japan Aerospace Exploration Agency (JAXA) accepted this project in 2009. We then prepared approximately 2000 FD sperm ampoules from 66 males and selected the 288 best ampoules from 12 males using a lot check (Fig. 27.3a). These ampoules were divided into six boxes, of which three were launched to the ISS in 2013 (Fig. 27.3b), and the remaining three were preserved on the ground (JAXA) in nearly identical conditions without being transported to the ISS. Subsequently, 1, 3, and 6 years later, the boxes were returned to the ground. The ground-control boxes were also returned from JAXA to the University of Yamanashi at precisely the same time. This study determined that space radiation had no effect on sperm DNA or fertility after preservation on the ISS for up to 6 years. Our results suggest that many genetically normal offspring were obtained without a decrease in success rate compared to the ground-preserved control group (Fig. 27.3c, d). Compared to a previous ground experiment in which FD sperm were exposed to X-rays, these results suggest that the storage of FD mouse sperm on the ISS for more than 200 years is possible without protection from space radiation [29, 40].

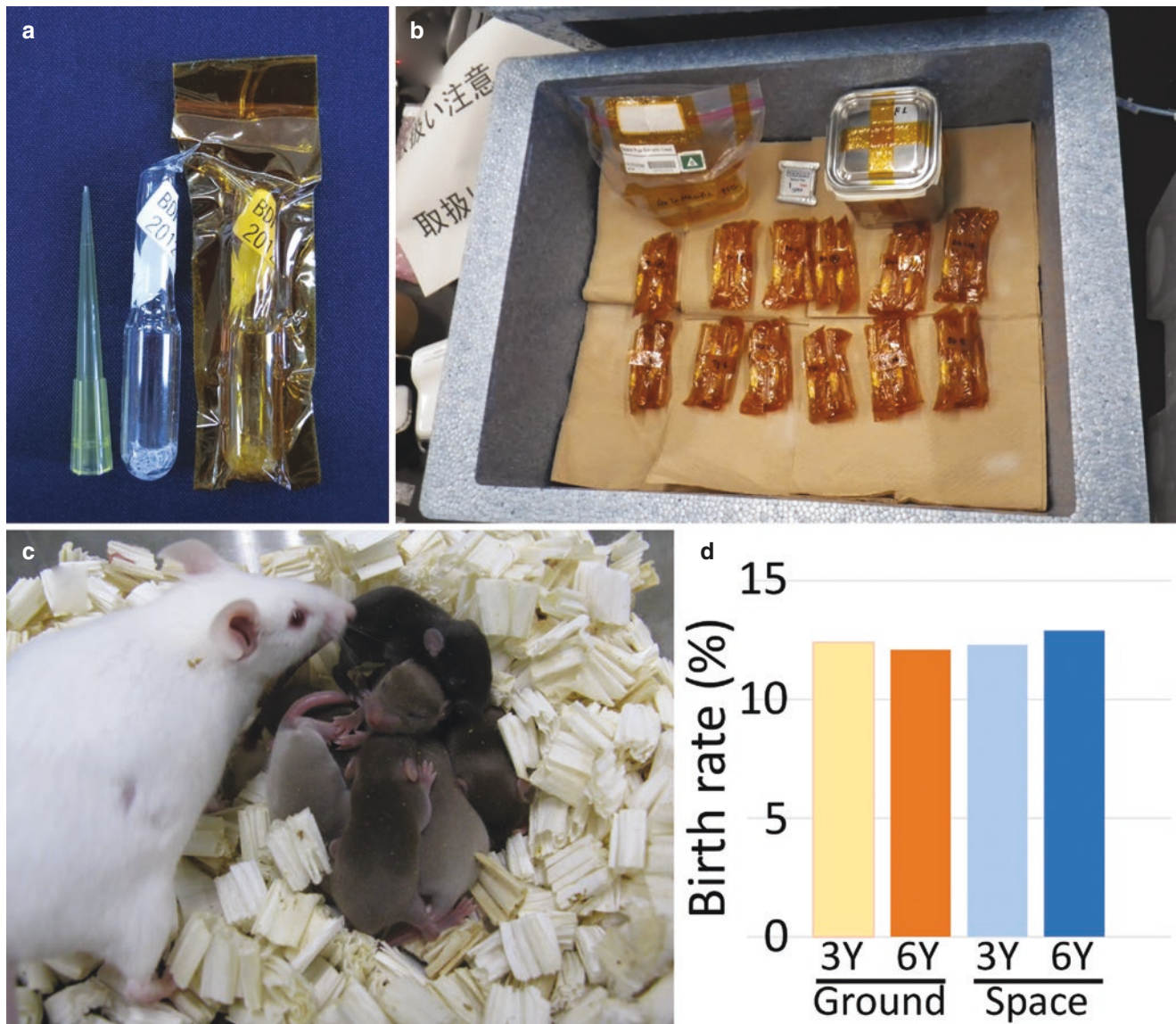


Fig. 27.3 Preservation FD sperm on the ISS. (a) The ampoule bottle containing the FD sperm was wrapped with Kapton polyimide tape so that the glass would not shatter if it broke. (b) One stainless steel box contained 12 male FD sperm and a radiation dosimeter. (c) Offspring

from space-preserved spermatozoa. (d) Birth rate of embryos fertilized with FD sperm stored for 3 or 6 years on the ground or space. Photo credit: Sayaka Wakayama, University of Yamanashi

27.5 Conclusion

We have been researching spermatozoa preservation at room temperature and the possibility of mammalian reproduction in space. Even after long-term storage at room temperature, there is no assurance that spermatozoa will reliably produce offspring. However, room temperature preservation is the best method for preserving genetic resources because it does not require LN_2 . However, to produce offspring, oocytes or embryos must also be preserved at room temperature, which has not yet been accomplished. Additionally, spermatozoa from elderly, immature, or infertile individuals cannot be

recovered. In order to address this issue, it is necessary to develop a method for preserving somatic cells through FD treatment. Somatic cells are not reproductive cells but can be collected anywhere in the body; their nuclei can be used to create offspring through cloning technology. After much trial and error, we recently succeeded in cloning mice from FD somatic cell nuclei [41].

During the 2011 earthquake in Japan, an unexpectedly large tsunami over 40 m tall triggered the explosion of a nuclear reactor, exposing the surrounding area to high-radiation levels [42]. Thus, global and severe disasters will occur more frequently than anticipated, and it will be diffi-

cult to find a permanently secure location on Earth. Extraterrestrial preservation of germ cells is a new option for our future to preserve genetic resources indefinitely in such a scenario. The freeze-drying of spermatozoa, embryos, and somatic cells is superior to traditional cryopreservation for this purpose. In the distant future, when we can demonstrate the reliability and integrity of space-preserved FD germ or somatic cells, underground storage on the moon, such as within lava tubes [43], could be one of the best places for long term or permanent genetic resource preservation due to their very low temperatures, protection from space radiation by thick bedrock layers, and complete isolation from natural disasters on Earth.

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Part V

Expected Outcomes and Quality Control

Continuous Quality Improvement (CQI) and Key Performance Indicators (KPIs) in Cryopreservation: Aspiring for the Best Results

28

Alison Campbell and Amy Barrie

Key Points

- Maintenance and continuous improvement in quality of cryopreservation require regular audit of practice against validated, effective operating procedures, benchmarking and the use of KPIs.
- Where available, cryopreservation KPIs should be established based on expert consensus publications, the scientific literature and using reference populations.
- Continuous quality improvement in clinical cryopreservation programs can be more challenging, and slower to progress, than in fresh practices, due to variable storage lengths and delays in collection of outcome measures.
- Published KPIs for cryopreservation may be outdated such that some competency levels are now too low. IVF laboratories should be aiming to attain the higher benchmark values.

28.1 Introduction

Cryopreservation is an integral and crucial part of medically assisted reproduction (MAR), enabling conservation of gametes and embryos to maximise efficacy and efficiency whilst providing a tool for long- and short-term fertility preservation and treatment. The use of cryopreservation of oocytes and embryos, in particular, has increased over the last decade—as vitrification methodology has become widespread and provided improved outcomes compared with previous, traditional methods. This has enabled advances and flexibility in oocyte banking for donation, and also for pre-implantation genetic testing, both supported by cryopreservation. Sperm cryopreservation practices and protocols, in

MAR, have remained largely unchanged over the last few decades but vitrification is being utilised in some cases.

Continuous quality improvements relating to cryopreservation practices for sperm, oocytes, and embryos require monitoring and audit of practice, and collection and analysis of high-quality data. Unlike fertility treatments, which utilise fresh embryo transfer, obtaining data from cryopreservation programs, in order to monitor and fine-tune laboratory practice and to continuously improve performance, takes longer due to the nature of the process, which includes varied durations of cryo-storage. In the case of fertility preservation, data collection can take significant time, such that a change in clinical practice may not be fully validated, or confirmed as an improvement, for many years. This brings challenges when measuring performance and striving for continuous improvement.

Attention to detail in cryopreservation of gametes and embryos is critical. The balance of cryoprotectant concentration, sequence and the timing of the steps within the operating procedure are finely balanced to minimise stress to the cells and to maximise safety, survival and viability.

Cryopreservation of gametes and embryos, whilst routine in MAR laboratories, is commonly considered one of the most complex and precise practices undertaken. For example, for the embryologist, vitrification of oocytes and embryos requires a high degree of composure, attention to optimal timings, pipetting within different viscosity media and associated microscopy skills; different, and arguably more technically challenging than standard (fresh) embryology practices. Individual practitioner monitoring audit and assessment is important for cryopreservation practices.

The Alpha consensus expert meeting proceedings, on cryopreservation key performance indicators and benchmarks, were published in 2012 [1]. A total of 14 KPIs were recommended, and benchmarks for each are presented. No recommendations were made regarding specific cryopreservation techniques or devices, or whether vitrification is ‘better’ than slow freezing, or vice versa, but it set a helpful, well considered, platform for fertility scientists worldwide.

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Since then, a broader performance indicator consensus was published by the ESHRE Special Interest Group on Embryology and Alpha [1]. Whilst not directly related to cryopreservation, this publication considered whether the 2012 KPIs required revision and proposed, in light of improvements in blastocyst

cryopreservation techniques, particularly, that the competency levels and benchmark rates for degeneration on warming should be lower than suggested in 2012. Such that they should be expected to be $\geq 90\%$ and $\geq 99\%$, respectively. Published KPIs are summarised in Fig. 28.1.

Fig. 28.1 Cryopreservation performance indicator values (adapted from Alpha consensus on cryopreservation [1])

KPIs Vitrification	Competence	Benchmark	
Oocyte			
Intact post thaw/warm 'survived'	70%	85%	95% for donors <30 years
Fertilisation	< 10% points lower than comparable fresh population		
Embryo development rate	Same as comparable fresh population		
Implantation rate	$\leq$ relative 30% lower than comparable fresh population		

KPIs Slow freezing or vitrification	Competence	Benchmark	
Zygote			
Intact post thaw/warm 'survived'	70%	85%	of morphologically normal zygotes
Cleavage	Same as comparable fresh population		
Embryo development rate Implantation rate	Same as comparable fresh population $\leq$ relative 30% lower than comparable fresh population		

KPIs Vitrification	Competence	Benchmark	
Blastocyst			
Survival rate	80%*	95%	*updated values 90% and 99%
Transfer rate	80%*	95%	
Implantation rate	$\leq$ relative 10% lower than comparable fresh population	Same as comparable fresh population	

*In light of improvements in blastocyst cryopreservation techniques, particularly, that the competency levels and benchmark rates for degeneration on warming should be lower than suggested in 2012. Such that they should be expected to be $\geq 90\%$ and $\geq 99\%$, respectively.

People	Environment	Practices
Patient	Air quality	Media constituents and concentrations
Donor	Temperature	Volumes
Embryologist	Equipment	Timings
Andrologist	Consumables	Assessment of gametes/embryos
Trainer	Humidity	Selection of gametes/embryos
Technician	Culture conditions	Quality control
Medic		Training and competency
		Medication protocol

Fig. 28.2 Factors which may influence success in cryopreservation and require attention or analysis for continuous quality improvement (non-exhaustive)

Every element of the cryopreservation practice matters. From the environment to the equipment, from the device to the media, from the selection of the gametes/embryos to the operating procedure and the practitioner, selection of the laboratory protocol is also vital and includes the volumes, temperatures, constituents and timings of each step in the critical process. Continuous quality improvement in cryopreservation should start with a demonstrable, sound, competency level and involve sustained monitoring and efforts to reach the realistic aspirational benchmark values described in the literature and shared by communication and collaboration within our field of reproductive science. Awareness of the high number of influencing factors can aid troubleshooting, should any of the KPIs not be met, as these can be worked through methodically. Figure 28.2 described some of the factors which may impact outcomes and require scrutiny for continuous improvement or troubleshooting.

Subjectivity within an IVF laboratory is challenging to overcome, as is defining ‘best practice’. Selection of gametes or embryos for cryopreservation is largely subjective and therefore can impact outcomes, such as survival, development and implantation. This needs to be taken into account when setting and monitoring performance indicators. Reference populations are particularly helpful for benchmarking to ensure that comparisons are as valid as possible [2].

28.2 KPIs and Oocyte Cryopreservation

The oocyte is a large cell with a limited surface area to cytoplasm ratio and, as such, is highly sensitive to structural or functional cryoinjury if cryopreservation is not optimised. Despite this challenge, oocyte cryopreservation has become increasingly successful over the last decade or so, as vitrification techniques have evolved and superseded traditional slow freezing; such that clinical outcomes have been reportedly very similar between fresh and vitrified-warmed oocytes

[3]. This significant achievement has brought a shift in the practice of oocyte banking, using vitrification, for donation and for fertility preservation—both for medical and non-medical reasons.

The success of oocyte cryopreservation (and also embryo cryopreservation) is highly dependent on the quality of the oocyte, which, in itself, is highly correlated with the age of the egg provider. For this reason, there is value in utilising outcome data from donor egg banks, to set indicators of quality and targets for improvement.

There are large published outcome data available from a number of donor egg banks worldwide. This enables IVF laboratories to readily benchmark their own performance against them and to establish KPIs and aspirational targets for key indicators. We also have a recent guideline from The Practice Committee of the American Society for Reproductive Medicine (ASRM) along with the now, decade-old, cryopreservation KPI Alpha consensus publication [4].

The ASRM’s evidence-based guideline recommendations of 2021, on oocyte cryopreservation, focus on the evidence available to predict the likelihood of live birth and clinical pregnancy, neonatal and obstetric outcomes, and on factors predicting reproductive outcomes. They concluded from their literature review that there was insufficient evidence to predict live birth rates after planned oocyte cryopreservation, and that, based on the limited data available, birth rates appear to be improved for women cryopreserving their oocytes at a younger age, compared with older counterparts. The expert panel also reported that there was also insufficient evidence that live birth rates were comparable between vitrified and fresh oocytes from donors. These findings should be considered when setting KPIs for oocyte cryopreservation practice, as data remains limited, and targets need to be realistic.

A report of the efficiency and efficacy of vitrification in over 35,000 sibling donated oocytes described fertilisation and embryo morphology significantly lower with vitrified oocytes, compared with fresh. However, when the efficacy of

warming was taken into account, reproductive outcomes (or recipients) became comparable indicating the effect of the efficiency of the vitrification and warming techniques, rather than damage to the oocyte by the processes [5].

Using donated oocytes as a reference population for KPIs and quality improvement is recommended.

28.2.1 Challenges and Choices

As described briefly, vitrification is a practically challenging process as, perhaps more than any other process in the IVF laboratory, it relies heavily on critical, measured and precise, timings of each step, coupled with progressive changes in the appearance of the oocyte/embryo in the specialised cryoprotectant media of varied viscosities, making visualisation of the translucent sample sometimes difficult. Training and competency attainment in oocyte vitrification may take time to achieve and can be delayed by the limited supply of material for training. Once competency has been demonstrated, regular performance of the process is required to avoid standards of proficiency slipping. Some laboratories favour having a small number of advanced practitioners dedicated to oocyte vitrification for this reason. High level training, competency assessment and review, and protocol-driven quality are essential for a successful oocyte vitrification programme.

With a number of seemingly similar suites of media and storage devices to select between, IVF professionals have difficult choices to make when deciding upon which method or system to use in their laboratory. This often involves the evaluation of several alternatives and always requires review of outcome measures in a regular basis.

Some of the key considerations when selecting a method for oocyte cryopreservation (some of which also apply to sperm and embryo cryopreservation) are as follows:

- Evidence of efficacy
 - Survival rates
 - Fertilisation rates
 - Onward development rates
 - Blastulation rates/euploidy rates
 - Implantation rates
 - Birth rates
- Training requirement and available support
- Ease of use/reproducibility
 - Device handling and labelling (e.g. open versus closed)
 - Storage space required
- Constituents of the media
- Shelf life
- Supplier reliability
- Certification
- Cost effectiveness
- Equipment and consumables required, e.g. open or closed straw devices
- Time per process
- Environmental conditions required, e.g. temperature
- Health and safety data

Automation of oocyte vitrification (as well as sperm and embryo vitrification) promises to provide reproducibility and more consistent results, by removing some or all of the human operator subjectivities and subtle differences in practice. The use of robotics to enable this for broad use is likely to be seen over the coming years.

28.3 KPIs and Sperm Cryopreservation

Since the first human live birth using frozen sperm in 1953, sperm cryopreservation has become common place for myriad reasons in fertility treatment and preservation. Sperm cryopreservation involves subjecting the cells to both permeating and non-permeating cryoprotectants and reducing the temperature of the resulting sample to enable long-term storage in liquid or vapour nitrogen. Through this process, sperm can be maintained in suspended animation whilst preserving the functional competence of their reproductive capacity. However, during the process of cryopreservation, sperm are subject to damage from multiple sources: thermal shock, osmotic shock and ice crystal formation [6]. This damage ultimately affects the function of the sperm upon thaw and use, evidenced through impaired motility [7], mitochondrial and membrane degradation [8] and increased DNA fragmentation [9].

In terms of functional parameters that can be assessed easily during routine semen analysis (motility, morphology and concentration), a multitude of evidence exists demonstrating a significant decrease in semen parameters comparing pre- and post-cryopreservation samples. Some report a loss of motility by up to 50% [7]. Furthermore, the assisted reproduction treatment outcomes have been shown to decrease when using cryopreserved sperm, including fertilisation rates [8]. However, unpublished data suggests that the effects of use of cryopreserved sperm may not be as damaging as has been previously demonstrated, with comparative fertilisation and clinical pregnancy rates between fresh and frozen sperm ICSI cycles ($n = 2386$). In addition, some suggest that the use of effective sperm cryopreservation prior to ICSI selects the most competent sperm for use in treatment, with cryopreservation acting as a selection method [10]. Variations in post-thaw survival and competence are likely to be due to methodological variations as well as pre-freeze parameters.

28.3.1 Pre-freeze Parameters

Samples with abnormal parameters have been shown to be particularly susceptible to damage from cryopreservation [11], and it is generally accepted that the success of the cryopreservation depends largely on the quality of the cells where poor quality sperm are less likely to survive cryopreservation [12]. When comparing ICSI cycles using fresh and frozen sperm, no differences were found in reproductive outcome where the sperm sample was normozoospermic in both groups; however, if the sample was asthenozoospermic prior to ICSI then the fertilisation rate was decreased when using frozen sperm but implantation rate remained unaffected [13]. In addition, high concentrations of non-viable sperm and leukocytes in the semen have been shown to detrimentally affect the sperm survival rate [9].

28.3.2 Methodological Variations

There are a number of methodological variations that have been investigated due to their potential influence on the effectiveness of sperm cryopreservation; cryoprotectant type, whether the sample is prepared before cryopreservation or not and the freezing technique (slow versus rapid). More recently, the device in which the sample is stored and sperm vitrification have been explored.

28.3.2.1 Cryoprotectants

There are a handful of cryoprotectants commonly used in sperm cryopreservation classified into two groups: permeable (including glycerol, dimethyl sulfoxide, dimethyl acet-aldehyde, propylene glycol, ethylene glycol) and non-permeable (such as raffinose, sucrose, albumin) [14]. Permeating cryoprotectants can be toxic if used in high concentrations as they enter the cells and replace the water whilst non-permeating cryoprotectants decrease the extracellular water freezing point and form a protective layer around

the sperm membrane [14]. Egg yolk was commonly used in sperm freeze solutions with success [15]; however, because this is animal-derived, the use of this is discouraged and substituted for chemically derived alternatives. Of particular importance the addition and removal of cryoprotectants in a stepwise manner ensure gradual osmotic changes and reduce cell damage as a result of osmotic shock [8]. There is an argument that basic questions remain unanswered with regard to sperm cryopreservation. Huanhuan and colleagues sought to answer these and concluded that human sperm should be cryopreserved by rapid freezing (discussed later) using 0.25 M sucrose or trehalose with osmolality 442–457 mOsm/kg followed by thawing at 37 °C for 2 min [16].

28.3.2.2 Sample Preparation

Whether the sample should be subject to preparation prior to cryopreservation should be considered. There is evidence to suggest that the seminal plasma offers a protective effect to the sperm during cryopreservation due to its constituents such as antioxidants, lipids, sugars, polyunsaturated fatty acids [17]. However, others have demonstrated superior post-thaw survival when the sample is prepared using swim-up [18] or density gradient [19] methods prior to cryopreservation. It may be that in those samples with a high concentration of non-viable sperm or leukocytes, the protection that the seminal plasma infers is outweighed by the damage from reactive oxygen species and so preparation prior to cryopreservation should be undertaken in a sample-by-sample basis (Fig. 28.3).

28.3.2.3 Freezing Technique

The slow freezing technique has been used since the inception of sperm cryopreservation and involves the progressive cooling of the sperm over a number of hours usually using a controlled rate freezer [8]. Using a controlled rate freezer allows for reproducibility, and this freezing technique is simple and removes the need for an operator for the duration of

Fig. 28.3 Author proposed cryopreservation performance indicator values

KPIs Vitrification	Competence	Benchmark	Comments
Oocyte			
Intact post thaw/warm 'survived'	80%	95%	Egg provider age is a key influencing factor
Fertilisation (ICSI)	≤ relative 5% lower than comparable fresh population KPIs		
Clinical outcomes	Same as comparable fresh population KPIs		

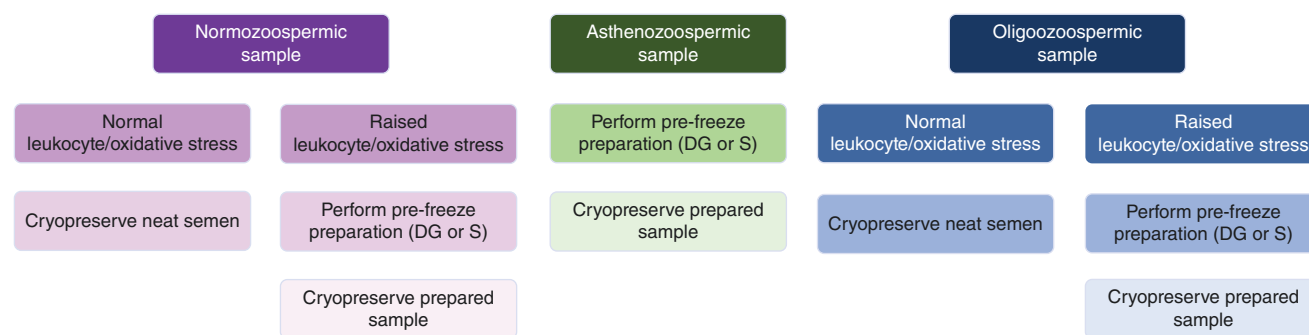


Fig. 28.4 Suggested sample preparation according to sample parameters, oxidative stress and leukocyte level

the process. However, there is evidence that this method can encourage ice crystal formation and therefore lead to sperm damage [20]. Rapid freezing was proposed in the early 1990s and involves the exposure of the sample to nitrogen vapour for less than 30 min before plunging directly into liquid nitrogen. Although this method is much quicker, the reproducibility is poor and the rate of freezing can neither be monitored nor controlled. In recent years, vitrification has been assessed as a method to improve post-thaw recovery rates and reduce DNA fragmentation levels. This method, which is essentially rapid freezing as described above but with the use of sucrose as a cryoprotectant, has yielded superior post-thaw progressive motility (34.7% vs 16.6%) and significantly reduced DNA fragmentation rates (14.6% vs 47.3%) [21].

28.3.2.4 Storage Device

Predominantly, straws or ampoules are the device used for sperm cryopreservation. The superiority of one or the other remains undetermined. Functionally, straws are preferred in terms of space required for storage, increased surface area to allow effective sperm cryopreservation as well as the ability to heat seal meaning the risk of contamination with other samples is negligible. However, the increased surface area also makes straws susceptible to warming during handling. In a recent study, the effectiveness of heat-sealed straws was compared with the newly developed heat-sealed ampoules. This analysis revealed that the sperm recovery rate (percent total motility post-thaw/percent total motility pre-freeze) was significantly higher in the ampoules (32.57% vs. 19.99%).

Alternative devices have been suggested, primarily for storage of samples with low sperm numbers. In the past, the storage of single sperm on devices such as Cryoloop [22] and Cryotop [23] was investigated as well as within empty zona pellucidae [24] and a Volvox globator sphere [25]. More recently agarose capsules [26], synthetic hollow hyaluronan microcapsules [27] and 'Cryopiece' [28] have been investigated with varying success. There is not yet a superior device, and more trials are needed [29] (Fig. 28.4)

28.4 KPIs and Embryo Cryopreservation

Embryo cryopreservation has been common place in ART for many years and has increased in volume in recent years. An analysis of embryo cryopreservation in the US between 2004 and 2013 revealed that embryo cryopreservation has increased dramatically since 2010 from 15.6% to 40.7% in 2013 [30]. This same pattern has been seen in Europe where it has been estimated that cryopreserved cycles contributed to 32% of all transfers in 2011 compared to 28% in 2010 [31]. This is likely due to the introduction of vitrification, the rapid freezing of embryos.

28.4.1 Survival Rates

According to the most recent consensus meeting of expert professionals, the benchmark cryosurvival rate for blastocysts following vitrification is 90% or above with the gold standard being 99% or above [32]. For the same parameters, the survival rate suggested by the Alpha consensus meeting is 95% [33]. As well as survival rates for vitrified blastocysts, the Alpha scientists recommend survival rates for slow frozen cleavage embryos and blastocysts and vitrified cleavage stage embryos as 85%. It is expected that survival rates will vary depending on the population under assessment as well as methodological variations as discussed below. See Fig. 28.1 for a summary of published cryopreservation KPIs.

28.4.2 Methodology Variations

28.4.2.1 Open Versus Closed Systems (Also Applicable to Oocytes)

The use of either open (direct contact with liquid nitrogen during vitrification) or closed (indirect contact with liquid nitrogen during vitrification) has been an area of discussion for many years. During vitrification, rapid cooling is required to ensure adequate cryopreservation and ensure optimal

cryosurvival; therefore, it is thought that direct contact with liquid nitrogen, i.e. use of an 'open' system, is optimal. However, the risk of contamination must be considered when an open system is used, and although contamination via these means has not yet been documented, many opt for a closed system to remove this risk entirely. In a recent systematic review and meta-analysis, no difference in cryosurvival, positive pregnancy rate, implantation rate, clinical pregnancy rate, miscarriage rate, ongoing pregnancy rate, live birth rate, cancellation rate, and multiple pregnancy rate was found in four prospective and three retrospective analyses when comparing open versus closed vitrification systems [34]. All data used in this meta-analysis was considered moderate quality; however, the authors recommended that further studies were required though it was concluded that the cooling rate of closed systems was lower compared to open counterparts which should be a consideration when choosing a system to use in the laboratory.

28.4.2.2 Slow Freezing Versus Vitrification

Slow freezing was the preferred method of embryo cryopreservation for many years and briefly involves the controlled decrease in temperature in three phases; firstly, the samples are cooled at a rate of 2 °C/min to -7 °C, the samples are then manually seeded to induce ice crystal formation followed by further cooling of 0.3–1 °C/min until the samples reach -40 °C to -70 °C. At this point, the samples are then plunged directly into liquid nitrogen [35]. In contrast to slow freezing, vitrification is far less labour and time intensive but exposes embryos to more toxic cryoprotectants and requires highly competent scientists. This method solidifies the samples in a glass-like state (*vitreum* meaning glass in Latin) using high concentrations of cryoprotectants and rapid cooling and warming rates. In a recent systematic review and meta-analysis, it was determined that a higher clinical pregnancy rate and live birth rate per embryo transfer were obtained with embryo vitrification compared with slow freezing [31]. However, the quality of evidence was low and was from just three randomised controlled trials. Further analyses are required to conclude which methodology for embryo cryopreservation is superior, nevertheless, the time and consumable saving associated with vitrification means this method is favoured in the absence of convincing evidence to the contrary.

28.4.2.3 Cleavage Versus Blastocyst Cryopreservation

Whether embryos should be utilised as either cleavage embryos or blastocysts remain highly investigated with an active randomised controlled trial underway to determine the non-inferiority of cleavage stage versus blastocyst-stage embryo transfer (PRECiSE trial) [36]. In a Cochrane review, it was concluded from low quality evidence that the live birth

rate following fresh blastocyst transfer was higher than cleavage stage transfers [37]. In relation to cryopreservation specifically, an analysis of 14 articles revealed that more large for gestational age deliveries were observed after blastocyst compared with cleavage stage transfer in frozen cycles; however, there was no difference in low birth weight, very low birth weight or congenital anomalies between the two groups [38]. In addition, comparable survival rates have been demonstrated between cleavage and blastocyst stage vitrification; 94% versus 96–98% [39]. Finally, significantly fewer subsequent transfers using frozen blastocysts are required when compared to cleavage stage embryos [40].

28.4.2.4 Blastocyst Collapse

Many investigations regarding the use of artificial collapse prior to cryopreservation were conducted a number of years ago, and there is a paucity of recent evidence to support this methodology. This is important because cryopreservation methods have markedly improved over the last 10 years. A recent meta-analysis identified six publications for inclusion, only one of which was prospective. This analysis demonstrated superior survival and pregnancy rates of artificially collapsed blastocysts; however, in the prospective study, although an increased survival rate was observed, the pregnancy rate was not enhanced when blastocysts were artificially collapsed prior to vitrification [41]. In a further prospective observational study using time-lapse incubation, 69 embryos subject to artificial collapse prior to vitrification were compared to 72 embryos that were not. There was no difference in survival or live birth rates in this investigation [42]. It is clear that more, high quality evidence is required before artificial collapse can be determined as beneficial or not to the efficacy of blastocyst cryopreservation.

28.4.3 Benchmarks and Gold Standards

Although an area of intense investigation, considering the current evidence available it is recommended that vitrification using an open system for blastocyst cryopreservation be employed to achieve optimum survival and subsequent pregnancy rates. Blastocyst collapse is an area that lacks high quality evidence and, as such, should not be adopted as common practice. Further, well-designed prospective randomised controlled trials are required to assess the effectiveness of artificial collapse especially given the requirement for an additional, invasive manipulation of the blastocyst and the associated risks of this unproven procedure. When considering key performance indicator benchmarks and gold standards, the population to which the measure is to be applied must be considered and control limits adjusted as required. Suggested target, control and action limits can be found in Fig. 28.5.

	Slow freezing cleavage stage	Slow freezing blastocyst stage	Vitrification cleavage stage	Vitrification blastocyst stage
Survival rate target	85% ^a	85% ^a	95% ^a	95% ^a 90% ^b 98.5% ^c
Survival rate control limit	75%	75%	85%	89% ^c
Survival rate action limit	<75%	<75%	<85%	<89% ^c
Pregnancy rate target^d	The same as for the comparable population of fresh embryo transfers			
Pregnancy rate control limit	10% below the rate for comparable population of fresh embryo transfers			
Pregnancy rate action limit	>10% below the rate for comparable population of fresh embryo transfers			

Fig. 28.5 Suggested target, control and action limits for cleavage stage and blastocyst stage slow freezing and vitrification. (a) according to Alpha scientists consensus meeting (2012). (b) according to ESHRE

special interest group consensus meeting (2017). (c) according to CARE Fertility UK data. (d) applicable to all measures of pregnancy rate including biochemical, implantation, clinical and live birth rate

28.5 Using KPIs to Improve Continuously

28.5.1 Setting the Bar and Monitoring Performance

Published benchmarks and KPIs for cryopreservation are available although, as discussed, some may be considered outdated. Scientific leaders, depending on the culture of the MAR clinic, or group, may have different attitudes towards targets and competency levels. Some will be satisfied with maintenance of results which are considered sufficiently close to the published competency level, whilst others will aim for the stars—using aspirational targets and continuously driving their teams to raise the bar. For this reason, it is recommended, in agreement with the Vienna consensus concept, that, for each KPI, there is a limit below which performance is a concern and should be investigated; a competency limit. And, that there is also an aspirational target, which represents top class performance, which should be attainable, at least within a good prognosis reference group of patients. Control limits are calculated by estimating the standard deviation (sigma) of historical data and are commonly used for performance monitoring within IVF laboratories over time. Users can specify the number of standard deviations from the mean to set limits to highlight which measure are out of control or not meeting required standards.

The Care Fertility Group use red, amber, green ratings for their KPIs; which consider large historical data, published KPIs and guidelines, as well number of procedures

performed. A result (in a given month/period) which is within the red zone immediately triggers a structured investigation. Amber is used to direct focus and monitor trends, particularly in the following period of assessment, and green results in no immediate action—as is considered satisfactory. In addition, there is a purple rating (the Care group's brand colour), which identifies performance which is considered 'world class', and above the typical aspirational target.

Below is an example of the ratings for embryo post-warm survival rate KPI:

Embryo Survival Rate	100% (purple)	>98.5% (green)	89-98.5% (amber)	<89% (red)
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Monitoring of performance is varied and can be very simplistic, sophisticated, fully automated or anywhere in between. It is essential that performance monitoring is well considered, in terms of the variables assessed and that minimum numbers and reference populations are considered. There are multiple methods and tools available for this important exercise and as long as there is rigour and routine, they can be equally effective as each other.

Audit of practice against standard operating procedure (SOP) is an essential part of continuous quality improvement, as practice drift can negatively impact outcomes and prevent effective fine-tuning and improvement. It also ensures that all operators within the team are performing the

task in the same way and that the data being collected is therefore consistent and reliable.

Clear concise, visual SOPs, rather than pages of complex text, can make audit easier to perform, particularly if the auditor is lay.

Developing a culture of continuous improvement is important if regular progress is to be made. In MAR, some of our practices are highly successful and so we are now looking to find marginal gains.

Developing such a culture involves an open mindset of staff, a willingness to learn, to study, to share and to change. Change does not come easily to some and, as discussed, can be particularly challenging when associated with cryopreservation, as results of the changes can take time to come through due to the variable periods of storage.

28.6 Conclusion

Cryopreservation is an essential part of MAR. Improved methodologies in the cryopreservation of gametes and embryos have facilitated and enabled the effective treatment of subfertile patients and the preservation of fertility, in many scenarios. An efficient and effective cryopreservation program requires high standards and detailed monitoring to provide continuous quality improvement. All aspects of the process are critical to its success. From staff training and competency assessment to selection and refinement of SOP, several key indicators of performance and quality have been identified and described, within this chapter, to aid us as professionals. Guidelines are somewhat outdated but many key performance indicators proposed around 10 years ago still remain valid. The future is likely to bring us at least marginal gains in cryopreservation outcomes, from survival rates to live birth, as we continue research in cryobiology and introduce more automation into our practices and have the facility to more rapidly analyse large and complex datasets using machine learning to identify best practice.

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Handling and Assessing of Human Oocytes and Blastocysts After Vitrification and Warming

29

Laura Conversa, Ana Cobo, and Marcos Meseguer

29.1 Introduction

In recent years, the safety and successful outcomes achieved with vitrification have led it to be considered the best approach for cryopreservation of human oocytes and embryos, allowing its application in the daily practice of assisted reproduction clinics.

Vitrification takes place when a solution highly concentrated in solutes is exposed to low temperatures using very fast cooling rates. Its application in gamete and embryo preservation involves addition of a high concentration of cryoprotective agents (CPAs), which replace most of the intracellular water (Fig. 29.1). Direct immersion in liquid nitrogen leads to the cytoplasm acquiring a viscous consistency with a glassy structure, which largely prevents detrimental effects such as dehydration and the formation of ice crystals inside the cells. Warming is carried out by directly placing the samples from nitrogen into the culture media at 37 °C. Then, cryoprotectants are gradually diluted, enabling progressive rehydration of the oocyte or embryo.

Once the integrity of the sample has been confirmed, it is cultured under standard conditions to obtain a reliable assessment of its survival hours later. Its subsequent viability will depend on its capacity to be fertilized give rise to adequate pre-embryonic development, and finally result in gestation and live birth (LB).

Current vitrification protocols provide similar efficiency to that of fresh samples in terms of survival rates and clinical results [1, 2]. Vitrification of donor oocytes offers a survival rate of around 90% and gives gestation rates of 60%, while in the case of oocytes from infertile patients, the survival rate is about 88%, and gestation is achieved in 40% of the cases, due to the poorer quality of the oocytes [3]. In the case of vitrified embryos, the survival rate after warming is around 95%. They can be frozen at any stage of development, but it is preferable on day 5/6 to allow them to self-select and thus freeze a smaller amount [4].

Despite these good results, the biological effects of preserving cells at low temperatures are complex and, in many respects, still unknown. So much so that, on many occasions, good quality oocytes and some of the best morphologically classified embryos before vitrification do not maintain their characteristics when they return to room temperature. Especially in the case of embryos, these unexpected events increase the difficulty of selecting the best one for transfer.

This review describes the factors affecting the clinical outcome of vitrification cycles, as well as some biomarkers related to the quality of thawed oocytes and embryos and their potential to generate a LB. We will also discuss the possibility of applying embryo selection models to vitrified-warmed blastocysts.

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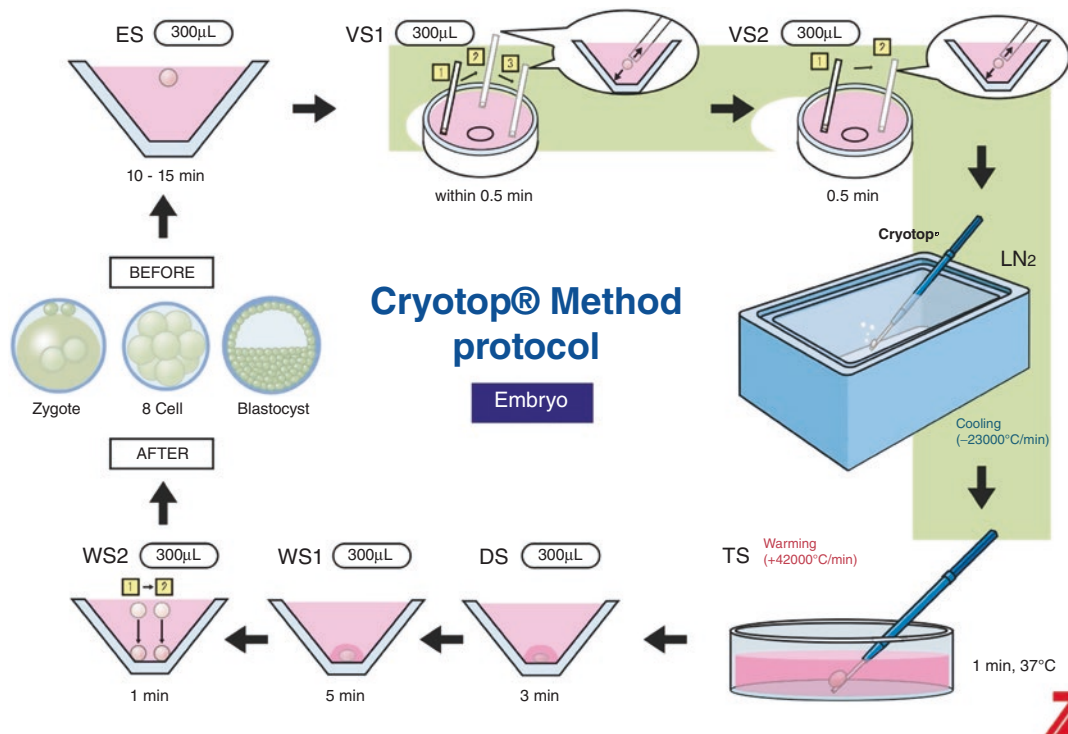


Fig. 29.1 Embryo vitrification and warming procedure (extracted from “Cryotop® Safety Kit—Vitrification Protocol for Cryotop® Method” by Kitazato)

29.2 Factors Affecting Reproductive Result After Vitrification

29.2.1 Clinical Factors

Vitrification has been widely applied in assisted reproduction clinics since it was initially used to store oocytes from egg donation until they are matched to recipients. This strategy has eliminated some of the drawbacks of fresh donations, as well as broadened the variety of phenotypes and blood types. Oocyte vitrification is currently applied under many other indications, including women at risk of losing their ovarian function due to gonadotoxic treatments or bilateral oophorectomy, and patients at risk of premature ovarian failure, such as those with low ovarian reserve or endometriosis. Moreover, oocyte banking is a very useful strategy in poor responders to maximize the chances to achieve a successful clinical outcome. The natural depletion of ovarian reserve resulting from age-related fertility decline is the reason why a growing population of women wishing to postpone childbearing is turning to oocyte vitrification as a means of fertility preservation.

Embryos are also routinely vitrified in the clinic, either to preserve those that have not been transferred for their use in a future cycle, or while awaiting the results of preimplantation genetic diagnosis of biopsied embryos. Embryo freezing is also applied when conditions are not suitable for transfer,

as occurs when there is a risk of ovarian hyperstimulation syndrome, the level of progesterone is excessively high, or the characteristics of the endometrium may hinder implantation.

Recent studies show that oocyte and embryo survival and reproductive prognosis after vitrification can be impaired by clinical indication, but also by patient age at the time of oocyte retrieval. Success rates are significantly higher when patients are aged 35 years or younger versus patients older than 35 years at oocyte collection [5]. Poorer clinical outcome was reported in cancer patients, low responders, and endometriosis patients when compared to healthy women in similar age groups [6].

29.2.2 Harmful Effects of Vitrification

Although vitrification of oocytes and embryos seems to offer similar results to those obtained in fresh cycles, its real impact on the different cellular structures is still unclear. During the process, the integrity and competence of oocytes and embryos can be compromised by the exposure to CPAs and their removal, sudden temperature changes, cooling to sub-zero, and long-term storage. The potential damage to female gametes and embryos is closely related to their cryobiological properties, such as sensitivity to cooling, permeability to water and CPAs, sensitivity to the chemical toxicity

Table 29.1 Damages of vitrification on oocytes and embryos. *qPCR* Quantitative polymerase chain reaction, *mt* Mitochondria, *mtDNA* Mitochondrial DNA, *IVF* In vitro fertilization, *DEGs* Differentially expressed genes

Structure/function	Study reference	Study design	Detection assay	Main results/findings
Cytoskeleton	Bogliolo et al. [7] (sheep oocytes)	CPA exposed, vitrified, and control oocytes	Raman and confocal microscopy	Abnormal actin distribution pattern in CPA exposed and vitrified oocytes
	Dalcin et al. [8] (sheep embryos)	Frozen, vitrified, and fresh embryos	Confocal, light, and transmission electron microscopy	Disorganized actin filaments and heterogeneous cytoplasm in cryopreserved group
Meiotic spindle	Tamura et al. [9] (mouse oocytes)	CPA exposed oocytes vs. control group	Immuno-cytochemistry	Dynamical changes during CPA addition, and excessive repolymerization of spindle microtubules after warming
	Jung et al. [10] (mouse oocytes)	Vitrified vs. fresh oocytes	Immuno-cytochemistry; comet assay	Abnormal spindle configuration and chromosome arrangement and stability, and higher DNA fragmentation in vitrified group
Mitochondria	Nohales-Corcoles et al. [11] (human oocytes)	Vitrified vs. fresh oocytes	FAD/NAD(P)H autofluorescence; potentiometric dye	Increased oxidized state, but normal mitochondrial distribution and membrane potential in vitrified oocytes
	Hara et al. [12] (cow embryos)	Vitrified vs. fresh embryos	qPCR; ATP assay	Reduced mt number, mtDNA integrity, and ATP content in vitrified embryos
Zona pellucida	Wiesak et al. [13] (cow oocytes)	4 groups: vitrified/fresh, immature/mature oocytes	ZP dissolution into chymotrypsin; IVF and embryo culture	Triggering of ZP hardening and impairment of in vitro developmental competence in vitrified oocytes
Gene expression	Wang et al. [14] (cow oocytes)	Vitrified vs. fresh oocytes	RNA sequencing	102 DEGs of cell cycle, metabolism, fertilization
	Gutierrez-Castillo et al. [15] (cow embryos)	Vitrified vs. fresh embryos	RNA sequencing	732 DEGs involved in adhesion, metabolism, stress response, cell proliferation

of CPAs, and tolerance to volume changes due to osmotic effects.

Some studies have indicated that vitrification may affect oocytes and embryos at the subcellular level, as it could induce changes in ultrastructure, reactive oxygen species (ROS) generation, gene expression, and epigenetic status (Table 29.1).

The cytoskeleton plays a determining role in oocyte maturation and early embryonic development, as well as in the maintenance of cell structure, intracellular transport, and cytokinesis. Cell volume changes caused by CPAs affect the integrity and functionality of the cytoskeleton, as evidenced by abnormal spindle configurations, misplaced organelles, and disorganized microfilaments [7, 8]. In the oocyte, the meiotic spindle temporarily depolymerizes below 37 °C and, although it reappears after warming its conformation can be altered leading to abnormal chromosome segregations [9, 10].

The number of mitochondria, their amount of genetic material, and ATP content are reduced with vitrification, which could be the cause of the increased ROS levels observed in warmed oocytes and embryos [11, 12].

Zona pellucida plays a key role in fertilization and in blocking subsequent polyspermy mediated by the cortical reaction. During oocyte vitrification, CPAs cause transient increases in calcium in the oolemma, in some cases triggering exocytosis of cortical granules, compromising sperm penetration [13].

Cryopreservation has been shown to negatively affect the expression of genes related to oxidative stress, metabolism, ubiquitination, apoptosis, cell cycle progression, and some membrane-related genes involved in adhesion and sperm–oocyte interaction. Also, DNA integrity is impaired by this technique according to some studies [14, 15].

Studies in non-human species report changes in DNA methylation and histone acetylation patterns, whereas no epigenetic changes have been observed in vitrified human oocytes and embryos [16, 17].

Most likely due to the effects described above, pre-vitrification morphological features are often not maintained post-warming, so accurate evaluation after this procedure is required to achieve a successful reproductive outcome. This problem is even more pronounced in blastocyst stage embryos, which often collapse immediately after thawing, making it necessary to keep them in culture for a period to evaluate them more accurately.

29.3 Assessment of Vitrified Oocytes and Grading of Derived Embryos

29.3.1 Quality Variables of Vitrified-Warmed Oocytes

The ability of vitrified oocytes to recover after warming and their potential for further development depend to some extent

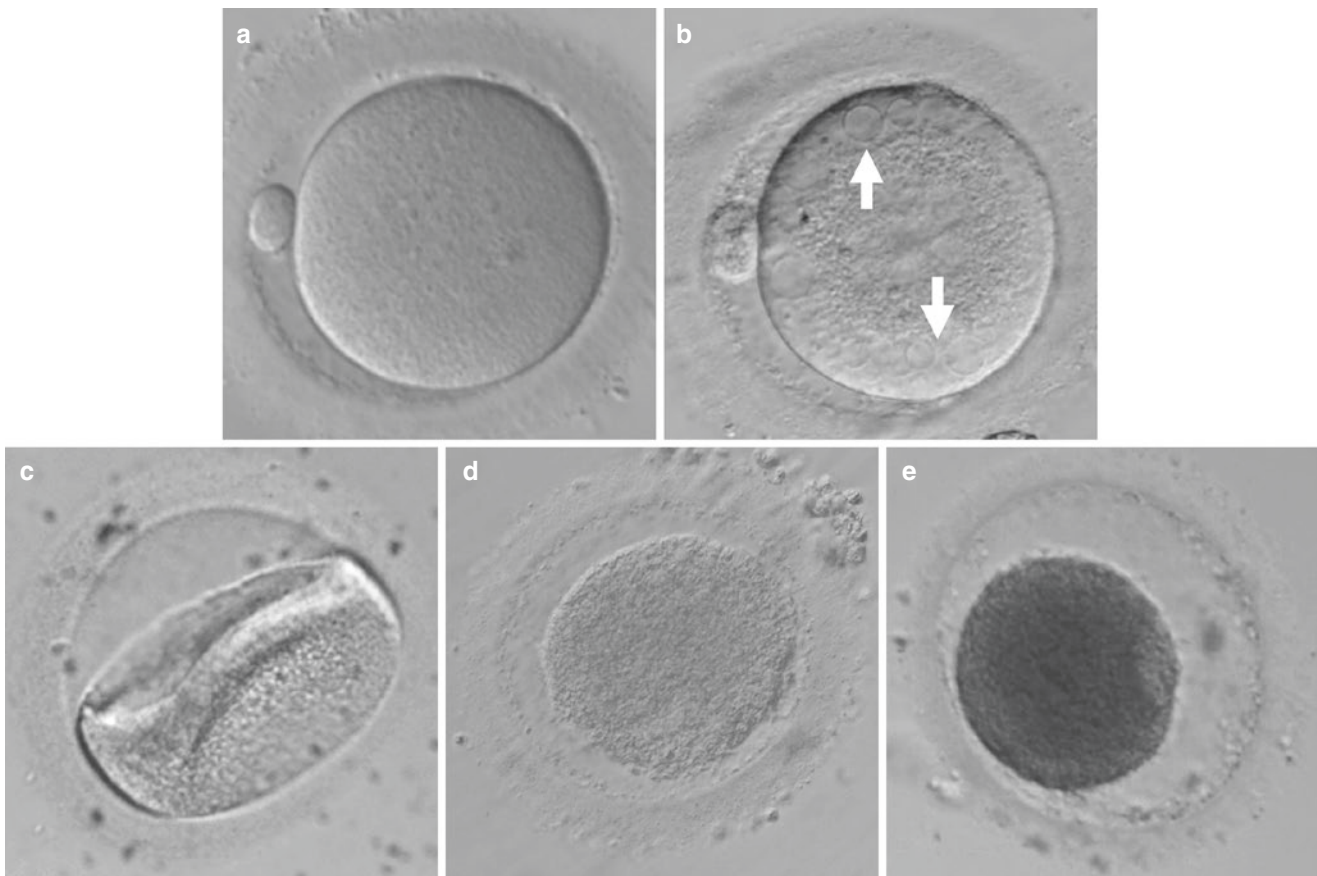


Fig. 29.2 Morphological changes of oocytes after vitrification and warming. (a) Normal. (b) Vacuolated (arrows) and granular cytoplasm. (c) Degenerated, biconcave disc-shaped. (d) Degenerated, dull-colored cytoplasm. (e) Degenerated, dark and shrunken

on the clinical conditions of the patient. Age at the time of egg retrieval, indication for vitrification, body mass index (BMI), hormone levels, or the ovarian stimulation protocol employed are some of the determining factors.

Given the possible impact of the effects described in the previous section, the first step after warming of vitrified oocytes is to evaluate their survival. Thus, the integrity of the plasma membrane and the ZP is checked by observing their continuity and regularity. Cytoplasm appearance is also examined, which should be bright, homogeneous, and not excessively granular. Vitrification could induce the appearance of vacuoles in the cytoplasm, which a priori does not indicate degeneration, but may ultimately lead to reduced competence of the oocyte for fertilization and/or reduced embryo development. Degenerated oocytes after warming are easily recognizable because their cytoplasm becomes dull in color, or they darken and shrink, or take a biconcave disk shape (Fig. 29.2).

The subsequent viability of the oocyte will depend on its capability to be fertilized, give rise to adequate embryonic

development, and ultimately result in gestation and live birth. Fertilization should be evaluated about 16–18 h after intracytoplasmic sperm injection (ICSI), when the maternal and paternal pronuclei should be visible in the cytoplasm and the second polar body has already been extruded. Once vitrified-warmed oocytes have been successfully inseminated, their embryonic development can be assessed by considering their morphology and dynamics. Morphokinetic data provided by time-lapse technology or proteomic profiling determined by analysis of spent culture media are other potential non-invasive sources of information about embryonic status during development.

29.3.2 Grading of Embryos Derived from Vitrified Oocytes

Morphological evaluation has always been the main method of embryo assessment. Generally, embryos are examined under an inverted microscope at 400× magnification and

evaluated according to established selection criteria at specific times of development, usually set as day 3 and day 5/6. The quality of a 3-day embryo will be reflected in its symmetry and cell number, while fragmentation and multinucleation are signs of poor prognosis. According to several studies, day-3 morphology is a good predictor of aneuploidy.

Due to the advantages of prolonged culture up to the blastocyst stage, such as self-selection and better synchrony with the endometrium, quality at day 5/6 will be a more reliable indicator when choosing the best embryo for transfer [18]. Good quality blastocysts have a large, rounded, and tightly packed inner cell mass (ICM), and a continuous, cohesive trophectoderm (TE), composed of numerous sickle-shaped cells. In addition, an advanced expansion degree is related to a higher implantation rate. To standardize the criteria among assisted reproduction clinics, different grading systems that include the most relevant morphological variables according to the literature have been proposed, as those by Gardner and Schoolcraft or ASEBIR (Spanish Association for the Study of Reproductive Biology).

Although this evaluation system is a fast, simple, and non-invasive method, it has certain drawbacks. Opening the incubator to view the embryos could compromise stable culture conditions, besides the fact that punctual and static observations entail the loss of information throughout development.

Time-lapse technology represents a useful tool to overcome many of these limitations. Automatic monitoring systems offer the possibility of obtaining objective and accurate information about the whole embryo development without altering its culture conditions. Additionally, it provides a large amount of quantitative morphokinetic data, including the onset, duration, and interval between cell divisions. Pronuclei formation and disappearance, fragmentation, and blastocyst expansion and contractions, among others, can also be recorded with such a system. Its main limitation is that embryos cannot be rotated, which makes observation difficult in case of overlapping blastomeres or increased cytoplasmic fragmentation.

In the last decade, many studies have focused on the search for objective and quantifiable morphokinetic parameters capable of estimating embryo viability and implantation potential and thus improving the results of IVF cycles (Table 29.2). In 2011, Meseguer et al. successfully correlated the timing of the cleavage-stage events t5 (time at 5-cells stage), S2 (second division synchrony or t4-t3), and cc2 (second cell cycle length or t3-t2) to implantation, and then developed a hierarchic model for embryo selection

based on a time-lapse algorithm with the use of the embryo-scope [19]. They also described some aberrant morphological events associated with poor implantation potential, such as direct uneven cleavage from 1 to 3 cells (cc2 <5 h), asymmetry of blastomeres after first cell division, and multinucleation at the 4-cell stage. Parameters such as cc2 or S2 are also useful for predicting embryo ploidy, which has also been related to other variables such as t5-t2, tSB (blastulation starting time), tB (full blastocyst stage), tEB (expanded blastocyst), and tHB (hatching blastocyst) [23, 24]. Live birth is the ultimate goal, and recently it has been found that the position of pronuclei and their fading time, the absence of multinucleation in 2-cell phase, t2, cc2, and tM (morula formation) are morphokinetic parameters able to predict this endpoint [26, 27].

The culture medium where the embryo develops could be a source of information about its protein and metabolic status, so some researchers have tried to define and validate new non-invasive biomarkers of gestational success present or absent in the plate, that is, secreted or metabolized by the embryo. Different protein patterns have been observed depending on the implantation potential and embryo quality, as increased secretion of HLA-G and IL-6 is associated with gestational success [28, 29]. It is also possible to measure the level of ROS in the culture medium, which informs us about the oxidative status of the embryo [30].

Note that morphokinetic parameters, the proteomic profile, and their relationship with embryo viability and clinical outcomes have been extensively studied. A comparative study on morphokinetics showed that embryos from vitrified oocytes had later early cleavage divisions and a shorter compaction stage than those from fresh oocytes, but no differences were observed in time to blastocyst and treatment outcomes [31]. However, the secretome of embryos generated from vitrified oocytes are largely unexplored field that could increase our knowledge about the impact that this technique has at the subcellular level on human oocytes.

29.3.3 Double Vitrification

Despite the possible negative effects of vitrification and warming already described, vitrification of either 3-day or blastocyst stage embryos derived from vitrified oocytes has been shown to have no impact on delivery rates [32]. In the case of embryo re-vitrification, when both freezing are done at day 3, live birth rates are not affected. However, vitrifying on day 3 and re-vitrifying at the blastocyst stage decrease the chance of success [33].

Table 29.2 Morphokinetic markers with clinical relevance. *cc2* Second cell cycle length or t3-t2, *S2* Second division synchrony (t4-t3), *S3* Third division synchrony (t8-t5), *tPNf* Time of pronuclear fading, *t2/3/4/5/8* Time at 2/3/4/5/8-cells stage, *tM* Time of morula formation, *tSB* Blastulation starting time, *tB* Time at full blastocyst stage, *tEB* Time of expanded blastocyst, *tHB* Blastocyst hatching time

Study reference	Study design	N	Primary endpoint	Morphokinetic parameter	P-value
Meseguer et al. [19]	Retrospective; morphokinetic parameters of implanted vs. non-implanted embryos	247	Implantation	cc2 (≤ 11.9 h) S2 (≤ 0.76 h) t5 (48.8–56.6 h)	0.006 0.016 <0.0001
Goodman et al. [20]	Prospective; daily morphological screening vs. additional time-lapse kinetic monitoring for selection	2092	Implantation	cc2 (<5 h) S2 (0–1 h) tSB (<100 h)	0.05 0.02 <0.01
Cruz et al. [21]	Retrospective; blastocyst rate and morphological features for cleavage-stage embryos, graded according to their morphokinetics	834	Blastocyst formation	cc2 (12.4 ± 0.3 h) S2 (0.6 ± 0.2 h) t2 (26.8 ± 0.2 h) t3 (39.2 ± 0.4 h) t4 (39.9 ± 0.4 h)	0.006 0.031 <0.001 <0.001 <0.001
Desai et al. [22]	Retrospective; kinetics of embryos reaching blastocyst stage and/or implantation	648	Blastocyst formation	cc2 (10.4 ± 4.5 h) S2 (2.4 ± 4.7 h) S3 (10.2 ± 8.7 h) tPNf (24.8 ± 2.6 h) t2 (27.2 ± 3.6 h) t8 (62.1 ± 9.8 h)	0.02 <0.0001 <0.0001 0.001 <0.0001 >0.0001
Minasi et al. [23]	Retrospective; morphokinetic differences between euploid and aneuploid blastocysts	928	Euploidy	S2 (1.7–3.5 h) t4 (39.3–40.6 h) tSB (102.2–104.6 h) tB (108.8–111.5 h) tEB (117.0–120.5 h) tHB (131.2–135.2 h)	0.004 0.002 0.007 <0.001 <0.001 <0.001
Nogales et al. [24]	Retrospective; differences in the cleavage time between types of embryo chromosomal abnormalities	485	Euploidy	t3 (34.7–40.5 h) t5-t2 (>21 h)	<0.001 <0.001
Azzarello et al. [25]	Prospective; pronuclei morphology and kinetics analysis	159	Live birth	tPNf (>20.8 h)	0.003
Rienzi et al. [26]	Retrospective; live birth prediction by euploid blastocyst morphodynamics	830	Live birth	tM (<80 h)	0.04
Sayed et al. [27]	Retrospective; morphokinetic variables related to implantation and live birth	2827	Live birth	cc2 (9.3–12.7 h) t2 (<25.4 h)	<0.01 <0.001

29.4 Assessment of Vitrified Blastocysts

29.4.1 Handling of Vitrified-Warmed Embryos Until Transfer

Blastocyst stage embryos are chosen for warming based on their pre-vitrification morphology, embryonic stage, and day of development. Thus, embryos with better quality ICM and TE, at advanced stages, and vitrified at day 5 rather than day 6, should be given preference to be transferred earlier. Vitrified blastocysts often collapse after warming, so they need to be cultured for a period to incorporate water into the blastocoel until they almost completely recover their pre-vitrification volume. For this reason, some researchers recommend assessing embryo survival and quality not only just after warming, but also at least 3–4 h then, a time after which the blastocyst is able to carry out this re-expansion, and the integrity and viability of the ICM and TE can be clearly determined (Fig. 29.3). Once this time has passed, blastocysts with more than a half dark, granular appearing cells

and large areas of degeneration are classified as non-surviving. Some of them take longer to re-expand, and also the increase in blastocyst size that occurs during re-expansion could be difficult to estimate because it is time-dependent and may change during contractions [34].

29.4.2 Quality Variables of Vitrified-Warmed Blastocysts

As happens with oocytes, recovery of warmed embryos and their clinical outcome will depend somewhat on the clinical factors of the patient, including the type of ovarian stimulation protocol used. Nevertheless, there are other frozen blastocyst-specific predictors, such as blastocoel re-expansion, which has been described as the most important indicator of vitrified blastocyst potential, as it is related to the osmotic and metabolic condition [35, 36] (Table 29.3). In 2017, Coello used time-lapse incubators to measure several variables of the morphological dynamics of the devitrified

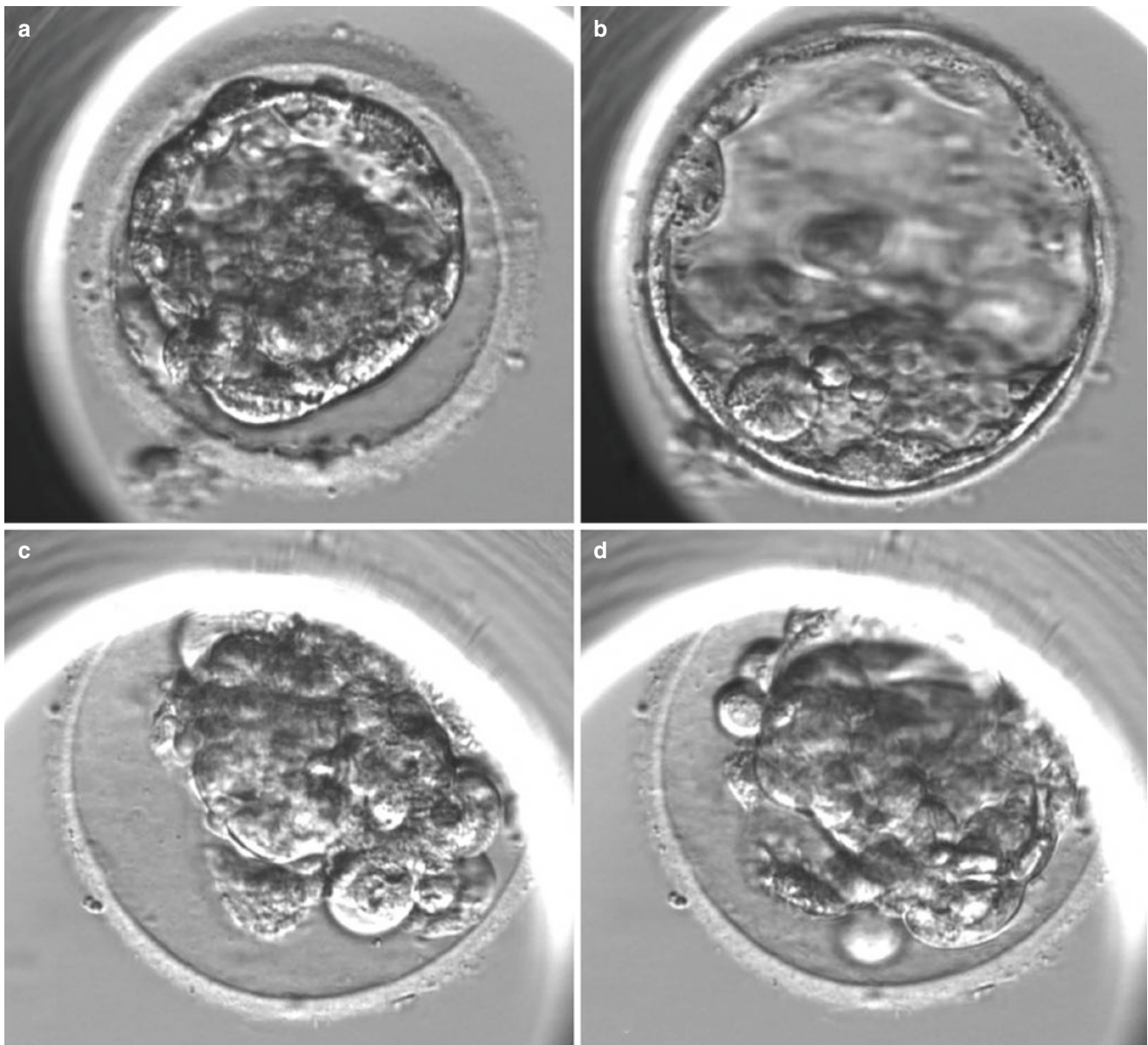


Fig. 29.3 Blastocyst re-expansion after vitrification and warming. (a) Good quality blastocyst 0.5 h after warming. (b) Good quality re-expanding blastocyst 4 h after warming. (c) Poor quality blastocyst 0.5 h after warming. (d) Poor quality non-re-expanding blastocyst 4 h after warming

blastocyst, such as the initial and minimum thickness of the zona pellucida (ZP), the initial and maximum blastocyst area, and the presence of re-expansion and contractions. By generating an embryo grading algorithm, she found that a larger blastocyst area after re-expansion and a thinner ZP are good predictors of clinical outcomes [39]. In the current year, Allen and colleagues found that fully re-expanded blastocysts yield significantly higher LB rates than partially re-expanded and collapsed blastocysts [43]. Other authors report that high-quality embryos also take less time to re-expand [37, 38, 41]. On the other hand, it has been found that the presence and number of blastocoel contractions are not related to the implantation rate [39]. Some of the negative

predictors described are high granularity of the cytoplasm and necrotic foci.

The main pre-vitrification parameters related to the possibility of achieving a LB are the quality of TE and the degree of expansion. This last one correlates with a lower survival to vitrification, but with a higher rate of implantation and RNV. This problem can be solved by performing artificial shrinkage of embryos before vitrification, which in turn decreases the time needed to re-expand. Delayed blastulation has been correlated to lower outcomes regardless of the morphologic quality [40, 42].

The effect of trophoctoderm biopsy used in preimplantation genetic testing on blastocyst cryosurvival has also been

Table 29.3 Quantitative markers predicting implantation potential after transfer of vitrified-warmed embryos. *TE* Trophectoderm, *tSB* Blastulation starting time, *tRE* Start of blastocyst re-expansion, *tCRE* Time to complete re-expansion

Study reference	Study design	N	Primary endpoint	Marker	P-value
Ahlström et al. [34]	Retrospective; morphological predictors for live birth of frozen-thawed blastocyst transfer cycles	243	Live birth	Expansion degree Re-expansion degree TE quality	0.0041 0.0033 0.002
Yin et al. [37]	Retrospective; morphology and re-expansion speed association with implantation and clinical pregnancy	327	Clinical pregnancy	Re-expansion speed (>50% after 2 h)	Not reported
Du et al. [35]	Retrospective; blastocoele expansion and re-expansion, and ICM and TE grades as predictors of live birth	370	Live birth	Re-expansion degree	0.02
Desai et al. [36]	Retrospective; blastocyst features predictive of successful pregnancy and live birth	669	Live birth	Re-expansion degree tSB (day 5)	<0.001 <0.001
Ebner et al. [38]	Prospective; morphokinetics of blastocoele re-expansion and hatching as predictors of implantation, clinical pregnancy, and live birth	144	Live birth	tRE (0.69 ± 0.45 h) tCRE (2.16 ± 0.94 h)	0.003 0.04
Coello et al. [39]	Retrospective; morphological dynamics, quantitative morphological variables related to implantation, and a hierarchical model using the most predictive parameters	429	Implantation	Initial area ($>9900 \mu\text{m}^2$) Max. area ($>141,597 \mu\text{m}^2$) Initial ZP thickness ($\leq 18 \mu\text{m}$) Min. ZP thickness ($\leq 12 \mu\text{m}$)	0.04 0.004 0.02 0.03
Zhao et al. [40]	Retrospective; predictive value of blastocoele expansion, ICM, and TE grades on clinical pregnancy outcome	1154	Clinical pregnancy	Expansion degree	<0.001
Hwang et al. [41]	Retrospective; effects of long-term and short-term post-warming culture, correlation between culture duration and clinical outcomes	168	Clinical pregnancy	tCRE (long-term: 2.19 ± 0.63 h; short-term: 1.17 ± 0.29 h)	0.003 0.018
Coello et al. [42]	Retrospective; most predictive pre-vitrification parameters of survival and live birth	11,936	Live birth	TE quality tSB (day 5)	<0.001 <0.001

analyzed. This practice has not shown any association with embryonic degeneration after warming [44].

29.4.3 Embryo Selection Methods

Although embryologists now have large datasets available to decide which is the best embryo for transfer, intra- and interobserver agreement is quite poor due to the subjectivity of viability, morphology, and timing assessments. Variations in culture conditions between laboratories make the criteria for evaluating embryo quality even more different for each center. To deal with this problem, several embryo selection models have been developed in recent years.

The first models were based on conventional statistics and used logistic regression analysis to find variables related to the clinical outcome [19]. Recently, researchers have begun to build machine learning models based on artificial intelligence (AI) algorithms. They are provided with inputs to automatically learn to annotate embryo cleavage times, classify embryos, or predict an outcome [45]. Further, deep learning is a subfield of machine learning, which is based on the unsupervised training of artificial neural networks (ANNs) with a large amount of unlabeled information [46].

To date, very few researchers have employed AI on vitrified-warmed embryo data with the aim of predicting survival to vitrification or reproductive success [47]. In 2021, iDAScore® ranking software, based on a deep learning con-

volutional ANN, has been shown to be as useful or even better than traditional embryo assessment or annotation-dependent scoring tools in the prediction of clinical pregnancy after single vitrified-warmed blastocyst transfer [48]. Moreover, Alegre used the embryo morphological grade and annotations of post-warming dynamics as input data in ANNs for LB prediction [49]. This is an approach to how images, videos, and other kinds of data of the period between the warming procedure and embryo transfer can be input into these AI models to obtain new post-warming predictive markers that the human eye is not able to detect.

29.5 Conclusion

Although vitrification is the best option for oocytes and embryo cryopreservation, their viability is not always ensured. The low oocyte survival cannot yet be explained, so research efforts should focus on this point. Also, establishing a rigorous evaluation process for warmed embryos in the 4–6 h until transfer may help to clarify whether freeze-thaw damage is a potential explanation of unexpectedly poor outcomes from embryos deemed good quality according to pre-cryopreservation evaluations. Furthermore, AI-based evaluation of these blastocysts would allow clinics a greater time window to thaw more embryos or cancel the transfer, decreasing the time constraint of post-thaw embryo viability assessment common to the typical cryocycle workflow.

29.6 Author Perspective

Undoubtedly, vitrification can be recognized as one of the latest major advances in the field of assisted reproduction, but certainly, the next step would be the rigorous standardization of all aspects surrounding vitrification and warming, including decision-making related to the oocytes and embryos involved, to achieve complete consistency between different laboratories. This goal could be accomplished through the development and implementation of deep learning algorithms capable of predicting survival and clinical outcome from heterogeneous data. In the case of vitrified embryos, it would be ideal to generate an ANN linking time-lapse videos of the pre-vitrification and post-warming periods, proteomic and metabolic profiles, and non-invasive preimplantation genetic testing obtained from the spent culture medium, with the probability of obtaining a live birth. It would also be possible to minimize human error through the design of automated devices that successfully perform the vitrification and warming processes. Another unexplored potential area of research could be the analysis of extrinsic variables in the work environment that may affect clinical outcomes.

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Health of Offspring Following Cryopreservation of Oocytes and Embryos

30

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Key Points

- Women pregnant after frozen embryo transfer have an increased risk of hypertensive disorders in pregnancy including preeclampsia compared with fresh cycles. Moreover, the risks of hypertensive disorders in pregnancy seem to increase further in programmed frozen cycles compared to natural frozen cycles.
- Children born after frozen thawed embryo transfer have a lower risk of preterm birth and low birth weight than children born after fresh embryo transfer but a higher risk than spontaneously conceived children.
- The higher risk of macrosomia and large for gestational age in children born after frozen thawed embryo transfer compared to fresh embryo transfer and spontaneous conception may have a negative effect on long-term health. However, studies so far have been inconclusive.
- Vitricification might damage the eggs, which may pose health risks for the offspring. Information collected so far at birth and up to the age of 6 years has shown no health differences between children born after use of frozen and non-frozen (or fresh) oocytes.

30.1 Introduction

The rate of frozen/thawed embryo transfer (FET) cycles has increased ever since the first baby was born after this technique in 1983 reported by Trounson and Mohr [1–3]. Particularly after vitricification of human blastocysts was undertaken at the beginning of this decade, FET has shown growing success rates mirroring pregnancy and live birth rates after fresh embryo transfers [3]. In the US and Australia/New Zealand, the proportion of FET cycles exceeded the rate of fresh ET cycles in 2015, while Europe in 2017 still performed more fresh cycles [1]. According to the CDC, 78.8% of the cycles in the USA in 2019 were FET cycles [4]. FET has obvious advantages herein facilitation of single embryo transfer and freezing of surplus embryos to avoid ovarian hyperstimulation syndrome (OHSS) and further to allow diagnostics prior to preimplantation genetic diagnostics (PGT) [5]. By combining the GnRH agonist trigger and a freeze-all strategy, FET has almost eliminated late-onset OHSS [6, 7]. Even more important, the improved cryo-techniques have promoted the use of single embryo transfer, hence reducing multiple birth rates from above 30% to less than 10% after assisted reproductive technology (ART) without affection overall live birth rates [8, 9]. Simultaneously a significant decline in preterm birth rates after ART has been observed, and a drop in the rate of ART children with cerebral palsy [10]. Thus, FET is a key tool to reduce multiple births, thereby reducing maternal complications and improving short- and long-term health of ART children. However, concern has been raised if the cryo-techniques may cause mechanical, chemical, or thermal stress to the oocytes and embryos affecting pregnancy and offspring.

The literature on obstetric and perinatal outcomes after cryopreservation is comprehensive and shows different risk profiles in singletons born after frozen and fresh embryo transfer and natural conception, while the literature on long-term health is modest. The causes for the differences observed between frozen and fresh embryo transfer may be

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due to the cryo-techniques themselves, but parental factors cannot be excluded. To disentangle between these influencing parts is a challenge but can be done in sibling studies, where the same mother gives birth to children after various ART procedures. Hence the maternal factors are kept steady, and the contribution from different ART methods per se can be explored.

Further, the clinical indications for oocyte and embryo cryopreservation are wide including egg donation for both. For oocyte cryopreservation, the indications are gonadotoxic treatments, fertility preservation in healthy women, and unplanned unavailability of spermatozoa at the time of oocyte retrieval and for religious reasons when embryo cryopreservation is not allowed. Embryo cryopreservation is performed in case of surplus embryos after IVF/ICSI, risk of OHSS, uterine pathology, endometriosis, and PGT. Hence patient cohorts vary tremendously, which may cause bias if not adjusted for. Other causes of bias are the cryopreservation techniques, including slow freeze or vitrification and cleavage stage or blastocyst freezing and warming, and also the vitrification techniques and culture media differ. The aim of this chapter is to summarize the current literature on obstetric outcome and short- and long-term health of children born after cryopreservation of oocytes and embryos.

30.2 Obstetric Outcomes Following Frozen Embryo Transfer

30.2.1 Obstetric Outcomes After Frozen Thawed Embryo Transfer Versus Fresh Embryo Transfer

A recent meta-analysis from 2018, including five cohort studies and 39,501 pregnancies after frozen embryo transfer (FET), found a higher risk of hypertensive disorder of pregnancy (HDP) after FET than fresh embryo transfer (relative risk, (RR) 1.29 [95% CI 1.07–1.56]) [11]. The increased risk of HDP after FET was confirmed in a Nordic cohort study using a sibling design (odds ratio [OR] 2.63 [95% CI 1.73–3.99]). The study was based on data from the Committee of Nordic ART and Safety (CoNARTaS), and 396 mothers having siblings, one from FET and one from fresh embryo transfer were included [12]. Furthermore, the risk of preeclampsia has been found increased (based on three studies and 4447 pregnancies, RR 1.79 [95% CI 1.03–3.09]), and the risk of OHSS decreased (based on seven studies and 5111 women, RR 0.42 [95% CI 0.19–0.96]) after elective FET (eFET) in a recent meta-analysis compared with fresh embryo transfer [13]. In recent years, a shift toward the eFET-strategy has been highly debated as pros argue that eFET can prevent an impairment of the endometrium and ovarian hyperstimulation caused by high levels of sex ste-

roids and multiple corpus lutea in the fresh cycles due to gonadotrophin stimulation. On the other hand, the cons focus on the prolonged time to embryo transfer and, consequently, time to pregnancy, including the increased risk of preeclampsia and high birth weight found in the offspring after FET [14]. Five large RCTs, published from 2016 to 2020 and comparing pregnancy and live birth rates after eFET and fresh embryo transfer, have reported obstetric results as secondary outcomes [15–19]. One study included 1508 women with polycystic ovary syndrome (PCOS) and found eFET to be associated with a higher risk of preeclampsia (OR 3.12 [95% CI 1.26–7.73]) [15]. On the contrary, three other studies (including 2157, 782, and 460 women) found no increased risk of preeclampsia after eFET. However, only normo-ovulatory women were included in these studies [16–18]. Finally, the fifth study was based on normo-ovulatory women and blastocyst transfers and confirmed the three-fold increased risk of preeclampsia in eFET pregnancies as was also observed in the RCT only including women with PCOS (Tables 30.1 and 30.2) [15, 19]. Three of the four studies, which included only normo-ovulatory women, have been combined in a meta-analysis finding the risk of preeclampsia increased after eFET (RR 1.68 [95% CI 1.03–2.75]) but a similar risk of pregnancy-induced hypertension compared with fresh cycles [16, 18–20].

30.2.2 Obstetric Outcomes After Vitrification Versus Slow Freezing and Prolonged Cryo-Storage Time

At the beginning of this decade, a new rapid freezing technique called “vitrification” replaced the original way of cryopreserving embryos, “slow-freezing.” This new technique, together with prolonged embryo culturing from 2 to 5 days, enhanced the survival rate of the frozen and thawed embryo from previously 50–60% to more than 95% [29–33]. An efficient cryopreservation program is key in ART settings to enhance elective single embryo transfer and thereby reduce multiple births. Furthermore, eFET is central in preventing OHSS in women at risk of this severe complication. Inadequate data are available comparing obstetric outcomes in pregnancies after transfer of a vitrified or slow-frozen embryo due to the almost simultaneous implementation of blastocyst culture and vitrification of surplus embryos. One Nordic cohort study comparing 3650 children born after vitrified blastocysts (Day 5 embryos) and 8123 children born after slow freeze day 2–3 embryos found comparable risks of HDP, placenta abruption, placenta previa, and postpartum hemorrhage but an increased risk of induction of labor and caesarean section (adjusted OR [aOR] 1.14 [95% CI 1.03–1.27] and aOR 1.17 [95% CI 1.05–1.30], respectively) [34].

Table 30.1 Characteristics of studies presenting risk estimated of obstetric outcomes in pregnancies after FET

Randomized controlled trials comparing eFET versus fresh embryo transfer				
Author (year published)	Number of included women	Country	Included women	FET cycles
Chen et al. [15]	1508	China	Women with PCOS	Programmed FET
Shi et al. [16]	2157	China	Norm-ovulatory women	74.2% NC-FET 25.8% programmed FET
Vuong et al. [18]	782	Vietnam	Normo-ovulatory women	Programmed FET
Wei et al. [19]	1650	China	Normo-ovulatory women	62.5% NC-FET 36.4% programmed FET 1.2% other*
Stormlund et al. [17]	460	Denmark	Normo-ovulatory women	mNC-FET
Meta-analyses Jin et al. [20]	Including results from Shi, Vuong, and Wei	China	Normo-ovulatory women	NC-FET and programmed FET
Retrospective cohort studies comparing singleton deliveries resulting from FET with 0 versus 1 corpus luteum				
Author (year published)	Birth cohorts	Country	Number of included singleton deliveries and definition of groups	
			0 corpus luteum	1 corpus luteum
Saito et al. [22]	2013	Japan	Hormone replacement cycle <i>n</i> = 10,235 Cycles not described	NC-FET <i>n</i> = 6287 Clomiphene citrate, human menopausal gonadotropin or FSH
Ginström Ernstad [21]	2005–2015	Sweden	Programmed FET <i>n</i> = 1446 Estrogen and progesterone with or without suppression with a GnRH agonist/antagonist	tNC-FET <i>n</i> = 6297 No exogenous hormones Stimulated cycles <i>n</i> = 1983 mNC-FET (hCG-trigger), ovulation induction (clomiphene citrate or letrozole with/without hCG), gonadotropin stimulation (FSH or human menopausal gonadotropin with/without GnRH agonist/antagonist), luteal support (progesterone with/without hCG)
Jing et al. [23]	2013–2016	Sweden	Programmed FET <i>n</i> = 787 ^a Estrogen and progesterone	NC-FET <i>n</i> = 2802 ^a No exogenous hormones in the follicular phase, luteal phase with vaginal progesterone
Saito et al. [24]	2013–2016	Japan	Hormone replacement cycle <i>n</i> = 75,474 Estrogen and progesterone	NC-FET <i>n</i> = 29,760 hCG-trigger or luteal support
von Versen-Höynck [25]	2011–2017	USA	Programmed FET <i>n</i> = 94 Estrogen and progesterone	mNC-FET <i>n</i> = 127 hCG-trigger and luteal phase with vaginal progesterone SC with known subfertility, IUI, mNC-FET <i>n</i> = 290 Clomiphene, letrozole, or gonadotropins
Asserhøj et al. [26]	2006–2014	Denmark	Programmed FET <i>n</i> = 357 Progesterone and/or estrogen with or without suppression with a GnRH agonist/antagonist	NC-FET <i>n</i> = 779 With or without hCG-trigger
Makhijani et al. [27]	2013–2018	USA	Programmed FET <i>n</i> = 391 Estrogen and progesterone with suppression with a GnRH agonist	NC-FET <i>n</i> = 384 No exogenous hormones in the follicular phase, luteal phase with vaginal progesterone
Wang et al. [28]	2013–2018	China	Programmed FET <i>n</i> = 4162 Estrogen and progesterone	NC-FET <i>n</i> = 10,211 With or without hCG-trigger, luteal phase with vaginal progesterone

FET Frozen embryo transfer, eFET Elective frozen embryo transfer, NC-FET Natural cycle-FET, tNC-FET True natural cycle-FET, mNC-FET Modified natural cycle-FET, SC Spontaneous conception, IUI Intrauterine insemination, FSH Follicle-stimulating hormone

*Both singleton and multiple deliveries, adjusting for plurality

Table 30.2 Risk estimates of studies presenting obstetric outcomes in pregnancies after FET

	HDP Risk estimate (95% CI)	Preeclampsia Risk estimate (95% CI)	Severe preeclampsia Risk estimate (95% CI)	Placenta previa Risk estimate (95% CI)	Placenta abruption Risk estimate (95% CI)	Placenta accreta Risk estimate (95% CI)	PPH Risk estimate (95% CI)	CS Risk estimate (95% CI)	PPROM Risk estimate (95% CI)	GDM Risk estimate (95% CI)
Chen et al. [15] <i>Programmed FET versus fresh embryo transfer</i>	Rate ratio ^a 1.97 (0.68–5.71)	Rate ratio 3.12 (1.26–7.73)								
Shi et al. [16] <i>NC-FET and programmed FET versus fresh embryo transfer</i>	Rate ratio ^a 0.75 (0.24–2.35)	Rate ratio 1.36 (0.77–2.42)								Rate ratio 0.79 (0.43–1.44)
Yuong et al. [18] <i>Programmed FET versus fresh embryo transfer</i>	Relative risk ^a 0.60 (0.14–2.49)	Relative risk 2.00 (0.18–21.97)					No significant difference	No significant difference		Relative risk 0.71 (0.23–2.23)
Wei et al. [19] <i>NC-FET and programmed FET versus fresh embryo transfer</i>	Relative risk ^a 1.27 (0.53–3.04)	Relative risk 3.13 (1.06–9.30)		Relative risk 1.25 (0.41–3.80)			Relative risk 6.20 (0.78–49.37)		Relative risk 0.87 (0.59–1.28)	Relative risk 1.27 (0.84–1.94)
Stormlund et al. [17] <i>mNC-FET versus fresh embryo transfer</i>	eFET: 2/57 (3.5%) Fresh: 2/55 (3.6%) <i>P</i> = 1.00 ^a	eFET: 4/57 (7.0%) Fresh: 4/55 (7.3%) <i>P</i> = 1.00					eFET: 3/57 (5.3%) Fresh: 3/55 (5.5%) <i>P</i> = 1.00	eFET: 12/57 (21.1%) Fresh: 20/55 (36.4%) <i>P</i> = 0.07		
Saito et al. [22] ^b <i>Hormone replacement cycle versus NC-FET</i>								aOR 1.64 (1.52–1.76)		
Ginström Erstad (2019) ^f <i>Programmed FET versus a: tNC-FET b: Stimulated cycles</i>	a: aOR 1.78 (1.43–2.21) b: aOR 1.61 (1.22–2.10)			a: aOR 0.71 (0.36–1.37) b: aOR 1.00 (0.46–2.16)	a: aOR 0.93 (0.37–2.35) b: aOR 0.56 (0.16–1.99)		a: aOR 2.63 (2.20–3.13) b: aOR 2.87 (2.29–3.60)	a: aOR 1.39 (1.21–1.60) b: aOR 1.27 (1.08–1.50)		
Jing et al. [23] ^d <i>Programmed FET versus NC-FET</i>	aOR 1.86 (1.38–2.50)							aOR 1.67 (1.37–2.04)		
Saito et al. [24] ^e <i>Hormone replacement cycle versus NC-FET</i>	aOR 1.43 (1.14–1.80)			aOR 1.00 (0.64–1.56)	aOR 1.58 (0.43–5.88)	aOR 6.91 (2.87–16.66)		aOR 1.69 (1.55–1.84)	aOR 1.87 (0.82–4.28)	aOR 0.52 (0.40–0.68)

von Versen-Höyneck (2019) ^f <i>Programmed FET</i> versus <i>a: mNC-FET</i> <i>b: SC with known subfertility, IUI or mNC-FET</i>	a: aOR 3.55 (1.20–11.94) b: aOR 2.73 (1.14–6.49)	a: aOR 15.05 (2.59–286.27) b: aOR 6.45 (1.94–25.09)							
	aOR 1.87 (1.17–3.00)	aOR 2.40 (1.43–4.02)	aOR 1.10 (0.46–2.62)	aOR 1.19 (0.45–3.16)	aOR 2.24 (1.68–2.99) ^h	aOR 1.52 (1.15–2.01)	aOR 1.19 (0.81–1.74)		
	aOR 2.39 (1.37–4.17)		aOR 1.50 (0.57–3.89)	aOR 1.11 (0.12–10.15)	aOR 2.98 (0.25–34.95)	aOR 1.44 (1.04–2.01)	aOR 2.39 (1.37–4.17)	aOR 1.69 (0.95–2.99)	No significant difference
Asserhøj et al. [26] ^g <i>Programmed FET</i> versus <i>NC-FET</i>			No significant difference	No significant difference			No significant difference		No significant difference
Makhijani et al. [27] ⁱ <i>Programmed FET</i> versus <i>NC-FET</i>		aOR 2.55 (2.06–3.16)			aOR 2.94 (1.44–5.99)				
Wang et al. [28] ^j <i>Programmed FET</i> versus <i>NC-FET</i>									

FET Frozen embryo transfer, *eFET* Elective frozen embryo transfer, *NC-FET* Natural cycle-FET, *mNC-FET* Modified natural cycle-FET, *SC* Spontaneous conception, *IUI* Intrauterine insemination, *aOR* Adjusted odds ratio, *CI* Confidence interval, *BMI* Body mass index, *IVF* In vitro fertilization, *ICSI* Intracytoplasmic sperm injection, *FSH* Follicle-stimulating hormone, *HDP* Hypertensive disorders in pregnancy, *PCOS* Polycystic ovarian syndrome, *PGT* Preimplantation genetic testing, *LH* Luteinizing hormone, *PPH* Postpartum hemorrhage, *CS* Caesarean section, *PPROM* Preterm prelabor rupture of membranes, *GDM* Gestational diabetes mellitus

^aOnly pregnancy-induced hypertension

^bLogistic regression analyses adjusted for maternal age, culture duration, child's sex, number of embryos transferred, use of assisted hatching, and cause of infertility

^cLogistic regression analyses adjusted for maternal age, BMI, parity, smoking, child's sex, year of birth of child, chronic hypertension, maternal educational level, years of involuntary childlessness, cause of infertility, IVF/ICSI, freezing method, culture duration, and number of gestational sacs

^dLogistic regression analyses adjusted for BMI, endometrial thickness on translate day, basal FSH level, culture duration, the day of development of frozen embryos, plurality, and HDP (when appropriate)

^eLogistic regression analyses adjusted for maternal age, culture duration, number of embryos transferred, use of assisted hatching, and cause of infertility

^fLogistic regression analyses adjusted for maternal age, BMI, parity, history of hypertension, PCOS, and diabetes mellitus (pregestational and gestational)

^gLogistic regression analyses adjusted for maternal age, parity, child's sex, year of birth of child, IVF/ICSI, culture duration, and single embryo transfer

^hNot adjusted for culture duration

ⁱLogistic regression analyses adjusted for maternal age, BMI, parity, smoking, diabetes mellitus, chronic hypertension, cause of infertility, number of embryos transferred, and PGT

^jLogistic regression analyses adjusted for maternal age, BMI, cause of infertility, primary infertility, IVF/ICSI, number of embryos transferred, endometrial thickness, number of antral follicle count, blood pressure, baseline FSH, LH, and total testosterone, fasting glucose level

30.2.3 Obstetric Outcomes After Different Cycle Regimens in Frozen Thawed Embryo Transfer Cycles

A FET cycle can either be performed in a natural cycle (NC-FET), a stimulated cycle, or a programmed cycle. NC-FET is used for regular cycle women, and the endocrine environment is kept physiological, and the transfer of the frozen/thawed embryo is being either timed by (1) the peak in luteinizing hormone (LH) and/or (2) by monitoring the size of the growing follicle by frequent ultrasound scans or (3) timed by hCG administration for ovulation induction. One corpus luteum is present in a NC-FET. Stimulated cycles use low doses FSH or letrozole and result as well in one or two corpus luteum. In a programmed FET cycle, also called artificial FET or substituted FET, the endometrium is primed by estradiol in the follicular phase, and additional progesterone in the luteal phase and no corpus luteum is created contrary to a NC-FET or stimulated cycle because of the suppression of the hypothalamic–pituitary axis. The programmed cycle is used for logistic reasons or for women not presenting a regular cycle.

The different endometrium preparation protocols are suggested to influence the maternal adaption to pregnancy, including obstetric outcomes, and the presence of the corpus luteum is thought to play an important role. However, the mechanisms are not fully understood [35–40]. In early pregnancy, the corpus luteum is the primary source of hormones like estrogen, progesterone, relaxin, and vascular endothelial growth factor (VEGF), which is crucial for, among other things, placenta development, vascular function, and circulatory adaptations in pregnancy [36–41]. Several cohort studies have compared obstetric outcomes after different FET protocols (Tables 30.1 and 30.2) [22–28, 41, 42]. Current studies agree on an increased risk of HDP, [23–27, 42], preeclampsia, [26, 28, 41], and caesarean section after programmed cycles [22–28, 42]. Moreover, programmed cycles seem to be associated with an increased risk of postpartum bleedings (Tables 30.1 and 30.2).

30.3 Perinatal Outcomes Following Frozen Embryo Transfer

30.3.1 Perinatal Outcomes After Frozen Thawed Embryo Transfer Versus Fresh Embryo Transfer

Several large cohort studies, as well as systematic reviews (SRs), have evaluated the perinatal outcome after FET ver-

sus fresh embryo transfer in singleton pregnancies [11, 43–48]. Two recent SRs and meta-analyses [11, 46], including 26 and 31 studies, respectively, are presented in Table 30.3. Embryos were frozen either at cleavage stage, i.e., day 2–3 or at blastocyst stage, i.e., day 5–6, and with either slow freezing or vitrification as cryopreservation method [11, 46]. Replacement of embryos was performed in a natural, stimulated or programmed cycle [11]. The risks of preterm birth (PTB) (<37 weeks), very preterm birth (VPTB) (<32 weeks), low birth weight (LBW) (<2500 g), very low birth weight (VLBW) (<1500 g), and being born small for gestational age (SGA) were lower after FET than after fresh embryo transfer. The risks of high birth weight (>4000 g), very high birth weight (>4500 g), and being born large for gestational age (LGA) were higher after FET than after fresh embryo transfer. The meta-analysis by Maheshwari et al. showed similar rates of admission to neonatal intensive care unit, birth defects, and perinatal mortality [11]. There was no substantial change over time for perinatal outcomes [11].

30.3.2 Perinatal Outcome After Elective Frozen Thawed Embryo Transfer Versus Fresh Embryo Transfer

Selecting the best quality embryos for fresh embryo transfer while cryopreserving less good quality may result in two morphologically different populations of embryos with different risks of any adverse outcome. Few studies compare adverse outcomes between good and poor quality embryos; however, there is no indication of more adverse outcomes in children born from poor quality embryos [50]. Yet, an appropriate comparison group to children born after fresh embryo transfer may be children born after eFET (“freeze all” strategy).

Several RCTs have been published, summarized in two SRs comparing eFET versus fresh embryo transfer, with live birth rate and OHSS as the main outcomes and obstetric and perinatal outcomes as the secondary outcomes (Table 30.3) [13, 49]. Roque and colleagues evaluated (PTB), mean birth weight, and birth defects and found no difference between eFET and fresh embryo transfer [13]. A more recent Cochrane review included eight trials [49]. An increased risk was reported after eFET for neonatal hospitalizations, for a baby born LGA, and singletons had a higher mean birthweight. There were no differences in PTB rate, perinatal and neonatal mortality rate, in the risk of a baby being born SGA, or with a birth defect. The quality of evidence was low or very low for most outcomes.

Table 30.3 Perinatal outcome after frozen thawed embryo transfer versus fresh embryo transfer (reference group)

Author, study design	Inclusion criteria, study period	Preterm birth <37 weeks	Preterm birth <32 weeks	Low birth weight <2500 g	Very low birth weight <1500 g	Small for gestational age	Large for gestational age	Macrosomia >4000 g	Macrosomia >4500 g	Admission to NICU	Perinatal mortality	Birth defects (minor and major)
Maheshwari et al. [11] SR	Cohort studies and randomized trials, any FET vs fresh ET. Singletons 1984–2016	No of studies	No of studies	No of studies	No of studies	No of studies	No of studies	No of studies	No of studies	No of studies	No of studies	No of studies
		Fresh ET	Fresh ET	Fresh ET	Fresh ET	Fresh ET	Fresh ET	Fresh ET	Fresh ET	Fresh ET	Fresh ET	Fresh ET
		Risk ratio (95% CI)	Risk ratio (95% CI)	Risk ratio (95% CI)	Risk ratio (95% CI)	Risk ratio (95% CI)	Risk ratio (95% CI)	Risk ratio (95% CI)	Risk ratio (95% CI)	Risk ratio (95% CI)	Risk ratio (95% CI)	Risk ratio (95% CI)
		20	12	20	13	10	7	3	4	5	12	6
		78,386/	68,927/	78,250/	71,218/	53,418/	51,719/	48,026/	55,313/	3703/	25,203/	25,789/
		202,236	184,377	201,794	189,008	89,044	86,544	113,241	164,542	15,862	77,280	107,692
		0.90	0.85	0.72	0.76	0.61	1.54	1.85	1.86	0.99	1.01	0.92
		(0.84–0.97)	(0.74–0.97)	(0.67–0.77)	(0.69–0.82)	(0.56–0.67)	(1.48–1.61)	(1.46–2.33)	(1.58–2.19)	(0.84–1.18)	(0.87–1.16)	(0.78–1.08)
Sha et al. [46] SR	Cohort studies and randomized trials. Any FET vs fresh ET. Singletons and twins End data September 2017	No of studies	No of studies	No of studies	No of studies	No of studies	No of studies	No of studies	No of studies	No of studies	No of studies	No of studies
		Fresh ET	Fresh ET	Fresh ET	Fresh ET	Fresh ET	Fresh ET	Fresh ET	Fresh ET	Fresh ET	Fresh ET	Fresh ET
		Risk ratio (95% CI)	Risk ratio (95% CI)	Risk ratio (95% CI)	Risk ratio (95% CI)	Risk ratio (95% CI)	Risk ratio (95% CI)	Risk ratio (95% CI)	Risk ratio (95% CI)	Risk ratio (95% CI)	Risk ratio (95% CI)	Risk ratio (95% CI)
		25	14	21	15	11	5	NR	NR	NR	Singletons and twins	NR
		165,195/	156,194/	126,462	156,001/	58,630/	41,667/	NR	NR	NR	17	NR
		148,388	127,009	126,462	135,169	93,446	32,264	NR	NR	NR	84,564/	NR
		0.94	0.81	0.74	0.78	0.63	1.53	NR	NR	NR	128,945	NR
		(0.91–0.96)	(0.75–0.87)	(0.69–0.79)	(0.72–0.84)	(0.60–0.66)	(1.47–1.59)	NR	NR	NR	0.88	NR
				Twins 7				NR	NR	NR	(0.79–0.99)	NR
				1828/				NR	NR	NR		NR
				6923				NR	NR	NR		NR
				0.89				NR	NR	NR		NR
				(0.82–0.96)				NR	NR	NR		NR
Roque et al. [13] SR	RCTs on eFET vs fresh ET. Singletons and multifetal pregnancies Jan 2016 to March 2018	No of studies	No of studies	No of studies	No of studies	No of studies	No of studies	No of studies	No of studies	No of studies	No of studies	No of studies
		Fresh ET	Fresh ET	Fresh ET	Fresh ET	Fresh ET	Fresh ET	Fresh ET	Fresh ET	Fresh ET	Fresh ET	Fresh ET
		Risk ratio (95% CI)	Risk ratio (95% CI)	Risk ratio (95% CI)	Risk ratio (95% CI)	Risk ratio (95% CI)	Risk ratio (95% CI)	Risk ratio (95% CI)	Risk ratio (95% CI)	Risk ratio (95% CI)	Risk ratio (95% CI)	Risk ratio (95% CI)
		4	NR	Mean birth weight 4	NR	NR	NR	NR	NR	NR	NR	2
		All 4727		All 1489								All 2363
		1.13		MD 127								0.88
		(0.93–1.36)		(–3 to 257)								(0.46–1.69)
Zaat et al. [49] SR	RCTs on eFET vs fresh ET. Singletons and multifetal pregnancies From inception to Sept 2020	No of studies	No of studies	No of studies	No of studies	No of studies	No of studies	No of studies	No of studies	No of studies	No of studies	No of studies
		Fresh ET	Fresh ET	Fresh ET	Fresh ET	Fresh ET	Fresh ET	Fresh ET	Fresh ET	Fresh ET	Fresh ET	Fresh ET
		Risk ratio (95% CI)	Risk ratio (95% CI)	Risk ratio (95% CI)	Risk ratio (95% CI)	Risk ratio (95% CI)	Risk ratio (95% CI)	Risk ratio (95% CI)	Risk ratio (95% CI)	Risk ratio (95% CI)	Risk ratio (95% CI)	Risk ratio (95% CI)
		3	NR	Mean birth weight 5	NR	3	3	NR	NR	3	2	3
		1962/		Singletons 127		1962/	1962/	NR	NR	1962/	1137/	973/
		1978		(77–178) g		1978	1978	NR	NR	1978	1153	816
		1.15 ^a		Multiples 4		0.82 ^a	1.96^a			1.37^a	2.27 ^a	1.08 ^a
		(0.89–1.50)		(–21 to 120) g		(0.65–1.05)	(1.51–2.55)			(1.07–1.75)	(0.65–7.84)	(0.65–1.78)

Significant differences are marked in bold. *FET* Frozen thawed embryo transfer, *ET* Embryo transfer, *MD* Mean difference, *NICU* Neonatal intensive care unit, *NR* Not reported, *SR* Systematic review

^aPeto Odds Ratio

30.3.3 Perinatal Outcome After Frozen Thawed Embryo Transfer Versus Natural Conception

A large Nordic cohort study, including almost 5 million singletons, compared perinatal health after fresh embryo transfer, FET, and natural conception using both conventional population-based analysis and sibling analysis [51]. Infants born after FET had higher mean birthweight and increased odds of being born LGA compared to spontaneous conception. Children born after fresh embryo transfer had lower birthweight and increased odds of being born SGA compared to spontaneous conception. Conception by either fresh embryo transfer or FET was associated with increased odds of PTB compared to spontaneous conception. These findings were observed both within siblings and in the population-based analyses.

30.3.4 Perinatal Outcome After Vitriification Versus Slow Freezing

A multicentre Chinese cohort study on cleavage stage embryos with propensity score matching (resulting in 297 matched singletons) showed that vitrification had similar risks of adverse perinatal outcomes of PTB, LBW, SGA, LGA, and macrosomia, as slow freezing [52]. Also, a population-based Australian study on blastocyst transfer after vitrification ($n = 4955$) and slow freezing ($n = 2065$) found similar perinatal outcomes [53]. A Nordic register study explored vitrification of blastocysts (3650 singletons) versus slow freezing of cleavage stage embryos (8123 singletons) and found a higher risk of PTB (aOR 1.33) after vitrification [34].

A recent SR, including 13 studies, compared frozen blastocyst transfer with frozen cleavage stage transfer on obstetric and perinatal outcomes [54]. Singleton pregnancies following frozen blastocyst transfer were associated with a higher risk of being born LGA (RR 1.17; 95% CI 1.08–1.27), PTB (RR 1.13; 95% CI 1.03–1.24) but a lower risk of being born SGA (RR 0.84; 95% CI 0.74–0.95) and a lower risk of perinatal mortality (RR 0.70; 95% CI 0.58–0.86). The quality of evidence was low or very low.

30.3.5 Perinatal Outcome After Prolonged Cryo-storage Time

Two recent meta-analyses evaluated the safety of storage time for cryopreservation lasting up to 8 years. Most of the included studies used vitrification as cryopreservation

method. No increase in birth defects was found [55, 56]. No other perinatal outcomes were reported.

30.3.6 Perinatal Outcome After Different Cycle Regimens in Frozen Thawed Embryo Transfer Cycles

Several recent SRs and cohort studies have shown that the cycle regimens in FET influence the perinatal outcome, i.e., the risk of adverse perinatal outcomes is less in natural (with ovulation and development of a corpus luteum) compared to programmed FET cycles [42, 57–59]. Compared to natural and stimulated frozen cycles, programmed frozen cycles were associated with a higher risk of postterm birth (aOR 1.59; 95% CI 1.27–2.01 and aOR 1.98; 95% CI 1.47–2.68, respectively) and macrosomia (aOR 1.62; 95% CI 1.26–2.09 and aOR 1.40; 95% CI 1.03–1.90, respectively) [42].

30.4 Long-Term Outcomes of Children Born After Frozen Embryo Transfer

The most consistent findings concerning perinatal outcomes are that FET results in babies with a higher mean birth weight and higher rates of macrosomia and being born LGA compared to fresh embryo transfer and spontaneous conception. These findings are important as macrosomia and LGA are not only associated with perinatal and neonatal complications as asphyxia, birth trauma, hypoglycemia, and mortality at birth [60] but also long-term sequelae as psychiatric disorders, childhood malignancies (leukemia), breast cancer, cardiovascular disease, and type 1 and 2 diabetes [61].

30.4.1 Growth and General Health

In an early Swedish cohort study, 255 children born after FET were matched with 255 children born after conventional IVF with fresh embryos, and 252 children from spontaneous conception and compared growth and health during the first 18 months [62]. Growth features were similar for both singletons and twins in the three groups. There were no differences in the rate of major birth defects or prevalence of chronic diseases.

However, a more recent Norwegian population-based study found that children born after FET had larger ultrasound measures during pregnancy and were taller and heavier in the first 2 years than those born after fresh embryo transfer [63]. At 17 years of age, there were no clear differences in anthropometric measurements.

30.4.2 Neurodevelopmental Disorders and Cognitive Development

Similar risk for cerebral palsy, autism spectrum disorders and intellectual disability for FET and fresh embryo transfer were observed in a recent SR [64]. A Danish study using standardized national tests found that adolescents conceived after FET had similar school performance at 15–16 of years of age compared with those conceived after fresh embryo transfer [65].

30.4.3 Diabetes, Cardiovascular Disease, and Obesity

A large registry-based study from Sweden ($n = 11,211$ children born after FET, mean age at follow-up 7 years) suggests that children born after FET may have a higher risk of type 1 diabetes [66]. In a subgroup analysis, an association was found between FET and type 1 diabetes ($n = 45$ events) (adjusted hazard ratio [HR] 1.52; 95% CI 1.08–2.14 and 1.41; 95% CI 1.05–1.89 for FET versus fresh and FET versus spontaneous conception, respectively). Increased risk of obesity for children born after FET compared to fresh embryo transfer (based on 78 events, 14,348 children) (adjusted HR 1.33 95% CI 1.02, 1.73) was found in a Danish-Swedish cohort [67]. No increased risk for cardiovascular disease was observed in the adjusted analysis (based on 27 events, 17,870 children with a mean age at follow-up 7 years). The number of children with type 2 diabetes was very low [67].

30.4.4 Cancer

A Danish population-based registry study found higher risk of childhood cancer after FET ($n = 3356$ children) than after spontaneous conception, but the result was based on only 14 cases, mainly leukemia (HR 2.43, 95% CI 1.44–4.11) [68]. Studies on childhood cancer after ART were recently summarized in a SR, concluding that FET may be related to a higher risk of pediatric cancer (RR 1.37; 95% CI 1.04–1.81) [69].

30.5 Children Born After Oocyte Vitrification

Ultra-rapid cooling and warming rates, high concentrations of cryoprotectants, and low volumes are necessary for the vitrification procedure. The osmotic cell dehydration and the

replacement of the intracellular water with permeable cryoprotectant obtaining a “glass” state and the rehydration with dilution of the cryoprotectant during the warming process have raised concern regarding the cryo-injury, i.e., DNA damage, meiotic spindle disturbances, chromosome abnormalities, and developmental competence of embryos resulting from vitrified oocytes. Further vitrification kits vary and require different cryoprotectants and incubation timing, and lastly the vitrification procedure is highly operator dependent. This section is a summary of the current knowledge on the short- and long-term health of the children born after oocyte cryopreservation. Studies including slow freeze of oocytes were excluded.

30.5.1 Short-Term Health in Children Born After Oocyte Vitrification

The short- and long-term health of children born after oocyte vitrification from 10 existing cohort studies all including more than 25 children conceived with vitrified/warmed oocytes have been summarized in Table 30.4. The cohort studies are very heterogeneous and are a mix of studies including donor and autologous vitrified oocytes, and the indications for oocyte vitrification varied from fertility preservation prior to gonadotoxic treatment (medical reason), fertility preservation to store fertility in non-oncological patients (non-medical reason), oocyte donation, avoiding embryo cryopreservation due to religious causes or prohibition of embryo freezing by law, and lack of semen on day of OPU. Obviously, the female age at OPU and at delivery varied considerably between and within studies with the use of donor and autologous oocytes. Only two studies included a control group consisting of children born after fresh oocytes.

The first review on children born after oocyte vitrification including more than 900 babies showed no increased risk of birth defects after the procedure [74]. Only in 452 pregnancies, the multiple pregnancy status was reported and in many of the studies singleton and multiple birth was a mix, hence the quality of the reporting of perinatal outcomes was poor [74].

A very recent systematic review included 13 studies whereof none were randomized controlled trials and two were case reports [81]. A total of 4159 babies were included in the review all presenting neonatal outcomes with multiple birth rates lingering from 2.6% to 60%. The following outcomes were included: caesarean section rate, gestational age, Apgar score, birth weight, congenital anomalies, and health outcomes. Overall, no significant differences were observed in any of the short-term outcomes between children born after conception with vitrified-warmed oocytes and fresh oocytes [81].

Table 30.4 Short- and long-term outcomes after oocyte vitrification with both donor and autologous oocytes. Case reports were excluded. *GA* Gestational age, *BW* Birth weight, *PTB* Preterm birth, *VPTB* Very preterm birth, *CS* Caesarean section, *OBS*

Author (year)	Country	Children from oocyte vitrification (n)	Controls (n)	Multiple pregnancies (%)	Child outcomes	Gestational age [70]	Birth weight [70]	Congenital anomaly	Long-term health
Chian [71]	Canada Columbia Mexico	200 Probably autologous oocytes as collected after ovarian stimulation	NA	17%	Pregnancy complications, delivery mode, GA, BW, Apgar score, cong. anomalies	37 weeks	2784 g (SD37g)	2.5%	NA
Cobo [72]	Spain	28 from OD cycles	NA	30.5%	Congenital anomalies	NA	NA	No	NA
Nagy [73]	USA	26 from OD cycles	NA	60%	Congenital anomalies	NA	NA	3.8%	NA
Noyes et al. [74]	Review of birth reports	392 Mix of OD and autologous oocytes	NA	No	GA, BW, Congenital anomalies, Epigenetic disorder	39 weeks reported in only 4 babies	3318 g (SD201g)	1.5%	NA
Cobo [75]	Spain	1027 32.1% from OD cycles	1233 from fresh oocytes 12.4% OD cycles	NA	PTB (<37 weeks) VPTB (<34 weeks) CS, BW, Height, Apgar score Birth defects Admission to NICU (days) Perinatal mortality	38.2 weeks	2859 g (2818–2901 g)	1.7%	NA
Levi Setti [76]	Italy	560 Autologous oocytes from infertile patients with superovulatory oocytes	NA for outcomes	13.7%	Congenital anomalies	NA	NA	1.3%	NA
Cobo [77]	Spain	1674 From OD cycles	NA	27%	GA, mortality rate, cong. anomalies	37 weeks	2990 g	0.0005%	NA
Nagy et al. [78]	USA	57 babies From OD and autologous cycles	NA	45.6%	PTB, child health at birth and 1-year, cong. anomalies	26 born preterm 5 singletons 21 multiples	NA	3.5%	1-year old: 97% had reached developmental milestones
Van Reekem et al. [79]	Belgium	72 From OD cycles All ICSI	41 children from OD cycles born after use of a fresh oocyte All ICSI	NA	GA, PTB, BW, Apgar scores, Neonatal admission, Cong. anomalies, Anthropometric and health at 2 years of age	38.9 weeks	3200 g	Major: 2.8% Minor: 5.6%	2-year old: Growth and health of children born after oocyte vitrification are similar to children born after use of fresh oocytes
Takeshige et al. [80]	Japan	116 Autologous oocytes Chemotherapy (medical indication), or postponement childbearing (non-medical indication). Male partners for TESE	Average data issued by the Ministry of Health, Labor and Welfare of Japan	2.6%	Follow-up 6 years of age Questionnaire data Cong. anomalies, growth, physical, and mental development	4/110 (3.6%) preterm reported only in singletons	2970 g (SD 0.42 g) Only in singletons	1.7%	6-year old: Growth and mental health are similar after oocyte vitrification and Japanese average national data

Gestational age at delivery, PTB and VPTB rates varied a lot between studies which is not surprising as the multiple birth rates differed. In the 110 singletons from vitrified oocytes in the Japanese cohort, the preterm birth rate was 3.6% equal to the preterm birth rates from the national Japanese statistics [80]. In the Belgian study, the preterm birth rate was 11.1% and 7.3% in the vitrified vs. the fresh oocyte group, respectively [79]. In the large Spanish study with multiple birth rates above 20% in both groups, the PTB rate (<37 weeks) was 22.7% vs. 21.8% in vitrified vs. fresh oocyte group and for VPTB (<34 weeks), the corresponding figures were 6.1% and 4.6% showing no statistical differences [75]. Similarly, for birth weight most of the studies present a mean birth weight of groups that are a mix of singletons and twins. In the two studies presenting birth weight only for singletons, the mean birth weight ranged from 2970 to 3200 g which did not differ significantly from the control groups [79, 80]. Overall cohort sizes, control groups and multiple births rates varied considerably making firm conclusions regarding PTB and low birth weight after oocyte vitrification very difficult. Further on the proportion on oocyte donation pregnancies also varied and it is well known that oocyte recipient pregnancies have higher risk of HDP and adverse obstetric outcomes [82].

The rate of birth defects varied from 0 to 5.6% mostly reflecting the mode of data collection on birth defects in the different studies. In the systematic review, overall 51 babies out of the 4159 (1.2%) included had an anomaly [81]. This rate is much lower than the comparable rate of 3% in the US national register and lower than the 3.4% major malformation rate in ART singletons and 2.9% malformation rate in the non-ART population observed in the Nordic cohort studies [83, 84]. Thus, a firm conclusion on the rate of malformations after vitrification of oocytes is difficult to draw.

30.5.2 Long-Term Health in Children Born After Oocyte Vitrification

Only two longitudinal cohort studies on health of 2- and 6-year old children born after vitrified/warmed oocytes have been published from Belgium and Japan, respectively [79, 80]. The Belgian study included 72 children born after oocyte vitrification and 41 children born after use of a fresh oocyte. All included children were born after oocyte donation (OD) cycles with the use of ICSI and oocyte retrieval between 2010 and 2017 and a fresh embryo transfer [79]. Anthropometrics, BMI, and waist circumference at the child age of 2 years were comparable between the vitrification and fresh oocyte group, also after adjustment for treatment, and maternal and neonatal characteristics. The rate of chronic illness, hospital admission, and surgical procedures were comparable between the vitrification and fresh group.

The Japanese study included 116 babies (110 singletons and 3 sets of twins) born after oocyte vitrification and warming followed by ICSI performed over a 20-year period from 2000 to 2020 [80]. Questionnaires were sent 11 times from birth to the age of 6 years to the parents at regular intervals from 3 to 72 months after the child's birth. Based on the reported data, development of motor function and mental status was evaluated as primary outcome. They found mental health and physical development herein motor function and anthropometric measurements in singletons to be similar to the nationally conceived children reported in average data issued by the Ministry of Health, Labor and Welfare of Japan [80]. A major limitation of the study was the low response rates that fell throughout the study with 49 out of 116 respondents (42%) at the first questionnaire 3-months after delivery and only 17 out of 116 (15%) responded to the 6-year follow-up questionnaire. Nagy et al. followed the children up to one-year of age and 97% of the children where the mothers responded had at that time achieved the developmental milestones, but the study included no control group [78].

Although all three studies showed reassuring development in children born after oocyte vitrification at 1, 2 and 6 years of age, databases with short- and long-term surveillance of the children born after this advanced technique are highly recommended.

30.6 Conclusion

The increasing use of oocyte and embryo cryopreservation has made the knowledge on the health outcomes in pregnancies and children even more important. Compared with fresh embryo transfer cycles, women pregnant after FET have an increased risk of HDP including preeclampsia. There is no indication of altered obstetric outcomes after vitrification compared with slow freezing techniques. However, only very few studies have compared these two cryopreservation methods. Current evidence indicates an increased risk of HDP, caesarean section, preeclampsia, and post partum bleeding in pregnancies after programmed FET compared with NC-FET.

Compared with fresh embryo cycles, cryopreservation is associated with lower risks of PTB, LBW, and SGA. Increased risks after FET include higher mean birth weight, higher rates of macrosomia, and LGA; however, there is no indication of any increased risk of birth defects. These risks are higher in programmed FET cycles than in natural cycles. The higher risks of macrosomia and LGA are also applicable when compared to spontaneous conception. The underlying pathophysiology of the increased risk of LGA/macrosomia is not known but potential explanations are the freezing-thawing procedure, differences in intrauterine environment, or epigenetic changes during early embryo/fetal develop-

ment. Whether the increased risk of LGA/macrosomia is associated with more long-term health risks, i.e., cancer, psychiatric and cardiometabolic diseases remain uncertain. There is limited data on long-term follow-up of children born after cryopreservation as embryos. The impact of their higher birthweight on future health needs further evaluation in large studies with older children and longer follow-up time.

Vitrification and slow freezing seem to result in comparable perinatal outcomes. For short- and long-term outcomes in children conceived with vitrified and warmed oocytes, the literature is still scarce and existing studies are very heterogeneous with lack of control groups and a mix of singletons and multiple pregnancies including various proportion of egg donation pregnancies. A cautious conclusion is that it does not seem that vitrification of oocytes increases the risk of adverse short- and long-term health outcomes in the children.

Overall short- and long-term health outcomes in the offspring after cryo-techniques are reassuring though obstetric risks and birth weight profiles differ with FET, fresh embryo transfer, and spontaneous conception. Certainly cryo-techniques should only be used when necessary and when used we may avoid programmed cycles due to the high risk of preeclampsia and use natural or stimulated FET cycles whenever possible. In pregnancies after oocyte donation where programmed cycles are unavoidable, we should consider using aspirin from gestational week 12 to lower the risk of preeclampsia.

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Elective and Onco-fertility Preservation Outcomes After Oocyte Vitrification

31

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31.1 Introduction

Oocyte vitrification with the aim of fertility preservation is nowadays an established approach for both medical and social reasons. It is no longer considered experimental since it is a reproducible, safe, and effective technique, with consistent results according to the available evidence [1]. The procedure itself is very widespread, being easily accessible since most of the clinics provide the technique nowadays.

Vitrification is an ultra-rapid cryopreservation process describing the solidification of water solutions without ice crystal formation due to the high cooling rates obtained when samples are directly plunged into liquid nitrogen, providing high survival rates and a clinical outcome similar to that of fresh oocytes [2].

The indication of oocyte vitrification for fertility preservation would be any women at high risk of ovarian failure. It should be considered as part of the management of those cancer patients in reproductive age who are going to be treated with chemotherapy or radiotherapy. But also for some medical conditions such as advanced immunological diseases that can receive chemotherapy, some genetic conditions that may lead to ovarian insufficiency or those patients who may need repetitive ovarian surgery due to endometriosis or any other benign pathology that may reduce the ovarian reserve and consequently lead to premature ovarian failure [3]. Despite this, the most frequent diagnosis nowadays among patients who vitrify their oocytes is age. Oocyte vitrification has especially become an indication, or at least a recommendation, for those healthy women without any medical indication that just want to delay motherhood, due to a lack of partner, late marriage, financial barriers or frequently

some career aspirations, and in whom the effect of age becomes relevant.

The potential of the vitrification process and the excellent clinical results it offers are well known, so the establishment of gamete banks for egg donation programs or to maintain and postpone the reproductive capacity of patients is not discussed. Despite this, there are different aspects of the process that may concern specialists and patients or their relatives that would determine the lack of referrals for fertility preservation treatments. These are perceived sometimes as limitations and could be due to different aspects related to the high estradiol levels reached with the controlled ovarian stimulation (COS), especially in breast cancer, or the chronology of the stimulation that may delay the start of chemotherapy. The use of letrozole and random start protocols for COS have shown to be the solutions. But also some concerns about their efficacy, safety, or costs.

Definitely, since oocyte vitrification was established, a new population of patients wishing to delay motherhood started demanding it electively for social or medical reasons. It is important to be aware about the efficacy of the technique and the chances of success before freezing for these banks to be useful and to avoid creating unreal expectations at the time of counseling, as pregnancy is not guaranteed and prognosis will depend on the number of available mature oocytes and their quality and age [4].

31.2 Efficacy of the Vitrification Process

Fertility preservation is a relatively recent discipline that started to be considered a few years ago in the treatment of cancer affecting young patients but also for those women who wished to delay motherhood electively. Oocyte cryopreservation is currently the fertility preservation technique of choice [3]. It is the time now when patients have started to use their cryopreserved material, although limited information exists regarding the real efficacy of oocyte vitrification

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especially in the population of cancer patients, with most of the publications consisting of small retrospective series.

Efficacy is highly related to age and the number of available mature oocytes and their quality. Pregnancy is not guaranteed with this type of treatment, just a limited number of IVF attempts, depending on the availability of oocytes. Similarly to fresh oocytes, IVF outcome using warmed oocytes is highly dependent on age at the moment of vitrification. Age, as a factor affecting success rates, will determine and affect the ovarian response and survival of the oocytes and clearly will condition the clinical outcomes, in terms of live birth, which is the main goal of these procedures.

The effect of age on fertility is mainly due to the reduction of the ovarian reserve at the time bad quality oocytes increase and the rise of the percentage of chromosomically abnormal embryos. This is an important point to consider when recommending a number of oocytes to vitrify, as cycle outcome strongly depends on the number of embryos replaced into the uterus and the number of available embryos will decrease with age [5].

One of the strategies to increase the number of oocytes in case of low responders could be to repeat different cycles of ovarian stimulation, in separate cycles or in the same one. This is the DUO-Stim protocol, consisting in two consecutive stimulations within the same cycle, starting the second stimulation immediately after the oocyte retrieval. This is important to consider for low responders and oncological patients, whose benefit is to shorten the stimulation process and increase the yield of oocytes [6]. This is possible with random start protocols, based on the existence of different waves of follicle development along the cycle that can be stimulated as in conventional COS, with similar outcomes regardless of starting stimulation as conventionally, in late follicular phase or in the luteal phase [7].

31.3 Differences Between Both Groups

Since oocytes vitrification was established and spread, there is a huge demand for it from patients delaying motherhood, especially for social reasons, and thus the real efficacy of the technique needs to be assessed. Most of the available studies until very recently consisted of small retrospective series. Cobo et al. recently analyzed in a retrospective study the results of their fertility preservation programme, probably one of the largest series published which included more than 6300 patients who vitrified their oocytes electively or due to oncological reasons, with more than 8000 cycles. Clinical results and outcomes were compared between both groups, although they are two different populations [8].

Oncological patients are mostly young, most of them childless, while elective freezers are usually older, 4 or

5 years more on average. Oncological patients have to start an ovarian stimulation usually suddenly, sometimes with short periods to complete it before chemotherapy starts and sometimes without the best conditions to carry it on due to some dominant follicles or a short recruitment, and often without another opportunity to repeat the stimulation. Social freezers can prepare the stimulations better and carry it on several times to accumulate oocytes if necessary, although the main difference is that they usually make the decision about vitrifying their oocytes at later ages [8].

Stimulation protocols may be also different, especially for breast cancer patients, with the use of the aromatase inhibitor letrozole and lower gonadotrophin doses as the elective protocol trying to avoid high estradiol levels. Anastrozole is another third-generation aromatase inhibitor but with lower response than ovulation inducing agent letrozole. Tamoxifen acts as a selective modulator of estrogen receptors and may also be used as ovulation inductor in breast cancer patients, but it does not improve estradiol levels, or the number of oocytes obtained with letrozole [9]. Oktay et al. compared different regimens for hormone-sensitive tumors with tamoxifen alone and with gonadotrophins and letrozole with gonadotrophins, and the estradiol / number of oocytes ratio was clearly favorable to the use of letrozole [9].

Mean storage time usually is shorter in social freezers, as oncological patients need a longer period to ensure recovery or some breast cancer patients, who need to take tamoxifen usually for a few years [8].

Return rate may be another problem when evaluating the efficacy of oocyte vitrification as it is usually low, i.e., 12% in the elective PF group and 7.4% in the onco group in Cobo's analysis, with the possibility that many of these patients may become pregnant spontaneously or may never use these oocytes, making difficult to analyze its cost effectiveness [8].

All these may be an important bias when comparing the results between both groups and therefore the efficacy of the technique.

31.4 Results in Both Groups

Results for oocyte vitrification are consistent, and success rates are similar to those obtained with fresh oocytes. The prognosis of the IVF cycle will depend on the number of available mature oocytes and also their quality, highly influenced by age, as cycle outcome strongly depends on the number available embryos [10, 11].

The efficacy of the oocyte vitrification procedure is highly related to age and the number of oocytes, and success only may be considered when the patient achieves at least a live newborn, which is the main goal of these treatments. Since

these two groups are different populations with their own characteristics, the comparisons between them are sometimes difficult as results may be different in both groups and thus the efficacy not comparable.

There are different questions or situations to consider when recommending a number of oocytes, as the number of attempts they are willing to do or the number of children the patient's desire. With the usual delay of motherhood, oocyte vitrification may be a great resource when thinking in a few children, as the first one may easily come, but the second one may be more difficult. It is also very important to consider the age of the patient at the moment of the vitrification. Age conditioning the quality of those oocytes and consequently the survival, the percentage of chromosomically abnormal embryos, and the percentage of low responders, who may need several stimulation cycles. The pretended storage time is also important, as it may be late for a further stimulation in case there is no success with no longer possibility for a new IVF cycle.

The relation between age and number of oocytes is an important point as the number of available embryos will decrease with age since chromosomal abnormalities become more relevant, with risk of no available normal embryo to transfer [12].

Cobo et al. stratified cancer patients (OFP) and elective freezers (EFP) in two groups, <35 and >35 years, and observed in their comparison comparable number of retrieved and vitrified oocytes, similar oocyte survival (83.9% vs. 81.8%; NS), but lower for younger (≤ 35 y) OFP patients (81.2% vs. 91.4%; $P < 0.05$). Ongoing pregnancy (57.7% vs. 35.7%) and live birth rates (68.8% vs. 41.1%) were higher in EFP patients ≤ 35 than the OFP matching age patients ($P < 0.05$). CCPR (65.9% vs. 42.8%), COPR (57.7% vs. 35.7%), and CLBR (68.8% vs. 42.1%) were statistically higher for the EFP patients ≤ 35 years than for the OFP age-matching women ($P < 0.05$). Similar results were found for older group (>35) [8].

Since the final objective is a live birth and CLBR highly related to the number of embryos replaced, results will increase in terms of IVF outcome after the cryotransfer of surplus embryos.

Any other factor that acts on oocyte/embryo quality and outcome and, therefore, cycle prognosis may be responsible for declining results of women with previous cancer since live birth rates using own oocytes are lower, but not with egg donations [13].

31.5 Survival and Clinical Outcome

Survival of oocytes is related to oocyte quality, an age-dependent factor which, despite possibly serving as a selection filter, may overshadow the procedure's benefits. When

analyzing the efficacy of oocyte freezing, it is important to point out that similarly to clinical results, the survival data after vitrification and warming in older patients would not be as high as in young patients. Cobo et al. found in their study that after introducing the age in the logistic regression analysis in the elective freezers group, the effect of the indication appeared relevant and the effect of the age was clear, where survival was 4 times higher in younger women (<35 vs >40 years) and LBR 6 times higher [8].

Niederberger and col. published in 2018 in a special issue of the journal *Fertility and Sterility* called 40 years of IVF data comparing survival rates and clinical outcome in different groups: egg donors, younger and older poor responders, and younger and older elective freezers. Ovarian response, survival rate, and clinical outcome were comparable in the egg donation group and in the younger elective freezers group. For young poor responders, survival rates were lower and quite similar to the older groups, with clinical pregnancy rate and CLBR lower than expected for their age. Probably these patients are already infertile patients with impaired quality of oocytes [14].

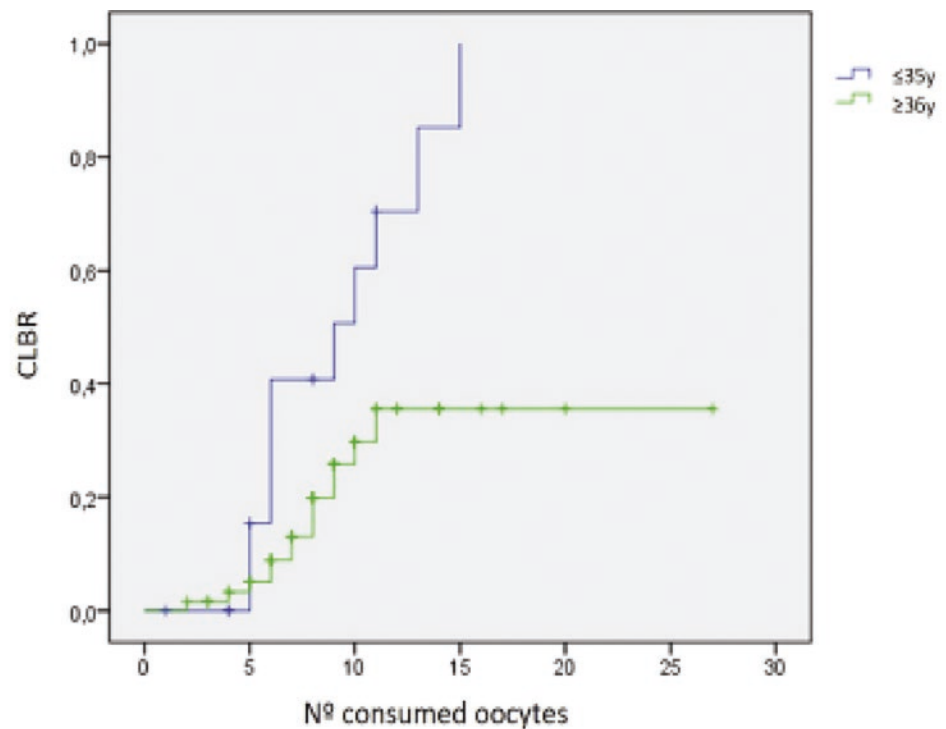
31.6 Number of Oocytes and Live Birth

The ideal number of mature oocytes that should be retained for vitrification is a controversial issue. Cobo et al. [4] analyzed this point in a population who elected to cryopreserve their oocytes due to social FP because of delayed motherhood, lack of a partner, or having an associated medical condition other than cancer due to age-related fertility decline. They found that age-conditioned clinical outcomes and the probability of having a baby depended on the number of oocytes consumed. Patients aged over 36 years had a significantly lower CLBR despite using the same number of oocytes. The gain in the ≤ 35 year group was 8.4% per additional oocyte and reached a CLBR of 85% with 15 oocytes. In the ≥ 36 year group, the gain was much lower per additional oocyte, and the plateau reached 11 oocytes with a 35.6% CLBR (Fig. 31.1) [4].

In 2017, Goldman published a model conducted with 520 fresh cycles to analyze the live birth predictions by age and number of mature oocytes retrieved. Each curve showed the percent likelihood that a patient of a given age would have at least one live birth based on the number of mature oocytes retrieved and frozen. The probability of having a baby using 20 oocytes was 37% for patients aged 40 years, while it was 90% for patients younger than 35, comparable to the 94% of the donors' group [15].

In 2016, Doyle published a study conducted in 128 elective freezers trying to predict the probabilities of having at least one, two, and three live-born children according to the number of mature oocytes cryopreserved and age at vitrifica-

Fig. 31.1 Cumulative live birth rate according to age (<35 vs ≥36) and number of oocytes consumed. Cobo et al. Oocyte vitrification for elective FP. Fertil Steril 2016; 105(3):755–764



≤35 years old		≥36 years old	
Nºoocytes	CLBR (IC95%)	Nºoocytes	CLBR (95% CI)
5	15,4 (-4.2-35.0)	5	5,1 (-0.6-10.7)
8	40,8 (13.2-68.4)	8	19,9 (8.7-31.1)
9	50,6 (31.6-79.6)	9	25,8 (12.7-38.9)
10	60,5 (34.5-89.5)	10	29,7 (15.2-34.2)
15	85,2 (60.5-100)	11	35,6 (18.4-52.8)

tion. They found that in the younger group (30–34 years), the probability of having one livebirth using 20 oocytes was 80%, while in the older than 40 years group, the probability decreased to 40%. For group 35–37 years, it was quite similar to the young one; and for the 38–40 years group, it decreased to 60%, and much lower if they pretended a second or third child, especially for older groups. The oocyte to baby rate, which informs about the oocyte to live-born child efficiency, was in overall 6.4% (30–34 years, 8.2% efficiency; 35–37 years, 7.3% efficiency; 38–40 years, 4.5% efficiency; 41–42 years, 2.5% efficiency) [16].

Comparable data were observed by Cobo et al. [10] in their analysis of their ovum donation program compared to a number of autologous cycles classified by ages. The oocyte to baby rate in the donation program was 6.5%, similar to the <35 years group, and especially lower in the older than 40 group ((30–35 years, 6.6% efficiency; 36–37 years, 6.1% efficiency; 38–39 years, 5.6% efficiency; >40 years, 1.8% efficiency) [10].

In overall, 15 oocytes would be needed to get a live birth in case of donors or young women and 1–2 oocytes more for the older groups up to 40, while in patients older than 40, this number of oocytes would increase up to 55 oocytes [10].

They conclude that 10–15 oocytes would give a 40–70% chance of having a baby in patients under 35 years, and even higher with a greater number of oocytes; however, this correlation is not so clearly observed after the age of 35 years due to the effect of age on oocyte quality and the increase in aneuploidies.

31.7 Age

Age is another factor that contributes to the procedure's success as survival rates lower with it. The outcome of IVF with vitrified oocytes is, as for fresh oocytes, highly dependent on maternal age at the time of freezing, but not related to the vitrification process itself [17]. To face low survival rates

when bad oocyte quality is suspected, embryo cryopreservation could be a solution if the patient has a partner as survival rates seem higher. Women's age beyond 35–37 years is a negative factor and is accompanied with lower survival and pregnancy rates, which become more marked after the age of 40. The cumulative live birth rate (CLBR) lowers significantly as the patient's age increases [11]. The number of metaphase II oocytes in older patients with a larger number of embryos needed to achieve live birth is higher, and the results in this group for cancer patients may be conditioned.

31.8 Are Results Affected in Cancer Patients?

A controversial point is that of the ovarian response and the clinical results in cancer patients, or at least in some of them, since there are studies that show a worse ovarian response to stimulation. There are those who find differences in the response to stimulation only in specific types of cancer, or who may need higher doses of gonadotrophins depending on whether or not there is systemic involvement, or decreased levels of AMH in LH and NHL. There are different authors who think so while others find no differences [18–21].

This possible worse response to ovarian stimulation has been associated with carriers of the BRCA1 mutation, which has been linked to poorer ovarian reserve [22], telomere length and telomerase activity [23], the cancer itself and multisystem involvement [24] or factors related to the stimulation itself and the start of it.

At IVI, we analyzed this point and observed that patients with breast cancer stimulated with letrozole presented a significantly worse response compared to a control group [25]. In a deeper analysis of the data from this study stratifying by number of oocytes obtained, no differences were observed between cancer and control group or letrozole and non-letrozole group, except for the “very low response” group, with a higher percentage of patients in the cancer or in the letrozole group, which corresponded to breast cancer patients (Table 31.1).

This means that a higher percentage of worse prognosis patients could be in this cancer group, and so later the clinical results are observed. Thus, results could be conditioned by the sample and the characteristics of the patients who enter the study. It would be interesting to analyze inclusion criteria in every fertility programme in all the studies respect AMH or low ovarian reserves in order to avoid discarding bad prognosis patients from the studies.

The intention of Cobo et al. [8] in the mentioned study was to analyze further this point in cancer patients and to perform a deeper analysis of the results of their fertility preservation programme with a larger sample and results divided by age-groups. They still found worse clinical results in the young cancer patients group compared to elective freezers.

But similar results were observed for both groups in patients over 35 years [8].

It would be interesting to confirm or not the existence of this specific group that will respond worse and determine if there is really an associated reason, as well as clarify or rule out the possible influence of the disease on the response.

31.9 Safety in Cancer Patients

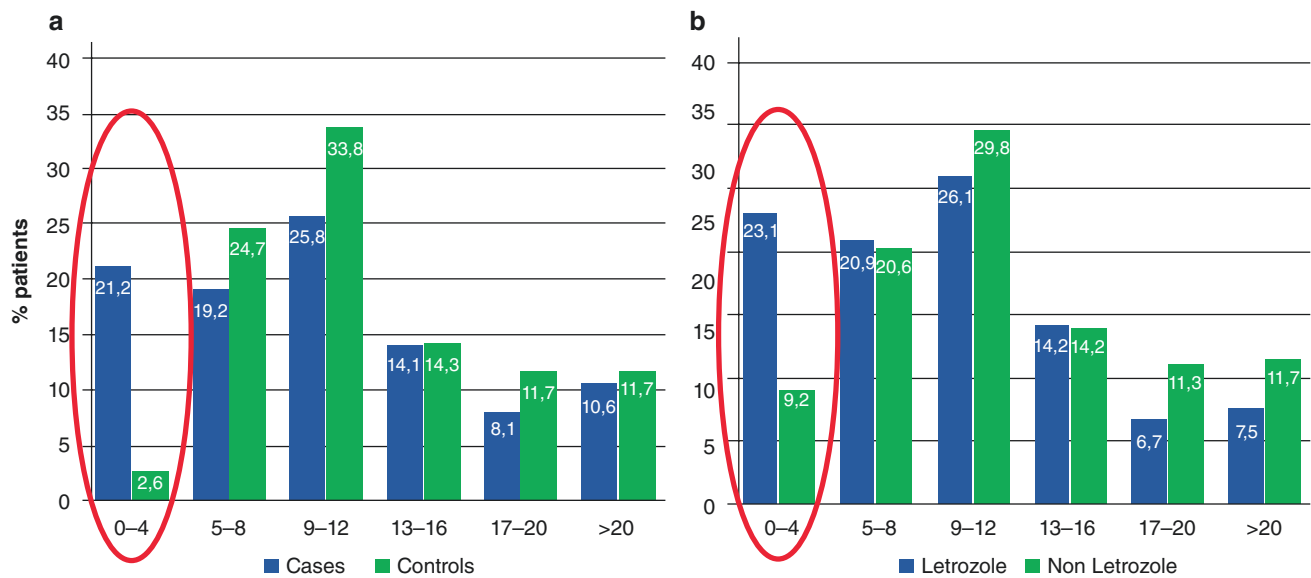
Safety is an important point when considering the efficacy of the procedure, especially for hormone-sensitive tumors such as breast cancer. In 2008, Azim and col. reported no increased recurrence risk when using letrozole and gonadotrophins for COS in breast cancer patients for the purpose of fertility preservation compared with patients who decided not to undergo oocyte cryopreservation [26]. These findings were regardless of one or two ovarian stimulations being completed to increase oocyte/embryo yields, although this is often difficult to perform given the short time before chemotherapy [27]. This possibility highlights early referral of cancer patients for fertility preservation.

But long-term safety had to be assessed, and similar results were observed after longer follow-up periods, with no increased recurrence rates and disease-free survival compared to controls, regardless of estrogen receptors or BRCA mutation status and whether the stimulation was carried out before or after surgery [28]. Other non-randomized prospective long-term follow-up studies have not found an increase in the percentage of recurrences or a decrease in disease-free survival in breast cancer patients who vitrified oocytes as a fertility preservation technique.

In the same way, more than 21 years follow-up studies that analyzed the evolution of patients who underwent ovarian stimulation for IVF treatments did not show an increased incidence of breast cancer compared to the normal population [29]. Even a decreased risk of death of up to 41% was observed in pregnant women after breast cancer compared to non-pregnant women, especially if they had negative nodes. This study would confirm the fact that pregnancy in women with breast cancer seems safe and would not compromise their survival. Therefore, the fact of suffering from breast cancer would not justify denying the opportunity of a future conception [30].

The impact of reproductive treatments on breast cancer risk is largely unknown. Some studies suggest a possible correlation while others have not found a definitive association [31]. Mammary benign and malignant cell lines have been used to assess the risk of the gonadotropins used to carry out the ovarian stimulations with the aim of avoiding any action of the existing *in vivo* estrogens. It has been observed after *in vitro* studies of these cell lines that exposure to FSH, LH, and hCG at doses similar to those used in IVF is not associated with growth in any of these epithelial cell lines. The

Table 31.1 Percentage of patients stratified by number of oocytes obtained. (a) Cancer and control groups. (b) Letrozole and non-letrozole groups. *Fertil Steril* 2012; 97:930–934



potential risk, therefore, would be associated with indirect estrogen-dependent processes in the physiology of these mammary cells [32].

Regarding offspring, the health of children and the obstetrical evolution of pregnancies conceived with vitrified oocytes are similar to those observed in our population of those conceived with fresh oocytes. Nor was there any apparent increase in adverse obstetric and perinatal outcomes in children conceived with vitrified oocytes. These outcomes endorse the safety of the technique [3, 33]. The incidence of other complications, including anemia, diabetes, pregnancy-induced hypertension, low birth weight, preterm premature rupture of membranes, and preterm birth rates is also comparable [33]. Live birth defects do not seem to increase after luteal phase stimulation compared to conventional COS [34].

31.10 Reproductive Counseling

Fertility preservation for oncological reasons is beyond all discussion, and young patients in reproductive age should be considered part of cancer treatment. Everybody agrees with this idea, although counseling should be individualized based on multiple factors, such as the patient's age, ovarian reserve status, type of cancer treatment and urgency to start chemotherapy, and associated risk of gonadal failure [2].

Different oocyte vitrification process aspects concerning its safety or the chronology of the stimulation that may delay chemotherapy may lead patients to forgo fertility preservation and must be clarify to them and their relatives at the time of discussing or recommending the procedure. Most of these aspects have already been solved, such as using letrozole to avoid high estradiol levels after COS, GnRH antagonists to

minimize the effect of progesterone or by starting COS randomly, irrespectively of the menstrual cycle phase, and without compromising its efficacy. Information about the safety of COS is also crucial regardless the number of cycles performed.

When analyzing the efficacy of oocyte freezing, it is important to point out that similarly to clinical results, the survival data after vitrification and warming in older patients would not be as high as in young patients, and physicians must be careful to avoid creating unreal expectations [35]. It is crucial to know real information about the possibilities of success according to the patient's age when recommending and deciding on a fertility preservation approach.

Cancer patients should be informed about their reproductive prognosis in accordance with the number of available oocytes, which could limit the number of embryos and, consequently, pregnancy attempts. These results would also support the idea of elective fertility preservation at young ages for all women who delay motherhood, independently of cancer problems, and there would be more chances of successful IVF cycles and future egg donation treatments would not be that necessary.

In adolescent patients, oocyte cryopreservation is physiologically feasible starting at puberty, considering the particularities of this population.

Oocyte vitrification is not the only available fertility preservation technique. It is the procedure of choice for elective freezers, but for cancer patients, other procedures such as ovarian cortex cryopreservation are possible. This is the main indication for prepubertal girls or for those situations with no available time to complete the ovarian stimulation. Although efficacy is higher with oocyte vitrification, it has good results with the benefit of, unlike oocyte vitrification,

no limit in the number of cycles and therefore the possibility of achieving a spontaneous pregnancy at least while the graft is active [36].

31.11 Conclusions

- Fertility preservation is an effective procedure that can ensure future fertility in both elective freezers and cancer patients. Oocyte vitrification is the only fertility preservation procedure not considered experimental nowadays. It should be recommended for any patient at risk of ovarian failure, although the most frequent indication is the effect of age in people delaying motherhood.
- It is very important to give patients real information about the chances of success before vitrifying their oocytes, especially based on their age, letting them know that pregnancy is not guaranteed and trying to avoid unreal expectations.
- Early referrals are important in oncological patients to have enough time to complete the ovarian stimulation or even carry out several cycles in order to increase the availability of oocytes.
- The objective of an ovarian stimulation for later oocyte vitrification with the aim of fertility preservation is to obtain the highest number of oocytes and at least in oncological patients in the shortest time.
- Clinical results and outcome after oocyte vitrification are similar to those obtained with fresh oocytes and therefore highly dependent on age at the moment of vitrification and the number of available mature oocytes.
- Markers of success after vitrification for fertility preservation are number of oocytes and survival and clinical results, and age affects all these markers.
- 10–15 oocytes is the desirable number with better results in terms of live birth. 36 years could be suggested as a threshold for better and worse outcomes.
- It is discussed if oncological patients may have worse results. Maybe not for all oncological patients but for a selected group.
- Pregnancy in women with a history of breast cancer has been shown to be safe and does not compromise their overall survival. Hence breast cancer patients should not be denied the opportunity of fertility preservation based on this argument.

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Part VI

Multiple Vitrification-Warming and Biopsy Procedures on Human Embryos

Expected Outcomes and Quality Control: Fertility Preservation in Pre-pubertal Girls

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32.1 Introduction

A variety of conditions, both benign and malignant, can cause the loss of potential fertility in pre-pubertal girls. Genetic disorders are the underlying cause in 10% of cases of premature ovarian insufficiency (POI). Girls with genetic disorders causing gonadal dysgenesis or dysfunction (e.g. Turner syndrome (45XO) or the presence of the Fragile X premutation (*FMRI*)) may have pubertal delay and/or POI at a very early age, long before they would be in a position to consider starting a family. Pre-pubertal girls may also require fertility preservation due to iatrogenic reasons: treatments for childhood cancer, including chemotherapy, radiotherapy, and certain pelvic surgery can put ovarian function at risk, whilst gonadotoxic medication for non-malignant conditions such as haematological conditions (e.g. those requiring a stem cell transplant for sickle cell disease) and for rheumatological conditions can also affect future fertility.

In the case of childhood cancers, rates have been rising over the last few decades; however, survival rates are also increasing due to improvements in cancer treatments. More than 80% of patients with childhood cancer will now survive more than 5 years from diagnosis [1]. The result of these medical advances means that there are an increasing number of childhood cancer patients surviving to adulthood. A proportion of these patients will go on to have treatment-induced subfertility and therefore would be suitable candidates for pre-treatment fertility preservation. Unfortunately, there are

currently limited fertility preservation options available for pre-pubertal girls compared to post-pubertal females. This chapter will detail why this is and summarize our current knowledge regarding fertility preservation options for pre-pubertal girls.

32.2 Ovarian Development

The formation of the female's population of oocytes, contained within primordial follicles constituting the ovarian reserve, begins early in the post-implantation embryo and is a highly regulated process. A small number of primordial germ cells are created, which go on to form oogonia. The oogonia proliferate rapidly, forming germ cell nests within the gonadal ridges, before entering meiosis and becoming oocytes. The oocytes arrest their meiotic progression at the end of prophase I and, from around 18 weeks gestation, the germ cell nests start to break down and form primordial follicles, consisting of one oocyte surrounded by a single layer of granulosa cells.

Our current understanding is that females are born with a fixed non-renewing pool of primordial follicles with approximately 300,000 follicles within the foetal ovary by 5 months gestation, there is a steady loss of primordial follicles with increasing age, such that by the time a girl is 13 years old, she only has on average approximately 180,000 follicles remaining [2]. This unavoidable decline continues following puberty, with ongoing continuous growth activation of a small proportion of the primordial follicle pool and cohorts of immature follicles recruited to later stages of growth, but with gonadotropin-regulated selection of one follicle to become dominant and ovulate per month [3]. The rate of decline slows with increasing age, until menopause occurs when the ovarian reserve reaches around 1000 follicles. It is widely believed that females have a fixed ovarian reserve, i.e. they have a finite supply of oocytes. Thus, the size of an individual's pool of non-growing follicles (NGF) will determine their reproductive lifespan. Mathematical models have been

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created illustrating the “normal” rate of NGF recruitment and decline potentially determining the age of natural menopause [2]. Although ovarian reserve is largely related to age, factors such as genetics, illness, smoking, and BMI can also have an effect. Anti-Müllerian hormone (AMH), produced by the granulosa cells of early growing follicles, can be measured in serum and used as an indirect marker of ovarian reserve [4].

Post-puberty maturation of follicles within a cohort requires increasing dependence on gonadotrophins. As gonadotrophins are at very low levels in the pre-pubertal girl, their ovaries contain only immature follicles, although smaller antral follicles are present throughout childhood [5]. There may well also be a process of eliminating poor quality oocytes/follicles throughout childhood and puberty: the immature ovary contains a high proportion of morphologically abnormal oocytes within primordial follicles, and these become rare in adulthood [6]. While the mechanism that results in this is unknown, it may account for the higher risk of aneuploidy in the pregnancies of teenage women [7]. The lack of more mature follicles in the pre-pubertal ovary restricts fertility preservation options for this group. Unlike in post-pubertal females, where oocyte and embryo cryopreservation are possible, retrieval of mature oocytes from pre-pubertal ovaries has not been described, nor has in vitro maturation of immature oocytes, although this is becoming more widely incorporated in combined approaches to fertility preservation [8]. Thus, the mainstay of fertility preservation treatment for pre-pubertal girls is ovarian tissue cryopreservation (OTC), where immature follicles are stored alongside their essential supporting somatic cells.

32.3 Which Pre-pubertal Patients Are at Risk of Infertility?

Assessment of infertility risk is essential in allowing the correct cohort of pre-pubertal patients to be offered fertility preservation treatments. As discussed above, certain genetic disorders can lead to POI, and fertility preservation may be a feasible option for this group of girls. With regard to iatrogenic causes of infertility, both chemotherapy (especially alkylating agents) and pelvic radiotherapy can be gonadotoxic via different mechanisms (Table 32.1).

It is logical that chemotherapeutic cytotoxic drugs, which affect DNA synthesis in dividing cells, could have gonadotoxic effects and, because females are born with a fixed ovarian reserve, oocytes cannot regenerate like other cell types might. As well as oocyte-specific DNA damage, there may be other mechanisms by which chemotherapy

Table 32.1 How different cancer treatments can be detrimental to fertility, stratified by risk

Treatment	Mechanism of gonadotoxicity/infertility	Risk to fertility
Chemotherapy		
Alkylating agents (e.g. cyclophosphamide and procarbazine)	Affects DNA synthesis Cyclophosphamide causes increased follicular recruitment and NGF “burn out”	Higher risk
Platinum analogues (e.g., cisplatin)	Crosslinks DNA to prevent cell division	Medium risk
Anthracyclines (e.g. doxorubicin)	Intercalates with DNA to prevent synthesis	Medium risk
Vinca alkaloids (e.g. vincristine)	Affects microtubule formation	Lower risk
Radiotherapy		
Total body irradiation	Destroys NGFs and impairs folliculogenesis Detrimental effects on uterine growth and vascularity	Higher risk
Abdominal or pelvic radiation	Destroys NGFs and impairs folliculogenesis Detrimental effects on uterine growth and vascularity	Higher risk
Cranial radiation	Damage to hypothalamus/pituitary	Lower risk

could be detrimental to ovarian reserve: increased activation and recruitment of follicles may lead to premature depletion of the NGF cohort, whilst damage to the supportive somatic cells of follicles or to the ovarian vasculature may lead to indirect follicular loss [9]. It is important to remember that cancer treatment regimens often involve the use of multiple cytotoxic agents and treatment modalities, which means it can be difficult to assess the effect of individual agents. Additionally, use of gonadotoxic treatments is not limited to cancer alone: some non-malignant conditions, particularly haematological disorders such as sickle cell anaemia and thalassaemia, may require myeloablative treatment and stem cell transplantation, and consideration of fertility preservation in this cohort of patients is important.

With respect to radiotherapy, the US Childhood Cancer Survivor study has shown that those who receive 5–10 Gy to an area of the body including the ovaries have a relative risk (RR) of 0.56 of ever conceiving compared to their unaffected siblings [10]. If the exposure is greater than 10 Gy, then the RR was reduced further to only 0.18. However, even doses of less than 2 Gy will destroy half of the oocyte population [11]; therefore, estimating the dose of radiation to which an individual’s ovaries have been exposed may help predict the timing of development of POI. Irradiation can also be detrimental to the uterus, by impairing growth and uterine circulation, leading to an increased risk of miscarriage, preterm

birth, and intrauterine growth restriction [12, 13]. This effect on uterine function is inversely correlated with patient age, with younger patients being most affected. Cranial irradiation can lead to hypogonadotrophic hypogonadism due to damage of the hypothalamus and pituitary, though that is fully treatable with gonadotrophin administration, and thus in itself is not an indication for a fertility preservation intervention.

In addition to the cause of infertility, the intrinsic health status of patient may affect ovarian reserve. Some studies have shown that young girls with cancer may have lower AMH levels than age-matched controls, even pre-treatment [14]. Patients who are unwell at the time of diagnosis may not be fit enough to undergo a fertility preservation procedure. Those pre-pubertal patients with suspected malignant contamination within the ovaries are not currently suitable fertility preservation candidates. Furthermore, availability of appropriate staff is essential for fertility preservation options to be offered in a timely manner pre-treatment. If local expertise and the necessary close links for rapid referral are not available, then these patients will miss out on their opportunity to preserve their future fertility [15].

32.4 What Fertility Preservation Options Are Available for Pre-pubertal Girls?

Whilst post-pubertal females have the possibility of undergoing oocyte or embryo cryopreservation before treatment starts, these are not viable options for the pre-pubertal cohort. Instead, the principal fertility preservation option is ovarian tissue cryopreservation (OTC). This option involves harvesting and cryopreserving of ovarian tissue which can be used

for autografting at some point in the future when fertility is desired. In comparison to the well-established IVF protocols used for oocyte and embryo freezing, OTC for adult women has been categorized as an ‘innovative’ treatment (thus with sufficient evidence of safety and efficacy to move it from ‘experimental’, but not fully ‘established’) [16], despite over 130 live births being reported worldwide from reimplantation of cryopreserved ovarian tissue [17]. The extremely limited evidence based in pre-pubertal girls means that the ‘experimental’ label is still appropriate. The benefits of reimplantation of ovarian tissue also extend beyond restoration of fertility: use of the tissue for restoration of endocrine function and successful induction of puberty has also been reported; however, there are concerns that may lead to wastage of oocytes within a precious resource of tissue [18].

Although OTC is the main fertility preservation strategy available to pre-pubertal girls, there are other potential treatment options (Fig. 32.1). A preventative approach to ovarian damage is the ultimate fertility preservation approach: in those having pelvic irradiation alone, oophoropexy (or transposition of the ovaries to higher up within the pelvis/abdomen) can protect the ovaries from a higher radiation exposure [16]. Pelvic shielding during radiotherapy has also been used but has limited benefit [19]. Furthermore, studies have looked at the possibility of using GnRH agonists to protect ovarian function, mostly in post-pubertal females with breast cancer [20]. However, the evidence for this is not robust in other settings, and there is none for pre-pubertal girls; even in adult women it is not recommended as an alternative to more established fertility preservation techniques [16, 21]. Novel techniques using cryopreserved ovarian tissue are being developed but are not in clinical use currently; these are discussed in more detail later in the chapter.

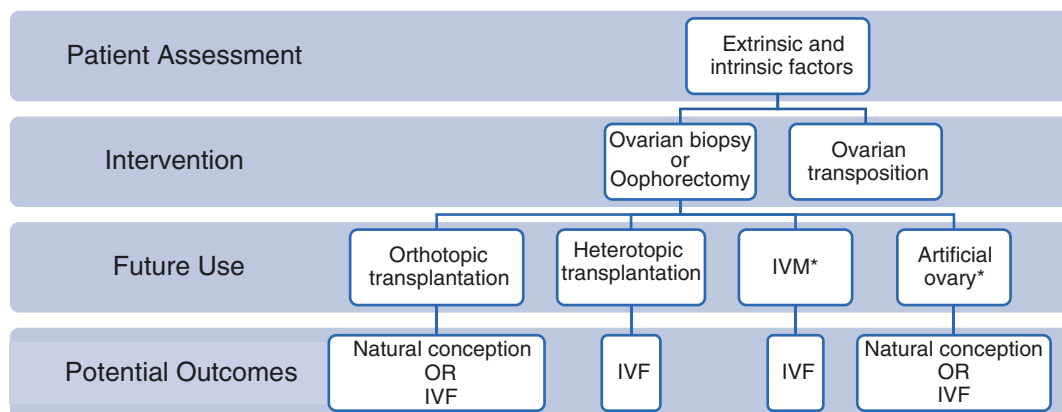


Fig. 32.1 Overview of fertility preservation options for pre-pubertal girls. IVM = in vitro maturation. Theoretical future uses are denoted by *. Adapted from [22]

32.5 What Does Ovarian Tissue Cryopreservation Entail?

The key aspect is surgical removal of strips of ovarian cortex or a whole ovary during an operative procedure. If the patient requires surgical intervention (e.g. central line insertion or tumour biopsy), OTC can be performed at the same time, to reduce the risk associated with repeated operations [16]. Ovarian tissue harvesting is usually performed laparoscopically to reduce operative risk and improve post-operative recovery. As the aim is to store primordial follicles, only the ovarian cortex is required to be removed. This has the advantage of leaving the rest of the ovary in situ, allowing an anatomically suitable area for reimplantation of the tissue at a later date. Alternatively, it may be appropriate to remove an entire ovary, e.g. in very young patients where in situ dissection of ovarian cortex may not be technically feasible, or where there is near certainty of immediate POI from treatment. Unilateral oophorectomy is also widely used where the service organization supports transport of tissue from the operating centre which is relatively local to the patient, to a more central tissue bank for processing and storage. The tissue is then slow frozen and stored in liquid nitrogen. While vitrification of ovarian tissue is possible, and indeed pregnancies have been reported following its use [23], most centres continue to use the well-established controlled rate freezing approach. Orthotopic transplantation of the thawed ovarian tissue into the medulla of the remaining ovary or into a peritoneal pocket adjacent to the ovaries is best practice [16]. This allows the possibility of either natural conception or pregnancy via IVF. Heterotopic transplantation to more accessible sites, such as the abdominal wall, has also been utilized [24] but is not as successful [19] and necessitates the use of IVF. The majority of patients will show evidence of ovarian activity and resume menses within 4–9 months, but the transplanted tissue has a variable and unpredictable duration of function, from just a few months to more than a decade [25].

There are various complications that can occur with OTC, and patients and parents should be counselled appropriately. Firstly, the required surgery to retrieve the ovarian tissue carries inherent risks, such as anaesthetic complications, haemorrhage, infection, and visceral damage. The risk of some operative complications may be further increased by the patient's underlying health condition; for example, immunosuppression will increase the risk of infection but carrying out the procedure at the same time as another required operation minimizes any additional risk. There are also valid concerns regarding reintroduction of malignant cells within the transplanted ovarian tissue, which is especially relevant in haematological malignancies. There have, however, been no reports to date of any patients experiencing relapse sec-

ondary to reimplantation [19]. Processes can be put in place to mitigate this risk: samples of the harvested tissue should be sent for histopathological assessment, with tissue not being suitable for reimplantation if any malignant cells are found. A combination of molecular and xenografting approaches was used to successfully support replacement of ovarian tissue in a young woman treated for acute myeloid leukaemia, resulting in live birth [26]. Furthermore, in diseases with a known molecular diagnosis, RNA analysis of frozen tissue can be performed to assess the likelihood of malignant cell contamination. A recent case report demonstrated the validity of this approach: a 14-year-old girl with Philadelphia chromosome positive acute lymphoblastic leukaemia (ALL) underwent OTC and wished reimplantation 15 years later [27]. The tissue was analysed for the BCR-ABL transcript which demonstrated a low likelihood of malignant cell seeding, and therefore transplantation was performed. The patient subsequently conceived twice, once by IVF and once naturally, with one livebirth and one pregnancy ongoing and no relapse of her disease.

32.6 Who Should Be Eligible for Fertility Preservation Treatment?

Given that the OTC procedure is invasive, experimental (in girls) and only a small proportion of pre-pubertal girls requiring cytotoxic treatment are at significant risk of POI and infertility, it is imperative that appropriate patient selection processes are followed (Fig. 32.2). The only validated selection criteria to date for OTC are the Edinburgh criteria, originally published in 2005 [28, 29]. These criteria suggest that pre-pubertal patients should only be considered for OTC if they:

- Have had either no previous chemotherapy or radiotherapy, or only mild, non-gonadotoxic chemotherapy
- Have a realistic chance of surviving 5 years
- Have a high risk of POI (>50%)
- Are negative for HIV, syphilis, and hepatitis B

Use of these criteria accurately identified those under 18 years old at time of diagnosis who would go on to develop POI: the 15-year probability of selected patients suffering POI was 35% compared to only 1% of those who did not meet the criteria [29]. The growing body of evidence for OTC and increasing availability of measures of ovarian reserve since these criteria were published may now allow their refinement.

As part of these criteria, a risk assessment from the perspective of fertility is essential at the time of diagnosis. Both intrinsic and extrinsic factors must be considered. As discussed above, the intrinsic health status of the patient can

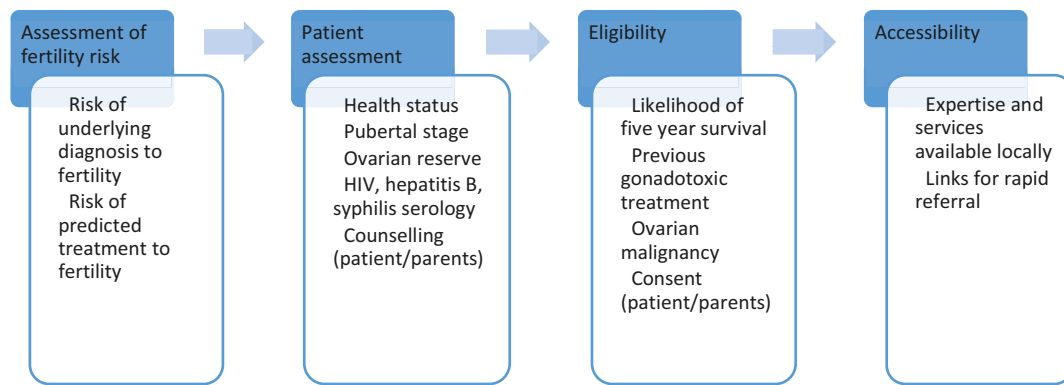


Fig. 32.2 Pathway for assessing which patients should be offered ovarian tissue cryopreservation [22, 32]

both affect ovarian reserve and increase the likelihood of operative complications during OTC. Mediastinal masses can make anaesthesia more difficult, whilst blood disorders may cause immunosuppression or coagulopathy, thus increasing the risk of infection and haemorrhage, respectively. An assessment of the patient's pubertal status is necessary to ensure that the correct fertility preservation strategies are discussed. Assessment of the patient's ovarian reserve is important: in the post-pubertal population, this is best performed by measuring serum AMH levels and/or early follicular phase antral follicle count (AFC). In the pre-pubertal population, this is challenging. AFC and other possible measures such as FSH and oestradiol are not informative, leaving AMH the only feasible measure. The current value of testing AMH in pre-pubertal girls lies in the ability to assess ovarian damage by comparing pre-treatment and post-treatment measurements [30]. However, although evidence exists that AMH can be used in the diagnosis of post-treatment POI in females of all ages, post-treatment AMH levels have yet been proven to be predictive of reproductive lifespan or fertility [31]. Those patients who already have POI (e.g. in some genetic disorders) are not suitable for any currently available methods of fertility preservation. All patients with an underlying genetic disorder at risk of POI should undergo genetic counselling prior to OTC [16]. Finally, from an intrinsic point of view, the age of the patient and their ability to understand the implications of her diagnosis and/or treatment on fertility and agree to fertility preservation is also an important consideration, with informed consent usually having to be obtained from parents/guardians.

With regard to extrinsic factors, the main aspects of note are (a) the gonadotoxicity and urgency of any necessary treatment and (b) the availability of expertise in OTC. The risk to infertility can be stratified according to the dose and type of treatment required (Table 32.1). European-wide rec-

ommendations suggest that OTC is a 'moderate recommendation' for pre-pubertal girls undergoing high-dose alkylating agents (cyclophosphamide equivalent dose of ≥ 6000 – 8000 mg/m²), ovarian irradiation, or haematological stem cell transplant (HSCT) but is not recommended in other childhood cancer treatment scenarios due to the risk-benefit ratio [32]. The degree of damage to the ovaries is also dependent on the age of the patient and their initial ovarian reserve, although data supporting that generally derive from adult women with breast cancer [31]. Data show that increasing age at time of treatment is correlated with increasing likelihood of POI [33], and women with higher pre-treatment AMH levels have higher levels post-chemotherapy [34], whilst those with lower pre-treatment AMH levels had a slower rate of recovery in AMH levels following completion of their treatment [35]. Prognostic models are available to predict post-treatment amenorrhoea using pre-treatment AMH levels and patient age [36]; however, this is specific to women with breast cancer, and further research is required to widen such predictive models to other disease processes and age groups. In addition, the uterine factor should be considered: if uterine function is likely to be significantly impaired by treatment, patients and/or parents should be counselled regarding the obstetric implications and whether surrogacy may be required in the future.

Whilst it is essential that the multidisciplinary team involved in treating the patient is aware of any potential risk to fertility and counsel the patient and her parent(s) appropriately, it is equally important that there is accessible expertise in performing OTC, which is still sporadic worldwide. OTC requires not only an experienced surgeon and clinical proficiency, but also appropriate laboratory expertise and capacity for cryopreservation and storage, all within a fertility preservation programme with country-specific regulatory approvals [16].

32.7 What Are the Known and Expected Outcomes?

Given the field of fertility preservation is a fairly recent development and the likely long interval between ovarian cryostorage and subsequent replacement in this population, there are limited data regarding uptake and long-term outcomes of OTC within the pre-pubertal population. This emphasizes the importance of appropriate counselling of patients and parents to allow realistic expectations of what it still an experimental technique. In our own experience in Edinburgh, where the procedure has been offered to girls and young women since 1996, over an almost 16-year-period, 8% of girls and adolescents under 18 years old diagnosed with cancer met the Edinburgh selection criteria with the uptake being just over 60% [29]. Another well-established centre, at the Karolinska Institute, had an uptake rate for fertility preservation treatment of 87% in a cohort of under 19-year-old girls requiring a haematological stem cell transplant (HSCT), with 82% of these patients undergoing OTC [37]. Reimplantation has been performed in 11%, all of whom underwent IVF and conceived; the pubertal status of these patients at the time of cryopreservation was not reported.

Regarding pregnancy following reimplantation of tissue harvested pre-pubertally, to date there is only one case report of a successful livebirth following reimplantation of ovarian tissue which was obtained pre-pubertally. Tissue was harvested from a 9-year-old girl with beta thalassaemia and was subsequently reimplanted 14 years later, and successful IVF was performed [38]. There has also been a report of a patient with sickle cell anaemia requiring HSCT who was in the early stages of puberty (pre-menarche) when her tissue was cryopreserved [39]. Reimplantation was performed 10 years later, and the patient subsequently spontaneously conceived a successful pregnancy. Additionally, as previously described, there has been one life birth and one ongoing pregnancy in a patient with high-risk ALL whose tissue was harvested at the age of 14; however, it is important to note that the pubertal stage of this patient at time of OTC was not reported [27]. Given the data discussed above regarding the possibility that oocyte quality may actually be reduced in the very young [40], it will be vital to see longer term data regarding the success of reimplantation of pre-pubertal and early pubertal tissue in terms of pregnancy outcomes. An important consideration in women wishing reimplantation of their tissue is their medical fitness for pregnancy. Many will have increased obstetric risks due to treatment: anthracyclines are associated with the development of cardiomyopathy [41], whilst issues with uterine exposure to radiation have already been discussed. Pre-conceptual counselling and obstetric-led antenatal care are recommended [16].

With regard to potential alternative treatment options using cryopreserved ovarian tissue, innovative research is ongoing [16]. It may be possible in the future to support the in vitro development of competent oocytes from the primordial follicle stage by creating a system in which in vitro activation of cultured ovarian cortex, in vitro growth of follicles, and in vitro maturation of oocytes can occur safely. The derivation of mature, metaphase II oocytes from unilaminar follicles has now been described for adult human ovary [42]. It may also become possible to derive human oocytes from stem cells in the future: this has now been achieved in the mouse, with both germ and somatic components of the ovarian follicle derived from somatic stem cells [43]. To allow in vivo folliculogenesis, artificial, or bioprosthetic, 'ovaries' may someday allow the reimplantation of isolated early follicles without transfer of any potential malignant cells. The use of any of these proposed options would clearly require thorough scrutiny of both safety and ethical considerations.

32.8 Patient Follow-Up

Given how recently OTC has been offered as a fertility preservation option, the vast majority of pre-pubertal patients who have undergone OTC have not required or requested reimplantation to date. It is therefore imperative that long-term follow-up is performed for this cohort of patients in order to ensure it is an effective and safe treatment option for them. Data on site of reimplantation, time to commencement of menses, spontaneous versus IVF pregnancy rates, live-birth outcomes, the long-term health status of conceived children, and duration of graft function should be recorded. Vitally, any relapse of malignant disease should be investigated to assess whether transplantation of the ovarian tissue may be the cause. Reassuringly, among adult patients who have had ovarian tissue reimplanted, none has experienced a transplant-related relapse and in those who conceived following reimplantation, there has been no increased risk of congenital abnormalities in their offspring to date [16, 32].

In summary, OTC is the principal fertility preservation option for pre-pubertal girls who are at high risk of POI. The coming years will allow analysis of its effectiveness and hopefully see further refinements, either in vivo or in vitro.

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Fertility Preservation in Transgender Patients

33

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33.1 Introduction

Gender identity is self-identification as male or female or other gender. Other gender identities may include gender neutral, non-binary, gender fluid, and gender queer.

The transgender (TGD) or gender diverse identity includes those whose gender identity is at odds with the birth-assigned gender, based on chromosomes, hormones, organs, and secondary sexual characteristics [1]. Approximately 0.5% of the world's population (around 35 million people) identify as TGD or gender diverse [2, 3].

The gender reassignment journey typically involves initially a physical and social transition (change in physical appearance and gender roles), followed by medical and surgical gender-affirming interventions.

Whilst gender-affirming care improves the quality of life of TGD individuals, it also represents a clear threat to their future reproductive potential. Gender-affirming surgery that leads to removal of gonads (bilateral oophorectomy/orchiectomy) renders the individual unable to have a genetic offspring, whilst removal of the uterus (hysterectomy) removes the ability to carry a pregnancy. Gender-affirming hormone treatment (GAHT) aims to suppress the sexual characteristics of the original gender and induce the evolution of secondary physical characteristics congruent with the desired gender [4]. It is well known to be detrimental to the overall gonadal function [5]. As there is little research on the potential recovery of gametogenesis after discontinuing hormonal treatment, it is justifiable to account for the worst scenario which assumes that the initiation of GAHT could have an irreversible impact on the TGD person's fertility. Even if that is not always the case, long-term discontinuation of the hormone treatment is not likely an acceptable option for many TGDs, as it is viewed as a backward step in their transition journey.

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Given the risk of irreversible medically or surgically induced impairment of reproductive potential, major medical societies recommend fertility preservation counselling prior to commencing gender-affirming treatment [6, 7]. However, this standard of care has not been universally achieved yet. TGD individuals face significant barriers to parenting, with poor access and inadequate provision of counselling, contributing to low utilisation of fertility services with potential for decisional regret in the future [8].

There are significant challenges when providing reproductive counselling and care for TGD individuals. Some of these challenges stem from the TGD's young age and lack of familiarity with medical services and procedures. Many clinicians also feel unequipped to deal with TGD patients and how they should adapt mainstream fertility care to suit the expectations of this particular group. Unsurprisingly, some of the major recommendations revolve around improved training and awareness amongst clinicians, so that they can be competent in supporting TGD people [9]. It is also worth noting that there is limited research to guide crucial steps of the fertility journey of TGD people. As a result, best practice is often extrapolated from experience with their IVF counterparts or cancer patients who also seek fertility preservation.

The aim of this chapter is to discuss the challenges around TGD fertility preservation, through critically appraising the up-to-date scientific evidence.

33.2 Literature Review of the Evidence

We performed an electronic search of Medline and the Cochrane Central Register of Trials (CENTRAL) up to April 2022, using the following keywords: (transgender OR gender reassignment OR trans-men OR trans-women OR trans-folk OR trans-folk) AND (IVF OR ICSI OR fertility OR eggs OR oocytes OR sperm OR in vitro). We restricted the search to articles written in English. A secondary search of the reference list of retrieved articles was also performed, to ensure that important relevant articles were not missed. Two authors independently evaluated the retrieved articles for relevance. In addition, we searched for guidance published by international

Reproductive Medicine societies, on the topic of management of TGD individuals who seek fertility preservation or assisted conception treatment.

33.3 Uncertainties Peculiar to Trans-gametes

TGD people who store eggs or sperm are relatively younger than their cis-gender counter parts who are cryo-preserving gametes due to cancer or social reasons [10]. Many of them will present as minors (before the age of 18 years). In addition, some will have already commenced their GAHT before storing gametes.

33.3.1 Effect of Gender-Affirming Hormone Treatment (GAHT) on Gametes

The World Professional Association for Transgender Health (WPATH) guidelines state that, unless medically contraindicated, patients considering gender-affirming surgery are required to complete at least 1 year of GAHT before surgical intervention [11]. Consequently, many TGDs are keen to commence GAHT as soon as possible and, therefore, may already be on this regimen when presenting for fertility preservation.

There is ample evidence of the overall effect of GAHT on the gametes; this may vary from no effect to significant suppression of gonadal function [12]. The available evidence indicates that the sperm may be more vulnerable to the effects of opposing exogenous hormones, than the eggs (Table 33.1). Age is a recognised factor influencing the impact of GAHT on spermatogenesis, with older trans-women being prone to more serious sperm defects [11, 13].

In the absence of concrete evidence, most recommendations regarding GAHT use around fertility preservation are based on expert opinion and a commonsense approach. If trans-women are willing to discontinue GAHT for 10–12 weeks before storage (which is approximately how long it takes for testicular germ cell differentiation), this may improve their chances of freezing better-quality sperm [13]. Similarly, if they were to discontinue GAHT prior to surgical gonadectomy, it may improve their chances of finding mature spermatozoa in the surgical specimen [13].

In trans boys, some research indicates that testosterone-based GAHT use may render the ovaries polycystic in morphology (PCOM). This may lead to higher number of eggs harvested [14]; it is important to acknowledge though that other confounding factors, such as higher stimulation doses prescribed to fertility preservation subjects, could also con-

tribute to the higher oocyte yield [14]. However, PCOM is not a universal finding on TGDs receiving GAHT [15, 16].

The reproductive effect of GAHT on oocyte quality can be clinically ascertained by reviewing the live birth rates specific to the trans-males that had used GAHT around fertility preservation. Unfortunately, there is very limited knowledge in this area and more research is urgently required.

33.3.2 Effect of Young Age on Gamete Maturity

TGD people are seeking fertility preservation at an increasingly young age, with a significant proportion of them being adolescents. There is very limited published experience regarding the maturity and fertility potential of the gametes harvested from trans-folk in their early teens. Although there is no obvious biological reason to believe that they are physiologically different from their cis-gender counterparts, our current knowledge is mostly extrapolated from research on cis-gender cancer patients and non-trans fertility preservation cases [17].

Sperm maturity can be assumed when the trans-girl is able to ejaculate or a certain stage of puberty has been reached (Tanner Stage 3: Enlargement of penis (length at first); growth of testes; darker, coarser, and more curled hair that begins to extend laterally; 7–8 cm growth spurt per year) [18]. For trans-boys, regular menses are used as evidence of a mature reproductive system and are expected to result in successful stimulation and retrieval of mature oocytes. However, the hypothalamic–pituitary–ovarian axis will likely have already activated during the earlier pubertal stages. Therefore, ovarian stimulation is likely to be effective for even young trans-teens (13 years or more), although higher strength of stimulation may be required and lower oocyte numbers may be retrieved [19].

33.4 Preparation for Treatment

33.4.1 Initial Consultation

Various studies particular to the TGD population have highlighted challenges during the fertility preservation process, such as the experience of ‘gender dysphoria’ symptoms, as well as difficulties in accessing their gametes in the future [20–22]. Therefore, it’s crucial that the fertility specialist utilises the initial consultation wisely, by gaining the patient’s trust and addressing their current and future concerns. As the amount of provided information can be overwhelming, a systematic approach to conveying this knowledge during the consultation should be adopted (Table 33.2).

Table 33.1 Reported effects of gender-affirming hormone therapy (GAHT) on the gametes

Exogenous hormones (current or recent use)	Clinical and metabolic changes	Hormonal effects on gametes	Gonad macroscopic changes	Gonad histopathological changes
Oestrogen	Breast tissue growth Reduction of facial and body hair Thromboembolic risk [52] Dyslipidaemia [5]	Reduced semen parameters (may be reversible) [12]	Reduced testicular volume (≥ 5 years of GAHT use) [5]	Variable impact on spermatogenesis [12] Atrophy, fibrosis [5] Hyalinization of seminiferous tubules Maturation arrest Sertoli cell phenotype [5] Leydig cell phenotype [5]
Testosterone	Amenorrhea Fibrous transformation of breast glandular tissue Ovarian reserve (AMH levels and antral follicle count) comparable to cis-gender controls [14] Oocyte numbers and maturity comparable to cis-gender controls [14] Dyslipidaemia	Uncertain impact on developmental potential of oocytes Preserved developmental capacity of ovarian follicles [53] Risk of impaired development of in vitro matured oocytes [54] Embryo developmental delays in hyper-androgenic PCOS women (similar effect in trans-men who take testosterone?)	Polycystic ovarian morphology [53] Follicular cysts Decreased ovarian volume (prolonged testosterone exposure)	Normal distribution of cortical follicles Retained ovarian function Normal or polycystic ovarian histology [53] Atrophy, fibrosis Normal in vitro maturation Normal chromosome and spindle pattern [53]
GnRH-analogues (puberty blockers)	Suppressed hypothalamic–pituitary–gonadal axis Puberty delay or arrest Some uncertainty if effects are fully reversible [55]			

33.4.2 Screening

The sex of the TGD person's future partner determines what reproductive route they will need to adopt to use their gametes for having a baby. As this is often unknown at the time of fertility preservation, it is sensible to allow for scenarios where future surrogacy will be necessary. This often requires that the TGD individual undergoes extensive screening, similar to the screening required for gamete donors [23]. This typically includes both diagnostic tests and a physical examination. Whilst physical examination is often unwelcomed and troubling for TGD patients, they need to be made aware that screening at the time of preservation will ensure that future regulatory obligations and requirements are met.

33.5 The Preservation Process

33.5.1 Sperm Preservation

The mainstay of sperm preservation is freezing of sperm produced via self-ejaculation, and this is still true for the trans-female group. Assessment of sperm maturity can be readily performed in an ejaculated specimen and is likely to reveal mature spermatozoa [13]. However, some transgender individuals find masturbation psychologically distressing; or

occasionally experience erectile or ejaculatory dysfunction due to use of GAHT, even though they are considered sexually mature [13]. For those individuals, penile vibratory stimulation or electro-ejaculation could be medically appropriate, though not necessarily acceptable [22]. Another challenge is that these specialised techniques require a dedicated clinical setup which may not be readily available [22].

If an adolescent trans-girl is not able to ejaculate for reasons of sexual maturity, the clinician may recommend waiting a while. This, however, may not be an acceptable option to some, who may feel the urgency to commence GAHT in order to stall further sexual development. In these cases, the clinician needs to ascertain how likely mature sperm will be retrieved surgically. In vivo spermatogenesis tends to develop from Tanner Stage 3 onwards, under the influence of increasing intra-testicular testosterone levels [13]. Therefore, the odds of successful sperm extraction from individuals who have attained this stage in puberty were high, provided that GAHT has not been commenced. An increased testicular volume (≥ 10 mL) is also considered a useful predictor of successful sperm retrieval [24]. In contrast, if puberty blockers have been commenced earlier (Tanner Stage 2), complete spermatogenesis is usually not present, and spermatozoa are not available for cryopreservation [13]. In a separate study, trans-girls who initiated GAHT during Tanner Stage 2–3 had

Table 33.2 Information to be provided during the initial medical consultation (Bourn Hall trans-oocyte preservation checklist)

Egg storage (trans-female to male)
Consent forms
Screening bloods, virology, Zika assessment
Fitness to undergo egg collection
Discuss impact of gender-affirming hormones on ovarian response, collected egg numbers
Mental health during and after fertility preservation process
Availability of counselling
Risk of ovarian hyperstimulation syndrome, symptoms, management strategies
Risk of low ovarian response, no eggs collected, no guarantee the frozen eggs will result in a baby in future
Issues regarding funding treatment (now and in future)
Limited data about effect of hormone therapy on future reproductive ability and outcomes
Discuss ovarian tissue cryostorage
Keep the clinic informed of last known contact details
Future use of the frozen eggs
Risk of failed thaw, failed fertilisation (especially with low egg numbers)
Need ICSI for fertilisation
Expected chances of having a baby depends on egg quality (depends on age at egg harvest) and egg numbers
Future reproductive scenarios (depending on future partner/circumstances)
Any person providing eggs to another person is a 'donor', needs extra screening, consents and counselling before egg freezing
Discussion about future circumstances (funding, type of treatment, need to assess any issues affecting the welfare of the unborn child)

just immature germ cells in their orchidectomy specimens [13].

The challenge remains in pre-pubertal trans-girls, where the only option is testicular tissue cryopreservation [25]. Whilst this procedure should be discussed, it should be noted that future re-transplantation is only feasible for individuals with residual testes. In cis-gender cancer survivors, immature testicular germ cells transplanted into the rete testis have been shown to colonise and restore spermatogenesis [26]. As most trans-women will be on GAHT for life, and many will undergo bilateral orchidectomy, re-transplantation may not be a realistic option. In vitro maturation techniques could allow use of previously stored immature sperm cell lines for assisted conception (without depending on re-transplantation); however, in vitro spermatogenesis has currently only been tried in animal models [13, 27]. Based on the available evidence, both ASRM and ESHRE highlight that testicular tissue cryopreservation should be considered an experimental practice [28]. Of course, it may still be possible to store testicular (or ovarian) tissue at the time of surgical gonadectomy, in hope that future technology will allow its use without needing re-transplantation, to have a baby. This practice of electively cryo-storing cells or tissue is already in use within other medical fields; for example, umbilical cord tissue and cells are often stored at the time of

childbirth, even with limited clinical potential at present (but with the promise of many more available applications in the future) [29]. On the other hand, it is difficult to justify performing a stand-alone surgical procedure on a pre-pubertal TGD with sole intention to extract and store testicular tissue, without having a clear plan how this tissue will be utilised in the future—except perhaps in the context of a research trial.

33.5.2 Oocyte Preservation

No matter the reason behind a recognised risk of ovarian failure; be it advanced age, cancer or in the case of trans-men; gender-affirming hormone therapy, fertility preservation with oocyte (or embryo) cryopreservation is known to be a safe and effective option. However, there has been little in way of research looking at how to tailor the stimulation process in this specific population. A sensible approach is to adopt similar protocols as for cis-gender individuals looking to preserve fertility for medical or social reasons.

In research, stimulation regimens have used mainly antagonist protocols with no special alteration for the trans-gender groups [14, 15, 30]. Antagonist regimes are certainly advantageous for keeping the risk of Ovarian Hyperstimulation Syndrome (OHSS) as low as possible, as these patients are often young with above-average ovarian reserve. Use of a GnRH-agonist trigger will further reduce the risk of OHSS and may also expedite symptom resolution after oocyte retrieval. However, trans-males on long-term GAHT may be at a higher risk of agonist trigger failure, in view of a suppressed hypothalamic–pituitary axis [31].

The gender dysphoria felt by these patients due to the exacerbation of unwanted hormones and/or discontinuation of testosterone therapy is also a recognised concern. The expected lower rise of oestradiol levels with the antagonist protocol (compared to agonist protocols) may, therefore, moderate any gender dysphoria symptoms. In addition, clinicians may want to consider the use of aromatase inhibitors such as letrozole, alongside gonadotropin stimulation to limit oestradiol levels' elevation, similar to protocols used for fertility preservation in breast cancer patients [6, 32]. This can be particularly useful in adolescents, where the use of letrozole can help minimise breast budding [33]. Luteal-phase or random-start stimulation has been used for expediency in cancer fertility preservation cases and has demonstrated similar oocyte retrieval outcomes [34]. Technically, it can be used for TGDs as well, since there is no fresh transfer involved; nonetheless, the degree of urgency is not the same as with cancer individuals, so many clinicians may not feel it is justified to utilise these protocols.

There have been anecdotal reports suggesting that it is still possible to achieve successful ovarian stimulation and blastocyst formation whilst continuing with testosterone

therapy throughout treatment [32]. However, there are no large longitudinal series evaluating the success and safety of this practice. It is, thus, recommended to discontinue testosterone therapy between 2 and 6 months before the start of ovarian stimulation [14, 32]. A practical rule is to wait for return of regular menses before committing to ovarian stimulation, as this heralds recovery of the hypothalamic–pituitary axis after discontinuation of testosterone therapy. In practical terms, however, it often comes down to balancing the priorities of the clinician (to wait for as long as possible) against patient preferences (to stimulate as soon as possible).

Whilst oocyte vitrification is the preferred option for post-pubertal trans-men, ovarian tissue cryopreservation (OTC) is the only option available for pre-pubertal trans-boys [25, 28]. This technique has moved away from its experimental status to an established option for fertility preservation in Europe but remains experimental in the United States of America [35]. It is also important to note that this practice is largely endorsed in an oncological setting. ESHRE has stated that OTC should *not* be recommended as a primary procedure in transgender men but rather as an experimental option at the time of gender reassignment surgery [6]. It is understandable why this technique has been seen as an attractive option, since over 130 live births have been reported worldwide from cryopreserved ovarian tissue in cis-gender women [36]. However, as auto-transplantation is a necessary step, this is very unlikely to be a suitable option for most trans-men [25]. Furthermore, there are no studies evaluating the safety or effectiveness of this technique in the transgender population [6].

33.6 Predicting Live Birth from Cryopreserved Trans-gametes

The direct evidence on the future reproductive potential of stored trans-oocytes is limited, with sparse studies reporting only a handful of pregnancies [14, 15, 22]. Only few trans-male patients undergo the embryo transfer themselves; the overriding majority decide to transfer the embryo into their cis-gender female partners.

Early case reports followed by a handful of retrospective studies have shown that trans-men exhibit comparable fertility preservation outcomes to age-matched cis-gender women [14, 15, 30, 37]. One of these studies went further, demonstrating that trans-men's outcomes are comparable with the 'fertile' cis-gender female population (the control group in this study consisted of egg donors) [30]. In summary, although outcome data on the reproductive potential of trans-oocytes are limited, preliminary findings suggest that they lead to high rates of live birth [14, 22].

There is even less evidence on the reproductive potential of cryopreserved trans-sperm. Case series indicate success-

ful ICSI outcomes using cryopreserved sperm from transgender women [38, 39].

The lack of large-scale studies in this area leads many clinicians to extrapolate future success rates from the general IVF population. Nevertheless, it is important to keep in mind that there are differences in the reproductive dynamics of the trans-folk in comparison with the standard IVF population. Young age and a presumed fertile status may somewhat upgrade the expectations from future assisted conception. In the case of trans-women, lower quality of the sperm at the time of storage and the routine use of frozen-thawed sperm may downgrade expectations. However, ICSI will often address these concerns; therefore, ICSI statistics 'borrowed' from the general population are reasonable to use for counselling trans-females [40].

In trans-males who are not on GAHT, the most comparable group is single women who have had a baby using previously stored gametes for social reasons. There are few prediction models tailored to live birth after social oocyte preservation [41, 42]. While these models still partially rely on IVF statistics from the general population, they do provide reliable live birth statistics based on the number of mature oocytes stored, which can be used for counselling trans-folk presenting for fertility preservation.

33.7 The Psychological Aspects of Trans-fertility Preservation

The psychological aspects of fertility preservation may be particularly challenging to a TGD individual. This is not just related to the TGD's young age and expectations. Significant anxiety may be unnecessarily generated, simply because the fertility services—facilities and staff—have not been optimised to manage TGDs in the first place. The healthcare professionals may also feel anxious treating TGDs, as they may have had limited experience with managing TGDs or patients who are very young (minors).

TGD individuals are thought to be vulnerable, as many of them have been exposed to child abuse, bullying and mental health issues (anxiety, depression, self-harm, suicide) [43]. Some may also be at risk of experiencing eating disorders, substance misuse, joblessness, food deprivation, incarceration, and HIV [43].

TGDs presenting for fertility preservation are typically in their teens or early 1920s—much younger than their IVF counterparts—and this could well be their first interaction with a 'medical' service. This can, by definition, be a daunting experience. They may also find it challenging to analyse future fertility objectives, which could complicate making fertility decisions. Although around two-thirds of young TGDs would like to become parents in the future, only a small minority (7%) have expressed disappointment if

biological parenthood was not possible [44]. This implies that, although having a consultation with a fertility specialist is important, not all TGDs referred for fertility preservation will commit to the process. Nevertheless, it should also be considered that, at the time of presenting for fertility presentation, most TGDs are quite young and tend to focus on the transition, rather than future family building, and these priorities may change with time [45]. It is therefore relevant to explore these uncertainties with the TGD person and to minimise the risk of future regret if fertility preservation is not pursued. Unsurprisingly, at the time of presenting for preservation, many TGDs will not have clear preferences about the sexual orientation of their future partner, which is clearly relevant to how the TGD person can have a family in the future. It is, therefore, important that fertility specialists plan the preservation process allowing various future reproductive scenarios. This will minimise future distress—and litigation—if access to the gametes is not straightforward. Training on how to deliver TGD care will instil confidence amongst staff and help optimise fertility preservation pathways [9].

It is important that face-to-face encounters happen in a quiet, private environment, where TGDs can express their views and concerns with confidence. When addressing a TGD person, it is helpful to use their stated name and preferred pronoun, as this will help develop rapport [46]. This is likely to be different from their legal or birth name. It is important not to assume that the TGD has chosen a binary—male or female—identity. It is essential to maintain patient dignity and adequate privacy during an examination; if possible, offer a choice of gender of the examiner as requested by the individual [8]. Moreover, consideration should be given to minimising the frequency of intimate examinations (consider transabdominal scans instead of transvaginal scans) and performing any necessary intimate examinations/procedures (such as oocyte retrievals) under a general anaesthetic. This is particularly important for the ones who are sexually inexperienced.

Although most fertility settings are not per se designed for TGD people, there are steps that fertility clinics can take to establish trans-friendlier facilities. These include setting up a dedicated gender-neutral restroom, including transgender people within the clinic's instructional materials and displaying posters or pictures that show that the facility is inclusive of gender diversity [47].

Particularly for minor TGDs, engaging the parents is a vital part of affirmative care [46]. Typically, parents who have accepted their child's TGD identity are keen to be involved and will support the preservation process. Parental consensual behaviour is likely to reduce the TGD person's anxiety. However, some caution is required that interaction remains with the patient, unless the TGD person is obviously too immature to understand and partake at the consultation.

Having said that, immaturity should not be assumed just on the basis of young age.

No doubt many TGDs will already have been under the care of a psychologist, for managing their gender dysphoria and/or any associated mental health issues. Nevertheless, providing additional support and counselling around the fertility preservation process are highly recommended, particularly for trans-males [48]. There is accumulating experience that, compared to their IVF counterparts, trans-male TGDs react less favourably to the IVF medication and the acute fluctuation of endogenous hormones [25]. The reported side-effects are usually of the hormonal type and could be as distressing to them as was the case with their original natural hormones. Even vaginal bleeding—occurring before and after ovarian stimulation—is a well-known stressor for trans-male TGDs. It is also unknown whether the process of ovarian stimulation and oocyte retrieval may have any medium-to-long term effects on TGD individuals, particularly of the psychological kind. Research is, thus, warranted to understand the psychological impact of the fertility preservation process on trans-males.

33.8 The Legal Aspects of Trans-fertility Preservation

It is important for reproductive medicine physicians to keep up-to-date with national legislation affecting transgender individuals, as this may be relevant to the provision of care especially for the younger trans-folk seeking fertility preservation. Few such legal cases have been under the media spotlight recently. A 2020 UK legal case raised concerns whether transgenders under 16 years of age are likely to be mature enough to understand the implications of using puberty blockers and, in turn, consent to their use [49]. Although this notion has now been challenged by the Court of Appeal, cases like this highlight the ongoing complexities of decision making and obtaining informed consent from minor transgenders [50].

Regardless of geographical differences in healthcare legislation, there are two main challenges for the clinicians when treating transgender children: (i) assess if the child is competent to consent and (ii) evaluate the 'best interests' of the child [51]. Considering that reproductive medicine clinicians routinely manage only adult patients, it is not surprising that even the most experienced ones will not be accustomed to obtaining consent from transgender minors. This process involves making individualised assessments whether a minor is deemed 'competent' to understand the process, benefits, and risks of fertility preservation. The clinicians may also be uncertain where parental wishes would fit legally within the decision-making process, in cases where the minor is not deemed 'competent'. Most regulatory

guidance places great emphasis on the child's 'best interests', when deciding whether to offer treatment. Evaluating what constitutes 'best interests' of a transgender minor involves ascertaining the child's needs and priorities, parental opinion, and potentially the opinion from other parties (gender identity specialists, psychologists, etc). As a significant minority of transgender individuals have been diagnosed to be in the autistic spectrum, evaluating competence and 'best interests' can be even more challenging for such minors [43, 45].

The medical risk involved when retrieving gametes also tends to weigh differently on those decisions. For example, a clinician may consider in the best interests of the child to store sperm that can be produced by masturbation, but not via electro-ejaculation or surgical extraction. Likewise, a clinician may not consider in the best interests of a non-competent-to-consent child to proceed with an intensive course of artificial hormones, followed by an oocyte retrieval—that can uncommonly lead to serious complications—even if there is parental consent. The degree of urgency to proceed with fertility preservation is often perceived differently by the transgender minor, the parents, and the clinician. It may be difficult for the clinician to delay the fertility preservation process, in hope that a non-competent minor patient becomes competent to consent, particularly when the parents are often happy to provide consent on behalf of their child. Gender identity specialists are much more confident navigating through such consent issues for minors and will likely have made their own assessment of competence. However, the burden still lies with the reproductive medicine clinician to make their own assessment, as they are the ones who are most familiar with the process of fertility preservation that they have been asked to provide. It is recommended that reproductive medicine clinicians seek expert input on matters of consent taking from minors, particularly when there is no clear national regulatory guidance available.

Another challenge for assisted conception clinics, with potential legal implications, revolves around offering surgical procedures—oocyte or sperm retrievals—to minors. Providing surgical care for minors requires a specific organisational set up, which includes staff who are properly trained to care for, can provide safe post-operative recovery and deal with medical emergencies in minors. ART clinics would be at risk of litigation if they were to treat minors without being fully compliant with the relevant regulatory requirements.

Focusing on the legal and regulatory considerations pertaining to storage, it is known that these may differ between countries. In addition, there may well be a different regulatory framework around storage of tissue for autologous or non-autologous transplant, compared to storage of gametes. As a consequence, many standard assisted reproductive technology (ART) clinics may not be able to store reproductive

tissue, primarily because of regulatory restrictions rather than limitations around technology. Finally, the rapidly unravelling medical and scientific advances in this field may well offer new ways for TGDs to become parents in the future; therefore, it is important that legislation also evolves to include these new technologies, to ensure that the legal status of the future parents and offspring is promptly assured.

33.9 Future Reproductive Scenarios

The future method of pregnancy for TGD patients depends entirely on the type of gametes frozen and the sex of their future partner. A key factor is whether there is a 'uterus' available within the future relationship and, obviously, whether the partner with a uterus is willing to carry the pregnancy. If not, then surrogacy will be required.

It is obvious that some form of fertility treatment is necessary to obtain this pregnancy, whether it be IUI or IVF/ICSI. IUI could be considered in the case of trans-sperm and a female partner and whilst it may be an easier, less intrusive, and cheaper option, it may not always be recommended. This is because unlike 'standard' donor sperm, trans-sperm may be 'finite' in quantity; therefore, opting for a more successful treatment like IVF can be beneficial and practical.

Donor gametes to fertilise the trans-gametes may also be a necessity. For example, in situations where the partner is the same gender as the trans-individual or if they are unable to provide their own (e.g. premature ovarian/testicular failure, advanced age, and failed surgical retrieval). In these circumstances, counselling regarding using donor gametes as well as screening and the matching process should all be discussed well in advance.

33.10 Should Trans-folk Fertility Services Be Provided Only by Specialist Centres?

Having highlighted the challenges around caring for transgender individuals seeking fertility preservation, it is worth considering whether there is a place for a dedicated trans-specialist ART clinic, in contrast to the local ART clinics which primarily serve the local infertile population. Table 33.3 summarises some of the notable differences between these two organisational settings. Nevertheless, it is difficult to predict how healthcare around transgender fertility preservation will evolve in the future. It is likely that factors such as local access to ART, availability of governmental funding, national healthcare regulations and legislation will influence the decisions around providing ART care to this minority group. Currently, it is important that ART clinics develop their clinical pathways, training opportunities, and

Table 33.3 A specialist trans-assisted reproductive technology (ART) clinic versus the standard ART clinic

	Trans-ART clinic	Standard ART clinic
Trans-tailored care	Develop in-house expertise (gender-neutral facilities and staff who are confident in managing trans-folk and in using trans-friendly terminology) Multiple in-house disciplines (reproductive medicine scientists and clinicians, gender identity specialists, andrologists, psychologists, and legal advisers)	Smaller patient volumes may hinder the development of local expertise (could be partly overcome by developing specialism among selected staff)
Travel requirements	Likely substantial travel distance to the clinic	Access to local clinic is likely to be straightforward
Treating minors	In-house expertise in managing transgender minors. Includes assessing minor's competence to consent and providing surgical care and facilities that are appropriate for minors. Develop in-house tissue preservation facilities and protocols	Unlikely that a general ART clinic will have clinical pathways and resources to provide regulation-compliant surgical care to minors Unlikely for a general ART clinic to offer tissue preservation
Research	Promote in-house trans-research (including adequately powered intervention trials)	Difficult to procure large enough population samples (observational research may be possible if trans-ART data are collected at the national level)
Gamete donation/surrogacy	Develop in-house transgender gamete donation bank and pathway Offer surrogacy	Not all ART clinics offer donation services Minority of ART clinics offer surrogacy

organisational settings so that they facilitate a safe and fulfilling fertility preservation journey for their local transgender population.

33.11 Conclusion

Through the existing ART technology, transgender individuals have the choice of a genetic offspring in the future. As experience in trans-fertility preservation is increasing, it is becoming apparent that this group of patients also has custom needs and expectations. Fertility preservation for trans-folk is still considered a rather recent addition to the ART curriculum, which means that there is limited research to

guide best practice. It is not surprising that the expertise about this process is 'borrowed' from standard ART practice, as well as fertility preservation protocols that are already in place for malignancy or social preservation cases. It is anticipated that, with increasing awareness, ART clinics will be seeing more cases of trans-preservation. In view of the complex clinical, psychological, and legal challenges surrounding trans-preservation, it is paramount that ART staff receive regular training on the management of this particular group of patients.

Authors' Perspectives

Key Points

- All trans-folk should be offered fertility preservation counselling, ideally before commencing gender-affirming hormone treatment.
- Standard sperm and oocyte cryopreservation techniques can be used for trans-folk, with only minor adaptations in clinical management.
- Although there is accumulating experience with testicular and ovarian tissue cryopreservation for pre-pubertal individuals, these techniques may not be suitable for trans-folk who undergo surgical castration. However, with technological advances, cryopreserved gonadal tissue may become readily useful for ART purposes in the future.
- There is limited direct evidence on the long-term reproductive potential of the trans-gametes, particularly when gender-affirming hormone treatment has been used around the time of preservation.
- There are psychological and legal considerations peculiar to trans-folk, especially trans-minors.
- Structured training around trans-fertility preservation should be available to all the fertility clinic staff that deal with trans-folk.

Potential Areas of Research

- To evaluate the long-term reproductive outcomes (including health of the offspring) from using trans-gametes which may have been exposed to gender-affirming hormonal treatment.
- To study the adverse psychological effects of ovarian stimulation on trans-folk and the modalities that can moderate these effects.

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Fertility Preservation in Prepubertal and Adolescent Males Facing Gonadotoxic Therapies: Recent Developments and New Insights

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34.1 Testicular Development and Spermatogenesis

34.1.1 Foetal and Infantile Period

The testis is formed during the first trimester of foetal life and undergoes key developmental changes that determine reproductive function and fertility in adulthood. This includes a critical period, known as the Masculinisation Programming Window (MPW), during which testosterone production is essential to programme the future development of the male reproductive tract [1, 2]. Reduced exposure to androgens during this period can result in the development of male reproductive disorders, which include cryptorchidism, hypospadias, testicular cancer, and infertility [3]. Foetal life is also a critical time period for germ cell development within the testis. A transition of the germ cell population takes place during which primitive gonocytes, expressing markers of pluripotency, differentiate into spermatogonia. By birth, the majority of the germ cells have completed the transition to spermatogonia [4]. Germ cells reside in the seminiferous cords (which form tubules at puberty) of the testis alongside

Sertoli cells, which provide the main support for germ cell development throughout life.

In the first 6 months of life, there is a period of hormonal activity during which germ cell and Sertoli cell number increase significantly [5]. This so-called mini-puberty leads to a doubling of testicular volume [6, 7]. During the infantile period, the remainder of the gonocytes differentiate to spermatogonia, failure of which can result in the development of testicular germ cell tumours [8].

34.1.2 Childhood

Prepuberty has traditionally been regarded a quiescent stage for testis development, given that the reproductive hormone axis is inactive. However, germ cell and Sertoli cell proliferation occurs during this period [6, 9]. Sertoli cell proliferation mainly occurs during infancy and in the period leading up to puberty [10], and this results in elongation of the seminiferous cords [11]. Since Sertoli cells cease proliferation after puberty and mature Sertoli cells can only support a limited number of germ cells in adulthood, the prepubertal phases of somatic cell proliferation are of major importance for future fertility [12].

Germ cells also proliferate at a relatively low rate during childhood [13]. During prepuberty, the germ cell population consists of spermatogonia, including SSCs, which possess the ability to self-renew or differentiate to undergo spermatogenesis from puberty onwards. Whilst occasional primary spermatocytes and, in rare cases, spermatids may also be identified from as early as 4 years of age, spermatozoa are not produced during prepuberty [14]. The presence of these more advanced germ cell types is believed to represent incomplete spermatogenesis, ending with cell death and therefore these cells cannot be considered viable gametes in the context of fertility preservation. Therefore, the SSCs represent the key spermatogonial population that must be preserved during prepuberty in order for future fertility to be possible.

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34.1.3 Puberty

In males, normal puberty begins between the ages of 9 and 13 years and lasts for between 2 and 4 years. Puberty is heralded by re-activation of the reproductive hormone axis with a rise in gonadotrophins (luteinizing hormone and follicle stimulating hormone) and testosterone production. This leads to the development of secondary sexual characteristics and an increase in testicular volume as a result of increased Sertoli cell proliferation [10]. As puberty progresses, Sertoli cells enter mitotic arrest and undergo further maturation to form terminally differentiated cells, which results in the formation of the blood–testis barrier [15]. Spermatogonial proliferation is responsible for the increase in seminiferous tubule diameter, which in turn produces a significant and clinically appreciable further increase in testis volume, resulting in a normal adult testicular volume of ≥ 12 mL. During the onset of puberty, initial waves of differentiation from spermatogonia to spermatocyte take place, although these spermatocytes will subsequently degenerate. As puberty progresses, the spermatogonia become capable of completing spermatogenesis [6]. Initiation of full spermatogenesis can precede the ability to produce an ejaculate and therefore viable sperm may be present in the testis before the individual is able to produce a semen sample [5, 16]. The onset of sperm production (spermarche) is important in the context of fertility preservation in adolescent males, but there is a wide variability in age at spermarche. Whilst semen samples may sometimes be obtained from boys as young as 12 years of age [17, 18], the precise timing of spermarche is difficult to determine from clinical and biochemical assessment. Testicular size and development of secondary sex characteristics are also highly variable at the onset of spermarche and in some cases may occur when little or no pubic hair has developed and when the testicular volume has increased only slightly [16]. Spermaturia has been used as an indicator of spermarche, and sperm has been identified in the urine of 5% of clinically prepubertal boys, compared with 50% of boys at Tanner stage II–III for pubic hair [16]. During early puberty, serum hormone levels are low and are not useful in predicting the onset of sperm production [19, 20]. Whilst correlations between spermaturia and clinical parameters have been established [21], they are not able to distinguish between those who can bank sperm or those who would require testicular tissue cryopreservation to preserve SSCs. Once spermatogenesis is established, it may be possible to obtain sperm using surgical techniques even if

the individual does not have the physical or emotional maturity to produce a semen sample.

34.2 Which Patients Are at Risk of Infertility?

In high-income countries with easy access to modern treatment modalities and supportive care, survival rates for Children and Young Adults (CYA) diagnosed with cancer are now around 80%. However, achieving these impressive cure rates is associated with significant treatment related morbidity with approximately two-thirds of survivors experiencing at least one co-morbidity and about a third of patients being affected in terms of fertility [22].

The high mitotic rate of the germinal epithelium within the testes makes it very vulnerable to the effects of both chemotherapy and radiotherapy. The Leydig cells are somewhat less sensitive to treatment than the germ cells and so testosterone production, pubertal progression, libido, and normal sexual function may be maintained despite the failure of sperm production.

Cancer treatment for CYA is usually multimodal, with complex chemotherapy, radiotherapy, and immunotherapy being used contiguously. It is the treatment rather than the underlying disease that primarily leads to infertility. The cyclophosphamide equivalent dose (CED) formula provides a way of estimating the gonadotoxicity of the alkylating agent component of chemotherapy treatment regimens and thus the risk of infertility [23].

All CYA cancer treatments have the potential to impact testicular development and function, the question is whether this damage is reversible or permanent. The most common chemotherapy agents associated with permanent damage are alkylating and alkylating-like drugs (Table 34.1). These drugs are mostly cell cycle independent and act by damaging the DNA affecting both rapidly dividing cells and those with lower proliferation rates such as the spermatogonial stem cells (SSCs). Whilst there is no ‘safe dose’ for these gonadotoxic drugs, azoospermia occurs with increasing frequency above a CED 4 g/m² [23]. Radiation similarly has a very damaging effect on the germ cells of the testes with doses as low as 2 Gy potentially leading to permanent azoospermia [24].

The focus of modern treatment regimens is to sustain and if possible improve survival whilst reducing toxicity, but in current practice there are still a number of tumour types where gonadotoxic treatments are frequently used (Table 34.2).

Table 34.1 Risk factors for male fertility. *CED* Cyclophosphamide equivalent dose indicates relative gonadotoxicity of alkylating agents. For example, 4 g/m² (CED = 1.0) cyclophosphamide is considered to have the same toxicity as 16.4 g/m² (CED = 0.244) ifosfamide. *NA* Not available, *TBI* Total body irradiation

Chemotherapy	CED	Additional information
Cyclophosphamide	1.0	Azoospermia increasingly likely once cumulative cyclophosphamide dose reaches 4 g/m ²
Ifosfamide	0.244	
Procarbazine	0.857	
Dacarbazine	NA	Less toxic to testes than procarbazine
Temozolomide	NA	Significantly reduces sperm number and quality in ejaculate
Chlorambucil	14.286	
BCNU (carmustine)	15.0	
CCNU (lomustine)	16.0	
Melphalan	40	
Busulfan	8.823	
Treosulfan	NA	Probably less toxic to testes than busulfan
Thiotepa	50	
Cisplatin	NA	Cumulative dose >400 mg/m ² high-risk azoospermia
Radiotherapy		Immunotherapy
Testes direct or scatter dose >1 Gy		Long-term effects not yet available for many agents
Total body irradiation (TBI) >1 Gy		
Hypothalamic–pituitary axis >30 Gy		

Table 34.2 Tumour types frequently associated with high risk of treatment-related infertility

Brain tumours
Tumours that require craniospinal radiotherapy that includes the hypothalamic–pituitary axis >30 Gy
Medulloblastoma
Pineoblastoma
Atypical teratoid/rhabdoid tumour
Ependymoma
Glioblastoma
Intracranial germ cell tumours
Sarcomas
Ewings sarcoma
Osteogenic sarcoma
Rhabdomyosarcoma
Non-rhabdomyosarcoma
Synovial sarcoma
Clear cell sarcoma kidney
Neuroblastoma
High-risk disease and relapse
Nephroblastoma
Hepatoblastoma
Lymphoma
Hodgkin's—Stage 4 requiring cyclophosphamide and dacarbazine/procarbazine
Burkitt lymphoma—high-risk groups
Anaplastic large cell lymphoma
Leukaemia
Relapse treatment with haematopoietic stem cell transplant

34.3 Fertility Preservation Options in Children and Adolescent Males

For children and adolescent males facing infertility as a result of gonadotoxic therapies, there are options for fertility preservation which include established and experimental approaches (Table 34.3). These include modification of treatment regimens to reduce exposure to gonadotoxic therapies. For example, substitution of dacarbazine for procarbazine has been shown to reduce gonadotoxicity in patients with lymphoma without negatively impacting patient survival [25]. However, changes to treatment regimens usually occur over relatively long periods of time and this is rarely an option in an individual patient basis. Shielding the gonad from radiotherapy-induced gonadotoxicity may also be an option when radiotherapy to the pelvis or gonad is planned. Agents to protect the testis from chemotherapy-induced damage are also under investigation, although there are currently no such agents used in clinical practice [26]. For pubertal males who are facing gonadotoxic treatment where preventative measures are not possible, established options include semen cryopreservation or surgical extraction of sperm.

34.3.1 Semen Cryopreservation

The easiest and least invasive option for sperm cryopreservation is via masturbation. In younger males, they may not have attempted this yet and expecting success at obtaining an

Table 34.3 Summary of options for fertility preservation in young males

Established	
Gonadal shielding	Lead shielding protects the gonad from radiotherapy-induced damage
Modify treatment regimen	Substitution of cytotoxic agents for alternatives with less gonadotoxicity
Cryopreservation of semen	Suitable for postpubertal patients. Sperm obtained from masturbation, electroejaculation, or penile vibratory stimulation
Testicular sperm extraction	Suitable for established puberty, sperm obtained from surgical retrieval
Experimental	
Ferto-protective agents	Drugs that can be administered in addition to gonadotoxic therapy to reduce testicular damage
In vitro spermatogenesis	In vitro culture of testicular tissue obtained from patients prior to treatment. Requires subsequent ART with generated sperm
Tissue transplantation	Re-transplantation of testis tissue obtained from patients prior to treatment. Requires subsequent ART with generated sperm
SSC transplantation	Injection of SSCs extracted from cryopreserved tissue into the testis of patient. Potential to restore natural fertility

ejaculated semen sample with good quantities of sperm is highly unlikely [27]. Asking a young adolescent, newly diagnosed with a cancer, to attend a reproductive medical unit to try and masturbate in one of their private rooms when they have never masturbated before can be very traumatic and unlikely to result in any success. In such cases, a semen sample may be obtained using an electroejaculation procedure (EEj) under a general anaesthetic. This involves inserting an anal electrode into the rectum in contact with the region of the prostate and seminal vesicles. Electrical stimulations are administered in order to produce ejaculation as previously described [28]. An embryologist in theatre would assess the sample to decide if it was suitable for cryopreservation and if not, a testicular sperm extraction (TESE) can be performed under the same anaesthetic. In general, this procedure takes less than 5 min to perform and can be carried out during the same anaesthetic used for a bone biopsy or large line insertion. Risks are minimal as there is no incision and there should be no delay to starting chemotherapy after this procedure.

Ejaculated samples then undergo phase-contrast microscopy according to WHO criteria. If no sperm are seen initially, samples are centrifuged at 300 g for 15 min, and the pellets resuspended in minimal volume of supernatant. Where only immotile sperm are found initially, theophylline can be used to induce motility and detect viability. Ejaculated sperm are then frozen by adding 7% glycerol cryoprotectant in equal volume to the semen and aliquoting into 0.5 mL high-security heat-sealed straws. These straws are incubated for 10 min at room temperature before cooling.

34.3.2 Surgical Sperm Retrieval

If males are unable to masturbate or electroejaculation has not resulted in an ejaculated sample containing healthy sperm, the next step may be to carry out a surgical sperm retrieval (SSR). Normally, this would involve a general anaesthetic but can be carried out with local anaesthetic and sedation in appropriate patients if deemed unfit for full anaesthetic. There are different types of surgical sperm retrieval, ranging from the least invasive PESA (percutaneous epididymal sperm aspiration) to a full micro TESE (microsurgical testicular sperm extraction).

PESA involves passing a 19-gauge butterfly needle through the skin into a full epididymis, held between the thumb and foreskin. In general, this is successful in men who are obstructed (i.e. post-vasectomy) and not in young patients with no pathological obstruction. A further problem is that in general it results in a very low yield of sperm being obtained. The aim with FP is to save enough sperm for multiple cycles of IVF, and this is less likely using this method.

The next step in the surgical pathway is a TESA (testicular sperm aspiration). Again, this is a percutaneous procedure with a large bore needle or biopsy gun going through the testicle skin. The procedure is less invasive than an open procedure; however, the limitations for TESA include low volumes of sperm extracted, decreased chance of success if there is poor spermatogenesis, and increased rates of bleeding and haematoma within the testicle, which cannot be seen and managed at the time as this is a blind procedure.

TESE (testicular sperm extraction) involves a small (3 cm) surgical incision on the midline of the scrotum, and the testicle is delivered. A 1 cm opening on the tunica albuginea allows for a large biopsy of seminiferous tubules. The procedure is carried out with the embryologist in theatre analysing the tissue during the surgical procedure. By doing this, it is possible to quickly identify cases where no sperm are present and if necessary move to the final potential surgical option, a MicroTESE [29]. This involves bi-valving the testicle and using an operating microscope to look for healthier, fuller appearing tubules which are more likely to contain sperm. Using the high-powered operating microscope also allows for identification of blood vessels and decreases the chance of bleeding and haematoma. The perfect scenario involves having access to a specialist, urologist able to carry out all of these procedures and an embryologist present in theatre so that the least invasive procedure can be trialled with a step-wise approach to the different potential options. If no sperm are found, then the final step during the same surgical procedure can be to take a further biopsy for testicular stem cell cryopreservation. The scrotal incision is small, absorbable sutures are used, and oncology treatment can still begin the next day once the wound has been reviewed.

It has been shown that success of sperm retrieval in post pubertal males in an oncological setting is roughly 60% [30]. Those who were unable to ejaculate have the highest success rate of sperm retrieval at 78%, whereas 75% of cryptospermic males were able to have a significant volume of sperm cryopreserved after surgery. In men who were able to ejaculate but found to be azoospermic, 40% had a successful sperm retrieval prior to starting their oncological treatment [30]. These are significant numbers in a group of males who without intervention would go on to their treatment and have minimal options for fertility in their future.

34.3.3 Testicular Tissue Cryopreservation

Whilst obtaining sperm is a well-established method of fertility preservation in males, for prepubertal boys, who do not yet make sperm, the approaches are limited. The experimental options for prepubertal boys involve removing testicular tissue for cryopreservation in order to preserve SSCs. The cells or tissues could then be used to generate sperm *in vitro*

or by transplantation of testicular tissue or cells back into the patient after they have completed their cancer treatment, although these approaches have not yet been proven to work in humans [31].

Testicular tissue cryopreservation aims to preserve the viability of SSCs for future use to generate gametes or restore fertility. The two primary approaches to tissue cryopreservation are slow freezing and vitrification. Slow freezing can be further classified into controlled slow freezing or uncontrolled slow freezing. Controlled slow freezing involves the use of programmable freezers in which the rate of reduction in temperature can be carefully controlled and recorded. This has traditionally been the most frequently used method for testicular tissue cryopreservation [32]. The reduction in temperature is typically in the range of $\sim 1\text{--}2^\circ\text{C}/\text{min}$ with periods of maintaining the temperature before slowly reducing the temperature and retaining the tissue in liquid nitrogen (reviewed in [33]). In some protocols, seeding may also be performed during the process. This involves applying tweezers (or similar) to the cryovial at the level of the meniscus to enhance the dehydration of the cells and reduce internal ice crystallization.

More recently, alternative slow freezing using semi-controlled methods has been increasingly used [34]. Tissue pieces are placed in cryovials containing cryoprotectant. Cryovials are then placed in an insulated container containing isopropyl-alcohol, which are known to reduce the temperature by $\sim 1^\circ\text{C}/\text{min}$ when placed in a -80°C freezer. Once the vials have reached -80°C , they can be placed into liquid nitrogen for long-term storage. The advantages of such an approach are that this requires less time and space as well as being less expensive than controlled rate freezing. Overall, a study comparing controlled and semi-controlled cryopreservation methods using human adult tissues has shown no clear difference in terms of apoptosis, germ cell proliferation, and spermatogonial survival [35]. As an alternative to slow freezing, vitrification may be used to freeze testicular tissue. This technique involves ultra-rapid cryopreservation with the aim of avoiding intracellular ice crystal formation and damage to the cell membrane. Vitrification has been used for immature testicular tissue and has been shown to support spermatogonial survival and proliferation after xenografting [36, 37]; however, this approach is not commonly used in current practice [38].

The choice of cryoprotectant is important in order to reduce the temperature at which intracellular ice crystals form and to preserve cellular integrity and cell-to-cell interaction. Dimethyl sulphoxide (DMSO) is the most frequently used cryoprotectant across centres offering testicular cryopreservation to patients [38], and this has been shown to be efficient in preserving prepubertal human testicular tissue [33]. Addition of sucrose, a non-permeating cryoprotectant, has also been reported to improve spermatogonial proliferation after thawing [39].

Whilst testicular tissues are now being collected from prepubertal boys due to receive gonadotoxic treatments in several centres worldwide, there are currently no established clinical options to generate sperm or restore fertility using the stored tissues. This remains an active area of clinical research, and the potential future options for patients are likely to include *in vitro* spermatogenesis, testicular tissue grafting, or SSC transplantation.

34.3.4 Transplantation of Testicular Tissues

Transplantation of testicular tissue back to the patient after completion of their treatment may be an option in the future for those who have cryopreserved tissue before gonadotoxic therapy. Animal studies involving many different species have provided proof-of-principle that functional gametes capable of producing offspring can be generated from transplanted immature testicular tissue (reviewed in Hutka et al. [40]). Sperm have also been generated in xenografts using testicular tissue from immature non-human primates grafted into mice [41]. Sperm generated from xenografted testicular tissues can be used in ICSI for generation of offspring, demonstrating the functional capacity of the gametes [42]. To date, spermatocytes are the most advanced germ cell type identified after xenografting human immature testicular tissues and no sperm have been generated [37, 39, 43, 44].

However, a study involving Rhesus Macaque has recently demonstrated the full proof-of-principle that testicular tissue can be taken from an immature non-human primate, and autologously transplanted back after the animal has been treated with sterilizing chemotherapy. The transplants were able to undergo spermatogenesis, and sperm were retrieved that were capable of producing offspring [45]. Similar studies involving autologous transplantation of human prepubertal testis tissue have not so far been reported.

34.3.5 Spermatogonial Stem Cell Transplantation

Similar to whole tissue transplantation, SSC transplantation can generate functional gametes capable of producing offspring in animal models. This approach involves isolating SSCs from cryopreserved immature testicular tissue and transplanting them directly into seminiferous tubules of a recipient. This approach has been shown to successfully restore natural fertility in mice [46] and to permit the generation of offspring in several other species using sperm generated from transplanted SSCs [47]. Full spermatogenesis has also been achieved in a primate model of SSC transplantation [48]. In this study, SSCs were autologously transplanted back into the testis of juvenile or adult recipient testes that

had been depleted of endogenous germ cells. Spermatogenesis occurred in the majority of recipient testes, and donor-derived sperm were identified in the ejaculate of the host. Functional capacity of the sperm was demonstrated by ICSI. Injected sperm were capable of fertilizing Rhesus monkey oocytes leading to the development of blastocysts [48]. Whilst autologous transplantation of testicular cells, presumed to include SSCs, was performed in 5 men with Hodgkins lymphoma, the outcome of this study has not been reported and it is presumed that fertility was not restored in these patients [49].

34.3.6 *In Vitro* Spermatogenesis

Transplantation of tissue or SSCs may not be an option for all patients who have stored testicular tissue. For example, the risk of reintroducing malignancy may be a significant factor, especially for haematological malignancy. Methods to generate sperm from testicular tissue *in vitro* may be required in such cases. Two-dimensional germ cell culture systems have demonstrated generation of functional haploid cells from spermatocytes [50, 51], and more recently from germ cells (primarily SSCs) obtained from the testes of cryptorchid men [52]. However, no such studies have been published using germ cells from prepubertal patients. The somatic cells are known to play a crucial role in supporting germ cell differentiation and therefore recent attention has focused on development of whole tissue-based approaches such as tissue explants and 3D organoid systems [53, 54]. Proof-of-principle has been provided by studies using prepubertal mouse testicular tissue, which showed complete spermatogenesis in tissue explants cultured on agarose blocks [55]. Sperm obtained from these explants were capable of fertilizing oocytes using ICSI and resulted in live offspring. Whilst the static culture system produces post-meiotic germ cells with relatively low efficiency, microfluidic culture systems have been shown to improve the yield of post-meiotic cells from fresh prepubertal mouse testicular tissue [56].

Meiotic, post-meiotic and haploid germ cells have recently been reported following organotypic culture of prepubertal human testis tissue [57–59]. However, the precise identity of these cells and whether they have the functional capacity to fertilize eggs and produce healthy offspring remain to be determined.

34.4 Approach to Fertility Preservation in Child and Adolescent Males

The approach to fertility preservation in young males due to receive highly gonadotoxic treatment involves assessment of pubertal status and selection of the most appropriate inter-

vention (Fig. 34.1). In patients with a high likelihood of sperm in the testis, attempts to obtain sperm from semen or surgical extraction can be attempted. In prepubertal patients and those in whom it is not possible to extract sperm, a biopsy to obtain testicular tissue for cryopreservation may be performed. Some of the considerations in applying this approach are highlighted in the following cases.

34.4.1 Prepubertal Case

A previously fit and well 5-year-old boy presented to the GP with a month's history of intermittent vomiting, increased lethargy, and a two-day history of being unable to ride his bike due to poor coordination. He was fully vaccinated and had no previous serious illnesses and was afebrile. The GP arranged urgent admission to the local children's hospital.

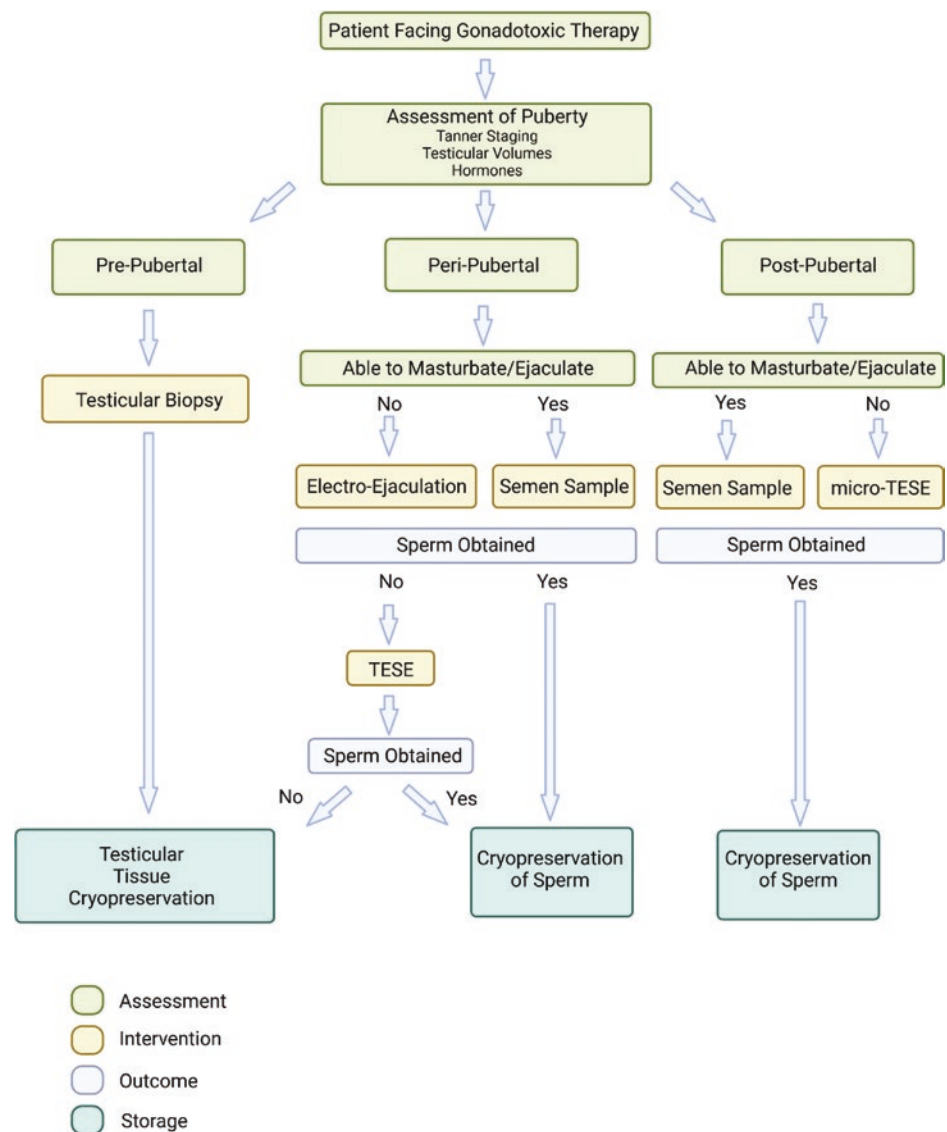
On examination at the hospital, the child was well grown with normal BMI and height for age, he had a wide-based gait, poor hand eye and motor coordination, and blurring of the optic discs. An urgent CT scan revealed a large posterior fossa mass with significant pressure effects and evidence of raised intracranial pressure. The working diagnosis was medulloblastoma. At emergency surgery, the tumour was resected, and subsequent histology confirmed the diagnosis of classical medulloblastoma—standard risk on molecular markers.

At the treatment planning discussion, the parents were given the diagnosis and treatment plan, which would be as per national guidelines, craniospinal radiotherapy followed by chemotherapy including lomustine, cyclophosphamide, and cisplatin which have a high risk of future infertility. The family requested a referral to the specialist children and young adult fertility preservation team. Given this young man's age and prepubertal status, the only fertility preservation option available to the family was cryopreservation of testicular tissue.

Key considerations: The cancer treatment proposed would cause direct damage to the germ cells in the testes with high risk of future fertility. Craniospinal radiation also has a high risk of damage to the hypothalamic-pituitary cells that produce hormones (LH and FSH) needed to stimulate the testis to enable pubertal development and then mature sperm production. These factors need to be weighed against the requirement for surgery under general anaesthetic to remove testicular tissue and the early phase nature of testicular cryopreservation. Whilst there is good evidence for retention of viability in cryopreserved testicular tissue, there have not yet been any human births as the majority of patients with tissue stored are still too young to start a family.

In England, the storage of testicular tissue is governed by the Human Tissue Authority (HTA). The parent/person with parental responsibility can consent for storage of reproduc-

Fig. 34.1 Approach to fertility preservation in child and adolescent patients with cancer due to undergo gonadotoxic therapy. TESE—testicular sperm extraction



tive tissue for a young person who is not capable of providing informed consent and there is no time limit for storage. This contrasts with storage of sperm which is governed by the Human Fertilisation and Embryology Authority (HFEA) where the donor themselves must be able to give consent, and there are storage time limits.

The future use of cryopreserved testicular tissue to restore fertility in humans will depend on research developments but if tissue is to be auto-transplanted as occurs with stored ovarian tissue, the issue of micro-metastatic disease within the stored tissue will need to be addressed. In the case of brain tumours, as in this case study, metastases outside the central nervous system are extremely rare but this is not the case for other tumour types.

The family chose to proceed with testicular surgery which was organized to occur at the same time as insertion of the central venous line required for his chemotherapy. Testicular

tissue was obtained and successfully cryopreserved and will remain in storage until the patient wishes to use it to restore fertility.

34.4.2 Adolescent Case

A 13-year-old male was referred to the teenage and young adult haematological unit with a diagnosis of osteosarcoma within the left distal femur. The diagnosis was made after a three-month history of pain within the left thigh, initially put down as secondary to excessive sporting activity.

The young man attended the reproductive lab to try and produce a semen sample. This was attempted after discussions with his father, who explained how to masturbate, but unfortunately in addition to being quite distressing, was unsuccessful in producing sperm. He was then referred on to

the local fertility preservation urologist, who after careful history and examination, found that the boy was peripubertal and had not started masturbating regularly. Examination revealed Tanner stage 4, with testicular volumes on 10–12 mls bilaterally. Baseline early morning bloods, performed within 2 days of histological diagnosis, showed a testosterone 15 nmol/L and a FSH 1.8 nmol/L, consistent with late puberty. The family were extremely interested in pursuing fertility preservation, particularly as the chemotherapy regimen for this oncological diagnosis has a high chance of infertility.

Emergency theatre capacity was arranged within 48 h, in addition to organizing both an embryologist and the stem cell retrieval team to be present for the procedure. Under a general anaesthetic, during the same session as a central line insertion, an electroejaculation was initially carried out. This involved 15 stimulations at 15 Hz; however, no ejaculate was produced. This is a common finding in peripubertal boys not masturbating frequently. During the same theatre session, the procedure was converted to a TESE, which involved a 2 cm incision on right testicle. A small piece of this tissue was given to the embryologist who was in the operating theatre and motile sperm were identified (3–6 sperm per microscopic field, 1 twitching seen every 2–3 microscopic field using a small laboratory microscope). The stem cell team was therefore not required and no testicular tissue was given to this group.

The testicular biopsy was taken to the embryology laboratory for further analysis; a 0.5 cm piece of tissue was macerated in 0.5 mL sperm wash media. The final analysis was a sperm concentration of 1×10^6 /mL sperm seen, with 26% motility. This allowed 6 vials of testicular tissue to be frozen. In general, it is estimated that each vial should provide enough sperm for 1 assisted reproductive cycle of IVF in the future. In addition to the large biopsy given to the embryologists, a small biopsy was also sent to the histo-pathologists, which showed an average Johnson score of 9.8, consistent with complete spermatogenesis.

A department which can provide a specialist urologist, embryologist, long-term funding for cryopreservation and fast access to emergency theatres gives patients the opportunity to undergo FP quickly, often at the same time as concomitant procedures minimizing general anaesthetics. There are a number of different options open to these young patients, which can give them the chance of parenthood in the future when their oncological treatment is long finished.

34.5 Conclusion

Consideration of the options for fertility preservation in child and adolescent males requires an understanding of testicular development and function during pre- and peri-puberty, in

addition to the potential gonadotoxicity of proposed treatment regimens. Careful assessment of pubertal stage and physical/emotional maturity is key to identifying the most suitable option for fertility preservation. For patients that have undergone spermatogenesis, obtaining sperm is an established option to preserve fertility, whereas for those who are prepubertal prior to gonadotoxic treatment, testicular tissue cryopreservation to preserve SSCs is an option that is becoming increasingly available. Strategies to restore fertility using cryopreserved prepubertal tissues are rapidly advancing, although these are not yet available in clinic.

Key Points

- Options for fertility preservation in males are dependent on the pubertal status of the individual
- Testicular tissue cryopreservation to preserve SSCs is increasingly offered to prepubertal boys prior to gonadotoxic therapies, albeit clinical application of this procedure remains experimental
- Adolescent males unable to have semen cryopreservation may be offered surgery with TESE or testicular tissue cryopreservation to preserve SSCs

Areas for Future Research

- Optimization of cryopreservation protocols to preserve viable SSCs in prepubertal and SSCs/sperm in adolescent patients
- Develop methods to generate spermatozoa from SSCs in cryopreserved testicular tissues
- Determine robust criteria for selection of adolescent patients to optimize potential for storage of sperm

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Part VII

Guidelines, Regulations and Best Practices

Setting Up a Cryopreservation Lab and Selecting Media, Disposables, and Systems for Vitrification

35

Kristen A. Ivani

35.1 Introduction

Cryopreservation of both embryos and oocytes plays several critical roles in an assisted reproduction cycle. It allows for the storage of supernumerary embryos after fresh embryo transfer, supporting the goal of elective single embryo transfer, and provides for optimization of uterine receptivity for frozen embryo transfers after PGT as well as freeze-all cycles due to OHSS risk or poor endometrial lining. Oocyte cryopreservation allows for fertility preservation prior to gonadotoxic treatment, planned oocyte cryopreservation for delayed childbearing, donor egg banking, storage of supernumerary oocytes when more oocytes were retrieved than the desired number to inseminate, or in a cycle when no sperm are collected.

35.2 History of Oocyte and Embryo Cryopreservation and the Transition from Slow Freezing to Vitrification

The first documented pregnancy from the transfer of a frozen-thawed eight-cell embryo was reported by Trounson and Mohr [1]. It wasn't until 1986 that the first successful pregnancy from cryopreserved oocytes using a slow cooling with rapid warming was reported by Chen [2]. The world's first egg bank was established in 1994 at the Royal Women's Hospital in Melbourne, freezing eggs for women with cancer [3]. Since that time, scientists have worked to improve survival, efficiency, and consistent success for both embryos and oocytes.

Early work with cryopreservation of embryos utilized a slow cooling process in which the temperature was reduced

gradually from room temperature, generally at a rate of -0.1 to -0.3 °C, to -30 °C or lower before plunging and storage in liquid nitrogen at -196 °C. This controlled freezing rate combined with a low concentration of cryoprotectants avoids the formation of intracellular ice crystal formation. Water is replaced by cryoprotectants to reduce cell damage due to intracellular ice formation. In contrast, vitrification is the process of freezing so rapidly that the water molecules don't have time to form ice crystals, and instead the cell(s) and surrounding solution instantaneously solidify into a glass-like structure. Vitrification uses a high concentration of cryoprotectants (CPAs), low volumes, and very rapid cooling rates ($15,000$ °C— $30,000$ °C) before plunging into liquid nitrogen. Successful vitrification results in reversible metabolic arrest in the embryo, maintenance of the structure of the embryo itself (including the DNA), consistently high survival rates, normal embryo growth after thawing, and highly reproducible results. Most vitrification protocols for both oocytes and embryos utilize some variation of Kuwayama's [4] original protocol (15% ethylene glycol, 15% DMSO, and 0.5 M sucrose) with modifications in cryodevice, dishes, medium volume, and exposure time to cryoprotectants. Several studies have shown that vitrification and warming provide better clinical outcomes than slow freezing for oocytes as well as embryos [5–9], and over the last 15 years, vitrification has been accepted as the standard of care. Additional advantages of vitrification over slow freezing include less liquid nitrogen required, simple protocols, increased time efficiency, and no need for expensive equipment.

35.3 The Laboratory Environment

The ideal IVF laboratory is designed to support the growth and development of gametes and embryos. A wide range of factors can affect laboratory outcomes including air quality (VOCs, particulates, humidity, temperature, air flow and air

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exchanges, and cleaning materials used in the lab), laboratory design (paint, flooring, casework, proximity to loading docks, gas closets, sterilizing apparatus), contact materials, (dishes, pipets, pipet tips, cryodevices), equipment (hood temperature and air flow, incubator gas, humidity and temperature, microscope optics), media (culture, vitrification, warming), laboratory staff (training and competency), and the patients themselves (age, stimulation, reason for infertility, and duration of infertility) [10]. It is imperative that we control the environment as much as possible to minimize outcome variability.

35.4 The Cryopreservation Bench

Extreme care should be taken to provide a controlled area for vitrification and warming. While most laboratorians will perform vitrification and warming in a flow hood, it is not necessary. In fact, the fan should be turned off to prevent excessive air flow which can impact osmolarity of media when using small drop sizes. The cryopreservation area should be set up ahead of time with all equipment and supplies easily accessible (Fig. 35.1). The liquid nitrogen bath should be immediately adjacent to the bench to facilitate easy transfer of the cryodevice between dishes and liquid nitrogen, thus minimizing the length of time cells are exposed to the final cryoprotectant solution. Timers should be used for every step—do not guess! Pipettors should be calibrated annually as part of the lab's QC program to ensure accurate dispensing of media drops. Pipet tips used for moving oocytes/embryos should be rinsed to prevent sticking as well as minimizing media cross-over into the next drop.

The microscope will optimally be set up with oblique illumination contrast where light is projected at the specimen

from a sideways, slanting angle to reveal features with higher contrast as compared to when viewed with brightfield illumination. This gives the oocytes an almost three-dimensional appearance, facilitating visualization of changes occurring during the vitrification/warming procedures including cryo-damage. This is especially important when depositing the oocytes or embryos into the vitrification solution (VS) drop as well as when the oocyte/embryo releases from the cryodevice into the thawing solution (TS) as they may float out of sight. While it may be tempting to focus up close on the oocyte/embryo during the vitrification/warming process, it is important to have a wider field of view to facilitate visualization of the vitrified material throughout the process.

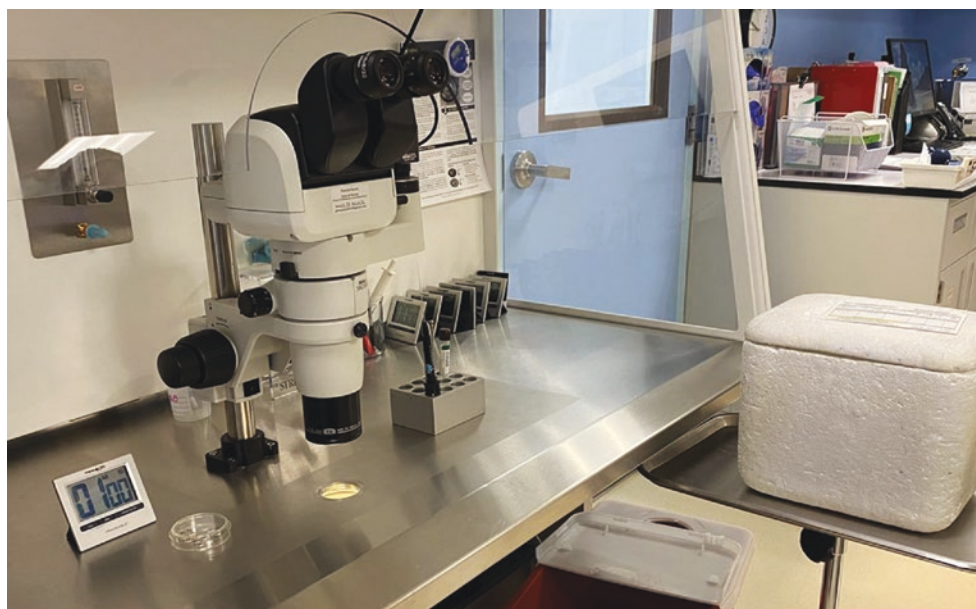
Vitrification procedures should be performed at room temperature (22–27 °C). The work surface itself should be maintained at room temperature for the vitrification and warming procedures. The one exception is the plunging of the device into the TS which should be done at 37 °C and will be discussed in more detail. One can use a 37 °C warming plate on a room temperature hood for this step. Alternatively, if a 37 °C surface is used, dishes can be placed on an elevated surface to provide a temperature closer to room temperature.

35.5 Supplies

35.5.1 Vitrification and Warming Media

Vitrification typically includes a two-step process. During the first step, the oocytes/embryos are exposed to an equilibration solution (ES) composed of low concentration of CPA. The function of the ES is to dehydrate the cell(s) and then allow for re-equilibration before exposure to the vitrifi-

Fig. 35.1 Cryobench setup (K. Ivani photo)



cation solution (VS). The VS contains a high concentration of cryoprotectant as well as a non-permeating CPA and, and exposure to VS results in additional cell shrinkage and the formation of a glass-like state before plunging into liquid nitrogen [11].

Vitrification and warming kits are available commercially or they can be prepared in house. While commercial kits are more expensive, they are more consistent, readily available, and easy to use. Most commercial vitrification/warming kits are very similar in composition and contain DMSO and ethylene glycol as the permeable cryoprotectant agents that serve to dehydrate the cells. Sugars, typically either sucrose or trehalose, are the most used non-permeating cryoprotectants. They do not enter the cells but rather act to draw out more water by osmosis during the vitrification process.

Historically, manufacturers of vitrification kits recommended that the partner warming kit be used when the patient was ready to use the oocytes/embryos. This can present challenges for embryologists who receive oocytes and embryos from other clinics, change their cryopreservation protocols, or are unable to purchase kits that are no longer manufactured or are not available for use in all areas. It is expensive for a clinic to stock kits they do not normally use (though this kit cost can be paid by the patient), the kit may expire before use, and it can be time-consuming for the lab staff to secure thawing/warming protocols from the clinic that froze the materials. In response to these constraints and because of the similarities between kit composition, Parmegiani et al. [12, 13] and Canosa et al. [14] proposed that different commercial warming kits could be combined into a new “Universal Warming” protocol and observed no adverse effect on cryosurvival, pregnancy, and live birth rates of vitrified and even slow frozen human oocytes, donor or autologous, as well as blastocyst embryos. The authors encouraged embryologists worldwide to share their experience using alternative warming kits to build a robust argument in favor of such a universal warming protocol.

35.6 Open vs Closed Devices

Oocytes and embryos have been vitrified using one of two types of devices: open or closed. An open device, while allowing for exceptionally high cooling rates of 20,000 °C/min, enables direct contact between the oocyte or embryo and liquid nitrogen. Examples of open devices include the Cryotop [15], Cryotech [16], Cryoleaf [17], Vitri-Inga [18], VitriGuard [19], and open pulled straws [20]. Closed devices, including the High Security Vitrification straw [21], CryoTip [22], Rapid-I [23], Vitrisafe [24], and MicroSecure straw [25] may not be able to provide for the same high cooling rates, but because the oocyte/embryo does not come in direct

contact with liquid nitrogen, the risk of contamination by exposure to liquid nitrogen during vitrification and storage is eliminated. A systematic review and meta-analysis by Cai et al. [26] showed that for oocytes and embryos, there was no significant difference in survival, fertilization, clinical pregnancy, live birth multiple pregnancy and miscarriage rates for open versus closed vitrification devices. Similarly, no differences were found by Porcu et al. [27].

35.7 Can Embryos/Oocytes Transmit COVID-19?

Concern over the risk of contamination was re-ignited in March of 2020 when the world was suddenly faced with a global pandemic, COVID-19. Could the SARS-V2 virus be transmitted via embryos or oocytes? There has never been a documented case of contamination by liquid nitrogen that resulted in disease transmission in the ART lab. One of the biggest defenses against potential viral infection of oocytes and embryos is the number of dilution steps between oocyte retrieval and embryo transfer. Pomeroy and Schiewe [28] estimated the dilution factor to be about 1 million to 1. If you consider the addition of vitrification and warming, the dilution factor could be higher than 1 trillion to 1, making it likely impossible for a free-floating virus to be passed along with an oocyte or embryo.

Historically, oocytes and embryos were cryopreserved with the protective zona pellucida barrier mostly intact. Current ART practices often involve the cryopreservation of embryos that have had the zona breached with a laser to collapse the embryo prior to vitrification. As the use of PGT increases, embryos are sometimes vitrified completely zona-free. Embryologists may want to increase the number of dilution steps with these embryos to minimize any risk of contamination during vitrification. Alternatively, sterile liquid nitrogen [29] or sterile liquid air [30] can be used, and liquid nitrogen baths can also be changed between patients if desired.

35.8 Disposables

Vitrification and warming can be performed in several different dish types including 60 mm Petri dishes, four well plates, or dishes specially designed for warming (Fig. 35.2). Careful consideration should be given to the pipets used to move oocytes and embryos during vitrification and warming. Manufactured micropipettes are sterile, non-breakable, will not scratch dishes, are compatible with all pipettors, and can be purchased in a variety of sizes depending on the need. It is important to choose the pipet tip appropriate for the task. For example, a 150 µm tip is appropriate for oocyte vitrification where minimal media carryover is required, and excess VS



Fig. 35.2 Dishes commonly used for warming (K. Ivani photo)

medium is removed from the cryodevice prior to plunging. A 300 μm tip would be more appropriate for blastocyst vitrification and warming due to the embryo's larger size. The 300 μm tip also works well for oocyte warming as warmed oocytes should be handled gently to reduce shearing forces.

35.9 General Protocol

Detailed review of vitrification and warming protocols will be reviewed elsewhere in this publication. Briefly, vitrification is performed at room temperature and involves a brief washing step prior to exposure to the equilibration solution. Exposure to ES is best done in a gradual manner and may be performed by merging drops or adding new medium to an existing drop. Oocytes and embryos will shrink when placed into the VS; this will be followed by a re-expansion to approximately 90% of the original size. The oocyte/embryo is then moved to the VS solution where the final dehydration step will occur. Timing is critical as extended exposure to VS will have detrimental effects on subsequent survival and viability. The oocyte/embryo is then loaded onto the cryodevice and plunged into liquid nitrogen, pulling the device through the liquid in the direction of the curved surface to ensure full exposure of the drop containing the oocyte/embryo to the liquid nitrogen. The cryodevice is carefully capped while submerged under the liquid nitrogen.

The first step in the warming procedure is the most critical and must be carried out at 37 °C to allow for adequate heat transfer. Subsequent steps are carried out at room temperature. The cap is removed from the cryodevice while still submerged in liquid nitrogen. The cryodevice is plunged into the thawing solution (TS) for 1 min. It is important to always keep the oocytes/embryo in view. The oocytes/embryo will then be transferred to the dilution solution (DS) for approximately 4 min. Following DS, the oocytes/embryo will be transferred through two incubations in washing solution. Finally, the oocytes/embryo will be transferred to a dish of culture medium containing 20% (v/v) protein for recovery prior to ICSI or embryo transfer.

Recently Manns et al. [31] introduced an “ultra-fast” blastocyst warming technique that involves warming blastocyst embryos in TS at 37 °C for 1 min before placing them in culture medium, eliminating the need for the dilution and warming solutions. Preliminary results showed no significant difference in blastocyst survival or re-expansion (after 24 h) between the ultra-fast and standard blastocyst warming protocols. Liebermann et al. [32] compared clinical outcomes of 833 FETs using a one-step rehydration protocol vs a standard multi-step protocol. They observed similar blastocyst survival rates between the two groups and a higher ongoing pregnancy rate with the one-step rehydration protocol. Future studies will reveal if there are any differences in live birth rates.

35.10 Training, Competency, and Quality Management

Oocyte and embryo vitrification and warming, as with any other facet of IVF, require strict quality management, thorough staff training, and regular competency evaluation. While the efficacy and success of these procedures are certainly dependent upon the patient population, overall quality management, training, and competency are essential to a successful cryopreservation program. The quality management system of the laboratory is composed of (1) quality control, a series of activities to ensure the laboratory is functioning normally; (2) quality assurance, a program designed to identify problems within the laboratory; and (3) quality improvement, a program designed to improve the laboratory's performance, efficiency, and effectiveness [33].

Vitrification and warming are highly technical procedures and as such require a validated protocol using solutions and devices that provide consistent and repeatable results. Vitrification and warming solutions may be purchased commercially or be homemade. Thorough training of embryologists using immature, discarded, or donated oocytes is required prior to working with clinical cases. Critical vitrification steps include timing from retrieval to vitrification, proper preparation of dishes to avoid pH and osmolarity changes, careful pipetting to avoid media carry-over, loading the device and removing excess media, plunging into liquid nitrogen, proper capping of the cryodevice, and subsequent transfer to the storage tank. Critical warming steps include safely removing the cap, rapid transfer from the liquid nitrogen to the TS, proper pipetting through the warming solutions, and subsequent transfer to the culture media until ICSI or transfer. Warmed oocytes should be placed into media containing 20% protein to recover prior to ICSI. Once technical competency has been acquired, ongoing competency evaluation is key to prevent protocol drift. Competency should be evaluated every 6 months during the first year working in the laboratory, and at least annually after 1 year [34].

One key part of training for successful vitrification and warming is to understand the function of the cryoprotectant and to know how the egg/embryo should react when exposed to different media. This gives the operator the knowledge to determine if an oocyte needs more time in the ES. Training should include repeated setting up of the microscope for optimal visualization, dealing with bubbles in the media when looking for the oocyte/embryo, proper loading onto the cryodevice within the specified time, and capping and uncapping under the liquid nitrogen.

Key performance indicators (KPIs) are used to measure specific processes within the IVF Lab and are an integral part of the laboratory's Quality Management Program. KPIs must

be measurable and reproducible and must reflect the effectiveness of the IVF laboratory. Fabozzi et al. [33] described three types of KPIs specific to the IVF Lab: structure-related (equipment and consumables), process-related (management of the IVF lab), and outcome-related (the result of lab practices). While clinical parameters can often confound outcome KPIs, they are nevertheless necessary to define effectiveness of the vitrification and warming procedures. Development and adherence to KPI monitoring facilitate early detection of potential problems and allow for corrective action before outcomes are affected. KPIs for oocyte vitrification and warming may include survival rate, fertilization rate, embryo utilization rate (embryos achieving developmental competency consistent with transfer or cryopreservation), number of embryos frozen from warmed oocytes, implantation rate, live birth rate, and, for some programs, euploidy rate. Similarly, KPIs for vitrified/warmed blastocysts include survival (overall and by embryologist), implantation, and delivery rates.

35.11 Documentation and Record Keeping

Chain of custody should be of utmost importance in any cryopreservation program. The cryodevice should be clearly and permanently labeled with the patient's name, a unique identifier (often the medical record number), date of cryopreservation, and contents. Proper witnessing of each step is essential. This can be done using a second observer or with an electronic witnessing system. Proper documentation of the storage location should include the tank number, canister number, and a unique cane identifier. Automated systems include the use of traceable radio frequency identification (RFID) tags on each specimen. Regardless of the storage system used, redundancy and safety should be top priorities.

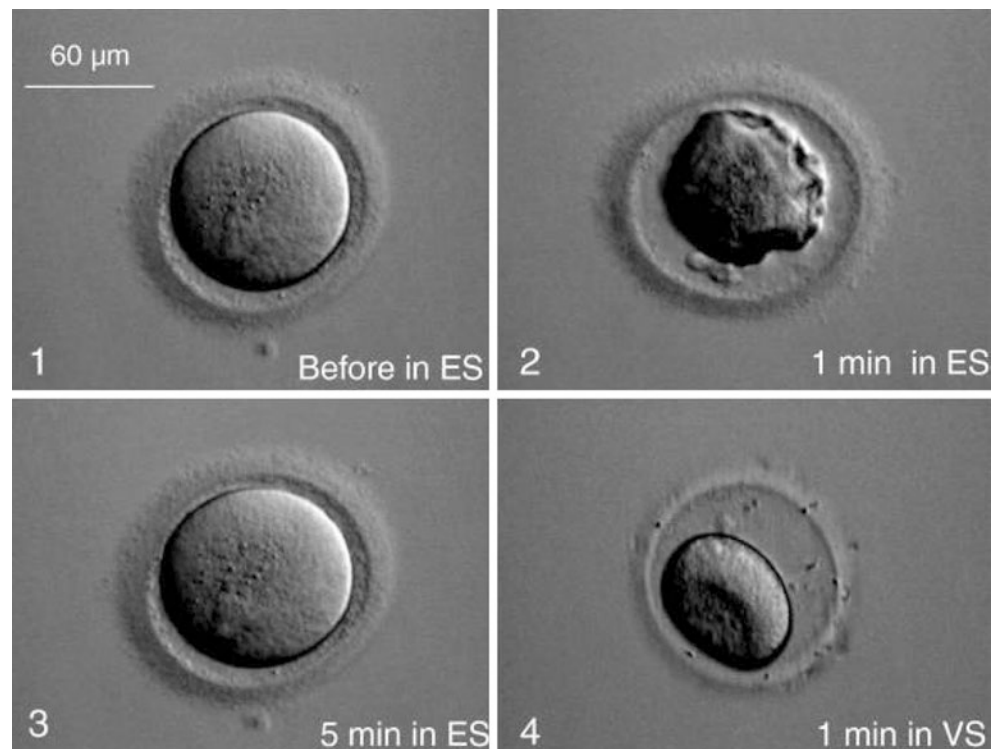
35.12 Tips and Tricks: Vitrification

An entire chapter could be written on tips, tricks, and pitfalls of vitrification and warming. There are many possible contributors to vitrification and warming success, including quality of the oocytes/embryos, protocol, timing of events, cryodevice, temperature, patient characteristics, media and cryoprotectants, drop volume, whether the embryo is biopsied or not, and embryologist skill level.

1. Set up the workstation, so all supplies and equipment are ready for use (Fig. 35.1).
2. The liquid nitrogen bath should be always full to avoid passing the loaded cryodevices through a liquid nitrogen phase slowing the cooling rate.

3. Temperature affects the permeability of the CPA into the cell(s) and will impact outcomes if not set correctly. Follow the kit manufacturer's instructions. Vitrification steps should be carried out at room temperature (22–27 °C). Be aware that the microscope's light source can generate heat. Having the liquid nitrogen bath too close to the stage can also impact temperature.
4. Drop volume is critical. Do not be tempted to use smaller than recommended volumes as this affects the environment around the oocyte and may have a negative impact on outcomes.
5. MII oocytes should be evaluated carefully before vitrification. Any oocytes with dark cytoplasm, vacuoles, granular areas, or abnormal polar bodies should be excluded from vitrification. This is especially true for oocytes destined for a donor egg bank. If the oocytes are being vitrified for autologous use, any imperfections should be documented.
6. Mark the loading side of the cryodevice with a permanent pen. This makes it easier to know which side of the device contains your oocytes/embryos.
7. If using a Petri dish, the lid allows more working surface than the bottom of the dish.
8. Do not pipet the VS drops until just before use to prevent evaporation.
9. Oocytes will react immediately when placed into the ES by shrinking (Fig. 35.3) [35] followed by re-expansion. You should observe approximately 90% re-expansion by around 8–10 min before moving to the VS drops. If you do not observe any shrinkage, especially for donor oocytes, consider excluding these oocytes from the batch.
10. The duration of exposure of oocytes to VS should not exceed 75 s; however, the oocytes should remain in the VS for a full 60 s before loading. Excess exposure to the high levels of CPA in VS will be detrimental to the oocytes.
11. Prime your pipet with VS medium before picking up the oocytes to be sure the oocytes will move freely within the pipet tip. Keep the oocytes in view in the pipet tip.
12. Oocytes will float and become almost transparent in the VS. It is important to keep the oocytes in view and gently place them back down to the bottom of the dish until they attain neutral buoyancy and settle to the bottom.
13. When loading the oocytes onto the cryodevice, deposit the oocytes near the black mark at the end of the cryodevice, extend the drop away from the oocytes, and gently aspirate excess VS, leaving the oocyte in minimal volume of VS (Fig. 35.4). If the oocyte is accidentally aspirated while drawing down media, carefully express the oocytes back onto the cryodevice and repeat the step. The best way to avoid this is to practice this step and then practice some more.
14. The size of the VS drop is critical. If the drop is too small, the oocytes may be difficult to remove from the cryodevice upon warming. If the drop is too large, the cooling rate may be impacted, and the oocytes may dislodge from the cryodevice immediately upon plunging

Fig. 35.3 Watch for oocyte reaction in ES and VS (From Chian, et al. JARG 2014). (a–d) The oocyte will exhibit a crenated membrane upon exposure to ES. After several minutes the oocyte will re-equilibrate. Subsequent exposure to VS will dehydrate the oocyte in preparation for loading on to the cryodevice and plunging into liquid nitrogen



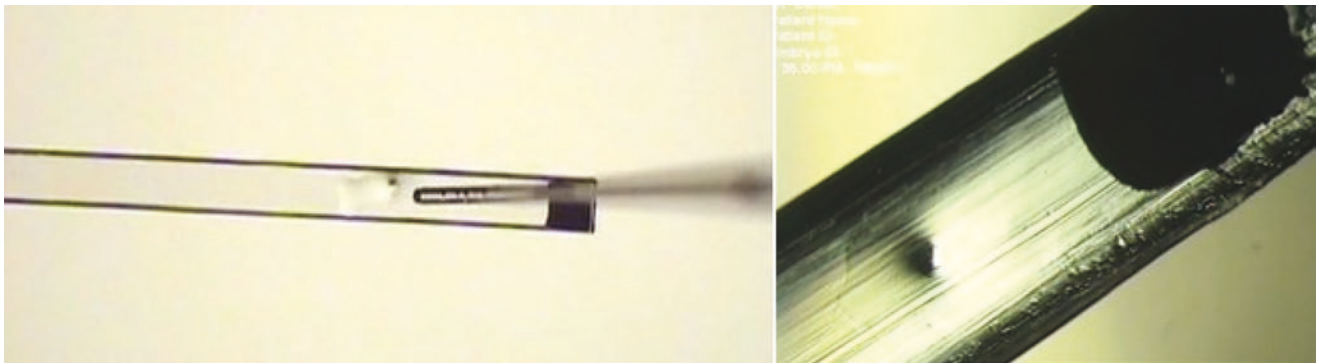


Fig. 35.4 Loading the oocyte onto the cryodevice and removing excess VS. (Photo courtesy of Wayne Caswell)

- into the TS, making locating them in the large volume of media more challenging.
15. The cryodevice should be plunged into the liquid nitrogen bath: (1) to avoid evaporation of the tiny media drop and (2) to increase the cooling rate.
16. The cryodevice should be pulled slowly through the liquid nitrogen and swirled gently to avoid the Leidenfrost effect. This occurs when the warmer cryodevice and medium contact the liquid nitrogen. The liquid first in contact with the device vaporizes, creating an insulating layer which may slow the rate of heat transfer.
17. The cryodevice caps should be cooled under liquid nitrogen prior to capping. The cap should be carefully applied with a gentle twisting motion keeping the tip of the cryodevice completely submerged. Any bending or scraping of the cryodevice while capping may cause breakage of the device or shearing damage to the oocytes.
18. The labeled cryocane with attached goblet and cane cover should be prechilled in the liquid nitrogen bath prior to loading the vitrified samples. Double check the number of devices for the patient and transfer the cryodevices to the goblet under the liquid nitrogen. Carry the liquid nitrogen bath to the storage tank, locate the predetermined canister, and quickly transfer the cane to the storage tank. Only trained personnel should be allowed to transfer cryopreserved specimens in and out of storage tanks.
2. Artificial collapse of the blastocyst prior to vitrification, performed with a microneedle, sucrose in the medium or laser pulses between two trophectoderm cells, has been proposed to improve blastocyst survival rate after warming. Indeed, several groups reported higher survival rates after artificial collapse [36]. A recent meta-analysis by Boyard et al. [37] suggested that while blastocyst survival and clinical pregnancy rates were higher in the artificially collapsed group when compared to non-collapsed controls, there was no difference in either implantation or live birth rates.
3. Special handling is required for zona-free blastocyst embryos as they may be sticky (especially if previously biopsied) and may float more irregularly during passage through vitrification solutions.
4. The biopsy procedure itself does not appear to affect embryo blastocyst survival or clinical pregnancy outcomes.
5. While there is not a clearly defined critical time to vitrify blastocyst post-biopsy, the embryologist must wait until the biopsied trophectoderm cells have been tubed before initiating vitrification.
6. It is not necessary to draw excess VS medium off the cryodevice prior to plunging in the liquid nitrogen. In fact, this may be detrimental in that too little medium can result in the blastocyst adhering to the cryodevice at warming making it more difficult to remove and possibly impacting survival and pregnancy rates.

35.13 Special Considerations for Blastocyst Vitrification

1. Most of the same tips and tricks apply to blastocyst vitrification with a few additional considerations. Evaluate blastocyst quality prior to vitrification.

35.14 Tips and Tricks: Warming

1. The TS medium and the dish(es) for TS must be warmed to 37 °C for at least 2 h and up to 24 h in a non-gassed incubator prior to use. Prepare multiple dishes if planning to warm multiple devices so you do not have to wait

for re-warming. A 35 mm Petri dish containing 4.0 mL TS works well. The larger volume of TS supports the rapid warming rate required for optimum survival.

2. Set up the workstation, so all supplies and equipment are ready for use.
3. Confirm the number of oocytes to be warmed and how many oocytes are on each cryodevice.
4. Perform all steps at room temperature except for the first step into TS.
5. Position the liquid nitrogen bath containing the vitrification devices close to the workstation.
6. Cool the forceps in liquid nitrogen and always keeping the end of the cryodevice under the liquid nitrogen, gently twist the cap and remove it from the cryodevice.
7. Bring the TS dish to the liquid nitrogen bath (Fig. 35.5) and in one smooth movement rapidly transfer the end of the cryodevice into the TS while moving the dish to the stereomicroscope stage. Alternatively, one may choose to position the dish on the microscope stage and bring the cryodevice to the dish. While this may facilitate visualization, the oocytes are exposed to room temperature air for a longer period than if the dish were positioned near the liquid nitrogen bath for the transfer.
8. As you remove the cryodevice from the liquid nitrogen, any residual liquid nitrogen on the cryodevice warms up and expands to vapor, possibly resulting in bubbles in the TS upon plunging. If this is an ongoing issue while training, try slowing down the transfer of the device from the liquid nitrogen bath to the TS giving the liquid nitrogen time to evaporate. Alternatively, you can gently flick prior to plunging into the TS to dislodge any liquid nitrogen that might be on the cryodevice.
9. Locate the oocytes on the cryodevice in the TS. Keep in mind that they may have floated off the cryodevice.
10. If the specimen does not release from device try gently swirling device in dish. Alternatively, you can gently tap the thick end of the cryodevice or rub the cryodevice along the bottom of the dish to cause vibration to dislodge the specimen. You can also use the tip of the pipet to gently blow the specimen off the device. As a last resort you can use the pipet tip to remove the specimen from the cryodevice, but you may risk damage to the specimen.
11. When moving oocytes into the next drop of medium, preload the tip with approximately 3 μ L medium from the next drop and then pick up your oocytes, move them to the next drop and then expel the media on top of the oocytes.
12. Look for signs of cryodamage which can be in the form of chemical, thermal, or mechanical disruption of the oocyte (Fig. 35.6).
13. Once thawed, oocytes should be transferred to culture medium containing 20% protein until ready for ICSI.

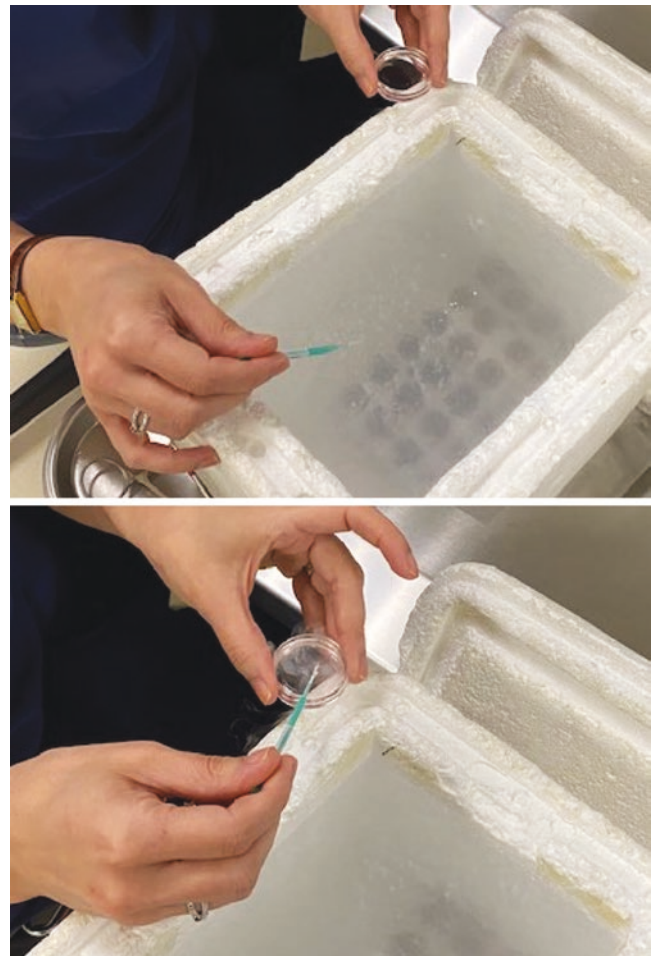


Fig. 35.5 Bring the TS dish to the liquid nitrogen bath and quickly plunge the cryodevice into the warmed medium. (K. Ivani photo)

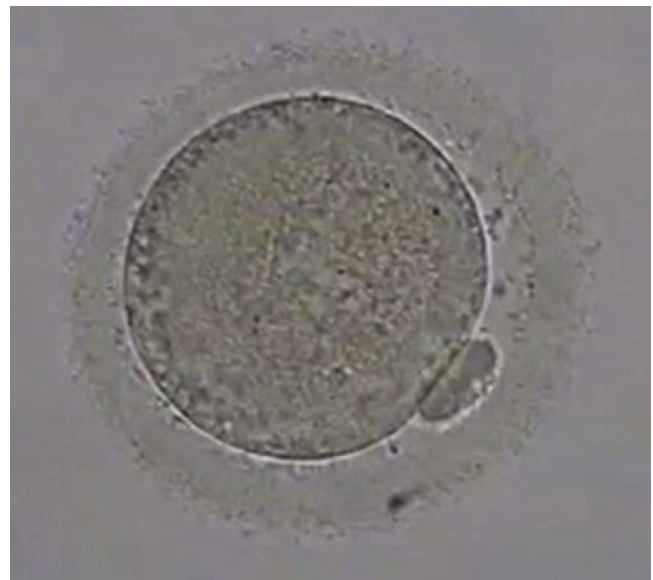


Fig. 35.6 Cryodamage/cortical granulation (Photo courtesy of Wayne Caswell)

35.15 Special Considerations for Blastocyst Warming

1. As for oocyte warming, the TS should be pre-warmed in a 37 °C non-gassed incubator for at least 2 h prior to warming.
2. Because the blastocyst is loaded onto the cryodevice in a larger volume drop than used for oocytes, the embryo may float off the cryodevice immediately upon plunging into the TS. Be prepared to scan the dish quickly to locate the embryo.
3. Zona-free embryos require special handling to avoid sticking to the pipet tip or becoming trapped in the meniscus of a warming drop or in the interface between medium and oil.
4. As discussed previously, artificial collapse of the blastocyst embryo is common practice prior to vitrification to reduce the risk of ice crystal formation in the blastocoel fluid. One measure of successful vitrification and warming of blastocysts is post-warming re-expansion of the blastocoel cavity. Does this mean that embryos that have not expanded by transfer time are less likely to result in a successful pregnancy? Allen et al. [38] evaluated the degree of blastocoel re-expansion immediately before embryo transfer as fully re-expanded, partially re-expanded $\geq 50\%$, partially re-expanded $< 50\%$, and collapsed. They observed that post-warming expansion correlated with cycle outcomes and that a higher re-expansion grade was associated with higher live birth rate, even when controlled for PGT-A. They noted that embryos with poor post-warming re-expansion still resulted in a live birth rate of 11.4%. A similar study by Guinco et al. [39] suggested that blastocyst embryos that survive thawing have similar ongoing pregnancy rates regardless of amount or rate of re-expansion. Both groups concluded that failure to re-expand should not justify discarding an embryo.

35.16 Future Directions

As freeze-all cycles (with or without PGT) combined with single embryo transfer are becoming more common, the demand for successful vitrification and warming of oocytes and embryos is growing concurrently. Vitrification is a time-consuming, highly technical procedure with often inconsistent results between embryologists and between clinics. The oocytes/embryos may be subject to osmotic shock and chemical toxicity due to cryoprotectants as well as mechanical damage due to incorrect manipulation during pipetting, loading, plunging into LN₂, and capping and uncapping of the

vitrification device. Excellent pipetting skills are required to ensure safe transfer between drops, and proper placement of the oocyte/embryo onto the device in a very small volume of fluid. Finally, a lack of standardization of protocols and vitrification devices across clinics results in inconsistent results. Automation of both the vitrification and warming procedures would increase efficiency while reducing the variation in performance and the risk of human error.

The ideal automated vitrification system would be easy to use, would automate all steps of the process, provide optimal cooling and warming rates of greater than 20,000 °C/min, load the oocyte/embryo in $< 0.1 \mu\text{L}$ volume, avoid any mechanical damage along the way, work with an open or closed system, alert the embryologist to any mechanical failure, take a short time to complete, and work with the lab's existing cryostorage system. In addition, the machine must be affordable, and the consumables QC tested, readily available and within the lab's budget.

One such instrument is the Gavi (Genea Biomedx; Sydney, Australia). Gavi is a semi-automated closed-type vitrification system that can be used for vitrification of oocytes, cleavage stage, or blastocyst embryos. The embryologist loads the embryo into the Gavi pod and presses start. Gavi controls timing and exposure to media using robotics to automate changing solutions around the oocyte/embryo and allowing gradual diffusion and exposure to the vitrification solutions. Once dehydration is complete, the Gavi seals the pod and plunges the cassette containing the pod into liquid nitrogen, swirling gently. Gavi can vitrify four blasts at a time in a span of 15 minutes. The liquid nitrogen reservoir can be detached from the unit, and the cassette containing the Gavi pods can be transferred to the cryotank for storage.

Gatimel et al. [40] performed a retrospective analysis of 282 patients comparing manual vitrification using the Irvine-CBS with semi-automated vitrification using the Gavi. They measured intact survival (100% of blastomeres intact) and positive survival (at least 50% of blastomeres survived) of day 2 or day 3 embryos as well as implantation rate and clinical pregnancy rate. The authors also assessed inter-operator variability and completion time. There was no significant difference between the two vitrification methods in intact survival rate, and both shared comparable implantation and pregnancy rates. In addition, they noted that for trained embryologists, there was low inter-operator variability between the manual and the semi-automated technique. Of particular interest, the authors noted that the Gavi did not reduce the time required for multiple embryo vitrification when compared to manual vitrification, and that the cost of semi-automated vitrification was three to four times higher than that of manual vitrification (excluding the price of the Gavi instrument itself).

When Miwa et al. [41] compared automated vitrification using the Gavi to manual vitrification of human blastocysts

using the Cryotop device in a retrospective study, the authors observed no significant difference between the two vitrification systems with regard to embryo survival, clinical pregnancy rates, miscarriage rates, gestational age, cesarean section rate, or birthweight in any age group. It is important to note that while the Gavi does automate some steps of the vitrification procedure, there are still manual steps that can result in variability: filling the pod with medium and loading the embryo into the pod as well as the entire warming procedure.

Casciani et al. [42] expressed caution about introducing any automated technology into the routine IVF workload, describing three requirements that should be met prior to integration. First, the intervention should address a clinical issue. Second, the intervention should be proven safe and to do no harm. And third, randomized-controlled trials should be carried out and the data generated be evaluated for the benefits of the intervention. There is no doubt the first requirement has been met: as mentioned earlier, automation of vitrification would reduce performance variability, potential mechanical damage, and risk of human error. However, at this writing, there is insufficient evidence suggesting that existing automated vitrification systems have been adequately evaluated to be deemed safer, more efficient, and more effective than manual vitrification protocols. Automation in the IVF lab involves the marriage of biology, engineering, and computer science. It is very unlikely that such automation will replace the embryologist we know today; however, that embryologist's role may change from an expert in manual skills to technical operator, ensuring quality control, instrument maintenance, and troubleshooting.

35.17 Conclusions

Vitrification and warming of oocytes and embryos are highly technical and completely manual at the present time with several critical steps that can drastically affect survival and outcomes including maintenance of optimum temperature, timing of individual steps, and appropriate loading volume. There is a learning curve, and the importance of rigorous training cannot be overlooked. Ongoing competency assessment is essential to prevent protocol drift, and the use of vitrification KPIs should be implemented into the lab's overall quality management program to monitor individual and clinic performance. Continued efforts toward automation of vitrification and warming should result in processes that are more consistent and more efficient with reduced risk of human error; however, only further studies will prove if such automated systems are superior to current manual vitrification and warming methods.

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Cryopreservation and Storage of Reproductive Tissues: Good Practice Guidelines

36

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36.1 Introduction

Cryo-storage tanks are liquid nitrogen containers designed for the reliable storage of biological samples at cryogenic temperatures. Such tanks are stainless steel or aluminum cylindrical vessels with multiple layers of insulation to maintain cryogenic temperatures. A vacuum layer protects against thermal loss and reduces the rate that liquid nitrogen boils away. As the liquid nitrogen boils away, it is released as a gas through a loose-fitting stopper, usually Styrofoam, or in more sophisticated models, through automatic venting. The narrow neck of these vessels also helps to reduce nitrogen evaporation rates. Cryo-storage tanks come in a variety of sizes and configurations to suit different storage needs. Maintaining a constant source of liquid nitrogen into such tanks is imperative, either through manual filling or through automatic filling. Some tanks exclusively use the liquid phase of nitrogen, a temperature of -196°C . Other tanks take advantage of the vapor phase of nitrogen, with storage at -150°C but no lower than -130°C .

Within the setting of an IVF laboratory, typical samples stored include sperm, oocytes, embryos, testicular tissue, ovarian tissue, and all of these tissues from cancer patients.

The number of samples will vary according to the patient but generally, an increased volume in cryo-storage of oocytes, embryos, and sperm on-site at IVF programs and at commercial biorepositories has been seen, as embryo cryopreservation has moved to the center of a patient's IVF treatment. This is driven in part by the success of embryology laboratories to produce robust cohorts of embryos often leading to surplus embryos, as well as the increased utilization of freeze-all cycles (both embryos and oocytes) [1].

Each cryo-storage tank can hold hundreds of samples. Laboratories are responsible for sustaining a constant source of liquid nitrogen to maintain temperature of the cryo tanks. Additionally, there must be constant vigilance to ensure the function and operation of the tanks. Failure to do so can result in catastrophic events. Such events can lead to the uncontrolled warming of hundreds of patients' reproductive tissues. This is an event that every laboratory must strive to avoid at all costs. The risk and liability of storage of such specimens are an unavoidable burden to IVF programs and laboratories, therefore rigorous policies and procedures to oversee the safety and security of cryo-storage tanks and their contents are necessary.

Recent high-profile catastrophic cryo-storage tank failures and other near-miss tank failures made headline news in the US. Unfortunately for the patients and for the programs in question, the samples warmed to room temperature and were no longer viable. While it is not widely known how many actual tank failures occur per year, these recent instances brought great scrutiny to our field and to the requirements and recommendations around cryo-storage. In response to recent concerns about cryo-storage, accrediting agencies such as the College of American Pathologists (CAP) drafted enhanced requirements for CAP-accredited IVF laboratories to follow. Further, the ASRM Practice Committee drafted a committee opinion on best practices for cryo-storage [2].

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36.2 Minimum Requirements

IVF laboratories in the US must be accredited by either CAP or TJC (The Joint Commission) if they are affiliated with IVF clinics that are members of the Society for Assisted Reproductive Technology (SART). CAP has established minimum requirements for maintaining cryo-storage of reproductive tissues, which all IVF labs including non-SART member programs should refer to as minimum requirements for best practices [3]. These include monitoring of temperature and liquid nitrogen (LN₂) tank levels, alarm systems connected to cryo-storage tanks, appropriate LN₂ supplies on-site, and maintenance of adequate policies and procedures for the utilization of LN₂, including adequate storage and safety requirements.

36.2.1 Temperature and LN₂ Tank Level Monitoring

To ensure the proper functioning of cryo-storage tanks, strict quality control on the temperature and the LN₂ levels are required. Cryo-storage tanks are designed to maintain stable LN₂ volume over time, but slow, constant evaporation occurs through the lid [4, 5]. Laboratories must have written procedures to monitor and record the measurement of LN₂ levels and to maintain them within an acceptable range. Liquid nitrogen levels can be measured electronically and/or manually. While the first one provides continuous monitoring through a temperature/level probe, the second should be performed at least three times per week, before filling tanks, by visual inspection and measurement of LN₂ levels. These verifications allow for the evaluation of the LN₂ rate loss, providing information on which cryo-storage tanks maintain nitrogen efficiently and whether they should continue in operation or be retired.

36.2.2 Alarm Systems

Complementary to monitoring the temperature and measuring the LN₂ levels, cryo-storage tanks must have alarm systems that are monitored 24 h/day and are activated when established measurements are out of range [3, 4]. Laboratories must ensure the proper functioning of alarms by testing the system at least quarterly. This can be performed by creating a controlled fail and confirming that the system is activated, and an alarm is received properly. A written procedure must be in place, and personnel must be trained and prepared to respond to audible alarms when the personnel is in the labo-

ratory and to remote alarms (telephone dialing system) if the personnel is not present. These systems ensure that personnel can act in a timely manner to resolve emergency situations.

36.2.3 Appropriate LN₂ Supplies on-Site

Laboratories should have extra LN₂ supply and backup tanks for situations such as tank failures or the need to freeze specimens in case of emergencies.

36.2.4 Storage

Cryo-storage tanks must be handled and stored in a well-ventilated area/space to provide a safe working environment. Even though LN₂ is a non-toxic gas, it evaporates in large quantities at room temperature, leading to a reduction of oxygen concentration in the area where the tanks are located. This reduction can be hazardous as it can cause asphyxiation, leading to serious injuries or even death [6].

36.2.5 Safety Data Sheet (SDS) and Appropriate Signage

All laboratory personnel must be aware of the SDS for LN₂. This document contains important information about this chemical, such as potential hazards during manipulation, first aid measures in case of exposure, and precautions for safe handling. Appropriate signs indicating the presence of LN₂ in the cryo-storage and tank supply areas must be easily visualized [7].

36.2.6 Personal Protective Equipment

Liquid nitrogen can be hazardous when not handled properly. It is paramount that all personnel with access to cryo-storage tanks are aware of the general precautions during use and follow adequate requirements for personal protective equipment. Direct contact of liquid nitrogen with skin and eyes is one area of particular concern while handling LN₂. Due to the susceptibility of the liquid to splash when manipulating cryo stored specimens, a face shield or safety goggles must be used to protect the eyes and face, together with insulated thermal gloves to prevent skin tissue damage. Personnel must also wear safety shoes and long sleeves while handling or operating cryo-storage tanks [6].

Table 36.1 Quality control and quality assurance steps

1.	Daily monitoring of cryo-storage tanks
2.	Alarms that detect rise in temperature and/or decrease in liquid nitrogen level
3.	Alarm system are electronically hardwired, on backup generator or UPS or “in the cloud”
4.	Once critical level reached the remote alarm system calls/texts/emails/audibly alarms lab personnel
5.	Extra storage tank on-site filled with liquid nitrogen for transfer of samples if catastrophic tank failure
6.	Ambient room oxygen monitor, preferably hard-wired vs. battery
7.	Appropriate signage, staff training, personal protective equipment
8.	A clear written response plan and phone tree for both working and non-working hours

36.2.7 Appropriately Trained Personnel

Laboratory personnel handling cryo-storage tanks and performing vitrification or similar procedures must receive information and training on the properties of LN₂, as well as on the risks and precautions involved in working with LN₂ and cryo-storage tanks. Laboratory staff should not be allowed to handle LN₂ before full training is provided.

It is strongly recommended that laboratories storing reproductive tissues in liquid nitrogen cryo-storage tanks follow the storage requirements of the lab accrediting agency, such as CAP or TJC. There may also be state and local laws that apply and should be followed. Table 36.1 summarizes quality control and quality assurance steps that are required and/or recommended as described above.

36.3 Access to Cryo-Storage Tanks

Entrance to rooms where cryo-storage and LN₂ tanks are located should be protected by secure doors, and access should be limited to authorized laboratory staff with proper training [2]. Cryo-storage tanks should be stored in a well-trafficked area or in a room in the vicinity of the IVF laboratory, ideally with a window to provide visibility. These allow for routine surveying of working conditions and for evaluating potential risks to personnel's safety before entering the room [4]. A camera system installed on-site can provide the additional continuous ability to monitor the conditions in the room for personnel safety. The organization of the storage room should also permit easy access to the tanks for their operations, monitoring, and refill.

36.4 Manufacturers' Recommendations

Cryogenic tanks used for the storage of reproductive tissue in IVF laboratories have different types, sizes, and storage capacities, providing flexibility depending on the laboratory layout. The most common type of cryo-storage unit in IVF laboratories are standard vessels filled manually with LN₂, but large-capacity storage units that permit automatic filling are also commercially available [2]. If stored on-site, it is fundamental to follow strict procedures for installation prior to initial use as recommended by the cryo-storage tank manufacturer. Moreover, proper validation and quality control are essential before any specimen is stored in a new cryo-storage tank. Proper functioning should be ideally assessed for 30 days or at least a minimum of 7 days prior to being placed into service [2].

Commonly used in IVF laboratories, small tanks (dewars) can hold approximately 30–75 L of LN₂ and usually require frequent manual fill [8]. However, with the increase in the number of cases in large programs and the necessity to store more reproductive tissue, the use of large high-capacity tanks has expanded. These tanks are usually replenished by an autofill system using a secondary source of LN₂ supply that maintains the LN₂ supply pressure and flow within a narrow range, which is critical for the proper operation of the tank. This system requires regular inspection to confirm its proper functioning as technical problems may cause malfunctions [9].

Manufacturers of the LN₂ storage tanks provide warranty statements that are usually limited to defects or malfunction of the equipment for a specific period. The laboratory personnel should be familiar with the warranty terms established by the manufacturer and ensure that proper maintenance is implemented, and potential required repairs are timely completed [8]. Once the warranty is over, personnel should continue to follow the manufacturer's recommendations for the tank's maintenance and the established timeline for the tank usage and retirement. If the manufacturer does not provide information on the estimated lifespan of the tanks, it is critical to perform continuous monitoring and preventive maintenance to evaluate the conditions of the tank. The laboratory should have a protocol in place to determine when tanks should be retired based on the evaluation of the tank integrity, years of usage, the capacity of the tank to retain liquid volume over time (static holding time), the information provided on the preventive maintenance reports, and frequent visual inspection of the tank.

Tank failure due to loss of vacuum between the inner and outer container requires extreme attention as it could lead to

a rapid increase in the rate of liquid nitrogen evaporation and, consequently, to catastrophic increase in the temperature inside the tank. Frequent visual inspections of the outside of the tanks are crucial to ensure the physical integrity of the tank and the proper functioning of the vacuum chamber. In the case of small-capacity tanks, the neck region is a critical area. The weight of these tanks is carried by their neck, and excessive motion or jolting of this area makes it particularly susceptible to cracks [5, 10]. If cracks extend to the vacuum chamber, they could lead to the compromise of its operation and lead to tank failure.

The lack of neck welds in large-capacity tanks makes cracking less common in these units. However, they should also be visually assessed for potential vacuum failures. The detection of condensation on the external part of the tank or water pooling can be signs of loss of tank vacuum pressure. Similarly, the presence of ice and frost on the tank demands particular attention. The formation of ice plugs around the neck of the tanks might disrupt pressure relief and lead to explosive ruptures that can compromise the integrity of the lid and/or the neck of the tank [9].

Since increases in temperature can impact the state of cryo-stored specimens significantly, every laboratory must have written protocols in place for potential failures of cryo-storage tanks and an immediate plan of action to avoid catastrophic losses [11].

36.5 Liquid Versus Vapor Storage

Cryopreservation of reproductive tissue can be accomplished using two different categories of liquid nitrogen storage tanks: liquid-phase storage tanks (samples stored at -196°C) and vapor-phase storage tanks (samples stored at $\leq 150^{\circ}\text{C}$). Liquid-phase cryo-storage tanks avoid temperature variation via the direct contact of the tissue with liquid nitrogen, providing increased safety in case of a shortage of LN_2 . The prevention of an abrupt increase in temperature provides extra time for laboratory personnel to respond to emergencies and allows for the relocation of reproductive tissue to a different backup tank before reproductive tissue is damaged.

On the other hand, vapor phase-storage tanks maintain the specimens in a nitrogen gas atmosphere at very low temperatures throughout the entire storage area, while maintaining the integrity of the tissue in a similar way as those that are maintained in liquid-phase storage. This type of tank is normally used for virus-positive and potentially infectious tissue storage, as the nitrogen gas environment theoretically reduces the risk of cross-contamination compared to LN_2 tanks [12]. Vapor phase-storage tanks are also used in shipper tanks when transporting samples between laboratories and in auto-fill long-term storage repositories. While storage

in liquid nitrogen is typical for IVF laboratories in the US, the latest artificial intelligence semi-automated technology for cryo-storage is vapor storage, bringing it more in line with non-IVF cryo-storage globally.

36.6 Risk Management

Risk management of cryo-storage identifies risks and mitigates risks. Communication is key, specifically among laboratory personnel, with the laboratory director, and with the medical director, who is often ultimately held responsible for any loss of patient samples. Have a disaster preparedness plan printed and posted so all laboratory personnel are aware of what steps should be taken in an emergency [13]. This plan should be formulated with program leadership including the laboratory director, medical director, and program administrator. To create a disaster preparedness plan, start by creating checklists to ensure attention to the small details, routine inspection and testing of the alarm system, and robust documentation of what is being done. Table 36.2 provides a guideline for what should be included in the checklist used to create a disaster preparedness plan for managing risks of cryo-storage.

36.6.1 Assessment of Risk

A visual tool to assess risk effectively is a bowtie diagram, commonly used in industry to logically follow a risk from its cause to a critical event and finally to the consequences [14]. A bowtie diagram depicts multiple threats that can escalate to a loss of control of a hazard such as liquid nitrogen and in turn, progress to a variety of negative consequences. A bowtie diagram is an elegant and effective way to identify risks and methods for risk reduction. This visual aid may be easier for staff to follow and when training staff.

The obvious risk from a tank failure is to the cryo-stored samples which may be lost if they thaw to room temperature. Tanks can fail due to a breach of the vacuum seal, or a mal-

Table 36.2 Checklist for risk management of cryo-storage

Frequency of QC	Action
Daily	Physically check tanks for frosting, icing, water condensation, pooling, cracks around tank neck
Weekly	Measure LN_2 level 3 times and fill tanks 1 time
Monthly	Review daily and weekly QC for possible accelerated evaporation over time indicating possible tank retirement
Monthly or quarterly	Test alarm and response phone tree
Bi-annual or annual	Test generator with load including automatic transfer switch

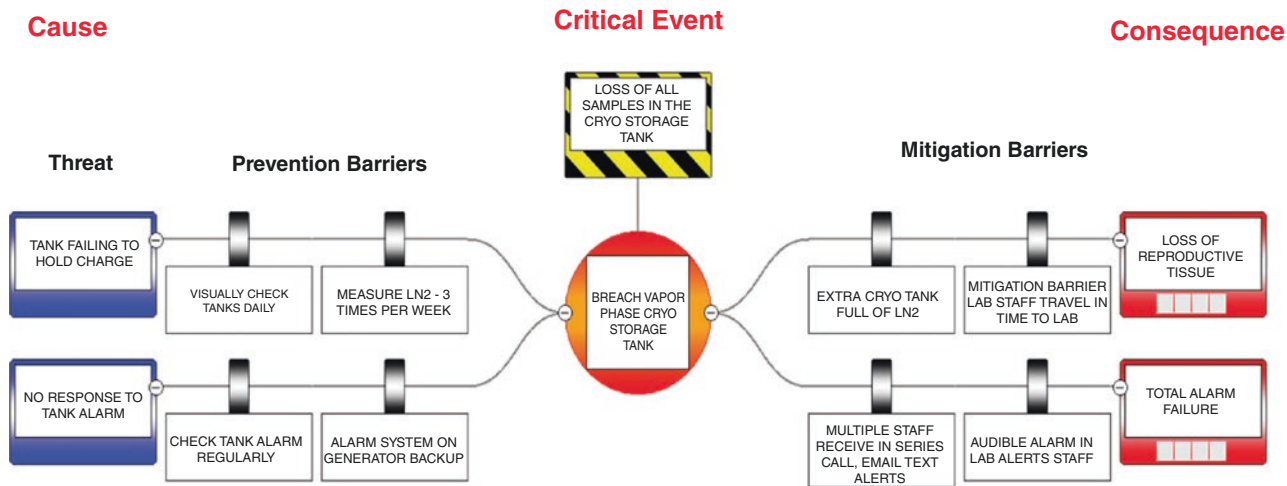


Fig. 36.1 Loss of samples in Cryo storage tank

function of an auto-fill solenoid or sensor. If a tank fails, the alarm should alert personnel but an alarm may malfunction due to power outage or unrecognized issues due to failure of testing. Tissues may be lost if laboratory personnel are not able to travel to the laboratory to rehome the samples to another tank, or if there is no other tank with available liquid nitrogen and space for rescued samples. Laboratory personnel may also be exposed to risk by inadequate personal protective equipment such as gloves, face shield, eye goggles, and shielding of skin. The tank storage room may not be well-ventilated or have an oxygen monitor to ensure all personnel are alerted if oxygen levels fall dangerously. Personnel that are inadequately trained or are not evaluated annually for competency may introduce risk. Figure 36.1 depicts a bowtie diagram that assesses the risk of a catastrophic tank failure in the IVF laboratory.

Several possible causes or threats to a catastrophic tank failure have been identified such as a tank failing to hold a vacuum or no response to an alarm. Prevention barriers in place include visual checks of the tanks daily, measuring liquid nitrogen several times per week, alarm system checks, and generator backup. The critical event or hazard is a breach in the liquid or vapor phase of the cryo-storage tank. The consequences of a tank breach are a loss of all reproductive tissues in the tank.

Risk reduction or mitigation barriers of such a catastrophic consequence include such items as having an extra cryo-tank on-site full of liquid nitrogen, setting up the alarm at a low enough temperature allowing personnel to travel to the laboratory in time to rescue reproductive tissues, setting up the alarm to contact multiple lab personnel by email, phone call, and text. Also recommended is a hardwired system vs. wireless to ensure the alarm can call out if the internet goes down.

If continuous alarm monitoring is in place, it is recommended that the web portal with all equipment listed includ-

Table 36.3 Pearls and perils of cryo-tank maintenance

Pearls	Perils
Check tanks daily	Don't turn off the alarm
Measure tanks 3× per week or continuously	Don't ignore red flags during daily tank check
Have continuous alarm 24/7	Don't forget to update alarm response list
Test alarm monthly or quarterly	Don't miss alarm testing
Have capability of backup LN2 tank	Beware of alarm fatigue
Have a disaster preparedness plan	

ing cryo-tanks be accessed daily by lab personnel and weekly by lab director to ensure all probes are online, temperatures of tanks are within acceptable limits and the system is operational. Table 36.3 provides a quick reference of dos and don'ts for cryo-tank maintenance.

36.7 Conclusion

Cryopreservation of reproductive tissues is central to the IVF patient's treatment. Cryo-storage in liquid or vapor of human reproductive tissues involves risk of tank failure and inadvertent thaw to room temperature. Alarm systems mitigate risk of undetected tank failure but catastrophic events are still possible. Managing cryopreserved reproductive tissues in an IVF laboratory requires skilled personnel and specialized equipment. This document can be used by laboratories as guidance for developing clinic specific tissue storage and management policies and procedures. Any tank that exhibits a loss of LN₂ that is faster than expected should be replaced as soon as possible. However, it is understood that adherence to even the most rigorous policies and procedures cannot prevent a rare catastrophic event from occurring.

36.8 Authors' Perspectives

Complicating cryo-storage of reproductive tissues are a number of issues facing IVF labs today. Insurance coverage for IVF programs that store reproductive tissues on-site may involve higher premiums or even loss of coverage due to prohibitive risks of tank failure. Gaps in the disposition process of reproductive tissues may include loss of contact with patients, unclaimed tissues, or breach of contract regarding discard of cryopreserved tissues due to potential legal ramifications, which can lead to semi-permanent cryo-storage adding to the burden on the laboratory personnel for maintaining tanks. Shipping cryopreserved tissues off-site to a commercial biorepository is an option but this involves risks in transportation, extra costs in labor and in shipping. Supply chain issues and the increased demand for new storage tanks by busy IVF laboratories can lead to shortages and stresses on already overburdened on-site biorepositories which are rapidly running out of storage space.

Key Points

- Policies and procedures for safe cryo-storage of human reproductive tissues is guided by best practices in the literature and requirements by accrediting agencies.
- Cryo-storage in liquid nitrogen tanks on-site in IVF laboratories is standard of care though lack of space and tank failure remain concerns.
- State and local laws should be followed in addition to each IVF program policy regarding legal ramifications for cryo-storage and disposition of cryopreserved tissues.

36.9 Potential Areas of Research

Automated platforms have been created recently to manage, identify, and store cryopreserved reproductive tissues. Artificial intelligence and semi-automated systems may become more commonplace in the IVF laboratory. Noncryogenic preservation by desiccation and storage at ambient temperatures is being developed for human embryos which will mitigate the risks that come from cryo-storage in liquid or vapor nitrogen [15].

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Transportation of Frozen Gametes and Embryos: Good Practice Guidelines

37

Don Fish

37.1 Background

Tissue Specimen Logistics may be the least thought about area of focus when it comes to risk mitigation in the fertility industry yet is a fundamental necessity of the daily workload with inherent associated risks. The use of technology combined with well-thought-through policies, procedures, and training of staff are all key ingredients to managing your tissue's logistics.

37.2 Technology

It is prudent to note that technology evolves very quickly and some of that mentioned herein is likely to be superseded during ensuing months/years to solve problems more adequately. That being said, there are some critical requirements that should be used for tissue specimen logistical operations regardless of the age and era we are in.

- *Adequate shipping container* capable of maintaining temperature colder than -150°C . The most widely used piece of equipment for this is called a dry shipper. A dry shipper allows cryogenically preserved tissue to be transferred at appropriate temperatures well within the typical transport window, and without being classified as dangerous goods.
- *Temperature Monitoring device* capable of measuring, recording, and reporting the temperature throughout the life cycle of the shipment. There are several options on the market for performing this function with the best devices showing “Near Real-Time Data” and up to the minute reporting with notification alerts when there is a deviation from the defined acceptable ranges.

- *GPS* (global positioning system) devices may be used to track the location of the shipper as a redundant function in case carrier tracking systems encounter errors, loss of package, separation of dry shipper from the package itself, or incorrect delivery location. There are several options on the market for performing this function with the best devices showing “Near Real-Time data” and up to the minute reporting, route deviation notifications, long battery life (some rechargeable), and are built into the same device with temperature monitoring.
- *LIMS* (*Lab information management system*) should be used for a variety of reasons and should include current and historical data on the location of each tissue specimen over time, tissue device IDs and serial numbers (including clerical corrections), and the condition on how a tissue is received or shipped.
- Electronic scanning devices such as barcodes and RFID should be applied to each individual storage device (vial, straw, goblet, and such) to allow for quick and redundant verification. While barcodes and RFID technologies have been around for decades, some manufacturers have already begun integrating RFID into the plastics of vials and soon to be egg/embryo storage devices/straws. RFID allows for scanning to be performed much easier than small barcodes and allows the reading of multiple devices (hundreds and/or thousands) of devices to be scanned in just a few seconds. Because of the speed and ease of RFID, it is a promising technology in the fertility field.

37.3 Equipment Validation

Like any piece of critical equipment, each item listed above should be qualified via installation qualification, operational qualification, and performance qualification in accordance with the regulatory requirements and manufacturer's instructions.

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Additional validation of the equipment should be performed at routine intervals (at least annually) with more regular quality control inspections (daily, weekly, bi-weekly, monthly, etc.).

As part of the validation and ongoing QC process, trending reports should be utilized with defined acceptable ranges to identify and address potential issues before they become catastrophic failures.

As an example, dry shippers should undergo initial validation to ensure they meet the hold time defined by the manufacturer and show no signs/symptoms of vacuum failure. The shippers should also undergo re-validation at least annually, as well as every time there is a suspected issue with the shipper (signs/symptoms of failure or does not meet fully charged weight after being charged for 24 h). Every time there is a validation, the hold time should be recorded and a trending report on the dry shipper should be created. This trending should be reviewed, and appropriate action should be taken to allow the shipper to be placed back into active use or to take out of use permanently.

37.4 Shipping

The key steps in shipping a tissue is outlined below with each section being critical in the safe transport of tissue to its new location.

37.4.1 Receiving a Request for Transfer

When a patient reaches out to request a transfer of tissue, it is important to gather key information. It is recommended that each clinic establish a Request for Tissue Transfer form and associated SOP to outline all information needed from the patient and to begin and approve the process. At a minimum, the request form should provide the following information.

- Patient Name
- Patient Date of Birth
- Type of tissue stored
- What type of tissue is being requested to transfer
- Number of tissue devices to transfer (patient may choose to ship some and store some)
- Where the tissue is being transferred to
 - Clinic Name
 - Clinic Address
 - Phone Number
 - Contact Person Name, Phone number, and email address
- Signature of the patient requesting the transfer
- Date of Request and Signature

- As applicable, the patient's partner information may also be required.

37.4.2 Internal Document Compiling and Completion

Upon receipt of the patient's transfer request, there are several documents that may require review and approval prior to the release of tissue. These may include, but are not limited to the following:

- Chart and Lab results review
- Tissue Authorization Release form
- Consent forms
- Account Payment Status updates and approval forms
- Additional required forms that are specific to the clinic operational policies and procedures

37.4.3 Review and Approval of Transfer Shipping Request

It is important that each of these documents (as applicable) be compiled, reviewed, and approved by appropriate staff before the tissue is released for shipment to another clinic. All reviews and approvals should be appropriately documented on the individual forms and/or in the patient chart as applicable.

37.4.4 Coordinating Shipment with Other Clinics

The clinic that your patient has requested a transfer to likely has to perform all or many of the same documentation review and approvals as described above. Therefore, it is critical that you coordinate with the receiving clinic to ensure they are prepared to receive the tissue before shipping. A list of items that should be provided are covered in the "Receiving" section below.

It should also be noted that many clinics can only ship or receive shipments on specific days, often due to staffing. It is important that one coordinate to ensure the receiving clinic are available to receive on the specific day/time the shipment is expected to arrive, and subsequent days should there be a delay in shipping.

37.4.5 Order Creation

Once all the information and documentation has been reviewed and approved, it is recommended that a Shipping

Order Document be created (either physical or electronic) to adequately provide critical information to the person(s) physically preparing the tissue for shipment.

37.4.6 Review Shipping Order

When receiving a shipping order, one should review it for completeness prior to preparing the order. Some things that are essential to each shipping order and thus needed to review and consider are as follows:

- Patient Name
- Type of Tissue being shipped
- Shipping Date
- Shipping Service (which courier is being used for a specific purpose and location)
- Shipping Address with contact information (Name, email, and phone number)
- Ship-from Address with contact information (Name, email, and phone number)
- List of item/serial numbers (or other applicable identifying marks) to be shipped
- The temperature requirement condition that items should be shipped in (-20°C , -40°C , -80°C , -137°C , or colder)
- The brand name of the business shipment should be distributed under (businesses often ship under multiple brands)

Any errors or values not populated should be addressed and resolved prior to shipping the tissue package.

37.4.7 Selecting an Appropriate Shipping Container

Dry shippers come in various shapes and sizes to accommodate large numbers of tissue devices (typically with shorter lengths of refrigeration hold time) or a small number of tissue devices (typically with larger lengths of refrigeration hold time). It is important to properly define policies and procedures to indicate which dry shippers (or other various shipping containers) should be used for specific purposes based on the number of tissue devices being shipped, the value of the tissue, and the length of time needed to deliver to the receiving facility.

Best practice: Move small numbers of tissue devices at one time with the maximum refrigeration hold time available and ship with the fastest delivery time available to mitigate risk. Additionally, when shipping the last tissue devices that a patient has, ship in separate packages on a different date/time.

When selecting a dry shipper, ensure that the shipper has been properly “Charged” with liquid nitrogen according to manufacturer’s guidelines. In most cases, this requires filling the dry shipper with liquid nitrogen until the liquid is somewhat stable and is not boiling off in a rapid and volatile state. Once filled, the dry shipper should remain with liquid nitrogen in it for at least 24 h prior to shipping.

In some cases, manufacturers have provided shorter charging times of 2 h when proper conditions exist. Be sure to verify the manufacturer’s guidelines on the appropriate charging time and adjust your procedures and facility practices accordingly.

37.4.8 Equipment QC

Dry shippers (and any shipping container) should undergo quality control verification to ensure the shipper is functioning properly and is in adequate condition to ship.

Prior to performing dry shipper QC functions, pour the liquid nitrogen out of the dry shipper. To ensure the majority of liquid nitrogen is removed, completely invert the shipper, and allow the liquid to drain for 1 min or until the liquid is removed. If the liquid nitrogen is not removed thoroughly, QC readings may result in false data indicating a dry shipper is fully charged, when in fact it may not be.

Perform these QC functions before shipping.

- Inspect the dry shipper for obvious damage and ensure there are no signs or symptoms of vacuum loss. Signs and symptoms of vacuum loss may include the following.
 - Excessive condensation on the outside of the shipper when it is not being filled with liquid nitrogen. This may also show up as a puddle of water on the ground at the bottom of the shipper after some time has passed.
 - Frosting or ice build-up on the shipper in any location.
 - Leaking of liquid nitrogen when the shipper is sitting upright.
 - Hissing sounds that may indicate a leak in the vacuum of the container.
- Weigh the dry shipper to ensure it meets manufacturer’s guidelines for being “Fully Charged.”
 - Manufacturer’s guidelines typically provide the weight of a fully charged dry shipper for relative comparison.

If the dry shipper shows any signs/symptoms of vacuum loss or does not pass the acceptable ranges defined by the manufacturer as being fully charged, do not use that specific dry shipper for shipping. Place it in a segregated area to undergo re-validation and approval before it can be placed back in use.

Then select a different dry shipper and go back through the same process of selecting a dry shipper and the ensuring QC process.

If you are using temperature dataloggers and/or GPS functions, perform the following QC prior to shipping.

- Verify that the unit turns on and is communicating information properly.
- Check the battery life on the unit so that it will last the entire shipment journey.
- Check the function and communications of the device.
 - If it is a GPS device, make sure a GPS reading occurs before shipping.
 - If it is a temperature monitoring device, make sure that the temperature is reading.

Best Practice: Create policies and procedures based on manufacturer's guidelines to outline the requirements of the QC of acceptable ranges for staff to readily identify and verify when performing these functions.

37.4.9 Tissue Device Verification

The one single thing that you absolutely cannot do in the fertility industry is ship the incorrect tissue device. Even though it may be understandable for humans to make these types of errors, it is simply not acceptable as a function of business in the fertility industry. Therefore, businesses must make extraordinary efforts in defining policies/procedures, training staff routinely on those procedures, and creating multiple redundancies in the verification process to reduce such occurrences to zero.

The best solution for verifying tissue devices for distribution include multiple human blind verification steps combined with electronic verification via scanning. Human minds, regardless of staff competency, will misinterpret alpha-numeric sequences, so enhancements of that process via the use of electronic scanning can be extremely useful in preventing these errors.

Therefore, the minimum verification should consist of a two-step/two-person blind verification of the label serial number and/or other distinct labeling system. All verification of tissue devices should take place at cryogenic temperatures colder than -150°C and submerged in liquid nitrogen whenever possible to ensure viability of each tissue is not compromised during this process.

Best Practice: Two-step physical verification combined with two-step electronic verification

- First Physical Verification
 - Staff member #1 should use the shipping order to locate the tissue and physically verify the labeling on the device to ensure 100% accuracy.

- First Electronic Scan
 - Staff member #1 should utilize an electronic scanner (barcode, RFID, or other device) to scan the storage device ID (barcode, RFID, or other) into the LIMS systems.
 - The LIMS system should reference the order item and determine if the correct tissue item was picked. If so, the system will allow the staff to move forward. If not, the system will alert the staff of the issue, and will require additional resolution before proceeding.
- Second Physical Verification (completely blind verification)
 - Staff member #2 will verify the tissue independently of knowing what staff member #1 picked. They will verify the labeling on the device to ensure 100% accuracy with the order placed.
- Second Electronic Scan
 - Staff member #2 should utilize an electronic scanner (barcode, RFID, or other device) to scan the storage device ID (barcode, RFID, or other) into the LIMS systems.
 - The LIMS system should reference the order item and determine if the correct tissue item was picked. If so, the system will allow the staff to move forward. If not, the system will alert the staff of the issue, and will require additional resolution before proceeding.

37.4.10 Transferring Tissue

When transferring reproductive tissue to a dry shipper, it is important to ensure that tissue devices are transferred with great care and in respect to ambient temperature exposure.

For sperm and testicular tissue, which is more robust, exposure to ambient temperature should be minimized and transferred to the dry shipper as quickly as possible, typically less than 2–3 s. The lid of the dry shipper should be immediately placed into the shipper upon transfer of the tissue.

For egg and embryos, any exposure to temperatures warmer than -150°C can have catastrophic consequences causing micro-thawing subsequent damage to the tissue. When transferring these types of tissues (and dependent on the device being used), ensure that tissues are transferred while submerged in liquid nitrogen. For devices that are stored in a goblet, this means that the lower portion of the goblet should contain liquid nitrogen before transferring. Then followed by the lid of the dry shipper being placed immediately into the shipper.

Once the lid is put into place, secure the lid to the shipper with a zip tie or other locking mechanism that is easily removed when the shipper is delivered to the recipient.

Best Practice: As technology allows, all reproductive tissue should never be exposed to temperatures warmer than -150°C .

37.4.11 Selecting a Courier

The type of courier you choose when shipping reproductive tissue is essential to a successful transfer to ensure the tissue is viable when it is received at the other clinic.

When to use a common courier (USPS, FedEx, and UPS type of service)?

It is recommended that a common courier be used ONLY when transferring reproductive tissue that is easily replaced and of low value.

Example of such tissue include but are not limited to the following.

- Donor Sperm provided from a commercial cryobank that is easily replaceable at very minimal cost
- Research Tissue (Sperm, Eggs, Embryos) that have no commercial or sentimental value

Common couriers are typically used for a cost-efficient method of quickly transferring packages domestic and internationally. These courier methods are readily available with multiple physical locations and easily accessible websites to schedule and ship packages with relative ease.

However, the ease of use and cost-effectiveness is routinely offset by the risk of quality in using this type of courier. It is not uncommon to experience mishandling, shipping delays, lost packages, and damaged packages when using this type of courier. Great caution should be taken when choosing to use such a courier when shipping precious reproductive tissue.

When to use a Dedicated Medical Courier Service (Airspace, MNX, Quick International, etc.)?

It is recommended that a Dedicated Medical Courier Service be used when transferring reproductive tissue that is NOT easily replaced and of high value.

Example of such tissue include but are not limited to the following.

- Client Depositor Sperm, Eggs, and Embryos that are not easily replaceable and because of their high value.
- Donor Eggs, Blastocyst, and Embryos because of their high value and effort to create may not provide for easy replaceability.
- Donor sperm obtained from a cryobank that is sold out and is not replaceable.

37.4.12 Package Labeling and Inserts

Ensure the shipping label printed (or was handwritten) correctly by reviewing the ship-to address/contact information, the ship-from address/contact information, the service type and delivery date, the weight of the package, etc. before applying to the package and sealing it for shipment.

Also ensure that lab results and other critically required chart information are included in the shipment.

37.4.13 Tracking Until Delivery

Tracking the delivery to the end user is extremely critical as packages are often re-routed, lost, and/or have missed scans of the package throughout the shipping process. It is important to track the shipment at least daily and at the scheduled delivery time to verify the delivery is on track. If there are any delays, follow-up with the courier to determine the appropriate resolution in locating and delivering the package. If you have included a GPS and temperature monitoring device, verify the GPS location and temperature at least daily. Some systems will allow you to set up alerts and notifications, both of which are greatly beneficial and allow for proactive responses and risk mitigation steps to be taken.

In addition to the above stated, documentation of all tasks may likely be the most beneficial to you. Remember, "If you didn't document it, you didn't do it." Having the proper documentation available for reference and auditing is an essential part of any business and especially so in the fertility industry.

37.5 Receiving

The key steps in receiving a tissue package are outlined below with each section being critical in adequately documenting the condition of the tissue upon arrival and when it was placed into long-term storage at your location.

37.5.1 Approval to Transfer Tissue

Prior to receiving an order, it is best practice to get all pertinent information regarding the tissue and the patient before agreeing to transfer to your facility. The following items should be reviewed and verified to meet your company's storage requirements.

- Patient name and contact information
- Type of tissues for transfer

- # of tissue devices to be stored (# of vials, straws, goblets, canes, etc.)
- Type of devices used (vials, straws, goblet, cane, etc.)
- ID information and numbers listed on the goblet, cane, vial, straw, etc.
- Freeze reports
- Infectious disease testing results
- Other applicable lab reports

37.5.2 Coordinate Shipment

Once the approval to transfer tissues have been provided, the coordination of the transfer must be defined. Who will provide the dry shipper and package? What courier will be used? What are the patient's expectations for the transfer? What date/time works best for both transferring facilities? All of these are questions that need to be answered prior to shipping.

37.5.3 Upon Receipt

Once you receive the shipper with reproductive tissue, the following items should be performed and documented.

- Verify and document the condition of the package, the tracking number, the courier ID, and such for future reference.
- Verify and record if the dry shipper was received in a secure manner with a zip tie or other locking mechanism that secures the lid to the shipper. If a locking mechanism was received, remove from the dry shipper, and proceed with the following steps:
- Verify and record the temperature of the internal dry shipper.
 - This can be performed with a datalogger that is attached to the device (if it has a digital display showing the temperature) or by using any NIST certified thermometer with a probe capable of reading down to -200°C .
 - If the temperature is measured to be warmer than -150°C , notify a manager and potentially a medical director before performing the next steps (or follow standard operating procedures).

If a tissue device is completely thawed, a medical director may opt for additional testing prior to re-freezing.

If a tissue device is colder than -130°C , a medical director (or SOPs) may provide guidance to continue with the normal practice and simply report this issue.

Likewise, if the temperature is recorded at any value warmer than -150°C , follow-up evaluation and documentation may be needed in a variety of ways specific to the policies and procedures of a business entity.

- Verify and document the physical condition of the shipper for obvious damage and/or signs/symptoms of vacuum loss.
 - If there are any signs/symptoms of vacuum failure document those conditions, quickly move the tissues in accordance with the following steps and notify a manager for next steps in evaluating the received tissue.
- Fill the shipper with LN2 (expel gas in the fill hose or use a secondary container to fill first... and then fill the shipper). Important: Filling the dry shipper without first expelling the warm gas inside the hose will expose the tissues to temperature warmer than -150°C and may cause detrimental harm.
- Transfer tissues into an appropriate cryogenic container with LN2 to submerge the tissues in accordance with "Transferring Tissues" section above regarding the proper handling during the transfer process.
- Verify tissues (two-person verification with electronic verification as well) and check against inventory list in accordance with the guidance provided in the section "Tissue Verification" above.
 - Make special note of any inaccuracies on the cane ID, Goblet ID, or other device ID (Vial, straw, etc.) and record/report those issues at your earliest convenience for follow-up and inventory reconciliation.
- Transfer into long-term storage tank (Fully validated and monitored to store tissues)
 - Follow guidance provided in section "Transferring Tissues" above.
- Update the manual log and electronic database with tissue ID, location, and any pertinent lab information accompanying the package.

37.6 Storage Tracking in Long-Term Storage

Tissues are moved throughout time to various locations commonly due to status changes, infectious disease, and type of tissues (quarantine, infectious, non-infectious, donor tissue, patient tissue, and such). It is important to keep track of these locations via manual and/or validated electronic system as a reference and for future auditing.

37.7 Commercial IVF Shipping Companies (IVF CRYO, Cryoport, etc.)

As demand for IVF continues to grow exponentially and access to care increases, ancillary services such as IVF shipping solutions offer great options to fertility clinics to reduce workload, reduce cost, and mitigate risk.

These companies typically focus on the shipping aspect and are true experts in the field. They may work directly with patients or clinics and provide a full service to include the following:

- Coordination with the patient.
- Coordination with fertility clinics to ensure all documentation is in place before shipping.
- Coordination with fertility clinics on the days available to pick-up/receive.
- Provide a full shipping solution that includes a dry shipper, attached shipping labels, temperature monitoring,

GPS, and complete logistics follow-up and problem resolution.

The greatest benefits of using one of these companies are described as follows:

- Reduce legal liability by outsourcing the risky business of shipping reproductive tissue to a third party
- Provide a great shipping service to the patient
- Reduce the workload of embryologists which allows them to focus on fertility treatments instead of shipping services

Given the inherent risk with shipping reproductive tissue, the expertise needed, and the time/cost of performing these tasks to the high standard required for success, it is strongly recommended that fertility clinics consider the use of commercial shipping companies.

The Importance of Ovarian Tissue Transportation to Reproduction Outcomes

38

Janice de M. V. Vilela, Ellen C. R. Leonel,
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38.1 Introduction

Ovarian tissue cryopreservation and transplantation (OTCT) is now considered an established procedure for fertility preservation [1]. Up to now, more than 200 live births have been described after OTCT application [2, 3]. However, cryopreservation requires specialized equipment, well-trained personnel, and a specifically designated space to manipulate and freeze the ovarian cortex. In this sense, only large hospitals can maintain these facilities. One way to increase access to ovarian tissue cryopreservation is by improving transportation, allowing small clinics and less developed countries to offer this service to their patients. Therefore, the ovaries can be removed in any location and sent to facilities where they will be cryopreserved and stored until it is time for transplantation [4–6].

However, transportation of ovarian tissue (OTT) is not a simple process. The ovary is a complex organ with different cells and structures essential for the survival and growth of the follicles. Currently, media used for ovarian tissue transportation, for instance, alpha-minimum essential medium (α -MEM) [7], Leibovitz-15 (L-15) [8], IVF medium [9], HEPES-MEM [10], or Custodiol® [6], were developed with different intentions or designed for other organs. There are only a few studies regarding OTT parameters, and no standard protocol has been developed yet. Thus, it is vital to better understand how different parameters affect the ovary

so we can tailor a specific protocol and improve fertility results.

38.2 What Do We Know?

There are not many studies regarding OTT, especially in human tissue. Previous studies have confirmed that transportation of human ovarian tissue from the surgery room to the cryobank may be adequately performed, avoiding loss of reproductive functionality [9, 11]. Based on that, in some countries, groups of physicians and researchers have organized transportation networks. FertiPROTEKT project is an association of German surgical centers that work together with a central cryobank that receives, prepares, and stores ovarian tissue until transplantation is required [12]. They validated Custodiol®, a medium for transportation of abdominal organs, for up to 22 h of OTT. Similarly, the Danish fertility network has described a transportation model in which “the patient stays, and the tissue moves” which, according to the group, considerably benefits the patients. Such a model consists of establishing a network in which the excised tissue is transported at a low temperature for up to 24 h. This group states that at least transplantation to 80 patients after transportation and cryopreservation of tissue was performed, and, in all patients, the ovarian function was reestablished, confirming the efficiency of such a model [13]. These networks are constantly evolving and growing throughout the world. However, the methods for OTT vary from center to center, and there are not many reports on which medium was used.

A systematic review analyzed data from 46 different studies, from humans and animals, to understand how temperature, media composition, and duration of transportation affect the ovary [14]. There were only four studies evaluating OTT in human ovarian tissue against 42 in nine different animal species. Understanding the effects of OTT in these species (Table 38.1) may provide important insight for humans as well. A summary of the main outcomes from these studies is shown in Fig. 38.1.

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Table 38.1 Best conditions for humans and animals from transportation studies

	Best time								Best medium								Best temperature with minimal damage								Total
	4 h	6 h	7 h	12 h	18 h	24 h	26 h	48 h	MEM+	Saline solution	Coconut water	ACP medium	TL-Hepes	PBS	Use of antiapoptotic drugs	Use of additives	4 °C	10 °C	15 °C	20 °C	25 °C	37 °C	38 °C		
Human					1	1	1										1							4	
Cow	1				1	2										1	1	1	2	1				6	
Cat		1		1		4		1								1	1							7	
Goat	1	1		2		2				1	1			1		1	6							6	
Sheep		2				2			1						1	2	1							5	
Dog				1		1			1			1	1				3					1	1	6	
Horse	1	1	1			1							1			1	1					1		5	
Pig		1		1	1											2					2			4	
Pecari tajacu						1						1												1	
Mouse	1												1			1								1	
Iberian red deer																					1			1	
Total	4	6	1	5	3	14	1	1	2	1	1	2	1	3	1	5	17	1	2	1	3	2	1	46	

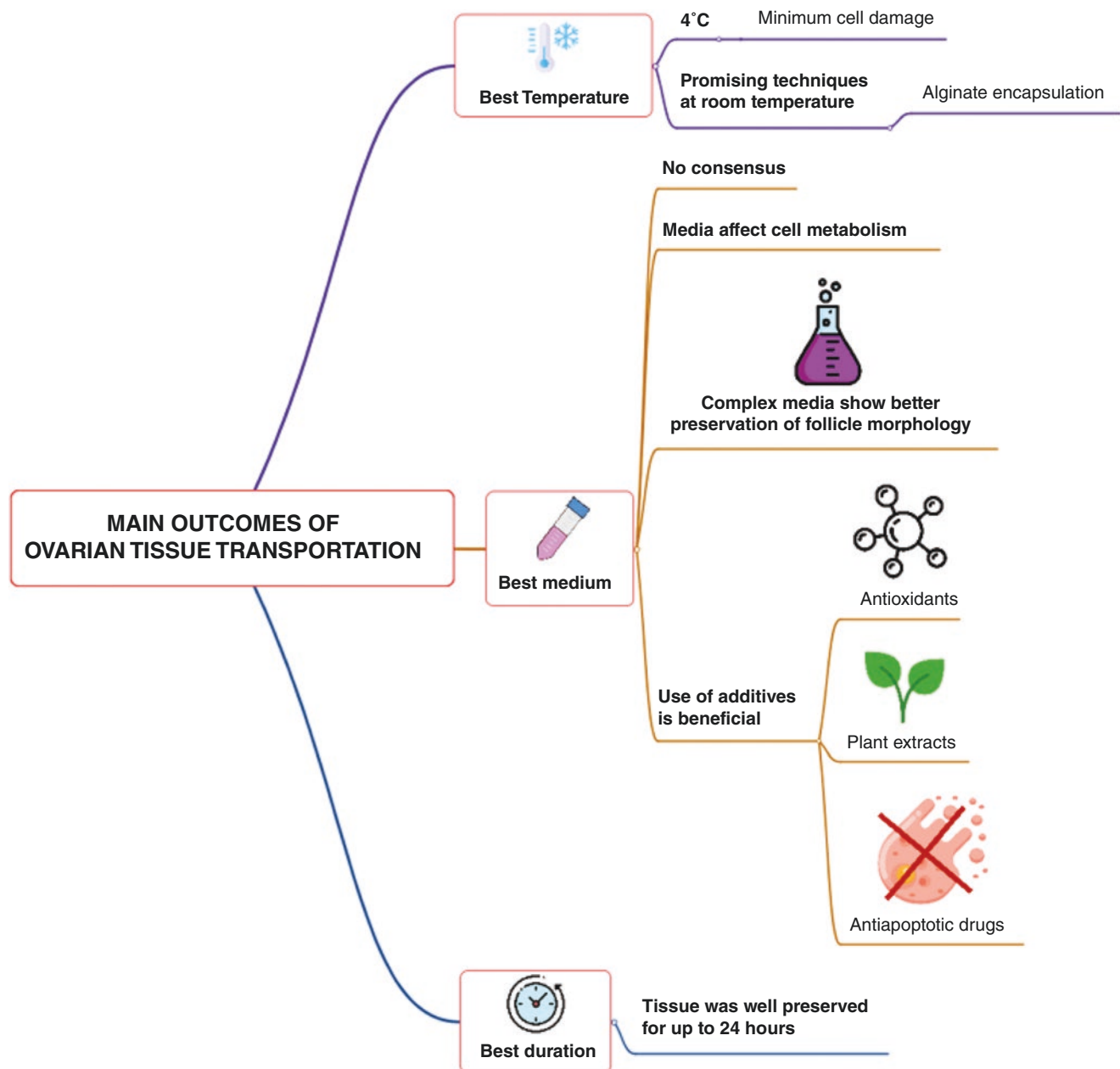


Fig. 38.1 Main outcomes of ovarian tissue transportation from different studies. (Icons from [flaticon.com](https://www.flaticon.com/). Mind map was created using EasyMapper software)

38.2.1 Studies on Human Ovaries

38.2.1.1 Effects on Ovarian Tissue

The four studies evaluating human OTT analyzed the effect of duration or temperature of transportation. In some cases, analysis of tissue morphology after transportation did not reveal any morphological damage. Use of Brama I medium at 4 °C after 26 h transportation [11] or L-15 [15] at the same temperature for 24 h did not reveal any differences in follicle morphology between transported and fresh tissue. Another study described an increase in follicle viability of cultured

tissue pieces previously cooled at 5 °C in L-15 for a 24 h period in comparison to cultured fresh samples [16]. Signs of oxidative stress, estradiol decrease, and increased malondialdehyde secretion have been described after transportation in Celsior medium at 4 °C for 24 or 48 h followed by vitrification and in vitro culture [17].

These studies in human OTT showed that the ovarian tissue could be safely transported at 4 °C for up to 24 h. However, none of the media in these studies are used in clinical practice preceding cryopreservation, highlighting the value of this subject for future research [14]. A more recent

study validated OTT of premenopausal women at 4 °C using Custodiol®. They analyzed follicle morphology and survival, as well as apoptosis, oxidative stress, glucose uptake, and anti-Müllerian hormone (AMH) production in 2 mm biopsies after 4 days of transport, cryopreservation, and 4-day in vitro culture and observed an increased cell viability in the tissue after thawing, showing no damages to human ovarian tissue transported for up to 48 h [18]. More studies are needed to understand and improve transportation techniques.

38.2.1.2 Effects on Oocyte Maturation and Fertilization and Embryo Production

Recently, the impacts of transportation medium and conditions on human oocyte maturation rates have been assessed. Human ovarian tissue transported in L-15 medium did not show significant differences in the rates of oocyte maturation nor in that of pronuclear stage embryos obtained after the tissue was stored for 15 min at 37 or 1–3 h at 4 °C [19]. Similar results were described when tissue was transported in by IVF-flushing medium for 10 min at 37 °C, 3 or 5 h at 4 °C, showing similar oocyte maturation rates between the groups [20].

Another study described an increase in the pH of the HEPES medium in comparison to the histidine after 4–5 h of transportation at 4 °C, which did not impact the maturation ability of the retrieved oocytes since maturation rates were similar after applying both media [21]. Contrarily to these results, the oocyte maturation ability obtained from human ovarian tissue transported for 5–6 h at 4 °C in IVF-flushing medium and for 10 min at 37 °C in α -MEM was negatively impacted [22].

While these studies report remarkable insights on OTT, evaluating follicles using in vitro culture or oocyte maturation may lead to bias, as this process does not support the interactions between cells and with the extracellular matrix, which are essential for follicle growth and development [23, 24]. Investigating transportation protocols by techniques that resemble the physiological environment of ovarian tissue will provide us with more reliable information on understanding how this process affects fertility, hence the importance of performing transplantation of cryopreserved ovarian tissue after OTT as a form of OTT assessment.

38.2.2 Studies on Animal Ovaries

38.2.2.1 Effects on Ovarian Tissue

Bovine ovarian tissue has shown maintenance of morphologically normal follicles compared to fresh tissue, after 18 h of transportation at 4 °C or for 6 h at 20 °C [25]. Studies in ovine ovarian tissue were the first to confirm that smaller

pieces have a better chance of preservation of follicle morphology than whole or half ovaries [26], also showing a higher capacity of in vitro growth after a 24 h of cold storage. MEM supplemented with insulin–transferrin–selenium, hypoxanthine, glutamine, bovine serum albumin, and FSH was reported as a good alternative to preserve sheep ovarian tissue for up to 6 h at 35 °C [27]. Interestingly, the use of extracts from plants, such as black mulberry and timber tree, showed an increase in the percentage of morphologically normal follicles and lower percentage of DNA fragmentation in sheep [28] and goat [29] ovaries.

Cat ovarian tissue has been stored for up to 48 h in Dulbecco's PBS at 4 °C, showing significantly increased rates of granulosa cell DNA degradation already at 12 h [30]. Another study revealed that no taphonomic alterations were present when compared to fresh tissue [31]. More recently, another group stored cat ovarian tissue for 24 or 72 h in MEM at 4 °C before vitrification. When comparing cooled to fresh tissue, the authors encountered similar viability rates, as demonstrated by neutral red staining. Still, mitochondria, plasma, and basal membrane integrity were preserved only in the fresh and 24 h stored follicles, but not for 72 h [32].

In dogs, ovarian fragments stored for 24 h at 10 °C in a coconut water-based medium showed better preservation of follicles morphology than those stored in PBS [33]. However, the transportation of canine ovarian fragments in MEM for 12 h at 4 or 20 °C did not impact follicle morphology, as revealed by histological analysis [34].

In equine ovarian tissue, transportation of fragments at 4 °C for 4 h in PBS yielded a higher rate of morphologically normal follicles than in MEM [35]. Another study comparing the impacts of transportation of small fragments (2 × 2 × 12 mm) and whole ovaries revealed that fragmentation allows better preservation of follicle morphology after 24 h of storage in α -MEM at 4 °C [36].

In mice, Kamoshita et al. [37] described the first report of reduced fertility after ovarian tissue storage followed by transplantation. Curiously, despite no significant morphological differences observed between stored (Dulbecco's modified Eagle's medium) and fresh follicles, the rates of embryo implantation and live births reduced after 8 h storage of the tissue at 4 °C. This finding highlights the importance of applying not only morphological analysis when evaluating the impacts of different transport protocols but being the reestablishment of tissue functionality as the definitive landmark.

In summary, animal studies confirmed that OTT at low temperatures reduces follicle damage. However, transportation at low temperatures involves specific shipping conditions that add challenges and increase the cost of tissue shipping. In this sense, new techniques have been studied to develop a protocol that allows the tissue to be stored at room

temperature (RT). Atelerix®, a new preservation solution that encapsulates cells and tissues in alginate, has been recently validated for storage of sheep ovaries for up to 24 h at RT, maintaining the viability of follicles similar to tissue stored at 4 °C [38].

38.2.2.2 Effects on Oocyte Maturation and Fertilization and Embryo Production

In bovine ovarian tissue, applying PBS, saline, or Ringer's solution suggested that better preservation, in either medium, occurs at 20 °C for up to 5 h, but storage between 10 and 15 °C for 24 h provided higher rates of maturation and blastocyst formation [39–41]. Another study showed improvement in blastocyst formation with the addition of epigallocatechin gallate and glutathione in the storage medium [39].

The addition of melatonin to a storage medium composed of PBS improved ovine embryo quality after ovaries were maintained at 4 °C for 24 h [42]. Follicles isolated from goat ovarian tissue stored at 4 °C for 4 h in a HEPES-buffered MEM medium showed higher meiotic resumption rates and higher early antral follicle diameters than those held at 33 °C after 18 days of *in vitro* culture [43].

From three studies evaluating oocyte meiotic competence in cats [44–46], one described a higher rate of meiosis resumption when storage was carried out at 4 °C, in comparison to 38 °C [45]. After 48 h at 4 °C, decreased MII rates were a consensus among all authors [44–46]. The viability of cumulus–oocyte complexes can still be increased with superoxide dismutase, which also improves embryo production, increases expression of Bcl-2, and reduces the expression of Bax, an apoptotic regulator [47]. While a previous study suggests that dog ovarian tissue shows higher maturation rates when transported at 4 °C [48], other authors suggest it is more sensitive to low temperatures [49, 50]. Still, the estrous phase may impact transportation more than temperature, as shown by higher maturation rates of follicles collected from ovaries obtained in the follicular or luteal phases in comparison to anestrus ovaries in the same transportation conditions [51].

In swine ovarian tissue, studies suggest storage should be performed for a short time (<6 h) at 25–35 °C since oocyte viability and maturation rates have been shown to be reduced at low temperatures in saline solution [52, 53]. This is probably because pig oocytes show high lipid concentrations, which provide higher sensitivity to low temperatures [54]. The storage of pig ovarian tissue in PBS induces a decrease in follicle viability overtime at 4 °C, despite the fact that follicle morphology remains similar to fresh controls for up to 12 h [55].

38.3 Developing a Tailored Transport Procedure for Ovarian Tissue

38.3.1 What We Can Learn from the Transportation of Organs for Transplantation

The collection, preservation, and transplantation of an organ from a donor have a significant impact on its homeostasis. This impact is expressed in the length of time it takes for normal organ function to return after transplantation. Ischemia and hypothermia are the most common causes of organ injury during recovery, preservation, and transplantation. Organ preservation techniques help to reduce this damage and increase graft survival and function [56].

The damage in transplanted organs has two phases of ischemia: the warm, from when blood supply is interrupted until the organ is immersed in hypothermic solution; and the cold ischemia, which is the time the organ is preserved in hypothermia until its transplantation. Warm ischemia causes rapid ATP depletion, lactic acid buildup, a reduction in pH, proteolysis, lipolysis, and lipid peroxidation [56]. Hypothermia, on the other hand, reduces metabolism 12 times [57], lowering oxygen requirements during the ischemia phase [58]. Yet, it does not entirely cease metabolic activity [59, 60]. Cold ischemia generates ATP and ADP depletion, resulting in damage such as metabolic disturbances, edema, autolysis, and oxidative stress, typically seen following graft reperfusion [61].

Different solutions have been developed for organ preservation to prevent cellular edema, delaying the destruction of the cells, and maximizing their functions after the reestablishment of revascularization [62]. In this sense, intracellular or extracellular solutions can be used with many additives to avoid organ failure after transplantation. Substances like glutathione, adenosine, and allopurinol can be added to preservation solutions to prevent this issue. Citrate solutions act as buffers, allowing the extracellular environment to remain stable. Histidine and amino acids, such as tryptophan and alpha-ketoglutarate, stabilize cell membranes, and together with mannitol, they act as antioxidants [56].

Current preservation solutions contain substances with buffering capacity and antioxidant properties. Custodiol®, a medium initially developed for cardioplegia, has been clinically used for heart, kidney, and liver transplantation with great success rates. UW (University of Wisconsin) Solution has shown successful results in pancreas transplantation, with storage for up to 24 h [63]. For the heart and lungs, different preservation solutions are more effective. Crystalloid hyperkalemic media are normally used for heart preservation at 4 °C for up to 4 h. The lungs appear to support a more

extended ischemia period, functioning well after 9–10 h of preservation using Euro-Collins solution before grafting [56, 64].

From different reports on the preservation of various organs, each of them requires particular substances to prevent function loss following transplantation. In this sense, different additives, buffers, antioxidants, amino acids, and other chemicals are selected with careful consideration of the needs of individual organs. This logic must be extended to the ovary to improve ovarian function and fertility outcomes after grafting.

38.3.2 Ovarian Tissue Metabolism During Transportation

Ovarian tissue ischemia after transplantation has been shown in different reports [10, 65–69]. Human ovaries xenografted to mice showed hypoxia duration of 3–5 days, while partial pressure of oxygen stabilized 10 days after transplantation, when the tissue is revascularized [67]. This 10-day period of anaerobic metabolism causes the cells to consume their internal energy storage, accumulate metabolic by-products and export protons [69, 70]. The main implications of ischemia-revascularization in grafted ovarian tissue include a shorter graft life duration, empty follicles following aspiration, and poor responder hormone profiles [71].

It is important to note, however, that ischemia begins immediately after the tissue is removed from the patient and continues until the tissue is cryopreserved [4, 72]. Degeneration of the vasculature in the fresh tissue frequently occurs during inadequate transportation before cryopreservation or transplantation of the tissue [10, 67–69], and metabolic activity will continue to occur at low temperatures before cryopreservation. Lactate release was detected in media [24] and follicular fluid [53] after the transportation of ovarian tissue in animal models, suggesting that cells were relying on internal energy sources rather than nutrients in the medium. These findings indicate that even at low temperatures, ovarian tissue metabolism is influenced by storage, which could have an impact on fertility following transplantation. In fact, lower embryo implantation and live birth rates were reported after 8 h storage of mouse ovarian tissue at 4 °C [37], highlighting the importance of tailoring a storage protocol specifically for the ovary.

Studies on ovarian follicle metabolism show that primordial germ cells require twice as much glucose and pyruvate than growing follicles, releasing lactate when in activity [73]. Follicles become glycolytic as they develop [74, 75], consuming amino acids, fatty acids, and glycerol to produce ATP [76, 77]. Nevertheless, although follicle metabolism has been intensely investigated [78], they are one of the many

different structures that belong to the ovarian cortex [79]. Different ovarian cell populations play key roles in follicle survival and development [78, 80, 81]. Only a few studies analyzed ovarian tissue metabolism during transportation [17, 24, 36, 43, 46, 53, 82]. Stromal cells, while not directly responsible for fertility, play an important role in follicle activation and development. Thus, it is vital that more studies on ovarian tissue metabolism are performed to better understand the whole tissue dynamics, which will impact the transport of the tissue.

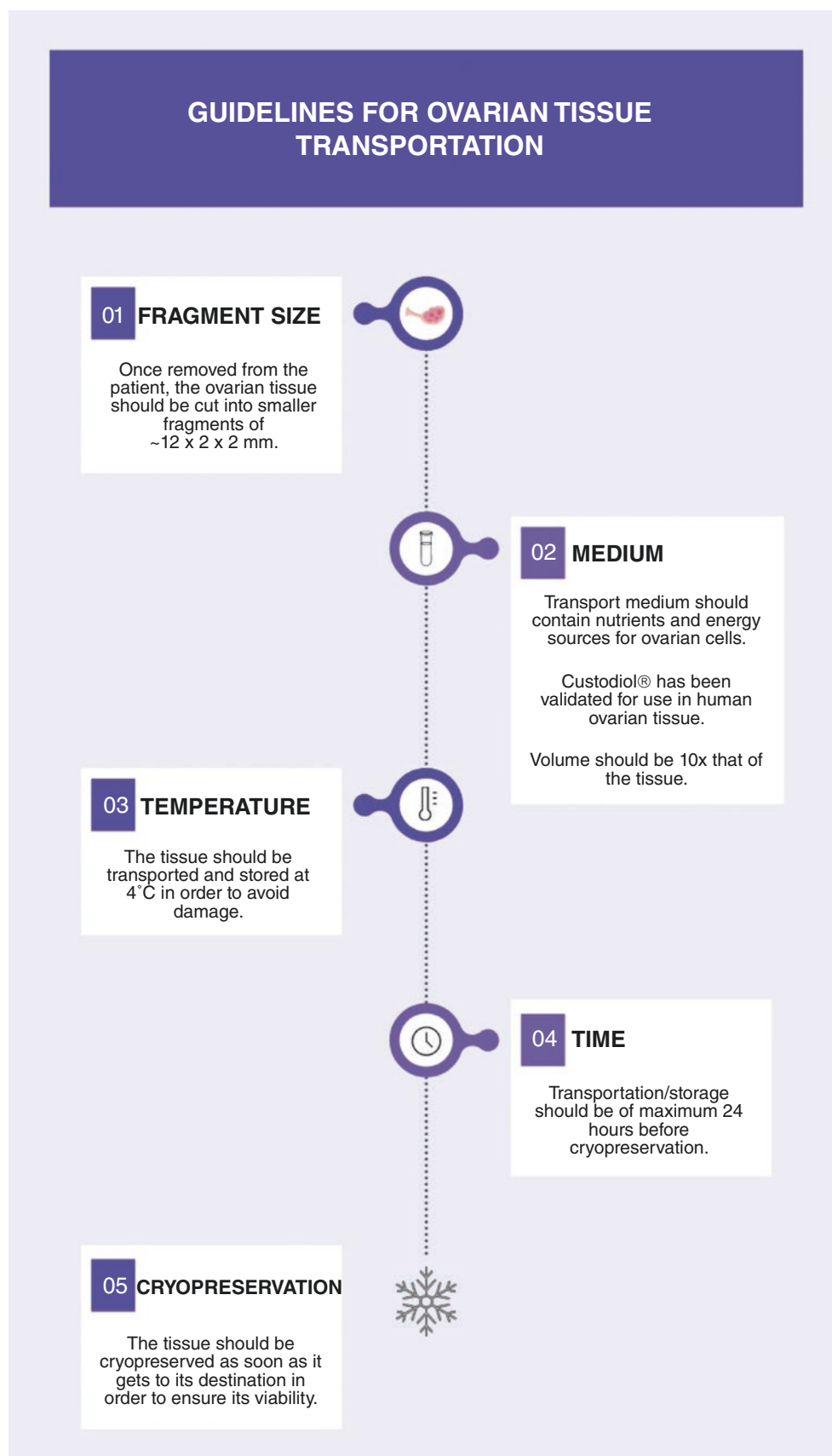
38.4 Conclusion

Over the years, much research has been carried out to understand how cryopreservation and transplantation can affect the ovarian follicle population and, consequently, reproductive function. On the other hand, studies in different animal models showed that the condition (duration, temperature, and medium) in which the ovarian tissue is kept between its collection and cryopreservation could influence post-transplantation follicle survival and growth, oocyte developmental competence, and even embryo quality and implantation. Although these studies demonstrate that transportation is critical to ovarian tissue performance, human OTT remains relatively overlooked. Current guidelines for OTT (Fig. 38.2) are based on information gathered from the few studies regarding this topic. However, due to the increasing number of networks for OTCT, it is crucial now to focus on developing an optimal strategy to transport human ovarian tissue and establishing adequate guidelines to ensure that it will not decrease the fertility potential of the patients.

Authors' Perspectives

- Key Points
 - Media composition, temperature, and time of transportation affect follicle morphology, viability, metabolism, and fertilization.
 - More attention needs to be given to ovarian tissue transportation.
 - Fertility networks are essential to increase access to OTCT.
 - It is necessary to create guidelines for human ovarian tissue transportation.
 - More studies need to be performed to develop a protocol tailored to the human ovary.
- Potential Areas of Research
 - While there are several studies on the description of cell response to ischemia, every tissue exhibits different tolerance to cold ischemia depending on its nature and demands after transplant. Hence, in order to develop a transportation medium that addresses the

Fig. 38.2 Guidelines for ovarian tissue transportation, considering the most recent findings [14]



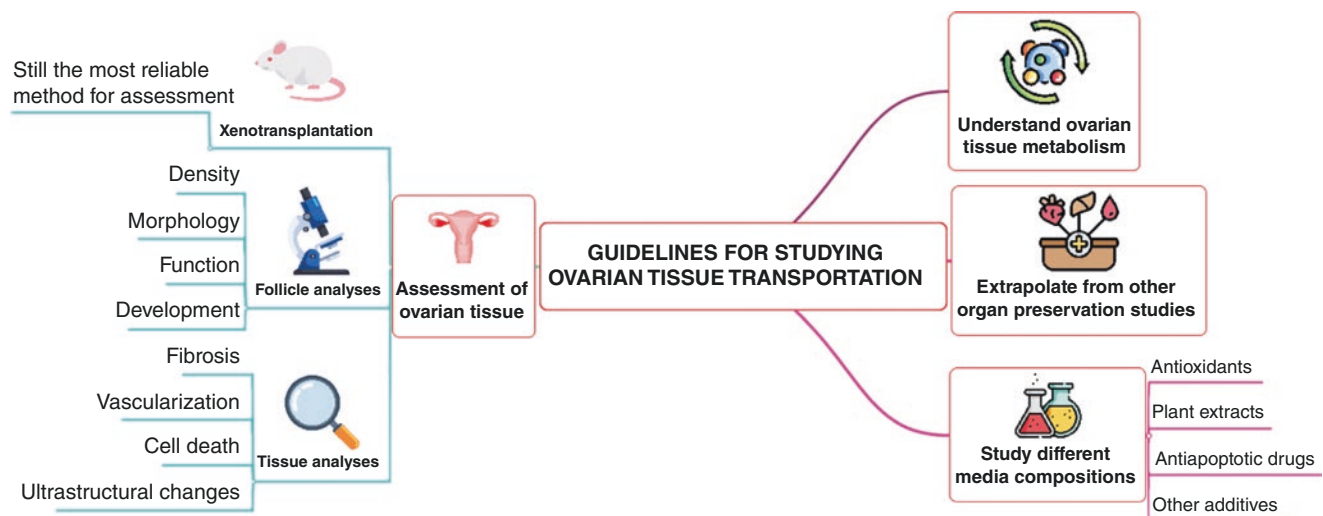


Fig. 38.3 Guidelines for studying ovarian tissue transportation. (Icons from [flaticon.com](https://www.flaticon.com/). Mind map was created using EasyMapper software)

requirements of the ovarian tissue, it is crucial first to understand the physiopathology of the ovarian cell and follicle cold ischemia and protection. To develop a medium that meets the needs of ovarian tissue, future areas of research should, therefore, focus on the knowledge of the metabolic activity in the ovarian tissue kept at 4 °C and how to supply the required metabolites to support cell survival and function during OTT (Fig. 38.3).

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Disposition of Cryopreserved Gametes and Embryos

39

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Key Points

- Cryopreserved gametes and embryo disposition options must be discussed before cryopreservation.
- Cryo storage facilities and patients both have an obligation to maintain an open line of communication during the storage period.
- Cryo storage facilities must have a plan for determination of specimen abandonment and specimen disposition.

39.1 Introduction

Throughout this book, readers are provided with evidence-based best practices to cryopreserve gametes, embryos, and reproductive tissues with the intent to optimize their reproductive potential when they are thawed. These advances have enabled infertility care providers to confidently prescribe gamete or embryo cryopreservation for medically indicated or planned fertility preservation. With improved survival and implantation rates, embryo cryopreservation has shifted from being an add-on or adjunct part of the in vitro fertilization (IVF) process to an essential element as providers advocate for single embryo transfer or prefer to avoid fresh embryo transfer altogether [1]. While we celebrate these advances, placing gametes and embryos in suspension with essentially no measurable half-life or biological expiration date may result in unanticipated consequences including emotional stress for patients suffering from decisional conflict and risk for centers providing long-term storage. Patients may face the challenge of determining the cryopreserved specimens' disposition when the decision has been made or the option is no longer available to utilize the gametes or embryos for family building. When patients can no longer be contacted or they fail to pay for storage services, centers pro-

viding long-term storage must determine the cryopreserved gametes or embryos disposition.

The first births from artificial insemination using cryopreserved human semen were reported in 1953 and nearly four decades have passed since the first report of a pregnancy resulting from the transfer of a cryopreserved human embryo, yet the state of the law regarding disposition of cryopreserved gametes and embryos is probably best described as "sparse" [2–4]. In light of the lack of a solid legal framework for cryopreserved gamete and embryo disposition and dispositional authority, infertility centers providing cryopreservation services utilize disposition agreements that have been developed in response to case law, and lessons learned from unanticipated patient disputes and specimen abandonment. The disposition agreement is incorporated into either the cryopreservation consent or cryopreservation contract, either of which describe the responsibility of each party to participate in the communication plan, duration of storage services, storage fees, expectations for timeliness of fee payment and disposition options in the event of death, dissolution of marriage or abandonment [4].

This chapter will review disposition options, pre-cryopreservation actions that may impact future disposition options and the issues infertility care providers need to address with patients before they have their gamete or embryos cryopreserved. We will discuss the process of consent and/or cryopreservation agreement review, when the agreement should be signed and, if storage is provided, considerations for subsequent storage contracts. Ideally, the cryopreservation agreement allows patients and their infertility care providers to optimize the benefits of gamete and embryo cryopreservation while mitigating burden and risk associated with the specimens with reproductive potential that has been suspended in time.

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39.2 Setting the Stage for Storage Success

39.2.1 Establishing a Cryopreservation Storage Plan

With increasing storage demands and heightened awareness of the risks of associated with specimen loss due to storage tank failures, centers providing cryopreservation services may want to tailor their storage options to the indication for cryopreservation [5, 6]. Centers that traditionally provided onsite storage for all specimens, may find they no longer have the capacity to continue to provide onsite storage for every patient or for the entire duration of the storage period. Off-site storage services allow programs to transfer the burden and risks of storage to an independent contractor. The liability for shipping is commonly assumed by the off-site storage facility. This option may be appealing for specimens cryopreserved for fertility preservation as it relieves the center providing cryopreservation services from the need to maintain a relationship with patients who may never return to the program for additional care but require periodic contact for invoicing and collection of storage fees and eventually, determination of specimen disposition. Off-site storage also may be a good solution for patients who initially presented to the center for infertility care but have not returned to the center subsequent treatment cycles over the course of a defined amount of time. If this is an option, centers should analyze their patient data for time to return for care and cryo storage space to determine how much time they will provide onsite storage before requiring patients to ship their cryopreserved gametes or embryos to an off-site facility.

Centers need to weigh the benefits of off-site storage against the obstacles and risks. The center performing cryopreservation must inform the patient of the risk of loss or

damage to specimens during shipment. Shipment of cryopreserved specimens requires many steps and dedicated staff members who have been trained in specimen shipping and receiving (Table 39.1). By not having the samples onsite, the center must have procedures and policies that to ensure patients are aware of their responsibility to notify the storage facility that the specimen(s) are to be released and shipped to the infertility care center. Failure to have a reliable process that ensures specimen(s) arrive in time for the treatment cycle could lead to cycle cancellation or demand extraordinary efforts by some or all parties to have the specimen(s) transported for just in time delivery.

Regardless of the storage location, the center must have policies and procedures for inventory management of cryopreserved specimens as they will need some duration of storage at the center. For an interesting summary on the evolution of cryo inventory management, readers are advised to read the review by Pomeroy and Marcon [7]. They reflect on the days when centers attempted to handle these tasks with a paper-based system, very similar to the way libraries would index cards to track the comings and goings of books. Fortunately, most centers now employ a relational database or, better yet, their electronic medical record to manage their cryo inventory. If onsite storage is provided, the center also must have and a reliable system for maintaining contact with patients for the entire storage period.

39.2.2 Establishing Limits for Onsite Cryo Storage

39.2.2.1 Intended Parent Age

For centers providing onsite storage, any maximum age limits for the treatment of patients or intended parents should be

Table 39.1 Steps involved in cryopreserved specimen transport

Cryopreservation center shipping specimens out	Centers receiving specimen (may or may not be performing warming and patient care)
Cryo devices are labeled, placed in storage, and entered into the laboratory's inventory management system.	Upon receipt of a request from the patient, review the following and determine whether to receive the specimens for future patient treatment and/or storage: • Specimen records • Post-thaw test results (sperm only) • Protocol used for cryopreservation and warming • Infectious disease test results
Confirm patient has signed a medical record release of information form and specimen release consent.	Notify patient that the shipment has been approved or denied.
Provide the following to the receiving center: • Specimen records • Post-thaw test results (sperm only) • Protocol used for cryopreservation and warming • Infectious disease test results	Upon approval of the shipment, correspond with the courier and schedule a date for specimen receipt. If the center allows patients to prepare for treatment prior to specimen receipt, ensure that the shipment arrives in advance of when the specimen will be needed for patient treatment.
Correspond with the courier and schedule a date for specimen pick up	

Table 39.1 (continued)

Cryopreservation center shipping specimens out	Centers receiving specimen (may or may not be performing warming and patient care)
Add the specimen ship out to the laboratory schedule	Add the specimen ship into the laboratory schedule
On the day, the dry shipper is scheduled to arrive, assign one staff member to have the primary responsibility for packing the shipment. This staff member has the following duties: • Contact the courier if the shipment has not arrived as scheduled. • Once the dry shipper arrives, confirm that the dry shipper is charged. The presence of frost on the exterior of the dry shipper or absence of LN2 vapors after removing the shipper's plug are indications of tank failure. • Remove the inventory to be shipped from storage and transfer it to a liquid nitrogen bath. • With the assistance of witness, review the specimens, goblet, and cane labels to confirm they match the inventory ordered to be shipped. • Transfer the cane loaded with the inventory scheduled to be shipped to a pre-chilled sleeve in the dry shipper. • Record the specimen shipment in the laboratory's inventory management system.	On the day, the dry shipper is scheduled to arrive, assign one staff member to have the primary responsibility for receiving the shipment. This staff member has the following duties: • Contact the courier if the shipment has not arrived as scheduled. • Once the dry shipper arrives, confirm that the dry shipper is charged. The presence of frost on the exterior of the dry shipper or absence of LN2 vapors after removing the shipper's plug are indications of tank failure. • Fill the dry shipper with liquid nitrogen. • Transfer the inventory to a liquid nitrogen bath. • With the assistance of witness, review the specimen, goblet, and cane labels to confirm they match the center of origin's records. • Assign a storage location to the specimens and transfer them to that location. • Record the specimen receipt in the laboratory's inventory management system.
After the shipment is received by the courier, confirm the shipment has arrived at its destination through E-mail or the courier's tracking system.	Notify the courier the empty dry shipper is ready for pick up.
	Notify the patient of the shipment's arrival.
	If not already on file, request the patient's signed cryo storage agreement from the clinic.

reflected in the storage agreement to ensure storage services will conclude on or before the patient or intended parents reach the center's age limit. Additionally, the center may want to forego onsite storage altogether for patients who will reach the age limit shortly after cryo banking. Our center has an age limit for women undergoing treatment and, until recently, age served as the only limit for the duration of cryo storage provided storage fees were paid. The age-limited storage policy has led to many difficult embryo disposition discussions with patients who struggle with embryo disposition decisions. They would rather pay the storage fees than to select an alternative disposition for their embryos. Fortunately, a policy change has been implemented that will limit the duration of onsite storage relative to the cryopreservation date. It is our hope that this should eliminate the difficult discussions that follow many years of storage. This policy also should help us free up precious storage space.

Regretfully, our program did not establish an age or time limit for patients banking sperm. A policy for shipping samples cryopreserved for long-term storage to an off-site facility was implemented several years ago, but long-term storage services continue to be provided to patients who banked sperm prior to the policy change. It has been our experience that fee increases and letters requesting patients consider shipping their samples to an off-site storage facility have had minimal impact on our inventory of cryopreserved sperm that has been in storage for over a decade.

39.2.2.2 Duration of Storage

Cryo storage duration does not appear to affect the reproductive potential of gametes or embryos [8]. So how long should a program provide onsite storage of gametes and embryos? What are our patients' expectations? If we limit onsite storage before the patient has completed family building, how many shipments between the infertility care center and off-site storage facility can be made without significantly increasing the burden of transportation risk [9]? These are some of the questions that come to mind when pondering how long a center should or capable of providing onsite storage. For those practicing without federal mandates that limit storage duration, one universal limiting factor that can guide cryo storage planning is the capacity to safely store cryopreserved specimens in the center. The space for storage tanks, number of storage tanks, liquid nitrogen supply, monitoring capabilities, and personnel dedicated to management of the inventory and specimen disposition must be taken into consideration when developing the center's storage duration policy.

39.2.2.3 Storage Based Upon Preimplantation Genetic Test Result

The use of preimplantation genetic testing (PGT) to select embryos for transfer has grown significantly during the past decade. Centers reporting their IVF cycles to the Society for Assisted Reproductive Technology used PGT during 17% of

cycles managed in 2014 and 44.9% of the cycles performed in 2018 [10]. The actual number of cycles using PGT jumped by 225%! Most cycles involve cryopreserving embryos after they are biopsied and transferring embryos deems suitable for transfer in a subsequent transfer cycle. As a result, centers may find themselves storing embryos that, based upon practice policies, have been deemed unsuitable for future transfer. The disposition decision for the healthcare provider prescribing PGT may seem evident, but it may not necessarily be clear for the patient.

A recent anonymous survey of patients who had recently used or considered using PGT for single gene mutations or chromosomal testing reported asked participants about their perspectives on the use of surplus embryos that had known genetic problems [11]. Responses to disposition options were independent of each other. Embryo discard was an acceptable option for 62% respondents, 77% agreed with donation to research, 29% felt it reasonable to donate the embryos with known genetic problems to another family, and 49% would keep them in storage for future use. While PGT can give patients a sense of empowerment over the uncertainties of their reproductive futures, but the disposition of embryos with the genetic condition that caused the loss of a child can be a source of emotional distress and grief [12]. The infertility care providers must discuss the plan for disposition of the cryopreserved embryos that the patient and practice mutually deem unsuitable for transfer.

39.3 Cryopreservation Consents, Storage Agreements, and Pre-cryopreservation Considerations

The consent process must involve a discussion between a healthcare provider from the center providing cryopreservation services and the patient. During the discussion, the healthcare provider should assess the patient's understanding of the purpose of cryopreservation, the potential to utilize the cryopreserved gametes or embryos for future family building and risks associated with cryopreservation and storage. The informed consent should be used as a tool to guide the discussion and a place to document the conversation and the patient's decisions. Patient's decisions should be based upon knowledge of the benefits and risks of gamete or embryo cryopreservation in the context of their values and priorities [13].

The conversation should start with verbal review of a written consent for cryopreservation. Ideally, the patient should have read the consent prior to this discussion. The information in the consent should capture the following:

- The cryopreservation processes
- Anticipated and unanticipated risks of cryopreservation on the gametes or embryos
- Where the specimens will be stored
- If the specimens are to be shipped to an off-site facility, the consent should include disclosure and acknowledgment of the risks involved with specimen transport
- Specimen disposition in the event of death or abandonment or embryo disposition in the event of divorce, death, or abandonment

A separate storage agreement or contract between the storage facility and patient should be reviewed and signed prior to cryopreservation. The items listed below should be described in a storage contract and not embedded in a consent for treatment:

- If fees are charged for storage, a description of the fees, frequency of billing, consequences of unpaid storage fees or, and storage options if the storage center closes
- Duration storage services will be provided
- A description of the communication plan for both the patient and the storage center during storage

The items to be covered in the cryopreservation consent and storage contract are numerous and some of the topics are complicated. Unfortunately, the topic of specimen disposition typically falls at the end of the time-limited, single conversation. Khorshid and Alvero (2020) reviewed current consenting processes and ethical considerations for patients seeking embryo cryopreservation [14]. They concluded that an informed consent process that addresses cryopreserved embryo disposition only at the point before cryopreservation is flawed and should be replaced with a longitudinal, narrative consent process with disposition discussions at multiple points throughout patient's reproductive journey. As they point out, it is important to recognize that patients seeking IVF after an extensive period of infertility will likely have some knowledge about the steps involved in treatment, but their goal is to have a baby. After years of struggle, it may be difficult for them to comprehend that they may have cryopreserved embryos in storage after they have completed their family building. One could envision a similar model for fertility preservation patients with gametes in storage although the contact would likely not occur in association with clinic visits unless the patients were returning to use their gametes.

For patients urgently seeking fertility preservation due to an anticipated gonadotoxic medical treatment, the abundance of information provided at their initial consultation can be overwhelming, leaving little room for consideration about the disposition of their cryopreserved specimens [15]. Similar to the infertility patient whose goal is to have a baby, the fertility preservation patient's goal is to preserve their fertility before they undergo treatment that will allow them to return to good health. The ASRM practice committee rec-

ommends that a team approach to patient counseling be used for fertility preservation patients undergoing gonadotoxic therapy or gonadectomy [16]. Ideally, patients should have the opportunity to meet with physicians, nurses, mental health professionals to discuss fertility preservation options. Unfortunately, the urgent nature of fertility preservation may not allow sufficient time to consider the disposition of specimens at the time of cryopreservation.

39.3.1 Sperm Cryopreservation

Sperm cryopreservation is the established method for fertility preservation for post-pubertal individuals anticipating gonadotoxic medical treatment, deploying for military service and those born as male but are planning on undergoing gender affirmation through hormone therapy and surgery. It is standard practice to cryopreserve sperm used for anonymous sperm donation, often used for directed sperm donation and may be recommended for an intended parent who may not be available to collect sperm for intrauterine or in vitro insemination. Cryopreservation also may be used to preserve sperm that has been surgically retrieved in advance of an in vitro fertilization procedure. The center providing cryopreservation services must have a cryopreservation agreement that includes specimen disposition options and storage plan for each of these scenarios. When cryopreservation services are provided for minors, the consents and storage agreements must accommodate proxy decision-makers [17].

39.3.1.1 Infectious Disease Testing

The options for disposition of the cryopreserved sperm may be dictated by actions taken prior to sample collection. Before cryopreserving sperm, patients must be informed of testing requirements set forth by the local or off-site storage facility. For example, storage facilities in the United States typically require infectious disease testing for antibodies to HIV 1 and 2, Hepatitis B surface antigen, Hepatitis C antibodies, but do not require the level of testing for infectious diseases required by the AS Food and Drug Administration for determination of donor eligibility [18, 19]. If the center providing cryopreservation services does not routinely determine donor eligibility for all individuals cryopreserving sperm, the patient must be informed that there are different levels of screening. If they forego screening to determine donor eligibility, procreative use of the sperm will be limited to use by a sexually intimate partner who would carry the pregnancy. It is important for the patient to understand that failure to determine donor eligibility before sperm cryopreservation may prohibit future transfer of embryos created with the sperm to a gestational carrier.

39.3.1.2 Cryopreserved Sperm Utilization

When sperm is being cryopreserved for use by a sexually intimate partner for intrauterine insemination or in vitro fertilization, the center may want to obtain informed consent from the partner to document they have been informed of the level of infectious testing performed at the time of sperm cryopreservation. The center also must have a policy for obtaining consent from the patient to permit their partner to access their cryopreserved sperm for intrauterine insemination or in vitro fertilization. Specimen release may require a new consent from the patient for each insemination event or there may be a single specimen release consent signed at the time the specimen was cryopreserved. If a center relies upon the latter, the patient must understand that they must notify the storage center if they wish to retract their consent for sperm release. The risk with this scenario is that the patient may have forgotten their responsibility to retract their consent and may not be aware that their partner (current or former) is using the cryopreserved sperm while undergoing infertility care.

39.3.1.3 Cryopreserved Sperm Disposition

As discussed previously, the consent for sperm cryopreservation must address specimen disposition in the event the patient abandons the specimen, dies, or loses the capacity to communicate their consent [20]. Sperm disposition options may include:

- Specimen discard
- Bequeath the sperm to their partner for posthumous reproduction
- Specimen donation for research (additional consent required)
- Identification of a proxy disposition decision-maker

39.3.2 Oocyte Cryopreservation

Like sperm cryopreservation, oocyte cryopreservation is the established method for fertility preservation for post-pubertal individuals anticipating gonadotoxic medical treatment and those born as female but are planning on undergoing gender affirmation through hormone therapy and surgery. As oocyte cryopreservation techniques improved, donor oocyte banks emerged, giving recipients a readily available source of anonymously donated oocytes [21, 22]. In recognition of the natural depletion of ovarian reserve, healthy women who wish to delay parenthood may opt to undergo controlled ovarian stimulation and oocyte cryopreservation for planned fertility preservation [23]. Oocyte cryopreservation also may be utilized in the setting of an in vitro fertilization procedure where either the patient or law restricts the number of oocytes

inseminated to avoid generation of supernumerary embryos or the partner's sperm is not available for insemination [24].

As with sperm cryopreservation, there are numerous indications for oocyte cryopreservation. The center providing cryopreservation services must have a cryopreservation agreement that includes specimen disposition options and storage plan for each of these scenarios.

39.3.2.1 Infectious Disease Testing

The conversation regarding infectious disease testing and whether to perform with patients cryopreserving oocytes will be like patients banking sperm. However, for patients undergoing oocyte cryopreservation prior to gonadotoxic therapy or gonadectomy, a future pregnancy may not be safe or warranted, requiring a gestational carrier to facilitate family building with the oocytes. The ASRM Practice Committee advises centers to routinely recommend donor eligibility determination at the time of oocyte cryopreservation to keep use of a gestational carrier a viable option [16].

39.3.2.2 Cryopreserved Oocyte Utilization

The center performing the oocyte thaw for in vitro insemination must obtain a consent from the intended parent. If there is a second intended parent, both parties should complete the in vitro fertilization consents.

39.3.2.3 Cryopreserved Oocyte Disposition

The consent for oocyte cryopreservation must address the patient's specimen disposition wishes in the event the patient abandons the specimen, dies, or loses the capacity to communicate [20]. Oocyte disposition options may include:

- Specimen discard
- Bequeath oocyte(s) to their partner for posthumous reproduction
- Specimen donation for research (additional consent required)
- Identification of a proxy decision-maker

39.3.3 Embryo Cryopreservation

Embryo cryopreservation may be performed for single patients or, more commonly, two intended parents. The embryos may result from donor gametes or gametes from one or both intended parents. The center providing cryopreservation services must obtain consent from the intended parent(s) and, when two intended parents are involved, their mutual embryo disposition decisions. Embryo cryopreservation is typically prescribed in association with in vitro fertilization procedures performed for infertility care. As oocyte cryopreservation methods have improved, the use of embryo

cryopreservation as a strategy to preserve a woman's reproductive potential during planned or medically indicated fertility preservation is discouraged in recognition of the potential loss of the patient's autonomy should they cryopreserve embryos and allows them to choose the sperm source in the future when they return to use the oocytes for family building [25, 26]. Additionally, cryopreservation of oocytes avoids the moral and ethical challenges of discarding embryos in the event the patient does not return to use the cryopreserved specimens.

39.3.3.1 Infectious Disease Testing

In the United States, donor eligibility must be determined for individuals who undergo IVF for creation of embryos intended to be transferred to a gestational carrier [19]. For cases where embryos are created for transfer to an intended parent, non-donor gamete providers, the testing must satisfy the infertility care center's infectious disease testing requirements, but not meet requirements for donor eligibility determination [27]. Unlike gamete cryopreservation, the lack of donor eligibility determination of the sexually intimate gamete providers at the time of in vitro fertilization, does not impact future embryo disposition options including transfer to a gestational carrier or embryo donation.

39.3.3.2 Cryopreserved Embryo Utilization

The center performing the embryo thaw for transfer must assure that consent for embryo warming/thawing has been obtained from the intended parents prior to removing the embryo(s) from storage. If the cryopreserved embryos resulted from an IVF procedure for which two intended parents consented, both individuals should be required to sign a consent for embryo warming for transfer.

39.3.3.3 Cryopreserved Embryo Disposition

Consent forms should address the future disposition of cryopreserved embryos. Patients should indicate their disposition preferences in the event of divorce, death of one or both intended parents and embryo abandonment. This conversation will not necessarily follow the pattern of the gamete cryopreservation discussions due to the likely involvement of two individuals, their moral and ethical views of the cryopreserved embryo, and focus on family building may obscure patient's ability to contemplate the disposition of surplus embryos [14].

Depending on federal, state, and local laws, embryo disposition options may include:

- Specimen discard
- Bequeath the embryo(s) to their partner
- Specimen donation for research (additional consent required)

- Identification of a proxy decision-maker
- Directed donation to an embryo recipient
- Anonymous donation to and embryo recipient

39.4 Specimen Disposition

39.4.1 Continued Storage

Maintenance of the cryopreserved specimens in storage is not the final disposition option, but it is often the choice of patients who have not completed their family building. During the storage period, the storage facility must ensure that a contract or agreement is on file that describes the duration of storage, storage fees, the patient's responsibility to maintain contact and final disposition instructions. If fees are assessed for the storage services, the contract should describe expectations for storage fee payment, the center's protocol for management of delinquent accounts and specimen disposition when storage fee payment is not received and all options to contact the patient have been exhausted.

Storage centers often rely upon the cryo storage fee invoice as a tool to assure routine, periodic contact with the patient. Caution should be applied when making this assumption as our center has encountered situations where patients were oblivious to the fact that storage services were continuing to be provided. Several years ago, this resulted from our center's institution failing to bill for storage. More recently, patients have claimed to not be aware of the storage fee invoices because the fees have been paid by healthcare insurance providers and the patients ignore the statements of benefits sent by their insurance provider. Due to these experiences, centers may need to assess the effectiveness of cryo storage billing as a means of communication with their patient population.

39.4.1.1 Specimen Abandonment

When payment for storage fees is not received or communication from the storage center to the patient(s) yields no response, the center must have a plan that includes multiple strategies to locate and contact the patient(s). The strategies for contact may include all or some of the following actions:

- Send a letter to the last known address.
- If available, communicate through the center's electronic medical record patient portal.
- Attempt to contact by email.
- Make a defined number of attempts to contact by telephone (weekdays, evenings, weekends), leaving a minimum of (number to be determined by the center) voice mail messages.
 - If the number is no longer active, call the emergency contact.

If a couple has embryos in storage, the steps listed above should be taken for both intended parents. For situations where the patient banking gametes is minor, their parent or parents should be contacted. If all attempts at contact fail, a final letter sent to the last known address by certified, restricted delivery, registered mail will give the center evidence of delivery attempts or a record of receipt.

After all options for communication have yielded no result, the center may claim the cryopreserved specimens abandoned. The center must have a policy that states how much time must pass before they proceed with a disposition other than continued storage. If no directive was provided by the patient, the center will employ the default disposition option described in the original cryopreservation consent.

39.4.1.2 Patient Death

Upon receipt of notification or discovery that a patient with cryopreserved specimens has died, the center should refer to the cryopreservation consent and/or storage agreement for specimen disposition directives. If no directive was provided, the center should attempt to contact the deceased patient's next-of-kin or emergency contact to determine if specimen disposition directives were described in the patient's will.

39.4.2 Discard

Cryopreserved specimen discard may sound like a straightforward disposition option, but patients may desire alternatives to the standard practice of removing the specimen from storage and disposing it in biological waste. An anonymous survey of international IVF laboratories revealed that there is no universal standard for the practice of disposing cryopreserved embryos [28]. The majority of laboratories reported removing the specimens from storage and placing the cryo device in the biological waste bin, but some respondents described handing the thawed devices to the patient or shipping the specimens scheduled to be discarded to another company that disposed of the embryos for the patient for a fee.

In the event specimen discard is not permissible by law or by program policy, or acceptable to the patient based upon religious or ethical views, patients who do not desire alternative disposition options after completing their family building may be open to the option of compassionate transfer [29]. A compassionate embryo transfer is performed at a time when pregnancy is highly unlikely to occur and when pregnancy is not the intended outcome. Patients who chose this option see it as a more respectful way to dispose of embryos rather than having the laboratory discard them. This disposition option requires scheduling a time for the transfer, potentially additional fees for the service and a small but, nevertheless, real risk of an unanticipated pregnancy [30].

39.4.2.1 Planned Specimen Discard

The storage center must have procedures in place for planned cryopreserved specimen discard. The procedures should include:

- Requirement for a consent for discard from the patient storing gametes or intended parents with cryopreserved embryos.
- Communication with the patient(s) notifying them that their consent was received and specifying the timeframe during which specimen discard will occur.
- A minimum of two laboratory staff members must complete the following steps:
 - Review the consent.
 - Sign off on the plan to discard.
 - Perform the discard (one handling the specimen, the other serving as a witness).

39.4.2.2 Discard Due to Patient Death or Specimen Abandonment

If specimen discard is performed due to abandonment or patient death, document review before scheduling the specimen discard should involve a member of the billing and administrative staff, the laboratory director and laboratory staff. The following is a description of the document review:

- The billing and administrative staff will review the patient's medical record and account records.
- The laboratory staff member reviews the patient's medical records, searching for evidence of communication.
- The laboratory director performs a review similar to that described above. If satisfied that the specimens are to be discarded, they sign the checklist and approve the discard.

39.4.3 Donation to Research

Gametes or embryos that were cryopreserved for reasons other than donation for research may be donated for research upon the conclusion of cryo storage. The ethical issues surrounding the study of human embryos and gametes are complex and oversight of the research process is essential. The American Society for Reproductive Medicine (ASRM) recommends use of the Common Rule/Internal Review Board (IRB) framework for research involving embryos, even when the facility conducting the research falls outside of structures in which the framework is legally required [31].

The donor's consent for specimen donation for research must be obtained using a separate, IRB-approved consent. The consent must include statements about the intended ben-

efits and description of the research study. It should clearly state that the specimens will not be used for fertility treatment. The consent process should be led by a healthcare team member who is able to explain the research and answer the potential participant's questions. To avoid the risk of coercion, the individual obtaining the consent for donating the cryopreserved specimens to research should be someone other than the patient's healthcare provider.

If the center opts to limit consents to specific research projects, they will be required to maintain contact with potential donors and seek their consent for donation when a project is actively recruiting donors. Alternatively, the IRB may approve an embryo donation for research consent that covers a broad range of projects. The consent should list potential research studies and provide an itemized list of research that is prohibited. The benefit of this approach is that consent for donation to research may be obtained at the time the patient or intended parents are ready to commit to this disposition option. If such a consent is employed, investigators will be required to obtain additional approval for their specific project, indicating the number of specimens that will be utilized from the donated specimen repository.

Given the amount of information that must be reviewed as the patient prepares to have gamete or embryo cryopreservation, it is difficult to add review of specimen donation for research consents to the process when this is not the intent of specimen cryopreservation. Centers must take this into consideration before they offer specimen donation to research as an option in the event specimens are abandoned or the patient dies. For our center, we are unable to offer "advanced planning" for specimen donation for research because our IRB requires consent for donation to research at the time the patient or intended parents have decided to discontinue storage.

39.4.4 Donation to Others for Reproductive Use

39.4.4.1 Gametes

Directed donation of cryopreserved gametes may be offered as disposition. Patients who wish to have the option of directed donation of their gametes must undergo donor eligibility determination at the time the gametes were cryopreserved. When directed donation in the event of their death is selected as an option, the recipient should be identified in the original cryopreservation consent, a subsequently filed consent and/or the patient's will.

If the patient is alive at the time, the directed gamete donation option is employed; an additional consent should

be obtained for donation, specifying the rights of the donor and recipient as well as the donor's release of claim to the specimens.

39.4.4.2 Embryos

Anonymous or directed donation of cryopreserved embryos may be offered as a disposition option. If the embryos were created for two intended parents, both must consent to the donation. If the embryos were derived from donated gametes, the gamete donor(s) must have given consent to future embryo donation.

If the storage center is not affiliated with an anonymous embryo donation program, there are private organizations that facilitate embryo donation. Patients should be made aware of these organizations so they may determine if embryo donation is a path they wish to pursue.

For situations where sexually intimate partners provided the gametes and donor eligibility was not determined prior to embryo cryopreservation, donor eligibility may be determined at the time the intended parent(s) decide to donate their embryos. In the United States, a minimum of 180 days must pass after cryopreservation before screening and testing of the donors is performed. The embryos must be accompanied with a label stating: "Advise recipient that screening and testing of the donors were not performed at the time of cryopreservation of the reproductive cells or tissue but have been performed subsequently" [32]. If the embryo donors are not available or refuse to undergo the required testing or screening, donation is still possible, but patients must be counseled regarding the risk of transferring the embryos and the label accompanying the embryos must state: "Not evaluated for infectious substances. WARNING: Advise recipient of communicable disease risk."

If one or both intended parents donating the embryos are alive at the time the donation option is employed, an additional consent should be obtained for donation, specifying the rights of the donor, recipient, and the donor's release of claim to the specimens.

Items to be addressed in the embryo donation consent include:

- Release of claim to the embryos and relinquishing all rights to any child resulting from transfer to an embryo recipient.
- The time period for which the donated embryos will be maintained in storage.
- In the event embryos are not selected by a recipient and remain in storage beyond the defined time period:
 - A description of the center's plan to communicate with the donors to seek an alternative disposition.

- The center's disposition plan should the embryos remain in storage at the conclusion of the storage period.

39.5 Conclusions

Advances in cryopreservation techniques, increased awareness of the need for and availability of these services for fertility preservation and optimization of embryo culture environments have led to a high demand for cryopreserved specimen storage. Patients who have cryopreserved specimens in storage must maintain a relationship with their infertility care provider or cryo storage facility until they determine they will no longer use the gametes or embryos for their own family building. Centers providing storage also have an obligation to maintain a relationship with the owners of the specimens. Determining the specimen disposition may be difficult as the patient's perspective and conceptualization of the gametes or embryos that remain suspended in time may have changed during the storage period. Discussion and documentation of preferred, alternative disposition options in the cryopreservation consent is an important initial step to prepare patients for decisions they may face several years later. Continued engagement with patients to discuss their disposition options throughout the storage period may facilitate patient decision-making and reduce the frequency storage centers are left to exercise their dispositional authority for abandoned cryopreserved specimens.

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European Regulations for Reproductive Tissue Banks

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Key Points

- The EU Tissues and Cells Directive (EUTCD; Directive 2004/23/EC) with all its directives and amendments regulates all aspects of an IVF cycle from procurement, to distribution of cells and tissues.
- In the EU all institutions, processing cells and tissues are referred to as tissue establishments.
- EU tissue establishments need authorization and licensing by the national competent authorities which perform on site audits in a 2-year rhythm.
- The “Guide to the Quality and Safety of Tissues and Cells for Human Application” by the European Directorate for the Quality of Medicines and HealthCare (EDQM) serves as basis for national competent authorities.
- Once a year, tissue establishments have to report all its activities to the competent authorities.
- All tissue establishment must have a functioning quality management system including tools for risk analysis, traceability and coding, donor administration and reporting serious adverse reactions and events.
- All processes have to be performed according to standard operation procedures, but the techniques itself are not regulated in detail.
- Air quality in the lab is strictly regulated.

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40.1 Introduction

The European Union (EU) is an international organization with currently 27 member states. In principle, it constitutes of three main institutions which are called the European Commission, the European Parliament, and the Council of the EU. These three authorities act in cooperation to produce legislation that is binding throughout all member states of the EU.

In 2004, the EU has passed the EU Tissues and Cells Directive (EUTCD; Directive 2004/23/EC) which sets out requirements regarding the quality and safety of tissues and cells for human application [1]. Over the years, several directives were adopted that provided further details concerning the specific technical requirements (Directive 2006/17/EC and its amendment 2012/39/EU), traceability requirements (2006/86/EC), quality and safety of tissues (Directive 2015/566), and coding (Directive 2015/565) [2–6].

While in general the EUTCD has a broader scope, dealing with a multitude of tissues and cells, some paragraphs focus specifically on reproductive cells. The EUTCD is currently under investigation and revision to address potential shortcomings that were identified in a previous evaluation of the Directorate General for Health and Food Safety of the European Commission (DG SANTE) to which ESHRE with its position paper (<https://www.eshre.eu/Europe/Position-statements/EUTCD>) contributed actively [7].

Additional important input regarding the performance of European tissue banks comes from the Council of Europe (CoE, not to be confused with the previously mentioned Council of the EU) which is an entirely separate body from the EU. The CoE focuses on democracy and human rights and since access to good quality healthcare and medicine is a basic human right this institution is constantly working on health-related topics including medically assisted reproduction (MAR). The agency of the CoE which realizes such health-related projects is called the European Directorate for the Quality of Medicines and HealthCare (EDQM). Amongst its tasks the EDQM develops technical guidance on medical

treatments, which can be used as a reference by national competent authorities, clinicians, and embryologists. The publication of the CoE particularly covering MAR is the “Guide to the Quality and Safety of Tissues and Cells for Human Application.” ESHRE experts wrote the two chapters specific for MAR and fertility protection. Currently, the fifth edition of the EDQM guide is in progress [8].

All units offering either the whole MAR portfolio or only a section of the same (e.g., cryobanks) have to follow the EUTCD and its realization is controlled biannually by the respective competent authorities of the individual states. Of note, the above-mentioned guide serves as guidance for the inspectors auditing European tissue establishments. However, it has to be clarified that European countries outside the EU will not have such rigorous controls and are not obliged to follow the EUTCD. Although there are vigorous efforts to harmonize quality and risk control in the field of MAR within the whole of Europe still national legislation is the legal basis in some non-EU countries.

This chapter focuses on EU law and deals with the requirements set by the EUTCD as well as the EDQM guide and their actual impact on tissue establishments and cryobanks in particular.

40.2 General Requirements

By definition, in the EU all institutions processing cells and tissues are referred to as tissue establishments irrespective of the fact whether they are set up as a private clinic, as university unit or simply acts as sole cryobank, for example, exclusively storing and distributing frozen-thawed or vitrified-warmed cells and tissues. All tissue establishments need authorization and licensing by the national competent authorities. Without a valid tissue establishment license number, no institute is allowed to collect, process, store and distribute cells/tissues, or to interact with third parties (e.g., import or export of cells and tissues).

One academic staff member per tissue establishment has to be designated as “responsible person” in a sense that she/he is legally liable for guaranteeing that the work is done in compliance with the EUTCD, for organizing all audits, for dealing with the non-conformities after the audit, and for reporting any substantial changes to the IVF lab activities. It is not said that the person in charge has to be the lab director as long as this person fulfils the following criteria: 2 years of practical experience in MAR and having a “diploma or other evidence of formal qualifications in the field of medical or biological sciences on completion of a university course or a course recognized as equivalent by the Member State concerned” (EUTCD, Article 17/1a). Although at first glance this definition would leave some room for interpretation, the reality is that the responsible person has to have a PhD

degree. However, for rest of the staff members who are involved in cell and tissue processing, cryobanking, and distribution of the same, it is sufficient that these colleagues are “appropriately qualified” and have passed relevant training. All work in IVF labs as well as cryobanks has to be carried out on the basis of a quality system taking into consideration good practice (e.g., ESHRE Guideline Group on Good Practice in IVF Labs, 2015) [7, 9].

Once a year tissue establishments have to report all its (recorded) activities related to the procurement, processing, storage and distribution of sperm, oocytes, embryos, and testicular/ovarian tissue to the competent authorities. Serious adverse events (e.g., the untoward leakage of a cryo tank) and serious adverse reactions (e.g., contamination of cells via liquid nitrogen) need immediate notification of the competent authorities. Needless to say, any action has to be taken to analyze the cause and the ensuing outcome. If possible, for example, in case that a used frozen sperm donor was later diagnosed with a hereditary disease, the affected product should be recalled and/or excluded from further distribution.

Signed agreements with other tissue establishments must be drawn up which in case of termination of activities will regulate and ensure that the stored cells/tissues are safely transferred between the IVF units and that there is no harm to the patients.

40.3 Risk Management and Validation

Another aspect of an existing quality management system is the identification and prioritization of potential hazards in order to control them so that the probability of such a threatening scenario is kept to a minimum [10]. Common methods to evaluate risk factors are HACCP (Hazard analysis and critical control points), FMECA (Failure mode, effects and criticality analysis), and FMEA (Failure mode and effects analysis) as highlighted in the EDQM guide [11, 12].

To keep risks at subcritical level, tissue establishments in Europe have to ensure that new equipment is qualified accordingly. Usually, a proper design qualification (DQ) would identify the most superior equipment which then undergoes further qualification steps such as installation (IQ), operation (OQ), and performance qualifications (PQ). The latter should involve a comparison with generally accepted laboratory KPIs, a process which would allow to establish levels of competence and aspirational benchmark [9]. The process of implementing a newly developed automated vitrification device may serve as a hypothetical example. After careful evaluation of all pros and cons, a tissue establishment decided to go for the device of a given company A (DQ). The vendor would not only install the device according to internal specifications but would also provide a

test report showing that all presettings (e.g., timing of vitrification process, temperature) are met (IQ). It would be up to the personnel then to verify the validity of this report (OQ) which could be done by using temperature probes, checking vitrification graphs, taking exact times, and so forth. When the automated vitrification device is ready for clinical application, its performance has to be controlled after a certain period of time or treatment cycles (PQ). If this provisional control reveals that the chosen KPIs (e.g., survival rate of vitrified and warmed embryos, implantation per warmed embryo) are within a targeted acceptance range or are at least as good as the KPIs achieved with non-automated vitrification, the equipment can be considered as qualified.

Likewise, the introduction of new techniques into clinical use should follow a usual pattern if appropriate. In case of the implementation of a completely new method (like lyophilization of sperms would be), the validation process would definitely require more steps as compared to a scenario in which an existing technique is only slightly modified (e.g., artificial blastocoel reduction in expanded blastocysts in order to increase survival rate). Harper et al. [13] have published a proposal on how such a validation of a new technique could look like. In principle, they suggest a sequence of steps involving hypothesis-driven research, testing hypotheses in animal models, translating into human, evaluating results in small-scale single-site trials, expanding to large-scale multi-center clinical trials, and finally assessing the clinical (and cost) effectiveness. As mentioned above, the effort to put up with might vary depending on the degree of novelty of the new technique.

40.4 Donor Recruitment

In the EU as in the majority of all other European countries, MAR treatment is allowed with partner as well as non-partner gametes [14]. Which patients actually can benefit from partner or non-partner donation is regulated in national legislation. Out of 47 European countries, MAR is performed in 44 (94%), with 39 (89%) of them having specific legislation on MAR. The following figures may bring to mind the heterogeneity in national legislation across Europe: donated sperm, donated oocytes, simultaneously donated sperm and eggs, and/or donated embryos can be used for MAR in 41 (93%), 38 (86%), 32 (73%), and 29 (66%), respectively. Thirty countries (68%) offer treatment to single women and 18 (41%) to lesbian couples [14].

Whenever fresh or cryopreserved donated gametes or embryos are used, the principle of voluntary and unpaid donation should be followed. However, expenses “related to the donation can be reimbursed, and loss of earnings may be compensated” [8]. Advertising, on the other hand, is illegal and false or misleading promotion could lead to prosecution.

In terms of donation, tissue establishments should remain non-profit organizations at any time.

It is regulated in the EU that individuals (where treatment is allowed) or couples submitted to MAR have to get proper medical consultation. Several topics have to be touched on: national legislation, withdrawal of consent, chances of success, risks of MAR, description of treatments, and detailed costs to name but a few. In MAR, fully informed written consent for couples using own gametes or embryos (fresh or cryopreserved) is mandatory; in case of the usage of non-partner cells and tissues special care has to be taken that not only both partners but also the donor signs the respective consent forms. Since MAR treatment usually comprises a series of treatment cycles, signatures are either needed for each treatment or should be valid until birth of a baby or a predefined date (national law might regulate that). Particularly, in the case of cryo cycles consent for warming/thawing should be available for each attempted treatment; this would prevent the beginning of a treatment cycle by either of the partners without the knowledge of the other. At the end of treatment, the destiny of cryostored gametes and embryos has to be put down in writing. National law will regulate donation to other couples, donation to scientific research or destruction. In those countries in which posthumous donation is allowed, the fate of remaining gametes and embryos should be specified in advance in case that one partner dies.

In partner donation (conventional IVF or ICSI with own gametes), both partners should be screened before treatment is started. This would involve assessment of ovulation, ovarian reserve, tubal patency, and uterine anomalies in the female partner and at least one diagnostic semen analysis plus screening for genetic abnormalities (in case of azoospermia, cryptozoospermia, or severe oligozoospermia) in the male counterpart. Non-partner donors need a more detailed screening (e.g., including family history). Apart from clinical and psychological examinations, these donors have to face laboratory testing (screening for infectious diseases), blood group typing, genetic testing (karyotype, common recessive genetic mutations, and assessment of sperm quality or ovarian reserve).

40.5 Donor Testing

As mentioned in the previous paragraphs, donor testing is not only relevant for non-partner donation but also for couples who live in an intimate physical relationship. These cautionary measures should minimize the risk to transmission of severe genetic or infectious diseases. Screening for infectious diseases is strictly regulated in the EUTCD [7]. In partner as well as in non-partner donation it is obligatory to screen both partners for anti-HIV-1, anti-HIV-2, HBsAg

(HBV surface antigen) and anti-HBc (HBV core antigen), anti-HCV as well as syphilis. From urine samples additional chlamydia testing by a nucleic acid test/technology (NAT) is required. Other testing is recommended but not obligatory in case one or both partners live or originate from high-prevalence areas for certain viruses (e.g. HTLV-1, Zika virus).

In the EU, there is a fixed period of time in which the results of blood and urine testing is acceptable for the tissue establishments. First, screening in partner donation must be done less than 3 months prior to the first donation (which equals the beginning of the first treatment cycle). The results are then valid for 2 years before testing has to be repeated. In non-partner donation, however, blood testing has to be done “at the time of donation.” After a quarantine of ≥ 180 days after donation, testing has to be redone. Quarantine and retesting can be avoided if at each donation (e.g., if a sperm donor donates multiple times within a given timeframe to increase the number of available samples in the cryobank) serology testing is done with NAT for HBV, HCV, and HIV. Recent literature has shown that at least testing for chlamydia trachomatis (no NAT available) could be done less frequently a fact that will be published in the upcoming version of the EDQM guide [8, 15].

Although for oocyte donation the term “at donation” could be seen more flexible, for example, as the first day of stimulation, reality shows that the competent authorities of most EU countries insist on the fact that blood sampling for infectious diseases has to be done on the day of ovarian puncture [8].

It is important to clarify that any detected seropositivity for the above-mentioned diseases does not exclude the donated gametes and embryos from being used between partners [7]. Of course, a system has to be in place to prevent the risk of contamination (partner and personnel) and cross-contamination (e.g., embryos from other couples stored in the same cryo tank). Such robust processes to minimize the said risk are not only limited to sperm preparation in case that the male partner is seropositive but should also involve separate equipment (e.g., centrifuge, incubator, or incubator segment) and adequate timing [9]. The latter refers to the common behavior to treat seropositive patients as last couple of the day followed by a specific hygienic workup.

40.6 Procurement, Processing, and Cryopreservation

While there is no restriction in the techniques used for the collection, processing, and storage (cryopreservation) of gametes and embryos, it has to be guaranteed that these important steps are performed as determined in the respective standard operation procedures (SOPs) that have to be

established in the frame of an existing quality management system. It is for example not regulated whether slow freezing or vitrification is used in order to cryopreserve cells and tissues although good clinical practice would indicate that vitrification is the means of choice (not necessarily valid for sperm, zygotes, and gonadal tissue). Likewise, there is no obligation to vitrify using a closed system and open system carriers can equally be approached.

EUTCD regulates air quality in rooms where tissues and cells are processed (e.g., IVF, ICSI) and distributed (e.g., fresh or frozen embryo transfer). “When these activities include processing of tissues and cells while exposed to the environment, an air quality of Grade A” is required. This air quality refers to the flow bench in use and not to ambient air in the room (which has to have a quality of Grade D). However, for processes in which Grade A environment would be detrimental (e.g., oocyte collection, insemination of oocytes) due to cooling effects, a less stringent environment is specified [1, 16, 17].

These requirements generally improved the overall results since optimal air quality provides cells and tissues a stable environment. Recent data indicate that outdoor air and chemical pollution adversely affect IVF results. Several studies have reported decreased rates of clinical pregnancies in IVF cycles; however, threshold values of Volatile Organic Compounds (VOCs) should be established. Temperature, humidity, and pressure should be maintained in between optimal range and air quality should be monitored periodically by swabbing and by analyzing air samples. Critical parameters must be monitored and recorded to demonstrate compliance with specified storage conditions [16, 18].

The Cairo Consensus recommends technical and operational requirements as well as aspirational benchmarks for air quality in IVF laboratories. Current directives have different specifications regarding air quality control; the EUTCD focuses on particulate air only and meanwhile the Brazilian directive focuses on VOCs in addition to particulate air. Specific regulations mandating air quality control are limited, and solid evidence is still lacking regarding the thresholds and quality requirements. Hence, most relevant contaminants affecting indoor air quality and aspirational benchmarks should be identified [1, 16, 17].

A HVAC-system (heating, ventilation, and air conditioning) is the main unit providing acceptable indoor air quality. In addition, HEPA-filtration (air pressure, circulating air, high-efficiency particulate air), ULPA-filtration (ultra-low particulate air), and VOC-filtration (Volatile Organic Compounds) are main parts of air filtration systems within critical areas. An effective air filtration system is essential to constantly meet key performance indicators. Removal of air particles is achieved with the use of HEPA filters which is designed to eliminate 99.9% particles greater than $0.3 \mu\text{m}$ (e.g., bacteria, mold spores); however, HEPA-filtration is not

capable of reducing VOCs. On the other hand, VOCs, which are constantly generated by materials and cleaning products used in the laboratory, are mostly removed by chemical filtration with activated carbon and potassium permanganate. Several air cleaning solutions are commercially available with variable efficiency to control air quality. There are several aspects affecting the air quality, including outdoor ambient pollution, size of the laboratory, number of staff working, furniture used, and laboratory equipment [16]. Positive pressure and HEPA filters are common in IVF laboratories processing cells and tissues; however, there is no consensus and guidelines may vary between different countries.

40.7 Storage

There is no doubt that gametes, embryos, and gonadal tissue, if cryopreserved and stored under controlled (and appropriate) conditions do not deteriorate even after long-time storage; hence, in principle this is possible but national legislation may declare a maximum storage period.

As for the other processing techniques in cryopreservation, no structured details on storage limits, storage temperature, and storage devices are mandatory as long as it is guaranteed that all processes follow the internal SOPs which should be based on good clinical practice.

Evidence that cross-contamination between seronegative and -positive embryos stored in the same vessel is lacking [19, 20]. Indeed, the virus load in culture media or liquid nitrogen was undetectable after warming of blastocysts of seropositivity [20]. However, due to the mere fact that viral and microbiological pathogens can survive in liquid nitrogen, the EUTCD is very clear in its phrasing. In detail, storage of gametes or embryos of sero- or chlamydia-positive patients/couples have to be kept in a separate container [7, 21]. Depending on the respective national competent authorities, this could—in extreme—lead to the scenario that every single infectious disease (or any combination of the same) would need its own separate cryo tank. In case that results from blood testing are not available at the time of storage (e.g., in case of acute fertility protection measures), a quarantine tank has to be used. Cleaning of cryo containers is not regulated by the EUTCD but recommended [21].

40.8 Traceability and Coding

Tissue establishments in the EU have to guarantee the traceability of human cells and tissues but is not limited to those. It is a fundamental prerequisite to verify compliance with safety/quality standards and appears also relevant for media, consumables, medication, donors, and recipients (both partner and non-partner).

According to the EUTCD and the EDQM guide, all media and consumables have to be fit for purpose which usually means that only CE-marked products should be used and patient- or donor-derived supplements (e.g. maternal serum as protein source) have to be avoided. Traceability shall apply to lot numbers, expiry dates, and other relevant data of all media/consumables coming into contact with cells and tissues. To be more precise, these records should include all consumable material from donation (e.g., sperm collection cup) over processing (e.g., hyaluronidase, ICSI needle) to storage and distribution (e.g., vitrification carrier, freezing kit, transfer catheter). Of note, records (paper or in electronic form) have to be archived for 30 years after clinical use [7, 8].

Traceability should be developed further in all processes from procurement to distribution of cells/tissues (including import and export). All consumables (e.g., sperm cup, culture dish, vitrification device) have to be labeled (permanently) and have to undergo witnessing steps in order to prevent the lab staff from mixing up samples. It is, however, not specified whether a well-defined 4-eye-principle would do or if a barcode- or radiofrequency-based system should be applied. Key processes, such as oocyte collection, insemination, embryo transfer, or warming of vitrified blastocysts, would require double witnessing [22]. The EDQM guide stresses that each tissue establishment should have an accurate and effective system in place that will allow to uniquely identify, trace, and locate cells and tissues during each individual step of the treatment. Such an identification program should ensure that the main information of donors (partner and non-partner) and their respective reproductive cells and tissues are available at all times [8].

Sperms, oocytes, zygotes, and embryos from non-partner donation require specific coding for those countries that are regulated according to EU directives. It is important to highlight that the urge to align with traceability and biovigilance requirements should not compromise the demand in non-partner donation to guarantee anonymity between recipient and donor and vice versa (also valid for newborns). Of course this has to strictly comply with national legislation.

Tissue establishments will automatically keep internal records of donors, the donation itself and the donated cells or tissues so that it is ensured that all of which is identifiable and uniquely labeled. Problems (e.g., the risk of file duplication) may arise, however, once gametes or embryos are moved from one clinic to another. Definitely, applying global standards to the coding of cells or tissues would minimize if not eliminate such a risk of identification failure. Within the EU, it was decided to go for the Single European Code (SEC) which allows to incorporate or combine both existing codes the ISBT 128 system and the Eurocode [23, 24].

The implementation of the said coding system into MAR centers seemed to be quite a challenge. A SWAT analysis

performed by the ESHRE Special Interest Group Safety and Quality in ART revealed that it has obviously strengths (guaranteed traceability, uniformity in the identification coding, ease of generation), but they also recognized several weaknesses, such as the incompatibility with some codes already used, time-consuming transcription, or the length of the SEC [25].

The SEC consists of two elements: a donation (origin of cells/tissues, tissue establishment code, internal 13-digit donation number) and a product identification sequence (type of cells/tissues, internal product code with identifier of the coding system in use). There are some exceptions which do not require the application of the SEC in order to prevent from impracticality (e.g., cells/tissues considered for partner donation or if these remain in the same tissue establishment). Exports within and from the EU have to be accompanied by the SEC and must only be managed by tissue establishments certified by the national competent authorities.

40.9 Serious Adverse Reactions and Events

All deviations from SOPs that might have a negative effect on the quality and safety of cells/tissues should result in “serious adverse reactions and events” reporting (SARE) to the national competent authorities. Examples of real-life SARE reporting can be found in the Notify Library (www.notifylibrary.org) or in the EDQM guide [8].

Serious adverse events (SAEs) can be experienced at literally every step of clinical or laboratory processes. As an example, SAE reports could deal with the loss or mix-up of gametes and embryos, embryonic arrest due to the usage of an expired culture medium or the leakage of a cryo tank. It is not meant to report every single damaged oocyte after ICSI.

Serious adverse reactions (SARs), on the other hand, are all those events that potentially affect the donor or recipient. Usually, these reactions would be related to procurement (e.g., oocyte collection) and distribution of cells and tissues (e.g., insemination, embryo transfer). Typical SAR reports record cases of OHSS, vaginal bleeding, ovarian torsion or infection but are not limited to these. Even the birth of a child with a genetic disease after gamete donation would come within this category.

40.10 Conclusion

It has to be admitted that the EUTCD with all its directives and amendments regulates nearly all aspects within the course of a fertility treatment or fertility protection without intervening with national legislation. There is no doubt that the implementation of Directive 2004/23/EC raised the qual-

ity in European tissue establishments by simply insisting on state-of-the-art SOPs within the frame of an appropriate quality management system [1].

Also, biannual inspections by the national competent authorities helped to improve the performance of the tissue establishments although it should be mentioned that these inspections had to go through a process of settling-in. At the beginning (in 2008), audits were characterized by an atmosphere of intolerance. On the one hand, inspectors were neither trained nor aware of the challenges of MAR, and on the other hand, the personnel working in IVF clinics was not willing to accept changes to processes that worked well in their experience. With increasing training and knowledge of the inspectors and the noticeable effect of their measures pushed through, the situation improved somewhat and meanwhile it can be called a fruitful co-existence.

However, soon after execution of Directive 2004/23/EC it could be realized that certain provisions that might be relevant for areas like transplantation medicine are impracticable or in worst case not valid for MAR (e.g., size of the SEC does not fit on dishes, validity of blood test results, air quality during processing). This led to amendments of some of the Directives and in general to an improvement of the situation. Still some shortcomings, particularly valid for MAR (e.g., transgender and same sex-couples not mentioned, no EU-wide registry on MAR treatments and non-partner donors, no harmonization of inspection's quality, little involvement of MAR experts, no full disclosure of new or modified reagents), are obvious and it is much to be hoped that ESHRE's position paper will be noted and well reflected in the upcoming new edition of the EDQM [1].

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Ethical Issues Related to Cryopreserved Reproductive Specimen

41

Heidi Mertes

Key Points

- There are strong ethical reasons to make fertility preservation measures available to those whose fertility is threatened and who consider parenthood to be an important part of “the good life” and of their desired life trajectory.
- While doing so, there are, however, a number of elements that require careful consideration in order to ensure that an intervention aimed at improving people’s quality of life does not result in harm, undue pressure, or injustice.
- As is the case in all medical decision-making, it is important that patients are informed in a complete and unbiased manner and that the values and desires of the patients are respected, even if they conflict with societal norms or physicians’ opinions.

41.1 Introduction: Why the Appeal of Freezing Reproductive Specimen?

When making ethical analyses of new technologies, we often-times resort to balancing moral pros and cons. In reproductive medicine, these pros and cons mostly align with benefits in terms of respecting people’s reproductive autonomy and disadvantages in terms of risks to either the patients or the resulting offspring, contravening the obligation of medical professionals to do no harm. When moving away from the micro-scale of the specific patient, towards the macro-scale of society, distributive, and social justice issues also need to be taken into consideration: can we justify spending health care resources on fertility preservation and will we be able to obtain equity in access to cryopreservation of reproductive material?

Before focussing on the potential ethical hurdles, let us first have a look at the benefits of cryopreservation for those

whose tissue is stored, in order to get a clear view on what weight is on that side of the balance. Freezing of reproductive tissue and gametes is directed at fulfilling the desire for genetic parenthood. As this desire is widespread in the general population (e.g. [1–4]), the importance of being able to reproduce is seldom questioned. Moreover, the suffering that accompanies infertility and infertility treatment is well documented [5–7]. In that sense, the benefits in terms of patient wellbeing are clear and will easily justify minor risks to the patient or offspring. Yet, for those applications in which risks or other ethical concerns are more prominent, a couple of arguments could be brought forward for why we should not take for granted that (retaining the option of) having genetic offspring legitimises all forms of fertility preservation.

First and foremost, it is important to keep in mind that not everyone has a desire for genetic parenthood and that therefore we cannot assume that reproductive specimen need to be cryopreserved for all those whose fertility is endangered. Especially infertility specialists will have to be conscious of the fact the patients they commonly see in the fertility clinic are not representative of the broader population but is a pre-selected sample of people with a clear desire for genetic parenthood. If a young cancer patient signals that she does not want to cryopreserve oocytes or ovaries despite being scheduled for gonadotoxic treatment, she is therefore not necessarily misguided. Younger generations are increasingly indicating that they have no desire for parenthood [8] and the group of voluntarily childless people—although still a minority—is steadily growing [9].

Second, we might even—somewhat controversially—state that those who think that parenthood will increase their wellbeing are more likely to be misguided, as empirical research does not support this widespread belief for the average person [10]. This observation may lead to the argument that rather than offering people invasive, burdensome, and/or expensive fertility preservation options to those with a moderate desire for parenthood, they would be better off with psychological counselling.

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For the purpose of this chapter, it suffices to say that respect for a person's reproductive autonomy is one of the key factors in making sure that cryopreservation of reproductive tissue is performed in the most ethical way. Applied to the first point (about not everyone having a desire for genetic parenthood), this means that if patients decide not to cryopreserve tissue or gametes after being well-informed and after thorough deliberation, this decision should be respected. The condition of being well-informed does imply that physicians discuss the different options with the patient, but in a non-directive way. For relatively mild and non-burdensome interventions (e.g. sperm freezing), we might imagine that patients who have no current desire to have children originally decide not to cryopreserve sperm, but after being informed about the precise procedure and price nevertheless decide that they might want to bank sperm on the off chance their desire changes in the future. Applied to the second point (that parenthood does not necessarily improve wellbeing), respect for reproductive autonomy implies that as long as the risks are within reasonable limits, we should respect people's desire for parenthood, even if empirical research suggests that those desires are irrational [11].

In some instances, somewhat surprisingly, people opt for cryopreservation of reproductive cells or tissue although they do not have a strong desire for parenthood (but are rather ambivalent), and despite the fact that the cryopreservation procedure entails physical and/or financial burdens. For example in the context of sperm and oocyte cryopreservation, this has been reported [12–14]. Such decisions can be driven by “anticipated decision regret”, in the sense that people take into account that although they do not currently have a strong desire for parenthood, they foresee that this may change in the future, and do not want to limit their future options. Whether this is a smart way of dealing with uncertainties, or a sign of how people internalise the societal discourse around the importance of parenthood and the common belief that it is just a matter of time before the desire for parenthood will kick in [15], remains open for debate.

Also in the context of paediatrics, one attempts to take (currently unpredictable) future desires into account. The “open future argument” can be invoked to argue for an *a priori* preference to undertake fertility preservation measures when possible. The “right to an open future” [16] refers to the fact that children have certain “rights in trust”; rights which they cannot yet exercise as they are not yet competent, but which they will be able to exercise once they reach adulthood. When others (typically parents) make decisions regarding the child that restrict its future options in life, they are as it were closing down potential paths although it should be up to the child—once it reaches adulthood—to make a decision about which path to follow. Therefore, if too many

paths are closed down, their future autonomy—and thus their right to an open future—is not respected. Bringing this argument back to cryopreservation of reproductive tissue, it can be argued that as reproduction is an important path in many people's lives, a refusal by parents to cryopreserve reproductive tissue is an infringement against the child's right to an open future. Again, this is an argument that needs to be contextualised as it is not absolute, but one of several considerations. For example, if parents of an adolescent boy with a desire for parenthood who is sure to lose his fertility after a particular type of cancer treatment, refuse to consent to banking sperm (e.g. because they object to masturbation), this argument will carry more weight than when parents of a toddler refuse to consent to testicular tissue cryopreservation, given that in the latter case the procedure is much more invasive, experimental, and there is no information about the preferences of the child.

Besides respect for reproductive autonomy and keeping future options open to ensure that autonomy can be exercised, there are also more practical reasons that can explain the appeal of freezing reproductive specimen. Embryo freezing, for example, is done to avoid repeated oocyte pick-ups and research into oocyte cryopreservation got a sudden boost around 2004, when freezing embryos was forbidden by law in Italy. Also, it is remarkable that the appeal of freezing reproductive tissues appears to be greater when it is aimed at remedying iatrogenic subfertility, rather than “natural occurring” subfertility. This became clear in the very different discourses around oocyte cryopreservation in the context of oncofertility versus the context of women wanting to take preventive action in the face of age-related fertility decline [17, 18]. Whereas the procedure was welcomed in the former context, it was heavily scrutinised in the latter although it is far from evident that there is a moral distinction justifying such differential perception.

41.2 First, Do No Harm

Having outlined the potential benefits of cryopreservation of reproductive tissue, we now need to assess the potential harms and disadvantages, in order to be able to check under which circumstances the harms would outweigh the benefits and thus render cryopreservation undesirable or even immoral. Setting aside more general societal concerns and feminist objections regarding the reinforcement of pronatalist and traditional family discourse by stressing the importance of fertility preservation, there are at least two parties that may experience more direct harm as a result of fertility preservation efforts: (a) the person whose tissue is being cryopreserved (whom I will call the “patient” as a shorthand although they are not necessarily ill), and (b) offspring resulting from the cryopreserved tissue.

41.2.1 Potential Harms to the Patient

In adult patients, the most discussed concern is related to a postponement of urgent treatment for female cancer patients, with conventional ovarian stimulation delaying cancer treatment 2–6 weeks [19]. Although the development of random-start ovarian stimulation (with comparable results to conventional ovarian stimulation protocols) and *in vitro* maturation of oocytes have reduced this risk, oocyte cryopreservation still comes with a delay, especially when back-to-back stimulation cycles are performed in order to increase the number of oocytes obtained. The removal of ovarian or testicular tissue causes less delay of treatment, but it is a more invasive procedure and involves a risk of reintroduction of malignant cells. The latter concern is mainly relevant for leukaemia, non-Hodgkin's lymphoma, and ovarian cancer. The former concern can be mitigated by combining tissue removal with another surgical procedure, so that it does not require an additional anaesthesia, but standard risks of surgical procedures such as infections and other surgical complications remain.

A second category of concerns relates to specific patient categories, in whom establishing a pregnancy may come with more risks than in others. For example, pregnancies in Turner patients are considered to be high risk, due to cardiovascular anomalies and particularly the increased prevalence of aortic dissection (which is most often lethal) in this population. While this need not mean that all Turner patients should be excluded from fertility preservation, it does plead for a thorough and multidisciplinary risk assessment, charting the individual risks for a particular patient [20].

In the context of prepubertal and peripubertal patients, it should be taken into account that the chances of success in restoring fertility after cryopreservation of reproductive tissue remain uncertain, which makes it more difficult to justify the surgical procedure to remove tissue. Also, when tissue is removed, it is of utmost importance to clearly communicate to patients and/or their parents or guardians what the tissue will be used for (research or potentially treatment). As previously argued, there is a danger that in the paediatric context, where storing gametes is oftentimes impossible before starting gonadotoxic treatment, an imperative for freezing tissue arises, as it is presented as “the only option” of preserving a child's fertility [21]. However, this is only true for those patients whose treatment or condition will most certainly lead to immediate sterility, while many patients will have a low or medium risk of sterility. For this group, careful monitoring of reproductive potential (e.g. ovarian reserve), with the possibility of banking gametes once they reach adulthood may be a more appropriate course of action, depending of course on the individual context.

A general concern for all categories above, and also for women cryopreserving oocytes for age-related fertility

decline, is that patient expectations may not be well aligned with reality and that potential risks and limitations may be downplayed. Several phenomena could be at play here. First, the therapeutic misconception may lead parents of young cancer patients to believe that the primary use of removed tissue will be the treatment of their children once they become adults, whereas at the time of writing, human prepubertal testicular tissue has only been used for research, not for treatments. Second, the so-called technological imperative can induce people to use medical technologies (such as cryopreservation) because they are available, rather than because there is a clear need. The already-mentioned psychological mechanism of anticipated decision regret can add to that. All of these mechanisms create a context in which people can be easily persuaded to cryopreserve reproductive specimen, even when that is not in their best interest. For example, cryopreservation of oocytes for age-related fertility decline can be a welcome plan B for women who currently still have a reserve of good quality oocytes, but fear subfertility by the time they find a partner to embark on parenthood. However, marketing strategies selling oocyte cryopreservation as a plan A to postpone parenthood without mentioning the limited utility and efficiency rates are doing a disservice to women's reproductive autonomy [22].

41.2.2 Potential Harms to Offspring

Although well integrated into fertility clinics worldwide, vitrification of reproductive tissue is a relatively recent procedure. Several systematic reviews and meta-analyses have shown reassuring data regarding feared cryogenic damage in embryos, oocytes, and sperm [23–25], but continued follow-up of offspring is necessary, as is more research into the effects of vitrification on ovarian and testicular tissue.

An important ethical consideration is that cryopreservation of reproductive tissue always involves a three-step process: harvesting the tissue, storing the tissue, and using the tissue. Sometimes, tissues or cells are cryopreserved based on the hope of being able to retransplant the tissue or extract viable cells from them (which may need to be matured *in vitro*) although at the time of freezing these treatment options are not yet possible. A foreseeable problem is that patients may return to use their reproductive tissue before there are enough reassuring preclinical trial data available to warrant the step to clinical trials—let alone widespread introduction into the clinic. In such circumstances, the fertility specialists in whose hands the patients have placed their trust, may be torn by two irreconcilable responsibilities: the responsibility to ensure that a treatment is safe and effective before introduction into the clinic, and the responsibility to (try to) restore the patient's fertility. For example, if a male childhood cancer survivor would desire to become a genetic

parent through using his cryopreserved testicular tissue, the most promising approach may be to regraft tissue, spermatogonial stem cells (SSCs), or PGCLCs. However, in such cases fertilisation may occur *in vivo*, making it impossible to check the embryo for (epigenetic) abnormalities and thus potentially leading to the birth of children with congenital abnormalities. As long as there are no (sufficiently) safe and effective treatment options available for patients, it is therefore of utmost importance to communicate this well to the patients from the onset and not to confuse hope and reality when talking about potential future options, so that patients do not feel abandoned or deceived when there is in fact no treatment option when they return to use their cryopreserved tissue.

41.3 Pitfalls in Informed Consent

An ethical implementation of a procedure not only requires an appropriate risk-benefit balance, to satisfy the condition of proportionality, it also requires due respect for patient autonomy and on a wider scale it needs to be in line with the so-called distributive justice: there should be a fair balance between risks and benefits in society. In this section, an important aspect of respect for patient autonomy, namely informed consent, will be discussed, as it is particularly challenging in many decisions regarding cryopreservation of reproductive tissue. The traditional requirements of a valid informed consent are that the patient (a) is sufficiently informed, (b) understands the information, (c) consents or declines voluntarily, free from external pressure, and (d) is competent. The contexts in which reproductive tissue is cryopreserved, pose challenges for each of these requirements. These challenges do not necessarily render an informed consent impossible but warrant vigilance.

41.3.1 Sufficiently Informing the Patient

Several studies have documented that many patients having undergone treatments affecting their fertility feel that their information needs were not met (e.g. [26–30]). Although this kind of research is always backward looking and therefore reporting on the past, rather than the present, it is important to learn from these studies and commentaries that there is a desire to be at least informed about fertility preservation options, even if one does not eventually decide to freeze reproductive tissue.

Counselling about fertility preservation is however not an easy task. In order to make a well-deliberated decision about whether or not to cryopreserve reproductive tissue, there are many different aspects to consider: the risk of subfertility or infertility (when will reproductive potential be decreased by

how much?); the risk of complications due to the harvesting of tissue; the impact on other treatment (e.g. for cancer patients); risks associated with using the tissue (e.g. reintroduction of cancer); the success rates after cryopreservation (tailored to the specific context of the patient); the chance of needing the reproductive tissue in the future (dependent on survival rates in oncological patients, age, reproductive aspirations,...); the cost of the procedure; the experimental, innovative, or established status of the procedure; different uses of the tissue (research, treatment, donation); the expertise of the treating physician; the options for posthumous use; etc. For some patients, all of this information will need to be digested *on top of* other disease-related information. Any fertility preservation measure will therefore need to foresee sufficient time for multidisciplinary consultations in which the patient is supported in assessing and weighing all this information.

41.3.2 Sufficient Understanding by the Patient

The potential problem of the therapeutic misconception (see above) and the limited time available to make a decision about whether or not to cryopreserve tissue may both hinder sufficient understanding by the patient. Also, patients may filter information to avoid cognitive dissonance. For example, transmen and transwomen may be less receptive to information about fertility preservation because it would delay their transition, while cancer patients may be blind for the risks of the cryopreservation procedure if they have a very strong desire to have children, or blind for the benefits if they are so overwhelmed by their cancer diagnosis that they cannot take in any additional information.

But there is another issue here, which has been less discussed in the specific context of fertility preservation. Not only the multitude of information and contextual constraints makes it challenging for patients to understand everything, but also the kind of information. Many of the items mentioned in the previous section involve risks and probabilities. These are hard to quantify and certainly to compare and weigh. Even if some risks can be quantified, it is a well-known phenomenon that risk perception is easily distorted by heuristics and other psychological mechanisms. Rationality is easily outpaced by intuition when processing risk information, leading to suboptimal decision-making by patients [31, 32]. While this phenomenon has not been extensively studied in the specific context of fertility preservation or even medically assisted reproduction, there is a lot these fields can learn from, for example, findings in the context of risk communication and risk assessment during pregnancy [33]. Jordan and Murphy have, for example, warned that “[r]isk communication patterns can lead a woman to a health care provider-desired decision”, leading to what they call

informed compliance, rather than *informed consent* [34]. Several tools and tips for the communication of risks in medical practice (countering potential distortions) are available (e.g. [35]). For example, it is advised that practitioners provide numerical data on risks, rather than vague terms such as “high risk” or “low risk”; provide the patient with absolute risks, rather than relative risks; state the odds from both a positive (e.g. chances of success) and a negative (e.g. chances of failure) perspective; use a consistent denominator (e.g. explain all risks/probabilities as a chance per 1000 patients, rather than a chance of “one in ...”) and very importantly: use visual aids [35–37]. All of these will increase understanding for the patient and hopefully lead to decisions in line with both the best information available and their personal values.

41.3.3 Voluntary Consent or Decline

This brings us to the third element of informed consent: the requirement that decisions are made free from outside pressure. First of all, it is important to stress that autonomous decision-making does not happen in a vacuum (arguably this is particularly the case for reproductive decision-making). In fact, it is oftentimes by discussing medical decisions with friends and family, that one comes to a well-deliberated decision in line with one’s own values and desires. In this sense, the concept of *relational autonomy*, rather than *individual autonomy* is what is to be pursued in the context of decision-making regarding cryopreservation of reproductive tissue [38]. However, while relational autonomy is considered to be an essential component of patient-centred care by some [39], others have pointed out that we should be careful not to conflate relational autonomy with undue pressure by family members [40]. The latter is certainly a possibility to be wary of in the specific context of fertility preservation, as reproductive decisions also affect other family members. Again, it will be very important to engage in thorough counselling of the patient to make sure that they are not pressured into undergoing or forgoing fertility preservation measures by their family members.

There are two additional potential sources of undue pressure: treating health care professionals and wider societal stereotypes and expectations. Both categories can exert pressure in either direction: towards or away from cryopreservation. Each health care professional will have their own opinion about the importance of (genetic) parenthood and thus the importance of preserving fertility through cryopreservation of reproductive specimen. Also, each will have a personal opinion about whether specific categories of people should be trying to preserve their fertility through tissue or gamete cryopreservation, for example, woman after their

natural reproductive lifespan, transmen and transwomen or people with a certain health condition. And third, each will look at the patient through the lens of their own specialty: while a paediatric oncologist may be reluctant to postpone treatment and subject the patient to an (additional) invasive surgery because they are worried about the impact on survival and short-term wellbeing, a fertility specialist meeting the same patient may be very adamant about the importance of offering tissue freezing because they in turn worry about the long-term wellbeing of cancer survivors who turn out to be infertile. While one cannot expect (or even hope for) the health care professional to be completely value-free when considering the best treatment plan for their patients, it is important that those impacting on the patient’s decision are well aware of their own beliefs, biases, and prejudices, so that they can prevent a negative impact on patient-centred care. Interdisciplinary consultation and collaboration can be an important help in this regard.

An important external source of pressure, which may be internalised by patients, are stereotypes and expectations that are widespread in society, for example, the expectation that long-term romantic relationships result in parenthood [41]. The empirical finding that many people want to have (genetically related) children leads to the expectation that people whose fertility is threatened will want to engage in fertility preservation, and sometimes even to a normative claim that people should have—or at least should want to have—children. If a patient without a desire for parenthood is confronted with these expectations, they may find themselves in a position where they need to justify why they do *not* wish to undergo cryopreservation of reproductive tissue, rather than the opposite. As mentioned above, it is important to also respect choices that are not in line with mainstream opinions regarding the importance of parenthood. A category of potential patients less likely to experience pressure towards cryopreservation are women considering freezing oocytes in anticipation of age-related fertility decline. Interestingly, although the desire for parenthood can be as powerful and as legitimate for a 37-year-old woman without medical problems (but fearing infertility due to her age) as for a 27-year-old woman about to undergo gonadotoxic cancer treatment, the possibility of cryopreserving oocytes generated a much more animated academic and societal debate when it was presented as a solution for age-related fertility decline than when it was presented as a solution for gonadotoxic treatment. Also in other contexts, there appears to be a strong negative attitude towards motherhood at a (reproductively speaking) advanced age [42]. When such attitudes are also present in those involved in counselling or supporting those who consider cryopreserving oocytes, they may exert an undue influence on the decision-making process.

41.3.4 Competence

Especially for children and young adults, there may be concerns regarding competence. Different jurisdictions have different ways of delineating when a minor is competent to consent to treatment or decline treatment. A general consideration, however, is that competence is not a black-and-white concept that is absent before a certain age and fully present after that age although legally specific cut-offs are often-times made. It is therefore important to also involve (legally) incompetent patients in the decision-making process [43]. By explaining the important elements of a fertility preservation intervention in language that is comprehensible for children, they may agree—or “assent”—to it, while the formal consent will need to be provided by the parents.

41.4 Priority Setting in Healthcare: Distributive Justice Issues

As cryopreservation of reproductive cells and tissues becomes more widespread, it will be important to follow up on utility rates: which percentage of the patients who cryopreserve reproductive tissue return to use it, and how many of them obtain their goal of achieving parenthood through their cryopreserved tissue? This information is important in order to better inform future patients about the benefits they can expect from freezing reproductive tissue. It will also help in establishing better inclusion criteria, so that the interventions are proposed to those most likely to benefit. Third, insight into the utility will allow for a better assessment of healthcare spending. Although coverage of fertility preservation through public or private health insurance is largely uncontroversial in the setting of, for example, oncofertility, this is not the case for oocyte cryopreservation for age-related fertility decline. As mentioned, the need for fertility preservation is assessed quite differently in that context due to socio-cultural norms, but also the utility is more questionable, in the sense that there is a very small window of opportunity between the moment when women are considered too young to cryopreserve oocytes (because they still have a reasonable chance of finding Mister Right in time) and the moment at which they are considered too old (because the quality of their oocytes has already diminished substantially). Also, the number of years women have between the time of freezing and the time they reach the upper age limit for embryo transfer is limited. While arguably not reimbursing egg freezing for age-related fertility decline, but reimbursing it for the so-called medical indications is a form of injustice, there may be reasons of limited utility that might justify this distinction although it should then also apply to other applications with low utility.

Besides the perceived injustice between those cryopreserving oocytes for medical versus age-related reasons, not covering age-related egg freezing by health insurance also creates injustice within the group of women who might benefit from this intervention, as it renders the option of oocyte cryopreservation only available to those with sufficient financial means [44–46]. However, as noted by Pennings [47] and De Proost [48], funding would mainly benefit a segment of society that is already—overall—a privileged group. If not through public health insurance, then what about coverage by private health insurance? Also there, red flags have been raised over silicon valley companies including egg freezing in their health insurance package, which may be perceived as limiting women’s reproductive autonomy, rather than increasing it [49].

41.5 Conclusion

In this chapter, roughly following the four traditional principles in biomedical ethics (beneficence, non-maleficence, respect for autonomy and justice), it is argued (1) that the potential benefits of cryopreservation of reproductive specimen in terms of patient wellbeing are important, given the importance many people attribute to (genetic) parenthood, (2) that there are, however, a number of potential harms, both for those who cryopreserve reproductive tissue and gametes and for those who result from it, (3) that obtaining a valid informed consent can be challenging, and (4) that considerations regarding distributive justice complicate discussions about reimbursement of certain fertility preservation measures.

Author’s Perspectives

- Key Points
 - There are strong ethical reasons to make fertility preservation measures available to those whose fertility is threatened and who consider parenthood to be an important part of ‘the good life’ and of their desired life trajectory.
 - While doing so, there are, however, a number of elements that require careful consideration in order to ensure that an intervention aimed at improving people’s quality of life does not result in harm, undue pressure, or injustice.
 - As is the case in all medical decision-making, it is important that patients are informed in a complete and unbiased manner and that the values and desires of the patients are respected, even if they conflict with societal norms or physicians’ opinions.
- Potential Areas of Research
 - Follow-up of patients (a) who decided to cryopreserve reproductive specimen, (b) who declined cryopreser-

vation, and (c) who were not offered cryopreservation in order to get a clearer view on the extent to which cryopreservation increases people's reproductive autonomy and increases their overall wellbeing, or the lack of cryopreservation decreases them.

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Legal Liability Landscape and the Person/Property Divide

42

Judy E. Stern and Susan B. Apel

42.1 Introduction

Freezing of sperm, embryos, and oocytes has become commonplace at IVF clinics. Nevertheless, the process of freezing gametes and embryos brings with it a host of legal complications that require planning and attention even before the process of any assisted reproduction including cryopreservation takes place. Clinics need policies and procedures for addressing the issues of long-term storage, decision-making, abandonment, loss, and parenthood in situations that can become complex and challenging. Consent forms need to conform to the current legal landscape and specific laws within different US states or countries. Staff need to understand these complexities before obtaining informed consent from patients and long before potential complications arise. Clinics should also consider advising patients to consult legal counsel before, rather than after, procedures take place.

In this chapter, we outline the major legal issues associated with cryopreservation of gametes and embryos. Because they are more complex, we focus primarily on embryo cryopreservation, however, issues for sperm and oocytes are also addressed. Over the years, there have been a variety of legal cases, the resolution of which have at times taken the field in widely different directions. We have thought about and taught about these issues for many years. In all that time, while the technology has changed, the legal issues have not and many of the same questions that we had when the techniques were first introduced persist today. In this chapter, we will attempt to address the underlying issues and to make suggestions based on our current knowledge. This is a template only, we cannot and will not resolve any of the many issues. Nothing in this chapter constitutes legal advice.

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The major topics we will cover below include the property/personhood debate and the status of frozen embryos, what to do with abandoned embryos, mishandling and loss of frozen embryos, freezing of gametes, what constitutes parenthood and the legal complications for procreation by same sex couples, posthumous reproduction, and genetic diagnosis. We end with discussion of the informed consent process as it relates to cryopreservation.

42.2 Frozen Embryos: The Property/ Personhood Debate

Embryos are formed from the fertilization of a single oocyte by a single sperm. As such, for a heterosexual couple using their own gametes, an embryo has genetic material from two adults each of whom has a stake in the resultant embryo. A preimplantation embryo is a collection of cells with the unique potential to develop into a human being. In contrast to a fetus, which is maintained within a woman's body, a preimplantation embryo during IVF is maintained either in a laboratory dish or cryostorage device and is separate from the body needed to gestate it to full potential. Because of this, embryos hold an unusual status within the law. As defined in *Davis v. Davis*, the first divorce case in the US to question the disposition of frozen embryos, embryos are "neither property nor persons but deserving of special respect" [1].

The *Davis v. Davis* divorce case and subsequent cases that have debated disposition of preimplantation embryos [1–6] have grappled with the definition of an embryo and how best to adjudicate conflicts over who should have control of frozen embryos. Defining embryos as property in a divorce case would mean that they could be divided equally between husband and wife according to preexisting law, fairness, and equity. Defining embryos as persons, specifically potential children, would give them different consideration including where they can best be cared for and nurtured and in this case whether they should be discarded or transferred to a uterus to

continue development. In the *Davis v. Davis* divorce case, and other subsequent cases, the debate as to whether the embryos should be destroyed or given the opportunity to become persons has been central.

Multiple factors enter into consideration as to the disposition of frozen embryos. One of these is whether a person having undergone IVF has the subsequent right to refuse to be a parent if that person doesn't want to be one. This is not as simple a question as it might at first seem because, in the course of undergoing the IVF procedure to begin with, a person originally entered into a decision to attempt to become a parent. Nevertheless, at the time of a divorce, these decisions are complicated by the fact that the couple will no longer be living together and working to raise a child as a team. On the other side of the issue is the possibility that the person who wants to transfer the embryos and bring them to term may have no other option than use of these embryos to become a biological parent. This could result from increasing age in women or a history of cancer or other medical condition that impaired fertility. Also of consideration if the embryos are transferred, is whether a person can be compelled to be a parent against their will. Either side of this question could be experienced by either males or females.

Questions have arisen about how courts would make decisions about frozen embryos. One early hypothesis posited that because IVF involves a considerable physical effort on the part of a woman including multiple diagnostic tests, controlled ovarian hyperstimulation, and egg retrieval while the man often does little more than masturbate a semen sample, decisions would be made in her favor, a hypothesis known as "sweat equity" [7, 8]. This idea has not panned out. In part this may be because some men undergo testicular biopsy or epididymal aspiration to retrieve sperm which results in their increased physical involvement. In part it has also been found that both men and women have significant psychological involvement in, and emotional reaction to, the IVF process [9, 10]. Instead, although legal cases have pinballed back and forth between different outcomes, most of these cases have been decided for the partner who doesn't want to procreate [1, 2, 5] and only in a minority of situations for the partner who wants the embryos transferred and brought to term [6]. In those latter cases, the court appeared to be persuaded by the fact that there were no other alternatives for the person (generally the woman) who wanted to procreate.

Underlying much of the decision-making has been consideration of whether the embryos should be considered property or persons. These considerations may also be influenced by state laws, as for example in Arizona, which recently enacted a law as a result of a 2014 frozen embryo case that directs judges to grant the embryos in a divorce case to the partner who will allow them to be "born" regardless of prior preferences expressed by the couple [11, 12]. As of this writing, this law has not been challenged.

42.3 Frozen Embryos: Abandoned Embryos

There are several options for the disposition of embryos that have been frozen as part of an IVF cycle. In the majority of cases, patients elect to return to the clinic and have those embryos transferred to them to achieve a pregnancy. There are also options for donating embryos to another couple or to research or to clinical quality assurance/training. Embryos that are no longer wanted for pregnancy can also be discarded. Under optimal circumstances, patients will return to the clinic in a timely manner and will either make use of their embryos or have them used for one of these other purposes. However, as anyone who works in an IVF laboratory for sufficient time has realized, eventually some people do not return for their frozen embryos. There are a number of ways in which lack of response is manifested. Sometimes the patients who produced the embryos can no longer be contacted because their address is no longer valid. Sometimes, there is a valid method of communication, but no response is forthcoming following multiple attempts to reach them. Sometimes, a couple will respond but they persist in wanting embryos stored long past the timeframe in which the clinic would prefer to store them. A couple may also disagree with each other on what to do with the embryos, or they may state that they are unable to come to a decision about the embryos. And sometimes, a couple wants their embryos stored but refuse to pay the storage fees. Any of these things can leave the clinic holding the embryos in legal limbo, particularly if no advance instructions have been given. Discarding the unclaimed embryos in these circumstances can lead to legal liability but keeping them in storage when protocols are in place that require discard can also result in legal jeopardy.

Professional guidelines [13] suggest development of a structured informed consent policy laying out the steps to be taken with regard to who makes decisions about frozen embryos for both the patients and the clinic. The process would include how and when decisions are to be made, and what to do if the embryos are abandoned. Setting up such protocols is an essential component of developing an embryo freezing program. The default for embryos that are unclaimed is generally to discard them since using them for embryo donation or research without the explicit permission of the progenitors could result in accusations of misuse. However, even discarding embryos can lead to accusations of mishandling and to significant liability unless proper policies are in place to do this. A clinic's abandonment policies should include clear guidelines on the maximum length of storage, protocols for how and when regular contact with patients should occur, responsibility of patients for keeping their contact information up to date, protocols for what will be done if no response is obtained after contact is attempted, and specific information on when embryos will be discarded follow-

ing non-compliance or failure to pay storage fees. It is not always the case that lack of response to communications about their embryos means that patients do not want them discarded. Making decisions about embryos is difficult both because there are ethical, religious, and other concerns regarding their potential for life, but also because discard can mean the end of attempts to conceive [14–16]. Thus, at times patients may simply not want to make a decision and instead opt for a passive refusal to reply [16, 17]. In a study of patient responses to requests for decisions at the Dartmouth-Hitchcock Medical Center we found that 18.9% of those with embryos left in storage at the end of the 5-year time limit simply let the time elapse and never replied. None of these patients ever followed up concerning these embryos [17]. In a different study at a Belgian clinic, patient decisions changed over time with consecutive mailings suggesting that the assumption that decisions made at the time of the IVF procedure itself will be consistent with the choices made after years in storage should be viewed with caution [18].

42.4 Frozen Embryos: Mishandling and Loss

Storing embryos also incurs the risk of legal liability due to mishandling of the embryos or their unintentional loss. Embryos can be mislabeled, transferred to the wrong recipient, or erroneously discarded [19, 20]. Laboratory equipment can fail, resulting in embryo death, as several cases in 2018 demonstrated [21]. Nothing in the consent form, and no amount of forewarning can protect a clinic from legal liability when these incidents occur. The 2018 cases involved malfunction of the cryostorage equipment at two separate centers, and both resulted in loss of a great many embryos affecting numerous patients. Patients who have put time, effort, money, and emotional capital into the fertilization and cryopreservation of embryos will respond to such a loss with a desire to obtain compensation. In addition, in one of these cases, a wrongful death suit was brought alleging that the embryos were children [21]. Though lawsuits are virtually inevitable in these situations, running a CAP or JCHO accredited laboratory that adheres to ASRM guidelines and has strict protocols for identification of specimens as well as documented double and triple checks on equipment, have the potential to reduce, though not eliminate, the amount of the damages awarded.

42.5 Frozen Sperm and Oocytes

Up to this point, we have been discussing frozen embryos, but gametes can also be frozen for future use. Freezing sperm and oocytes does not engender issues related to personhood

but issues of abandonment, mishandling, and loss are very similar to those of embryos. Generally, when sperm or oocytes are frozen for an individual, this is done because that person is at risk of losing fertility due to cancer, increasing age, or other medical conditions. Protocols for consent and laboratory excellence should not differ in these situations from those for embryos. There is less liability from freezing of donor gametes which, while their loss could result in monetary loss to the clinic that recruited, screened, and froze the donor gametes, do not initially “belong” to anyone but the clinic. The exception to this involves gametes procured by a couple for a second or third offspring using that same donor.

42.6 Who Are the Parents?

Although not specific to embryo freezing, as they can also occur in fresh cycles, questions arise in IVF cycles about parentage of children born from complex arrangements that develop from donor egg and sperm, donor embryo, and surrogacy. These complexities arise out of the separation of the biological and gestational aspects of reproduction as well as the intent of the parties with regard to the children born. Practitioners would be well advised to consider having patients consult with their own legal counsel before getting involved in these arrangements. In some cases, particularly those involving surrogacy, there are legal requirements that must be met in different states or countries, such as prior court approval, before these agreements can take place.

A particularly egregious example of the type of complications that can arise in these situations can be found in the Bimber surrogacy case [22]. This case involved a 62-year-old man, James Flynn, (the intended father) who entered into a surrogacy agreement with Danielle Bimber. The sperm were supplied by the intended father and the egg was supplied by a young egg donor because the presumptive mother, his female partner, was too old to supply one. Three embryos were transferred to Mrs. Bimber who acted as a gestational carrier and who gave birth to triplets. Shortly after delivering the infants, Mrs. Bimber became concerned about the suitability and commitment of the intended father and his partner and she, with the blessing of the hospital staff, took the babies home from the hospital, subsequently filing suit to keep them. The intended parents filed suit to have the infants returned to them. At one point in the proceedings, because a judge found that none of the parties to either suit could be designated as the legal “mother” (neither Mrs. Bimber nor the unmarried partner qualifying in this regard), the egg donor was asked by the intended father to get involved and request parental rights. When they were two-and-a-half years old, after years of legal wrangling in multiple states, the children who had remained with Mrs. Bimber and her husband were returned under judge’s order to Flynn. Legal actions

brought by Flynn asked for monetary compensation from Mrs. Bimber, the surrogacy agency, and the delivery hospital [23]. In a final irony, in 2011, Flynn himself died [24]. We are unaware of who then raised the children.

The above situation could have occurred without cryopreservation. But the involvement of multiple persons in the conception and gestation of these children is an example of the sort of complexities that can be facilitated by gamete and embryo freezing. Players in these sagas can be intended parents, sperm donors, egg donors, embryo donors, and gestational carriers. Legal arrangements usually clearly define the intended parents as the individuals entitled to raise and make decisions about the offspring. They usually define the donors and carriers as having no rights or responsibilities. But these “contracts” cannot a priori be expected to forestall or to stand up to challenges in court. Where there are conflicting definitions of parents, different courts will decide these cases differently. Further, states and countries will differ in the laws that these agreements are subject to and on how they interpret the information. Egg and sperm donors can also no longer be promised anonymity and some countries have eliminated anonymous donation altogether [25]. DNA testing and sibling registries have given children of these arrangements access to information that allows them to find biological parents. Even though the courts may not recognize parentage, gamete providers and surrogates may find themselves contacted and emotionally connected to these offspring in ways that no longer allow them to stay on the sidelines. Clinics freezing gametes for donation and those offering donor embryos and gestational surrogacy may wish to take these things into account and consider the information that they provide to each of the parties about the realities of their involvement.

The above questions of biological connection to offspring have ramifications in terms of who will raise the children. The questions also have ramifications for inheritance. These issues will be discussed further below under posthumous reproduction.

42.7 Procreation by Same Sex Couples

Adding to the complexities created by use of donor gametes and surrogacy is procreation by same sex couples. Until the Supreme Court decision in 2015 legalizing same sex marriage, not all states recognized the rights of same sex couples to marry and the rights of parentage for both partners is still in question in some parts of the USA. Same sex reproduction, by definition, generally involves the gametes of only one of the partners. In female–female couples, the egg might be donated by one partner and the pregnancy may be carried by the other, but this is not always the case. For male–male couples an egg donor and surrogate gestator are generally

required. While sperm from both partners in a male–male couple might be mixed for insemination, only one sperm will ultimately prevail to fertilize the egg. In these arrangements, one or both partners may not be considered a parent by the courts, and in divorce proceedings among these couples some states have historically recognized only the partner with the biological connection.

An example of the complications that could arise in these situations prior to the 2015 Supreme Court decision is a case involving a lesbian couple who had entered into a civil union and had a child using donor sperm in Virginia in 2002 [26]. The couple moved to Vermont where civil unions were legal. The biological mother, Ms. Miller, began to have second thoughts about her lesbian lifestyle and decided to leave that life, resulting in their civil union being dissolved in 2004, which left primary custody with Ms. Miller. Nevertheless, the partner, Ms. Jenkins, retained visitation rights under a Vermont court ruling. Ms. Miller moved back to Virginia where she became involved with religious fundamentalists and began preventing Ms. Jenkins from seeing their daughter. A Virginia court sided with Ms. Miller and prevented visitation rights for Ms. Jenkins; however, this ruling was subsequently overturned, and the custody issue was returned to the Vermont court’s jurisdiction. Vermont then granted sole custody to Ms. Jenkins after which Ms. Miller fled with the child to Nicaragua [26]. The action prompted a charge of kidnapping under which Ms. Miller was arrested in February of 2021 and pled guilty to kidnapping in 2022. The child, now grown, grew up in Nicaragua [27]. While the 2015 Supreme Court ruling made same sex marriage legal across the USA, individual laws in many states can still complicate issues of parentage. In addition, with the current makeup of the Supreme Court in 2024, the future of same sex unions across the country has again become uncertain.

Laws regarding parentage have been slow to change in the face of the 2015 Supreme Court decision. This has made it difficult for couples in some states to be legally designated as parents on birth certificates and/or to require that even the biological parent and/or the partner adopt their own child in order to have legal rights. This process can be costly and take time. Some states have adopted the Uniform Parentage Act, a model act which clearly defines how paternity will be defined and which in its 2017 iteration also addresses legal rights and obligations of other intended parents as well [28]. However, very few states have adopted this law and not all of the final laws developed from this model are the same across different states. The patchwork of laws in different parts of the country can lead to confusion and uncertainty. It is essential that clinics that engage in procedures that could result in complex parentage consult legal experts in their own states such that the information given to prospective parents is accurate. Of additional concern, even married couples with children, may find their rights questioned when traveling or

moving to another state and this should be made clear to patients in advance of treatment.

The situation of transgender parents is more complex still. A person can transition gender roles at any point in the use of their gametes. Practitioners should be aware that transition of a transgender person has been used to take action against that individual and to deny parental rights. There are resources available for those in this situation and clinics should, whenever possible, help to facilitate access to these resources. One such publication is a guide to parents and lawyers promulgated by the American Civil Liberties Union [29].

42.8 Posthumous Reproduction

Embryo and gamete freezing also enables reproduction by a person who is no longer alive. Embryos, sperm, or oocytes already in storage can be used to conceive viable offspring. In addition, people who are dealing with disease that could prove fatal, such as cancer, can freeze gametes or can freeze embryos fertilized from their gametes, prior to death. Occasionally, sperm or oocytes are harvested from a newly deceased person at the request of a family member. Freezing of gametes has also been done prior to deployment of military personnel in order to save these gametes in the eventuality of death or loss of fertility during combat [30]. A spouse or partner can arrange to use these previously frozen products at a later date. From a technical standpoint, use of cryopreserved gametes and embryos for procreation is uncomplicated. From a legal perspective, the process is fraught with multiple complications [31, 32].

One issue following posthumous reproduction is, again, the question of parentage. Getting the name of a deceased person on the birth certificate can be a complicated process. This is the case when the deceased person was alive at the time that the pregnancy was initiated. It can be far more complicated when a pregnancy occurs months or years after the biological parent's death. Getting the name of the deceased on the birth certificate can involve an application and documentation and will require legal representation by a knowledgeable attorney. One reason for this is financial. Once a person is designated as the parent, the child becomes entitled to inheritance of an estate and access to Social Security and other death benefits. The government has fought requests for death benefits as have family members [33]. An example of a complex case that arose from posthumous reproduction is the case of *Hecht v. Kane* [34] in which living children contested the use of their deceased father's sperm by his second wife even though he had frozen his sperm for her use. This case dragged through the courts for years until the wife was so old that she was no longer able to conceive using her husband's sperm.

42.9 Genetics and Genetic Diagnosis

Current practice of IVF often involves preimplantation genetic testing (PGT) either for aneuploidy (PGT-A) or diagnosis of a parentally carried abnormality (PGD). Most genetic testing is now done with embryo biopsy at the blastocyst stage followed by cryopreservation of the embryo(s) and their subsequent transfer during a frozen cycle once the genetic test results are known. Thus, genetic testing has become an integral part of many cryopreservation cycles. The issues that develop with these testing procedures can be classified as (1) risks of tort associated with misdiagnosis and transfer of an embryo that is not consistent with the expectations of the parents, (2) issues related to selection and the discard of unwanted embryos, (3) potential eugenic concerns and disability rights issues associated with the concept of "designer children," and (4) the potential for creation of savior siblings and the associated right of a child to an "open future" free of requirements to provide a service for a sibling. Some of these issues are ethical issues with no immediate legal ramifications and are well beyond the scope of this chapter; others raise legal concerns.

The most pressing legal issue for laboratories offering PGT is the need for clear and open communication with patients on the risks of misdiagnosis, incomplete diagnosis, and the effects of embryonic mosaicism on the subsequent offspring. The limits of the testing procedures should be very clearly stated in preliminary discussions and on consent forms. Uncertainty should be carefully conveyed when testing results are unclear. The fact that misdiagnosis is always a possibility, albeit a small one, should be conveyed. Information about the quality of the diagnostic services offered by third-party laboratories should also be communicated.

Several of the other issues associated with genetic testing circle around the difficult concepts of when life begins and whether choosing embryos based on diagnostic criteria is immoral or otherwise unacceptable. As a result of the 2022 Supreme Court decision in *Dobbs v. Jackson Women's Health* [35], the legal status of abortion in different US states is now uncertain. Currently, several states have proposed laws that define life as beginning at conception, and it would be no more legitimate in these states for an embryo with an abnormality to be discarded as one without abnormality. Louisiana, for example, has for years had a law that states that an "in vitro fertilized human ovum is a biological human being" with parents having the right to sue on its behalf. The law makes embryo discard illegal including, presumably, the discard of an embryo with unwanted genetic traits and requires any unwanted embryos to be held for adoption by a married couple [36]. There are also some troubling new laws being enacted that prohibit abortion based upon fetal abnor-

malities like Down syndrome [37]. Basically, these laws provide that if a doctor knows that an abortion is being conducted because the fetus has been determined to have Down syndrome, the doctor can be charged with a felony, lose his or her license, and be sued for damages. If states would outlaw abortion based on Down syndrome, they might certainly also decide that destroying embryos in the laboratory because they carry a genetic abnormality should also be outlawed. This could subject clinic personnel to similar charges. The risks to clinical personnel might even be greater because unlike an individual doctor performing an abortion, they could not deny knowledge of the reason for destroying certain embryos.

We will leave some of the more complex questions regarding abortion and choosing embryos based on non-medical criteria for another publication, but practitioners should be aware that since the Dobbs decision [35], the legal future of genetic diagnosis as well as any discard of embryos, whether or not they contain undesired traits, now hinges on decisions made by legislators and judges in each of the 50 US states. If a state enacts laws that define a human life as beginning at conception, the result may be that genetic testing of embryos as well as discarding of any embryo, could be considered illegal.

42.10 Informed Consent for Cryopreservation: Embryos

From the perspective of a clinic offering cryopreservation services, the most important thing the clinic can do to protect from finding themselves in the middle of one of the difficult legal entanglements cited above is to have a robust informed consent process. Typically, this process starts with a “consent form” that a patient signs prior to cryopreservation. Although the document that lays out the rights and responsibilities of the clinic and the patients with regard to cryopreservation is often termed a consent form, these documents are not consents in the traditional sense. They are more closely aligned with contracts that permit and proscribe conditions of future action. A traditional consent form for medical procedures lays out the risks of a procedure and allows the patient to make an informed autonomous decision on whether or not to undergo it. A contract lays out the rights and responsibilities of the various parties and develops a set of obligations for each. While a consent form is agreed to between the clinic and the patient, it says nothing about the agreements needed between patients and each other (i.e., a couple) or between patients and other interested parties (e.g., donors and surrogates). While these distinctions may seem minimal to clinical staff, they can have profound effect on the legal implications for failure to comply by one or more of the parties. The structure of a consent form, if inadequately

constructed, may render it ineffective in a court of law. Expert legal advice as to the structure of a clinic’s consent is thus extremely important.

SART/ASRM currently promulgates a model consent form for cryopreservation that can be used as the template for clinics to develop their own consent document [38]. In addition, the American Bar Association Model Act has information on elements that would be best to be included in IVF consents and contains many of these same recommendations [39]. A consent form should be developed along with legal experts familiar with specific state laws and procedures that will need to be followed. Major features of a cryopreservation consent should include (1) information on what cryopreservation entails including the freezing processes and its effects on the embryos, (2) risks to the embryos including the possibility that one or more may not survive the freeze thaw process, (3) obligations of the clinic to maintain the embryo along with clearly stated information on the risks that equipment can fail and embryos could be lost or misidentified in spite of reasonable efforts, (4) the location at which embryos will be stored including whether they will be shipped to another facility for storage and if so, risks of shipping, (5) options for use of the embryos including patient use, donation, or discard, these options should be clearly defined, (6) obligations of the clinic to contact the patient at predefined intervals along with the method for this contact (e.g., written letter, email), (7) responsibilities of the patient(s) to keep the clinic apprised of their current contact information, (8) definition of abandonment and the process by which embryos in storage would be considered abandoned including maximum length of storage, definition of failure to respond (e.g., number of contacts without reply that constitute failure), (9) limitations set by the clinic on future use of the embryos such as maximum age of the woman at the time of embryo transfer, (10) clinic procedures for situations in which a couple cannot agree on the final disposition of the embryo (this may include situations where one partner is unable to consent to use due to but not limited to death or disability), and (11) procedures to be followed if storage fees are not paid. Consent forms can be constructed to have patients choose options in advance by expressing their preference for particular decisions, such as disposition in the case of death or dispute, but it is important for practitioners to understand that such decisions may not be binding and that legal decisions can sometimes override the chosen options.

An excellent informed consent document is a good starting point, but such a document alone does not constitute informed consent. Obtaining adequate consent is a process that begins with walking the patient(s) through the major risks and issues, ensuring that they fully understand the processes, and checking that they have no additional questions and concerns [40]. In the case of cryopreservation, it is particularly important that patients understand their rights and

responsibilities with regard to responding to correspondence, keeping the clinic apprised of their address, and making decisions in a timely manner. Ensuring that patients understand all aspects of the process including the fact that embryos may not survive is particularly important as part of this process. Again, reminding patient(s) of the value to obtain their own legal advice is always advisable.

The process of dealing with frozen embryos also does not end when the consent is signed. When a clinic sets up protocols for subsequent contact and follow up of patients, they are responsible to follow their own protocols. Deviating from one's own protocols can result in the entire established process being considered null and void and result in the invalidation of the entire document. This can be particularly difficult in situations where patients can't make up their minds. It is tempting in these cases to give the patient leeway, to allow extra time for a decision to be made, or to otherwise change the protocol that has been established so that the patient "feels good" about the experience. Doing this can invalidate the entire process and can mean that the clinic is left with no option other than to store the embryos indefinitely.

42.11 Informed Consent for Cryopreservation: Sperm and Oocytes

The informed consent for freezing of sperm and oocytes has certain similarities to that for freezing embryos, but there are a few distinctions. One major difference between embryo and gamete freezing is that the issue of personhood is not relevant in the freezing of gametes. Nevertheless, gametes have the potential to be used to procreate and loss of function of these gametes can prevent a person from realizing their future fertility. Another difference between embryos and gametes is that decision-making for most gamete freezing does not require agreement between two parties as it does with embryos that contain the genetic material of two individuals. The exception to this is when eggs or sperm are frozen for a minor child in which case a parent may hold some decision-making authority. As with frozen embryos, the consent process for gametes must deal with what will be done in the eventuality that the clinic loses contact with the patient, or the patient fails to pay storage fees. As with embryos, protocols for contacting the patient and the processes that constitute abandonment need to be clearly defined. One odd difficulty that can arise is that it is not always clear to the facility holding the gametes that the owner of those gametes has died. It is even possible that insurance or a family member continues to pay the storage fees after the death without the clinic being made aware of the change in status. A parent may also want to make use of the gametes to procreate a

grandchild. Consideration of these possibilities in the consent process is important but may be very difficult as consent discussions, particularly for young people with cancer or other diseases, can be a very challenging process [31, 41].

Oocyte freezing for women who want to maintain their fertility into an older age when the quality of their oocytes will have declined also requires discussion. Because oocyte freezing is, at this time, a relatively new procedure, the ASRM recommends that providers specifically inform patients that the risks and benefits may not be known at present [42]. In addition, consent documents for this procedure should again specifically provide information on the possibility that the stored oocytes may not survive or may not fertilize or implant in a subsequent IVF procedure.

42.12 Summary

The successful incorporation of cryopreservation into IVF has been an extraordinary advantage to successful IVF cycles. Cryopreservation allows the ongoing storage and use of embryos for more than one cycle. This process increases success rates that can be obtained from a single retrieval and gives patients the opportunity to have more than one delivery from a single fresh cycle of IVF. Cryopreservation of gametes and embryos increases the options for use of donor gametes and embryos by facilitating their availability. It provides an option for posthumous reproduction, and it facilitates accurate and accessible genetic testing. However, cryopreservation is not without legal risk. There is risk to the progenitors in the possible loss of embryos and gametes, and it introduces multiple points of difficulty with regard to decisions that need to be made subsequent to freezing. Clinics require carefully constructed and consistent protocols to ensure that the decisions made concerning these valuable products are made with knowledge and forethought. Assuming these issues are considered in advance by the clinical staff, the advantages of these procedures can be enormous.

Key Points

- Decisions on disposition of frozen embryos following divorce have balanced the concepts of whether the embryos are persons or property. Although having often been decided in favor of the partner who does not want to procreate, this outcome is not assured even when the couple has expressed decisions to the contrary in advance of having embryos frozen.
- Competing interests can complicate who may be designated as a parent following gamete donation, surrogacy, or posthumous reproduction. Legal counsel should be obtained by all parties prior to entering into complex arrangements.

- Risk to embryos, sperm, or oocytes due to mishandling or equipment failure and misdiagnosis during PGT are examples of problems that should be fully explained to patients in advance of treatment.
- A comprehensive informed consent process that lays out risks and benefits as well as the potential legal complications is a critical component of a well-run embryo and gamete cryopreservation program.

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Preservation and Storage of Frozen Biological Material and Their Transport from the War Zone to Safe Zones

43

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Due to the invasive and destructive actions of the Russian Federation in Ukraine, the lives of many people and their families were at risk, and the frozen biological samples of tens of thousands of IVF patients were in danger. Due to the severe panic that prevailed on the first day of the war, many embryologists made great efforts to reach their clinics. Intense attacks in different parts of the country put many clinics in danger and prevented the transport of frozen biological samples to different regions. However, these samples were safely stored and protected in local areas. Although shortages of liquid nitrogen occurred throughout the process, clinics were able to meet these needs, thanks to collaboration and solidarity within the IVF community. It is very important that the patients' samples are not damaged or that such information is not accessed during this complex and grueling process.

In the first days of the unimaginable attacks of the Russian Federation, many frozen sperm, egg, and embryo samples from IVF patients were sent to the western parts of the country. On the other hand, some clinics managed to get their samples out of the country at great risk after intensive efforts and long journeys. While the local transfer of samples was carried out following the country's legal regulations through the exchange of documents and the transfer of materials act between the two clinics, this practice was somewhat different for samples shipped out of the country. For biological material to be moved out of the country, the patient to whom it belongs must present a power of attorney document in the presence of a notary public, which must be apostilled. In addition, the clinic sending the sample must issue an embryo-oocyte-sperm release form for each patient, and the clinic accepting the sample must issue a document accepting the sample for each patient. However, the urgent decision made

at the beginning of the war did not leave time or opportunity for this. Therefore, without the patients' consent, these biological materials were removed from customs as bulk biological materials in freezing tanks and registered in the host country's customs offices. While the materials traveled in private vehicles to another country accompanied by embryologists on the same day, another team communicated with the two countries and completed the paperwork. The samples were safely transported out of Ukraine in a controlled manner after days of travel.

While the samples were being transported away, another team informed all patients of their samples and provided face-to-face communication with them. The humane and empathetic attitude that patients displayed is crucial in this process. The biggest goal of an embryologist is to preserve the samples prepared after great efforts and hours of work and to transfer them to the patients. Ukrainian embryologists put all their efforts into this during the war and displayed an inspiring attitude. The most important point to note here is the readiness of IVF clinics for emergencies and unexpected situations. It was concluded that IVF clinics should have contingency plans for uncontrollable events such as war, fire, earthquakes, and floods, and that an appropriate SOP system should be established. In addition, the storage conditions of stored biological specimens and where they are kept are very important. As many clinics used very large storage tanks due to the large number of samples, it was impossible to move these samples to another location. This situation raised questions about the use of heavy transport tanks with many samples. The importance of having liquid nitrogen in reserve was shown to be beneficial, as was the ability to automatically control the temperature and nitrogen content of the sample tanks with a battery-powered data logger. In addition, it has been found that the metal cane and the single goblet system are better in terms of protecting and keeping the samples in order or reducing the risk of confusion compared to storing many patients' samples in different colored compartments just within the same goblet.

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There are up to 60 IVF clinics in Ukraine which gives IVF/egg donation services. The reality of the war show us several points which we need to seriously consider for clinic and cryo bank management. Mostly all of IVF Clinics are working with non-standard SOPs and without risk/management protocols. The main problem is in case of risk situation how to show quick action to monitor biomaterials or to relocate them.

At the moment up to 50,000 frozen embryos and 10,000 frozen donor oocytes relocated in 1 week with three long and difficult trips which took already 120 h! The main advantage of the clinic who made relocation type of storages were convenient to move even with regular car. Many clinics even if they had chance to move frozen biomaterial they couldn't consider while they were using big capacity of storages which need too much effort and organization for relocation.

The situation of relocation has two different realities or perspectives. The first perspective is risk of the movement for all cryo storages by personal decision without patient consent and acceptance letter. The force major situation and risk factors did not allow to organize this. The second perspective long distance and driving could bring risk of traffic accident, bombing on the road or difficulties on the check points. However to keep material in Ukraine were more risky than to relocate them.

This situation also taught us if we are working as an international clinic we need to have departments or management system which also trained well custom documentation and legislation. Who relocate the biomaterial they had international egg banking experience and they used this advantage to register all storages officially in Ukrainian and European customs.

However, all these situations showed one big reality as all IVF community in Ukraine were fighting for their patients and biomaterial of patients to save them by different way. The first month of the war ended and still all clinics kept safety of biomaterial as far as we know. Anyway, cryo banking should be considered more seriously and all clinics all around world should have action plans for different force major situations.

While the devastating war aims to destroy people's lives, their freedoms, and their hopes for the future, the incredible struggle people are waging against it is exemplary. The formation of the IVF community in Ukraine is the best example of this struggle. To be a part of such a formation and to meet these amazing people is the greatest experience one can have in life. It should be our greatest wish that these people can return to their normal lives and live in peace in their beautiful country as soon as possible, and these scientists should be supported in every possible way.

43.1 Challenges to Safe Keeping Cryo-Stored Specimen at the Time of Armed Conflict: Live Observation from the War Zone

Ulyana Dorofeyeva

It is well-known that the right to self-determination and the right to health are fundamental rights of human beings. Self-determination is meant, in its family dimension, as a right to be a parent, and it has its constitutional basis in Articles 8 and 14 of the European Convention on Human Rights.

Treatment of infertility is important demographically and socio-economically a worldwide problem for which adjuncts are used to solve reproductive technologies. In Ukraine, the procedure for using assisted reproductive technologies (ART) is defined in the order of the Ministry of Health of September 9, 2013, No. 787 "On approval Procedure for the use of assisted reproductive technologies in Ukraine." The Procedure for the Use of Assisted Reproductive Technologies in Ukraine contains the definition of assisted reproductive technologies, which are defined as methods of treating infertility, in which manipulations with reproductive cells, individual or all stages of preparation of reproductive cells, processes of fertilization and development of embryos before their transfer to the patient's uterus are carried out in conditions in vitro.

Currently, there are many types of ART in Ukraine, order of the Ministry of Health No. 787 defines such a type as a donation of gametes and embryos (a procedure by which donors provide their reproductive cells, gametes (sperm, oocytes), or embryos for use in other people in the treatment of infertility). Specialists of the state of Ukraine are constantly improving the legislative framework, and now a characteristic feature of the order of the Ministry of Health No. 787 is that the donation of reproductive material does not require the presence of a number of documents, including a contract, which were provided for by the order of the Ministry of Health of December 23, 2008, No. 771 "On the approval of the Instruction on the procedure for the use of assisted reproductive technologies," which was replaced by updated Order of the Ministry of Health No. 787, in which the contract was transformed into an informed voluntary consent for oocyte donation.

The approach under which gamete donation in Ukraine is anonymous should be considered positive. Also, it is positive that according to clause 5.19 of the Procedure for the use of ART in Ukraine, the selection of a sperm donor is based on phenotypic characteristics (information on age, height, skin, eye and hair color, etc. provided).

On the other hand, due to legal restrictions in many countries of the world, such types of medical care are not fully available. Therefore, for example, the dramatic majority of donated oocytes in Italy, UK, Ireland, etc., up to 93% over the 2015–2018 period, came from foreign oocyte banks or clinical providers. The possibility of having access to a biobank of cryopreserved oocytes donated from young women could be a highly useful alternative to using one's fresh but older oocytes in ART treatment. The existence of an oocyte bank in such circumstances would ensure many advantages. For these reasons mentioned above, medical tourism related to ART with emphasis on material obtained by donation has been developing in Ukraine in recent decades. The number of ART cycles in Ukraine has tripled over 10 years, and now exceeds 30,000.

However, on February 24, 2022, trouble came to Ukraine—Russian invasion in Ukraine. What can now assure doctors and patients in Europe and all over the globe that partners from Ukraine, in particular Ovogene, will continue to provide service and guarantees of high quality when using cutting-edge technologies? Ovogene is a company that operates in compliance with high ethical standards and according to globally recognized regulatory documents, in particular the European Parliament Resolution of 24 June 2021 about sexual and reproductive health and rights in the EU, in the frame of women's health (2020/2215(INI)). Also, the donor material is issued according to "Single European Code" or "SEC," a unique identifier that consists of two elements, a donation identification sequence, essentially indicating the origin of the cells, i.e., oocytes, sperm, etc. Accordingly, company employees decided to move all cryomaterial (which is over 8000 embryos and 10,000 oocytes) to the territory of the EU in compliance with all above-mentioned technological and legal requirements of the European Community.

In particular, the clinician Europe, has become a reliable licensed storage facility for genetically priceless cryomaterial. The cooperation between Ovogene and this medical institution is the appropriate guarantor of safety and high results of the medical service that Ovogene provides to patients from all over the world.

It is not an easy decision taken by a patient to go into a different country for fertility treatment. Many aspects should be considered and safety of the patient and staff should be on first place. Mainly patients were deciding to be treated in Ukraine due to legal circumstances for oocyte donation and surrogacy, high level of efficiency and communication and affordable costs. In a situation which happened in February the main challenge for patients was to get information about safety of their biological materials, as even simple communication with many centers was lost. As thousands of patients were at very similar situation, they began to join, create groups, share their experience, and help each other. That fact

that our clinic (bank) took a responsibility and moved the materials out of Ukraine into the EU, and that fact that a process was done right and in accordance with international regulations and high level of safety was highly appreciated by patients and gave an opportunity to continue a treatment at such complicated period.

43.2 The Experience of Ukrainian Embryologists and IVF Specialists During the War and Their Perspectives on Future

Daria Hudkova

As a result of destructive, pro-terrorist attacks initiated by the Russian Federation in our country and aimed at destroying the liberal structure of our country, serious problems have arisen in the union of ART, as in any field. Ukraine has become an important location for IVF treatment of foreign patients, particularly in terms of egg donation and surrogate mother programs, especially in recent years, due to compliance with the law. Ukraine has become the focal point of IVF patient tourism mainly due to the pricing policy, order of laws, and quality of IVF services offered. The fact that the practical skills of IVF doctors and embryologists are very good and the training they receive result in success in their intensive work pace plays a major role.

On the first day of the Russian Federation's attack, embryologists from nearly 80 IVF clinics in different cities of our country were working diligently to preserve patients' sperm, eggs, and embryo samples, considering the protection of their own lives and the lives of their families as secondary. However, the progressive process did not allow the IVF clinics to work, and the intensity of the attacks increased. In this process, many Ukrainian IVF doctors and embryologists did not leave their country and preferred to stay with their families. In doing so, they ensured continuity of storage and preservation, especially of frozen sperm, oocytes, and embryo samples in their clinics. They ensured that the samples were stored in optimal conditions. The decision made in Ukraine to keep the male population in the country allowed only female embryologists and IVF doctors to leave the country. In particular, female staff members who have children left their spouses and families in the country and traveled to different countries to provide their children with a decent future and security.

Embryologists who traveled to other countries and took the opportunities offered by many European countries faced difficulties in starting their work as embryologists in different IVF clinics due to limited financial opportunities, high

tax rates, and expensive accommodation. The same situation was even more problematic for IVF physicians. IVF doctors experienced more difficulties, such as the practice of equivalence of diplomas, not being able to work as specialists due to the legal regulations, and not being able to speak the language of the country they went to. The fact that Ukrainian IVF staff are adaptable to an intense work pace and have high work practice has increased their employment opportunities in IVF clinics in other countries. However, both the language barrier and the psychological traumas they have experienced, as well as the fact that they have left their families and loved ones behind, cause them great problems. Many IVF professionals, especially those with young children, have had difficulty finding someone to care for their children and have therefore been unable to find suitable work opportunities.

Although attacks by the Russian Federation continued in the aftermath, IVF clinics in Ukraine began operating and providing care to patients, albeit under difficult conditions. IVF professionals who stayed in the country began working for lower salaries due to the economic downturn and declining patient numbers. However, the fact that they were in their country and at home made them overlook this situation. More than 70% of embryologists and IVF doctors in the country still work in their country. The Russian Federation's

attacks have eliminated the possibility of foreign patients entering the country, and clinics have continued to treat patients who have been on the waitlist, especially in the surrogate mother program. In addition, treatment of domestic IVF patients has continued, and some IVF circulation has been achieved.

In addition to the hundreds of embryologists and IVF doctors who did not leave their country and worked hard, Ukrainian IVF professionals who had to travel to different countries for the future of their children and families set an important example for the whole world. It is obvious that Ukrainian IVF professionals do not receive enough support, especially from international organizations and platforms, and therefore face difficulties. It is the greatest wish of all of us that these successful and talented scientists find the appreciation they deserve and that IVF patients in different countries benefit from these scientists' contributions.

While Ukraine is fighting for its independence and claiming its land, the people in the country also feel obliged to do something for their future and that of their families. Our greatest wish is that international IVF organizations involve these scientists in various projects, that IVF clinics outside of Ukraine offer employment opportunities to Ukrainian IVF professionals on favorable terms, and that these scientists make their voices heard and share their experiences.

Part VIII

Industrial Breakthroughs in Cryopreservation and Cryo-storage

At-Home Sperm Banking Kits

44

Hui Chen, Qingsong Lei, Gaojian Li,
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44.1 Introduction

Sperm banking through cryopreservation is highly recommended for preserving fertility, particularly for men who need certain medical procedures such as chemotherapy, vasectomy, and other gonadotoxic treatments. Chemotherapy can cause various problems that lead to infertility, including direct damage to reproductive organs or the surrounding tissue, changing hormone levels, and/or psychological effects [1]. More than 6% of cancer patients are adolescents and young adults (age 15–39) worldwide [2]. Although the 5-year relative survival rate for male childhood cancer survivors is near 80%, the normal semen quality rate after cancer treatment is approximately 33% [3, 4]. Medical treatments such as exogenous testosterone therapy can negatively affect the hypothalamic-pituitary gonadal axis and inhibit the production of follicle-stimulating hormone and luteinizing hormone [5]. Other reasons for sperm cryopreservation are age, occupational hazards, or for posthumous sperm retrieval. According to the National Institute for Occupational Safety and Health (NIOSH), reproductive systems can be affected by a wide range of occupational hazards, such as cosmic ionizing radiation, circadian disruption (jet lag), solvents, formaldehyde, infectious source exposure, pesticides, animal waste, zoonotic diseases, physical strain, and long working hours [6]. Additionally, semen quality gradually decreases with increasing age [6].

To preserve fertility with cryopreservation, interested consumers must be able to access a clinical cryopreservation facility [2]. However, sperm collection at clinical settings can be time-consuming, uncomfortable, embarrassing, and stressful for men who are interested in sperm banking [7–11].

Moreover, sperm banking facilities are inadequate in number, providing limited access to a large portion of those in need for cryopreservation [11, 12]. At-home sperm banking kits for the transportation of semen specimens from users' homes to a remote specialized fertility preservation facilities offer a solution by providing a more convenient, efficient, private, and cost-effective experience [10]. Although sperm count, motility, and other sperm quality parameters are affected during the shipping and post-thaw process, it has been reported that losing 50% of a sample in quantity and quality is still acceptable for sperm banking [13]. For home-based sperm banking, the quality of the collection kit is crucial as protection of the semen sample during the shipping process is necessary to ensure fertilization potential [10]. In this chapter, we discuss key factors of home-based sperm banking and report the current state of the art in home-based sperm banking kits. We will also discuss emerging technologies for at-home semen sample collection and testing that can be used alongside sperm banking kits towards potentially improving at-home sperm cryopreservation.

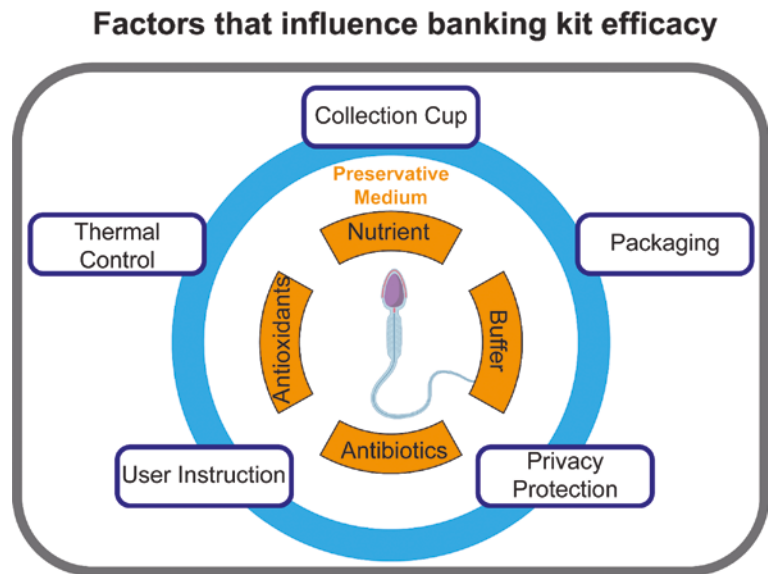
44.2 Key Factors in Home-Based Sperm Banking

44.2.1 Principles of Home-Based Sperm Banking

For any at-home sperm banking kit to successfully transport semen specimens from a user's home to a remote laboratory for andrological evaluation and cryopreservation banking, the most important concern is that sperm viability and fertilizing potential are maintained during the shipping process. There are many components of a kit that are important for effective banking, these include the semen collection cup for efficient sample collection, provided instructions for correct DIY-operation, and privacy controls, as shown in Fig. 44.1. The primary factors that determine the success of remote sperm banking are the transport medium, the thermal control

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Fig. 44.1 Schematic of the key factors of home-based sperm banking kits



components, and shipping time [14]. Transport time is mostly determined by the user's location and the public mailing system and should optimally be completed within 24 h as the motile subpopulation of spermatozoa declines over time [14–17]. During short-term shipping or storage, the influence of temperature on sperm viability is minimal if the temperature is controlled. It was found that sperm motility in washed normozoospermic samples could be retrieved up to 7 days after storage at either 4–6 or 25 °C but was completely lost after 12 days of storage at either 4–6 or 25 °C, with less mobility lost at 4–6 °C [18]. In fact, many current commercially available kits do not provide active thermal control elements, such as ice bags, for the transportation of semen samples to banking facilities [19–22]. Instead, they only provide thermal indicators to monitor accidental thermal shocks during shipping.

Preservative medium in at-home sperm banking kits plays a key role in protecting sperm viability during the shipping process. Unlike traditional cryopreserve medium, the preservative medium of at-home sperm banking kits does not need to protect sperm cells from possible negative factors generated during the freeze–thawing process, such as free oxidative radicals, cold shock, and osmotic shock. The major functions of preservative medium include (1) protecting sperm cells against possible damage by their own toxic by-products in seminal plasma, (2) stabilizing plasmalemma factors such as pH and osmolality, (3) providing nutrients, and (4) reducing growth of contaminant bacteria.

A variety of different preservative mediums have been used for short-term sperm preservation or shipping at ambient temperature. For instance, citrate yolk buffer has been demonstrated to preserve motility and various functional attributes of sperm of both normozoospermic and astheno-

zoospermic men at ambient temperature at a dilution factor of 1:1 [23]. Agarwal et al. also compared the performances of refrigeration medium (TEST Yolk Buffer, TYB), and human tubal fluid (HTF) as preservative medium in an at-home sperm banking kit by monitoring the pre- and post-shipment changes in sperm motility, membrane integrity, total motile spermatozoa, and recovery of motile spermatozoa [14]. They found comparable sperm motility, membrane integrity, and total motile spermatozoa in both mediums after simulated overnight shipping. All parameters decreased in both experiments but were still adequate for use with assisted reproductive techniques. Even a simple mixture of phosphate-buffered saline and glucose has been found to preserve a relevant fraction of human sperm for more than 1 week at room temperature with conserved sperm motility, viability, and mitochondrial membrane potential [24].

In most current commercially available at-home sperm banking kits, there are four major components included in the preservative medium: (1) buffering reagents, (2) antioxidant reagents, (3) antibiotics, and (4) nutrients, as shown in Fig. 44.1. In the following sections, we discuss the composition of preservative medium focusing on these four major components.

44.3 Preservative Medium Components

44.3.1 Buffering Reagents

The main function of buffering reagents in preservative medium is to stabilize the plasmalemma of sperm cells by avoiding pH and osmolality changes to conserve motility and fertility of spermatozoa over time [25]. A variety of buff-

ering reagents have been used for short-term storage and shipment of human semen samples, such as 2-[tris(hydroxymethyl)methylamino]-1-ethanesulfonic acid (TES) buffer, tris(hydroxymethyl)aminomethane (tris) buffer, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, phosphate-buffered saline (PBS) buffer, and citrate buffer [14, 26]. As an alternative approach, it has been reported that completely depleting ions in the sperm preservation solution is also effective to maintain the viability of human spermatozoa [27]. Riel et al. found that sperm cells stored in an electrolyte-free medium (EFM) composed of glucose and bovine serum albumin at ambient temperature maintained motility and viability for up to 4 and 7 weeks, respectively [27]. After 1-week storage, motility and viability of sperm in EFM was similar to that of cryopreserved sperm, with significantly higher DNA integrity, resembling that of fresh sperm [27]. The author's explanation for the phenomenon was that DNA damage by ions in the surrounding medium is avoided by the depletion of ions in EFM. The conductivity (0.4–0.5 mS/cm) and pH (7.0) of the EFM did not change during the 4 week of storage [27].

44.3.2 Antioxidant Reagents

Antioxidant reagents can counter-reactive oxygen species (ROS) produced by semen handling and the cryopreservation process which can damage sperm DNA integrity, mitochondria, plasma, and acrosome membranes [28]. It has been reported that adding antioxidant reagents to mammalian sperm samples during cryopreservation can better maintain sperm motility [29]. Common antioxidant reagents used in sperm storage include vitamin C (ascorbic acid), vitamin E (-tocopherol/Trolox), glutathione (GSH), quercetin, superoxide dismutase (SOD), catalase (CAT), and melatonin [30]. For example, it has been reported that melatonin, a neuroendocrine hormone with natural antioxidant activity normally present in human seminal fluid, could significantly increase sperm viability and membrane integrity ($P < 0.05$), and decrease lipid peroxidation damage during cryoprotectant storage [28]. However, the usage of antioxidant reagents for sperm storage without a chilling process is controversial since it is likely that the ROS level generated during short-term storage at ambient temperature is much less than that generated during the cryopreservation process. In a recent study of factors influencing sperm viability changes in short-term storage, it was found that antioxidant reagents including Trolox, coenzyme-Q10, did not influence sperm quality maintenance after 72 h storage at 22–27 °C [31].

44.3.3 Antibiotics

Semen collection and sperm manipulation for at-home sperm banking are not sterile processes, making bacterial contamination in sperm samples unavoidable. Contaminated bacteria not only directly compete with spermatozoa for nutrients but also can produce toxic metabolic by-products and endotoxins [32]. Some bacteria are harmful to the spermatozoa in a concentration-dependent manner. For example, *E. coli* can reduce sperm motility through sperm adhesion and agglutination [33]. Therefore, antibiotics are almost universally required in preservative medium for shipping or freezing semen. Commonly used antibiotics for sperm protection include ticarcillin (often used in combination with clavulanic acid under the designation timentin), streptomycin, amikacin sulfate, penicillin, lincomycin, spectinomycin, and gentamicin [34].

44.3.4 Nutrients

Nutrient reagents are important for sperm to maintain viability at ambient temperature, by supporting the basic metabolism of sperm cells. Commonly used nutrient reagents for sperm storage include glucose and some nutrient mixtures such as Ham's F-10 nutrient mix. It was reported that the storage time of viable sperm cells at room temperature could be doubled with glucose compared to using only phosphate-buffered saline (PBS) from 1 to 2 weeks [24]. Keshtgar et al. found that extracellular ATP did not influence sperm quality when Ham's F-10 nutrient mix was used for semen storage at ambient temperature [31].

44.4 Current Commercially Available Home-Based Sperm Banking Kits

The home sperm banking industry is still in an early stage, with most of the major companies appearing in the past decade. Therefore, there are only a limited number of banking kits available, many of them through startup companies, as shown in Fig. 44.2. Although cost and components vary, most currently available at-home sperm banking kits share many similar design philosophies. For example, almost all kits provide a preservative/transport medium to be mixed with collected semen samples to protect the viability of sperm cells during shipping. Additionally, most kits require 2–5 days of abstinence from the user for optimal sperm quality. Overnight shipping is almost universally required for these kits, which necessitates that the return shipping is done



Fig. 44.2 Commercially available home-based sperm banking kits. (a) Sparre.me kit (adapted from [22] with permission). (b) CryoChoice kit (adapted from [35] with permission). (c) Legacy kit (adapted from [36] with permission). (d) Fellow kit (adapted from [37] with permission)

Mondays through Thursdays. A detailed comparison is summarized in Table 44.1. In the following section, we discuss the seven major brands of at-home sperm banking kits on the market including NextGen, Sparre.me, CryoChoice, Legacy, OverNite, Ro, and Fellow, focusing on the kit contents, operation protocols, and related studies.

44.4.1 NextGen Kit

The NextGen® Home Sperm Banking kit is one of the earliest available kits on the market, developed by the Andrology Center of the Cleveland Clinic, Ohio. The sperm banking service offers an at-home collection kit and overnight shipping to the Andrology Laboratory for fertility preservation. The components of the NextGen® Home Sperm Banking kit are standardized, so that the semen sample temperature is

maintained during overnight shipment, and the quality of the sample is not compromised [14]. The standardized NextGen® Home Sperm Banking kit contains a specimen cup, a container containing preservative/transport medium (refrigeration medium), a cooling sleeve, and ice packs, along with the semen collection and storage agreement, patient questionnaire, offsite specimen collection documentation form and billing agreement.

The detailed user instructions can be found in the official website of NextGen [38]. In brief, after the kit has been received, the transport medium needs to be removed from the kit and refrigerated until it is used. The remaining components are stored in the freezer to keep the ice packs frozen within the Styrofoam container. Ejaculation is avoided for at least 2–3 days before the sample collection. It is advised that the semen sample is collected on any Monday, Tuesday, or Wednesday, in the late afternoon around 2–3 pm, or before

Table 44.1 Comparison of major at-home sperm bank kits on the market

	Kit price	Test included	Abstinent	Shipping timing	Prescription requirement	Preservative medium	Thermal control for semen sample	How many vials per semen sample	Cryogenic storage costs
NextGen	NA	NA	At least 2–3 days before the sample collection	Monday, Tuesday, or Wednesday, in the late afternoon around 2–3 pm	Yes	Refrigeration medium (TEST Yolk Buffer, TYB)	Cooling sleeve and ice packs	NA	NA
Spare.me	\$300	Full fertility testing report within 12 h	At least 2 days but no more than 5 days before submitting his sample	Monday through Thursdays	No	The preservation medium contains human serum albumin, sodium chloride, water, HEPES sodium salt, D-(+)-glucose, sodium bicarbonate, potassium chloride, calcium chloride, magnesium sulfate heptahydrate, sodium dihydrogen phosphate (monobasic), sodium pyruvate, gentamicin sulfate, taurine, HEPES free acid, tri-sodium citrate dihydrate, and ethylenediaminetetraacetic acid	No	3–6 vials	1 year free storage, \$145/year later
CryoChoice	\$595	Sperm count and motility testing	NA	NA	No	Preservative medium composition not available	No	NA	1 year free storage, \$149/year later
Legacy	\$195	Post-thaw analysis (result within 48 h), optional DNA fragmentation analysis	2–5 days before using the kit	Monday through Thursdays	No	Preservative medium recipe not available. Transport medium can be stored in the fridge for up to 14 days	No	Up to 8 vials	\$145 for yearly storage
OverNite	\$575	48 h semen analysis report	NA	Monday through Thursdays	No	Preservative medium composition not available	Temperature indicator	NA	1 year free storage, \$300/year later
Ro	\$199	48 h semen analysis report	NA	Monday through Fridays	No	Preservative medium composition not available	No	3 vials	1 year free storage, \$99/year later
Fellow	\$189	Sexually transmitted infections (STI) testing	2–5 days before producing the sample	Mornings or early afternoons between Mondays and Thursdays	No	Preservative medium composition not available	A temperature sensor and a gel pack	3 vials	\$140/year

the final dispatch schedule, and shipped immediately to avoid any delay in processing during the weekends. Prior to use, the transport medium needs to be removed from the refrigerator and thawed to room temperature for about 1 h. The transport medium needs to be added immediately after the sample is ejaculated into the specimen cup and mixed gently back and forth after tightly securing the specimen cup lid. The specimen cup is then labeled appropriately as per the directions provided and sealed inside the biohazard bag. The bag is then placed between the foam pieces in the kit box and shipped back to the laboratory.

The performances of two transport mediums—Refrigeration medium (RM) and Human Tubal Fluid (HTF), and five different combinations of ice packs were compared for NextGen Home Sperm Banking kit in a study [10]. The two transport mediums were comparable in maintaining the quality of the sperm samples. For the ice pack comparison, a cooling sleeve wrapped around the container and cushioned with solid ice bricks was better in maintaining the temperature of the container than the gel-based ice packs [10]. Although a decline in sperm motility was found after shipment, the observed motility and total motile sperm count was sufficient to be used for assisted reproductive techniques [10]. Preliminary studies of offsite (using NextGen® Home Sperm Banking Kits) versus on-site semen sample collection demonstrated that parameters such as sperm motility, total motile sperm, and cryosurvival rates of sperm by both these sample collection methods, from infertile patients and cancer patients, share a comparable outcome [10]. This finding suggests that the NextGen kit enables cancer patients to bank sperm efficiently [10].

44.4.2 Sparre.me Kit

Sparre.me was founded in 2014 and primarily sells a home sperm kit for male fertility cryopreservation [8]. Affiliated services of Sparre.me include lab processing, sample testing for sperm count and motility, and priority overnight round trip shipping [22].

The Sparre.me home sperm kit contains insulated styrofoam container, temperature stabilizer gel, thermal foil, and sperm preservation fluid [22]. Expanded polystyrene in the package is used for thermal insulation. The non-toxic chemicals in the leakproof viscous gel refrigerant in the gel pack allow for long-lasting cooling and efficient energy transfer. The gel keeps the sperm preservation fluid cool until the box is delivered to the consumer. The package is protected against humidity, moisture, oxygen, and other airborne contami-

nants using a high-quality aluminum thermal foil protector. The sperm is kept alive and stable during transportation due to the enhanced sperm protection medium (Table 44.1) [22]. An operation protocol similar to NextGen kit is applied, except that no ice bags are needed for return shipping with Sparre.me kit. Details of the instructions are available from Sparre.me website [22].

44.4.3 CryoChoice Kit

CryoChoice, with their processing partner SEBNJ, run an FDA-registered sperm bank. They claim over 50 years of experience in reproductive and cryogenic medicine and have reported more than 1000 successful inseminations [35]. The CryoChoice kit contains a sterile specimen cup, sperm preservative, collection worksheet, detailed collection and shipping instructions, and the pre-paid return shipping label and shipping envelope [35]. A detailed user protocol can be found on the official website of CryoChoice [35]. In brief, the sperm preservative needs to be thawed to room temperature, half an hour prior to sample collection. After collection, the sample should not be refrigerated, and the cup and all the kit materials are to be placed back into the CryoChoice box and shipped accordingly to the instructions provided in the kit to through the nearest FedEx shipping drop off location [35].

44.4.4 Legacy Kit

Legacy was founded in Cambridge, Massachusetts in 2018 [19]. The kit contains a sterile sample cup, a vial of transport medium, an ice pack, and different packaging items [36]. A detailed user protocol can be found in the official website of Legacy [39]. The Legacy transport medium can be stored in the fridge for up to 14 days. The user should stay abstinent for 2–5 days before using the kit. The sample can only be returned between Monday and Thursday. To register and prepare the kit, the customer should scan the QR code on the sample cup with a smartphone camera or visit the dashboard to register the kit. If transport medium was refrigerated, it should be removed from the fridge and allowed to reach room temperature before producing the sample (~45 min). To produce the sample, hands and genitals should be washed, then the sample is ejaculated directly into the sample cup, without lubricant. The sample is packaged with the included packaging materials in the original shipping box and shipped back to the company. The results will be available on the

online dashboard within 48 h after the sample is arrived at the lab. If the user's sperm passes the post-thaw analysis at the laboratory, it will be immediately stored in the company's cryo-storage facility.

44.4.5 OverNite Kit

The OverNite Male Kit was developed by Dr. Gail S. Prins and her team at the University of Illinois at Chicago Andrology Lab in 1994, in partnership with the University of Illinois at Chicago (UIC) Andrology Lab and ReproTech Ltd. [40]. The kit contains the complete instructions, a collection cup, a buffer medium, a bio-hazard bag, a temperature indicator, a tamper proof strip, a foam shipping package, a FedEx® air bill, and a semen storage agreement [20]. A detailed user protocol can be found in the official website of OverNite [41]. In brief, the user should produce the sample into the storage medium, and ship it back overnight between Monday and Thursday. Base price (\$575) includes collection kit, all lab testing, semen analysis and processing, overnight delivery, and 1 year of storage. After the first year, the annual storage fees including all vials are \$300. HIV 1&2 tests are required within 90 days to qualify for the standard storage rate. Users with no test results or those who have been responsively tested for an infection will be charged an additional \$138 annual storage fee.

44.4.6 Ro Kit

Ro company has partnered with the New England Cryogenic Center, founded in 1971, providing full-service cryogenics [21]. Because of the unique design of advanced sperm transport capsules, the Ro sperm kit is tamperproof, shock proof, and drop proof, ensuring sperm survival from extreme temperatures until it reaches the partner lab for analysis and cryopreservation [21]. A detailed user protocol can be found in the official website of Ro [21]. In brief, the user collects semen in the collection cup, then screws on the bottle cap and presses a button on the lid to release preservatives. The cup is returned to the kit and secured with the provided security tape. The kit is then returned to the central lab overnight. A semen analysis report will be generated within 48 h of arrival of the sample at the lab, evaluating sperm count, concentration, and motility. Each kit sample is divided into three vials that are stored, which gives the user three opportunities for future fertilization. In addition, a unique ID on the bottom

of the collection cup inside the kit ties the patient's lab results to their account with two-factor security authentication.

44.4.7 Fellow Kit

Fellow kit includes a sample container, a vial of preservative solution, an instruction manual, a temperature sensor, and a gel pack [37]. An operation protocol similar to Sparre.me kit is also used for the Fellow kit. A detailed user protocol can be found in the official website of Fellow [37]. In addition, the Fellow semen preservation service includes free mail-in sexually transmitted infection (STIs) tests, so that users can directly use cryopreserved samples at any fertility clinic, as most clinics require disease screening.

In a study of the Fellow mail-in semen analysis (SA) system, the authors compared the outcome of fresh (within 1-h) semen analysis (SA) with delayed SA testing and developed a methodology to predict the fresh SA results based on delayed SA results [15]. The authors collected 564 ejaculates from 164 normospermic participants and used 257 samples for the optimization of the prediction model, 203 samples to determine the model for predicting baseline SA, and 104 samples to validate the performance of the model. It was found that this SA testing system demonstrated a strong degree of correlation between fresh and delayed SA results, with a stable decrease in sperm concentration, linear motility, and morphology over time. With up to 52 h of observation and four subsequent SA measurements/ejaculate, the motility decreased at a rate of 0.39%/h, and normal morphology decreased at a rate of 0.1%/h.

44.5 Emerging At-Home Sperm Banking Technologies

The advancements in consumer electronics particularly smartphones, microfluidics, and computer programming especially artificial intelligence have allowed the development of rapid, sensitive, reliable, low-cost semen analysis technologies towards home-based semen analysis. Such point-of-care semen analysis technologies can potentially facilitate higher quality semen preservations by enabling home-based measurement of some of the most important semen parameters such as sperm concentration, motility, and even morphology. Here, we briefly discuss the emerging at-home semen analysis technologies such as paper-based and smartphone-based devices (Fig. 44.3).

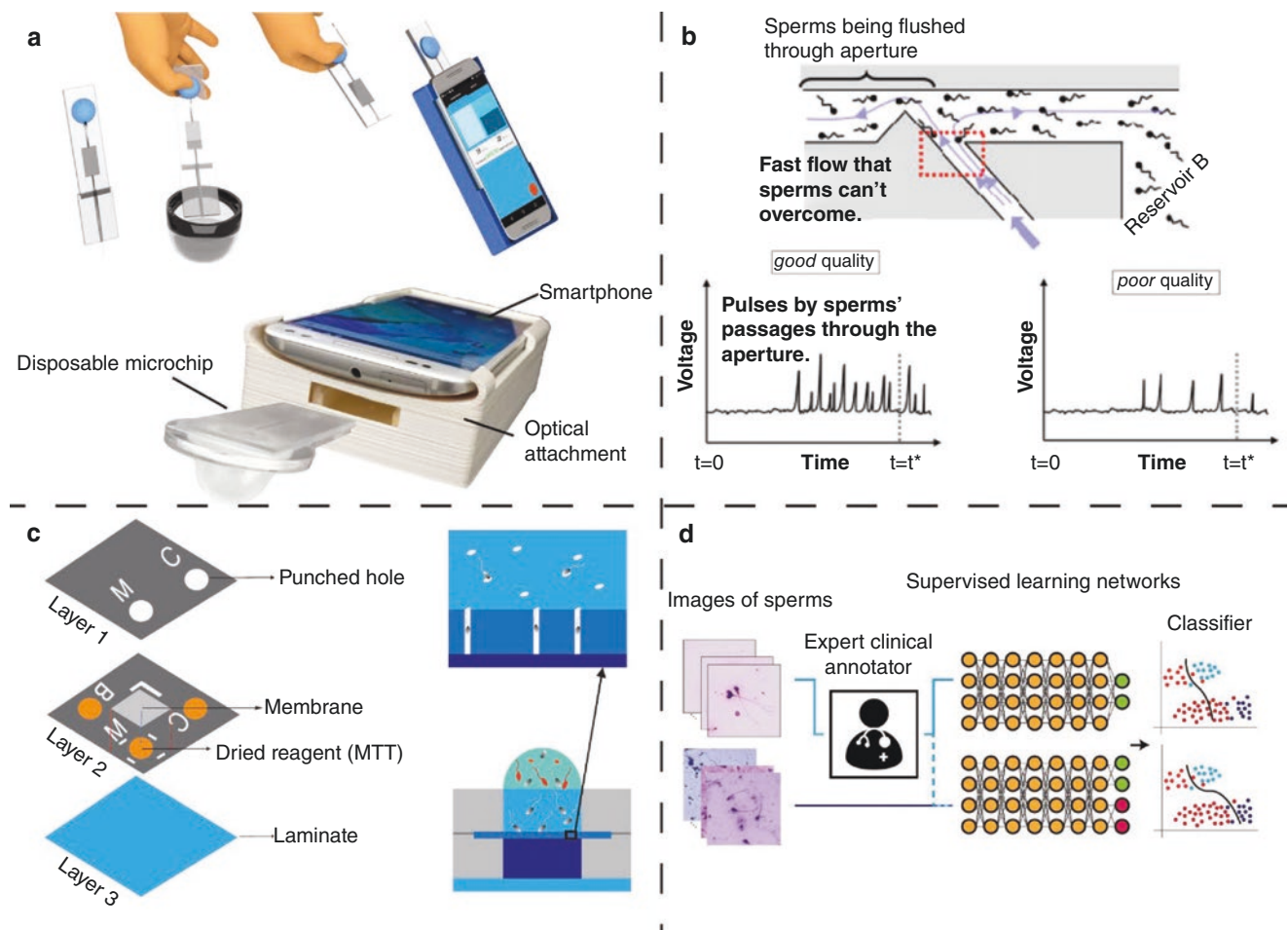


Fig. 44.3 Emerging at-home sperm banking technologies. (a) Schematic of smartphone-based device for home-based semen analysis (reproduced with permission from [46]). (b) Working principle of microfluidic device for the analysis of sperm concentration and motility

(reproduced with permission from [44]). (c) Schematic of an integrated paper-based semen analysis device [42]. (d) Schematic of an adversarial domain adaptive neural networks for sperm image analysis (reproduced with permission from [49])

44.6 Paper-Based Devices

Paper-based devices are attractive diagnostics for point-of-care and home-based biosensing of biological samples. In fact, some of the most successfully developed home-based diagnostics such as pregnancy tests are made of paper-based materials. Spermcheck was one of the first paper-based devices developed and commercialized for semen analysis that can only measure sperm concentration. Others have developed a paper-based semen analysis assay using wax printing with the ability to measure sperm viability and motility in addition to sperm concentration in less than 10 min [42]. A reaction between a yellow tetrazolium dye and the diaphorase flavoprotein enzyme present in active sperm causes the device to change color [42]. After enzymes in live sperm react with the dye, the device determines the concentration by measuring the colorimetric change from

yellow to purple [42]. Motile sperm must first successfully swim through narrow pores on a membrane filter and a viscous buffer before they can react with the dye, which means the colorimetric measurement also works as an evaluation of sperm motility. This assay was evaluated with only 17 frozen human semen samples. The outcome of the assay performance evaluation study was promising as they were able to correctly identify 1 out of 6 samples with low sperm motility [42].

44.7 Microfluidics

Microfluidics is the science and technology of miniaturizing microfeatures, channels, and reservoirs on microdevices to enable precise handling of minute fluidic samples, particularly biological samples for processing or testing purposes.

Microfluidics can have significant applications in semen cryopreservation and analysis. Numerous microfluidic-based systems have been developed for sperm sorting [43]. Here, we provide examples of microfluidic-based systems developed for semen analysis by measuring sperm concentration and/or motility. Chen et al. developed a microfluidic-based assay to measure motile sperm concentration and motility using resistive pulse technique without the need for any biomarkers or labels [44]. The system creates a flow field in which impurities are left behind by label-free sperm cells from liquid specimens swimming upstream. The sperm cells that manage to overcome the flow are discharged to an area where resistive sensing takes place. This design could help infertile patients with at-home male fertility screening and frequent sperm quality monitoring. To demonstrate the accurate assessment of sperm quality by the microdevice, Chen et al. used the device to measure motile sperm concentration while comparing the results to those provided by hemocytometer and the Sperm Motility Index (SMI) by the Sperm Quality Analyzer as two control methods. The test group was divided into two groups. The first group contained eight samples with progressively motile sperm concentrations ranging from 0 to $19 \times 10^6/\text{mL}$ (total sperm concentration from 0 to $76 \times 10^6/\text{mL}$). The second group contained four samples with motile sperm concentrations of 19, 17, 14, 7, and $4 \times 10^6/\text{mL}$. The result shows that the pulse number correlates well with motile sperm concentration in the samples examined by the microdevice. These samples' SMI values were also related to the number of pulses.

Another microfluidic device was designed by Chen et al. that quantifies total and motile sperm counts [45]. In this device, motile sperm must swim through a phase-guide barrier before mixing with buffer and centrifugation to determine sperm concentration. The microfluidic gadget is divided into two sections: the left section initially includes all of the sperm cells, while the right section contains clean semen buffer. Motile spermatozoa can freely swim across regions and reach equilibrium in a few minutes in this system, which was inspired by the traditional swim-up technique for isolating and collecting motile sperms. Because Brownian motion drives diffusion, dead sperms should stay in the left zone, while motile sperms should be able to swim randomly in both regions to reach dynamic equilibrium, i.e., 50% of motile sperms should travel to the right region. The chip was placed on a microscope (BX51, Olympus) soon after the initial contact of semen and buffer for dynamic equilibrium tests. The main channel's buffer region was recorded by video (720p by Nikon D5100) for analysis. The concentration on the bottom channel allowed for the acquisition and analysis of the majority of the sperm. Each sample received a total of 20 min of video. Each minute, the amount of sperm cells in the buffer region was manually counted on a screen. Roughly 15 min was required for dynamic equilibrium. The

results from the device are in agreement within 5% compared to a manual evaluation with a microscope in a Makler chamber [45].

44.8 Smartphone-Integration

Smartphone-based diagnostic tools have demonstrated their huge potential to revolutionize the field of POC testing by adding unprecedented convenience and accessibility in the past decade. To meet the urgent need of home-based sperm testing in conjugation with home-based sperm bank kits, Kanakasabapathy et al. developed a smartphone-based POC semen analyzer that is rapid, automated, and inexpensive [46]. The microchip contains a straight microfluidic channel and a bulb made of polydimethylsiloxane (PDMS) to create negative pressure to pull the semen sample into the microfluidic channel. With a small volume of less than 35 μL of semen sample loaded into a disposable microfluidic device, the smartphone-based diagnostic assay was able to measure the concentration and motility of the sperm cells. The developed smartphone application enables recording and analyzing a short video (1 s) of the semen sample placed on the microchip. The performance of the assay in qualitatively classifying fresh semen samples based on the WHO guidelines on sperm concentration (abnormal: <15 million sperm/mL) and motility (abnormal: $<40\%$ motility) was 98% when tested using 350 fresh patient semen samples at Massachusetts General Hospital [46]. No significant difference in the performance of the assay was observed when operated by trained and untrained users. Yo Sperm is a commercially available smartphone-based device for semen analysis developed and manufactured by Medical Electronic Systems (MES). This device can be used for qualitative assessment of semen samples based on a threshold on the concentration of motile sperm in a semen sample (6 million motile sperm/mL). It cannot, however, measure sperm concentration and motility of semen samples.

44.9 Artificial Intelligence

The advances in machine learning particularly for image processing over the past decade have enabled the possibility of developing point-of-care and home-based diagnostic/screening assays for disease detection or treatment monitoring. Such advances have allowed the development of smartphone-based optical systems enabled with deep learning frameworks for rapid, sensitive, and automated analysis of sperm morphology. The conventional method of sperm morphology assessment based on manual microscopic image-based sperm morphology grading is time-consuming, tedious, subjective, and labor-intensive. The recently devel-

oped deep learning frameworks have shown great promises in accurate sperm morphological assessment using both high-quality and low-quality images recorded using bench-top, bulky, expensive microscopes [47] and low-cost, portable microscopes such as smartphone-based optics [48, 49]. The neural network developed by Thirumalaraju et al. was evaluated using image data associated with 9 smeared patient semen samples available through the American Association of Bioanalysts Proficiency Testing Services by comparing the AI sperm morphology score results with the national average of the sperm morphology of the semen samples. The neural network had 100% accuracy in identifying normal and abnormal semen samples ($n = 9$) [47]. Kanakasabapathy et al. developed adversarial neural networks for analyzing lossy and domain-shifted datasets of medical images including low-quality sperm images recorded using a low-cost, portable standalone optical set up and a low-cost smartphone-based device [49]. The Bland-Altman analyses showed a mean bias of 2.465% (StD: 5.524%) ($n = 46$) for the standalone optical set up and a mean bias of -0.3012% (StD: 7.836%) for the smartphone-based optical device. No systematic biases were observed for any of these portable optics in measuring the sperm morphology score of the semen samples [49]. Such AI-enabled, portable, low-cost optics for point-of-care semen analysis can be used along with home-based sperm cryopreservation kits to provide more efficient sperm banking services.

44.10 Conclusion

Sperm cryopreservation is an effective approach to preserve fertility for men particularly those who need certain medical procedures such as chemotherapy, vasectomy, and other gonadotoxic treatments. The currently available at-home sperm banking kits provide convenient, private, and cost-effective sperm cryopreservation services particularly for those who are reluctant to seek conventional clinical banking due to long wait time, inconvenience, or social stigma. The composition of preservative medium is vital in the development and deployment of efficient home-based sperm banking kits to reduce any damage on the sperm cells during shipping from home to the laboratory for testing and cryopreservation. There is still enormous room for improving the quality of home-based sperm banking kits and sperm cryopreservation services through utilizing the recent advances in microtechnologies, consumer electronics, and computer programming. For example, microfluidic-based devices and systems and AI-enabled smartphone optics can further improve our at-home cryopreservation services by enabling point-of-care semen analysis through measuring semen parameters such as sperm concentration, motility, and morphology.

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Single Sperm Cryopreservation for Azoospermia Management

45

Arie Berkovitz and Vita Freger

Male factor contributes to 23–25% of infertile couples [1, 2]. Azoospermia, defined as the absence of sperm in the ejaculate, is identified in 10–15% of infertile men [3]. Some men present with cryptozoospermia, which is when spermatozoa are absent from fresh preparations, but are observed in a centrifuged pellet in the semen sample [4]. A more extreme condition, virtual azoospermia, refers to the occasional presence of spermatozoa, after an extended search, in the ejaculate of men diagnosed with azoospermia [5]. This condition is probably due to fluctuations in spermatogenesis [6]. Men with virtual azoospermia are usually referred for intracytoplasmic sperm injection (ICSI) treatment, in which the presence of one spermatozoon per oocyte can achieve pregnancy [7].

In cases of virtual azoospermia, it is uncertain whether sperm cells will be found on the oocyte pick-up (OPU) day. This situation may lead to the need for oocyte cryopreservation, a procedure that is no longer considered experimental. An alternative method is to freeze a few spermatozoa that

can be used for backup when none are found in the ejaculate (Table 45.1).

Azoospermia can be further categorized as obstructive azoospermia (OA) due to congenital or acquired causes that result in an obstruction in the ejaculatory pathway. Fine needle testicular sperm aspiration (TESA) is the preferred method for sperm retrieval, with almost 100% recovery [8]. Since these patients usually have normal spermatogenesis, the conventional method of slow freezing is effective.

Non-obstructive azoospermia (NOA) is the most severe form of male factor infertility, accounting for 5% of infertile couples [9]. NOA is characterized by impaired spermatogenesis ranging from hypospermatogenesis and maturation arrest to Sertoli cell-only syndrome [9].

Klinefelter's syndrome (KS) and Y chromosome microdeletions are the most common congenital causes of NOA [10]. Acquired causes of NOA include torsion, mumps,

Table 45.1 Summary of single-sperm cryopreservation methods

Author	Carrier	Sperm origin	Recovery rate	Motility rate	Fertilization rate	Pregnancy rate
Desai et al. [19]	Cryoloop	Oligo-terato ejaculate sample Epididymal testicular	68% 72%	73% NA	67% 58%	NA NA
Sereni et al. [20]	Droplets on plastic dish	NA	95%	NA	NA	NA
Peng et al. [21]	Cryoleaf	Epididymal sperm	99%	66%	61%	NA
Endo et al. [22]	Cryotop	Testicular sperm	94%	43%	64%	NA
Endo et al. [24]	Cell Sleeper	Testicular sperm	94%	56%	66%	58.3%
Araki et al. [26]	Hollow-core agarose capsules	Donor sperm	91%	84%	NA	NA
Tomita et al. [27]	Hollow hyaluronan-phenolic hydroxyl microcapsules	Donor sperm	95%	14%	NA	NA
Sun et al. [28]	Cryopiece	Testicular sperm	83%	48%	73%	4 births
Berkovitz et al. [29, 30]	SpermVD	Ejaculate testicular sperm	96.6	33%	59%	55%

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orchitis, cryptorchidism, and iatrogenic problems (chemotherapy and radiotherapy) [9].

For cases of NOA, testicular sperm biopsy for sperm recovery has been an acceptable method of treatment since 1997. Sperm recovery rates have been reported to be 36–64% [11] and even lower in testicular aspirates. Newer techniques involving testicular microdissection offer improved sperm retrieval rates, even when previous conventional testicular biopsy was unsuccessful. Regardless of the techniques used, sperm, if successfully recovered, are often weakly motile and present in small numbers, necessitating repeat surgical interventions if pregnancy is not achieved or if another pregnancy is desired. This is costly and can entail significant risks to the patient. Multiple testicular biopsies can lead to inflammation or hematoma at the biopsy site and to major complications, such as testicular devascularization and fibrosis [11]. Efficient cryopreservation of small numbers of sperm avoids surgical procedures and reduces the complications associated with repeated interventions.

In 1943, Hoagland and Pincus were the first to develop the concept of using liquid nitrogen (LN2) to freeze human sperm [12]. This practice has become routine in the Assisted Reproduction laboratory [13]. Slow freezing is the method most often used by sperm banks worldwide. Sperm is suspended in a mixture of 1:1 v/v sperm washing medium and a cryoprotective agent for 10 min at room temperature and then gradually cooled to -192°C [14, 15]. This method improves survival and motility after thawing by reducing deleterious effects such as ice crystal formation, dehydration, increased salt concentration and thermal shock. However, it requires a relatively large total liquid volume of 0.25–1 mL and is not appropriate when only a few spermatozoa are frozen, as in cases of virtual azoospermia, cryptozoospermia, or after testicular biopsy, due to the inevitable loss of spermatozoa upon post-thaw sample processing [15, 16].

Cohen et al. [17] first suggested an empty zona pellucida procedure for cryopreservation of small numbers of human spermatozoa. The first live births using human and hamster zonae for cryopreservation of epididymal and testicular spermatozoa were reported a year later [18]. The method is not currently in use because it depends on a biological carrier, with the potential risk of contamination and disease transmission. To overcome the challenge of using biological carriers, products that are used in the laboratory have been adapted or hollow capsules similar to empty zona pellucida have been fabricated to freeze small numbers of sperm cells.

In 2004, Desai et al. [19] proposed a novel Cryoloop method for freezing individual sperm. A nylon Cryoloop was developed to cryopreserve 5–10 ejaculated spermatozoa. For single-sperm cryopreservation, the magnetic wand holding the Cryoloop was placed in the micromanipulation pipette

holder so that the Cryoloop could be visualized under the inverted microscope during sperm loading. Spermatozoa were first isolated microscopically and pooled into a single microdrop of HTF-HEPES. The Cryoloop was then preloaded with a film of this 50:50 cryoprotectant mixture. The selected spermatozoa were picked up with a standard ICSI micropipette or embryo hatching pipette and deposited directly onto the cryoprotectant (Fig. 45.1). Once loaded, the cap with attached Cryoloop was fastened to a 1.5 mL cryogenic vial. The vial was vapor frozen for 2 min and then immersed in LN2.

During the thaw procedure, the Cryoloop was removed from the cryogenic vial and held over a sterile 60 mm dish with the loop touching the dish surface. A scalpel was used to cut the Cryoloop from its metal stem. A 2 μL aliquot of HTF-HEPES media with 6% plasmanate, pre-warmed to 37°C , was immediately pipetted on the Cryoloop and the dish was then overlaid with light mineral oil. Total sperm count and percentage motility were assessed. After thawing, the recovery and motility rates were 68% and 73%, respectively (Fig. 45.1).

The cryopreserved sperm could induce pronuclear formation when injected into human oocytes. Each nylon Cryoloop was also developed to cryopreserve 2–8 epididymal or testicular spermatozoa, and they achieved 72% recovery and 56% pronuclear formation.

The main disadvantage of a nylon Cryoloop is the surface film that carries the droplets with sperm. These makes the sperm loading technique complicated and the storage of frozen droplets unstable. Therefore, the recovery rate is relatively low. Clinical trials are needed to further explore this technique.

In 2008, Sereni et al. [20] cryopreserved individual testicular spermatozoa in a microdroplet with oil overlay in a plastic tissue culture dish. Cryoprotectant was added to the culture dish (Fig. 45.2). After thawing, 67% of motile sperm retained their motility. The sperm recovery rate was 100%. A total of 51 oocytes were treated across the six study samples, and in two of six cases, motile spermatozoa were injected. The fertilization rate was 18%. This method is simple and easy to control, but polystyrene culture dishes with microdroplets are problematic because their size and shape make them more difficult to store in LN2 for long periods.

In 2011, Peng et al. [21] demonstrated a novel method for freezing and recovery of individual or small numbers of spermatozoa. Small numbers of sperm were cryopreserved on a self-designed Cryoleaf (Fig. 45.3) under high humidity conditions, which prevents cryoprotectant evaporation. Individual spermatozoa were transferred into a 0.2 μL droplet comprised of 0.1 μL seminal plasma and 0.1 μL cryoprotectant. The Cryoleaf was subsequently sheathed with a casing and then slowly lowered (20 cm in 30 s) to 1 cm above an LN2 surface, exposed to the vapor for 2 min, and then plunged into the LN2.

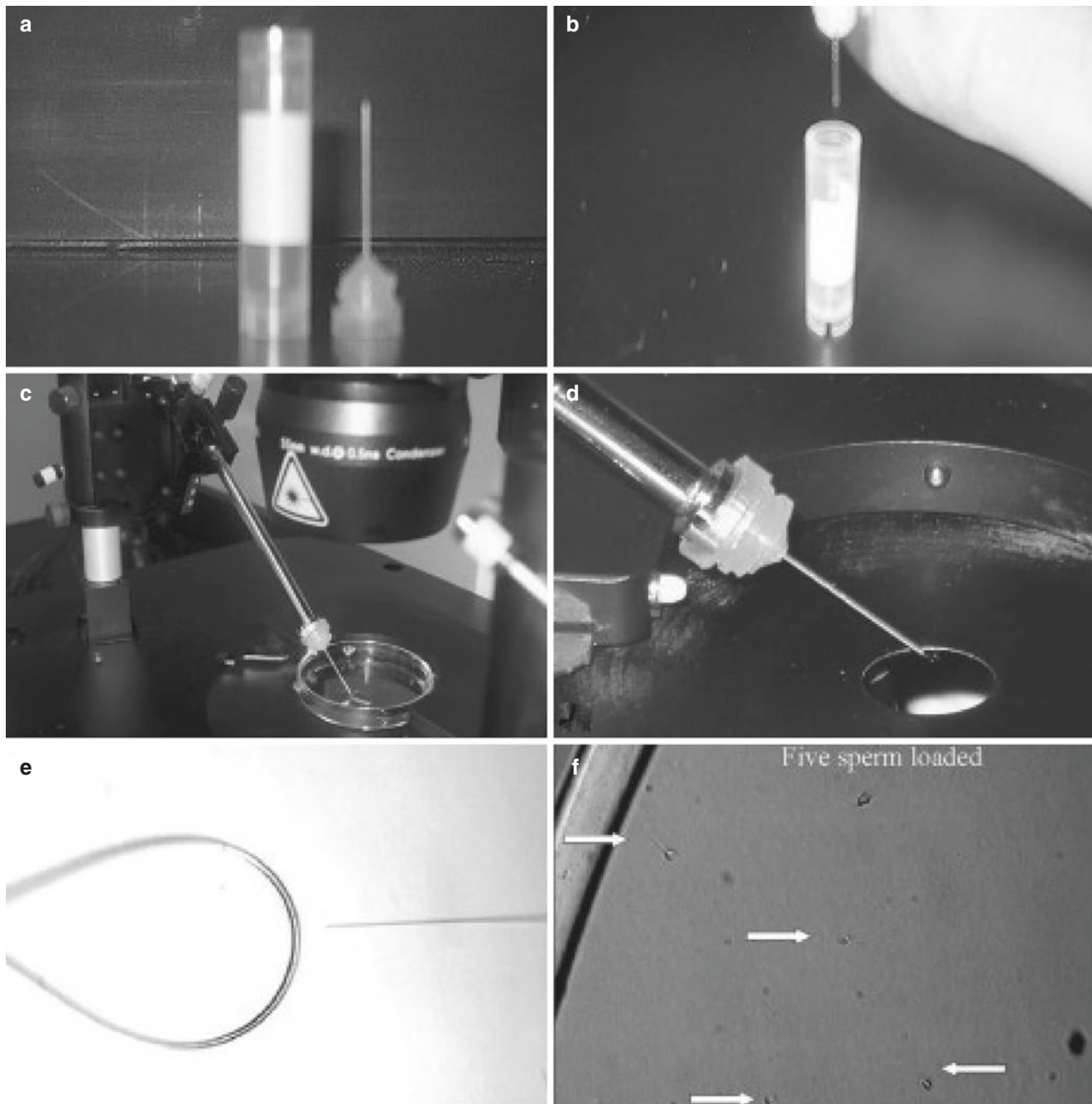


Fig. 45.1 (a, b) The Cryoleaf device, (c, d) Cryoleaf placed in the micromanipulation pipette holder so that it can be visualized under the inverted microscope. (e, f) Sperm loaded onto Cryoleaf with the aid of micromanipulation station and ICSI pipette

In the Cryoleaf method, the motility of recovered sperm obtained through percutaneous epididymal puncture was 93% after freezing and rewarming; ejaculated sperm had a motility recovery of 62%. Following ICSI injection, no differences in the rates of fertilization were observed between fresh and frozen-thawed spermatozoa, and the cleavage rates were similar [22]. Therefore, the cryoleaf method can effectively and conveniently be used to cryopreserve individual sperms. However, skilled operators are required and the

freezing process must be rapid to prevent evaporation of the droplets. The technical requirements of this technique limit its widespread use in clinical practice. No successful pregnancy has been reported using this technique.

Endo et al. [23] developed a simple novel verification technique for a single spermatozoon with the use of cryotop. The cryotop consists of fine polypropylene strips, a plastic handle, and a cover straw. It acts as a vitrification container for both embryos and oocytes.

Fig. 45.2 Description of the freezing procedure: (a) the testicular sperm suspension was centrifuged for 5 min at 1800 g in a 15 mL conical tube; (b) the sperm suspension was divided into 5 μ L drops in a 60 mm diameter dish; (c, d) 5 μ L drops of Sidney IVF Oocyte Wash Buffer medium were added and immediately removed to leave a “footprint” on the dish; (e) all the microdrops were coated with 5 mL of mineral oil; (f) the dish was covered and wrapped in transparent plastic film; (g) the dish was immersed into LN2 and stored until thawing

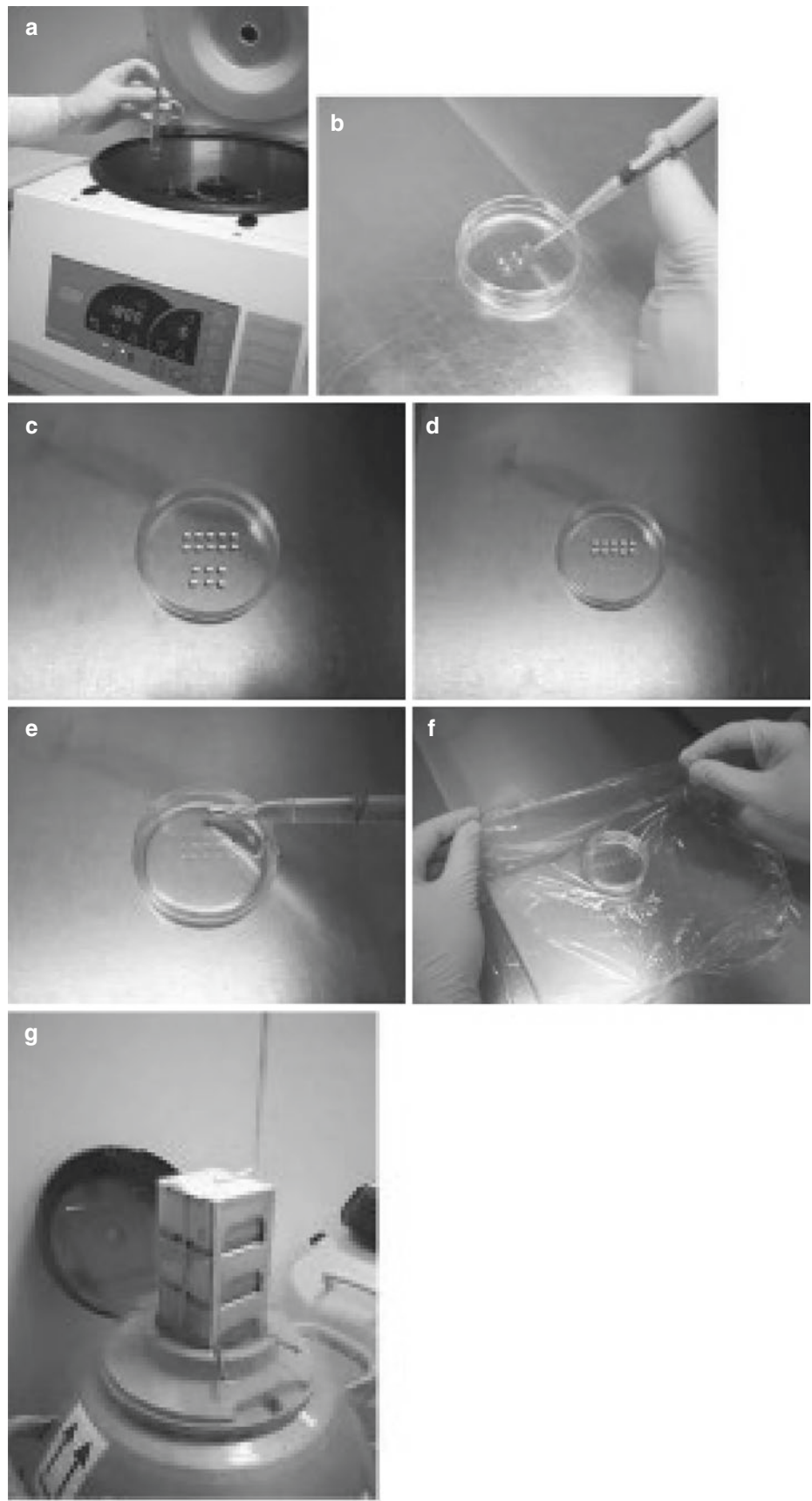


Fig. 45.3 A Cryoleaf bound to a handle by cotton thread is placed in a protective casing containing cotton wool at the bottom

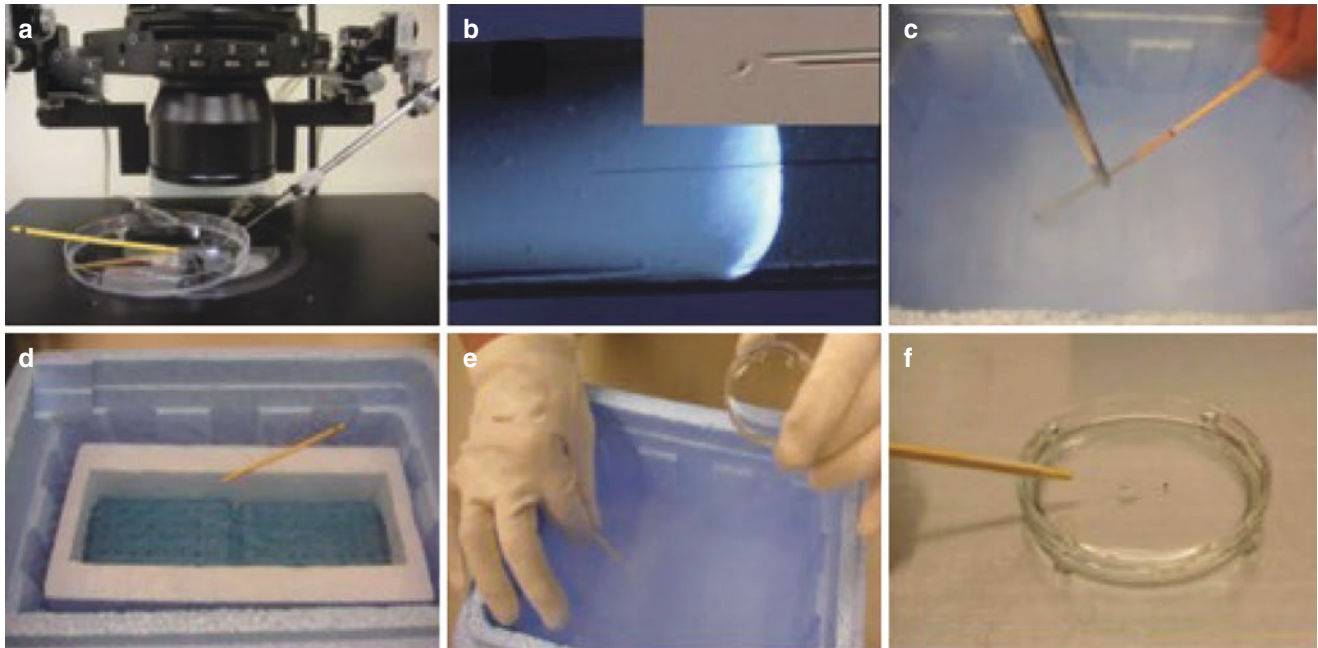
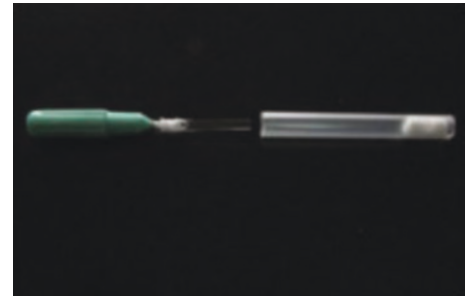


Fig. 45.4 Description of the freezing and thawing procedures. (a, b) A motile sperm was loaded into freezing medium on a Cryotop strip with the aid of a micromanipulator. (c, d) The Cryotop strip with a single

spermatozoon was frozen in LN2 vapor and then submerged in LN2. (e, f) For thawing, the Cryotop strip was taken out of LN2 and immediately placed in a droplet of medium, which was covered by mineral oil

Using an ICSI pipette, individual sperm cells were transferred at room temperature to a droplet of freezing medium (1 mL) on the cryotop strip. Immediately, the cryotop strip was placed 4 cm above the surface of the LN2 for 2 min and then directly exposed to sterilized LN2. The cryotop strip was covered with the hard plastic straw during storage in the cryotank. For warming, the cryotop strip was placed directly in a flat droplet of basic medium (2 mL), resuscitation was performed at 37 °C (Fig. 45.4).

Sperm from ejaculates and testis were frozen using Cryotop. The recovery and motility rates after thawing were 93% and 40%, respectively [7]. The authors used this technique clinically with two patients with severe oligozoospermia and NOA, respectively. The fertilization rate was 75% [24].

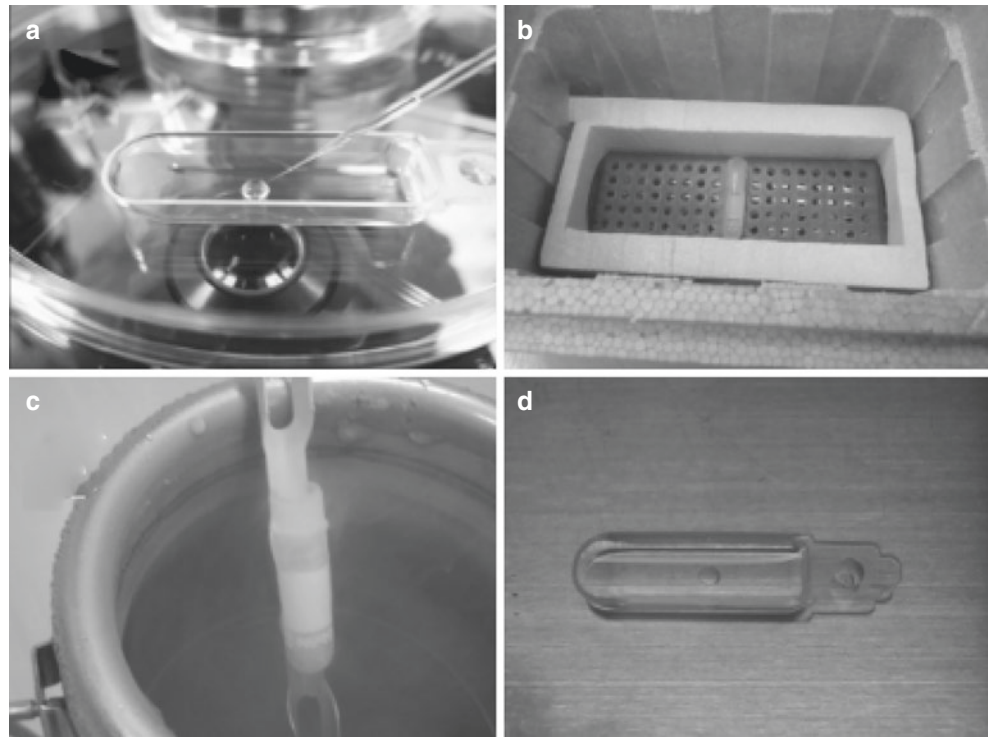
Although the Cryotop is suitable for cryopreservation of a few spermatozoa, the spermatozoa are loaded into a small

freezing drop on the top of the sheet using a manipulator in air. It is necessary to transfer spermatozoa quickly from the culture drop to the freezing drop before the drop evaporates. In addition, it was reported that sometimes it takes a long time to search for spermatozoa after thawing.

In 2012, Endo et al. [24] cryopreserved small numbers of spermatozoa using the Cell Sleeper, which is a closed system. The Cell Sleeper is a vial-based cell-cryopreservation container equipped with an inner tray. It was the first successful attempt at sperm vitrification with a small volume of droplets.

In the Cell Sleeper method, individual sperm were transferred using an ICSI pipette to a freezing medium drop deposited on the tray. The tray was immediately put into a vial and sealed with a screw cap. The vial was cooled in LN2 vapor for 2.5 min and then submersed in LN2. The Cell Sleeper was warmed at room temperature for 1 min and then

Fig. 45.5 Description of the vitrification and warming procedures using the Cell Sleeper. (a) Spermatozoa were loaded into the cryopreservation medium on a tray with the aid of a micromanipulator. (b) After placing the tray into a cryotube, the tray was cooled in LN₂ vapor. (c) The Cell Sleeper was mounted on a cane and stored in LN₂. (d) After warming the Cell Sleeper, the droplet on the tray was covered with oil and observed to retrieve the spermatozoa



the tray was removed from the vial, without delay. The sperm on the tray was immediately covered with oil and incubated at 37 °C for 2 min (Fig. 45.5). The sperm was retrieved individually from the Cell Sleeper.

This technique was applied clinically in a patient with NOA, resulting in the birth of a healthy boy. In addition, 12 spermatozoa from testis were vitrified. After thawing, 10 were successfully retrieved and injected individually into six mature oocytes; the fertilization rate was 83%. In 2016, Coetzee et al. [25] used Cell Sleepers to cryopreserve testicular sperm. The sperm recovery rate was 94% and the motility rate was 56%. Recovered thawed spermatozoa were injected into 179 mature oocytes and the fertilization rate was 66%. Embryo transfer was performed in 10 cases: 3 on day 3 and 7 on day 5, resulting in a per cycle pregnancy rate of 58.3%.

The Cell Sleeper method appears fast, easy, and simple to complete. Endo et al. [24] reported that the recovery search for sperm took approximately 30 min. This period is relatively long and may damage the sperm cell due to prolonged exposure to the cryoprotectant solution. In addition, microsurgery requires constant lifting and lowering of the ICSI needles; thereby, leading to higher risk of needle breakage.

In 2015, Araki et al. [26] designed hollow-core agarose capsules similar in size to mammalian oocytes. These were

used for cryopreservation for individual sperm. The hollow-core agarose capsules were produced using calcium carbonate and alginate acid water. The structures had an agarose wall (outer diameter, 80–120 μm; inner diameter, 60–100 μm).

A single sperm was injected into each capsule as per the conventional intracytoplasmic sperm injection (ICSI) method (Fig. 45.6). Then, the capsules were transferred into a drop of cryoprotectant solution. The cryoprotectant solution that contained the capsules was placed on polycarbonate or nylon mesh sheets. For freezing, a special tool was made using styrene foam (Fig. 45.7). The solution was gently frozen with LN₂ vapor and the sheet was immersed in LN₂ after 10–30 s.

After thawing, the sheet was transferred onto the drop of the medium and held there for several seconds to separate the capsules from the sheet. Sperm motility in the capsules was checked using an inverted microscope.

Although this study described the methodology, it is still being developed and recovery of sperm from the capsules was not described. Studies are needed to investigate whether the method can be used for patients. Additional sperm function testing data will be needed to assess a potential carrier for clinical applications.

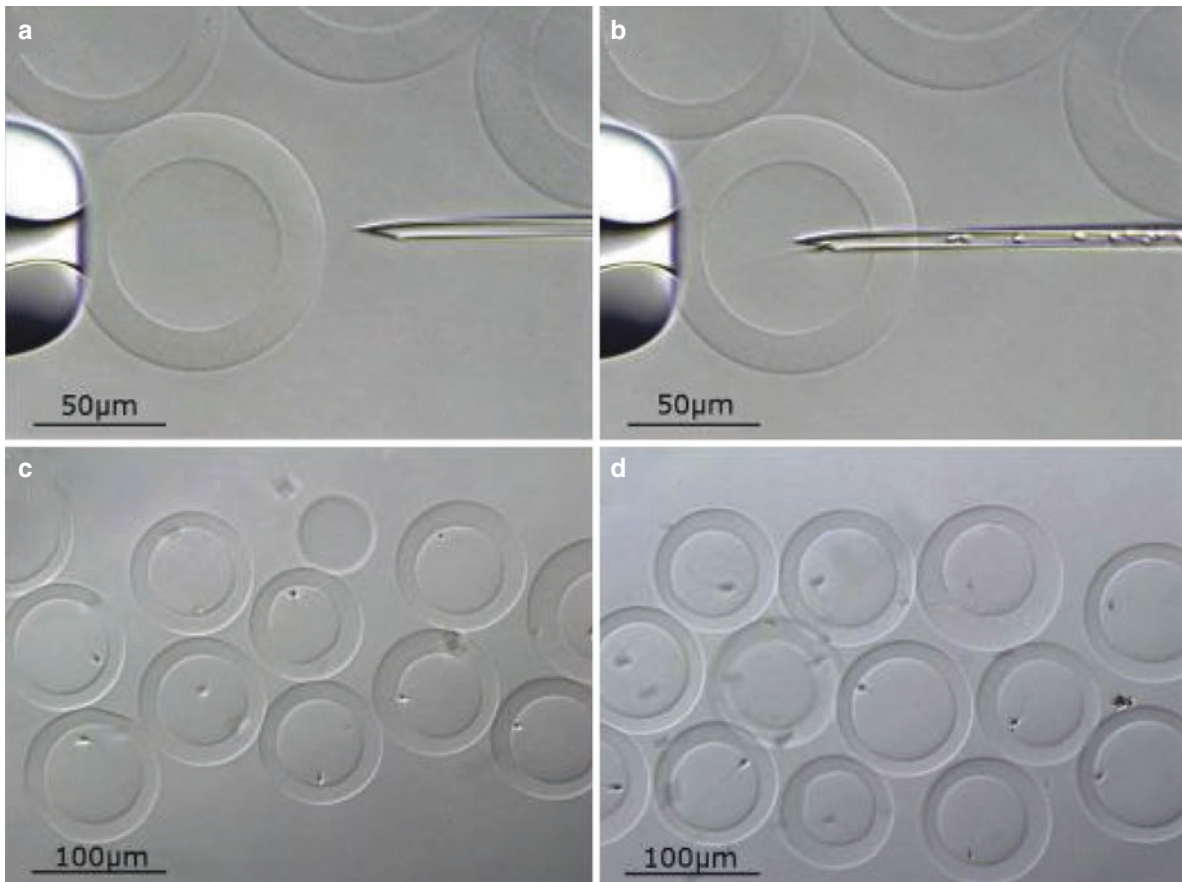


Fig. 45.6 Sperm injection into an agarose capsule. (a) Agarose capsule held by a holding pipette before injection. (b) Injection pipette inserted into an agarose capsule. A single sperm was injected into the

capsule. (c) Agarose capsules containing sperm before freezing. (d) Agarose capsules after thawing

In 2016, Tomita et al. [27] showed that hollow hyaluronan-phenolic hydroxyl (HA-Ph) microcapsules could be used to cryopreserve small numbers of sperm, with no adverse effects on sperm quality. HA microcapsules were fabricated using a system of water-immiscible fluid under laminar flow. Hollow HA microcapsules were prepared by decomposing the gelatin core of the capsule. Three sperm were injected into a hollow HA microcapsule using conventional ICSI procedures (Fig. 45.8). An HA microcapsule mimicking zona pellucida (HA-ZP) was frozen based on a rapid freezing method using sugar as cryoprotectant. The HA-ZP injected sperm were incubated in 10 μ L droplets of cryopreservation medium for 5 min at room temperature. Capsules were placed into Cryotop devices containing 1 μ L of cryoprotectant and were placed 4.5 cm above the surface of LN2 for 2 min, then plunged into LN2.

After thawing, the sperm were recovered in hyaluronidase-decomposing HA-Ph gels. Although the sperm recovery rate

was 95%, the rate of motile sperm (14%) was relatively low after resuscitation. It was shown that treatment with HA did not impair sperm motility or viability. The low motility rate was due to the freezing of the aggregated gelatin, which damaged the sperm plasma membranes.

The main disadvantage of using capsules is they are difficult to fabricate. Additional studies are needed to test the safety and efficiency of HA microcapsules before incorporating them into clinical practice.

Sun et al. [28] reported a novel, high-efficiency storage device, Cryopiece.

The Cryopiece was designed for cryopreservation of single spermatozoa or small numbers of spermatozoa. The device and cryopreservation procedure are shown in Fig. 45.9. The spermatozoa suspension was loaded into the microstrips in the dish covered with mineral oil and cultured for 1 h until the spermatozoa moved to the margins of the strips. The spermatozoa with normal morphology and higher

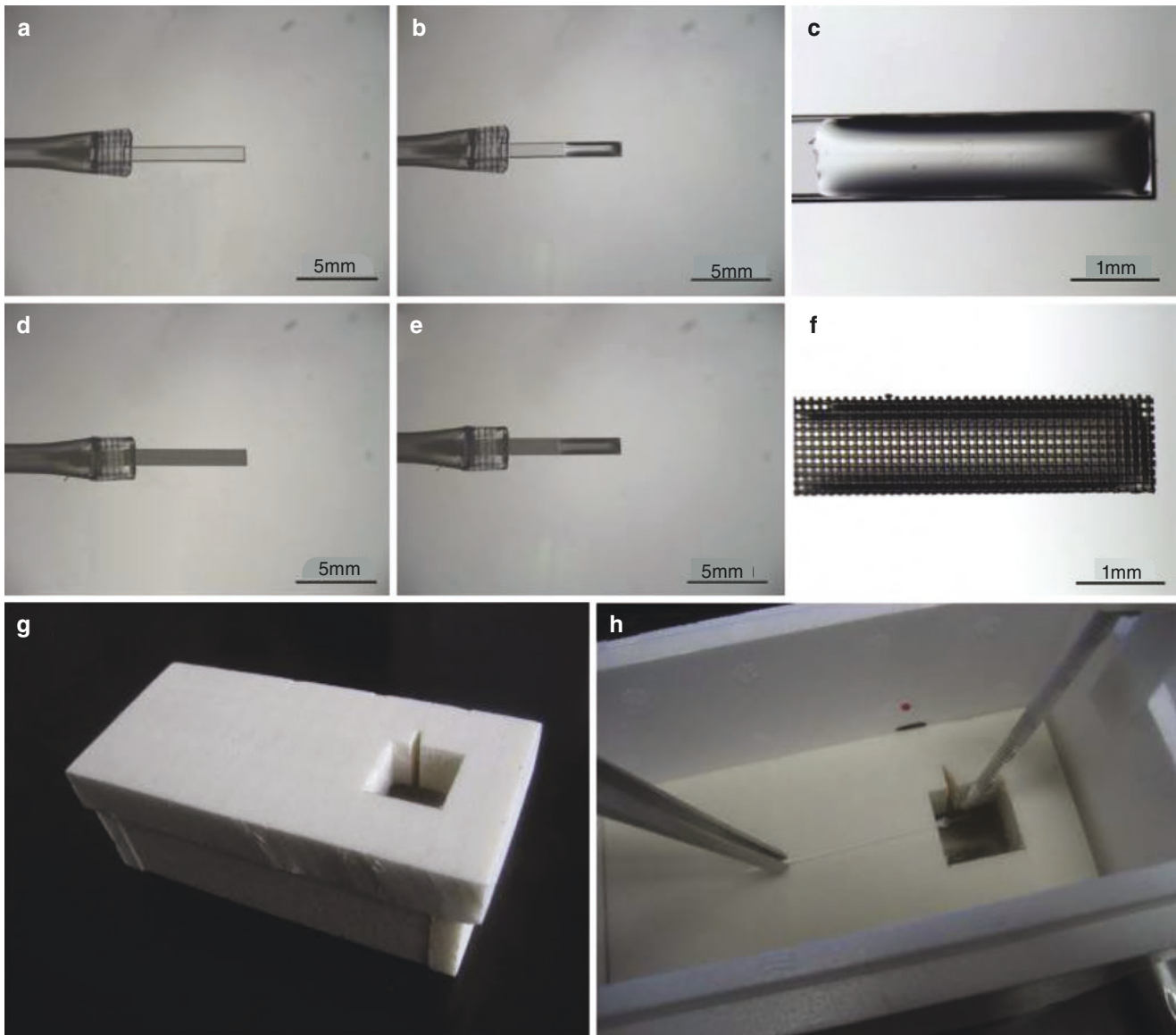


Fig. 45.7 Devices for freezing capsules. (a–c) A polycarbonate sheet fixed on a plastic straw. (d–f) A mesh sheet fixed on a plastic straw. (b, c, e, f) Capsules containing cryoprotectant solution on the top of the

sheets. (g) The special tool was made using a styrene foam board. It had a square 2.5-cm hole on the top of a styrene foam box. (h) The box floated on LN2 vapor within a larger box for freezing the capsules

motility were captured under a microscope using the ICSI pipettes on the micromanipulator and then loaded onto the indicated medium drops on the Cryopiece. After holding the piece over liquid nitrogen vapor for 15 min, they were put into a cell freezing tube and stored in an LN2 tank.

For thawing, the cryopreserved Cryopiece was pulled out of the freezing tube and placed at the bottom of a 35 cm dish and immediately covered with pre-warmed mineral oil (Fig. 45.9e). The thawed and recovered spermatozoa were prepared for ICSI.

After thawing, 83% of the spermatozoa completely recovered with 48% movement. Following ICSI, the fertilization rate was 73% and 19 (86%) zygote cleavage events were observed. Four healthy babies were born at term. The clinical data published included only four patients and additional clinical trials are needed.

In 2018, Berkovitz et al. published information about a new device for verifying small numbers of spermatozoa [29, 30]. The SpermVD allows quick and convenient vitrification of spermatozoa (or even a single cell). The

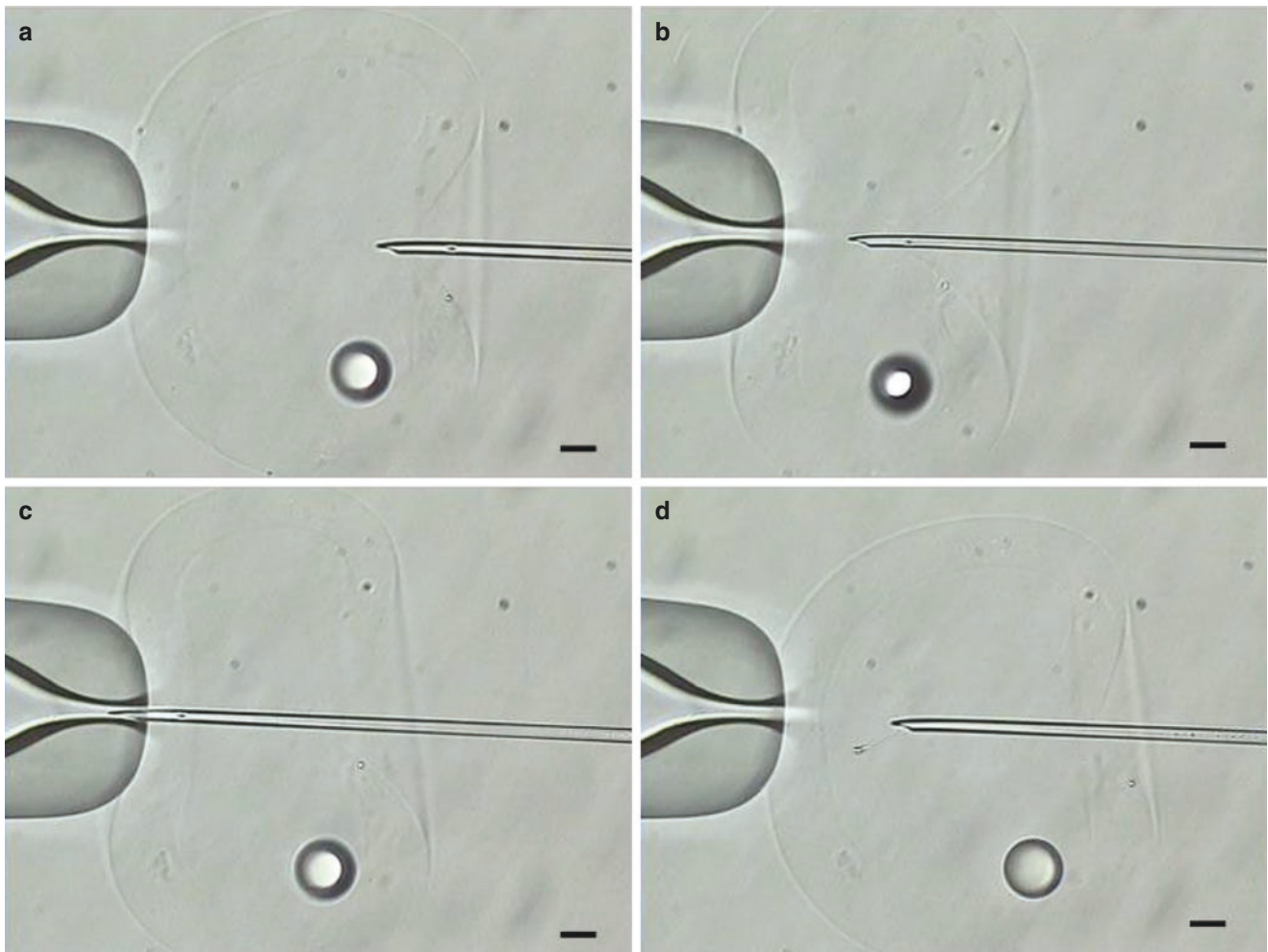


Fig. 45.8 Injection of sperm into a hollow hyaluronan-microcapsule mimicking the zona pellucida (HA-ZP) using a conventional intracytoplasmic sperm injection (ICSI) procedure. (a, b) HA gel was pushed by the injection needle. The capsule morphology was changed by the injection force, but the HA gel did not penetrate at position (b). (c) HA

gel penetrated in the position where the injection needle touched the opposite side of the HA gel. (d) The penetrated HA-ZP returns to a spherical shape. The small sphere in the capsule is a drop of mineral oil injected before sperm injection. Scale bars = 20 μm

thawing process is equally simple, as the small size of the droplets allows quick retrieval of the sperm, which may be used immediately for ICSI (intracytoplasmic sperm injection). SpermVD is injection molded from medical grade polycarbonate sterilized by gamma irradiation. Its dimensions are $26 \times 8 \times 7$ mm and it weighs 0.2 g (Fig. 45.10).

The instructions for the SpermVD follow.

Attention!

*The transfer of spermatozoa from SpermVD to sperm washing medium droplets should be started **immediately** after placing the SpermVD under oil.*

Sperm vitrification procedure:

1. **Collect individual spermatozoa into one or more droplets of sperm washing medium (Quinns™ Advantage Sperm Washing Medium, ref. ART-1006, Sage In-Vitro Fertilization, Inc. or a similar product) on an oil-covered (Oil for tissue culture, ref. ART-4008P, Sage In-Vitro Fertilization, Inc., or a similar product) SpermVD DISH (MFC Global, Ref. SVD-1002).**

**Anywhere where SpermVD dish is indicated, a standard IVF-compatible Petri dish can be used. All figures in this IFU relate to the use of the SpermVD DISH.*

2. Using sterile forceps, remove the SpermVD (MFC Global, Ref. SVD—1001) from its package and place it on

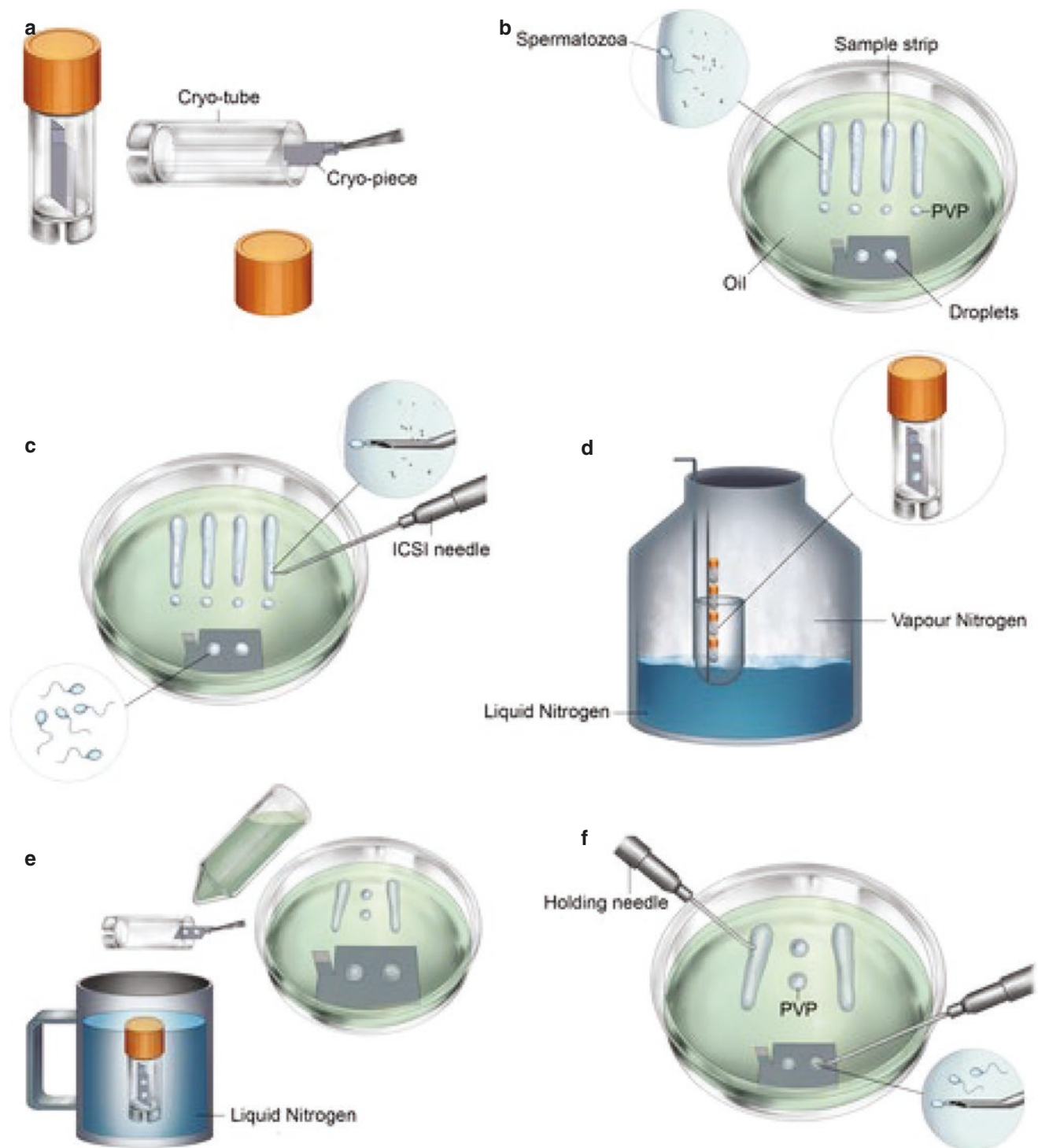


Fig. 45.9 A model of the Cryopiece-based freezing/thawing procedure. The Cryoloop device (**a**, **b**), Cryoloop placed in the micromanipulation pipette holder (**c**, **d**) so that it can be visualized under the inverted

microscope. (**e**, **f**) Sperm loaded onto Cryoloop with the aid of micromanipulation station and ICSI pipette

a sterile culture dish or dish cover. Do NOT cover with oil.

3. On the same dish cover prepare the cryoprotectant solution by mixing sperm washing medium and sperm freez-

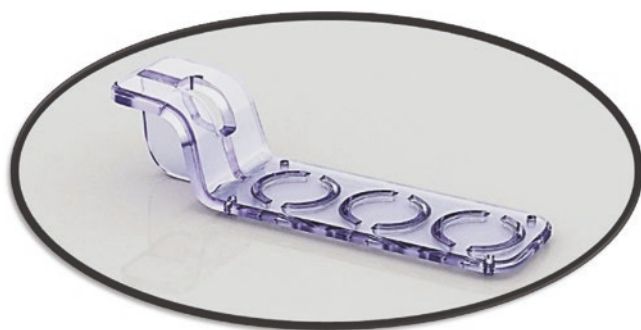


Fig. 45.10 The SpermVD. The post-thaw motility and recovery rates were 33% and 96%, respectively. Post-thaw, the fertilization rate was 59%, pregnancy rate 55%, and delivery rate 39%

ing medium (Quinns™ Sperm Freezing Medium, ref. ART-8020-12, Sage In-Vitro Fertilization, Inc. or a similar product) as per freezing medium manufacturer's protocol.

Attention!

Since washing medium evaporates faster than freezing medium, create a new mixture droplet for each additional SpermVD to avoid changes in concentration

4. Place 0.8–1 μ L droplets of the cryoprotectant mixture in the center of the marked areas on the SpermVD (Fig. 45.11.1)

Tip: Do not flatten the droplets so they are more stable and allow better sperm motility along the borders.

5. Immediately after preparation, gently place the SpermVD into the designated cavity on the SpermVD DISH containing spermatozoa (Fig. 45.11.2).

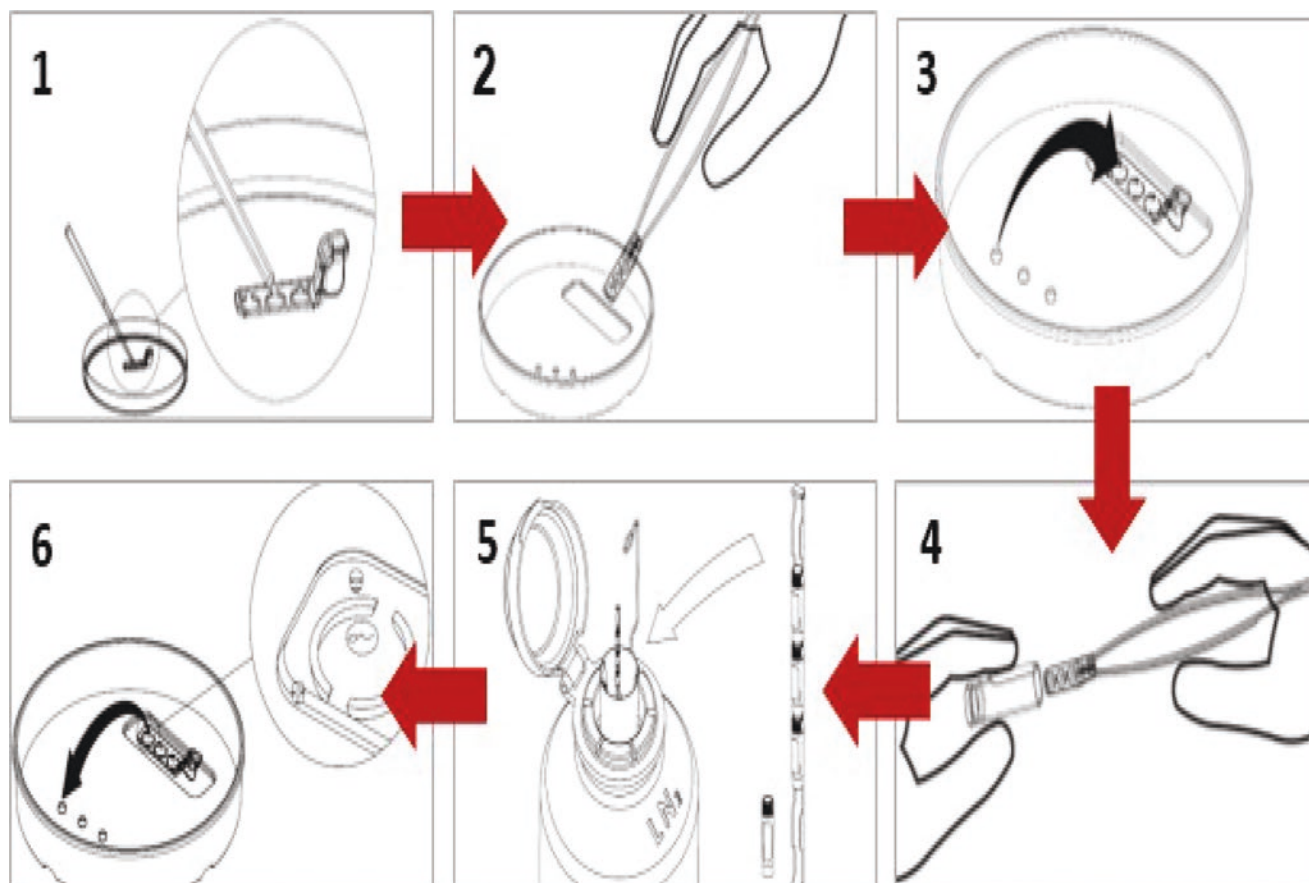


Fig. 45.11 Sperm VD procedure. Droplets of the cryoprotectant mixture are placed in the center of the marked areas on the SpermVD (Fig. 45.11.1). The SpermVD placed into the designated cavity on the SpermVD dish containing spermatozoa (Fig. 45.11.2). An ICSI injection pipette, mounted on a micromanipulator is used to transfer the spermatozoa from the collection droplets to the SpermVD (Fig. 45.11.3).

A labeled 1.8 mL or 3.6 mL cryovial is mounted on a cryorack. The SpermVD is transferred into the cryovial (Fig. 45.11.4). The cryorack is transferred into an LN₂ tank (Fig. 45.11.5). After thawing, the spermatozoa inside the droplets are transferred to a sperm washing medium droplet (Fig. 45.11.6)

- Using an ICSI injection pipette, mounted on a micromanipulator (Eppendorf or equivalent) under $20\times$ magnification, transfer the spermatozoa from the collection droplets to the SpermVD (Fig. 45.11.3). It is recommended to freeze no more than 15–20 cells per device, so the number of frozen spermatozoa suffices for an average oocyte yield of an OPU cycle.

Tip: For better control of progressively motile sperm, they can be transferred into a PVP droplet first and then into SpermVD droplets. Note that post-thaw motility might be affected.

Attention: SpermVD containing spermatozoa must be frozen within 8–10 min from the placement of the first cell.

- Mount a labeled 1.8 mL or 3.6 mL cryovial on a cryorack. Gently pick up the SpermVD from the SpermVD DISH and transfer it into the cryovial (Fig. 45.11.4). Leave any excess oil on the SpermVD, as it serves as an additional protective layer for the droplets.

Tip: Do not tighten the cryovial cap completely. It serves both to prevent accumulation of pressure inside the vial and to facilitate entry of LN2 which will prevent thawing if the vial is briefly exposed to ambient temperature.

- Carefully transfer the cryorack into an LN2 tank. The mounted vial can also be submerged into an LN2 bath and after making sure that a sufficient volume of LN2 entered the cryovial and covered the SpermVD, to transfer it to storage in an LN2 tank (Fig. 45.11.5).

Attention:

This is the most delicate phase. Take caution when placing the cryovial on the rack and lowering the rack into the LN2 tank. The droplets are stable but may be dislodged by an abrupt shake.

Warming procedure

- Remove the cryovial from LN2, carefully unscrew the cap, wait until LN2 inside vaporizes and let it thaw in a horizontal position at room temperature or on a heated surface or in 37°C water bath, until the oil covering the droplets is completely liquified.
- Remove the SpermVD from the cryovial and place it in the designated cavity on an oil-covered SpermVD dish with one or more extra sperm washing medium droplets in addition to your standard ICSI setup of microdrops.
- Search and locate the spermatozoa inside the droplets under $20\times$ magnification and transfer them to a sperm washing medium droplet (Fig. 45.11.6). Once all cells

have been transferred, it is possible to proceed with ICSI.

- The transfer of spermatozoa from SpermVD to sperm washing medium droplets should be started immediately after placing the SpermVD under oil.
- After use, dispose of the SpermVD and the SpermVD DISH in accordance with local regulations.

45.1 SpermVD Dish

The SpermVD Dish is an accessory for SpermVD designed to enhance and optimize the process of freezing and warming small number of spermatozoa (Fig. 45.12).

The accessory product provides several advantages over other dishes, commonly used for searching, preserving, heating, and injecting rare sperm:

- Stability**—The niche prevents the SpermVD shifting, when the dish is moved around, allowing easier and safer work for the embryologist.
- Focal plane**—The niche brings the SpermVD to the same focal plane as the surface of the SpermVD Dish, eliminating the need to adjust the height of the micromanipulator arm.
- CE Mark**—The SpermVD Dish is CE Marked and MEA tested, thus suitable to serve as an ICSI dish for all purposes.
- ICSI Dish too**—The location of the niche near the perimeter leaves enough room for a standard ICSI layout, so retrieved spermatozoa can be injected immediately.

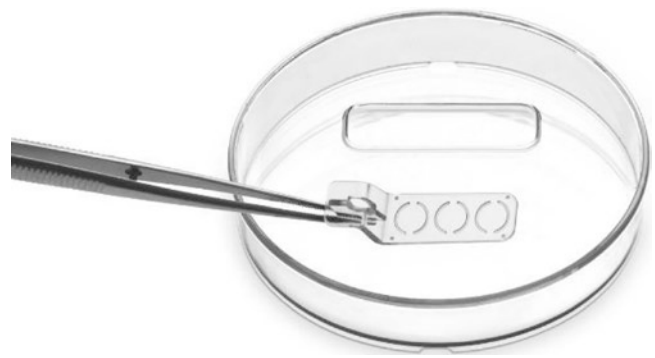


Fig. 45.12 SpermVD DISH. The SpermVD Dish is a 60 mm-wide Petri dish containing a niche-like indentation specifically designed to fit the SpermVD

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Keeping Cryopreserved Specimen Safe: Thermographic Imaging

46

Thomas Elliott

Key Points

- Thermography is an effective method to provide additional insight into the health and integrity of a cryostorage system.
- Thermographic monitoring provides feedback on the exterior temperature of cryogenic dewars which is a much more sensitive marker in the case of failure than measuring internal conditions (Fig. 46.1).
- By using thermography for relative temperature differentiation, very small differences in temperature can be visualized and dewars displaying slightly cooler temperatures can be removed from use and replaced with a dewar with a non-compromised vacuum.
- Thermography provides the ability to see the entire cryogenic storage at a facility remotely providing real-time information on the integrity of the vacuum.
- Infrared monitoring can provide an additional layer of protection over traditional or developing methods of cryogenic vacuum failure monitoring.

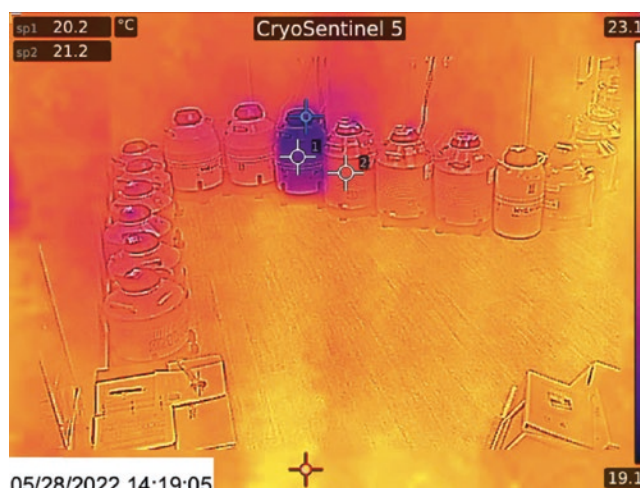


Fig. 46.1 Thermographic monitoring showing a single degree difference in the external temperature between two dewars

46.1 Introduction

46.1.1 Traditional Methods of Cryogenic Monitoring

The fundamental principle of cryogenic storage is limiting heat energy transfer by the use of a vacuum. A vacuum does not permit the transfer of heat energy as there is no vector within the vacuum to permit this. This allows a cryogenic liquid, such as liquid nitrogen, to be stored in relatively stable conditions inside a vacuum storage container for long periods of time, and thus allows the use of this liquid for effective cryogenic storage of samples. Vacuum cryogenic storage was invented by James Dewar,

a Fullerian Professor of Chemistry at the Royal Institution in London, in 1892. These dewars have been utilized from the early days of IVF to store sperm and excess embryos either short or long term. To a very large extent, these same style dewars are in use still today and along with modern manufacture of these containers have become very reliable. As a simple solution for long-term storage, there is very little that can go wrong with them; however, a vacuum leak is always possible [1, 2] and failure can be very costly [3] and so should be prepared for. The traditional method of monitoring for vacuum failure is internal temperature monitoring with a probe that is immersed into liquid nitrogen. The reasoning behind this is that as liquid nitrogen slowly evaporates, the level will decrease and eventually the probe will be in vapor phase nitrogen, which is warmer than liquid nitrogen. Internal temperature monitoring is easy to implement with Resistance Temperature Detector (RTD) probes with either a wired or wireless con-

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nection to a data gathering center (computer, Internet Cloud, etc.). Until recently, internal temperature monitoring was ubiquitous in the cryopreservation industry and is used worldwide.

46.1.2 The Importance of Vacuum Integrity

The integrity of the vacuum in cryostorage containers is of paramount importance to its functionality. The vacuum inhibits the transfer of heat through the walls of the container and thus enables the retention of the cryogenic liquid inside the container. If the vacuum is compromised, molecules of air can act to transmit heat and thus warm the interior of the storage container and in addition cool the exterior wall. The degree to which this process occurs is directly related to how much the vacuum is compromised. It is possible to have a very slow leak and the vacuum deplete over a long period of time. It is also possible for a sudden and catastrophic loss of vacuum. Recent experiments performed by Nagy [4], Pomeroy [5], Kelk [6], and Schiewe [7] have examined catastrophic vacuum loss by drilling a hole in the outer wall of the dewar or piercing a hole in the inner wall [4].

46.1.3 Signs and Symptoms of Vacuum Failure

During a vacuum failure, there are several signs and symptoms to look out for [8]. Awareness of these can help laboratory staff take precautions and act quickly in the event of vacuum failure.

1. The exterior of the dewar will cool. This can be observed by thermography, touch, or external temperature sensors. If the high humidity is high in the room, this could also lead to a degree of condensation, which is sometimes referred to as “sweating.”
2. There might be a hissing noise as the vacuum leaks out.
3. Liquid nitrogen vapor can exit from the top of the tank, cooling the surrounding air condensing water vapor in the air.
4. Frosting can occur. Depending on the location of the vacuum leak, the frosting can occur around the neck of the dewar as nitrogen vapor escapes and cools the surrounding area, or it could be towards the base of the dewar in the case of an internal leak where liquid nitrogen can leak into the vacuum space.
5. There is a decrease in the level of liquid nitrogen in the container. As the vacuum depletes and heat transfer increases, the liquid nitrogen will start to boil, turning from a liquid to a gas which then dissipates into the air.

46.1.4 Consequences of Vacuum Failure

If the vacuum in a dewar or storage container is lost, the liquid nitrogen within the storage container will also be lost. The length of time that this takes depends on the size of the cryogenic container [7], the level of liquid nitrogen within the container, the size of the hole (tiny crack vs a drilled hole) that caused the loss of nitrogen and the temperature of the room where the container is located. For a dewar, such as the commonly used MVE 47/11, the amount of time that can be expected between catastrophic vacuum failure and critical glass transition phase (approx. 125 °C) is about 19 h [6]. At temperatures higher than this, irreversible damage can occur in either oocytes or embryos, including complete loss.

46.2 Thermographic Imaging

46.2.1 What Is Thermographic Imaging?

Thermographic imaging, or thermography, is the use of an infrared camera to produce images that display infrared radiation that is either emitted, transmitted, or reflected as a color on the image or video. These thermographic images can be overlaid with visible light to produce an image that has more definition compared to infrared radiation images alone. Modern thermographic equipment can also determine a calculated temperature of an object based on infrared radiation and have the ability to measure field of view, region of interest or spot temperatures and various measurements within each of these areas including maximum, minimum, and average temperatures.

Thermographic cameras can be very sensitive to variations in temperature and the display of colors that represent temperatures within a field of view. This creates an easy way for a human operator to see fluctuations in temperature, especially when comparing objects within the same vicinity. The combination of the thermographic display, together with much more accurate relative temperature monitoring, can allow much smaller differences in temperature to be detected, for example, between two dewars if one were to be slightly cooler (Fig. 46.1).

46.2.2 Thermographic Imaging and Cryogenic Storage

While traditional monitoring methods of cryogenic storage utilize a change within internal conditions, usually a change in internal temperature of the level of nitrogen. Thermography uses external temperatures to assess potential vacuum failures. Research by Graham [8] showed that lid temperature went below 5 °C in every dewar type and type of failure with

12 retired various model cryogenic dewars, thus detecting and alerting to external drops in temperature is a valid additional safeguard for dewar failure in addition to traditional monitoring methods. Additionally, thermography can provide specific advantages over internal condition monitoring. Mainly the ability to monitor multiple cryogenic storage vessels at the same time (which provides a direct comparative method for observing external temperatures), and the speed of being able to determine a failure (external conditions change much faster than internal conditions in the event of a vacuum failure). Thermography also provides unique insight into the current conditions and health of the cryogenic storage system by way of a simple video interface (Fig. 46.2). This provides a real-time method for the exterior temperature assessment of each cryostorage vessel within the field of view. This video image can also be streamed online to provide staff with the ability to check in on the cryostorage at any time, day or night, and immediately see the conditions in the event of an alert. Thermography can also be used to evaluate the vacuum integrity of cryogenic dry shipping units which are considerably more challenging to validate vacuum integrity (Fig. 46.3).

46.2.3 Thermodynamics of a Cryogenic Dewar Vacuum Failure

If there is a breach in the integrity of the dewar vacuum, air molecules enter the space between the inner and outer wall of the dewar and can start to transfer heat energy. The transfer of heat energy implies two things for compromised dewars: (1) the cold from the cryogenic liquid can start to make the exterior of the cryogenic dewar decrease in temperature and (2) the heat from the room in which the cryogenic dewar

is located can start to be transferred to the inner wall of the dewar. The main concern of this is that the cryogenic liquid will start to boil and evaporate at a much faster rate than usual. As the cryogenic liquid boils and evaporates, the liquid nitrogen level decreases and once the level reaches a critical point the samples will be lost.

The effect of heat transfer is the effect on the temperatures inside the dewar. In the event of a catastrophic dewar vacuum failure, there is a sudden and dramatic exchange of heat energy. The effect of this is that the cryogenic liquid starts to boil, as already determined. In the case of liquid nitrogen there is an expansion rate of 694:1, so a massive expansion. This expansion forces the vapor phase nitrogen out of the top of the storage container/dewar and results in a shift in temperature gradient between the liquid and vapor phase. The result of this is a super cooled vapor phase inside the cryogenic storage dewar. This phenomenon can be easily observed by measuring the temperature at the top of a small thermos flask containing some liquid nitrogen, and dropping into the liquid a small heat source, such as a metal bolt. This causes a significant drop in temperature, even though it is still the vapor phase that is being measured.

46.2.4 Vapor Phase Nitrogen vs Supercooled Vapor Phase Nitrogen

It is important to remember that the temperature of vapor phase nitrogen can be different depending on the conditions. This response becomes very significant when we examine the ubiquitous method of monitoring nitrogen dewars around the world, which is internal temperature monitoring. Several experiments have been performed on cryogenic dewars where a hole is drilled directly into the outer wall of the dewar and



Fig. 46.2 A failing dewar showing clear signs of vacuum failure even at room temperature



Fig. 46.3 A failing dry shipper showing signs of vacuum failure with CryoSentinel thermographic monitoring

observing the effects, particularly with monitoring systems that utilize internal temperature probes. An experiment performed by Dr. Zsolt Peter Nagy [4] demonstrated that when drilling a hole in the exterior of a dewar the expected time that an MVE 47/11 style dewar, that had recently been filled, could retain liquid nitrogen was between 18 and 22 h. Additionally, when the internal probe alerted lab staff there was approximately 1 cm of nitrogen remaining in the bottom of the dewar. Dr. Kim Pomeroy also conducted a similar experiment showing that with their monitoring system, there was about 1 h between alerting staff and critical warming temperature. In both of these cases, it would be generally accepted that there was not enough time for lab staff to take action to save stored samples, especially if the alerts were sent after hours or the staff were not physically present at the facility. Therefore, it is critically important to realize that internal temperature does not directly correlate with liquid nitrogen depth in the event of vacuum failure. Also, the location that internal temperatures are measured can also lead to a variation in temperature. Due to these issues, internal temperature monitoring should be fully evaluated before relying on it as the sole source of vacuum failure alerts.

46.2.5 How Can Thermographic Monitoring Help?

Thermographic monitoring has the ability to measure the external temperatures of dewars and present data in a way that can be easily understood. This means that should a dewar's exterior temperature be colder than others around it, a user looking at the thermographic video will be able to see it. Additionally, comparative thermographic video is so sensitive that even small differences in temperature (0.5–1.0 °C for example) can be visualized (Fig. 46.1). This makes thermographic video a very valuable tool to monitor relative external temperatures of multiple dewars. It is not possible to simply monitor external temperatures with probes and achieve this sensitivity because the accuracy of each probe is not enough to determine such a small difference in temperature.

In an unpublished experiment by Brian LoManto and Kim Pomeroy, a dewar that had failed validation was filled with liquid nitrogen and allowed to equilibrate. It was placed next to other dewars that were currently in use (Fig. 46.2). Once equilibrated, the thermographic video of the dewar that had failed validation showed a slightly colder external temperature compared to other dewars. This temperature was still close to room temperature and would be very difficult to determine with any other method. The dewar was left in the view of the thermographic video system for a period of a few days until the dewar started to fail. The thermographic video clearly showed the decrease in temperature of the dewar, and this was highlighted by a dynamic color shift with further help to highlight the failing dewar.

46.2.6 Thermography as an Early Warning Alert System

As detailed above, the use of comparative thermography can indicate a compromised dewar or imminent failure of a dewar, and when combined with bi-daily QC, can provide reassurance on the integrity of the cryogenic storage system at a glance. This passive method can proactively spot potential issues a day, or even longer, in advance of a catastrophic failure and can also be enhanced by the addition of active thermographic temperature monitoring.

Depending on how the dewar fails, the exterior of the dewar will cool down. In the event of an external wall breach the external temperature might reach temperatures just below freezing to –4 °C. A failure of the internal wall where liquid nitrogen might leak into the space between the two walls can lead to a considerably colder external temperature.

Temperature thresholds can be determined to alert staff when conditions occur that could indicate a dewar vacuum failure. Once these parameters are crossed staff can be alerted, and they can immediately check the thermographic display and their other monitoring systems.

Thermography also gives insight into what is happening during the time of the failure. Many people think that the crack in the dewar might be visible, or that a colder area might be apparent. In practice, it can be seen that the dewar in its entirety cools down uniformly (in the event of an external vacuum leak). This observation can be understood by realizing that in the event of a vacuum failure it is the loss of the vacuum (the vacuum has no temperature and will suck air in) rather than the expulsion of cold air or nitrogen.

These observations had the added advantage because the thermographic video can be recorded and archived. Therefore, in the event of a vacuum failure, the timing and details can be re-examined, thus providing a complete picture and timeline of any issue.

As is the case in all systems, thermographic monitoring has both advantages and drawbacks. These pros and cons should be taken into consideration when selecting how to best manage the monitoring of your cryogenic storage system. It is the philosophy of the author that no one system is perfect and that redundancy in both method and communication technology is extremely important due to the nature and value of the samples being stored.

46.2.7 The Advantages of Thermographic Monitoring of Cryogenic Dewars

The most obviously beneficial advantages of thermographic monitoring are the ability to preempt a dewar failure and take proactive and preventative action to prevent the loss of cryogenically preserved reproductive material, the superior sensitivity of measuring radiation over internal condition

changes and the simplicity in which the thermographic data is displayed on a constant basis. Additional advantages pertain to the speed with which abnormal conditions indicating vacuum failure can be sent versus monitoring internal conditions.

The format of thermography also lends itself to specific advantages such as the ability to stream the thermographic video to outside of the cryostorage facility, so that staff can view the same video locally or remotely through any web-enabled device. Additionally, the thermographic video files can be stored or archived and referred to if there is ever an issue.

Another benefit of thermographic video monitoring is the ability to oversee multiple dewars, indeed, due to the ability of each thermographic camera to monitor multiple dewars and for cameras to be networked, it is possible to monitor a very large number of dewars effectively and affordably.

Thermographic monitoring can also offer benefits to individuals or corporations that need to monitor multiple cryostorage facilities or areas. Directors who manage multiple labs can see remote feeds and check on the cryostorage status at any time, corporations might want to have a centralized “command center” that can monitor labs in addition to local staff. This will also provide another layer of security.

46.2.8 Considerations of Thermographic Monitoring Systems

There are some considerations when adding a thermographic monitoring system to your facilities’ cryogenic storage. The dewars have to be in line of sight of the cameras, and it is possible that dewars could be inadvertently moved or blocked by another object and thus no longer able to be monitored. The room that the dewars are in needs to have a reasonably constant temperature and cold objects like windows or air

conditioning units can cause issues. It is often possible to select regions of interest to avoid these cold areas, but it is best to have a uniform room with a constant temperature of 18–22 °C. Another consideration is emissivity, which is the reflective properties of some materials, such as stainless steel. Emissivity can mean reflected temperatures of objects around the surface to be monitored are being measured instead of the actual temperature of the object. This can be fixed by covering the surface with a non-insulating material that is non-reflective.

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47.1 Introduction

In vitro fertilization (IVF) revolutionized the treatment of infertility, providing the extracorporeal development of a patient's embryos for intra-uterine transfer and, ideally, implantation, leading to a healthy liveborn child [1]. The application of controlled ovarian hyperstimulation improved the efficacy of IVF by enabling the collection of multiple mature oocytes during a single treatment cycle, as opposed to the normal menstrual cycle that yields a single oocyte. This presented an opportunity to shorten the time to pregnancy by allowing for the selection of the best developing embryo(s). In the *earliest* iterations of this treatment, it was accepted practice to transfer as many as four embryos back to the patient to improve the chance of conceiving in a given cycle [2, 3].

Even after a multi-embryo transfer, supernumerary, or “extra,” high-quality embryos could remain. Cryopreservation—the processes of freezing and storing these embryos—offers an excellent solution, enabling future embryo transfers without ovulation induction and oocyte retrieval, providing an ethical solution, obviating their languishing in culture or eventual discard. The refinement of these freezing protocols, and now the widespread adoption of rapid vitrification techniques have been shown to be a safe and effective option for supernumerary tissues, and now open the door as a primary treatment option [4].

While there is an abundance of scientific reports describing efforts to optimize cryopreservation protocols, i.e., the procedures for freezing and thawing the embryos, formulations for cryopreservatives, best equilibration intervals, optimal freezing ramps using controlled freezing machines, best

warming techniques—the actual *storage*, i.e., the methodology and systems for maintaining cryopreserved embryos safely and consistently at optimally low temperature, has not received the same attention [5]. The perspective may be that there is no “technology” involved in *storage*. The “process” seemed obvious and straightforward: fill an appropriate storage tank—a dewar—with liquid nitrogen and place the samples in the tank.

The seeming simplicity of cryopreserved storage, “cryostorage,” belies the array of inherent challenges that have emerged: consistent and effective monitoring of the integrity of the storage tank and levels of liquid nitrogen, maintenance of temperatures below the glass transition temperature, effective emergent responses to temperature elevations in tanks from falling liquid nitrogen levels, and the reliable logging and tracking of inventory. Indeed, some aspects of storage in liquid nitrogen-filled tanks have remained virtually unchanged since the invention of this container by James Dewar in 1892 [6].

As IVF and its array of assisted reproductive technologies have dramatically evolved and improved to produce more embryos per treatment cycle and are accessed by greater numbers of individuals for family-building, those numbers of tissues requiring cryopreservation have risen sharply. The therapeutic trend for some treatment cycles dictates embryo cryopreservation as the objective of or integral to the treatment, for example, “freeze-all” cycles for better synchrony with the uterus [7, 8], or cycles involving preimplantation genetic testing [9].

The advent of the cryopreservation method termed vitrification was decisive in bringing oocyte cryopreservation, “egg freezing,” to broad clinical practice. This offered fertility preservation to women challenged by gonadotoxic medical therapy or who wished to defer reproduction by “saving” their oocytes and staving off the inevitability of reproductive aging. Many IVF laboratories now add vitrified oocytes to their cryostorage inventory, dramatically increasing the rate of tissues going into storage, and now facing the realistic prospect of most of those tissues never coming out [10, 11].

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The bifurcation of the IVF clinic into distinct disciplines and specialties—the continued evolution and growth of the clinical labs, coupled with the burgeoning reality of managing a large cryostorage facility has brought significant challenges to the clinical team. The number of cycles performed has increased steadily over the years, and one can expect the numbers of patients needing treatment and requiring cryopreservation and storage to continue to rise [12]. An urgent need for innovation and investment in safely and efficiently building capacity to handle this increased volume is of utmost importance.

In addition to providing higher efficiency in time and motion, automated, machine-mediated performance of repetitive tasks can achieve consistency in handling that is desired and ideal in cryostorage [13]. The *robotic* deposition of specimens into and out of storage avoids the potential transient exposure to warmer temperatures—innocent exposure—that can result in cellular and tissue damage, or loss from mishandling. When complemented with software direction for specimen location within a tank, misplacements of specimens can be eliminated. Designing a system that uniquely identifies specimens, their containers, and then automates the logging, tracking, and tracing of those specimens would optimize inventory management. Continuous electronic monitoring of the storage containers with the earliest detection of any deviation from set conditions with the ability to provide a corrective action immediately provides the security in cold chain integrity for optimal quality assurance.

This chapter addresses the application of automation and robotics to the management of cryopreserved inventories generated in IVF laboratories, and how they can catalyze the safety and security of cryostorage for best practice. IVF laboratories have become biorepositories and demand the level of resources and instruments concomitant with that custodial responsibility for invaluable patient cells and tissues.

47.2 Evolution of the IVF Lab

Assisted reproductive technologies have evolved continuously and relentlessly in pursuit of the ultimate goal for patients: producing healthy live births through optimized gamete and embryo culture, fertilization rates, and embryo implantation potential. The ART catalog includes cryopreservation, assisted fertilization through Intra-Cytoplasmic Sperm Injection (ICSI), and embryo biopsy at the blastocyst stage for preimplantation genetic testing (PGT) for determination of chromosome copy number or look for single-gene mutations [14]. We are now employing artificial intelligence (AI) in our clinical decision-making and embryo selection [15]. We've developed incubators with cameras that take pic-

tures at regular intervals, obviating the need for us to take embryos out of their culture conditions for observation [16].

In addition to the introduction of technical and procedural enhancements, improvements in the design of both incubators (low oxygen environments) and culture media (mono and sequential culture mediums, ingredient tweaks) have been advantageous to embryo development [17, 18]. These advances catalyzed the transition from transfers of cleavage-stage embryos at the Day 3 or 8-cell stage to the Day 5 or blastocyst stage. Having better-informed selection among cohorts of embryos, in turn, galvanized the adoption of Single Embryo Transfer (SET), a greatly needed clinical advancement for safest pregnancies, by allowing expedient and confident transition away from multi-embryo transfers and their resultant high-risk multifetal gestations.

These technical innovations also drove human embryo cryopreservation, with more freezing occurring in treatment cycles and more embryos to freeze per patient. Improved culture conditions led to larger cohorts of embryos with good morphology and SET meant that more embryos would remain after transfer for cryopreservation [19]. With the increasing frequency of PGT prescribed as part of treatment, now performed using molecular techniques such as NextGen Sequencing, cryopreservation of all embryos after biopsy is required, adding to a laboratory's cryostorage [20].

Clinical considerations have also contributed to the higher frequency of cryopreservation. To mitigate any negative effects of ovarian hyperstimulation on the uterine lining, impacting the receptivity of the uterus to the transferred embryo, “freeze-all” cycles emerged to delay transfer to an un-stimulated cycle and synchronous environment for the transferred embryo [21]. Embryos typically develop to the blastocyst stage around day 5 of development but can occur as late as Day 7 and still be viable [22, 23]. This presents an asynchronous scenario where the uterus is losing its ability to allow the embryo to implant, and if the embryo is frozen and transferred back when the uterus is most receptive, this can allow for optimal conditions for both embryo and uterus, improving the chances for success [24].

In addition to innovations with ART, societal evolution has meant greater access to assisted reproduction by more patients. This is exemplified by family building by members of the LGBTQ+ community and the addition of oocyte cryopreservation—egg freezing—to the ART repertoire [25]. The latter has been a boon for female fertility preservation, whether to defy the loss of ovarian function after oncotherapy or to defend against the advance of time and diminished ovarian function [26].

The cumulative result of these factors is that owing to the central place occupied by gamete and embryo cryopreservation and storage in IVF treatment and the burgeoning number of specimens stored in IVF clinics, these laboratories have become biorepositories. Perhaps we never thought of

attaching this term to our inventories of frozen gametes and embryos, thinking that their time in our tanks was limited. Treatment cycles would consume them. We did not anticipate a time when cryopreservation was not adjunctive to the treatment but BECAME the treatment as evinced by PGT-A, fertility preservation or other freeze-all cycles.

47.3 Current Storage Models in IVF Laboratories

James Dewar (1842–1923) invented the vacuum flask while performing cryogenic research and this led to the modern containers we use today [27]. The double-walled vacuum dewar has proven a durable piece of equipment for holding liquid nitrogen, and in large-dimension versions, storing numerous cryopreserved specimens. IVF laboratories have typically adopted either portable tanks that are subdivided into 6–12 canisters, each suspended at the tank's opening above the neck, or high volume tanks that can, for example, hold 370 L of liquid nitrogen and have the capacity for thousands of specimens. Many laboratories that have preferred the smaller and mobile model of dewar have been challenged in recent years to meet the physical demands of their expanding cryo inventory. First, there is the requirement for physical space: the number of square feet occupied by the tanks. Some laboratories have outstripped their original designated space for their dewars, needing to stash tanks in places distal from the laboratory. This may be in different rooms, different floors, even different buildings, and in any available space, including next to file cabinets or lockers and under desks.

De-centralization of cryostorage, i.e., having dewars in an array of locations, presents the risk of inadvertently “missing” a tank when performing routine monitoring of the liquid nitrogen level for re-filling. A single location allows maximal security by having a dedicated, locked, authorized-access-only site for the entire inventory. An ideal design also requires a well-ventilated space with low oxygen alarms to prevent asphyxiation (from air displacement by LN2) [28].

Maintenance of the tanks can be intensely manual, requiring potential transposition of the mobile tank to an LN2 source, removal of the tank lids, measurement of the LN2 level using an immersed meter stick, and filling with LN2 as needed. While LN2 auto-fill mechanisms are available, one must be guarded about the possibility of components not functioning and the continued reliance on local, physical monitoring of the freezer [29].

Some laboratories with multiple tanks may have the unenviable, routine chore of moving the dewars to and from a gas/liquid cylinder room where LN2 can be dispensed. Manifold-mediated access to LN2 for replenishment of the dewars as needed alleviates the physical labor of taking dewars to the LN2 source, prevents specimen loss or damage from the

potential tipping over of dewars during transit or handling, and minimizes the physical stress on the dewars from the frequent movement and handling.

To monitor liquid nitrogen levels or internal temperature, the tanks can be outfitted with sensors integrated into an alarm system. This however presents another challenge, as the most commonly used dewar models for cryostorage in IVF do not come equipped with any monitoring capabilities. It is up to the laboratory to source, purchase, and install systems to allow for remote monitoring. A sensor can be installed by insertion into the dewar; sometimes they are placed into holes drilled through the lid, compromising the integrity of the insulation. Newer wireless options preclude the risk of wires detaching or breaking from the sensor/alarm during tank manipulation or movement, and batteries must be checked on a regular basis. As with all alarm systems, routine testing, and consistent arming of the system during sustained intervals when the laboratory is unoccupied will ensure that their sentinel function is fulfilled by alerting the laboratory team to any deviation in tank temperature or integrity demanding a response [30].

In the event an alarm is triggered, an embryologist must be able to identify the tank in the alarm state and be prepared to address the deviation to the cold chain, i.e., restore the temperature to an appropriate setpoint, or if the tank is no longer intact and cannot hold LN2 owing to rupture of the vacuum, trigger the emergency response plan and safely transfer the contents of the tank to a safe place.

Undoubtedly, this scenario is a source of profound dread to any embryologist: to confront an event of this magnitude alone and potentially in the middle of the night in one's laboratory. Despite this, it is the accepted responsibility of most embryologists to confront and manage this event, in all of its daunting challenges to composure.

47.4 Labeling of IVF Laboratory Specimens

Accurate, stringent, and consistent labeling of all specimens collected and stored in the IVF laboratory is essential. Each is a unique reproductive cell or tissue that is owned and designated for clinical use by an individual or a couple/family unit in pursuit of establishing pregnancy and subsequent live birth of a human being. The chain of custody for ownership must be guarded to ensure uninterrupted integrity.

Early models for labeling were varied. Handwritten information on the cryo carriers or devices, for example, vials and straws, or affixed labels with markers, could reasonably inspire concern for the possibility of fading or disappearing ink or detachment of the label. Adhesives for labels could fail, particularly if they had not been validated for cryogenic use. To address these risks, an array of labels is now available that, affixed properly, can ensure secure adhesion and

attachment to cryo carriers, with materials and inks designed to withstand long-term exposure to ultra-low temperatures. The information on labels is more frequently applied using printing machines, providing vital legibility that could be a challenge from handwritten models.

Another critical issue related to labeling was how useful or complete the information recorded on a specimen label is [29]. The scheme for frozen embryo labeling has evolved since the earliest era of cryopreservation when storage was in glass vials at some laboratories. The importance of a second unique identifier may not have been recognized as important for inclusion. In the delivery of medical care, the primacy of two identifiers for each patient and each patient specimen has been highlighted, respectively, by the Joint Commission, which sets the standards for hospitals and other healthcare organizations in the United States to ensure the reliable identification of an individual for whom a clinical service is intended (<https://www.jointcommission.org/standards/standard-faqs/home-care/national-patient-safety-goals-npsg/000001545/>) and by the College of American Pathologists, that sets the standards for clinical specimen collection and management (<https://elss.cap.org/elss/ShowProperty?nodePath=/UCMCON/Contribution%20Folders/DctmContent/education/OnlineCourseContent/2017/LAP-TLTM/checklists/cl-gen.pdf>). The American Society for Reproductive Medicine (ASRM) is a multidisciplinary organization dedicated to the advancement of the science and practice of reproductive medicine that has also published recommended practices for the management of assisted reproductive laboratories [31].

In the United States, Public Law 102-493, “Fertility Clinic Success Rate and Certification Act” of 1992, states that each clinic must adhere to a certification program, but no enforcement on the federal level is described. Each IVF laboratory should consult their respective states’ departments of health for clarity and direction. These certification standards are, however, required for membership into other organizations, and the majority of US clinics do subscribe to one of these programs. Other countries regulate and license IVF labs, and all labs have internal standard operating procedure manuals that typically include verbiage on labeling.

The date of cryopreservation is essential, as it identifies the treatment cycle from which the embryo(s) originated and allows reference to all associated data for the cycle and embryo, including the stage of development and morphologic grade—information that would inform the order of specimen selection for warming and transfer.

The absence or lack of confidence in a cryo specimen labeling system can render the specimens inappropriate for clinical use. At the minimum then, the information on a cryo specimen label should include two identifiers for the patient, one of which is unique (e.g., patient identification number, medical record number), and the date of cryopreservation. Additional information can include, as space on the label

allows the type of specimen (“embryo,” “oocyte”) and descriptors of developmental or maturation stage, morphology grade, or quality score. An example of a label’s content is Patient Name, Patient Medical Record or Clinic Identification Number, Cryopreservation Date, “MII oocyte” or “expanded blastocyst.”

47.5 Cryopreserved Inventory Management in IVF Laboratories

At the dawn of cryopreservation services in IVF laboratories, the low volume of specimens may have been compatible with minimalist approaches to the recording and reconciliation of inventory. These may have included simple logbooks, pages in ring binders, or index cards kept in a box organized with alphabetized tabs, or the information could have been recorded in a patient’s medical file or medical record for consultation on an individual patient-by-patient basis.

This wholly manual, analog system depended on meticulous attention to (1) creating a complete record of cryopreservation, i.e., how many and what type of specimens for a patient were cryopreserved on a specific date; (2) documentation of any event related to the disposition of a cryopreserved specimen such as warming for an embryo transfer cycle, discard, donation, or export to another laboratory or storage site, and (3) routine confirmation of the written inventory against the physical inventory of specimens—an audit of the stored samples to identify and resolve any inconsistencies. Omissions in any of these steps lead to an inaccurate inventory and can result in incorrect information being given to patients, creating intolerable loss of trust by the patients and disruptions in other departments, including the financial division that may be responsible for communication and the storage invoicing system.

Inherent to effective cryo inventory management is a reliable system for communicating with patients about their cryo gametes and/or embryos. This ensures that the patient can realize the clinical benefits of using their cryo specimens for treatment, i.e., maximizing the family-building opportunities and avoiding the ovulation induction and embryo creation and culture phases of a treatment cycle. Many clinics utilize billing for cryostorage as a way of consistently advising patients of their stored cells and tissues and as a documentation system to record the array of dispositions patients can exercise, for example, warming, discard, donation, personal acquisition, i.e., patients who take their specimens from the clinic to manage their disposition personally.

Failures or lapses in communication between clinic and patient about cryo inventory are disconcerting at best [32]. The problems that can arise from ruptures in communication cryostorage have been described and introduced the term, “embryo abandonment.” This refers to the absence of a response by patients in the effort to reach them to obtain their

continued instruction regarding their cryopreserved embryos. Conversely, patients may receive incorrect or untimely information about their cryo inventories. A well-designed process for sustained communication with patients regarding cryostorage coupled with robust informed consent can alleviate these stresses although the challenges to patients in making decisions about their frozen embryos are significant and have been described [33].

47.6 The Limits and Risks of the Current Analog Methodology for Cryostorage and Management

The largely unevolved, static platform for the storage of human embryos and oocytes, now created in high volume and at high frequency in IVF laboratories and comprising the same vacuum flasks and organization by canes and goblets, demands significant enhancements for safety and security. Even without two highly publicized negative incidents of cryo management leading to major loss of cryopreserved embryos and oocytes in IVF laboratories, the urgency to optimize the way that this invaluable resource to patients is safeguarded must be addressed [34].

In addition, consistent with the more mobile characteristics of modern society, patients more frequently request physical transfer and shipment of part or all of their cryo inventories to new clinics, sometimes at considerable (intercontinental) distances [35]. Best practices for these operations must also be described. Knowing their status in transit and being assured of their location would be singularly valuable information to both patients and the laboratories overseeing their shipment to and receipt at a new site.

The limits of current modalities in cryo management are (a) the physical space that is required for the storage tanks; (b) the time and effort required for maintaining safe temperatures within the tanks (below the glass transition temperature at which the specimens would begin to warm, endangering their integrity and developmental potential); and (c) the methods of logging and tracking the inventory, particularly in transit.

Ideally, laboratories can adopt and install the best instruments and practices for the safest cryostorage and maintenance [29]. IVF has evolved, making cryopreservation a central part of assisted reproductive technologies, and the expectation is that newer clinic and lab designs would be taking these considerations into account. With the increasing trends toward more complex and technology-intensive treatment cycles requiring more manipulation, for example, Intra-Cytoplasmic Sperm Injection, embryo biopsy (including preparation and handling), vitrification, oocyte, and embryo warming, the embryologists must *still* allocate time and energy to the maintenance of the ever-expanding cryo inventory. This entails the replenishment of liquid nitrogen

in the storage tanks, and often, the physical movement of multiple tanks—possibly dozens—to the source of liquid nitrogen, and the repetitive removal and replacement of lids.

The tanks are typically individually fitted with sensors and alarms that must also be inspected and tested. At regular intervals, the alarm system must be activated and tested to ensure that deviations from set points and levels are detected with signal dispatch and detection by the embryologist on-call.

Even routine handling of the cryo inventory entails the potential for loss or damage. The movement of specimens into or out of the tank is an opportunity for deviations from the intact cold chain: transient warming of the embryos or oocytes. Deposit into and retrieval from storage can be a routine and daily occurrence in an IVF laboratory with the integration of newly cryopreserved samples or samples imported from other laboratories or extraction for clinical application or preparation for shipping. When specimens are verified in advance of a clinical event, there may be unintended exposure to ambient conditions and warming, for example, raising a cryodevice out of a liquid nitrogen-filled goblet to read its label. Transferring specimens to or from a dry shipper also entails potential rupture in the cold chain. To mitigate against these temperature excursions and their negative effects on the inventory, a strong training program in handling cryopreserved specimens can be as important a part of the laboratory's cryobiology training as the vitrification and warming techniques.

Creation of cryo inventory necessitates best label creation, secure affixing to the cryo carriers, and entry of the inventory to potentially multiple sites, including cryo worksheets (records of cryopreservation), the patient's medical record (electronic or paper), a written logbook, and/or an electronic database. In some models, separate communication to the administrative or clinical departments of the clinic may also be part of the protocol to ensure communication has occurred concerning the cryopreserved specimens to the patient and clinical team.

The guidelines proffered by accrediting or regulatory bodies generally include language regarding checking the accuracy of the cryostored inventory, and audits at regular cadences are suggested. This presents an entirely new set of challenges for the laboratory—allocating the time and resources to physically go through each cryopreserved specimen, checking it against some written or electronic record to make sure the data was transcribed properly. A 2-way audit is best practice, meaning the written data should be checked back against the physical specimens to ensure specimens that are in the written inventory match what is physically there. Ultimately, the laboratory team must decide what the source of truth is if there is a discrepancy—if the specimen isn't located in the tank, but the written record indicates it should be—how to resolve? Had the specimen been warmed and the records not been updated? Was the specimen moved to a new

location and records not updated accordingly? Did the specimen fall to the bottom of the tank?

Notwithstanding all of the time and energy required to perform this task, by removing each specimen from the dewar to check against records, the laboratory now has introduced a scheme where potentially thousands of opportunities for error come into play [29]. There is no conferred benefit to the specimen (or patient) to regularly pull it out of storage just to make sure the details were written down correctly. Maintaining an accurate cryostorage inventory is an essential component of running a fertility clinic, as the potential liabilities of not doing so can be financially damaging, at best.

What methods and tools are useful in optimizing all aspects of cryostorage in the IVF laboratory? These would, ideally, address all of the aspects: accurately and securely identifying specimens, adhering to automated pick and place of specimens into and out of storage, remote monitoring and programmed maintenance of storage conditions, best practices in safely handling specimens, automatic logging and tracking of specimens with an immutable audit trail for clarity and visibility into the entire specimen history—who did what, when, and what were the environmental conditions at that time.

47.7 Automation and Robotics as a Solution for Cryostorage in IVF Laboratories

We reap the advantages and greater efficiencies from automation in multiple facets of daily life. We benefit from saving time and energy in routine tasks and their reliable precision in execution, as well as documentation and traceability of many ordinary transactions. Withdrawal of money from an Automatic Teller Machine (ATM), operation of household appliances, and the prospect of driverless vehicles—indeed, any task characterized by prescribed, repetitive movement—all of these exemplify the relegation of activities to a programmed machine. As well as realizing more time for alternative activities (leisure, self-improvement, altruism, or alas, more work), we expect that robotics can deliver the best performance of a task. We depend on its designed optimization, consistency, and reliability.

Cryostorage has a defined catalog of activities: cryodevice labeling, placement into a storage tank, listing in an inventory, regular monitoring of storage tanks for temperature and liquid nitrogen level, specimen withdrawal from storage, continuous specimen tracking through shipping until its delivery is confirmed, alerting of staff to an emergent excursion from setpoint, inventory audit, and reporting. This

array of processes lends itself readily to robotic management.

The introduction of robotics to the IVF laboratory is an established aspiration, with designs and patent applications already submitted for oocyte processing by hyaluronidase treatment [36], ICSI [37], and a microfluidics-based lab-on-a-chip [38]. An automated vitrification machine is already available [39]. While we await the exciting streamlining of operations in gamete and embryo culture these advances may bring, the deployment of a robotic solution for cryostorage has already been realized [40].

A robotic cryostorage for an IVF laboratory might entail a platform that provides:

- Software that controls the automation of tank maintenance
- Software that can integrate with the clinic's electronic medical record (EMR) system
- Software that uniquely identifies cryo devices, with patient/specimen information auto-generated from the clinic's EMR system to avoid transcription errors
- Automatic logging of all inventory actions where dispositions are updated in real time
- Automated reporting of cryo inventory
- Maintenance of cold chain through the controlled, timed movement of specimens into and out of storage
- Always-on remote monitoring of the entire platform that cannot be locally turned off
- Redundant systems built in to maintain connectivity
- Proactive monitoring of all platform telemetry to detect trends and report deviations
- ISO 27001 certified information security management system
- ISO 13485 certified medical device company credentials

Examples of a robotic cryostorage system, with software written specifically for embryology cryo management and integrated with different EMR systems, are shown in Figs. 47.1 and 47.2. In the systems below, the traditional goblet and cane (or visotube) container used for holding cryodevices is replaced by quarter-turn screw-capped containers with integrated RFID tags called a CryoBeacon, shown in Fig. 47.3. Two versions of this automated platform exist; in the version shown in Sect. 47.1, CryoBeacons are stored in grids/racks arranged in a carousel within the tank, accessed by a robotic grasper and arm for delivery through a window where the grid can be moved immediately to a built-in liquid nitrogen bath, ensuring intact cold chain. In the version shown in Sect. 47.2, CryoBeacons are picked and placed from a transport container to the storage tank. Controlled



Fig. 47.1 CryoRobot

access to the system is via software that requires the enrollment of authorized users and includes specifications of permissions and clinical tasks related to the specimens.

The advantages of maintaining an accurate inventory of the tank's contents are derived from the unique identity on the barcoded labels and the RFID-enabled cryodevice containers. Manual audits, with their inherently risk-laden requirements for handling, could be reduced or eliminated. The software can deliver a complete inventory report to authorized users. The tank contains multiple sensors reporting to a remote network operations center for 24-hour monitoring. This infrastructure provides notifications to the embryology team in the event of alerts and remote human support via video and audio communication if requested. Data have shown that this system has no deleterious effects on the post-warming survival of tissues [41] and has the opportunity to streamline workflow through automation of previously manual steps (assignment of storage location).



Fig. 47.2 CryoRobot Select



Fig. 47.3 CryoBeacon

The development of extensions of this platform can enable clinicians to give patients visibility into the system, ensuring they have direct access to the information regarding their cryopreserved specimens. This is likely to contribute to higher patient satisfaction and more engagement in their care through the reassurance of having access to this information.

47.8 Conclusion

IVF treatment cycles are increasing in complexity, with more frequent ICSI and PGT, and higher volumes of embryos, and now oocytes, to cryopreserve. The storage of these burgeoning inventories of frozen specimens is challenging for embryologists to meet—from the physical space for housing the storage tanks as well as the time and energy required for maintenance of the tanks and management of the inventories.

In the recognition that software and robotics can be designed to address current cryostorage challenges—in achieving safety and security in maintenance and handling during laboratory operations and shipping—a model in which these have been realized is presented. As IVF laboratories continue to become busier and cryopreservation continues to emerge as a central facet of assisted reproductive technology, manual and analog procedures and management may prove inadequate and impracticable, and intolerably risk laden.

Automation-complemented cryostorage, in addition to much-anticipated applications in assisted fertilization and embryo culture, will herald a new era of IVF in which embryologists can concentrate on applying their expertise through scientific knowledge, experience, and innovative design.

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Maximizing Donor Egg Efficiency: Artificial Intelligence and Genetically Certified Oocytes

48

Birol Aydin, Daria Hudkova, and Cihan Halicigil

48.1 Introduction

48.1.1 Methodology of Donor Egg Banking: A Brief History of Egg Banking and Donor Oocyte Cryopreservation

After the first successful oocyte donation in 1984, thousands of livebirths have been achieved through fresh donor eggs over the years [1]. Although embryo cryopreservation with slow freezing approach and frozen embryo transfers were implemented in the clinical IVF, early oocyte cryopreservation strategies, led to disappointing and unreproducible clinical outcomes until late 1990s [2–5]. Because of this reason, oocyte cryopreservation was accepted as an experimental procedure until 2012. By the successful incorporation of “vitrification” method to oocyte cryopreservation [6], it has become the gold standard by the advancements in the protocols, solutions, and the carrier systems [7]. This breakthrough enabled the option of fertility preservation and provided solutions to ethical, religious, and legal problems associated with embryo cryopreservation.

After incorporation of successful oocyte vitrification protocols to the clinical practice, donor egg banking became a part of ART services which resolves the drawbacks associated with fresh donor cycles. Donor egg banking facilitated the donation process in several ways such as providing a

wider choice of donors, eliminating the risk of cycle cancellation due to stimulation or endometrial synchronization problems with donor and recipient.

The successful and reliable clinical outcomes with the egg bank cycles have initiated a sudden shift from fresh donor cycles to frozen donor oocytes. In 2019, the SART reported a total of 4617 cycle starts that used donor oocytes. Only 1776 (38.5%) used fresh donor oocytes whereas 2841 (61.5%) used frozen donor oocytes [8]. Similar trends were observed in the countries which allow oocyte donation all over the world.

High standards and quality in donor egg bank provided services led to their registrations in different countries. Now some of the egg banks are legally providing services in different countries.

There is an increasing number of commercial donor egg banks across the world in the last couple of years. The market has reached to 2651.40 million dollars in 2021, and the estimate growth of donor oocyte market is expected to reach 3782.40 million dollars until 2028 [9].

This chapter will summarize the good practice approaches in donor egg banking.

48.1.2 Best Timing for Donor Oocyte Cumulus Denudation and Vitrification for Consistent Successful Clinical Outcomes

The oocyte maturation process starts with the trigger shot after the controlled ovarian hyper stimulation (COH). After 34–36 h, the follicles can easily be retrieved. Although the nuclear maturity of an oocyte can be easily assessed under the microscope, the cytoplasmic maturation process is an ongoing process unfortunately with no morphological indicator. Cytoplasmic maturation in oocytes is a complex process which involves extensive reorganization in the intracellular organelles and mitochondria [10]. This maturation

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tion process has an optimal window from the trigger to fertilization before the developmental potential of the oocyte starts deteriorating. This process is called “oocyte aging.” Although the literature still lacks convincing evidence, the common practice in the IVF laboratories is to complete the insemination process at ~42 h after the trigger for the best clinical outcomes in fresh IVF cycles. There are several studies in the literature outlining the importance of the optimal insemination window for IVF patients in fresh cycles [11–13]. This concept is also valid for oocyte vitrification and warming cycles.

The effect of cumulus cells and time of denudation on oocyte aging was previously studied. Mizuno et al. [14] compared the effect of prolonged incubation of OOCs before denudation in terms of IVF outcomes. In the study, immediate denudation had a negative effect on the number of good quality blastocysts.

Rienzi et al. [15] reported a preincubation of 3 h period before ICSI improving embryo quality. In a different study, although higher fertilization rates were observed by preincubation over 39 h, the implantation and the ongoing pregnancy rates were found to be the highest between 37 and 39 h after hCG trigger [12]. There was a significant decrease in the implantation and clinical pregnancy rates after 39 h. Yanagida et al. [11] reported that the oocytes retain the developmental potential between 1 and 9 h after oocyte retrieval.

One of the latest studies by Barcena et al. investigated the effect of oocyte aging on timing of ICSI in fresh and vitrified oocytes in donor cycles [16]. This study used a witnessing system to automatically collect the times from retrieval to ICSI and categorized deciles to see the effect of oocyte aging. This study failed to show the effect of timing on denudation or insemination with donor oocytes both with fresh and vitrified oocytes. Their explanation for this observation was majorly the effect of young donor oocyte’s ability to withstand the effects of aging for longer times. One of the drawbacks of the study was 93.5% of the cases took longer than 3 h to inseminate after retrieval.

In the literature, the timing of oocyte denudation and insemination for vitrified donor oocytes is still controversial. It is not easy to design a prospective randomized study that may reveal the effect of oocyte aging on the outcome because of the nature of donor oocyte banking. Usually, the frozen donor oocytes are used by different recipients in different centers which makes it hard to compare the outcomes even for the same donor’s eggs. The experience and success of the laboratory in the warming cycles is also one of the determinants in successful cycles (Fig. 48.1).

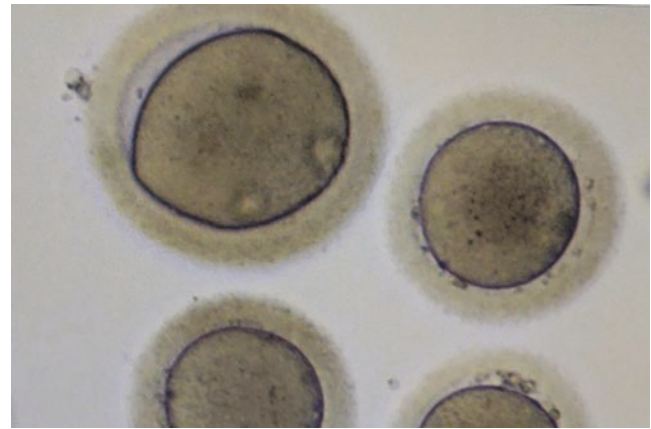


Fig. 48.1 Multicenter frozen donor oocytes outcome

48.2 Morphological Criteria of Donor Oocyte Quality

Morphological evaluation of the oocyte can be performed with invasive and noninvasive methods. In the invasive method, the oocyte morphology is evaluated by the embryologist under a stereo or invert microscope. The evaluation is based on the form of oocyte, cumulus quality, size, morphology, and number of polar bodies, perivitelline space diameter, and cytoplasmic granulation criteria. Whether this evaluation method is successful especially within the scope of oocyte freezing and storage determines the efficiency to be obtained in oocyte donation. The morphological evaluation of the oocyte with the noninvasive method is more detailed and yields results under the mathematical algorithm. The software-based artificial intelligence bases the morphological evaluation of the oocyte on mathematical measurements, and it is expected that the reference values will be matched with the measurements. For both methods, the main source of success may be by performing and monitoring donor stimulation in a good way, performing oocyte collection at the right time, and performing all tests of the donor in accordance with the criteria.

The oocyte collection time yielded different results in different scientific studies; and based on these results, the efficiency of oocytes collected at the 34th, 35th, and 36th h of the post-human chorionic gonadotrophin (HCG) trigger was examined. It should be known that only the morphological evaluation of the oocyte is not sufficient, and the time of denudation oocyte is very important for successful oocyte cryopreservation.

In the natural concept, the 40th h was determined as the optimal fertilization time. Therefore, the periods from oocyte collection to oocyte freezing and fertilization should be evaluated carefully.

48.2.1 Cumulus Complex Morphology

The first morphological evaluation should be performed on the cumulus complex during the oocyte collection process. Various studies have demonstrated that the fertilization of the cumulus complex cells does not affect the viability rates after blastocyst development and freezing of the oocyte [17]. However, the structure of the cumulus complex can affect the morphology of the egg, especially during the denudation process. The interaction between the cumulus complex structure and the nuclear maturation of the oocyte is important; therefore, the evaluation of the cumulus complex is especially important in terms of oocyte banking and oocyte freezing. While evaluating the morphology of the cumulus complex, the scoring system is evaluated between Grade 0 and Grade 4. In Grade 0 oocyte, the cumulus complex has dense layers and oocyte is not visible. In Grade 1 oocyte, the oocyte is visible; however, all lines of the oocyte cannot be evaluated. While the oocyte is clearly visible in Grade 2 oocyte, a large part of the oocyte lines can be evaluated. In Grade 3 oocyte, the main lines of the oocyte are clearly visible and the distinction between the corona layer and the oocyte can be evaluated. In Grade 4 oocyte, cumulus complex expansion is very convenient, and all morphological lines of the oocyte can be evaluated. In particular, the magnitude and density of the cumulus complex structure should be evaluated before the denudation process and, if necessary, the cumulus complex structure should be cut. The healthiest method to perform the cutting process is with an MEA-tested glass Pasteur pipette. In this way, the morphological damage that may occur due to the width of the pipette are minimized during the denudation process.

48.2.2 The Size of Oocyte

Different results have been obtained in the different studies on oocyte size. Some studies demonstrated that oocyte size was effective on maturation and embryonic development, while some studies concluded that no significant connection was found. The oocyte size is between 110 and 120 μm apart from the thickness of the zona pellucida. It varies between 140 and 150 μm with the thickness of the zona in normal morphology (Fig. 48.2). Considering the oocyte size in the selection of the pipette, especially during denudation and during egg freezing, would eliminate the mechanical damage to be caused by oocyte.

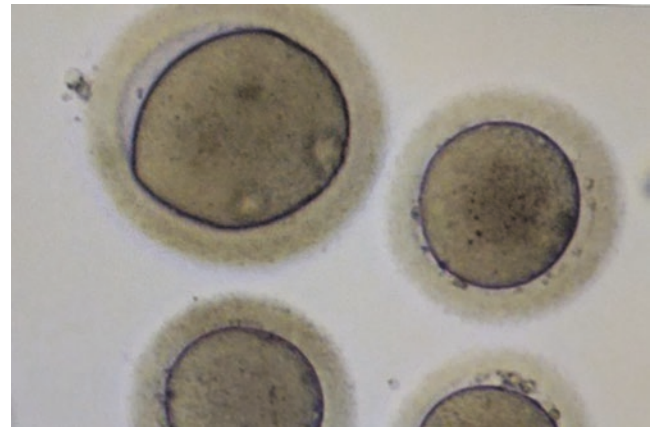


Fig. 48.2 Giant oocyte (left) next to a normal-sized oocyte (right; $\times 200$ magnification)

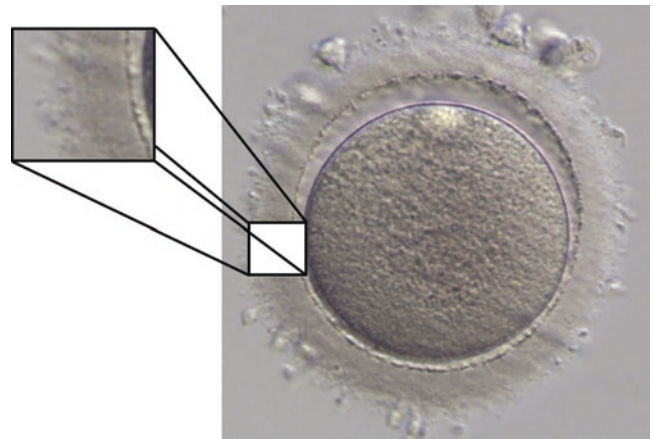


Fig. 48.3 Oocyte with normal zona pellucida with a clear inner layer ($\times 400$ magnification)

48.2.3 Zona Pellucida

The zona pellucida surrounding the oocyte is covered by ethereal granulosa cells. It is known that sperm penetration is more successful in oocytes with thin zona pellucida (Figs. 48.3, 48.4, 48.5, 48.6 and 48.7). Looking at the zona birefringence evaluated with the colposcopy method and the embryo efficiency obtained from this method, it was determined that higher implantation and pregnancy rates were obtained in cases with high zona birefringence [18]. In cases with low zona birefringence, implantation and pregnancy rates were quite low. In addition, our studies conducted in cases with low zona birefringence have shown that the viability rate of oocytes is lower due to the intensity of cryoprotectant concentration during vitrification or the rate of degeneration is high after ICSI procedure.

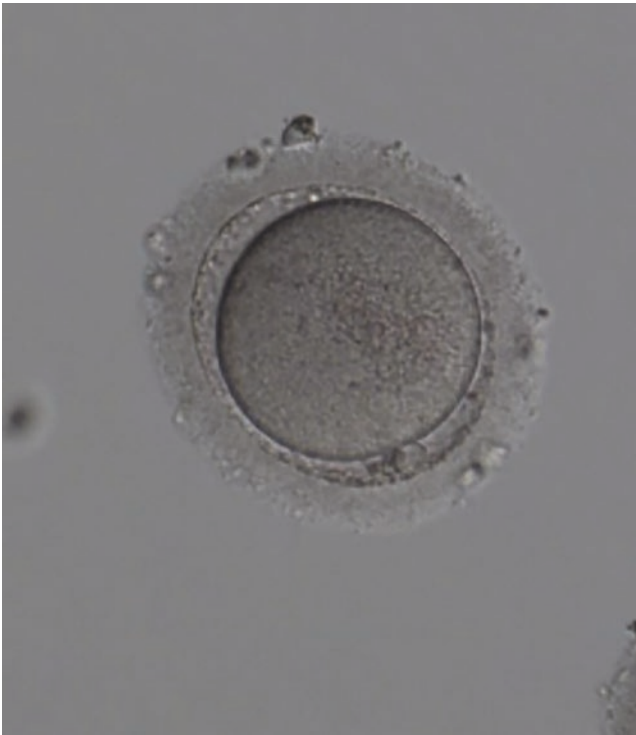


Fig. 48.4 Oocyte with normal zona pellucida ($\times 200$ magnification)

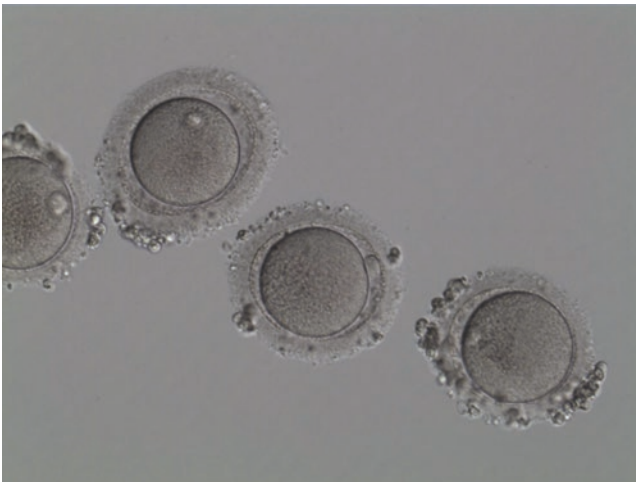


Fig. 48.5 Oocyte with thick and dense zona pellucida (top) next to oocytes with normal zona pellucida ($\times 200$ magnification)

48.2.4 Meiotic Spindle

The purpose of determining or measuring meiotic spindle is to understand whether the development of oocyte is regular after fertilization and especially to determine whether oocyte is suitable for oocyte banking (Fig. 48.8). Significant differences were found when the meiotic spindle measurements of oocytes were obtained before the ICSI procedure

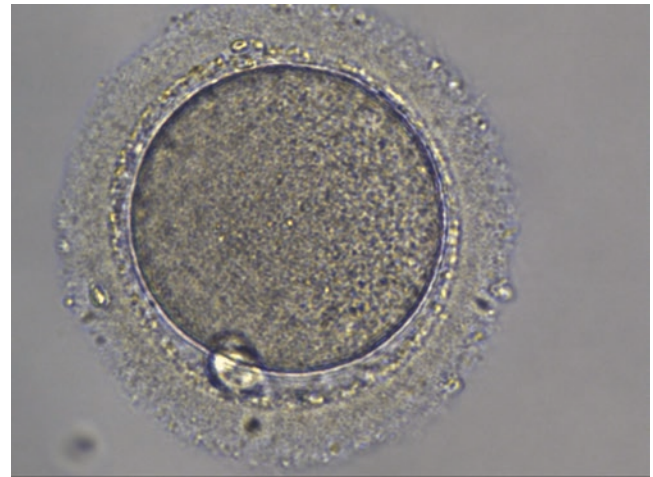


Fig. 48.6 Oocyte with visible inner layer ($\times 400$ magnification)

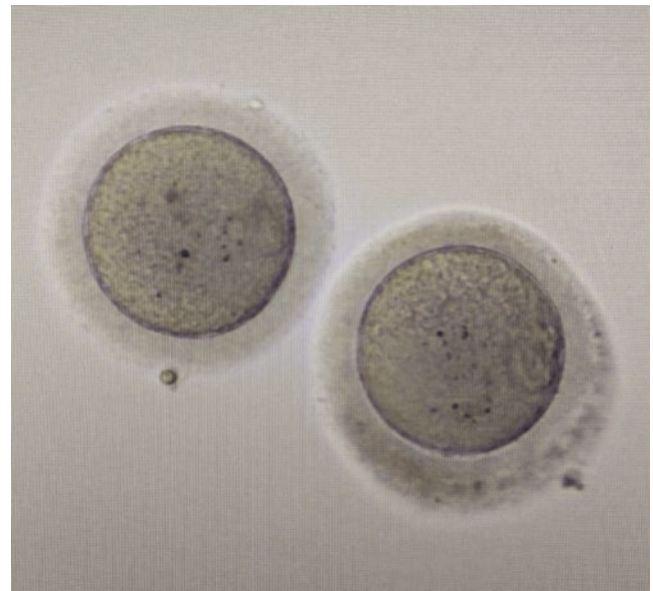


Fig. 48.7 Oocytes with thick and dense zona pellucida ($\times 200$ magnification)

and before the vitrification process and the efficiency values obtained both after ICSI and after oocyte dissolution were compared [19]. Particularly, the effects of abnormal values obtained in meiotic spindle measurements on embryo development have been determined. When the development of oocytes measured with smaller or larger than normal spindles after ICSI is evaluated, it is observed that more cleavage arrest embryos are formed compared to standard spindle measurements, while the blastocyst ratio is much lower. It was determined that many cleavage arrest embryos were formed after the ICSI procedure performed after oocyte dissolution in oocytes that could not be observed with spindle before vitrification.

Fig. 48.8 MII oocyte observed using polarized light microscopy with a visible meiotic spindle below first polar body ($\times 400$ magnification)

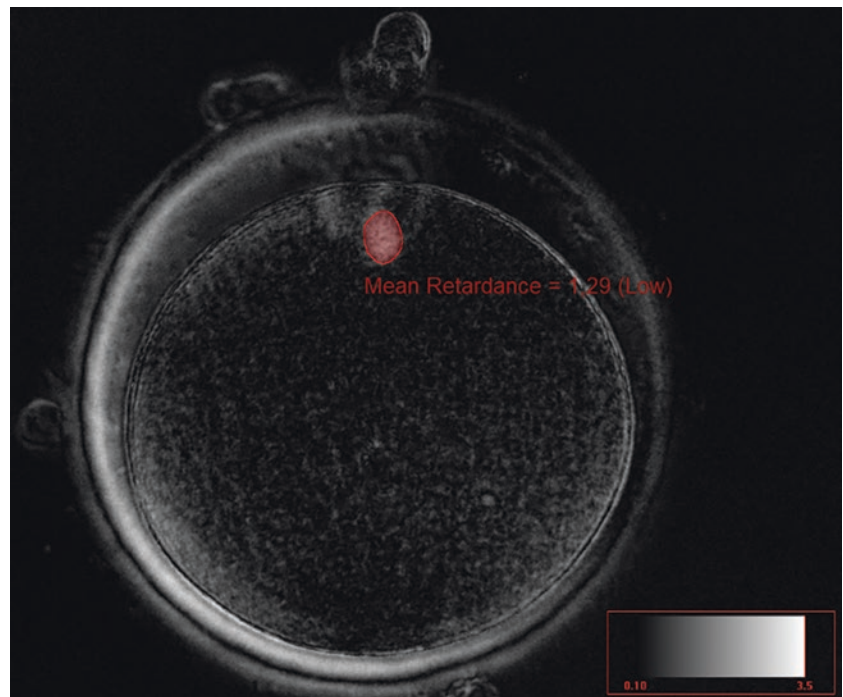
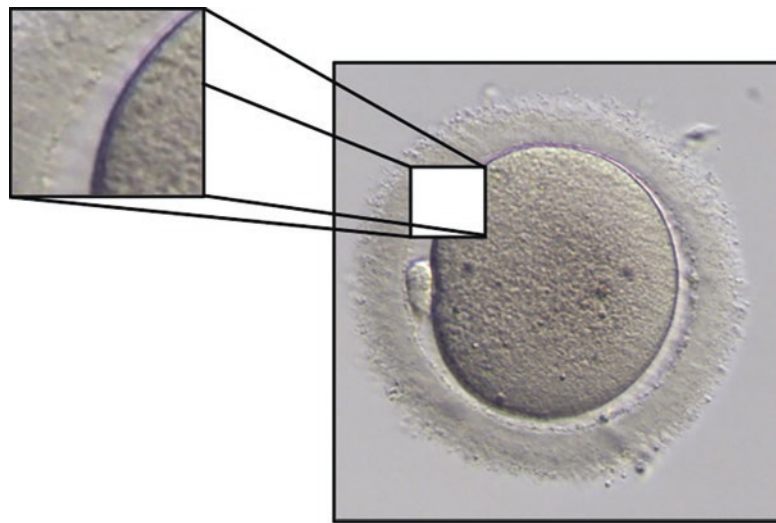


Fig. 48.9 Oocyte with normal perivitelline space ($\times 400$ magnification)



48.2.5 Perivitelline Space

Excessive or complete loss of perivitelline space width has a significant effect on the viability rates after thawing in oocyte vitrification. In cases with high perivitelline space amplitude, the high rate of cryoprotectant passage and the increase in the internal pressure of the oocyte increase the sensitivity of the oocyte. Mechanical errors that may occur especially during micromanipulation increase the risk of oocyte degeneration. In cases where perivitelline space is completely lost, these oocytes will yield very low viability rates after thawing, as the absence of sufficient cryoprotectant entry will cause loss of density in cytoplasm (Figs. 48.9, 48.10, 48.11, 48.12 and 48.13).

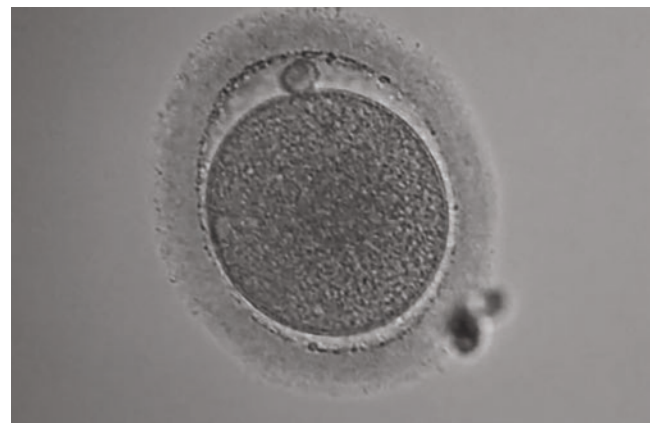


Fig. 48.10 Oocyte with large perivitelline space ($\times 200$ magnification)

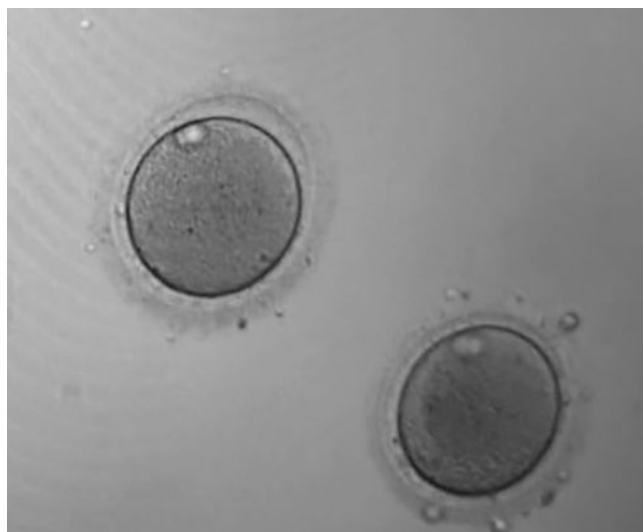


Fig. 48.11 Oocyte with normal perivitelline space (right) next to an oocyte with large perivitelline (left; $\times 200$ magnification)



Fig. 48.12 Oocyte with large perivitelline space ($\times 200$ magnification)

48.2.6 Shape

It has been determined that changes in the shape of oocytes have no direct effect on either viability rates after vitrification or embryo development. However, abnormal structures in the form of oocytes should be evaluated negatively in terms of oocyte banking and should not be included in the database (Figs. 48.14, 48.15, 48.16 and 48.17).

48.2.7 First Polar Body

The regular morphology of the polar body is an indication that the meiotic maturation of the oocyte occurs appropriately. Therefore, the oocyte to be selected especially in terms of oocyte banking should preferably have a single compact polar body. In this sense, the polar body can be used as an important biomarker in both the evaluation of oocyte aging and the evaluation of oocyte quality [20]. The degenerate polar body should be evaluated negatively for both oocyte maturation and advanced embryo development, and these

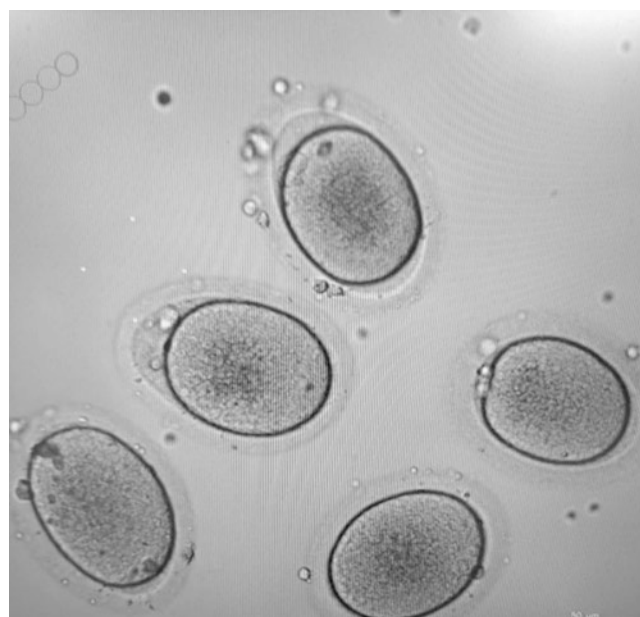


Fig. 48.14 Elongated oocytes inside an elongated ZP ($\times 200$ magnification)

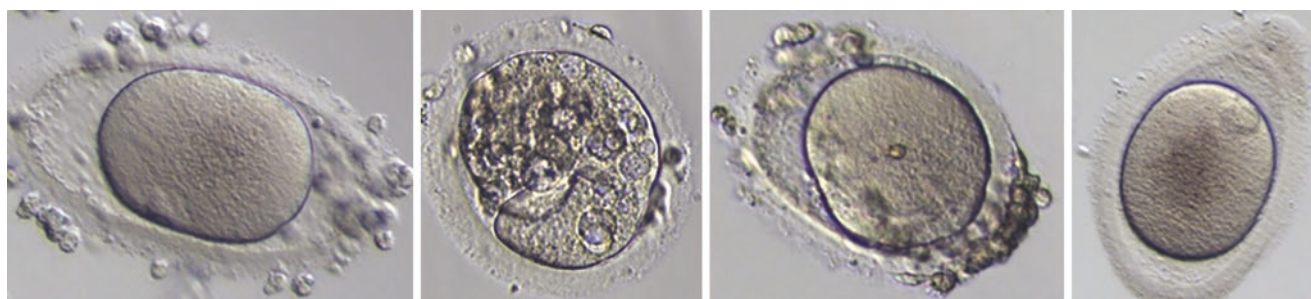


Fig. 48.13 Perivitelline space abnormalities ($\times 200$ magnification)

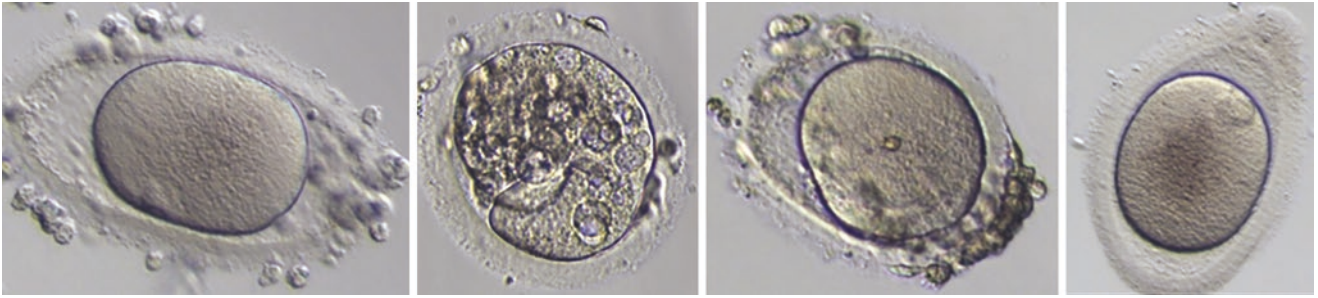


Fig. 48.15 Elongated oocytes inside an elongated ZP. Oocyte presenting multiple vacuoles in the cytoplasm (middle-left)

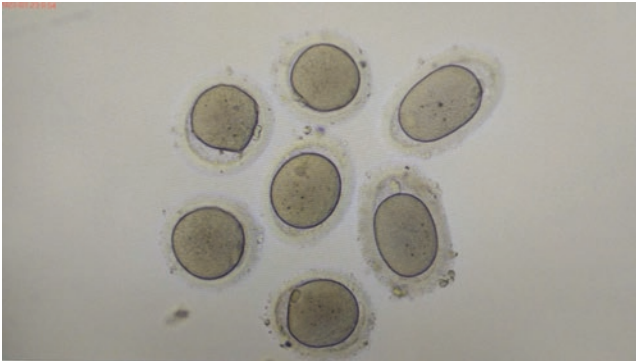


Fig. 48.16 Two oocytes with elongated shape (right) in a cohort of other round oocytes (left; $\times 200$ magnification)

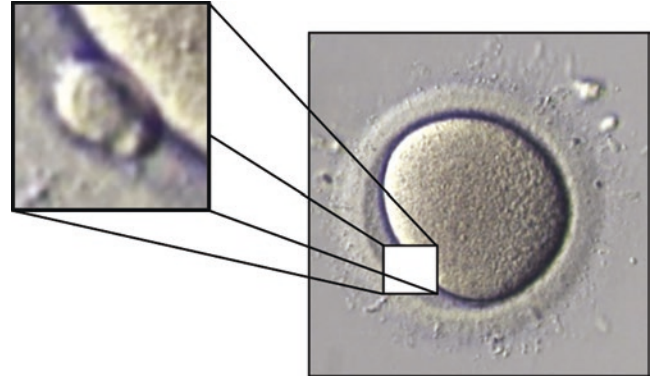


Fig. 48.18 MII oocyte with an intact polar body ($\times 400$ magnification)

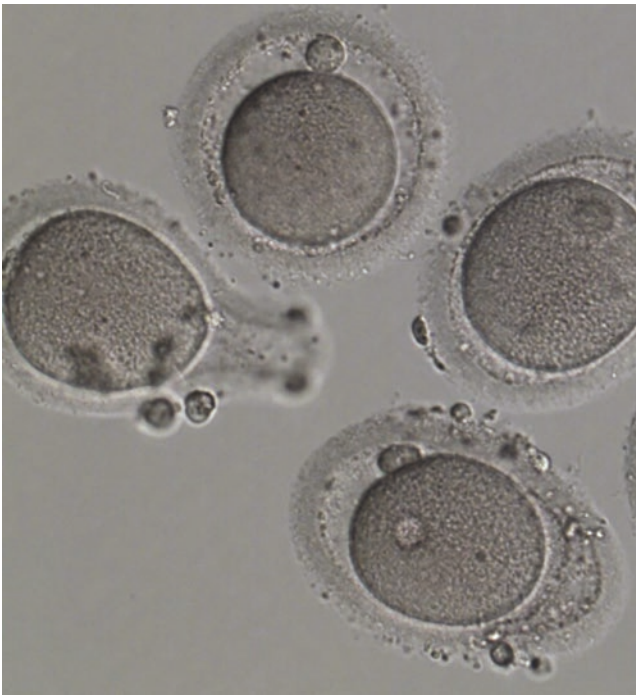


Fig. 48.17 Two oocytes with elongated shape (right and left) next to two oocytes with round shape (top and bottom). Oocytes also present large perivitelline space. Zona pellucida in two oocytes present irregularities (bottom and left; $\times 200$ magnification)

oocytes should not be used for oocyte banking. A significant decrease in fertilization and blastocyst rates was observed in oocytes with intact polar body structure. While it was observed that the viability rates after vitrification were low in oocytes with large polar body structure, there was no problem in the development of embryos obtained from oocytes with fragmented polar body structure. The polar body structure should be considered especially in the selection of oocytes in oocyte banking (Figs. 48.18, 48.19, 48.20, 48.21 and 48.22).

48.2.8 Cytoplasmic Changes

When the effects of vacuolization, SER formation, granulation, and color change in the oocyte on fertilization, blastocyst formation, and clinical pregnancies are investigated, it is observed that while there is a significant decrease especially in blastocyst formation and increase for biochemical pregnancy rates. Especially in oocytes with intense cytoplasmic granulation structure, it was observed that the viability rate may be low after vitrification. In addition, in oocytes with vacuole formation, the lower viability rate is observed after

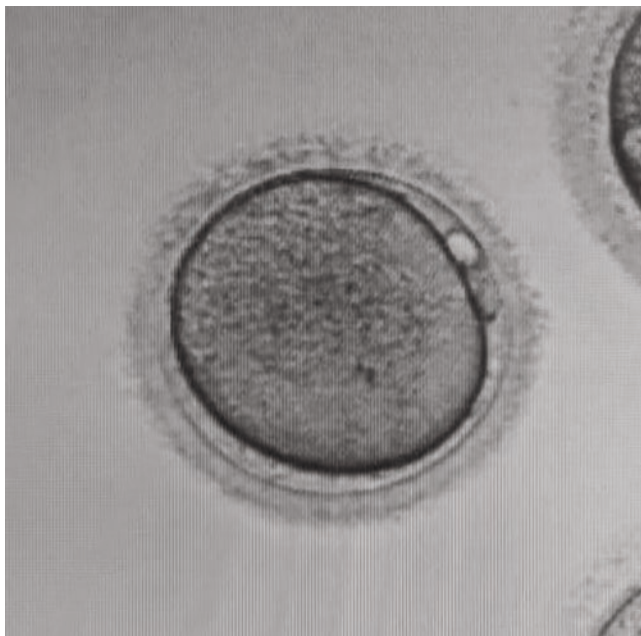


Fig. 48.19 MII oocyte with fragmented polar body ($\times 200$ magnification)



Fig. 48.22 MII oocyte with fragmented polar body and large perivitelline space ($\times 200$ magnification)



Fig. 48.20 MII oocyte with fragmented polar body ($\times 200$ magnification)

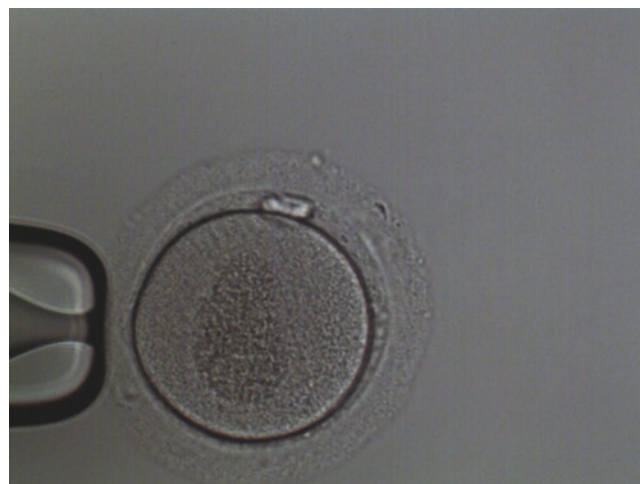


Fig. 48.23 MII oocyte showing a very large centrally located granular area occupying most of the cytoplasm. This granularity is typical of organelle clustering ($\times 200$ magnification)

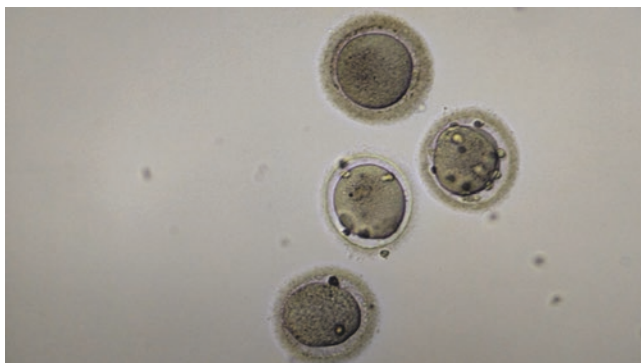


Fig. 48.21 Two oocytes with fragmented polar bodies (middle; $\times 200$ magnification)

vitrification, while SER and color change do not have a serious effect on viability rates after vitrification [21]. It will be an appropriate strategy not to evaluate the oocytes where cytoplasmic changes are observed, especially in oocyte banking (Figs. 48.23, 48.24, 48.25, 48.26, 48.27 and 48.28).

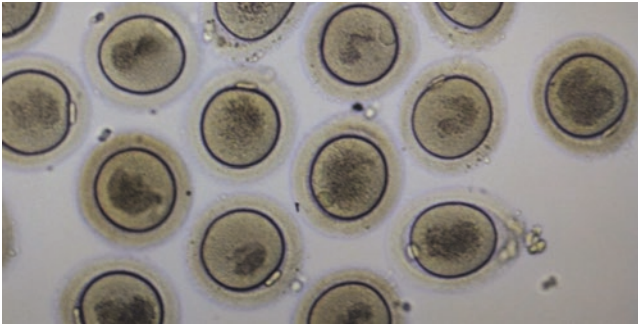


Fig. 48.24 MII oocytes showing a large centrally located granular area in the cytoplasm denoting organelle clustering ($\times 200$ magnification)



Fig. 48.26 Oocyte with several vacuoles distributed throughout the cytoplasm ($\times 200$ magnification)

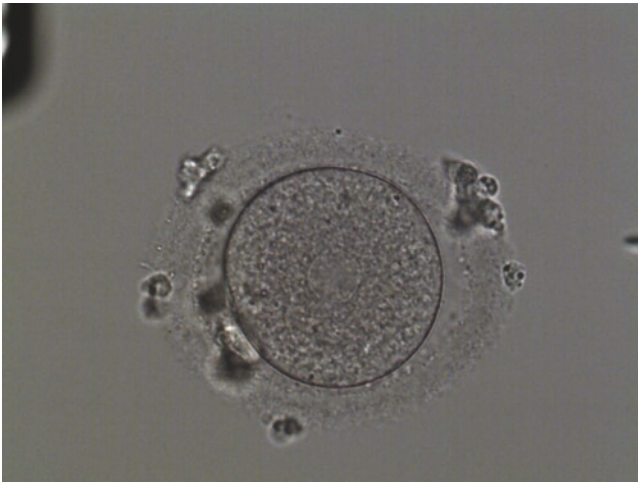


Fig. 48.25 MII oocyte showing an SER disc in the cytoplasm ($\times 200$ magnification)

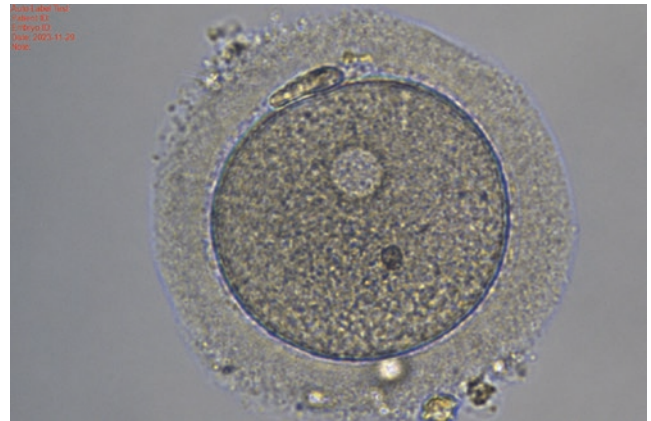


Fig. 48.27 Oocyte with a single small vacuole in the oocyte cytoplasm ($\times 400$ magnification)

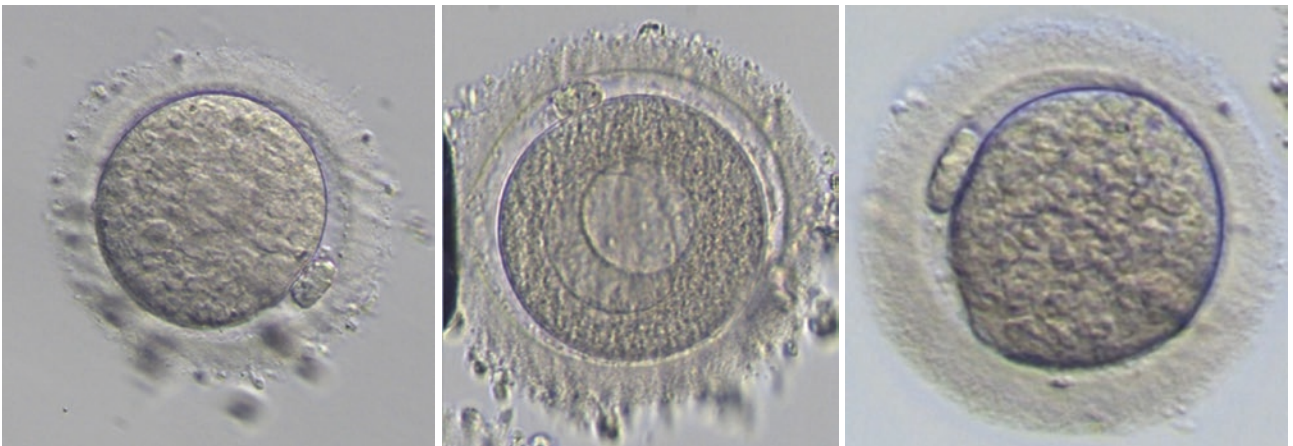


Fig. 48.28 MII oocytes with a high degree of cytoplasmic vacuolization/degeneration (right and left) and MII oocyte with two SER discs (middle; $\times 400$ magnification)

48.3 Artificial Intelligence (AI) Algorithm

48.3.1 AI-Based Morphological Selection Criteria

Correct determination of oocyte morphology directly affects the survival rate to be obtained after vitrification and the success of egg banking. Since the AI algorithm is built on three-dimensional mathematical measurements, the quality image to be taken from the oocyte will make morphological matches with the comparative analytical calculation of the AI software. While making the morphological evaluation of the oocyte with AI-based software, the clinical values of patients and the donors and the data obtained from previous procedures are evaluated. The number of oocytes, maturity values, embryo development and PGT/A results taken from patients or donors in previous cycles are evaluated by the system and the system decides which oocyte or embryo is suitable for the procedure. Especially in terms of oocyte banking, if the selective oocyte freezing method is used, it has been observed that both the survival rates are higher and the cleavage arrest rates after thawing are reduced to a very low level in the procedures performed with AI-based software. In this sense, the number of usable blastocysts taken from a small number of batch oocytes [6–8] in donor oocyte banking has exceeded 50%, and this has significantly increased the possibility of pregnancy and implantation of the patients. The algorithm developed with AI-based software will not only bring freedom of work to the embryology laboratory but will also provide great convenience in problem finding and solving.

48.3.2 Clinical: Age, BMI, Genetic Background, Family History

All candidates into oocyte donors were between 18 and 32 with a mean age overall 25.4 (± 2 years). Around 5% of the donors are below the age of 21 and 9% are over the age of 30. All donors are with proven fertility which means having at least one child of their own. BMI is between 18 and 25, minimum weight is 48 kg and maximum weight is 76 kg. When evaluating a potential oocyte donor, a comprehensive review of their past medical history is required. After screening and selection performed, all donors were healthy and gave no history to suggest hereditary or communicable disease. Information about family history and hereditary diseases should be available till the third generation. In case information is absent, candidate couldn't proceed into a donation process. The goals of screening are to ensure that the donor is not at risk for suffering an untoward event during the stimulation/retrieval process and to also ensure that the donor is not at risk of transmitting a possible blood-borne pathogen.

48.3.3 Evaluation of Oocyte for Cryopreservation

Egg freezing strategies based on different timings should be developed in oocyte retrieval procedures at the post-trigger 35th or 36th h. Taking the 40th h cut-off value in the natural concept as a reference, the ICSI procedure should be determined according to this value. After the oocyte retrieval process, the cumulus complex structure should be analyzed, and large cumulus should be cut before the denudation process. After the denudation procedure, the first morphological selection should be made under a stereo microscope and the maturation should be determined. After maturation, the second determination is made on the width of the polar body and perivitelline space. After the embryologist-based morphological selection, Artificial Intelligence performs the morphological selection based on the mathematical algorithm, and the clinical outcome from the patient or donor is also evaluated, and oocyte selection is made for vitrification. The zona pellucida, cytoplasm, and polar body parts of the oocyte are imaged clearly using a 20X lens and a real-time HD camera. Then, the meiotic spindle imaging of the oocyte is performed under a polarizer and 40x lens, and the zona birefringence value is determined and transferred to AI-based software. In this way, two separate 3D image viewing systems are loaded into AI software. The system determines the polar body size, inner and outer width of the oocyte, spindle size, perivitelline width, and zona birefringence value with the help of a mathematical algorithm and saves them in the sub-algorithm. In the next step, the clinical evaluation from the patient or donor is included in the calculations by the system and the algorithm marks the oocytes with potential morphology suitable for the vitrification process. These oocytes are evaluated by the embryologist and subjected to vitrification.

48.3.4 Time Optimization and Record for Equilibration Solution (ES) by AI

After the morphological evaluation of the oocytes is made by the embryologist with AI support, the selected oocytes should be subjected to Equilibration Solution (ES) and the vitrification process should be started. Although different vitrification solutions work at different timings and concentrations, it is basically recommended to expose oocytes to ES solution for 12–15 min. What is important here is to catch the time when the oocytes are fully expanding and to transfer the oocytes into the Vitrification Solution (VS) at that time. In AI-based timing evaluation, it was determined that different oocytes make total expansion at different times. The durations which some oocytes expand may be 8–10 min after they are taken into the ES solution. Taking the oocytes with this situation and subjecting them to the VS solution will both have a positive effect on the viability rates and will provide the opportunity to freeze a large number of oocytes in the time determined for the embry-

ologist. AI software starts real-time video recording and timing calculation when the oocytes enter the ES solution. During the time spent in ES solution, it gives a warning as soon as the oocyte is fully expanded and marks the oocytes under the video image. These oocytes are ready to be taken in VS solution. All the time in the ES solution is recorded and abbreviations can be made on the video as timing. In this way, especially in terms of safety and efficiency of oocyte banking, the question that may arise after thawing are eliminated.

48.3.5 Time Optimization and Record for Vitrification Solution (VS) by AI

First, the denudation pipette used must be changed before the oocytes are taken into the VS1. The oil residue that will come from the culture petri dish on the pipette will both affect the position of the oocyte on the pipette and degrade the clarity of the image required by the AI system. Oocytes are absorbed into a certain number of pipettes and placed in the VS1 in such a way that they are at the center, and the pipette is washed at least three times with VS1 solution. As soon as the oocytes enter the VS solution, the AI-based timing calculation starts. The oocytes are washed 15 times in VS1 solution and lined up in the center. The AI algorithm determines the sudden shrinkage of the oocytes, and at this time, the oocytes taken into the VS2 solution are washed 15 times at different points of the solution after the pipette is washed at least three times and loaded into the straw. While the total time spent in the VS solution varies between 1 and 1.5 min, the AI algorithm can determine whether the shifts in this time have any effect on oocyte viability and develop-

ment. According to the results obtained from here, the survival rate and productivity of the oocytes increase as the duration of 1.5 min is approached. The whole process is recorded with an AI time lapse camera.

48.3.6 Oocyte Expanding and Shrinking During Vitrification Process; Can We Optimize by AI Application?

The timing of expanding and shrinking of oocytes can be observed with the help of AI and an appropriate algorithm can be formed. The issue to be considered here is that not every oocyte can catch the same timing. Especially in oocyte banking, if many oocytes are to be frozen at the same time, it is very important to be able to set this timing. Optimum expanding and shrinking timings determined by the AI method are presented in the table.

48.3.7 Is There Difference Between Clinical Embryologist and AI-Based Morphological Oocyte Selection?

In embryologist-focused oocyte selection, embryologists usually decide on the image under the stereo microscope after denudation. Since the mathematical calculation is disabled in embryologist-focused oocyte selection, it is a choice based entirely on experience and the morphological image under the microscope. Morphological oocyte selection by AI method will eliminate the risks, especially in laboratories that have a busy schedule and make many cycles (Fig. 48.29).

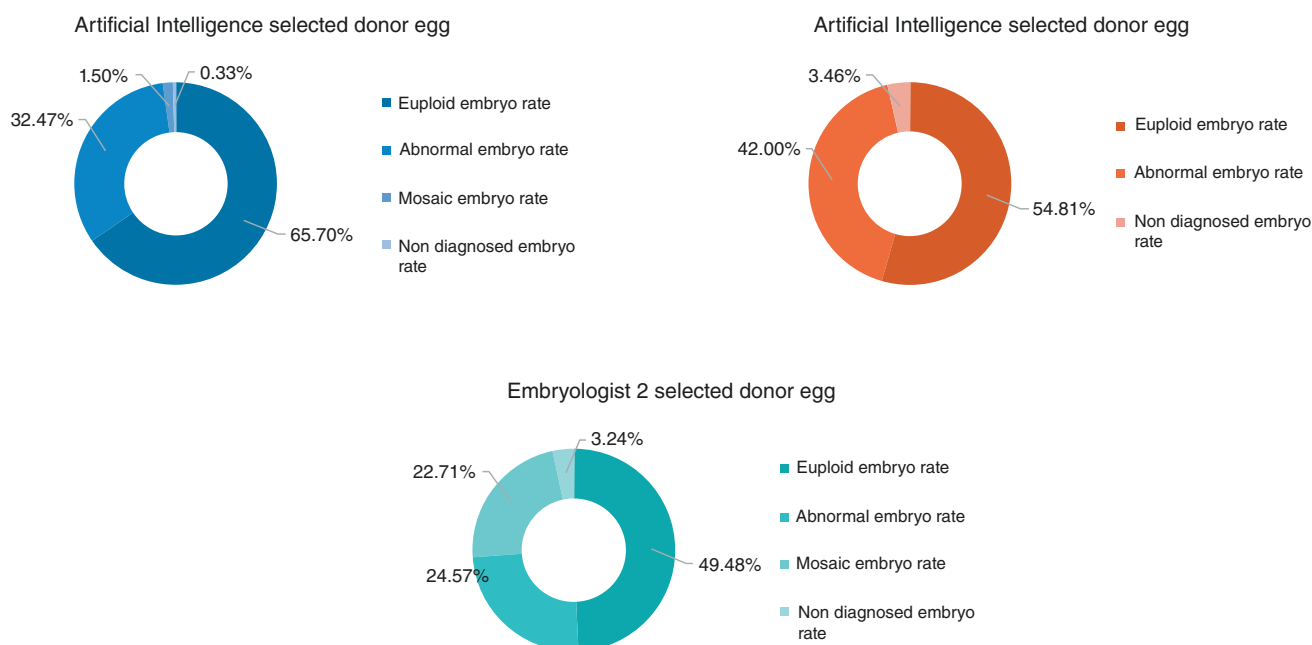


Fig. 48.29 Artificial intelligence morphological oocyte selection versus embryologist-based morphological oocyte selection data

The viability rates and blastocyst rates to be obtained from oocytes after thawing will be much higher. Studies have compared the morphological oocyte selection of clinical embryologists with at least 5 years of oocyte vitrification experience with the oocyte selection from sibling donors using AI software. The data obtained showed that there was a significant increase in favor of AI in both the viability rates and the obtained blastocyst rates.

48.4 Oocyte Aneuploidy Testing By NGS

1. Background of donor oocyte aneuploidy
2. The role of first polar body in donor oocyte
3. The first polar body can be used to produce normal offspring

48.4.1 Background of Donor Oocyte Aneuploidy

The use of donor oocytes, which are typically obtained from young age gamete donors, is an effective treatment option in cases of advanced motherhood age or decreased ovarian reserve. It is a common observation that there are higher pregnancy rates and lower miscarriage rates in donation cycles compared to the control groups. However, what is the real reason for that? Is it the low chromosomal error rate in young age oocytes? Or is it the presence of other cytoplasmic factors in these oocytes? In this case, the most important question to be answered is “how low is the chromosomal error when oocytes are obtained from young and healthy donors?”

In a study conducted by Sills et al. with 24 donation cycles, 24 chromosome PGT-A screening data (data obtained from WGA) of 284 embryos that could be reported from 305 embryos were compared retrospectively with the parent DNA obtained before IVF. In this way, it was ensured that mitotic and meiotic copy errors were distinguished; that is, it was determined how much of the aneuploidy was caused by donor oocyte. In the study, the age distribution of women providing donor oocytes was 24.0 ± 2.7 , [22]. While only 133 of the 284 embryos were identified as euploidy (46.8%), 151 were reported as aneuploidy. The results of the analysis of the origin of aneuploid embryos from both parents (oocyte donor and sperm source) revealed that 88.1% of the embryo aneuploids were of oocyte donor origin. Considering the entire chromosomal aneuploidy rate, it was demonstrated that embryos had a very high aneuploidy error even when oocytes obtained from young donors were used. Another result of this study was that a significant part of aneuploidies still originated from oocytes despite the young donor age.

In another study examining 169 donor cycles to compare the rate of aneuploidy in donor oocyte cycles with the general IVF population, the mean donor age was reported to be 26. Although the results of the study determined that the rate of aneuploidy was higher in the infertile IVF population compared to the donor population at a young age, it was noted that the prevalence of aneuploidy was still approximately 20% in the donor population. In conclusion, in this study, it was suggested that preimplantation genetic screening still provided a significant embryo selection method because the use of donor oocytes did not completely rule out the risk although it provided a lower rate of aneuploidy compared to the infertile population.

48.4.2 Role of First Polar Body in Donor Oocyte

Although polar bodies (PB1 and PB2) contain much smaller amounts of membranous material, mitochondria, ribosomes, cortical granules, and other cytoplasmic materials compared to the oocyte cells, they contain a genomic material that has completely the same structure as oocytes but in haploid form. This provides a great advantage to polar bodies in terms of reflexing the genetics of the oocyte. Polar bodies of human oocytes undergo apoptosis within 17–24 h following their formation and the resulting destruction products remain trapped in zona pellucida. In addition, some studies have revealed that the chromatin structure in PB2 is preserved with a neural membrane; and therefore, it is more permanent and remains intact until the early blastocyst stage.

PB1 and PB2 biopsies have very impressive advantages in PGT-M or PGT-A applications. The first of these is that the use of these structures, which undergo apoptosis without contributing to embryo formation, for diagnostic purposes is more ethically attractive compared to taking a biopsy from a fertilized embryo. Maternal meiotic errors are the most important cause of chromosomal aneuploids, and up to 80% of these meiotic aneuploids originate from first meiosis. Various studies have been reported since the first preimplantation genetic diagnosis of Verlinsky from PB biopsy [23]. Although FISH was the most preferred method in the diagnosis of chromosomal irregularities, it was quickly replaced by array CGH and PCR-based technologies. PCR technology increases sensitivity by 1000 times compared to FISH. However, amplification loss and contamination are important weaknesses of the PCR techniques. On the other hand, although CGH has reached widespread use, it has not been possible to capture ploidy disorders and balanced carriers with this method.

Polar body analyses do not always reflect oocyte and hence embryo; therefore, the value of PB biopsy remains controversial. The most typical example of this is that

approximately half of the single nucleotide errors detected by PB1 analysis may develop as a normal embryo, which can be accidentally discarded.

Although it has been demonstrated by CGH method that maternal meiotic aneuploidies can be accurately detected in embryos at the cleavage stage with PB analysis [24], another study concluded that PB analysis was insufficient in preimplantation genetic screening, and it did not accurately reflect the ploidy status and reproductive potential of the embryo [25].

On the other hand, clinical studies have revealed that PB biopsy is safe and does not affect embryo quality [26]. Polar bodies contain RNAs as well as genetic material, and it has been demonstrated that mRNAs found in oocytes can also be detected in polar bodies (Klatsky 2010). Therefore, polar body transcriptome analyses have the potential to evaluate the global gene expressions of mature oocytes (Reich 2011).

48.5 Can the First Polar Body Be Used for the Production of Normal Offspring?

Female infants are generally thought to be born with complete oocytes, and these primary follicles stored in puberty are activated. This means that human eggs may have aged to their 40s during fertilization. Upon completion of the first meiotic cleavage, the oocyte proceeds directly to the second meiotic cleavage. The remaining homologous chromosomes are aligned on the metaphase II spindle, and this haploid cell, which has discarded the polar body, is called metaphase-2 oocyte. It is a great advantage that the First Polar body is identical with nuclear DNA; and thus, it allows genetic testing of the oocyte. Numerical chromosome errors (aneuploidies), translocations, and monogenic diseases can be detected by PB analysis. The frequency of aneuploidy in oocytes increases sharply after the age of 35. In a 40-year-old woman, an estimated 50–70% of mature oocytes are affected by a chromosomal abnormality [27].

However, multicenter studies have shown that the number of chromosomally abnormal oocytes and embryos in young women is also high, and there are differences between individuals. In addition, 90% of the meiotic aneuploids detected in embryos were found to be of maternal origin [28]. This reveals the potential of oocytes to provide a very important prediction by testing with a polar body. However, PB tests also have significant challenges and limitations. The first of these is that the test provides limited information only with the mother. On the other hand, the first polar body test may not reflect the oocyte optimally for various reasons. In single cell PCR applications, an amplification failure occurs in 10–20% of cases and may lead to misdiagnosis. A major dis-

advantage of polar body diagnosis is that only the maternal genome can be investigated, and no explanation can be made about possible paternal factors. This may be acceptable for maternal autosomal dominant or maternal-derived X-chromosome diseases since all mutating oocytes result in a sick child, regardless of the genetic structure of the father. All mutating oocytes (statistically 50%) should be excreted in the diagnosis of recessive inheritance; however, the disease occurs only in 25% of the resulting embryos. This is because only 50% of the sperms are also susceptible to the disease.

Human oogonia enter first meiotic division during 12th week of gestation, thus being blocked in diplotene stage of prophase I until initiation of ovulatory cycles in female puberty. These cells have already passed through crossing-over and chromatid exchange and are ready to resume meiosis when the appropriate follicle will be recruited, but no earlier than in 10–11 years [29]. Preovulatory FSH and LH surge pushes primary oocyte to quit prophase arrest, complete meiosis with extrusion of the first polar body just to be blocked in metaphase of meiosis II. This unfertilized secondary oocyte will be able to fully complete genetic maturation only after sperm penetration and formation of a second PB [16]. Regarding the fact that realization of the meiotic division for these cells takes up to 20–50 years, it is not surprising that researchers were desperately trying to reveal markers of successful oocyte nuclear maturation for years. And of course, the tempting target to begin with is extruded genetic material of unfertilized mature oocyte—first polar body (PB1).

Morphologic assessment of the first polar body may be an easy auxiliary tool to sort donor oocytes before assignment to a bank. Ovarian stimulation may lead to the asynchronization of cytoplasmic and genetic maturation of the egg, and rapid maturation and subsequent prolonged pending before fertilization contribute to PB1 fragmentation [4]. Several studies investigated the correlation between PB1 morphology and fertilization rates, embryo development pattern, pregnancy, and implantation rates. Thus, Younis et al. demonstrated that oocytes with normal round or ovoid PB1 formed embryos that implanted more often in comparison to the embryos created from gametes with fragmented or large polar bodies [1]. Also, embryos derived from oocytes with fragmented polar bodies were prone to have lower morphology grades on cleavage stage and decreased blastocyst formation rate and so implanted less often than samples from eggs with intact PB1 [2]. As fragmentation of polar body may be attributed to the interval between the oocyte nuclear maturation and its morphological assessment, clinical protocols, and laboratory routine directly affect the incidence of such common dysmorphism, which is observed in 37% of retrieved eggs [3]. Consequently, degenerated or large PB1

that mainly arise from the asynchrony of nuclear and cytoplasmic maturation [6] or dislocation of meiotic spindle during meiosis are rarely observed and associated with poor fertilization rates [8]. Oocytes with extremely large polar bodies should also be excluded from the cohort of fertilized or frozen donor oocytes as these cells have a high risk to be aneuploid [15].

At the same time, other researchers found no persuasive connection between PB1 and egg competency as well as outcomes of the IVF cycle [9–13]. Ten et al. in their unique study with fertile donors' oocytes demonstrated that among all the structural abnormalities only dark cytoplasm negatively affected treatment results whereas first polar body fragmentation and shape didn't influence fertilization rate and embryo development [5]. Moreover, first polar body morphology and its positioning were not associated with embryo ploidy assessed with trophoctoderm biopsies NGS-testing [14].

As far as agreement regarding PB1 morphology and cycle outcome is not yet achieved, structural abnormalities of polar bodies may rather be the criteria to allocate donor oocytes to the pool of mild quality gametes than discard them [3].

Though morphological analysis of polar bodies may not be an excluding, but rather selective criteria for donor oocytes, these extruded cells can provide valuable data about oocyte genetic background. Single-cell analysis of polar body I may be a brilliant source of DNA to check the sibling oocyte for chromosomal abnormalities, presence of malicious gene variants, or even handling mitochondrial disease testing.

Polar body biopsy was initially introduced by the Verlinsky team in 1990 [17] and represents a safe and low-invasive technique that utilizes only by-products of meiosis for genetic testing with the maintenance of embryo integrity. Nowadays, a combination of trophoctoderm biopsy, new-generation sequencing, and effective vitrification resulted in routine blastocyst genetic evaluation in assisted reproduction. Still, polar body biopsy is the only available option for countries that have strong regulations restricting embryo biopsy [20] and a powerful selection instrument for the donor egg banks seeking the best gametes among the present cohort of cells. Usage of non-sorted donor eggs that bear chromosomal abnormalities may lead to the formation of genetically affected embryos, which may cause a decrease in treatment effectiveness or end up in even worse consequences: spontaneous abortion or birth of a child with a congenital disorder.

Donor eggs are retrieved from young and fertile women, and so their gametes are expected to have high developmental competency and normal chromosomal integrity. The expected rate of embryo euploidy with donated oocytes is about 70% [30] but may vary greatly in every individual donation cycle. Presumably, controlled ovarian stimulation with high dosages of hormones, exposure to inappropriate

culture conditions and manipulations, and intrinsic egg development and maturation features may compromise even donated gametes [28]. For instance, up to 88% of all the detected aneuploidies in abnormal blastocysts retrieved in oocyte donation cycles had maternal origin [31].

Overall, oocytes contribute to most embryo aneuploidies: up to 80% of chromosomal abnormalities found in embryos may be attributed to the mistakes in chromosome segregation during meiosis I. Moreover, segregation errors during the first meiotic division are more prevalent compared to meiosis II [29], making PB1 quite an informative target for genetic analysis. Three different mechanisms leading to aneuploidy in oocytes were identified: premature separation of sister chromatids, reverse segregation, and meiosis I non-disjunction. If premature chromatids separation typically associates with advanced maternal age, meiosis I non-disjunction errors are prevalent in younger woman cohorts including egg donors. This aneuploidy mechanism often impacts larger chromosomes that subsequently ends up in embryo arrest and failure of implantation. Extended diplotene arrest lasting decades and vulnerable recombination configurations of oocyte precursor cells are two major reasons why aneuploidy in human eggs is so common even in young oocyte donors [32]. Evidence of premature chromatid separation and non-disjunction events may be revealed during single cell testing of the first polar body of donor eggs, which enables embryologists to select the most competent samples for banking. Though some of these abnormalities may be repaired by consequential "compensational" meiosis II errors [32], that is not an event that is expected to happen in the cohort of high-quality donor oocytes. Moreover, a recent study by Ghevaria et al. suggests that germinal mosaicism and its consequence—premeiotic aneuploidy may attribute to the chromosomal imbalance in 10% of unselected oocytes. This type of error that emerges during early oogenesis even before meiosis starts increases the risk of oocyte aneuploidy and is irrespective of maternal age, and so attributable to the gametes of young and healthy donors as well [33].

As any other technique, PGT of polar bodies has its limitations and challenging aspects. Indicatively, it is more prone to mistakes due to possible sample contamination with cumulus cells, limited/single copy of appropriate DNA molecules, and degradation of collected material [27]. It is also should be considered that PB1 testing is not able to reflect embryo genetic consistency completely as it doesn't consider the input of spermatozoa, meiosis II errors, and mistakes that arise during mitotic divisions of embryo cells [28]. Still, the predictive value of the polar bodies testing with ANP-array comprised 22% for implantation of the cleavage-stage embryo and 40% for blastocyst implantation [18]. The pilot study of ESHRE for PB-based PGT with CGH-array revealed 94% concordances between polar bodies testing results and subsequent zygotes' genetic status [19].

Mendelian monogenic disorders also can be successfully detected on polar body biopsies, which was proven on over 9000 tested samples [21].

Although genetic testing of the oocytes may be beneficial for the selection of the most competent euploid cells, it also should be noted that laboratory routine and donor egg processing after thawing influence PGT results significantly. A multicentered study derived in 2017 revealed that embryo euploidy rate was associated with the referring center and donor age and was not dependent on testing technology and other clinical parameters. Reported euploidy rate ranged from 39.5 to 82.5% in separate clinics [30], supporting the idea that usage of the tested donor oocytes may be beneficial for the centers with high-quality egg-processing routes to maximize outcome of the cycle and compensate poor egg management in clinics with insufficient donor egg cycles experience.

48.5.1 Polar Body Aneuploidy Screening

PBB does not impact the morpho kinetic parameters of the embryo development and suggested no influence on embryo transfer, chemical pregnancy, and implantation events. Hence, PBB can be safely applied without the risk of impairing the reproductive potential of the embryo and can be highly recommended as safe and practicable approach to ensure transfer of euploid embryos for the patients, especially in countries with prevailing legal restrictions in PGT analysis (Figs. 48.30, 48.31 and 48.32).

- Aneuploidy is still an issue for young oocyte donor cycles (27.9%).
- Polar body aneuploidy screening increases implantation and decreases miscarriage rates.
- When both polar bodies were euploid, the resulting embryo was euploid in 74.7% (174/233) of cases.
- Aneuploidy rates were more than 25% in donors, which means 1 of every 4 embryos from donor oocytes may not implant due to chromosome abnormalities. Therefore, it is highly recommended to complement IVF with PGT when donor oocytes are used.

Human oogonia enter first meiotic division during 12th week of gestation, thus being blocked in diplotene stage of prophase I until initiation of ovulatory cycles in female puberty. These cells have already passed through crossing-over and chromatid exchange and are ready to resume meiosis when the appropriate follicle will be recruited, but no earlier than in 10–11 years [32]. Preovulatory FSH and LH surge pushes primary oocyte to quit prophase arrest, complete meiosis with extrusion of the first polar body just to be blocked in metaphase of meiosis II. This unfertilized secondary oocyte will be able to fully complete genetic maturation only after sperm penetration and formation of a second PB [29]. Regarding the fact that realization of the meiotic division for these cells takes up to 20–50 years, it is not surprising that researchers were desperately trying to reveal markers of successful oocyte nuclear maturation for years. And of course, the tempting target to begin with is extruded genetic material of unfertilized mature oocyte—first polar body (PB1).

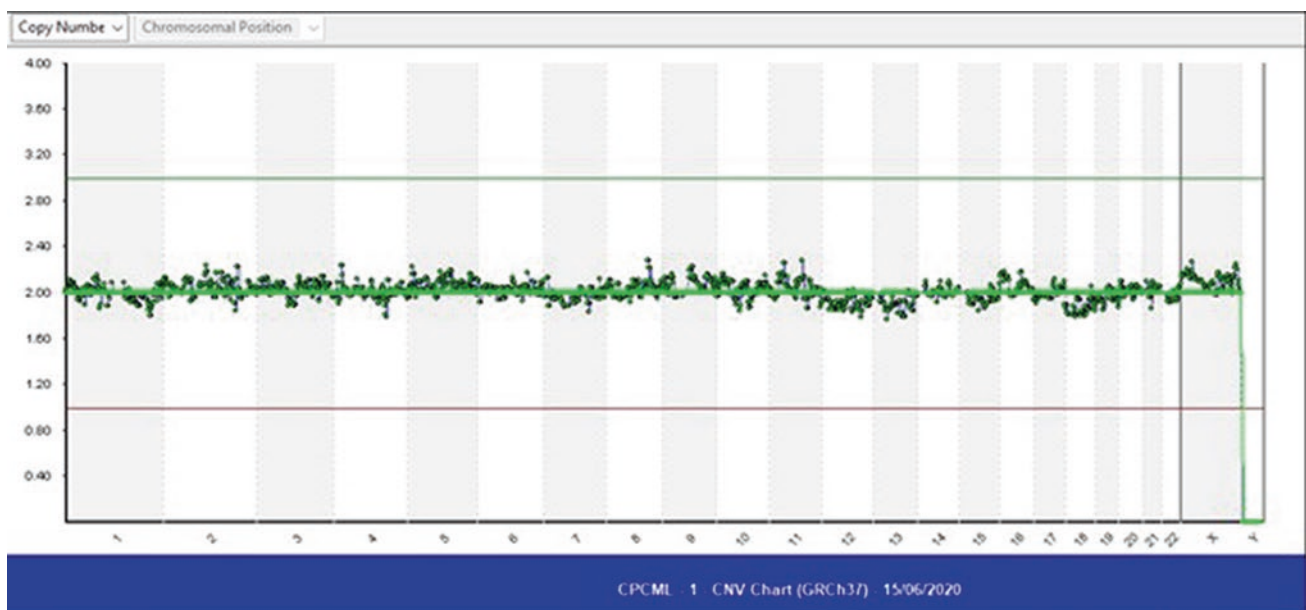


Fig. 48.30 Euploid oocyte profile by PGT-A

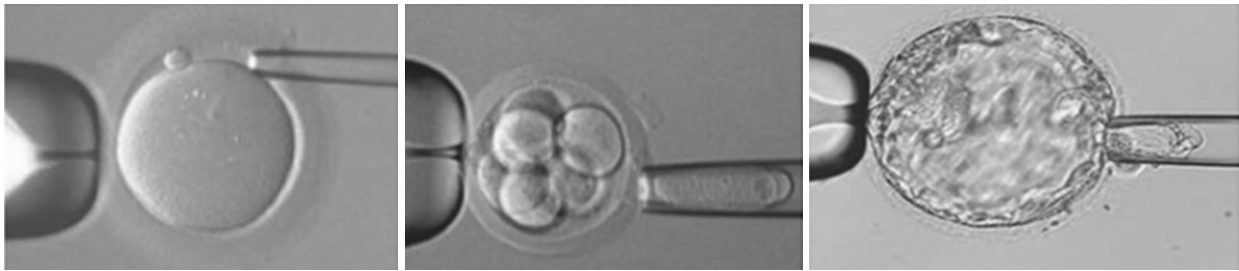


Fig. 48.31 Biopsy techniques

Fig. 48.32 Comprehensive genetic screening versus polar body screening. *CCS* comprehensive genetic screening, *PBS* polar body screening

Aneuploidy Rate	Donor :20% Patient :47%	Donor :30% Patient :65%	Donor : 27,9% Patient : 48,1%
Implantation Rate	CCS(+) : 42% CCS(-) : 23%	CCS(+) : 50,3% CCS(-) : 38.1%	CCS(+) : 61,4% CCS(-) : 50.1%
Miscarriage Rate	PBS (+) : 6,8% PBS (-) :14,1%	CCS (+) : 18.2% CCS (-) : 29%	CCS (+) : 8,1% CCS (-) :15.3%

Morphologic assessment of the first polar body may be an easy auxiliary tool to sort donor oocytes before assignment to a bank. Ovarian stimulation may lead to the asynchronization of cytoplasmic and genetic maturation of the egg, and rapid maturation and subsequent prolonged pending before fertilization contribute to PB1 fragmentation [33]. Several studies investigated the correlation between PB1 morphology and fertilization rates, embryo development pattern, pregnancy, and implantation rates. Thus, Younis et al. demonstrated that oocytes with normal round or ovoid PB1 formed embryos that implanted more often in comparison to the embryos created from gametes with fragmented or large polar bodies [31]. Also, embryos derived from oocytes with fragmented polar bodies were prone to have lower morphology grades on cleavage stage and decreased blastocyst formation rate and so implanted less often than samples from eggs with intact PB1 [30]. As fragmentation of polar body may be attributed to the interval between the oocyte nuclear maturation and its morphological assessment, clinical protocols, and laboratory routine directly affect the incidence of such common dysmorphisms, which is observed in 37% of retrieved eggs [34]. Consequently, degenerated, or large PB1 that mainly arise from the asynchrony of nuclear and cytoplasmic maturation [35] or dislocation of meiotic spindle during meiosis are rarely observed and associated with poor fertilization rates [36]. Oocytes with extremely large polar bodies should also be excluded from the cohort of fertilized or

frozen donor oocytes as these cells have a high risk to be aneuploid [37].

At the same time, other researchers found no persuasive connection between PB1 and egg competency as well as outcomes of the IVF cycle [38–42]. Ten et al. in their unique study with fertile donors' oocytes demonstrated that among all the structural abnormalities only dark cytoplasm negatively affected treatment results whereas first polar body fragmentation and shape didn't influence fertilization rate and embryo development [43]. Moreover, first polar body morphology and its positioning were not associated with embryo ploidy assessed with trophoctoderm biopsies NGS-testing [44].

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Polar body biopsy was initially introduced by the Verlinsky team in 1990 [45] and represents a safe and low-invasive technique that utilizes only by-products of meiosis

for genetic testing with the maintenance of embryo integrity. Nowadays, a combination of trophectoderm biopsy, new-generation sequencing, and effective vitrification resulted in routine blastocyst genetic evaluation in assisted reproduction. Still, polar body biopsy is the only available option for countries that have strong regulations restricting embryo biopsy [23] and a powerful selection instrument for the donor egg banks seeking the best gametes among the present cohort of cells. Usage of non-sorted donor eggs that bear chromosomal abnormalities may lead to the formation of genetically affected embryos, which may cause a decrease in treatment effectiveness or end up in even worse consequences: spontaneous abortion or birth of a child with a congenital disorder.

Donor eggs are retrieved from young and fertile women and so their gametes are expected to have high developmental competency and normal chromosomal integrity. The expected rate of embryo euploidy with donated oocytes is about 70% [46] but may vary greatly in every individual donation cycle. Presumably, controlled ovarian stimulation with high dosages of hormones, exposure to inappropriate culture conditions and manipulations, and intrinsic egg development and maturation features may compromise even donated gametes [47]. For instance, up to 88% of all the detected aneuploidies in abnormal blastocysts retrieved in oocyte donation cycles had maternal origin [48].

Overall, oocytes contribute to the vast majority of embryo aneuploidies: up to 80% of chromosomal abnormalities found in embryos may be attributed to the mistakes in chromosome segregation during meiosis I. Moreover, segregation errors during the first meiotic division are more prevalent compared to meiosis II [32], making PB1 quite an informative target for genetic analysis. Three different mechanisms leading to aneuploidy in oocytes were identified: premature separation of sister chromatids, reverse segregation, and meiosis I non-disjunction. If premature chromatids separation typically associates with advanced maternal age, meiosis I non-disjunction errors are prevalent in younger woman cohorts including egg donors. This aneuploidy mechanism often impacts larger chromosomes that subsequently ends up in embryo arrest and failure of implantation. Extended diplotene arrest lasting decades and vulnerable recombination configurations of oocyte precursor cells are two major reasons why aneuploidy in human eggs is so common even in young oocyte donors [22]. Evidence of premature chromatid separation and non-disjunction events may be revealed during single-cell testing of the first polar body of donor eggs, which enables embryologists to select the most competent samples for banking. Though some of these abnormalities may be repaired by consequential “compensational” meiosis II errors [22], that is definitely not an event that is expected to happen in the cohort of high-quality donor oocytes.

Moreover, a recent study by Ghevaria et al. suggests that germinal mosaicism and its consequence—premeiotic aneuploidy may attribute to the chromosomal imbalance in 10% of unselected oocytes. This type of error that emerges during early ovogenesis even before meiosis starts increases the risk of oocyte aneuploidy and is irrespective of maternal age, and so attributable to the gametes of young and healthy donors as well [49].

As any other technique, PGT of polar bodies has its limitations and challenging aspects. Indicatively, it is more prone to mistakes due to possible sample contamination with cumulus cells, limited/single copy of appropriate DNA molecules, and degradation of collected material [50]. It is also should be considered that PB1 testing is not able to reflect embryo genetic consistency completely as it doesn't take into account the input of spermatozoa, meiosis II errors, and mistakes that arise during mitotic divisions of embryo cells [47]. Still, the predictive value of the polar bodies testing with ANP-array comprised 22% for implantation of the cleavage-stage embryo and 40% for blastocyst implantation [51]. The pilot study of ESHRE for PB-based PGT with CGH-array revealed 94% concordance between polar bodies testing results and subsequent zygotes genetic status [25]. Mendelian monogenic disorders also can be successfully detected on polar body biopsies, which was proven on over 9000 tested samples [24].

Although genetic testing of the oocytes may be beneficial for the selection of the most competent euploid cells, it also should be noted that laboratory routine and donor egg processing after thawing influence PGT results significantly. A multicentered study derived in 2017 revealed that embryo euploidy rate was associated with the referring center and donor age and was not dependent on testing technology and other clinical parameters. Reported euploidy rate ranged from 39.5 to 82.5% in separate clinics [46], supporting the idea that usage of the tested donor oocytes may be beneficial for the centers with high-quality egg-processing routes to maximize outcome of the cycle and compensate poor egg management in clinics with insufficient donor egg cycles experience.

48.5.2 First Polar Body Biopsy in Donor Oocyte

48.5.2.1 Polar Body Biopsy Technical Requirements

When compared with the ICSI procedure, a different set up is required for the polar body biopsy procedure. The appropriate set up and equipment use will ensure that the polar body biopsy is performed smoothly and carefully. Here, it is

important that the angular balance in the injection arm of the invert microscope matches the angular feature of the biopsy pipette to be used. Regardless of the brand of microscope, the use of appropriate angular set up will reduce the duration of oocytes' stay outside. The equipment and materials required for polar body biopsy are listed below:

- Invert microscope with heating plate feature (with 10x, 20x, 40x, and 40x laser lens)
- Active or non-active assisted hatching laser system (active preferable)
- Micromanipulation system with 30° or 35° angle (depends on biopsy pipette)
- Holding pipette (30° or 35° angle)
- Biopsy pipette (30° or 35° angle and 19 µm inner diameter)
- Ca and Mg free biopsy solution (37 °C and 5–6% CO₂ equilibrated)
- 50 mm petri dishes for micromanipulation

48.5.2.2 Polar Body Biopsy, When and How?

Considering the last 10 years, the age of having a child has increased, especially with the social life factor, the increase in marriage age, the priority given by women to their career planning, and more effective genetic factors, and this has significantly increased the infertility rate. Since advanced age reduces the possibility of pregnancy, additional laboratory methods to be applied have gained importance. In particular, tests and techniques to eliminate the effect of genetic factors on pregnancy have gained great importance. It is very important to obtain a maximum number of blastocysts from a limited number of donor oocytes that can be taken due to financial balances in oocyte banking. Especially, with advanced age, chromosomal abnormalities in oocytes reach 60%, while this figure may be around 30–40% in donor oocytes. In other words, it is possible that 3–4 out of 10 donor oocytes to be taken will have chromosomal anomalies. In this sense, providing oocytes with appropriate chromosomal structure to the patients who will use oocyte donation will both increase the rate of pregnancy and obtaining blastocyst and reduce the risk of miscarriage. It is a fact that a

small number of blastocysts to be obtained from a small number of donor oocytes are transferred without PGT-A test, and many clinics and laboratories apply this procedure. It is possible to eliminate these risks with the polar body biopsy method.

48.5.2.3 Polar Body Tubing for Genetic Testing

After the polar body biopsy procedure is performed, the polar body sample should be transferred to sterile DNA tubes containing a maximum of 2 mL of sterile PBS. In order to reduce the risk of loss or adhesion of the polar body sample taken during this process, the sample should be washed well with an irrigation solution containing PBS + PVP and a different denudation pipette should be used for each polar body sample. The total volume in the tube should not exceed 2.1 mL after the sample is placed. The result to be obtained by PGT-A method with DNA amplification to be performed at this point will be much more appropriate.

48.5.2.4 The Combination of Polar Body Biopsy and Embryo Biopsy

Our studies have demonstrated that the aneuploidy ratios obtained from the polar body biopsy and the trophectoderm samples obtained from blastocysts using the same oocytes had a significant similarity with the aneuploidy ratios obtained from the PGT-A test (Fig. 48.33).

48.5.2.5 Does Vitrication Process Safe After Polar Body Biopsy?

After the polar body biopsy procedure is completed, duration of the oocytes in culture medium, width of the opening created by the assisted laser hatching method in the zona pellucida region of the oocyte and oocyte vitrication protocol to be used are very important. Especially after polar body biopsy, it is seen that oocyte fully preserves its vitality and there is no outflow of outward cytoplasm. Subsequently, oocytes should be incubated in a multi-gas incubator for 15–30 min, preferably in embryo culture solution. As there is an openness in the oocyte zona, the inner width of the denudation pipette to be used is quite important. For this type of process, a denudation pipette with a width of 200 µm and above should be used. After Equilibration (ES) and

OUTCOMES	DONOR 20–24 YEARS OLD	DONOR 25–29 YEARS OLD	DONOR 30–34 YEARS OLD
TOTAL NUMBER OF MII DONOR OOCYTES	714	554	568
TOTAL NUMBER OF SURVIVED OOCYTES	701 (%98,17)	551 (%99,45)	548 (%96,47)
TOTAL NUMBER OF EUPLOID OOCYTES	574 (%81,88)	424 (%76,95)	384 (%70,02)
TOTAL NUMBER OF ANEUPLOID OOCYTES	127 (%18,12)	130 (%23,05)	184 (%29,98)
3-4-5 AA,AB,BA QUALITY BLASTOCYSTS	391 (%68,11)	304 (%71,69)	264 (%68,75)
TOTAL NUMBER OF EUPLOID BLASTOCYSTS	347 (%88,74)	274 (%90,13)	226 (%85,60)
TOTAL NUMBER OF ANEUPLOID BLASTOCYST	44 (%11,26)	30 (%9,87)	38 (%14,4)
EUPLOID OOCYTES-EUPLOID BLAST MATCHING RATE	%89,21	90,06%	88,07%

Fig. 48.33 Polar body biopsy-trophectoderm biopsy euploidy matching rate

Vitrification (VS) solutions are brought to room temperature, oocytes should be left in ES solution with the help of a denudation pipette and a second pipetting or micromanipulation should not be performed. The oocytes should be kept in the ES solution for 5–7 min. This is because the oocyte zone is open and the cryoprotectant entry is fast. After 5–7 min, the morphological appearance of the oocytes should be controlled and after being sure that they are fully expanded, they should be kept in VS solution for 1 min and loaded into straws. The most critical point here is that the entire solution into the straw is not pulled after the polar body biopsied oocytes are loaded into the straw. This will increase the risk of degeneration and adhesion. 50% of the oocytes should be covered with the solution on the straw.

48.6 Conclusion

Oocyte cryopreservation by vitrification technique is the most modern and high efficiency method in ART and egg banking. The morphological criteria of the oocyte may design quality of donor egg banking. Morphological selection of the oocyte can be optimized by Artificial Intelligence (AI). AI-based software may choose effectively high morphological quality of donor oocyte and software can combine clinical and embryological data for the better outcome. Donor oocyte aneuploidy testing may use specially when recipients using small batch of oocytes. The PGT-A based oocyte selection can be cost-effective and more efficient for egg donation. AI based of morphological selection and genetically tested donor oocyte are new design of egg banking strategies for clinical perspective and patient perspective. Especially oocyte vitrification protocols may show difference regarding oocyte quality. Optimization of oocyte vitrification is the key point of successful egg banking. The studies show there are many criteria that may affect the survival rate and embryo outcome from frozen oocyte. The standardization of cryopreservation protocol is the most important step for successful oocyte banking establishment.

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49.1 Short History of Vitrification

Vitrification is a physical process whereby liquid is transformed into solid, amorphous glass. The transformation is due to the extreme elevation in viscosity from 1–10 poise to $\sim 10^{13}$ poise, at which point the liquid can be considered reverted to a glassy or vitreous state [1]. Vitrification avoids the process of crystal nucleation and ice crystal growth, thereby enabling a high-viscosity liquid to maintain its disordered molecular arrangement. Vitrification can be regarded as “the extreme case of supercooled water” [2, 3] and was summarized by Luyet, who reported that “some of the oldest investigations on subcooling were made by Gay-Lussac (1836) who observed that water can be subcooled to -12°C when enclosed in small tubes” [4]. Already in 1858, after spraying droplets of water less than 0.5 mm in diameter on a dry surface, Johann Rudolf Albert Mousson observed that the smaller the drops were, the longer they stayed subcooled [4, 5]. Yet, besides the importance of volume for achieving supercooling, it was soon understood that the cooling velocity and the concentration of the supercooled or supersaturated solutions, among other factors, might also influence crystallization rates. These applications resulted in the first cell that survived vitrification [6]. As Luyet wrote: “To avoid freezing, the temperature should drop at a rate of some hundred degrees per second, within the objects themselves” and “The only method of vitrifying a substance is to take it in the liquid or gas state and cool it rapidly so as to skip over the zone of crystallization temperatures in less time than is necessary for the material to freeze. Therefore, one should consider the crystallization velocity [4]. It is evident that when

the crystals grow faster one must traverse the crystallization zone more rapidly to avoid crystallization” [3–7]. Notably, the currently used and most effective cooling rate for oocyte and embryo vitrification is in the range of 100°C/s [8], as proposed by Luyet some 80 years ago.

The first important, successful attempt at vitrification to preserve biological samples was made in 1968, when human erythrocytes were vitrified in rapidly cooled, ~ 5.3 molar glycerol, and remained intact after rewarming [9]. The first successful recovery of living nucleated cells (mouse embryos) from a liquid nitrogen temperature was reported by Rall and Fahy in 1985 [10]. This stimulated research on the cryopreservation of complex tissues and of organs [11–17]. Additional reviews on the history of biological vitrification are available elsewhere [8, 15, 18–20].

Pegg reported that a gradual introduction of a solution containing 55% v/v DMSO while reducing the temperature reduced the toxicity of this high-concentration cryoprotectant [20]. Indeed, he reported on the successful preservation of guinea pig uteri in a supercooled state at -79°C (the temperature of subliming dry ice) with subsequent function after rewarming and removal of DMSO. Yet, this method poses an increased risk of chilling injury, as shown in cells exposed to low temperatures for prolonged periods of time [21, 22]. Following this work, Kemp et al. reported on the cryopreservation of rat, cat, and dog kidneys by perfusing them with 55% DMSO at temperatures down to -40°C , later storing them at -79°C [23]. Rapatz reported on the successful vitrification of frog hearts with EG of 11 M (EG) [24, 25].

49.1.1 Modern Vitrification

Fahy was the first to propose the extension of deep supercooling down to glass transition temperatures (T_g) [26, 27]. The T_g (glass transition temperature) of most vitrification solutions is at the change from a liquid to a solid state, which occurs near -120°C ($\pm 10^{\circ}\text{C}$) [28]. This method was motivated by the need to prevent thermal stresses, which lead to

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fractures in large-volume sample [29, 30]. Fracturing is also responsible for devitrification [19], which involves ice nucleation and crystallization upon the release of heat via the fractures. These, in turn, alter tissues, organs, and even cellular systems [29–36]. The probability of fractures (Pfrac) can be expressed with the following equation [37]:

$$P_{\text{frac}} = \eta \times CR (\text{or } WR) \times V,$$

where η is the viscosity; CR and WR are the cooling or warming rates, respectively; and V is the volume. As expressed in this equation, the risk of fractures of small biological samples can be eliminated by reducing the volume of the drops to a minimum and by decreasing the viscosity [cryoprotectant (CP) concentration] [minimum drop size (MDS) technique], as will be described next. Unfortunately, these solutions are not possible for large tissues and organs, as heat transfer is usually achieved by convection, which highly limits the cooling rate (CR) and warming rate (WR) in large tissues and organs. Therefore, the only option is the use of a high concentration of CPs, which causes chemical toxicity and osmotic stress [38–42]. These can be reduced by the introduction of additives, such as sugars, macromolecules, polymers such as polyvinyl alcohol (PVA) or polyglycerol (PGL), antifreeze proteins, ice blockers, or ice-nucleating agents [43–45]. As described earlier, heat transfer is limited when using liquid nitrogen. We and others have demonstrated the benefit of liquid nitrogen slush (-210°C , using the VitMaster device) in increasing the cooling rate extensively [46–48]. Recent studies have shown the benefit of electromagnetic irradiation [49, 50] and, more recently, of magnetic nanoparticle warming [51–53] (nanowarming) and warming with metal forms [54, 55] to achieve faster and more uniform tissue heating during recovery from the vitrified state.

As described, vitrification requires high viscosity, high cooling/warming rates, and a small sample volume [56–58]. The probability of vitrification (Pvit) can be described with the following equation [18, 59]:

$$P_{\text{vit}} = CR (WR) \eta / V. \quad (49.1)$$

In the past, slow freezing was the method used to cryopreserve oocytes and embryos. Many of the clinics today have adopted vitrification as the most successful method for cryopreservation of oocytes and embryos. There are two vitrification methodologies currently used: one involves plunging oocytes and embryos directly into LN in what is referred to as an open system [60], and the second utilizes the so-called closed system. However, the open system approach poses risks of contamination and cross-contamination [61–63] since it exposes oocytes and embryos to direct contact with potentially contaminated commercial liquid nitrogen. These risks were solved by introduction of the CLAIR, a device which produces clean liquid air [64]. A third approach, pro-

posed 30 years ago, reduces the sample volume to enable a reduction in CP concentration and an increase in the cooling rate [8, 18, 46]. This approach, termed minimum drop size (MDS), addresses the three requirements for successful vitrification. Currently, this method is successfully applied worldwide for oocyte and embryo vitrification [65]. The nucleation rate of intracellular ice crystals is a function of temperature and cytoplasm composition [66]. The classic theory claims that a stable ice nucleus forms by random clustering of water molecules [18, 67]. Therefore, nucleation is a statistical occurrence by nature, and thus, the more water molecules are present, the higher are the chances for nucleation to occur. Intracellular viscosity, which is dictated by water, salt, and CP content, affects both nucleation rates and crystal growth rates [66]. As explained, the key factors in preventing nucleation are sample volume and intracellular viscosity. Adrian et al. showed that a thin ($<1\ \mu\text{m}$) layer of pure water or dilute aqueous solutions resulted in vitrification upon immersion in liquid ethane or propane [67, 68]. In the past, the success of vitrification was mainly attributed to the high cooling rate [3]; however, this is an incomplete explanation, since once an ice nucleus has formed, the ice crystal propagation velocity is extremely high (submitted for publication). As mentioned above, the chance for ice nucleation to occur correlates with sample volume [18, 59]. Therefore, in a large volume, the ice propagation velocity (u), according to the Turnbull equation [69, 70], is inversely correlated with the viscosity (η) and directly proportional to the function of the system's supercooling ($f(\Delta T_r)$) and can be plotted as follows:

$$u \cong \frac{K_u}{\eta} f(\Delta T_r) \quad (49.2)$$

where K_u is a constant determined by the designed model [69, 70]. Ice crystal growth in water occurs at a velocity of $u = 0.158 \Delta T^{1.69}\ \text{cm/s}$. Although the Turnbull equation was presented many years ago, to our knowledge, information on the speed of ice crystal propagation in vitrification solution containing different concentrations of CPs remains to be investigated.

49.1.1.1 The Two Main Problems of Vitrification are Lack of Standardization and Time Consumption

The article of Tannus et al. (2018) [71] brought to the forefront an important issue in oocyte vitrification: the lack of reproducible results. The authors reported that in the same IVF lab and with the same vitrification protocol carried out by the same team of embryologists, the results from vitrification and rewarming differed dramatically. This article, therefore, provided impetus to the search for standardization of vitrification methodology since too many protocols and even subtle changes may ultimately have a profound impact on

the outcome. As explained earlier, the process of vitrification is the solidification of liquid material into a glass-like form without the formation of ice crystals. Its introduction and widespread adoption have really resolved two of the main reasons for oocyte damage during slow freezing: membrane chilling injury and the lethal effect of ice crystal formation. The chilling injury of the oocytes is avoided by the fast cooling and warming rates of the process, which basically outruns the time needed for membrane phase transitions to occur. Ice crystals are avoided due to the high viscosity of cryoprotectants in the solution and by keeping the biological samples in a small volume [minimal drop size (MDS)] and a high cooling rate [57, 72]. The terms cooling and warming shall be used for vitrification, while freezing and thawing are related to ice crystal formation and are appropriate for slow freezing. Many factors determine the success of oocyte vitrification; among them are the following: the composition of the equilibration solution [type and concentration of cryoprotectants (CPs)], the method of exposure (how many steps, time, and temperature of exposure), and the type of carriers utilized (open or closed system, surface, or tubes). At present, there are many carriers on the market, and some of them do not allow reaching of high cooling or warming rates (which for oocytes should be $\sim 20,000$ °C/min). The size of the drop containing oocytes just prior to immersion into liquid nitrogen (LN) is also very important. As previously reported, the concept of minimal drop size (MDS) of <0.1 μL is critical not only for increasing the cooling rate but mainly for reducing the probability of ice nucleation and glass fractions [18, 57, 73]. Finally, successful oocyte survival after vitrification is not attained if the warming process is not carefully considered. This step is very important, and, as we have shown recently, the distance between the LN and the warming solution as well as the volume and the composition and temperatures of the warming solution affect the warming rate and negatively impact the resulting oocyte survival [72]. The cooling and warming rates are important not only because of the probability of vitrification but also because of the chilling injury to which human oocytes are much more susceptible than embryos. As pointed out by Tannus et al. [74], an automatic procedure is necessary for reducing the human factor and lack of reproducibility during vitrification and warming. Given the increasing worldwide utilization of vitrification, time has arrived for a simpler (whether semi-manual or automatic) and standardized methodology as a crucial step for successful oocyte cryopreservation [73].

49.1.1.2 What is Required for Automation of Oocytes and Embryos

Cooling rate should be a balance between too slow cooling rate, which can lead to crystallization and chilling injury, to very high cooling rate that produces numerous ice nucleation that recrystallizes upon warming and/or fractures which can

cause devitrification [37]. For avoiding chilling injury in bovine oocytes, it was established that the cooling rate should be higher than $18,000$ °C/min (0.1 s from 30 to 0 °C). We also have shown that rapid cooling rate with high concentration of CPs in a volume of 1 μL produced fractioning [18, 75, 76]. However, if high cooling rate is combined with small volume (MDS) and rapid warming, then the risk of fractioning or recrystallization is minimized. As stated above, the volume in most of the cryocarriers is in the range of 0.1 μL (MDS), resulting in very high oocytes and embryos survival. Finally, we found that the exposure to equilibration solution (ES) and vitrification solution (VS) should be gradual. Exposing ovine blastocysts to the increasing and decreasing concentrations of the cryoprotectant solutions (by using many steps) was shown to be very successful and will be described in detail below. To achieve this gradual exposure to ES and VS solutions, the expanded ovine blastocysts were vitrified using a device called E.Vit. (Easy Vitrification) that produced hatching rates comparable to fresh control embryos [73, 77]. In terms of the cryocarrier, it is suggested that small straws such as those used with the E.Vit. device should be easy to operate and to store in a minimum storage space similar to those used today for manual vitrification (Cryotop, OPS, CBS Cryolock, etc.). To be considered fully automated, the procedure of vitrification should include also plunging the prepared sample (oocytes or embryos) in LN, without the need of constant presence of an embryologist. Likewise, the process of warming and its various steps should be part of the ideal automated device. Finally, as the main reason for automation is the reducing operation time, we should consider the total operation time including the preparation of the device and the time the device operates with single or multiple patients.

49.1.1.3 Different Strategies to Solve the Problem

There are two options for producing an automatic vitrification device: the first is to produce a robotic system that mimics the manual MDS techniques (Cryotop, cryolock, etc.) such as proposed by Sun (2017) in the USA (patent 9,723,831), and the second is to develop a carrier which allows the MDS technique such as the E.Vit. (WO2017122210A1). The E.Vit. (FertileSAFE, Ness Ziona, Israel) used for vitrification consisted of a 0.25 mL straw with a polycarbonate grid (capsule) with pore diameters of 50 μm inserted at the free end of a straw (Fig. 49.1). Once the oocytes or embryos are loaded in the straw, the solution of vitrification will enter the straw by capillary actions. Understanding how vitrification works and what influences and what kind of damages may occur during the processes of vitrification and warming have led to several developments in the field.

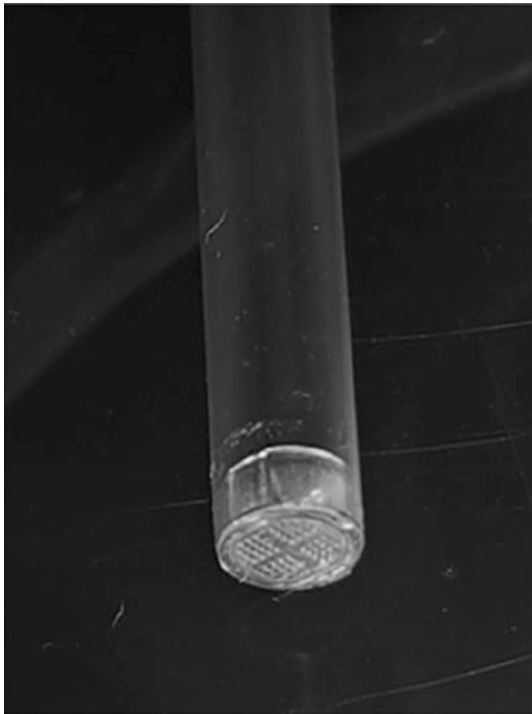


Fig. 49.1 The E.Vit. 1/4 cc straw with a capsule of 2 mm in diameters and 50 μ m pores

Among the first developments, it was the evaluation of different CPs: glycerol [18] DMSO [40], propylene glycol [78], ethylene glycol [79], antifreeze proteins [80–82], polyvinylpyrrolidone, sugars (sucrose, trehalose, glucose, etc.) [40], and proteins (albumin, starch, etc.). Then, after the minimal drop size was published, many new developments took place based on this principle for vitrification of oocytes. These included the creation of carriers such as the Cryotop and Cryoleaf (for a review, read [18]).

The sample's volume reduction also led to reducing concentrations of CPs, which are known to cause osmotic stress and are toxic at high concentrations to many cells [40].

Trying to increase the cooling rates was one of the most, and still is, challenging factor to overcome. The use of LN leads, of course, to high cooling rates; however, when a sample is placed in it, it causes the LN to boil creating an insulating vapor phase surrounding the sample until it reaches the LN temperature [72]. A device named Vitmaster (IMT, Ness Ziona, Israel) was developed to overcome this hurdle by applying negative pressure on the LN, so to reduce its temperature almost to -210°C . By creating LN slush, it is possible to avoid the boiling of the LN when sample is inserted [46, 72]. However, as was explained before, in order to avoid fractions or numerous ice nucleation, the high cooling rate should be associated with small volume and rapid warming rate.

As well as trying to solve problems that are associated with the vitrification/warming processes directly, other prob-

lems have risen due to the practicality of the procedures. One of the problems is the risk of contamination and cross-contamination [62, 63] through the LN, especially when vitrification is done in an open system, which has been proven to have superior results over closed system in regard to oocyte vitrification [37, 83]. There are currently several devices that offer clean LN such as the Elan 2 (MMR Technologies, San Jose, California, USA) which produces clean LN, the Nterilzer[®] (Nterilizer, Bologna, Italy) which sterilizes the LN using ultraviolet light [84], and the Veriseq[®] (Linde Gas, Schiedam, Holland) which filters gaseous nitrogen and liquefies it to produce sterile LN. As stated earlier, we have developed our own system called CLAIR, which simply creates filtered, sterile liquid air having the same temperature of LN (-196°C) [64].

Another problem that encumbered the vitrification success was related to the toxicity of the CPs. To minimize this, it is necessary to gradually transfer the oocytes or the embryos among several solutions with increasing concentrations, until reaching the final vitrification solution prior to plunging the sample into LN. Upon rewarming, there is the same need of gradual exposure but with declining concentrations of sugars. This has made the process cumbersome, and much more costly, requiring highly skilled personnel and responsible for differences in outcome across centers [71, 73, 85]. The need to standardize the process is therefore crucial. One of the new proposed devices to accomplish automation of the process is the Gavi (Genea Biomedx, Sydney, Australia) system, which is mainly for embryos since the cooling rates that it produces are low for avoiding chilling injury to the oocytes [86]. Another system that automates both the vitrification process including the insertion into LN and the rewarming process is named Sarah (FertileSAFE, Ness Ziona, Israel) [73]. Using this device, we vitrified oocytes and embryos successfully [73], as well as reproductive and nerve tissue [87]. There are also new publications regarding robotics systems for embryo vitrification [85].

49.1.1.4 Automatic (Sarah) and a Simplified Semiautomatic (E.Vit.) Cell Vitrification Device

To advance the use of oocytes, embryos, and tissue vitrification, we developed a system in which embryo vitrification, warming, and dilution can be performed within a straw. In vitro-produced (IVP) ovine embryos were vitrified using the “E.Vit.” composed of a 0.25 mL straw with a 50 μ m pore polycarbonate grid at one end. The automatic device (called Sarah, Fig. 49.2) used to vitrify oocytes and embryos consists of a vertical robotic handle where a special straw holder, that can load up to six straws, is attached. This robotic arm moves in a vertical plane (up and down), at predetermined time intervals, and by so doing carries the biological samples contained in the straws between different solutions (vitrifica-



Fig. 49.2 The automatic vitrification device Sarah by FertileSAFE

tion and equilibration) arranged into nine cups placed in a temperature-controlled metal carousel plate. The final station on the carousel plate is the one containing LN where the straws are ultimately plunged, and the entire cycle of vitrification is considered completed. The straws utilized in these experiments are 0.25 mL straws (CBS, L'Aigle, France). Prior to attaching the straws to the holder, the oocytes or embryos are first manually loaded into the straws, and then the straws are closed at one extremity by special capsules (50 μ m pores) (FertileSAFE Ltd., Ness Ziona, Israel). After the holder is placed on the robotic handle and the protocol has been selected from the touch screen, it is sufficient to press the "ON" button for the vitrification cycle to begin. Once the entire preparatory steps are completed, the holder plunges the straws into a special insulated vessel containing LN or sterile liquid air [64]. The special straw holder is then disconnected from the handle, and the straws can be stored as it is or they can be inserted into a 0.3 mL straw heat-sealed and then placed in LN tanks for long-term storage.

Results of mice and bovine oocytes [73] and ovine blastocysts [77] showed that oocyte survival, blastocyst re-expansion, embryo survival, and hatching rate were no different than fresh control. In addition, testicular tissue vitrification was done using E.Vit. straws [76]. The study led by Sergio Ledda [77] during his sabbatical in Australia was the most significant using the E.Vit. device. To advance the use of embryo vitrification technology in veterinary practice, ovine in vitro-produced (IVP) embryos were vitrified at an early blastocyst stage (EBs, n.65, 6 days post-IVF) and at the fully expanded blastocyst-stage FBs, n.168, 7 days post-IVF). Vitrification procedure done in E.Vit. constitutes a 0.25 mL straw with a capsule having 50 μ m pores inserted at one end (Fig. 49.1). Embryos at each stage (EBs and FBs) were divided into two subgroups and vitrified by one of the two methods: (1) Multistep (MS) group: a straw containing two embryos was sequentially loaded vertically into 1.5 mL

tubes containing six different vitrification solutions: 10, 20, 40, 60, 80, and 100% ES (100% ES being 7.5% DMSO + 7.5% EG + 20% FCS in TCM-199) (90 s each step) followed by 30 s each in 75% and 100% VS (100% VS is 18% DMSO + 18% EG + 0.5 M trehalose + BSA in TCM-199). (2) Two-step (TS) group: the straw (two embryos/straw) was loaded with 100% of ES (5 min) followed by 100% VS solution for 30 s. For both methods, at the end of the preparation steps, the straws were plunged directly into LN. Non-vitrified embryos were maintained in in vitro culture as a control group (n.102). Warming procedure consisted of placing the straws directly into 5 mL tubes containing 100, 50, and 25% WS (WS = 1 M sucrose in TCM-199 + 20% FCS) at 38.6 °C (for the first solution) and at RT for all the rest for 5 min each, before arrival into the holding medium. Embryos were recovered from the straws incubated at 38.6 °C in 5% CO₂ in air in TCM-199 + 5% FCS and evaluated for blastocoel re-expansion, embryo survival, and hatching rate at 2, 14, and 48 h post-warming. Blastocyst re-expansion (2 h) after warming increased as the developmental stage progressed and was not affected by the vitrification method. In fact, it was significantly ($p < 0.05$) higher for FBs vitrified in the DS and TS methods (77, 90% and 71, 25%, respectively) compared with the EB method (62, 5% and 48, 50%, respectively). At 24 h, survival rate of vitrified FBs was significantly higher ($p < 0.05$) in the DS system (95.35%) compared to those in TS (86.25%). Survival rates of FB embryos for both methods (DS and TS) were significantly higher ($p < 0.001$) than EB embryos vitrified in DS (56.25%) and TS (56.55%) methods. After 48 h of culture, the hatching rate for FBs vitrified in DS system (87.21%) was comparable with TS (77.5%) and control group (85.3%) but significantly higher ($p < 0.001$) than vitrified EBs in DS (43.75%) and TS (36.36%). In conclusion, this study shows that a high survival rate of IVP embryos can be achieved by this new in-straw vitrification and warming device "Sarah" with hatching rates in vitro comparable with control fresh embryos. This method has the potential use in direct embryo transfer in field conditions. We concluded that the most important features of an automated vitrification system should be the ability to produce high survival and viability of both oocytes and embryos by generating high cooling and warming rates with the reduction of the sample drop volume and by exposing the cells to the increasing and decreasing concentrations of the cryoprotectant solutions by using many steps to avoid the osmotic damages [77]. Furthermore, an ideal device should enable time and temperature control and reduce the vitrification working time required per patient. Disposable containers such as straws should be easy to label and easy to store in LN tanks without making changes in storage space. These features are all present in the Sarah device reported here. Currently, there is no methodology able to fully automate the vitrification process. Few publications have described solu-

tions for automated vitrification; however, the only commercially available semiautomated vitrification machine is the one marketed by EMD Serono [86]. The Sarah device is a simplified methodology that uses commercially available straws and is almost completely operator independent since it also includes the immersion into LN, thus providing flexibility to embryologists since the biological samples are vitrified by the device and kept in LN until placed in long-term storage. The Sarah device can operate up to 6 straws, and since each straw can load 5 oocytes (or embryos), a total of 30 oocytes/embryos can be vitrified simultaneously, thus shortening the time required to complete the entire task. This is an important feature, particularly for busy units. But the most important characteristic of the presented device here is the use of a very small capsule connected at the extremity of the straw, which allows the use of the minimal volume size of the samples. The minimal drop size is the most critical feature for a successful vitrification allowing the high cooling and warming rates of over 20,000 °C/min. Our results with fertilized oocytes (2PN embryos) are comparable with results obtained with other vitrification systems such as OPS and Cryotop [60, 65]. In summary, a key element for oocyte survival is a high cooling and warming rate, which is achieved by using small volume and a small-size carrier for the cells. Both these important requirements are present in the Sarah automatic vitrification device. This device has simplified the vitrification process due to two main advantages: (1) the embryos/oocytes are within the same straw during the entire vitrification cycle, from the initial exposure in ES medium until the final step being the plunging into LN or LN slush, and (2) the movements between the different solutions are done in an automated and precisely timed manner. This means that the time and the temperatures that the oocytes or embryos are exposed to in each solution are predetermined and very accurate. These two advantages eliminate the need for searching the embryos/oocytes, allowing the simultaneous transfer of multiple oocytes or embryos between the various solutions, and standardize the exposure time to each of the vitrification steps resulting in a consistent reproducibility of the process and results. It is anticipated that this new device by maintaining all the important features needed for successful vitrification such as rapid cooling and warming, small volume, small carrier, and relatively low concentrations of CPs and controlled temperature and time for each step would be a breakthrough for simplifying and standardizing.

49.1.1.5 Results of Semiautomated Tissue Vitrification

Another advantage of E.Vit. is the opportunity for moving multiple oocytes (or embryos) or tissues at once between solutions. Initial experiments [76] carried out with ovine prepubertal testicular tissue vitrified with E.Vit. showed cell membrane

integrity assessed after culture of 0, 2, and 24 h by trypan blue exclusion test. Controls consisted of non-vitrified tissue analyzed after 0, 2, and 24 h in *in vitro* culture (IVC). Expression of genes involved in transcriptional stress response (BAX, SOD1, CIRBP, HSP90AB1), cell proliferation (KIF11), and germ (ZBDB16, TERT, POU5F1, KIT) and somatic (AR, FSHR, STAR) cell-specific markers were evaluated 2 and 24 h after warming. Post-warming trypan blue staining showed the survival and gene expression of most cells.

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Key Points

- Cryopreservation of gametes, gonadal tissues, and embryos is integral to the provision of a clinical service in assisted reproduction and fertility preservation, necessitating the purchase of equipment, consumables, and services to maintain the viability of cells and tissues during long-term storage.
- Patients may be faced with uncertainty over what to do with their embryos remaining in cryo-storage, and fertility specialists may lack the time and training required to educate and counsel their patients in fully understanding their embryo disposition options.
- Embryo Options provides an online cryo-storage billing service to fertility clinics and a patient portal for patient education and communication, thereby facilitating embryo donation between donors and recipients.

50.1 Introduction

The provision of in vitro fertilization (IVF) in assisted reproduction treatment (ART) has become a standard fertility service throughout the world, with millions of babies having been born since its successful clinical application in 1978. With the advent of controlled ovarian hyperstimulation (COH), more oocytes may be retrieved during each IVF cycle, resulting in more embryos to increase the likelihood of conception. However, single embryo transfer (SET) is now accepted as the standard for good practice as it almost completely avoids the known neonatal and maternal risks of multiple conception [1]. Therefore, in most cycles employing COH, there will be more than just a single good-quality embryo available, and those remaining after an SET, so-

called supernumerary or excess embryos, are usually cryopreserved to maximize the live birth rate (LBR) per cycle. In more recent times, freeze-all cycles have been advocated as a means of segmenting fertility treatment, originally to avoid suspected cases of ovarian hyperstimulation syndrome and latterly to avoid any disruption to endometrial receptivity that may be a side effect of COH. However, the benefit of freeze-all cycles to the LBR remains controversial [2]. Regardless, the success rates of ART have significantly improved following both fresh and frozen embryo transfer (FET), including the cumulative LBR per cycle [3], most likely primarily as a result of improvements in embryo culture [4] and the introduction of vitrification as a means of cryopreservation [5]. Consequently, the number of embryos cryopreserved and maintained in cryo-storage around the world has increased over time [6]. Inevitably, this increases the weight of responsibility for fertility clinics in terms of administration, billing, risk mitigation, and staffing required for equipment maintenance, quality control, and inventory management [7]. Furthermore, patients are obliged to comply with the various regulations governing the cryopreservation of their embryos and, ultimately, must somehow navigate the complex issues regarding the fate of any embryos remaining in cryo-storage once they have decided that they no longer wish to use them for their own treatment.

50.2 Communication Between Fertility Clinics and Their Patients

Patients undergoing fertility treatment are in regular communication with their fertility clinic, from their initial consultation with their fertility specialist through to when they receive the result of their pregnancy test. In this respect, education and counseling are an ongoing requirement of patients throughout their fertility journey, to reassure them regarding any fears, doubts, and misconceptions they may have regarding their fertility treatment and the fate of their embryos during *in vitro* culture and following cryopreservation.

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Understandably, ongoing communication with those patients who have successfully completed their families diminishes significantly, mostly restricted to payment of ongoing cryo-storage fees [8]. Indeed, there is a tendency for fertility specialists to delay review of a patient's disposition options until the patient has reached the stage at which they are obliged to consider donation or disposal of their embryos remaining in cryo-storage, by which time they may be less engaged in the process [9]. However, patients require appropriate and timely information regarding the options for disposition of their embryos remaining in cryo-storage and expect support from their fertility specialists in reaching such decisions [8–10]. Unfortunately, fertility professionals may find themselves lacking the time and training necessary to educate and support their patients with all the emotional, moral, and medicolegal concerns regarding the fate of their cryopreserved excess embryos [7, 8]. Besides, well-planned and executed embryo donation programs are not yet commonplace [8, 9, 11]. Furthermore, if fertility clinics do not already have in place the appropriate infrastructure to support embryo donation, there may be a reluctance on their part to suggest this to their patients as an option. Indeed, clarity appears to be lacking in this respect since, although fertility clinics may report that they provide a clinical service in embryo donation, they do not necessarily offer their patients the counseling necessary to fully consider this as an option [12, 13]. This is compounded by the fact that up to 70% of patients delay deciding what to do about the disposition of their embryos remaining in cryo-storage for at least 5 years [9, 14]. Effectively, this is lost time during which patients could have received educational support and counseling and may not even be aware of all the options available to them.

50.3 Patients' Dilemmas, Deliberations, and Decision-Making

Typically, consent forms regarding fertility treatment are signed by both the patients and the fertility specialist explaining the process and taking their consent. However, the informed consent process tends not to consider the emotional difficulties patients may face when having to decide about the disposition of their excess embryos. Furthermore, during long-term cryo-storage of a couple's embryos, unforeseen circumstances might arise such as death, divorce, or separation. This is not to be confused with orphan or abandoned embryos, which relates to instances where communication between fertility clinics and their patients has been lost due to relocation, leaving their embryos in limbo [15]. To complicate matters, there is a great desire by those unable to have their own children to receive donated embryos, and embryos are always in demand for various purposes including training of staff, validation of different methods, and research into

novel approaches to improve clinical outcomes. Based upon a purely altruistic mindset, many couples would prefer to donate their embryos to those in need [9]. On the other hand, faced with having to decide whether to have their excess embryos thawed and discarded, patients may prefer to keep them in cryo-storage. Indeed, over 70% of patients changed their minds regarding the disposition of their embryos within 3 years of their cryopreservation [16]. In this respect, patients are most likely to be receptive to the concept of embryo donation once they have successfully completed their fertility treatment and have given birth to their desired number of children. Indeed, it has been reported that at least 15% of patients would be very likely to donate their embryos in the future [9]. However, these patients may need emotional and practical support to navigate the medicolegal and mental health aspects of embryo donation. Some adoption agencies offer support to embryo donors and recipients with drafting agreements, but they have been denounced by the American Society of Reproductive Medicine (ASRM) Ethics Committee for unnecessary imposition of costly and time-consuming adoption practices [17]. Indeed, patients may be confused by going down this pathway since the ASRM states that equating embryo donation to adoption of children is misleading and not ethically justifiable. Besides, fertility specialists may not feel comfortable referring their patients to adoption agencies as they are unable to assess the viability of donor embryos. Clearly, patients may be faced with a complex and difficult decision to make regarding the disposition of their excess embryos in cryo-storage with limited resources and support systems available to them.

50.4 CooperSurgical's Embryo OptionsSM

In essence, the Embryo OptionsSM platform has been designed to provide ongoing communication between an expanding network of fertility clinics and their patients to facilitate billing for cryo-storage and matching of embryo donors to prospective recipients. In practice, it is so much more than that, since it provides resources for embryo donation, as well as educational material for increasing patients' awareness of their embryo disposition options and processes. Indeed, since its inception in 2015, the ethos of Embryo Options is that provision of an appropriate online infrastructure for embryo disposition may enable fertility clinics greater freedom with which to actively engage in embryo donation for their patients. In this respect, Embryo Options provides a support structure for implementation and management of a successful embryo donation program. To enable these functions, Embryo Options has built a comprehensive online portal using an industry-standard technological platform that currently reaches over 373,860 patients, thereby assisting fertility clinics with donor engagement and embryo

donation. Ongoing functionality and stability of the online portal are maintained and cyber-secured by the CooperSurgical® software engineering team. The Embryo Options applications are hosted in a healthcare-compliant cloud environment, meeting all applicable regulatory and data safety standards.

50.4.1 How Embryo Options Supports Fertility Clinics

When fertility clinics opt to partner with Embryo Options, the partnership is with a solid organizational foundation, comprising the Embryo Options Team and IVF Center. Embryo Options provides a medical liaison or customer success managers who are fertility nurse specialists with extensive nursing or embryology experience, proficiency in training health professionals, and experience in educating patients. Medical liaisons train fertility clinic staff in managing an embryo donation program and provide resources for updating the center's website to include information regarding embryo donation, with links to the Embryo Options Patient Portal (EOPP) plus educational material, which is discussed in the following section of this chapter. Though there is a large network of fertility clinics using Embryo Options, each fertility clinic has its own customized "front end" to Embryo Options featuring their name and logo. Training is provided by medical liaisons in person or via virtual meetings and is aimed at specialists in reproductive endocrinology and infertility, IVF nurse third-party coordinators, embryologists, mental healthcare professionals, genetic counselors, and legal professionals specializing in reproductive medicine. Training covers the following topics:

1. Provision of counseling to patients at critical junctures in their fertility treatment journey
2. Support services for prospective embryo donors
3. Medicolegal, psychosocial, ethical, and practical issues surrounding embryo donation
4. Evidence-based embryo donation guidelines and regulations

Fertility clinic staff are trained to discuss the availability of donated embryos while counseling patients regarding their treatment options and providing information to prospective recipients. In this respect, fertility clinics are encouraged to follow professional society guidelines for preparing patients to become recipients of donated embryos. Embryo Options provides fertility clinics with protocols for referring donors and their recipients to legal professionals specializing in embryo donation. Prospective recipients of donor embryos may be

enrolled into the EOPP by their fertility clinic and can "reserve" the embryos of a suitable donor. They will then be instructed to schedule an appointment with the fertility clinic where the embryos are in cryo-storage so that the fertility specialist can review the match. Embryo Options also provides an online billing service via a secure online payment portal to facilitate payment of cryo-storage fees, thereby automating the annual billing process of fertility clinics. Each year that cryo-storage fees become due, the EOPP automatically prompts patients to receive further information regarding their options including their own usage of their embryos, embryo donation, donation for training or research, or thawing for disposal, as outlined by the fertility clinic.

50.4.2 How Embryo Options Supports IVF Patients

Embryo Options is a more patient-centered and less costly alternative to adoption agencies which should, theoretically, result in increased awareness of embryo donation. In effect, Embryo Options partners with fertility clinics to educate and assist their patients to make decisions regarding the disposition of their embryos that they have in cryo-storage. Indeed, it aims to increase access to embryo donation by expanding its existing network of fertility clinics, donors, and recipients and by increasing awareness of embryo donation programs among fertility specialists and their patients. The EOPP is a comprehensive, online educational resource for patients, which includes information about various aspects of cryo-preservation, ongoing cryo-storage, and embryo donation. Educational content includes medicolegal and mental health implications for those desiring to donate embryos, guidance on building a patient profile, downloading of consent forms, and how to communicate their interest in donating embryos to another couple. Patients receive timely, relevant education via standard patient engagement for billing, as outlined by the fertility clinic. Indeed, by managing patient billing and monitoring which educational materials are being viewed, Embryo Options can optimize its support of patient decision-making. Medical liaisons are available via an email and telephone contact center to provide discreet one-on-one patient education and counseling tailored to individual needs, as required. A separate module within the EOPP allows prospective embryo donors to build a profile of themselves with varying levels of detail, depending upon whether they choose "identified" or "non-identified" donation [18], including basic demographic information and a family medical history, with the option of including a letter of introduction and non-identifying photos.

The EOPP facilitates compatible matching between embryo donors and recipients as follows:

1. Should a patient submit a medical profile for embryo donation to another couple, an automated alert is sent to the patient's fertility specialist to reach out to their patient and initiate a conversation regarding that option.
2. Patients may then download their fertility clinic's informed consent for embryo donation.
3. The fertility clinic's Donor Embryo Coordinator (DEC) is alerted and prompted to complete the medical section of the donor's profile and provide their contact information to the patient.
4. Once the patient has given their informed consent and has been properly screened by their fertility clinic to donate embryos, the DEC may confirm their donation within the EOOP to reveal their profile for prospective recipients to view on the fertility clinic's matching site.
5. If the fertility clinic allows, donors may place special conditions on their donation including the following:
 - (a) Being informed of the outcome of the FET
 - (b) Requesting that updates be provided regarding any child born from their donation
 - (c) Outlining the circumstances for future contact, such as emergency medical situations
 - (d) Confidentiality preferences
 - (e) Requesting that recipients participate in a criminal background check
 - (f) Any preferences for any embryos remaining in cryo-storage following donation
6. Recipients can request a match with a donor which, if approved by the donor, instructs the recipient to contact the donor's fertility clinic's DEC to discover which steps to take next.
7. Once both donor and recipient have been cleared to participate in embryo donation and have given their informed consent, each party can be referred by the fertility clinic to separate legal counsel to facilitate an embryo donation agreement.

50.5 Quality Management of Embryo Options

Comprehensive quality management is achieved via evaluation of both process and outcome components through data review within the Embryo Options Master Administrative Portal using monthly benchmarks for all outcomes pertaining to the following key performance indicators:

1. Expansion of the Embryo Options network of fertility clinics
2. Fertility clinic utilization of Embryo Options services
3. Increased awareness of embryo donation
4. Facilitation of embryo donor and recipient matching

The EOOP automatically tracks information regarding patient activity, making it an extremely useful evaluation tool to review the numbers of patients with embryos in cryo-storage, with a cryo-storage bill imminent, and who have expressed interest in learning more about embryo donation. It also reveals the type of information accessed from the EOOP and the step in the embryo adoption consideration process that any patient is currently on.

50.6 Conclusion

Considering the vast number of embryos in cryo-storage around the world and the relatively few donor FETs performed each year, cryopreserved embryos are a much-underutilized resource. Recent studies suggest that patients may be more likely to make better informed and constructive choices regarding the disposition of their excess embryos if provided with complementary counseling and educational support [19, 20]. Fertility clinics and their patients also require support with navigating medicolegal and logistical considerations surrounding cryo-storage and disposition of embryos [21]. CooperSurgical's Embryo OptionsSM platform offers a comprehensive approach to help mitigate many of the obstacles to efficient cryo-storage and disposition of patients' excess embryos.

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LN2 Sterilization to Avoid Cross Contamination

51

Lodovico Parmegiani

51.1 Introduction

51.1.1 Contamination Risk Related to the Use of Liquid Nitrogen

Liquid nitrogen (LN2) is a key cryogenic agent in cooling, chilling, and food freezing. Because of its extremely cold temperatures, it is extremely useful in a wide range of applications. In food and beverage, LN2 is used for freezing and chilling or modified atmosphere packaging. In healthcare and life sciences, LN2 is used as a cryogen to freeze and preserve blood, tissue, and other biological specimens and to freeze and destroy diseased tissue in cryosurgery and dermatology.

LN2 is produced commercially from the cryogenic distillation of liquefied air or from the liquefaction of pure air-derived nitrogen using pressure swing adsorption. LN2 can easily become contaminated in the supply chain or in use. This risk was not perceived as dangerous until early this millennium, when studies reported that tanks containing cryopreserved human cells were infected with mold, fungi, bacteria, and viruses [1, 2]. Contamination creates potential risks for frozen biological samples, patients, and food quality.

51.1.2 Risks of Contamination Throughout Liquid Nitrogen During Vitrification and Warming and Cryostorage

During both cryopreservation and storage, particular attention should be paid to the avoidance of bacterial or viral contamination. Depending on the storage vessel type, both liquid nitrogen and nitrogen vapor may be used for cryostorage of human gametes and embryos. Storage in either phase can be

potentially hazardous because some pathogens can survive at cryogenic temperatures and may contaminate the frozen cells or surfaces of the cryostorage containers [3]. There have been no cases of disease transmission via transferred cryopreserved human embryos to date [4], but microorganisms at any stage during culture and transfer of embryos can ultimately reduce the chances of a successful pregnancy [5]. Money lost due to contamination in ART has recently been estimated at around USD 100 million per year, but this is only for cases where culture contamination is so evident as to interrupt treatment [6]. The problem is probably bigger than evidence suggests, with this cost being just the tip of the iceberg.

51.1.3 Liquid Nitrogen Transportation

Manufacturers produce pure LN2 with various types of certifications. Food-grade and medical grade LN2 are widely available in the USA and Europe. Due to its physical nature, LN2 cannot be hermetically packed or sealed, posing a risk to the purity of the gas in the supply chain. LN2 is usually transported by large tank trucks and then poured into various tank/vessels before final use.

LN2 and its vapors (NV) can potentially be a vector of infection. The contamination of LN2/NV can occur at any step from manufacturing to final use, including transport, storage, distribution, and preparation for cryopreservation. After the recent COVID pandemic, this risk can no longer be underestimated.

51.1.4 Purity of Liquid Nitrogen for Healthcare and Food and Beverage

Some applications require LN2 to be sterilized before use. In healthcare and life sciences, this problem was identified in the early days of vitrification for human IVF [7]. The recent COVID-19 pandemic underlined the importance of not

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underestimating the risk of LN2-mediated contamination [8]. In pharma manufacturing, LN2 is sterilized for specific applications, such as lyophilization when using nitrogen fog (VERISEQ ice fog nucleation—<https://ima.it/pharma/product-insights/lyophilization-process/ice-fog-nucleation/>).

In food and beverage, aseptic dosing has been a well-established technology for bottling drinks or packaging food for over 25 years (https://files.chartindustries.com/20624665_ChartDosersCatalog_2015.pdf). LN2 has a crucial role in packaging and bottling. It is used to pressurize and/or for inerting products and their packaging. Sterile doses of LN2 are introduced into aseptic containers prior to the seamer or capper. The LN2 gasifies and either is trapped in the containers to add rigidity or escapes with oxygen for inerting the headspace.

Regarding the option of sterilizing LN2 before use in food and beverage, this practice can also be a solution for preventing bans or blockages of import/export or the stop of manufacturing sites. In January 2021, the Chinese government suggested that the disease SARS-CoV-2, first detected in the central city of Wuhan in late 2019, came from abroad. The government highlighted what it says are discoveries of the coronavirus on imported fish and other refrigerated or frozen food such as ice cream. Coronavirus was found on ice cream prompting a recall of 29,000 cartons from the same batch, according to the government. The company which manufactured the ice cream was sealed, and its employees were put in lockdown (<https://www.nerilizer.com/index.php/blog/56-chinese-city-reports-coronavirus-found-on-ice-cream-scientists-warn-about-liquid-nitrogen-use>).

51.1.5 Contamination During Gamete/Embryo Transportation

Shipment of cryopreserved samples is complex, comprising multiple critical steps, as listed below [9]. What can be expected is that during transfer between storage vessels, moving from liquid to vapor phase of nitrogen and vice versa, the shipping dewars are exposed to multiple contamination agents, which are potentially harmful to vitrified cells and operators.

The steps involved in shipment of cryopreserved samples from one location to another are the following:

- At the center, performing the vitrification procedure and shipping:
 - Cryo-devices holding oocyte/embryos are plunged in liquid nitrogen bath.
 - Cryo-devices are moved from liquid nitrogen bath to a storage dewar.

- Cryo-devices are moved from storage dewar to dry shipper.
- Dry shipper is transported by plane as hold baggage or plane by an operator as cabin baggage.
- At the center, receiving the shipment and performing the warming procedure:
 - Dry shipper is opened and refilled with liquid nitrogen (method of refilling may vary and some or all methods may pose a risk!).
 - Cryo-devices are removed from dry shipper to liquid nitrogen bath, and samples are identified manually.
 - Cryo-devices are moved from liquid nitrogen bath to dewar for storage before warming.
 - Cryo-devices are moved from storage dewar to liquid nitrogen bath for warming.
 - Dry shipper is transported by plane as hold baggage or plane by an operator as cabin baggage.

51.1.6 In Situ Sterilization of Liquid Nitrogen (LN2) or Nitrogen Vapor (NV) Before Use

In this scenario, the solution to minimize risks related to potential LN2-borne contamination is to sterilize the liquid nitrogen “in situ” immediately before the use on cells, tissues, or food. There are a number of ways to do this.

51.2 Contamination Risk Related with the Use of Liquid Nitrogen and Nitrogen Vapor

51.2.1 Hypothetical Mechanism of LN2 and NV and Contamination

Liquid nitrogen (LN2) and its gaseous form nitrogen vapor (NV) are key cryogenic agents for cooling and freezing in the healthcare and food industries. They can, however, become contaminated by viruses, bacteria, and fungi [1, 10]. In 1995, a hepatitis B virus contamination of a cryostorage tank used for bone marrow stem cells caused acute icteric hepatitis B in six patients following transplantation [10]. Human ART is the only medical discipline where human gametes and embryos with the potential to produce live births are routinely stored in LN2/NV. Many embryo/oocyte cryopreservation techniques utilize “open” cryo-devices with direct contact with LN2/NV during cooling. Even with “closed” systems, accidental contact with LN2/NV may occur during storage and warming of material. The fact that infections traceable to cryostorage have not been reported after millions of embryo transfers may be due to innate

immunity in the female reproductive tract and reduced dosage of infective agents during laboratory procedures. However, the risk of infection is real, especially when new, highly infective agents arise. Airborne contaminants encounter LN2/NV and remain cryopreserved [1]. Coronaviruses, as well as other viruses, are cryopreserved in LN2/NV. The use of contaminated LN2/NV risks virus awakening and the contamination of thawing cells, environment, and operators.

51.2.2 Model for Cryo-Specimen Contamination

Human reproductive cell vitrification can be performed with specific open carriers, which show high efficiency especially with oocytes [11]. The risk of contamination of the culture medium at warming through contact between the cells and/or the outer surface of the open carrier cannot be excluded, if the LN2 used for vitrification/storage is accidentally contaminated [3, 12, 13]. When using open carriers, frozen microorganisms in LN2 adhere to the vitrified cells or to the carrier surface. At warming, the reactivated microorganisms contaminate the culture medium, and they may attach themselves to the oocyte/embryo zona pellucida if this is cracked [14]. Even with “closed” systems, accidental contact with liquid nitrogen and nitrogen vapor (LN2/NV) may occur during storage and warming of material. It is now clear that the real risk is not in the type of vitrification carrier but with the LN2/NV themselves. A box with 2 L of LN2 can contaminate worktops, operators, cells, and patients. In the immediate vicinity of tanks, dewars, dry shippers, and vitrification boxes, aerosol mist particles are present near the LN2 surface, due to condensation, evaporation, and floating particles [15]. Environmental nitrogen-mediated contamination can infect reproductive cells during handling or vitrification and warming, regardless of whether we are using “open” or “closed” carriers [16]. This concept finds momentum in the recent observation of ACE2 and CD147 receptors on the cell membranes of preimplantation embryos, despite the fact that human oocytes and embryos are considered to be poor vectors for infections [17].

51.3 Vitrification Carriers and Contamination Risks

In the early days of slow cooling, glass ampoules, loaded with an embryo and then heat sealed, were used. This was satisfactory, but the smallest hole in the glass could cause an explosion on thawing, with disastrous consequences. After this time, it became convention to use 0.5 cc straws for sperm and 0.25 cc straws for embryos, with various sealing methods, heat sealing, and PVA bungs being common. Early vit-

rification used 0.25 cc sealed straws, and it was not until Vajta published his success with open pulled straws [7] (effectively thinning down the straw walls and reducing straw barriers, allowing for a small volume, and avoiding the need for sealing) that an explosion of “vitrification carriers” followed. Many are still in the commercial market today. “Are you using an open or closed system?” is a common question when vitrifying. What are you using, and does it really meet your cells’ needs or comply with current legislation? Below is a list of the various carriers and their nomenclature.

51.3.1 Open Carriers Plunged in LN2

Extremely rapid rate cooling and warming rates can be achieved by using specific “open carriers” such as open pulled straw, Cryoloop, hemi-straw, Cryotop, Cryoleaf, Cryolock, Vitri-inga, Cryotech, Vitrifit, Fibreplug, and Vitrix [7, 11, 18–22]. Each company will recommend a volume for vitrification, and in general, any of the flat surface devices or those with a tiny hole or ring (allowing for a film of liquid) can take the smallest volumes (0.1–0.5 μ L). If plunged directly into LN2, this gives extremely rapid cooling rates. These type of “open carriers” allowing for <1 μ L are generally preferred for oocytes [23–27], the most sensitive reproductive cell. A blastocyst- or a cleavage-stage embryo can survive with some cells damaged, but with a mature oocyte, with just one cell and one metaphase spindle, there can be no compromise.

However, these systems cannot avoid the hypothetical risk of microorganism contamination during the vitrification procedure, if the LN2 is accidentally contaminated [12, 13]. Sterilization of LN2 before the vitrification procedure is recommended to ensure safe clinical application of these systems in humans. However, this does not prevent possible contamination from the tank, if the device is not fully sealed during storage. For example, a Cryotop can be stored in the tank “as is” or it can have a partially sealed “cap” placed over the loaded end of the device. Alternatively, it can be placed inside a straw (while both straw and loaded device are in the LN2), and if the unsealed end of the straw is raised just above the LN2, a heat sealer can seal the outer straw (semi-closed carriers). This can be a challenging procedure, but with sterile LN2 and proper containment, these devices can avoid contamination from all sources and thus be acceptable for clinical use.

51.3.2 Carriers Sealed in Straws Prior to LN2 Plunging: True “Closed” Carriers

Another option for vitrification is the closed carrier based on the “straw-in-straw” mode (high-security vitrification, HSV),

designed to isolate the inner carrier containing the cells/tissue against LN2 during the vitrification process by using a sealed external straw [28, 29]. The sealing occurs once the carrier is loaded with the embryo but before being plunged into LN2. This system avoids direct contact between specimens and LN2 and also any hypothetical risk of contamination, and it allows good results with zygotes, cleaved embryos, blastocyst, and ovarian tissue [30, 31]. However, the “straw-in-straw” system reduces the rate of cooling and is not routinely used in clinical oocyte cryopreservation.

51.3.3 Single Straw or Pods

As an alternative to straw-in-straw, other types of closed systems, such as CryoTip [32] or Cryopette [33], allow faster rates of cooling. These closed carriers consist of a very thin straw specifically designed to load cells with minimum volume of cryoprotectant solution and to be hermetically sealed (single-straw closed carriers), avoiding direct contact between cells and LN2. Unfortunately, their design means that these systems cannot avoid the transmission of microorganisms in the warming medium during the warming procedure, due to the previous direct contact during vitrification between the external surface of the carrier and the accidentally contaminated LN2 [34, 35]. This is because the contamination of cells occurs at 37 °C, when any cryopreserved microorganism found in the LN2 reactivates after thawing in the culture medium. Even though the cells are passed through two or three warming solutions, there is still the theoretical risk that some microorganisms will reach the IVF culture media; even though antibiotics are usually used in culture media, some microorganisms may be resistant to antibiotics and infect the culture. In these circumstances, the bacterial or viral particles released in the culture medium may attach themselves to the oocyte/embryo's zona pellucida, particularly if it is cracked [10, 12]. A procedure to decontaminate the straw is to quickly wipe the carriers with 70% ethanol for disinfection at warming [32]. However, the deactivation of all microorganisms can only be obtained by a 5-min contact between ethanol and carrier [36]; this prolonged contact time can damage human cells, which remain inside the carrier in the warmed vitrification solution rich in potentially toxic cryoprotectants [37]. These carriers would therefore not be the first choice for clinical applications, but the sterilization of LN2 for the vitrification and warming procedure would offer a reduction of risk.

Another closed carrier is the Gavi® Pod [38], which is automatically sealed within a robotic liquid-handling and sealing unit (Gavi®) prior to plunging into LN2. While the cooling and warming rates may not be as fast as the open straw methods, it has the advantage of having no contact with LN2. After unsealing, warming solutions are added

directly into the pod. This device has proven to be successful for human embryos but is not routinely used for oocytes.

51.3.4 Nitrogen Vapors/Supercooled Air Vitrification

Some authors have demonstrated that it is possible to vitrify reproductive cells through exposure to nitrogen vapors [39, 40]. Inserting the carrier containing the cells into the supercooled air of a straw for instantaneous vitrification and then sealing the open end of the straw (post-sealing method) to avoid direct contact with LN2 [40] have also been proven to be useful. It is important to point out that the supercooled air inside the straws is basically composed of nitrogen vapors owing to the nitrogen's rapid evaporation and molecular weight [41]. For this reason, any microorganism accidentally present in the LN2 can also pass to the nitrogen vapor phase [42]. This may lead to the hypothetical contamination of the oocyte/embryo and the inner carrier. This means that contamination issues associated with LN2 cannot be avoided when using supercooled air, and sterilization of LN2 before evaporation is again recommended to ensure safe clinical application of supercooled air systems [42].

51.3.5 Solid Surface Vitrification

With these systems, vitrification is performed by touching the loaded device onto a solid surface of a chilled block partially submerged in LN2 [43]. Following vitrification, either the specimen is partially sealed with a straw “sleeve” fitting onto the device or the device is inserted into a sleeve, which is then fully heat sealed. Since the air around the block is composed of nitrogen vapors [14], sterilizing the LN2 before evaporation guarantees safe clinical use.

51.3.6 Carrier Nomenclature and the “Sex of Angels”

In some cases, the nomenclature used to market the vitrification carrier is inaccurate and misleading. Some open or “semi-closed carriers” are described as closed. In a closed method, the oocytes/embryos are physically separated from the LN2 used for vitrification, and therefore there can be no contamination from microorganisms potentially present in the gas.

As already detailed in various studies [41, 42], in “semi-closed” systems (Rapid-I, Cryotop SC, etc.), vitrification occurs with the cell in direct contact with nitrogen vapor—or supercooled air as originally defined by Larman and Gardner in 2011—and the carrier is subsequently sealed for cryostor-

age [40]. Thus, the hypothetical risk of potential contamination during vitrification is exactly the same as for an open carrier. The continued use of this imprecise terminology is confusing for practicing embryologists, physicians, patients, and legislators. A plea for clarity and precision of usage to authors, journals, and manufacturers was recently launched, with the warning not to belittle this matter as a philosophical treatise about the “sex of angels” [44].

51.4 Method for Sterilization of Liquid Nitrogen (LN2) and Nitrogen Vapor (NV)

The need to guarantee the sterility of LN2 for vitrification is therefore critical, due to the directives on tissue manipulation (European Commission, 2006—<https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:294:0032:0050:EN:PDF>).

Sterilization can be performed by filtration, by heat, or by ultraviolet radiation. Raw filtration does not guarantee sterility and is not effective against smaller microorganisms. Heat sterilization is applicable only for N₂ gas. UV-C radiation is effective and efficient even against the smallest microorganisms, such as viruses (HIV, hepatitis, coronavirus, Ebola virus, etc.).

51.4.1 Filtration

Some authors reported the purification or filtration of LN2 [11, 24, 42]. However, at the present time, only one filter is specifically designed for LN2 (Ceralin online, Air Liquide, Spain, https://www.airliquide.com/sites/airliquide.com/files/aligal_drink_2_bulk_offer-i_commercial_brochure-sales_tool-soft_drink_preview.pdf). This is a ceramic filter with a pore of 0.1 µm. The manufacturer markets this product for both the healthcare and food and beverage sectors. As this device filters up to 0.1 µm, it is obviously inadequate for smaller microorganisms, such as viruses.

51.4.2 Heat Sterilization and Re-liquefaction

Heat sterilization is mainly used for aseptic dosing in food and beverage. Chart, USA, AseptiDoser (https://files.chartindustries.com/20624665_ChartDosersCatalog_2014_web.pdf) involves filtering the LN2 through microporous filters before heating it from −172 to 149 °C and holding this temperature for 30 min. Once the nitrogen has been sterilized, it takes 15 min to cool down to 65 °C before being recondensed and transferred into a holding reservoir as sterile LN2, which is ready to dose. The entire process takes ~1 h and 25 min.

51.4.3 Ultraviolet-C (UV-C) Rays

Some authors have hypothesized the possibility of sterilizing liquid nitrogen with UV [45, 46]. This method was developed by Parmegiani et al. who demonstrated that ultraviolet-C (UV-C) radiation can sterilize small volumes of LN2 from bacteria, viruses, and fungi [47]. The method is based on administering a UV-C dose of 660,000 µWs/cm², which deactivates all known microorganisms, thus easily obtaining sterile LN2 for an aseptic vitrification procedure and for any other application. Its first application in IVF paved the way for a method for on-site sterilization of LN2 based on international patents on sterilizing liquid nitrogen by ultraviolet radiation (UV-C) and traceability + registration (blockchain). With this method (Nterilizer, Italy—www.nterilizer.com), the LN2 is sterilized before use, eliminating any risk of infection. Certified Sterile Liquid Nitrogen (SLN2) has become the “gold standard” for any application from healthcare to food and beverage.

51.4.4 Traceability of LN2/NV Sterilization

Certification and traceability of liquid nitrogen must be provided, such as using an indicator of the sterilization status of the batch, in order to certify the successful sterilization of the lot, and then the procedure must be recorded. In the case of UV-C sterilization, Nterilizer uses specific UV-C labels and a proprietary custom web platform: data are sent from the sterilization site to dedicate servers using the HTTPS protocol, protected by an SSL encryption layer. Finally, data are made incorruptible using blockchain transactions.

51.5 Cryopreservation in the Age of COVID

51.5.1 Scientific Societies’ Guidelines

During the COVID-19 pandemic, the fertility scientific societies released guidelines for minimizing the risk of contamination in IVF laboratories.

The Italian society SIERR pointed out that liquid nitrogen and nitrogen vapor (LN2/NV) can be a source of infection during a pandemic involving a respiratory virus. They highlighted the need for safer and more protective measures, including LN2 sterilization, to minimize viral exposure during cryopreservation and cryostorage.

The Spanish society ASEBIR released recommendations to minimize the risk to reproductive cells at cryopreservation during the COVID-19 pandemic. In this document, ASEBIR suggested increasing the number of washes of oocytes and embryos with culture medium and making dilutions in liquid nitrogen before passing the sample to the final tank (<https://>

asebir.com/wp-content/uploads/2020/07/Recomendaciones-COVID_V7-1.pdf). The procedure for vitrification carrier washing with sterile LN2 was originally described by Parmegiani and coworkers (2012) [48]. This procedure is also recommended by the Practice Committee of the American Society for Reproductive Medicine in the “Recommendations for reducing the risk of viral transmission during fertility treatment with the use of autologous gametes” [49].

Finally, the World Health Organization (WHO) in the “WHO laboratory manual for the examination and processing of human semen” 2021 (<https://www.who.int/publications/i/item/9789240030787>) recommended some precautions to take to avoid or limit viral contamination:

- Sterilization of LN2 to prevent contamination—useful in case of vitrification where the sample is directly immersed in LN2.
- Periodic refilling of dewar storage flasks or tanks with sterile LN2 and annual decontamination of the cryotanks.
- Decontamination of frozen specimens by sterile LN2 before warming.

51.5.2 Articles Suggesting Precautions for the Pandemic

Recently, many authors have suggested implementing “good manufacturing” practices in ART to minimize the risk of LN2-mediated contamination. Maggiulli et al. [50] suggest the use of single-personalized-disposable vitrification containers; Arav [51] and Alteri et al. [52] recommend the sterilization of LN2 before use. Hickman et al. [53] and Shapiro et al. [54] advocate the washing of cryopreserved specimens with sterile LN2 before thawing/warming. Pomeroy and Schiewe [55], Scarica et al. [56], and Vajta et al. [57] in their recent articles encourage using precautions for the safe use of LN2, by sterilizing it before use.

51.6 Risk Management When Using Liquid Nitrogen

51.6.1 Changing Perspectives When Using Liquid Nitrogen in IVF Laboratories

De Santis et al. on behalf of SIERR, observed that the safety of cryopreservation in the context of reproductive medicine is a crucial topic to mitigate any risk of cross infection. For this reason, they suggested changing perspective when using LN2 in IVF labs, advocating overcoming the historic diatribe between open and closed vitrification carriers as LN2 and

NV themselves can potentially be a risk as a source of infection. Since the use of contaminated LN2/NV enhances risks of virus awakening and contamination of warmed samples, the local environment, and operators, LN2/NV is recommended [16].

51.6.2 Adoption of Single-Use Medical Devices for Personalized Vitrification/Warming

In a recently published detailed risk analysis, Maggiulli et al. suggest using sterilized LN2 and disposable liquid nitrogen trays to mitigate risk [50]. Washing the polystyrene boxes used in IVF laboratories for vitrification and warming is difficult, due to the porosity of the material, time consumption, and most of all difficulty in verifying. It therefore seems obvious that in the future, the vitrification and warming procedure being performed in single-use sterile containers will become the “best practice.” Furthermore, this disposable container should be a certified medical device, as it contains human reproductive cell to be reimplanted.

51.6.3 “Double Bagging” for Cryostorage

Some precautions can be routinely taken in ART laboratories to minimize the risk of cross contamination during cryopreservation. For example, cryostorage in hermetically sealed containers and the use of a secondary sleeve (straw-in-straw) have been recommended for both vitrified and slow-frozen cells [7]. Storage in a sealed container inside an LN2 tank has been shown to be both possible and safe by Parmegiani et al. who used aluminum cylinders [14], Parmegiani and Rienzi using sealed plastic visotubes [58], and Arav who used hermetical canisters [59]. Various carriers in the market nowadays ensure hermetic cryostorage using a sealed external straw or by a tight cap closure.

51.6.4 Tank Disinfection

LN2 and NV tanks used in IVF labs can contain frozen microorganisms [1, 2].

It must be pointed out that aerosol mist particles 1–5 µm in size are generated near the LN2 surface, up to a distance of 10–20 cm, which could be due to particle evaporation [15]. This could pose a risk to personnel who are handling contaminated LN2 containers. Periodic disinfection of cryocontainers or ensuring the sterility of factory-derived LN2 (Bielanski and Vajta) [60] is a risk mitigation strategy to be implemented to ensure safety for patients, personnel, and laboratories offering cryo-facilities [61]. Thus, periodic

refilling of storage dewars with sterile liquid nitrogen and annual decontamination of the cryotanks (which requires removal of the specimens) can help minimize the potential risk of cross contamination [9]. Care should be taken during cleaning to ensure that stored reproductive samples are not put at risk during removal from the cryotank [62].

51.6.5 Specimen Washes with Sterile Liquid Nitrogen Before Warming/Thawing

LN2 can be effectively sterilized with UV-C radiation [48], and the “vitrification carrier washing” with UV-sterilized LN2 before warming has been demonstrated to eliminate vitrification carrier contamination in extreme experimental conditions. In a 2021 study, Parmegiani et al. used a concentration of microorganisms over 10,000 times higher than any observed in LN2 IVF storage vessels, and the washing procedure fully removed the carrier contamination mediated by LN2 [47]. Scientific societies’ guidelines and various authors advice this procedure to eliminate viral pathogens [49, 53, 54, 61].

51.6.6 How to Set Up a Virus-Free Vitrification Program

In healthcare, blockchain can become a tool for addressing challenges around sensitive data sharing and traceability of medical and laboratory procedures. Recently, Parmegiani et al. described the first application of blockchain in IVF for incorruptible traceability of a “virus-free” vitrification/warming procedure based on the combined use of UV-C-sterilized liquid nitrogen (LN2) and CE medical devices (CE-MD) [63]. They reported 2346 Ethereum blockchain data transactions for IVF laboratory procedures mined from 01/10/2019 to 31/12/2021. The procedures were oocyte/embryo vitrification, warming, or handling in LN2 after cryopreservation. For each vitrification, warming, and handling, a UV-C-sterilized batch of LN2 was associated with the code assigned to the vitrification/warming procedure and with the lot number of the single-use sterile vitrification box (N-Sleeve). The clinical results obtained from warmed oocytes/embryos were observed as completion of this process. A blockchain-trusted “virus-free” vitrification/warming program was set up using a specially designed CE-MD N-Bath-System (Nterilizer, Italy). Each procedure was traced by the CE-MD’s software and a dedicated Web application. Finally, data were made incorruptible using Ethereum blockchain transactions. Before oocyte/embryo warming, vitrification carrier washing with

UV-C-sterilized LN2 was performed in accordance with recent international anti-COVID guidelines. Of the 2346 blockchain transactions, 1268 were vitrification and cryopreserved specimen handling procedures; 1078 transactions were frozen cell warming (308 oocytes and 770 embryos) performed on 799 patients. To date, 445 pregnancies have been obtained (pregnancy rate: 41% per cycle; 56% per patient), and 219 babies have been born. This is the first evidence of the application of blockchain in IVF, and many others will probably follow. Blockchain immutable records of LN2 sterilization combined with procedure codes and disposable lots represent incorruptible traces for “virus-free” vitrification/warming. During the pandemic period (December 2019–December 2021), 219 babies were born from “virus-free” cryopreservation procedures powered by blockchain.

51.7 Conclusion

The risk of using contaminated LN2 has been underestimated for many years. Nowadays, after the SARS-CoV-2 pandemic, even authors who more than 10 years ago considered the risk of cross contamination in ART cryotanks negligible now suggest sterilizing LN2 [55]. The solution to minimize the LN2 contamination risk is straightforward and easy to implement. It is hoped that LN2 sterilization will become a mainstream practice before the first cases of specimen cross contamination or operator infection occur in IVF. LN2 sterilization and adopting single-use medical devices for personalized vitrification/warming are best practices suggested by many authors and scientific societies, after the recent SARS-CoV-2 pandemic.

51.7.1 Author Perspective

In the modern ART laboratory, where each instrument, device, and medium is a certified medical device, performing vitrification and warming with old (often dirty) polystyrene boxes is not only an unnecessary risk, but also a poor advert for the IVF center.

If our laboratories had glass walls—and some IVF centers now do, as patients can view the lab through a window, similar to the open kitchens in top restaurants—what would patients think of these boxes? The recent pandemic has given us the input to refine the last step for the vitrification revolution, which is performing this technique with medical devices in aseptic condition, tracing, and recording the procedure, until such times as the next significant change—probably automation—takes place.

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52.1 Lab on a Chip

Microfluidics is the field that studies and manipulates fluids at millimetric and submillimetric channel scales (1–100 μm), i.e., microchannels and embodying a miniaturized format. This discipline has many applications and gave way to the name “lab on a chip” as it enables the concentration of different processes in one small space: a biochip [1].

The field has four precursors that catapulted the field forward: molecular analysis, biodefense, molecular biology, and microelectronics [2].

First came microanalytical methods—gas-phase chromatography (GPC), high-pressure liquid chromatography (HPLC), and capillary electrophoresis (CE)—which, in capillary format, revolutionized chemical analysis achieving high sensitivity and high resolution using very small amounts of liquid sample. This success gave way to the development of novel, more compact, and more adaptable platforms and the search for other applications in microscale formats.

On the biodefense side, after the Cold War, and to counter the threats of deployment of chemical weapons, the US Department of Defense supported a series of programs in the 1990s aimed at developing field-deployable microfluidic systems designed as detectors for chemical and biological weapons, which provided the main stimulus for the rapid growth of academic microfluidic technology development.

The third motivational drive came from the field of molecular biology. The explosion of the genomics field in the 1980s, and the necessity of high-throughput DNA sequencing, required analytical methods with much greater sensitivity and resolution than the ones previously contemplated in biology. Microfluidics offered approaches to overcome these challenges.

Last came the contribution from the microelectronics field. Because photolithography had been successful in sili-

con microelectronics and microelectromechanical systems (MEMSs), the hope was the same applied to microfluidics. However, in the context of microfluidics, glass has been largely replaced by plastics because those fabricated in glass were inappropriate and are not permeable to gases, a requirement when working with living tissue or cells. Furthermore, silicon, being expensive and opaque and although it provided chemical, thermal, and mechanical stability, was inappropriate and too rigid for pumps and valves.

Most research in microfluidic systems has been carried out with a polymer—poly(dimethyl siloxane) or PDMS—the properties of which are entirely distinct from those of silicon. PDMS is an optically transparent, soft elastomer, and these characteristics have made it a key material in the early stages of prototype development.

52.2 Cryopreservation in Assisted Reproduction

The first successful pregnancy following cryopreservation and thawing of an eight-cell embryo was reported in 1983 [3], and almost 40 years later, cryopreservation is a routine part of daily fertility clinic activities and has resulted in thousands of successful live births.

Cryopreservation is necessary for long-term storage of spermatozoa, oocytes, and embryos obtained from patients in clinical assisted reproduction clinics, for later use as part of an assisted reproduction treatment [4] or as elective fertility preservation [5]. Cryopreservation approaches, such as slow-rate freezing or vitrification, involve the use of cryoprotectants to avoid damaging ice crystal formation in the cytoplasm of oocytes or cells of the developing embryo [6]. Slow freezing, which uses specialized equipment (e.g., controlled-rate freezer), is necessary for slow and controlled cooling of samples and was the clinical standard for many years but had lower outcomes due to intracellular ice crystal formation [7].

Vitrification on the other hand does not need a controlled-rate freezer but however involves more sophisticated sample

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(oocytes and embryos) preparation to replace cellular water with cryoprotectant agents in several dehydration steps and precise exposure times of the cells to increasing concentrations of cryoprotectant agents (CPAs) (equilibration steps) followed by flash freezing in liquid nitrogen (LN2) in a minimal cell volume to achieve high cooling rates.

The elevated CPA concentrations suppress ice crystal formation, but it also inflicts osmotic pressure and has been shown to cause irreversible damage. To mitigate osmotic stress, the gradual (gradient)/stepwise increase in CPA concentrations is carried out.

This technology has rapidly been adopted and has replaced slow-rate freezing in IVF laboratories [8–10]; this stepwise protocol improves post-warming viability and developmental competency [11].

Cryoprotectants reduce the formation of damaging ice crystals in the intracellular and surrounding liquid solutions of oocytes or cells of the developing embryo. When frozen, these liquid solutions are converted into a glass-like amorphous solid that is free of any crystalline structures when plunged in LN2.

The addition and removal of CPA from cells can cause successive phases of shrinkage and re-expansion due to movement of water and CPA across the cell membranes. A common method for oocyte and zygote vitrification involves a three-step equilibration process in which the cells are manually pipetted into subsequently higher levels of permeable CPA concentrations [12]. This process allows the cells to shrink in stages, and re-expand, avoiding the minimum cell volume critical for viability. Exposure to impermeable solutes can be critical for success of vitrification, but impermeable solutes often do not allow for cell re-expansion. For this reason, exposure to impermeable solutes can be reserved until the final step to maintain as large a cell volume as possible.

Compared with murine samples, bovine samples can be more difficult to cryopreserve and are more representative of human in terms of dimensions. For bovine samples, a greater number of equilibration steps increases the rate of cryosurvival [13] and developmental competence [14].

However, the number of steps for loading and unloading CPAs cannot be unlimited due to practical restrictions in a high number of manual steps but should minimize abrupt osmolarity changes. Microfluidic technology can provide a promising solution to this impracticality by automating and standardizing these procedures. Microfluidic devices can facilitate various processes of the cryopreservation process, including providing precise control of fluid delivery and exchange, progressively delivering varying concentrations of CPAs, automating delivery of CPAs to/from cells, automating loading of cells, automating equilibration of cells, automating dehydration of cells, containing cells, immobilizing cells, uniformly exposing cells to

CPAs, real-time imaging of cells, and allowing manual retrieval of cells.

52.3 Advantages of Microfluidics

The small scale of fluids manipulated on biochips gives rise to different behavior of fluids (what is termed laminar flow or absence of turbulence). On large scales, fluids mix convectively, but in microsystems, when two fluid streams come together in a microchannel, they flow in parallel without turbulence and only mix by diffusion [15]. Although this requires development of specific devices to accomplish mixing (such as long and thin channels) when mixing is required, it has proven to be an advantage in many circumstances; the contact between both fluids is much more controlled and provides a high surface-to-volume ratio. Therefore, the benefit is the potential for precise control of concentration gradients by diffusion-based gradient generation [16].

Also, the microfluidic approach provides mechanical and biochemical advantages, which can be exploited in the assisted reproductive technology space using different types of reproductive cells (gametes) and embryos, and at different stages of the process, such as sperm sorting, insemination, embryo culture, and cryopreservation [17]. With respect to cryopreservation, microfluidics plays an important role in improving outcomes such as viability of the cells, as an automated microfluidic cryoprotectant exchange (CPA) approach can minimize osmotic shock (sudden changes in osmolality) during cryopreservation, which can be achieved by the more complex protocols that can be generated, for example a combined stepwise and linear protocol rather than just one simple protocol of a stepwise or a linear profile [16].

Lower osmotic shock can be measured in the low shrinkage rate achieved by microfluidics, which is correlated to better outcomes such as cellular viability and developmental potential by reduction of sublethal damage [18].

Some have reported that microfluidics schemes lower shear stresses originating from manual pipetting [19]. Therefore, instead of moving the cells from one place to another (as in manual methods), the sample is static, and fluids are delivered to and from the sample (held in either a chamber, a holding channel, or a well) [20]. In this way, and thanks to microfluidics, both physical damage (caused by osmotic stress) and chemical stress (due to CPAs) are reduced (Song et al. 2009).

Other advantages of microfluidics are the use of low volumes of reagents (and associated lower costs of each cycle of cryopreservation), low cost due to the disposable nature of the microfluidic platform, small size, and thus low footprint, which make this technology highly desirable where lab footprint is scarce. Lastly, integration of various stages of the cryopreservation procedure into one automated platform will

achieve standardization and increase both throughput and key performance indicators such as cryosurvival rates and other downstream metrics.

Among the main challenges of assisted reproduction labs are laborious and manual current technologies and lack of process automation. Most conventional manual methods offer very good efficiency rates, which does not offer a huge gap to bridge with innovative technology. For example, some of the well-established vitrification and warming protocols offer a high survival rate of approx. 90%. However, there is great variability between operators with different training levels, and automation will bring everyone to the same high survival results.

In addition, in recent years, there have been various studies that have led the way to consider not only survival rates, but also cell quality and developmental potential other than current reference values such as morphology and survival rate alone. This is something to consider if we want to improve upon the current protocols and to obtain improved outcomes.

52.4 Microfluidic Devices

A few examples of microfluidic platforms for oocyte and embryo cryopreservation using microfluidics have been reported in the literature [18, 20, 21]. For instance, Heo et al. (2011) developed a microfluidic device and a protocol to deliver cryoprotectant solutions to mouse oocytes to minimize toxic injury from cryoprotectant solutions and study oocyte volumetric responses. They were able to successfully load CPAs in a linear protocol with a volumetric change of less than 10%. The disadvantage of this device was that the oocyte was confined to a closed chamber preventing easy retrieval of the oocytes, which were held in semicircular holders of the chamber.

Furthermore, Pyne et al. [21], in a proof-of-concept study and using a digital microfluidic device (Fig. 52.1) applying voltages to move mouse embryo-containing droplets through

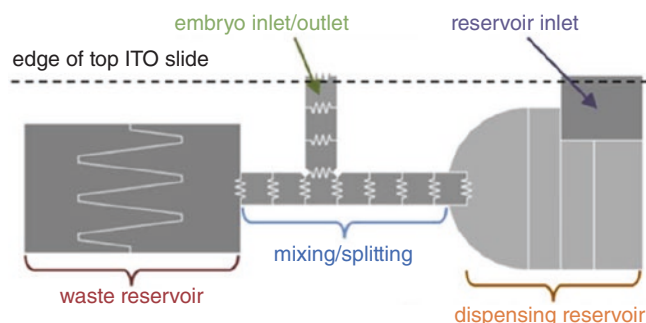


Fig. 52.1 Device schematic with regions for vitrification medium dispensing and for embryo loading/extraction. (Reproduced from [21])

a complete two-step vitrification protocol, successfully demonstrated that the whole process of sample preparation could be entirely automated, with similar survival and vitrification results compared to manual intervention. However, the disadvantage to this study was the accumulation of serum originating from the high serum concentration from the embryo media, rendering some droplets immovable.

Also, another research group at the University of Michigan [18] designed a microfluidic device and an automated microfluidic protocol for the murine and bovine model and achieved improved oocyte and zygote vitrification. The use of a slow and steady CPA protocol lowered shrinkage rate and osmotic stress, resulting in improved developmental competence and higher cell quality over the manual pipetting method. One disadvantage to this device was that, again, the samples were constrained to a chamber with no manual access.

The disadvantages of all the abovementioned microfluidic platforms, apart from not being user-friendly devices, relate to their unsuitability for clinical laboratory programs and operations given that they were designed as proof-of-concept devices and not as point-of-care medical devices.

52.5 Innovation and Industrial Breakthrough Using Microfluidic Devices

Recently, a fully automated microfluidic biochip for both human oocyte and embryo preparation prior to vitrification and warming has become the first one under serious development and validation for the purposes of seeking regulatory approval and commercialization (DaVitri) [22–24] (Fig. 52.2). The features that make this device innovative and an improvement over previous ones lie in its design and method of use: (1) an open-well system for the user to introduce and retrieve oocytes easily and for confinement of the oocyte to enable 100% recovery of sample, (2) even exposure of the oocyte to concentration gradients of cryoprotectant, (3) on-chip reservoirs for storage of cryoprotectant solutions and waste, (4) a biochip electronic control station that both seals and pressurizes the reservoirs, and (5) a proprietary method of mixing and controlled delivery of CPA concentration gradients to the sample for sample preparation prior to vitrification and prior to revival of cells.

Furthermore, this microfluidic biochip, in contrast to others, achieves loading and mixing of cryoprotectant solutions not by way of co-mixing fluids (co-flow) but by alternative injection of one solution or another using pumps for precise control of inflows as a function of time.

Preliminary results during the validation stage of this device, using mouse and bovine models, are promising:



Fig. 52.2 Both the microfluidic biochip (right) and its corresponding electronic control system (left)

Table 52.1 Mice oocyte cryosurvival and blastocyst development rates

Mice zygote vitrification			
Process	<i>n</i>	Cryosurvival rate	Blastocyst development rate
Manual	103	99% (102/103)	92.2% (94/102)
Automatic	107	99% (106/107)	85.8% (91/106)
		NS	NS

* $p = 0.02$, ** $p = 0.049$

Table 52.2 Mice oocyte cryosurvival, fertilization, and blastocyst development rates

Process	<i>N</i>	Cryosurvival rate (%)	Fertilization rate (%)	Blastocyst development rate (%)	Good-quality blastocyst rate (%)
Manual	86	NA	91.9*	83.7**	83.3
Automatic Vitri	105	100%	83.2**	83.2	79.7

* $p = 0.02$, ** $p = 0.049$

52.5.1 Mice Zygotes

Mice zygotes ($n = 210$) were divided into two groups: (A) manual control ($n = 103$) with standard vitrification using the Kitazato protocol and (B) automated group ($n = 107$), following a microfluidic protocol performed using Kitazato media. All the zygotes were stored on the same vitrification carrier device: the Kitazato Cryotop® device. After their storage in liquid nitrogen, both groups were warmed manually following the standard Kitazato thawing protocol, and Piezo-assisted intracytoplasmic sperm injection (ICSI) was later performed to evaluate embryonic development. Chi square test was performed to compare cryosurvival and blastocyst development rates between groups.

The results showed 99% cryosurvival rate (see Table 52.1) and 92.2% blastocyst development rate for the control group A ($n = 103$) vs. 99% and 85.8%, respectively, for the automated protocol, group B ($n = 107$). No significant differences were found between groups either for cryosurvival rate ($p = 0.998$) nor for blastocyst development rate ($p = 0.726$).

52.5.1.1 Mice Oocytes

Mice oocytes were vitrified using the device and warmed manually followed by Piezo-assisted intracytoplasmic sperm injection (ICSI) to evaluate embryonic development (see Table 52.2). Kitazato media was used for all experiments.

These promising results show similar blastocyst development rates to manual protocols routinely used in IVF laboratories.

52.5.1.2 Bovine

A total of 238 fresh bovine oocytes were vitrified with the DaVitri device (automatic vitrification group) and subsequently warmed manually. Also, 104 fresh bovine oocytes were vitrified manually and warmed with the device (automatic warming group). All were cultured for 1 h before checking cryosurvival rate.

The manual control group ($n = 25$) showed 92% cryosurvival rate, while the automatic vitrification ($n = 212$) and the automatic warming ($n = 104$) group showed 89% and 93.2% cryosurvival, respectively (see Table 52.3).

Table 52.3 Bovine oocyte cryosurvival rates and blastocyst development rates

Process	<i>n</i>	Cryosurvival rate	Blastocyst development rate
Manual	25	22 (92%)	NA
Automatic Vitri	238	212 (89%)	NA
Automatic Warming	104	97 (93.2%)	NA

52.5.1.3 Human

Although the device is still in the preclinical stage of development, there are plans to conduct validation in the human model (clinical trials) in the near future.

52.6 General Issues with Introducing New Technologies

What requirements must be fulfilled for microfluidics applied to cryopreservation to become a major new technology and go through the cycle of development from university laboratories to industrialization, regulatory approval, and commercialization, reaching clinics for patient benefit? It is clear that the field can cover real necessities and market gaps. As in any field, the problems it faces are those faced by most fields as they develop. The problem of capital investment and huge capital risks will likely only to be taken on by venture capital firms. A huge amount of capital is needed for product development, and the fact that microfluidics has not yet lived up to its early promises is not a surprise, and the reasons for the rate at which it has developed are a characteristic of new technologies.

Superior results, in terms of performance and key metrics such as pregnancy rates and live birth rates, are also aspects that will need to be demonstrated for the uptake of microfluidic technology in routine clinical practice. So far, most platforms discussed have been tested on animal models but, as with any new technology, will need to be validated using human oocytes and embryos in preclinical and clinical phases with the ultimate test to be done in randomized clinical trials to ensure safety and effectiveness. However, in principle, both the traditional manual method and the novel automated cryopreparation method would have comparable effectiveness if the former was performed by expert embryologists, with the automated method having slight advantages. As discussed above, automated methods which include microfluidic technologies increase cell viability by reducing physical and chemical damage [16, 18, 20] and more importantly by reducing variability between users of different degrees of expertise.

Another aspect to consider is the concept of the ages-old concept of “misonicism”—a fear of the new and the unknown. The manual traditional method is well established in ART clinics; thus, there will be initial reluctance to acquire new

platforms. However, once the technology has received regulatory approval and as more and more clinics acquire the technology and confirm effectiveness, barriers to uptake will slowly disappear.

52.7 Future Directions

Egg and embryo freezing is at the crossroads of all automated process in IVF. It is indicated for elective fertility preservation, for freezing embryos while performing PGT diagnosis, and for freezing excess embryos during single-embryo transfer, because freezing all embryos in high responders and transferring in a differed cycle increase live birth rates compared to fresh embryo transfers [25, 26].

The automation of egg and embryo vitrification will be coupled with other automation activities such as singulation and denudation, embryo culture, time lapse and AI embryo selection, spent media sampling for NI-PGT, and metabolomic analysis. All frozen eggs after thawing need to be fertilized via ICSI, and ICSI automation [27] would also require microfluidic chips for sperm sorting and loading to the injection pipette.

A problem encountered by many technologies that have been launched in ART, for example PGT-A [28], is that differences in operator technology, especially biopsy and DNA analysis, provide different results in different laboratories or even within the same lab [29]. The laboratory of the future will be fully automated, and that equipment will become a platform, a platform that would yield a solid baseline of standardized results from where to really test new methods and ideate without confounding factors produced by intra-operator variability.

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Part IX

Appendices: Protocols

Oocyte Slow Freezing Protocol

53

Carlotta Zacà, Giovanni Coticchio, and Andrea Borini

53.1 “Protocol” on “Human Oocyte Slow Freezing”

53.1.1 Materials

53.1.1.1 Materials for Slow Freezing and Rapid Thawing Procedure

Reagents for slow freezing and rapid thawing procedure:

- PBS Dulbecco’s phosphate-buffered saline (Sigma Life Science, Sigma-Aldrich)
- PROH 1,2-propanediol (Sigma-Aldrich, The Netherlands)
- Serum substitute supplement (SSS) (Irvine Scientific, Santa Ana, California)
- Sucrose $\geq 99.5\%$ MW 342.3 (Sigma Life Science, Sigma-Aldrich)

Equipment for slow freezing and rapid thawing procedure:

- Automated biological vertical cryo-freezer Kryo 360-1.7 (Planer) (Fig. 53.1)
- Precision balance
- Impulse-heat sealer (Fig. 53.2)
- Label printer (Brady)
- Timer (Fig. 53.2)
- Flexipet Adjustable Handle Set (Cook Australia)
- Metal forceps
- Liquid nitrogen and liquid nitrogen reservoir
 - Thermometer
 - Small sterile scissors

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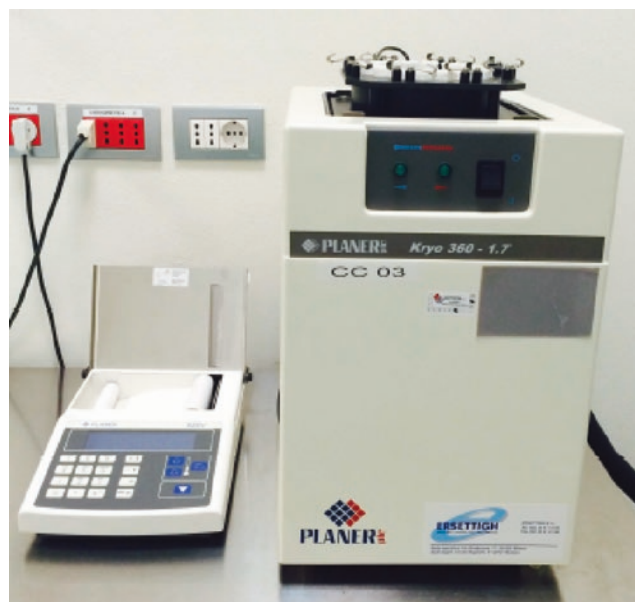


Fig. 53.1 Automated programmable vertical cryo-freezer Kryo 360-1.7 (Planer)

- Surgical gauze
- Water bath 30 °C

Disposables for slow freezing and rapid thawing procedure:

- Sterile tube (10 mL, Ref.2001, Falcon)
- Flask 50 mL (Falcon, Becton Dickinson, NJ, USA)
- Sterile serological pipettes (1 mL, 5 mL, 10 mL, Falcon, USA)
- 10 mL Syringe (BD Emerald, Becton Dickinson, Spain)
- Millex GV Filter Unit 0.22 μm , Durapore PVDF Membrane (Merck Millipore LTD, IRL)
- Flexipet-Denuding Pipette tips ID = 170 μm
- Multidish 4 Well plate Nunclon
- White adhesive labels, BMP21 (Brady, USA)



Fig. 53.2 Slow freezing equipment and disposables. From upper left: impulse heat sealer, timer. From lower left: sterile straw, insulin-type syringe 1 mL connected to a silicone tube, label (name, surname, sample number, date, couple code), and goblet

- Adapted syringe: silicone tube inner diameter 1.5 mm, outer diameter 3.5 mm connected to an insulin-type syringe 1 mL (Fig. 53.2)
- Sterile straws (CBS high-security embryo straw 0.3 mL, Cryo Bio System, Groupe I.M.V. Technologies, France) (Fig. 53.2)
- Weighted ID rod 30 mm for CBS high-security straw (Cryo Bio System, Groupe I.M.V. Technologies, France)
- Petri dish 35 × 10 mm (Ref. 353001, Falcon, Becton Dickinson, NJ, USA)

53.1.1.2 Slow-Freezing Solution Preparation

There are two freezing solutions for slow freezing of human oocytes: 1.5 M PROH in PBS (equilibration solution) and 1.5 M PROH in PBS plus 0.2 M sucrose (loading solution). Both the solutions need a 20% protein serum substitute supplement (SSS). Manage all the reagents under laminar flow, and reduce artificial light conditions.

1. Prepare the solution into a 10 mL tube.
2. Equilibration solution: mix 6.9 mL PBS and 1.1 mL PROH.

3. Loading solution: solubilize 0.2 M sucrose in 6.9 mL PBS plus 1.1 mL PROH.
4. Add 2 mL SSS to each solution and mix.
5. Filter both the solution into a new sterile 10 mL tube.
6. All the solutions are stored in the refrigerator at 4 °C for a maximum of 3 days since preparation.

53.1.1.3 Rapid Thawing Solution Preparation

There are four thawing solutions for rapid thawing of human oocytes:

- Solution 1: 1 M PROH in PBS plus 0.3 M sucrose
- Solution 2: 0.5 M PROH in PBS plus 0.3 M sucrose
- Solution 3: 0.3 M sucrose in PBS
- Solution 4: PBS medium

All the solutions need a 20% protein source supplementation (SSS). In order to obtain the four thawing solutions, it is necessary to prepare a stock solution. Manage all the reagents under laminar flow, and reduce artificial light conditions.

- First mix into a 50 mL flask: 14.5 mL PBS with 1.5 mL PROH (stock solution).
- Prepare the four thawing solutions into a 10 mL tube.
- Thawing solution 1 (1 M PROH/0.3 M sucrose): solubilize 0.3 M sucrose in 8 mL of stock solution.
- Thawing solution 2 (0.5 M PROH/0.3 M sucrose): solubilize 0.3 M sucrose in 4 mL of stock solution and 4 mL PBS.
- Thawing solution 3 (0.3 M sucrose): solubilize 0.3 M sucrose in 8 mL PBS.
- Thawing solution 4 (PBS): 8 mL PBS medium only.
- Add 2 mL SSS to each solution and mix.
- Filter all the solution into a new sterile 10 mL tube.
- All the solutions are stored in the refrigerator at 4 °C for a maximum of 3 days since preparation.

53.1.2 Methods

53.1.2.1 Slow-Freezing Procedure

- The oocytes are de-cumulated mechanically and enzymatically around 2 h after transvaginal retrieval to detect the metaphase II oocytes and undergo slow-freezing procedure.
- Place the solutions to room temperature (22–24 °C) before use. All dehydration steps must be performed at room temperature (RT). Just before starting, the cryo-freezer is turned on.
- One to maximum three oocytes are loaded per straw. Connect an adapted syringe to each straw, and prepare a weighted ID rod.
- Set up a 170 µm denuding pipette.

- For each oocyte or group of oocytes, dispense 0.6 mL of the equilibration solution in Well 1 and 0.6 mL of loading solution in Well 2 of a 4-well plate labeled with the patient's surname, name, and couple code.
- The denuded mature metaphase II oocytes are transferred from their culture media to the equilibration solution and incubated for 10 min. The countdown begins when the first oocytes are placed into the equilibration solution (Table 53.1).
- After this period, the oocytes are moved to the loading solution beginning with the first oocyte incubated. The passage between one solution and the other is carried out by collecting the oocytes with a 170 μm denuding pipette in a small amount of solution.
- Within 5 min since the first oocyte has been incubated in the loading solution, it is necessary to proceed in loading the oocyte into the straw (Table 53.1).
- First, start loading a small amount of loading solution, then a bubble air, subsequently loading solution containing the oocyte in the middle of the liquid, then another air bubble, and at the end another small amount of the same solution (Fig. 53.3).
- The straw is then separated from the adapted syringe. Insert each weighted ID rod into the straw, and seal it on both sides.
- The straws are placed into the automated cryo-freezer when the temperature is around 20 $^{\circ}\text{C}$.
- All the steps are carried out by two persons (the operator and a second person who assists the operator during the procedure).
- Automated freezing program: the sample is slowly cooled from +20 to -7°C at a rate of $-2^{\circ}\text{C}/\text{min}$ (Table 53.2). Manual seeding of the sample is performed at -7°C , and this temperature is maintained for 10 min. During this time, the straws are removed rapidly and not completely to induce ice crystal by touching the straw with very cold metal forceps in proximity of the first small amount of loading solution nearby the filter (Fig. 53.2). Then, the temperature decreases to -30°C at a rate of $-0.3^{\circ}\text{C}/\text{min}$ and then rapidly decreases to -150°C at a rate of $-50^{\circ}\text{C}/\text{min}$ (Table 53.2). An acoustic and visual signal signals the end of the freezing program. Then the straws are directly plunged into identified colored goblet (Fig. 53.2) in liquid nitrogen and finally stored into the dewar at -196°C .

53.1.2.2 Rapid Thawing Procedure

- Place the thawing solution to room temperature ($22\text{--}24^{\circ}\text{C}$). All thawing steps must be performed at room temperature (RT).
- Prepare one 4-well plate for each straw to thaw. With 1 mL serological pipette, dispense the thawing solutions into individual wells of the 4-well plate as follows: 0.5 mL

Table 53.1 Slow-freezing solution and incubation time

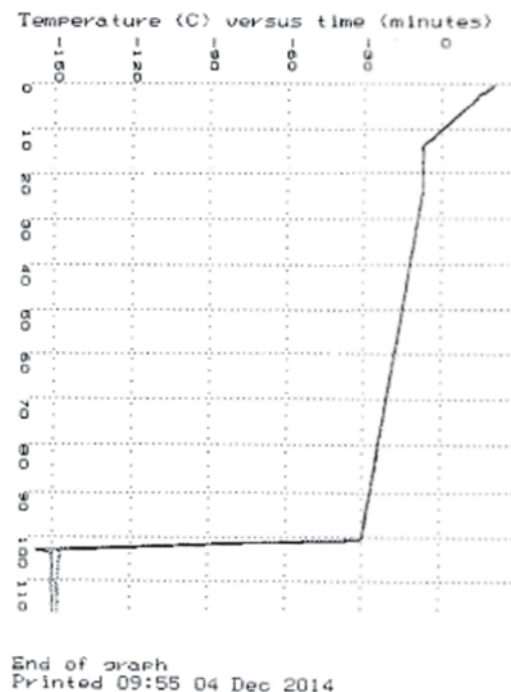
Slow cooling (differential sucrose concentration)		Volume (mL)	Incubation time	Temperature
Solution 1	1.5 M PROH +20% SSS	0.6	10'	RT
Solution 2	1.5 M PROH +0.2 M sucrose +20% SSS	0.6	5'	RT



Fig. 53.3 Schematic representation of oocyte loading in straw and correct position for seeding

Table 53.2 Temperature changes over time (speed) and thermal intervals of slow freezing curve (cryo-freezer printed output is shown below)

Ram p	Speed	Thermal Interval
1	$-2.0^{\circ}\text{C}/\text{min}$	$+20^{\circ}\text{C}$ to -8°C
2	Hold for 10 min (seeding)	-8°C
3	$-0.3^{\circ}\text{C}/\text{min}$	-8°C to -30°C
4	$-50^{\circ}\text{C}/\text{min}$	-30°C to -150°C
5	Hold for 10 min (plunge into LN_2)	-150°C



of thawing solution 1 (1 M PROH/0.3 M sucrose) in Well 1, 0.5 mL of thawing solution 2 (0.5 M PROH/0.3 M sucrose) in Well 2, 0.5 mL of thawing solution 3 (0.3 M sucrose) in Well 3, and finally 0.5 mL of thawing solution 4 (PBS) in Well 4.

- Prepare sterile gauze, sterile scissor, adjust syringe, Petri dish, Flexipet Adjustable Handle Set equipped with 170 μ m denuding pipette, timer, and 30 °C water bath.
- Remove the samples from the liquid nitrogen dewar, and immerse them in the liquid nitrogen reservoir.
- Start taking out the straw from liquid nitrogen, and keep it 30 s in air (room temperature).
- Immediately after this, put the straw for 40 s in a 30 °C water bath.
- Subsequently dry the straw gently with a surgical gauze, and cut the straw at one end with the small sterile scissors and attach it to the adapted syringe.
- Then cut the other extremity, and empty the solution containing oocytes in a Petri dish under the stereo zoom microscope to visualize immediately the oocytes.
- As soon as you see the oocytes, move them into the thawing solution 1 and incubate for 5 min. Start to calculate the time from this moment (Table 53.3).
- After this time, move the oocytes in thawing solution 2 and incubate for 5 min, and then in thawing solution 3 and thawing solution 4 for 10 min, respectively.
- At the end of the thawing procedure, evaluate oocyte's integrity with the inverted microscope (20X). The thawed and surviving oocytes are cultured until the insemination time.

53.1.3 Notes

- We recommend adding 2 mL of serum substitute supplement (SSS) after the freezing and thawing solutions have been filtered to prevent a possible protein retaining.

Table 53.3 Rapid thawing solution and incubation time

Rapid thawing (differential sucrose concentration)		Volume (mL)	Incubation time	Temperature
Solution 1	1.0 M PROH +0.3 M sucrose +20% SSS	0.5	5'	RT
Solution 2	0.5 M PROH +0.3 M sucrose +20% SSS	0.5	5'	RT
Solution 3	0.3 M sucrose +20% SSS	0.5	10'	RT
Solution 4	PBS + 20% SSS	0.5	10'	RT

- Freezing and thawing solutions must be stored at +4 °C, possibly protected from light because of their light sensitivity.

- The preparation date can be used as the freezing and thawing solution batch number.
- An insulin-type syringe without a needle is connected to a narrow silicone tube to connect the syringe to the straw during sample loading.
- The same pipette can be used to dispense the freezing and thawing solutions if you start with the weaker cryoprotectant solution.
- Before thawing, it is advisable to prepare a small container of liquid nitrogen in order to remove the straw from goblet.
- All the steps are carried out by two persons (the operator and a second person who assists the operator during the procedure).
- On the basis of your experience, you can freeze-thaw more than one straw/oocytes at one time, paying attention to calculate and to consider the different incubation time in the freezing-thawing solutions.
- During the oocyte loading into the straw, it is important to aspire the solution until it wets the filter to obtain vacuum.
- Slow-freezing protocol can be divided into two steps. In the first step, permeating cryoprotectants create an osmotic gradient that removes water from the cell by preventing the ice crystal formation. The oocyte dehydrated and shrunk then returns to the original volume; after that, the cryoprotectant enters the cell replacing water. The extent of shrinkage and swelling causes an osmotic stress that is minimized in the second phase of dehydration when non-permeating cryoprotectant is added. In this phase, the shrinkage rate is faster with a higher reduction of cell volume before freezing.
- With the slow-freezing procedure, when moving the oocytes on to the next step, collect them in a small amount of the surrounding solution and release them into the next solution. This makes the passage from one molarity to another less shocking.
- Likewise, during the thawing procedure, between steps, collect the oocytes in a larger amount of the surrounding solution and release them into the next solution, in order to make rehydration more gradual.
- At the end of the thawing procedure, rate all thawed oocytes. The survived oocytes have the same morphology as fresh oocytes, whereas degenerated oocytes appear dark and non-three dimensional.
- The insemination of surviving oocytes takes place no earlier than 1 to maximum 2 h after the end of the thawing procedure, and avoid exceeding a total culture time of 7 h and remember to recover meiotic spindle after thawing.

Human Oocyte Vitrification/Warming Protocol

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Ching-Chien Chang, Daniel B. Shapiro,
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54.1 Materials

54.1.1 Reagents

SAGE Vitrification Kit (ART-8026), CooperSurgical
SAGE Vitrification Warming Kit (ART-8030),
CooperSurgical
Embryo culture media [e.g., SAGE Cleavage Medium
(REF: ART-1026, CooperSurgical) with 15% Serum Protein
Substitute (REF: ART-3010, CooperSurgical)]
ICSI Cumulase (REF: 1612500D, CooperSurgical)
Mineral oil, embryo tested (e.g., Ovoil REF: 10029,
VitalLife)

54.1.2 Equipment

Incubator(s), dry, 37 °C, in an atmosphere of mixed gas
(6.5% CO₂, 5% O₂, 88.5% N₂) (e.g., K-system 225,
CooperSurgical)
Pipettors (P1000, P200)
Stripper pipette (e.g., Research Instrument EZ-GRIP)
Stripper pipette tips (e.g., Research Instrument EZ-TIP
290 µm, EZ-TIP 145 µm)
Stereomicroscope with a transmitted light base
Laminar flow IVF hood with a heating stage (setting tem-
perature at 37 °C)
Timer
Tissue culture dishes, 35 mm, as well as 4-well dishes
(e.g., Nunc)
Cryo-devices (e.g., Cryotop, Kitazato)
Styrofoam box for liquid nitrogen (8 × 10 × 12 inches)
Hemostatic forceps (straight jaw)

54.2 Methods

54.2.1 Preparation of Dishes

1. Prepare the oocyte collection dishes.
 - (a) Place droplets of 80 µL of SAGE cleavage medium with 15% SPS in the 35 mm Petri dish.
 - (b) Overlay the culture media with mineral oil.
 - (c) Prepare the oocyte collection and embryo culture dishes the day before the scheduled oocyte retrieval, and keep them at 37 °C incubator with an atmosphere of mixed gas (6.5% CO₂, 5% O₂, 88.5% N₂) overnight.

54.2.2 Oocyte Retrieval and Handling Before Vitrification

1. Oocyte retrieval is scheduled at 36 h post-trigger.
2. Cumulus cells are stripped at 38 h post-trigger using 145 µL stripper tips.
3. Sort the mature oocytes (showing one polar body) for oocyte vitrification.

54.2.3 Oocyte Vitrification

All the oocyte vitrification procedures are operated at room temperature (22–25 °C) and room atmosphere.

1. Prepare oocyte vitrification dishes: add 1.0 mL equilibration solution (ES) and 1.0 mL vitrification solution (VS) into 4-well dish.
2. Set up a drop of 100 µL of MOPS solution (WS) and 2 drops of ES (100 µL each) on a 4-well dish lid.
3. Merge the WS drop with the first ES drop, and then move the mature oocytes (~39–40 h post-trigger) to the merged drop for 2 min.

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4. Merge the second ES drop with the merged drop with oocytes for another 2 min.
5. Move the oocytes into ES well in a 4-well dish for another 8-min equilibration.
6. Move oocytes into VS well for 30 s (1 or 2 oocytes/time).
7. Start to load the oocytes onto the Cryotop with 145 μ m pipette tip (usually 30–40 s for loading).
3. Grab the handle of the Cryotop, and twist to release it from the cover. Keep the apparatus submerged in liquid nitrogen while removing the cover.
4. **Very quickly** remove the Cryotop from the liquid nitrogen, and plunge the end of the cryo-device into the TS.
5. Gently shake the Cryotop in the TS until the oocytes come off the device. Start the 1-min timer. Keep at 37 °C during this time. When the timer goes off, look at the oocytes to ensure that their membrane boundaries can be clearly seen. If not, gently pipet the oocytes until their membrane boundaries are evident.

54.2.4 Oocyte Warming

All the oocyte warming procedures are operated at room atmosphere.

1. Prepare oocyte warming dishes: add 4.0 mL 1.0 M sucrose solution (TS) into 35 mm dish, and warm up TS for at least 30 min at 37 °C.
2. Pour 1 mL of 0.5 M sucrose solution (DS) into each of the two wells of a 4-well dish. Also pour 1 mL of MOPS solution (WS) into each of the remaining two wells of the 4-well dish. Keep this 4-well dish at room temperature.
6. Move the oocytes from TS to DS with a 290 μ m stripper tip, and keep oocytes in the DS for 3 min at room temperature.
7. Move the oocytes from DS to WS DS with a 290 μ m stripper tip, and keep oocytes in the WS for 10 min at room temperature.
8. Transfer the oocytes to the pre-equilibrated culture dish (SAGE cleavage medium with 15% SPS).
9. Return the culture dish to incubator for 2–3 h before beginning ICSI.

Embryo Slow Freezing Protocol (Zygote/Cleavage/Blastocyst)

55

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55.1 Introduction

The embryo cryopreservation technique plays a crucial role in assisted reproduction techniques (ART) as it allows the preservation and storage of embryos in liquid nitrogen for a prolonged amount of time. The use of gonadotropins for ovarian stimulation has led to the recovery of increased number of oocytes, resulting to surplus embryos suitable for cryopreservation following embryo transfer. The introduction of the slow freezing technique in ART allowed the reduction of the number of embryos transferred in order to minimize the risk of multiple pregnancies. Besides, the cryopreservation technique is necessary in case of embryo transfer cancelling due to various conditions that reduce the chances for implantation.

Slow freezing is based on the dehydration of cells with the use of cryoprotectants and the gradual reduction of the temperature by using a freezing machine. A combination of two different types of cryoprotectants is used for slow freezing: permeable and non-permeable. Permeable cryoprotectants commonly used for slow freezing are glycerol, dimethyl sulfoxide (DMSO), and propylene glycol (PROH). They have low molecular weight, can permeate the cells through the plasmalemma, and their primary role is to equilibrate the cells and cause dehydration before freezing. On the contrary, non-permeable cryoprotectants such as sucrose do not cross the plasmalemma, remain out of the cells, and they serve to further dehydrate the cells prior to freezing. A modification to the protocol, with elevated concentration of sucrose and

an additional thawing step has provided higher cryosurvival of biopsied cleavage stage embryos [1]. Moreover, the use of glycerol as permeable cryoprotectant has been employed for the successful cryopreservation of blastocysts [2].

In slow freezing, the decrease of the temperature requires the use of a programmable rate freezer. Initially, the embryos are exposed to a cooling rate of 2 °C/min to around −6.5 to −7 °C followed by manual seeding. The manual seeding is one of the most important steps of the slow freezing protocol because it serves to initiate the ice crystal formation outside of the cells and at a controlled temperature, thus the intracellular ice crystals are avoided. Subsequently the cooling rate typically drops to −0.3 °C/min until a temperature of −30 °C is reached. During this slow process, the dehydration of the cells is being completed.

In 1998, a new technique for human embryo freezing has been developed, named as vitrification because the embryo remains in a glass-like vitreous state [3]. The main aspects of vitrification are the use of high concentration of cryoprotectants, low volumes, and ultra-rapid freezing and thawing rates. In contrast to slow freezing, the dehydration of the cells that is completed prior to cooling and the fast cooling rates do not allow the formation of intra-cellular ice crystals. Several studies have demonstrated the superiority of the vitrification over slow freezing protocol [4], as it provides higher clinical pregnancy and live delivery rates [5]. Although vitrification is currently the freezing method almost exclusively employed by majority of IVF clinics, slow freezing is still used by some centers who wish to not change their protocols and methods.

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55.2 Zygote and Cleavage Stage Embryo Freezing

55.2.1 Materials and Methods

Here we describe the slow freezing procedure using a widely used commercial freezing/thawing kit by Vitrolife. The formulation contains cryoprotectants according to Edgar et al.

[6]. The basic medium is buffered with MOPS to sustain optimum PH and contains Human Serum Albumin (HSA) with gentamicin as an antibacterial agent. According to the producer, all solutions are aseptically filtered, pH and osmolarity tested, and have passed assays for optimum mouse embryo development (expanded blastocyst within 96 h: $\geq 80\%$) and for the presence of bacterial endotoxins (<0.5 EU/mL). Although recent product descriptions of Vitrolife do not contain detailed information about media composition, it is supposed to be identical with those published from Edgar et al. except from some minor modifications (i.e., macromolecule supplementation).

55.2.2 Freezing and Thawing Solutions

1. ES (Equilibration solution: contains no cryoprotectant)
2. FS (Freezing solution: 1.5 M PROH +0.2 M sucrose)
3. TS1 (Thawing solution 1: 0.5 M sucrose)
4. TS2 (Thawing solution 2: 0.2 M sucrose)
5. ES (Equilibration solution: contains no cryoprotectant)

55.2.3 Equipment

1. Controlled rate freezer (Kryo 360-1.7, Planer) (Fig. 55.1)
2. Manual heat sealer for straws (Syms-Cryo Bio system) (Fig. 55.2)
3. Stereomicroscope (Olympus SZX10 with Tokai heating stage)
4. Inverted microscope (Olympus IX71 with Tokai heating stage)
5. Label printer (Brady BMP LabPal)
6. Laminar air flow workstation (Origio)
7. Timer
8. Cryomarker (Nalgene Cryo Marker)
9. Micropipette (Stripper, Cooper Surgical)
10. Battery powered pipette controller (Macroman, Gilson)
11. Metal forceps
12. Cooling rack for liquid nitrogen (Kitazato/Cryobath-Origio)
13. Liquid nitrogen tanks for long storage (MVE)
14. Special equipment for LN2 handling (Cryogloves, Visors, Oxygenometer)
15. Heating block
16. Laboratory scissors

55.2.4 Consumables

1. 4-well dishes (Nunc, Ref: 14444)
2. Sterile serological pipette (Falcon 1 mL—Ref: 356521 and 5 mL—Ref: 357543)



Fig. 55.1 Programmable freezing machine



Fig. 55.2 Cryo Biosystem sealer (Syms)

3. Insulin syringe 1 mL (BD)
4. 5 mL sterile round bottom tube (Falcon Ref:352003)
5. 15 mL sterile tubes with conical bottom (Falcon Ref:352095)
6. Small sterile surgical gauge
7. 60 mm center well culture dish (Falcon: 353653)
8. Denuding pipettes-175 μ m (V-Denupet, Vitromed)
9. Sterile clear rigid embryo straws (0.25 mL–133 mm) (Cryo Biosystem)
10. Sterile connection tubing for syringe (Cryo Biosystem)
11. Labels for Brady printer
12. Goblets for straws (13 mm diameter and 120 mm length)
13. Aluminum canes
14. Cane coder tabs

55.2.5 Slow Freezing Procedure

1. Aliquot 1 mL of each freezing solution ES and FS into 5 mL tubes and let it equilibrate for 1 h to room temperature. Close the caps of the tubes to avoid changes in solution osmolarity. Return the kit into the fridge and minimize the time you expose it to room temperature.
2. Switch off stereoscope's heating stage.
3. Prepare the straws with the appropriate labels (patients name, unique attempts code, date, number of embryos, number of straw).
4. Prepare a 4-well dish. Label the dish with the name of the patient and mark well #1 with ES and well #2 with FS.
5. Place the coder and the goblet into the cane. Write the unique code onto the coder and patient's name, ID, date of freezing, unique code on the goblet.
6. Prepare the micropipette and the syringe with the connection tube.
7. Switch on the freezer and the heat sealer.
8. Transfer the solutions from the 5 mL tubes into the marked wells.
9. Assess the embryo quality and note which embryo(s) are represented to each straw.
10. Transfer the embryos with the micropipette into the equilibration solution with minimum amount of culture medium and rinse several times. According to producer's instructions, the embryo(s) can stay in ES for maximum 10 min.
11. Transfer the embryos into the FS medium with minimum amount of ES and wait for maximum 10 min.
12. Load the straws using the 1 mL syringe with its connector according to the three-drop method. First aspirate small amount of FS, air, FS including embryos, air, and finally small amount of FS (Fig. 55.3). Avoid the formation of small bubbles into the drops.
13. Seal the tip of the straw using the heat sealer. Put the open edge of the straw into sealers aperture and push the button to start a welding cycle. A welding cycle lasts 4 s. Firmly, remove the straw from machine's apparatus and check the sealing.
14. Place the straw on the holder of the freezer. If several straws need to be placed on the holder try to align them to make the seeding easier.
15. Place the straw holder into the freezing machine and start the program.

55.2.6 Freezing Program

1. The embryos are slowly cooled from room temperature to -6.0°C with a cooling rate $2.0^{\circ}\text{C}/\text{min}$.
2. Hold at -6.0°C for 10 min to allow equilibration of the samples. To perform the seeding, immerse the tip of the

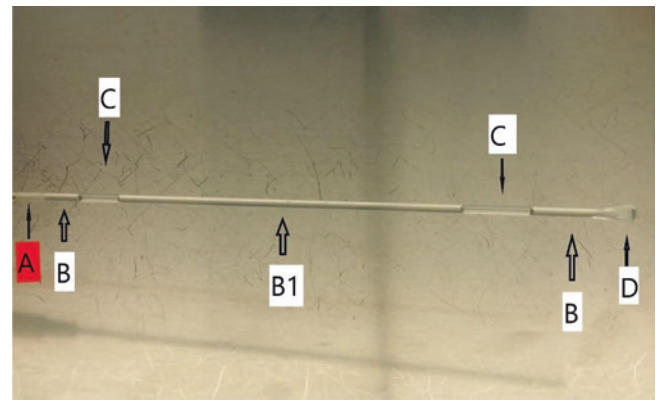


Fig. 55.3 A: Plug, B: Drops with FS solution, C: Air, B1: Drop containing the embryos, D: Seal



Fig. 55.4 Seeding procedure

metal forceps in liquid nitrogen for 2–3 min. Without fully removing the holder from the freezer, carefully touch the straw with the cold forceps to induce ice formation. The seeding should be performed away from the embryos and close to the plug (Fig. 55.4).

3. Slow cool at $-0.3^{\circ}\text{C}/\text{min}$ to -30°C .
4. Cool at $50^{\circ}\text{C}/\text{min}$ to -150°C and equilibrate for 10 min.
5. When the program has ended the machine alarms. Fill the cooling rack with LN_2 , remove the holder with the straws and immediately submerge it in LN_2 .
6. Place the ready to use cane into the cooling rack, carefully remove the straws from the holder, and carefully deposit them into the goblet.

All procedures should be performed under liquid nitrogen. To secure the straws, a second goblet can be inversely adapted onto the cane. Place the cane with the straws into the LN_2 tanks at pre-defined canister and record the tank and canister number for future tracking. Switch off the freezing machine.

55.2.7 Thawing Procedure

1. Fill the 15 mL conical bottom tube with HPLC grade water and warm at 32 °C.
2. Aliquot 1 mL of each thawing solution TS1, TS2 and ES into 5 mL tubes and equilibrate for 1 h to room temperature. Cap the tubes to avoid changes in solution osmolarity. Return the kit into the fridge and minimize the time you expose it to room temperature.
3. Prepare the center well dish and mark it with patient's name and unique code.
4. Prepare a 4-well dish. Write patients name, unique code and mark well #1 with TS1 and well #2 with TS2 and #3 with ES.
5. Prepare the equipment for embryo release and handling (sterile scissors, gauge, micropipette, syringe with its connector).
6. Dispense each solution to the matching well.
7. Put the prewarmed 15 mL tube into the heating block to maintain the temperature and remove the cap.
8. Remove the straw from the goblet and expose it to room temperature (air) for 30 s.
9. Place the straw in the 32 °C water for 45 s.
10. Remove the straw and wipe it with the sterile gauge to remove extra water from the outer wall.
11. Smoothly, cut the straw at the first air drop (between the last and the drop containing the embryos at the sealed edge of the straw), carefully place the tip of the straw into the center well and cut the straw at the second air drop (between the plug and the drop containing the embryos). The embryos are expelled into the center well dish. In case there is any retained solution into the straw, expel it with the syringe.
12. Identify the embryos as soon as possible and expose them to TS1 solution for 5 min.
13. Transfer the embryo to TS2 solution and expose for 5 min and then to ES for 5 min.
14. Finally transfer the embryos to a pre-equilibrated culture medium and rinse several times. Culture for at least 2 h before embryo transfer.

55.3 Blastocyst Slow Freezing

Today blastocyst slow freezing has been almost fully replaced by vitrification which offers >95% survival rates. When, for the first time, the extended culture to the blastocyst stage was achieved due to the culture conditions improvement, the classic PROH protocol could be efficiently used. Some commercial kits use glycerol and sucrose into their freezing/thawing solutions according to Menezo's protocol [7]. The commercial kit of Irvine is based on Menezo's protocol with some modification.

55.3.1 Freezing and Thawing Solutions

Basal medium and protein (Modified HTF-containing 21 mM HEPES buffer, supplemented with 12 mg/mL HSA and 10 µg/mL gentamicin sulfate) with

1. 5% glycerol (freezing solution 1)
2. 9% glycerol and 0.2 M sucrose (freezing solution 2)
3. 0.5 M sucrose (thawing solution 1)
4. 0.2 M sucrose (thawing solution 2).

The embryos should be exposed for 10 min at each solution (F1 and F2) during freezing and 10 min at each solution (T1 and T2) during thawing. The procedure of blastocyst freezing/thawing is similar with that of zygote/cleavage stage embryo.

55.3.2 Freezing Program

1. The embryos are slowly cooled from room temperature to −6.0 °C with a cooling rate 2.0 °C/min.
2. Hold at −6.0 °C for 10 min and perform the seeding.
3. Slow cool at −0.3 °C/min to −37 °C.
4. Hold 10 min and immerse directly to liquid nitrogen.

55.3.3 Additional Requirements for Blastocyst Freezing and Thawing

The method of artificial shrinkage of an expanded blastocyst before freezing has been demonstrated to be beneficial for the survival of the blastocyst as it reduces the total post-thaw degeneration [8]. Artificial shrinkage of the blastocyst is achieved by applying one to two pulses (2.0 ms) using the 1.48-µm diode laser (Octax). The laser pulses are applied at the junction between two trophectoderm cells, opposite to the ICM. After the collapse of the blastocysts, the freezing procedure is started.

The blastocysts require culture for 2–3 h post thawing to fully recover. During this time, the collapsed blastocysts regain their shape and re-expand. Post-warming blastocyst re-expansion and degeneration score are noted, as it correlates with pregnancy rates [9].

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Stepwise Vitrification and Warming Protocol (Standard Operation Procedure) of Embryo

56

Juergen Liebermann

56.1 Materials

1. HSV (High Security Vitrification Kit, Catalog # 022137; CryoBioSystem).
2. Heat sealer (CryoBioSystem).
3. Polycarbonate micropipettes, 170 and 140 μm end hole (MidAtlantic Diagnostics# KFPIP-1170-10BS).
4. Brady labeler (BMP 61).
5. Brady labels (PTL-19-427).
6. 90 $\times$ 15 mm Petri dish (Nunc # 150362).
7. Styrofoam container.
8. Visotubes 10 mm (IMV 5561).
9. Cryo-canes aluminum (ThermoScientific 5015-0001).
10. Cooper wire cutter (GB; GS-360; 0.80–2.6 mm).

56.2 Reagents

1. Sage Protein Supplement (SPS) (Sage).
2. Modified Human Tubal Fluid (HTF-HEPES) from Sage.
3. Vit-Kit-Freeze (Irvine Scientific #90133DSOC).
4. Vit-Kit-Thaw (Irvine Scientific # 90137DSOC).

56.3 Equipment

1. Dissecting stereomicroscope (Olympus SZX-12, Bausch Lomb or Leica) with warming stage.
2. Laminar flow hood (Origio).
3. Inverted microscope (Olympus IX-71).
4. Infrared 1.48 μm diode laser (Hamilton Thorne-Zilos or Lykos laser (Hamilton Thorne Research, Beverly, MA).

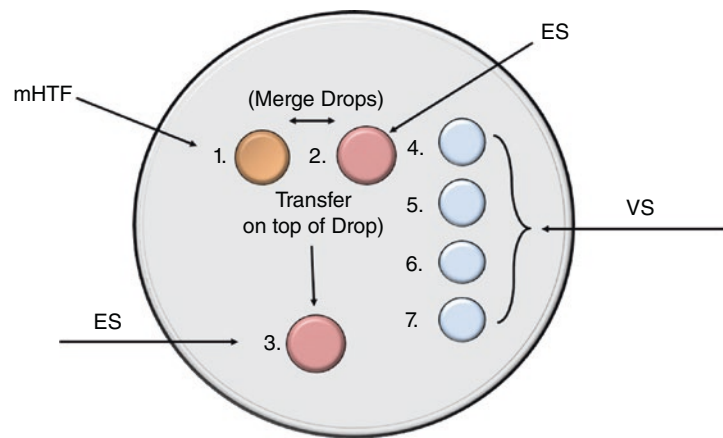
56.4 Stepwise Blastocyst Vitrification Procedure

Vitrification of blastocysts needs to be undertaken utilizing a “closed system” (HSV: High Security Vitrification Kit; CryoBioSystem, L’Aigle, France; FDA 510(k) clearance for cleavage-stage embryos in blastocysts) after a two-step loading with cryoprotectant agents at 24 °C. If assisted collapsing is done prior to vitrification, then the blastocyst needs to be put on an inverted microscope equipped with a laser system (ZILOS-tk, or Lykos, Hamilton Thorne); the junction of two trophectoderm cells in each blastocyst needs to be located, and one shot (100% power, 500 μs pulse length) was applied. Then the blastocysts can be moved back in the incubators for 5–10 min. Briefly, blastocysts must be placed in equilibration solution, which is the base medium [M199 with 20% serum supplement substitution (SSS) containing 7.5% (v/v) ethylene glycol (EG) and 7.5% (v/v) dimethyl sulfoxide (DMSO)]. After 8 min, the blastocysts need to be washed quickly in vitrification solution, which is the base medium containing 15% (v/v) DMSO, 15% (v/v) EG, and 0.5 M sucrose, for 60 s and transferred onto the HSV using a micropipette. Immediately after the loading of one or not more than two blastocysts in a 1 μL drop on the HSV, the straws can be heat sealed, then plunged in LN_2 , and secondarily stored inside 5 mL liquid nitrogen-prefilled canes (Visotube Rond, IMV, France). Each single step is described in detail below. The timeline for vitrifying blastocysts requires a total of 9 min (Fig. 56.1):

1. Aseptic techniques are required at all stages. For equilibration and vitrification procedures, ensure that the benchwarmer is at room temperature (~ 25 °C).
2. Take reagents from the refrigerator, and allow them to warm to room temperature.
3. Separate the blastocysts to freeze into a separate well. Bring this dish to the inverted microscope, and with the embryo positioned with the laser objective, use a single pulse to hit the blastocysts between two trophectoderm

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Fig. 56.1 Setup for the vitrification procedure on a plain 100 mm Petri dish lid surface. *ES* equilibration solution, *VS* vitrification solution, *WS* washing solution



cells to collapse the embryo. Place the dish back into the incubator for 5 min.

4. Label a petri dish with the patient's name under the lid as follows: HTF-HEPES, ES, and VS. Prepare 1 × 50 µL of HTF-HEPES, 2 × 50 µL of ES, and 4 × 50 µL of VS (*see* Fig. 56.1).
5. Brady label should include the patient's last name, first name, accession #, MPI#, date plus number, and type of embryos.
6. Before vitrification, use a Stripper tip with 200 µm end hole for loading the blastocysts on the top.
7. Fill Styrofoam container with LN₂.
8. Each sample that is vitrified will be done in a separate hood and verified by a second embryologist before proceeding. Vitrify good expanded/hatching blastocysts on day 5/6/7.
9. Remove embryos from culture dishes using a stripper tip into the HTF-HEPES (drop 1), gently aspirating to remove any traces of culture media.
10. Pipette from mHTF or WS (drop 1) to the other drop of mHTF or WS (drop 2), and immediately merge it with the first drop of ES (drop 3). Set the timer for 5 min.
11. When the time is up, transfer the embryos to the remaining drop of ES (drop 4). Set the timer for 3 min. Place the embryos on the top of the drop, and let them settle to the bottom.
12. Then load the blastocysts in a VS back-loaded stripper tip, and rinse through the 4 droplets of VS (drop 5–8); between each droplet, clean your tip.
13. Placement into the VS and loading of the cryotop should take less than 1 min, so that the total incubation time in 15% VS is 30 s. After 30 s, gently transfer them to the tip of the HSV by using a stripper tip to load the blastocysts in as small volume (less than 0.5 µL) as possible onto the edge of the stick.
14. Visually confirm placement.
15. Before loading, apply the label to the open end of the empty straw. Load the HSV stick into the empty straw,

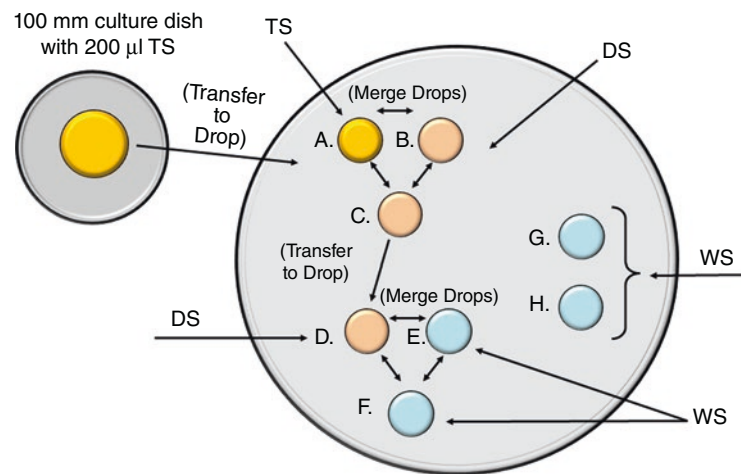
the side with the embryos first. Use the blue handle to make sure that the stick is in as far as it goes. Then, using the heat sealer, seal the open end of the stick, and plunge the whole straw into the LN₂. Place the straw in a pre-cooled aluminum cane for further storage.

16. Store at the cane in a nitrogen tank.
17. Make sure to record cane location on the freezing worksheet and cryo-inventory log.
18. Complete all paperwork, and recheck that all vial locations are logged into the Embryo Inventory.

56.5 Stepwise Blastocyst Warming Procedure

Important to be mentioned is that regardless of the day of cryopreservation of the embryo (whether day 5, 6, or 7), at thawing, blastocysts should be treated as if they had been frozen on the fifth day of development. To remove the cryoprotectants, blastocysts need to be warmed and diluted in a three-step process. With the HSV submerged in LN₂, the inner straw should be removed, and then the carrier with the blastocysts can be removed from the LN₂ and placed directly into a pre-warmed (37 °C) 200 µL drop of 1.0 M sucrose on a 90 mm dish lid surface. Blastocysts can be picked up directly from the HSV, placed in a fresh 50 µL drop of 1.0 M sucrose at 24 °C, and immediately connected with a 50 µL drop of 0.5 M sucrose. After 3 min, connect with another 50 µL drop of 0.5 M sucrose for 2 min. Then blastocysts can be transferred to a 50 µL drop of 0.5 M sucrose solution and connected with a 50 µL drop of wash solution for 3 min. Then connect to another 50 µL drop of wash solution for additional 2 min. Thereafter, transfer the blastocysts through the 2 × 50 µL of wash solution, and return into the transfer dish containing the culture medium (SAGE Blastocyst Medium, Trumbull, CT, USA) until transfer. Each single step

Fig. 56.2 Setup for the warming procedure on a plain 100 mm Petri dish bottom surface. *TS* thawing solution, *DS* diluent solution, *WS* washing solution



is described in detail below. The timeline for warming blastocysts requires a total of 10–11 min (Fig. 56.2).

1. Take reagents from the refrigerator, and allow them to warm to room temperature. All cryoprotectants are removed at 25 °C.
2. Place a 200 µL drop of TS on a dish lid surface, and place on a warming plate.
3. Label a petri dish (Nunc) with the patient's name under the lid as follows: TS, DS, and WS. Prepare 1 × 50 µL of TS, 3 × 50 µL of DS, and 4 × 50 µL of WS (see Fig. 56.2).
4. Before warming, use a stripper tip with 200 µm end hole for removing the blastocysts from the carrier.
5. Fill Styrofoam container with LN₂.
6. Confirm location and identification with a second embryologist before warming any HSV kit. Warm one kit at a time.
7. Each sample that is warmed will be done in a separate hood and verified by a second embryologist before proceeding.
8. With the HSV kit under LN₂, open the kit by cutting the outer straw using a wire cutter. Use the blue handle to remove the inner stick if needed.
9. Submerge HSV kit directly in the pre-warmed drop containing TS, which should be as close as possible to the LN₂ Styrofoam container. As soon as the HSV kit contents liquefy (within 1 min), try to locate the blastocysts before removing them with a stripper tip. After locating all the blastocysts, remove them from the tip, place them in the droplet of TS (drop A), and connect immediately with the first droplet of DS (drop B) for 3 min.
10. Then connect with the second droplet of DS (drop C) for another 2 min.
11. Then transfer the blastocysts to a fresh drop of 0.5 M sucrose (drop D), and connect immediately with the first drop of WS (drop E) for 3 min. Then connect with the second drop of WS (drop F) for another 2 min.
12. Finally dilute and rinse through a series of two wash drops of WS (G to H).
13. Place the blastocysts into a transfer culture dish, and put it in the incubator for subsequent culture.
14. Record the survival and appearance of all blastocysts. Update log with warm data, and notify the physician of result.

Human Ovarian Tissue Slow Freezing

57

Candice Kluyskens, Luciana Cacciottola,
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The protocol describes how to prepare ovarian cortical strips for cryopreservation using a slow-freezing protocol. **57.1 Devices**

Denomination	Brand/model
Planer® freezer	Planer Kryo 360-3.3 (Cryo Solutions) ^a
Dewar thermos	
20 cm Stainless steel tweezers	
Fridge/freezer	Liebherr Premium
Laminar flow hood	ESCO LA2 5A1
Cold plate	Buffet-7 ECKIG
High-security welder	SYMS III-CBS (IMV Technologies)
Label printer	Brady BMP51
Pipetus®	Hirschmann-Laborgeräte 612-1874
Fridge box	

^a Or cryochamber (model CC5S) by CryoLogic

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57.2 Reagents and Consumables

Denomination	Brand/model/supplier
Human albumin 20% for perfusion	CAF-DCF
Culture dishes, 90 × 17 mm	Nunc (150360) by NordicCell
Culture dishes with central well, 60 × 15 mm	Corning 353653 by VWR
Colored capsules for cryotubes	CBS (024108 to 13) by Fertipro
Joseph's scissors, right ($L = 15$ cm)	AES BC156
Sterile high-security cryotubes	CBS (023722) by Fertipro
Sterile glass blades	
Sterile needle 1.1 × 40 mm (yellow)	BD (301500)
CryoSure-DMSO	Wak-Chemie Medical GmbH Cryoprotectant
Leibovitz 15 (L15) medium with GlutaMAX	Gibco (31415-029) medium with amino acids, vitamins, inorganic salts, and phenol red (without antibiotics) by ThermoFisher scientific
Graph paper	
Waugh dissecting forceps (tweezers) 1 × 2 claws, 184 mm	AES BD670
Fine tweezers, straight without teeth, $L = 10$ cm	AES OC020
Sterile pipettes 10 mL	607180 Cellstar Greiner Bio-one
Sterile disposable scalpel n°24	Swann-Morton (0511)
Syringe 1 mL	BD (300013)
Syringe 2 mL	BD (307727)
Test tube cooler for freezing at -20°C	Nalgene (471-0001) by VWR
50 mL conical bottom tubes	16105 Vitrolife
Universal IVF Medium	100300 10A by CooperSurgical

57.3 Protocol for Ovarian Tissue Slow Freezing

57.3.1 Preparation

In the freezing room (Fig. 57.1):

- Switch on the Planer freezer to initiate cooling of the freezing compartment by liquid nitrogen (LN2) injection.
- Check LN2 levels if applicable, and ensure that the Planer freezer is well connected to the nitrogen circuit.
- Fill a Dewar thermos with LN2, and immerse 20 cm stainless steel tweezers inside to prechill them for later use (see Sect. 57.3.3).

In the laboratory for assisted reproductive medicine:

- Cool a fridge box with precooled freeze packs (optimal temperature $2-8^{\circ}\text{C}$).
- Prepare two conical tubes each with 15 mL Universal IVF Medium under a laminar flow hood (in sterile conditions) (for right and left ovarian biopsies).
- Place the tubes in the precooled fridge box.
- Transport the fridge box to the operating room.

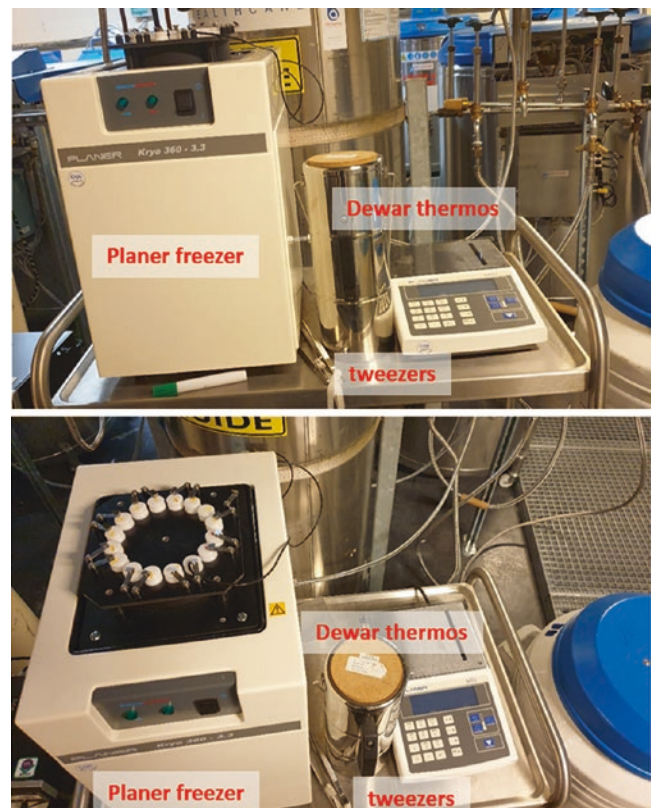


Fig. 57.1 Preparation in the freezing room

In the biobank clean room under a laminar flow hood:

- Prepare 25 mL freezing medium in a conical tube as follows: 22 mL L15 with GlutaMAX (using 10 mL sterile pipettes) + 2.5 mL DMSO 1.4 M (aspirating with a needle) + 0.5 mL human serum albumin (aspirating with a needle).
- Prepare two rinsing tubes each with 15 mL L15 medium with GlutaMAX.
- Prepare the cold plate for ovarian tissue manipulation with (see Fig. 15.2 from Chap. 15):
 1. A piece of graph paper.
 2. A large culture dish with sterile glass inside.
 3. Two central well dishes (one for L15 and one for freezing medium)
- Prepare the materials for cortical strip cryopreservation:
 1. The test tube cooler.
 2. Cryotubes with their colored capsules.
 3. The high-security welder.
- Cut the cortex with a scalpel into fragments measuring between 3–4 and 5–8 mm.
- Fill the first central well dish with a few mL L15 medium with GlutaMAX to rinse the ovarian fragments and 80% of the medulla.
- Label the cryotubes according to the number of fragments (between 1 and 3 fragments per cryotube), and place a colored and numbered capsule on the bottom of each.
- Fill the cryotubes with 800 μ L freezing medium (for the medulla, 80% is frozen in an additional cryotube, while the remaining 20% is fixed in formol together with a small fragment of cortex and sent to the anatomopathology department for histological analyses).
- Fill the second central well dish with freezing medium to rinse the ovarian fragments.
- Place the fragments inside the cryotubes with tweezers (184 mm or 10 cm).
- Weld the cryotubes with the high-security welder.
- Store the test tube cooler with the cryotubes in the fridge box.

After surgical removal of ovarian biopsies and transferal to the conical tubes, transport the fridge box to the clean room.

57.3.2 Manipulation (in the Biobank Clean Room Under a Laminar Flow Hood)

- Rinse the ovarian tissue inside the tubes prefilled with L15 medium with GlutaMAX.
- Transfer to the large culture dish (prefilled with a few mL L15 medium with GlutaMAX).
- Measure the biopsy by placing the graph paper beneath the culture dish.
- Gently dissect the medulla from the cortex using fine 10 cm straight tweezers without teeth and/or Joseph's scissors.
- Place the cryotubes in the holders of the Planer freezer (make sure that the meniscus of the medium is visible).
- Proceed with manual seeding when the temperature drops to -7°C (around 30 min after the freezing program starts); use 20 cm stainless-steel tweezers precooled in LN2 to pinch the cryotube at the meniscus of the medium (medium/air interface) until an ice nucleus appears inside the medium (see Fig. 15.3 from Chap. 15).
- At the end of the freezing procedure (around 2 h later), place the frozen cryotubes in a Nalgene box (Fig. 57.2a) in the storage container bank filled with LN2 (Fig. 57.2b).
- Check the curve printed from the Planer freezer (see Fig. 15.4 from Chap. 15).

57.3.3 Slow Freezing

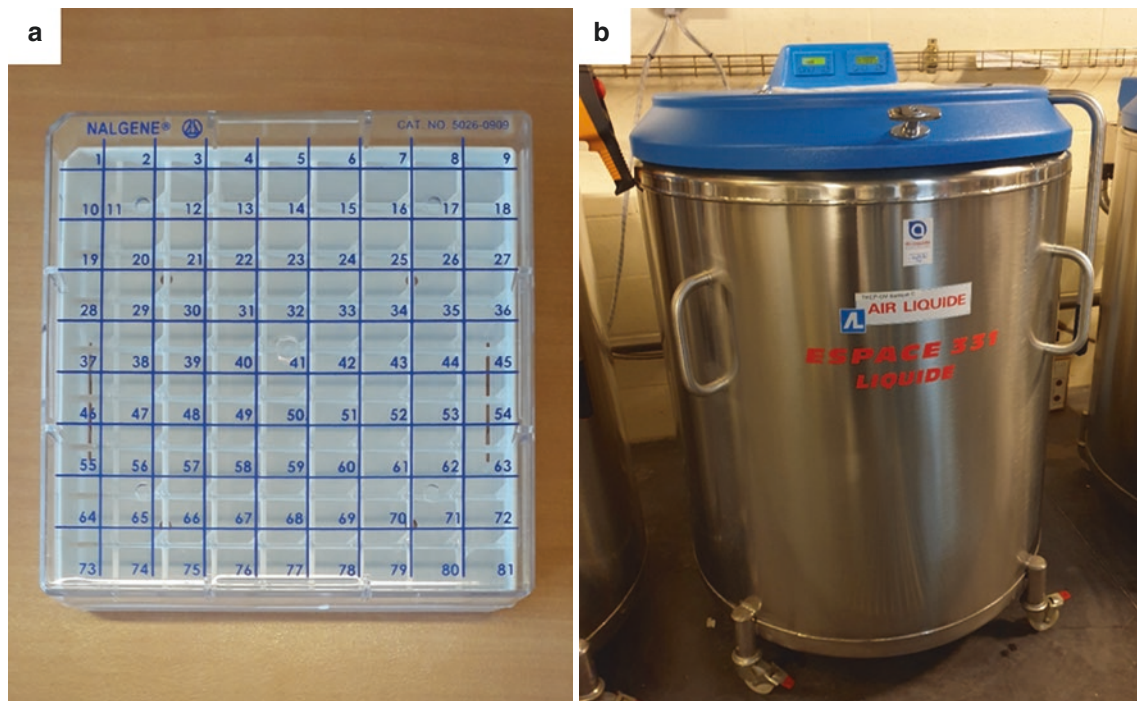


Fig. 57.2 (a) Nalgene box and (b) storage container bank filled with liquid nitrogen

Proper Maintenance of Dewars/ Cryostorage Tanks

58

Martine Nijs

Embryology and andrology laboratories provide storage of often irreplaceable reproductive tissues such as oocytes, embryos, sperm, and ovarian and testicular tissues, including tissues from oncology patients. In general, reproductive specimens are stored in the liquid phase of nitrogen during handling and transport to and from the cryostorage room (short-term storage). Long-term storage is established in the liquid or vapor phase of nitrogen [1]. Reproductive specimens are to be stored under stringent conditions, and a quality management system must be in place for cryogenic storage. Multiple standards and guidelines from prominent scientific societies like the ASRM committee's opinion on cryostorage of reproductive tissues are an excellent guide for developing clinic-specific management policies and procedures ensuring safe and reliable cryostorage [2]. The ISO 21973:2020 [3] standard describes the general requirements of cells for therapeutic use, including cryostorage during transportation.

In a nutshell, to prevent cross-contamination of cryopreserved specimens during cryostorage, good clinical and laboratory practice (GLP) consists in testing patients and donors for infectious diseases, using quarantine storage vessels for virus-positive patients, and using temporary quarantine for all samples until the test results are known. GLP also consists of the prevention of infections by regular cleaning of cryostorage short- and long-term Dewars and tanks as well as all devices and tools used during the cryopreservation and warming/thawing processes.

58.1 Cleaning and Performance Qualification for New Dewars and Storage Tanks

In general, a cryopreservation vessel is a double-walled, vacuum-insulated vessel made of aluminum with a fiberglass composite neck. This vessel should be used for liquid nitrogen only. The vessel should always be orientated in the upright position, to avoid damage to the vessel. If a vessel experiences a drop, hit, or blow, it can suffer immediate or premature vacuum failure that will result in a temperature increase. It is imperative that handling, maintenance, and cleaning instructions provided by the supplier should be strictly followed over time. The life expectancy of most liquid nitrogen Dewars is ten (10) years [1].

A new vessel and its packaging should be examined upon receipt for any evidence of damage (like dents and/or damage to the cork or neck). It is then cleaned with the standard cleaning procedure described below. The empty vessel is weighed, before being slowly filled with LN₂, closed, and weighed again. Excessive frost or sweating on the outside of the vessel during the first few hours indicates either a leak or loss or no vacuum. The vessel is weighed again after 2 h. If the weight is different, the vessel could be leaking or the vacuum wall damaged. This weighing check should be carried out as a regular maintenance procedure during the year. The performance qualification should also include a check of the evaporation rate. In this test, the weight of a full vessel is measured over multiple days to define the normal evaporation rate and compared to the normal evaporation rate provided by the supplier.

Yearly maintenance should include a check of this evaporation rate.

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58.2 Cleaning of Short-Term Dewars and Long-Term Large Storage Tanks

The Dewars are designed according to standard requirements for safety, durability, and performance. It is imperative that maintenance and cleaning instructions provided by the supplier be strictly followed over time. Mishandling of these basic instructions may lead to indirect damage to the cryopreserved specimen. In addition, a periodical cleaning procedure with solutions that do not react with aluminum or stainless steel is required for long-term maintenance of the container.

Before the cleaning, cryopreserved samples need to be transferred in a safe and temperature-controlled way to a similar cryostorage vessel. All transfers need to be witnessed, to guarantee the chain of custody and to avoid critical errors such as warming of samples or mistakes in positioning on the new tank.

The vessel is then moved outside the laboratory in order not to disturb the air with VOCs during the cleaning procedure. The vessel is emptied in an aired environment in combination with monitoring of oxygen levels in the environment (nitrogen can asphyxiate and/or cause burn injuries). Protective personnel equipment must be worn throughout this process. If deposits are present at the bottom of the tank, they should be identified and traced since these could be broken or lost cryostorage devices [4]. The vessel is then left to dry upside down.

A typical cleaning procedure of a small emptied stainless steel storage vessel includes cleaning the vessel inside and outside with sterile water followed by spraying the cleaning solution or fully filling the vessel with the cleaning solution. The cleaning solution is agitated in the vessel to ensure coverage of all surfaces. The vessel is then emptied, placed upside down, and left to dry completely before putting into service again.

Any household detergent or mild soap solution is suitable. Other cleaners and disinfectants that can be used safely include hydrogen peroxide, chlorine/water mixture, and denatured alcohol. The generally accepted practice of using 10% chlorine bleach with 90% water solution holds as the best method for decontamination. It is important that all sur-

faces being sanitized are thoroughly rinsed and that all cleaner solution residues are removed after cleaning.

The cleaning of large vapor phase or liquid nitrogen tanks is more complex. Specialized companies offer cleaning/decontamination services for large tanks, including the delivery of a temporary replacement tank for the storage of biological specimens. Always allow the units to dry completely before putting them into service again.

Bielanski et al. [5] identified bacterial and fungal spores in samples from sediments found at the bottom of the tank and in ice crystals formed on the lids of the tanks. It is therefore imperative that ice crystals and condensations are removed from the lids after each opening of the Dewars or tanks with a sterile cloth, to avoid dropping of the potentially contaminated crystals in the tanks.

The outside of all types of storage tanks should be cleaned with sterile water on a regular basis.

58.3 Dry Shippers

Dry shippers are used for shipping cryopreserved samples from one location to another. They contain adsorption material that is cooled with liquid nitrogen which is removed before loading and shipping. Multiple steps are involved in the shipping of cryopreserved samples, as described by Alikani and Parmegiani [6] in Table 58.1. At each step, a risk of contamination is present, and microbiomes can be transmitted to the samples.

Dry shippers can be cleaned in a similar way, using the cleaning products as advised by the supplier. The study on the effectiveness of biocides used for cleaning/disinfecting dry shippers by Bielanski and Vajta [7] identified sodium hypochlorite and peracetic acid as effective. Drying of the adsorption material will take a considerably longer time compared to a stainless steel Dewar. Replacing the adsorption material in the dry shipper on a regular basis could be an option. The life expectancy of standard dry shippers is five (5) years. A yearly evaporation rate test should be performed (as described above). Dry shippers should be cleaned on the outside before and after a shipping cycle.

Table 58.1 Steps involved in the shipment of cryopreserved samples from one location to another (from Alikani and Parmegiani [6])

At the center performing the vitrification procedure and shipping	At the center receiving the shipment and performing warming procedure
Cryodevices holding oocyte/embryos are plunged in liquid nitrogen bath	Dry shipper is opened and refilled with liquid nitrogen (method of refilling may vary and some or all methods may pose a risk!)
Cryodevices are moved from liquid nitrogen bath to a storage Dewar	Cryodevices are moved from dry shipper to liquid nitrogen bath and samples are identified manually
Cryodevices are moved from storage Dewar to dry shipper	Cryodevices are moved from liquid nitrogen bath to Dewar for storage before warming
Dry shipper is transported by plane as hold baggage or by an operator as cabin baggage or road courier (possible only in the case of short distances)	Cryodevices are moved from storage Dewar to liquid nitrogen bath for warming

The use of disposable dry shippers is becoming more and more popular, it should be noted that these have a holding temperature of -150°C and a shorter holding time as compared to dry shippers (5 days versus a minimum of 8 days).

58.4 Devices Used During Cryopreservation Processes

The temporary storage tanks (like small stainless steel containers or polystyrene boxes) used for transporting straws or devices between the long-term storage tanks or used during vitrification and warming at the IVF lab bench should be cleaned on a regular basis. This could be a daily practice or even done between different patients if deemed that a risk for transmission of infectious contamination is present. This cleaning is done after emptying the small vessel in a safe way, and by rinsing or spraying the empty small storage device with a cleaning solution, followed by rinsing with sterile water, and letting it dry upside down.

58.5 Materials and Equipment Used During the Cryopreservation Procedures

Materials and equipment used during the cryopreservation procedures and storage/removal of specimens need to be cleaned on a regular basis: these include forceps, tongs, canisters, canes, heat-sealing devices, and slow freezing equipment. Items used for personal protection like gloves, aprons, and visors are to be cleaned on a regular basis. Table 58.2 gives an overview of GLP measures for cleaning.

Table 58.2 GLP for cleaning of materials and equipment used during the cryopreservation process

	GLP cleaning
Surfaces	Standard cleaning products IVF-Andrology laboratory
Gloves, apron, measuring stick, cool reach	Standard cleaning products IVF-Andrology laboratory
Forceps, tongs	Heat or gas sterilization
Canisters—goblets—canes	70% ethanol OUTSIDE the laboratory/ storage room Rinse with sterile water
Temporary storage tanks	70% ethanol OUTSIDE the laboratory/ storage room Rinse with sterile water
Slow freezing device	Follow suppliers' guidelines
Heat-sealing device	Wipe with disinfectant before and after use

Cleaning procedures should be clearly described in standard operating procedures, these should be periodically reviewed/revised with new information. All cleaning and maintenance interventions should be logged. It is strongly suggested to establish a training course for all staff members on liquid nitrogen handling and cryostorage compliance including a periodical test for staff competency to simulate a critical catastrophic event in the cryostorage room.

If deemed necessary, swabs can be taken for microbial testing of the cryostorage Dewars and tanks.

58.6 Summary

Minimizing the risk of cross-contamination during cryopreservation and cryostorage is mandatory. The laboratory manager should develop specific protocols for regular cleaning of equipment, dry shippers, and short- and long-term storage Dewars and tanks.

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Aseptic Capillary Vitrification of Human Spermatozoa

59

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59.1 Materials and Methods

Except where otherwise stated, obtain all chemicals from Sigma (Sigma Chemical Co., St. Louis, MO, USA).

59.1.1 Spermatozoa Preparation

1. After informed consent, obtain ejaculates from patients with normospermatic samples (*see Note 1*).
2. Spermatozoa were collected by swim-up technique (*see Notes 2 and 3*).
3. Prepare solution 0.5 M of sucrose with bi-distillate water (*see Note 4*).
4. Before vitrification by direct plunging into liquid nitrogen, dilute spermatozoa 1:1 with 0.5 M sucrose at room temperature (*see Note 5*).
5. Ten μL spermatozoa suspension was filled in the capillary by aspiration (Fig. 59.1a) (*see Notes 6 and 7*).
6. After aspiration, the capillary was inserted into a 0.25 mL straw (Medical Technology GmbH, Bruckberg, Germany).
7. Seal the end of the straw after inserting the capillary (Fig. 59.1b). One end of the 0.25 mL straw was sealed in advance using a heat sealer (CryoBioSystem, Paris, France).

59.1.2 Spermatozoa Vitrification and Warming

1. Carry out vitrification by plunging the straw into liquid nitrogen as illustrated in Fig. 59.1c.
2. Before warming, fix tightly the end of the capillary, while the part of the straw containing spermatozoa is still submerging in liquid nitrogen as illustrated in Fig. 59.1d (*see Note 8*).
3. Disinfect the straw with 70% ethanol in the area of the marked end of the capillary.
4. The upper part of the 0.25 mL straw was cut off over the capillary's labeled end (Fig. 59.1d) with sterile scissors, as close as possible to the marked end of the capillary, just above the marked position, without touching the marked end of one.
5. Take the capillary with a conical bolt or forceps (Fig. 59.1d) from the isolating 0.25 mL straw.
6. The capillary was immediately immersed into an Eppendorf tube with the prewarmed basic medium for approximately 20 s (Fig. 59.1e) (*see Note 9*).
7. After warming, the capillary was fixed with an IVF pipette controller, and 10 μL of spermatozoa suspension was expelled from the capillary for the evaluation of spermatozoa quality.

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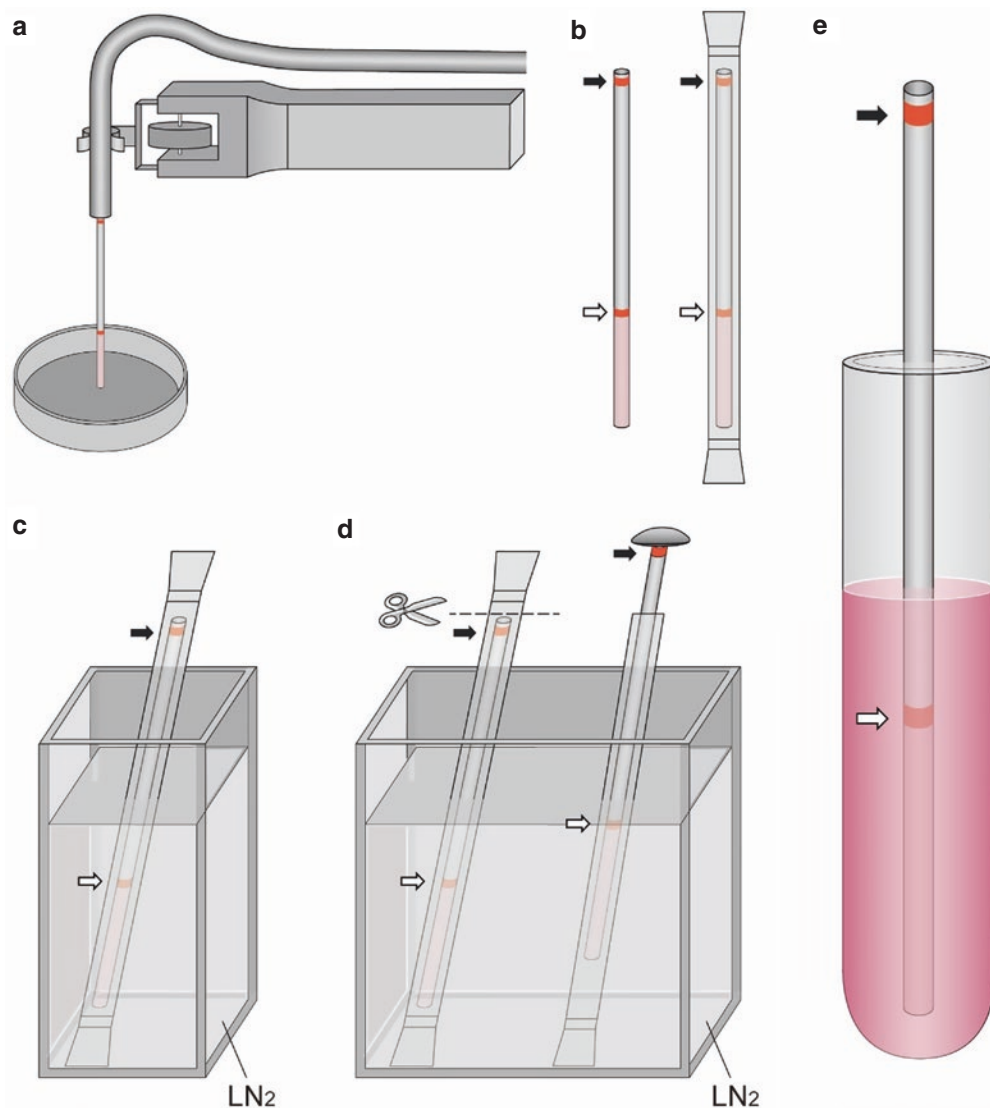


Fig. 59.1 Scheme of spermatozoa vitrification: (a) aspiration of 10 μ L spermatozoa suspension in the capillary, (b) capillary sealed in a 0.25 mL straw, (c) immersion of vitrification packaging system (0.25 mL straw) into liquid nitrogen (LN₂), (d) cutting of marked end of the packaging system and removing of the capillary from 0.25 mL

straw, (e) warming procedure: immersion of capillary with sperm suspension into pre-warmed-to -42 °C medium. Arrows: Marked aspiration position (empty arrows) and end cut-off position (solid arrows) of capillary

59.2 Notes

1. Spermatozoa analyses are performed according to published guidelines of the World Health Organization (WHO) [1]. All specimens used for this methodology of cryopreservation have the following quality criteria for spermatozoa concentration, motility, and morphology: more than five million spermatozoa/mL, more than 32% progressive motile ("a" and "b"), and more than 4.0% morphologically normal spermatozoa. Ejaculates con-

taining a minimum of 20×10^6 sperm/mL and displaying 70–75% progressive sperm motility were collected from 20 healthy men by masturbation, after a minimum of 48 h of sexual abstinence.

2. Swim-up was performed using a culture medium (human tubal fluid (HTF) [2]; Irvine Scientific, Santa Ana, CA, USA) supplemented with 10 mg/mL of human serum albumin (HSA; Irvine). After liquefaction, each ejaculate was prepared through a swim-up method and divided into equal aliquots.

3. Human tubal fluid (HTF) with 1% dextran substitutive supplement (DSS, Irvine Sci., Irvine, USA) or HSA will be the basic medium for spermatozoa preparation.
4. Prepare vitrification stock solution of 0.5 M sucrose (MP Biomedicals, Illkirch, France) with bi-distillate water (Berlin-Chemie, Berlin, Germany), pass through a 0.22 μ m filter (Millipore, Darmstadt, Germany), and then store frozen until use. Before the preparation of the vitrification medium, thaw 0.5 M sucrose and keep it at room temperature. With regard to sucrose (MW 342.3 g/mol), from experience, when a 0.5 M of stock solution is required, the amount of sucrose can be reduced pro rata, e.g., 171 g in a final volume of 1000 mL (requires approximately 880–890 mL buffer). To ensure that all the sucrose is dissolved, the solution should be shaken vigorously and left overnight before filtration [3].
5. The vitrification solution was prepared extempore as follows: 0.5 M sucrose was diluted 1:1 with HTF medium supplemented with 1% DSS or HSA, to achieve the final concentration of sucrose 0.25 M [4].
6. The package for all 10 μ L spermatozoa suspension was used in the form of 600 μ m capillaries from hydrophobic material (Vitromed GmbH, Jena, Germany) and the commercially supplied capillary of regular shape with a stable (fixed) diameter for storing samples. Before the manipulation, one end of all capillaries was labeled on the top to mark the “cut-off” position (Fig. 59.1, solid arrows) [5].
7. In brief, using a micropipettor (Eppendorf, Hamburg, Germany), aspirate 10 μ L of spermatozoa suspension and drop it into a 35 $\times$ 10 mm Falcon petri dish (Life Sciences, Durham, USA). The capillaries one by one were filled with 10 μ L of spermatozoa suspension by aspiration using an IVF pipette controller (Medical Technology GmbH, Bruckberg, Germany), and the position of the spermatozoa suspension was marked (Fig. 59.1, empty arrows).
8. The straws were transferred in a Styrofoam bath with liquid nitrogen in a perpendicular position. Only the labeled “cut-off” part of the capillary was above the level of liquid nitrogen (Fig. 59.1c). The upper part of the 0.25 mL straw was cut off over the capillary’s labeled end (Fig. 59.1d) with sterile scissors, as close as possible to the marked end of the capillary, just above the marked position, without touching the marked end of one.
9. For warming, a 2 mL centrifuge tube (Eppendorf, Hamburg, Germany) filled with 2 mL of basic medium was pre-warmed in the digital dry bath (Labnet International, Inc., Edison, USA) to 42 $^{\circ}$ C.

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