Development of in vitro maturation techniques for clinical applications

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In vitro maturation (IVM) refers to maturation in culture of immature oocytes at different stages that may or may not have been exposed to short courses of gonadotropins. The source of immature oocytes is an important feature for subsequent embryonic development, pregnancy, and healthy live births. 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. As the development of IVM treatment continues, an attractive possibility for improving the already successful outcome is to combine a natural-cycle in vitro fertilization (IVF) treatment with immature-oocyte retrieval followed by IVM of those immature oocytes. If the treatment processes can be simplified for immature-oocyte retrieval, different types of infertile women may be able to take advantage of these treatments. Mild-stimulation IVF combined with IVM treatment may represent a viable alternative to the standard treatment. Although IVM treatment is still considered to be experimental, it is now time to reconsider the IVM technology and its development. Mild-stimulation IVF combined with IVM may prove to be not just alternatives to standard treatments, but potentially first-line treatment choices. (Fertil Steril® 2017;108:577–84. ©2017 by American Society for Reproductive Medicine.)

Key Words: IVM, oocyte, immature, IVF, mild stimulation

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Initial attempts of human in vitro fertilization (IVF) in the 1930s used in vitro matured (IVM) oocytes (1–4) because it was impossible to obtain human in vivo matured oocytes. The landmark work on IVM of human immature oocytes was carried out in the 1960s (5, 6), and human IVF techniques were also established with the use of IVM oocytes (7–10). Therefore, one can assume that current advanced assisted reproductive technologies (ART) for infertility treatment are based on the development work of IVM.

In the 1970s, laparoscopy was introduced to collect human mature oocytes from preovulatory follicles (11), resulting in the possibility to retrieve in vivo matured oocytes for IVF (12). Although the first human live birth resulting from IVF was produced with the use of natural-cycle IVF (13), this procedure was gradually replaced by controlled ovarian hyperstimulation combined with IVF treatment because the number of oocytes retrieved determined the embryos available for transfer, which in turn directly affected the chance of successful pregnancy (14–16).

At the beginning, clomiphene citrate (CC) was used as a single ovarian stimulation agent (17–19). Subsequently, in combination with hMG, it was used to generate multiple follicle developments and to increase the yield to more than one oocyte (20–22). However, current standard protocols use gonadotropins (recombinant or hMG) combined with LHRH agonists to prevent the problem of premature ovulation with the aim of obtaining an average of 10–15 mature oocytes per retrieval. Although high-dose gonadotropin treatments may obtain more oocytes, this approach is associated with a number of adverse short- and long-term side-effects, including a greater risk of ovarian hyperstimulation syndrome (OHSS) (23). Thus, natural-cycle and mild-stimulation IVF as well as IVM treatments have become appealing options to infertile couples.

Today, given the efficiency of IVF and improvements in the culture system, natural-cycle IVF or mild stimulation may be more suitable for women undergoing IVF treatment. Several studies have shown that natural-cycle IVF treatment has advantages over standard-stimulation IVF treatment, particularly in the management of women with low ovarian reserve (24, 25). In contrast to standard-stimulation IVF treatment, the aim of mild stimulation is to develop safer and patient-friendlier protocols where the risks of the treatment are minimized. Interestingly, despite theoretic advantages, mild IVF treatment has not become a mainstream approach in the United States. A recent large retrospective study found a significant decrease in live birth rate associated with increasing FSH dose regardless of the number of oocytes

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retrieved (26), cautioning against high doses of FSH in IVF treatment cycles, albeit falling short of recommending mild IVF treatment. There is also evidence that mild stimulation or modified natural cycle protocols may have equal or even improved success rates compared with conventional IVF in women with a history of poor ovarian response (27).

Recovery of immature oocytes followed by IVM is a potentially useful treatment for infertile women. This method seems particularly effective for women with polycystic ovaries (PCO) or polycystic ovarian syndrome (PCOS)–related infertility, because there are numerous antral follicles within the ovaries of this group of patients (28–30). To date, IVM treatment has been mainly applied to women with PCOS and is not regarded to be applicable to all types of infertility with acceptable outcomes (31). It is clear that IVM treatment is not likely to replace the current stream of IVF treatments. In 2013, the Practice Committees of the American Society for Reproductive Medicine and Society for Assisted Reproductive Technology indicated that IVM should be performed only as an experimental procedure evaluating both efficacy and safety in carefully selected patients (32).

As we accumulate more experience and outcome data, natural-cycle IVF, mild-stimulation IVF, and IVM treatment may prove to be not just alternatives to standard stimulation treatments, but potentially first-line treatment choices (33). In the development of IVM treatment, one very attractive possibility for enhancing successful outcomes is to combine natural-cycle IVF treatment with immature egg retrieval followed by IVM of those immature oocytes (34). It has been proven that the use of IVM technology can thus be broadened to treat women suffering from all types of infertility with acceptable pregnancy and live birth rates (35–38). The aim of the present review is to share our views of the development of IVM technology.

DEFINITION OF IN VITRO MATURATION

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 metaphase II (MII) stage accompanied by cytoplasmic maturation to successfully prepare the oocyte for fertilization and early embryonic development (39). 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 germinal vesicle (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 hours 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. This is the biologic definition of oocyte IVM. IVM of human immature oocytes has developed as a clinical procedure a few decades after the first live birth from IVM oocytes (40). However, 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. Such differences include the source of immature oocytes that may not be at the GV stage owing to patient selections and different stimulation protocols. In some cases, the human immature oocytes were retrieved from follicles that have been stimulated for a few days with the use of gonadotropins to support moderate follicle growth and triggered with the use of hCG before oocyte retrieval. A recently proposed clinical definition of IVM is based on the size of follicles during the immature oocyte retrieval (41), but that proposed definition may be cumbersome and complicated. It has been criticized that a definition based on the size of follicles is not scientifically justified, and such a definition can lead to false results in interpreting the follow-up of children conceived with the use of IVM techniques (42, 43).

It is interesting to mention here that the first reports of pregnancies from IVM oocytes were from stimulated cycles where the retrieval of immature oocytes was followed by IVM and IVF (44, 45), in which the IVM procedure of immature oocytes was not based on the size of follicles. It seems to be difficult to clearly define the clinical meiotic status of oocytes, because there are different situations with patient selections and the sources. Therefore, the definition of clinical IVM should be defined according to the origin of immature oocytes to clarify for follow-up the outcomes derived from different sources of immature oocytes. Nevertheless, we think that clinical IVM treatment should be defined as IVM of any immature oocytes regardless of stage, from GV and MI to MII, for clinical application, because it involves the procedure of IVM for immature oocytes. It is important to point out for scientists, especially for basic scientists, that they should distinctly understand that the situation of clinical procedures is quite different from the laboratory procedures. It is not possible to evaluate the clinical procedures by evaluation of laboratory procedures for IVM of oocytes. The accumulation of our combined knowledge is to better understand the purpose of performing clinical IVM of oocytes and to use this knowledge for our health care.

SOURCE OF IMMATURE OOCYTES FOR IVM

As mentioned above, today's human IVF technology was developed with the use of IVM oocytes derived from surgical materials (8). Thus far, there is no exclusive IVM technology applied in the world (30). Since the first report of a pregnancy in a woman with anovulatory PCOS after undergoing IVM and IVF (46), several groups have made efforts to develop this treatment for infertile women with PCO or PCOS, because a large number of antral follicles in the ovaries are seen in infertile women with PCO or PCOS. This group of patients is sensitive to ovarian stimulation with the use of gonadotropins and has an increased risk of OHSS compared with women who have normal ovaries. The technique involves modified IVM treatment, with priming with the use of FSH or hCG before immature oocyte retrieval. It seems that the successful pregnancy rates with the use of IVM treatment correlated with the number of immature oocytes retrieved (47).

Ovarian stimulation with the use of FSH before immature oocyte retrieval has been applied, indicating that FSH before treatment promotes efficient recovery of immature oocytes and IVM (48). Recently it has been reported that FSH priming is beneficial for promotion of the maturation and quality of oocytes, leading to a higher embryo cleavage rate and lower rate of pregnancy loss (49). Theoretically, the use of FSH priming at the beginning of follicular or luteal phases may enhance more follicular development and the maturational competence of immature oocytes in vivo. It has been found that oocyte maturation rates were not different between the immature oocytes priming with and without hCG, but the time course of oocyte maturation was different (39). It appears that for oocytes retrieved from follicles in response to hCG, hCG may promote the initiation of oocyte maturation in vivo, because it has been reported there were LH/hCG receptors in the granulosa and cumulus cells from the antral follicles as small as 3 mm in diameter, suggesting a benefit to most follicles when using hCG priming (50). It has been demonstrated that the time course of oocyte IVM is hastened and the rate of oocyte maturation is increased by priming with hCG 36 hours before retrieval of immature oocytes from women with PCOS (51, 52). Therefore, it is possible that pregnancy rates may potentially be improved by means of priming with hCG before immature oocyte retrieval (53). This notion was confirmed by other reports (54–57).

It has been reported that in women with PCOS, the time course and maturation rate are different when the GV-stage oocytes are divided into different groups based on the pattern of cumulus cells after hCG priming (58). It seems that with hCG priming, not only does it promote initiation of the maturation process in some oocytes, but it also enhances some GV-stage oocytes from the small follicles to acquire maturational and developmental competence in vivo. Nevertheless, a recent review indicated that there is no conclusive evidence that hCG priming had an effect on live birth, pregnancy, or miscarriage rates in IVM treatment and that low-quality evidence suggested that hCG priming may reduce clinical pregnancy rates. However, those findings were limited by the small amount of data included, because no data were available on adverse events (other than miscarriage) or on drug reactions (59). Therefore, the authors could not adequately assess the safety of hCG priming, suggesting that further evidence from well designed RCTs is needed before definitive conclusions can be made about the role of hCG priming and optimal dose and timing.

How to inseminate the IVM oocytes, especially for the oocytes already denuded from cumulus cells? Is intracytoplasmic sperm injection (ICSI) an essential procedure for IVM oocytes? We have found that ICSI is not essential for IVM oocytes after denuding if the sperm parameters are normal (Chian et al., unpublished data from presentation O-061, 16th Annual Meeting of the European Society for Human Reproduction and Embryology, 2000). Interestingly, recent data reported that the normal fertilization rate was similar in both conventional IVF insemination and ICSI for IVM oocytes derived from GV and MI stages (60), indicating that although fertilization methods resulted in a similar abnormal fertilization rate in GV-derived oocytes, the multipronuclear formation rate was significantly lower with the use of ICSI than with the use of conventional IVF insemination. The authors concluded that both fertilization methods could be applied in GV-derived oocytes, but ICSI is favored in MI-derived oocytes because ICSI could prevent multipronuclear formation.

CLINICAL OUTCOME AND SAFETY OF IVM TREATMENT

As mentioned above, priming immature oocytes with the use of FSH or hCG before retrieval improves oocyte maturation rate and embryo quality as well as pregnancy rates when retrieved from infertile women with PCOS and normal ovaries, as well as in regular menstrual-cycling women. The source of follicles may be important for subsequent embryonic development, but the developmental competence of immature oocytes derived from the small antral follicles seems to not be adversely affected by the presence of a dominant follicle, developed in natural-cycle IVF combined with IVM, because there are in vivo matured oocytes and immature oocytes retrieved at the same time and in the same cycle. So far, it has been estimated that there are a few thousand healthy infants born after immature oocyte retrieval and IVM from women with PCOS (30, 61). In general, the clinical pregnancy and implantation rates per embryo transfer have reached ~35%–40% and ~15%–20%, respectively, in infertile women with PCOS after IVM of immature oocytes (30). If natural-cycle IVF combined with IVM is included, the clinical outcomes must be higher than those numbers.

Since the introduction of IVF and other ARTs for infertility treatment, the health of infants born from these techniques, including IVM technology, has been a major concern, especially for the extra period of culture in vitro of immature oocytes. In fact, IVM of the immature oocytes from GV via MI to MII stage normally requires 36–48 hours of culture in vitro (6, 39). Clinical IVM of the immature oocytes from MI to MII stage needs only 6–24 hours of culture in vitro (62). Interestingly, it has been reported that there was no significant difference in morphokinetics of embryos developed from oocytes matured in vivo versus in vitro, indicating that the embryonic developmental potential may not differ between oocytes matured in vivo and in vitro (63). There were insignificant differences for zona pellucida birefringence and meiotic spindle between the in vivo and in vitro matured oocytes (64). Furthermore, it has been indicated that the in vivo and in vitro matured oocytes showed normal ooplasm showing uniform distribution of organelles (65). Mitochondrial morphology appeared similar between the maturation conditions. Cortical granules were found typically stratified in a single, mostly continuous row just beneath the ooplasm in both in vivo and in vitro matured oocytes.

One study showed that optimized human IVM procedures have no significant effects on the establishment of maternal DNA methylation patterns at LIT1, SNRPN, PEG3, and GTL2 (66). There were no differences in oxygen consumption between embryos derived from in vitro versus in vivo matured oocytes, also indicating that there was no imprinting gene disorder in IVM babies (67). No statistically significant impact was found of IVM on chorionic villus and cord-blood DNA

TABLE 1

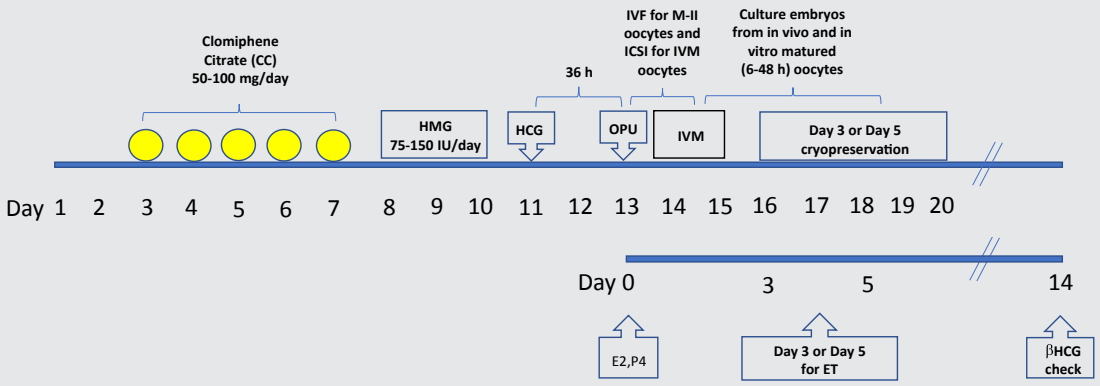
Obstetrical outcomes and congenital abnormalities in 1,421 IVM babies born from 1,187 pregnancies.

Variable	Singleton pregnancies (n = 960)	Twin gestation pregnancies (n = 221)	Triplet gestation pregnancies (n = 5)	Quadruplet gestation pregnancies (n = 1)
Pregnancies (n = 1,187)				
Gestational age (wk+d) at delivery, mean	37+4	36+5	35+2	29+0
Deliveries at >37 wk, n (%)	855 (89)	60 (27)	0 (0)	0 (0)
Deliveries at 34–37 wk, n (%)	82 (9)	132 (60)	5 (100)	0 (0)
Deliveries at <34 wk, n (%)	23 (2)	29 (13)	0 (0)	1 (100)
Newborns (n = 1,421)	**Singleton newborns (n = 960)**	**Twin newborns (n = 442)**	**Triplet newborns (n = 15)**	**Quadruplet newborns (n = 4)**
Birth weight (g), mean ± SD	2,965 ± 532	2,434 ± 365	1,968 ± 472	1,330 ± 84
LBW, n (%)	35 (4)	59 (13)	12 (80)	0 (0)
VLBW, n (%)	5 (1)	12 (3)	2 (13)	4 (100)
Apgar score at 1 min, median (interquartile range)	9 (7–9)	8 (7–9)	8 (8–9)	–
Apgar scores at 1 min <7, n (%)	133 (14)	31 (14)	0 (0)	–
Apgar score at 5 min, median (interquartile range)	10 (9–10)	10 (9–10)	8 (8–9)	–
Apgar scores at 5 min <7, n (%)	25 (3)	5 (2)	0 (0)	–
Incidence of congenital anomalies, n (%)	15 (2)	3 (1)	0 (0)	0 (0)

Note: Data reproduced from Chian et al. 2014 (31). LBW = low birth weight (1,500–2,500 g); VLBW = very low birth weight (<1,500 g).

Yang. Development of IVM techniques. Fertil Steril 2017.

FIGURE 1



Protocol for mild-stimulation cycle in vitro fertilization (IVF) combined with in vitro maturation (IVM) treatment. ET = embryo transfer; ICSI = intracytoplasmic sperm injection; MII = metaphase II; OPU = oocyte pick-up.

Yang. Development of IVM techniques. Fertil Steril 2017.

methylation at the studied developmentally important genes and interspersed repeats, suggesting that IVM-induced epigenetic changes in offspring, if any, would be relatively small in magnitude and/or infrequent (68). For human IVM, it seems that no definitive conclusion on imprinting establishment can be drawn, because well designed studies are currently not available. Therefore, epigenetic analysis should be performed in children born from pregnancies after IVM to draw definitive conclusions on the epigenetic safety of human IVM (69).

The number of live births from IVM oocytes has been increasing over the past three decades. It has been estimated that >5,000 IVM infants have been born worldwide (30). There are many concerns about IVM infants regarding obstetrical and perinatal outcomes as well as long-term development (70). Several studies have reported that the mean birth weight and the incidence of congenital anomalies seem to be similar to those with spontaneous conceptions or conceptions of infertile women undergoing IVF treatment (71–73). It was reported that 196 infants conceived after IVM of immature oocytes were not associated with an increased risk of adverse obstetrical or perinatal outcomes compared with children conceived by in vivo matured oocytes or children conceived by conventional stimulated ICSI cycles (74).

Recently 1,421 IVM babies born from 1,187 pregnancies from 31 IVF clinics in 22 countries were reported; the data were collected at the time of birth and include stillbirths but not pregnancy terminations (31). Of the 1,421 IVM infants born, there were 18 major congenital abnormalities (Table 1). However, that is similar to the prevalence of major birth defects in spontaneous conceptions per birth according to International Clearinghouse for Birth Defects Surveillance and Research (75). Based on these data, IVM may not pose any significantly increased risk of poor obstetrical outcomes or congenital abnormalities over those already accepted with the use of IVF or other ARTs.

DEVELOPMENT OF IVM TREATMENT

As mentioned above, immature oocyte retrieval followed by IVM was shown initially to be a successful treatment for infertile women with nonovulatory PCOS, because there are numerous antral follicles within the ovaries in this group of patients, making them prone to develop OHSS (52). In women, although only a single follicle usually grows to the preovulatory stage and releases its oocytes for potential fertilization, there are several small follicles that also develop during the same follicular phase of the menstrual cycle. It seems that ~20 antral follicles are selected and continued through to the preovulatory stages of development during each menstrual cycle (76). Two or three waves of ovarian follicular development in women during the menstrual cycle have been reported based on daily transvaginal ultrasonography, challenging the traditional theory that a single cohort of antral follicles grows only during the follicular phase of the menstrual cycle (77, 78). The quality and early embryonic developmental competence of immature oocytes after IVM are not detrimentally affected by the presence of the dominant follicle in the ovaries (79, 80). Based on this evidence, IVM technology has been further developed clinically.

Natural-Cycle IVF Combined with IVM

It has been demonstrated in women that atresia does not occur in the nondominant follicles, even after the dominant follicle is selected in the ovary during the follicular phase, because immature oocytes retrieved from nondominant follicles have been successfully matured in vitro and fertilized, and have resulted in several pregnancies and healthy live births (34). Therefore, one very attractive possibility for increase of success rate of natural-cycle IVF treatment is the combination of immature oocyte retrieval and IVM. If the mature oocyte from the dominant follicle and the immature oocytes from

the small follicles were collected as well, the chances of a pregnancy are greatly increased when we manage to mature these immature oocytes and produce several viable embryos.

It seems very important to prevent ovulation from the dominant follicle due to a natural LH surge when patients are treated with the use of natural-cycle IVF combined with IVM. Our experiences indicate that 10,000 IU hCG can be administered 36 hours before oocyte retrieval when the size of the dominant follicle reaches 10–14 mm in diameter. Most oocytes collected from the dominant follicles were at mature MII stage. A pilot study indicates that the clinical pregnancy rate can reach ~45%–50% per embryo transfer after natural-cycle IVM combined with IVM in a selected group of patients (35). It has been shown that more than one-half of infertile women who came to the infertility clinic for IVF treatment can be treated with the use of natural-cycle IVF with IVM or IVM alone when these treatments have been chosen primarily, and that natural-cycle IVF with IVM is an effective treatment, especially for women under the age of 35 years (36).

The strategy that has been successfully explored with the use of natural-cycle IVF is the one combined with IVM for women without PCOS, indicating that a selected group of ovulatory women can benefit from natural-cycle IVF with IVM with the result of acceptable pregnancy rates (35). More recently, we reported that natural-cycle IVF with IVM treatment might be offered to >50% of infertile women who are seeking infertility treatment, with results of >40% pregnancy rates (36, 37). Interestingly, it has been reported that although the clinical pregnancy rates are not different in terms of mature oocytes being retrieved or the time of egg retrieval with natural-cycle IVF combined with IVM treatment, the live birth rate is higher when the transferred embryos are produced from the in vivo matured oocytes (38). Therefore, natural-cycle IVF combined with IVM may be the most suitable treatment for younger women who have regular menstrual cycles.

Although natural-cycle IVF combined with IVM treatment together with IVM-alone treatments can achieve acceptable pregnancy and implantation rates in >50% of infertile women, most of the time immature oocyte retrieval from the small follicles encounters technical difficulty after the retrieval of mature oocytes from the dominant and leading follicles. Therefore, we have proposed the modified treatment based on natural-cycle IVF combined with IVM, namely, mild-stimulation IVF combined with IVM treatment.

Mild-Stimulation IVF Combined with IVM

Today, given the efficacy of IVF and improvements in the culture system, natural-cycle or mild-stimulation IVF may be more suitable for women undergoing IVF treatment. In contrast to conventional IVF treatment, the aim of mild stimulation is to develop safer and more patient-friendly protocols where the risks of the treatment are minimized. Mild stimulation is defined as administration of low-dose exogenous gonadotropins, a shorter duration in GnRH antagonist–cotreated cycles, or the use of oral compounds, such as CC, for ovarian stimulation, with the aim of retrieving fewer than eight oocytes (81, 82). Mild stimulation with the use of CC in combination with low doses of gonadotropins can be considered as a realistic option for good-prognosis patients undergoing IVF (83).

The protocol for mild stimulation combined with IVM treatment has been developed (Fig. 1). If the patient has more than several antral follicles seen on the day 2 baseline ultrasound scan, she is given 50–100 mg CC daily for 5 days. The second ultrasound is performed on day 8 to measure the size of follicular growth. At that stage, based on the follicular size, 70–150 IU hMG is administered daily for 3 days. Once a leading follicle reaches ≥18 mm and another reaches 16 mm in diameter, 5,000 IU of hCG is given; 36 hours after hCG injection, oocyte retrieval is performed with the use of a 19-G single-aspiration needle. The leading follicles are aspirated first and then the sub–leading follicles second to determine under a dissecting microscope whether they are mature oocytes. The mature oocytes can be inseminated with the use of IVF or, if the parameters of sperm quality are not normal, ICSI. To identify the immature oocytes, both the size of follicles derived from and the morphology of expansion of cumulus-oocyte complexes (COC) must be analyzed. COCs with few inner layers of compacting cumulus cells and a good pattern of outer-layer cumulus expansion can be denuded 4–6 hours after culture in fertilization medium or IVM medium to determine oocyte maturity. If oocytes are mature, they can be inseminated with the use of IVF or ICSI, and the remaining immature oocytes can be cultured in IVM medium for overnight culture. Those immature oocytes are checked again 24 hours after IVM; if they have become mature, they can be inseminated by means of ICSI with the use of the sperm sample prepared on the previous day, for which it needs to be kept at room temperature in a cap-tightened tube. Our preliminary data have shown that this mild-stimulation IVF combined with IVM treatment is a very effective treatment.

CONCLUSION

The source of immature oocytes is an important feature for subsequent embryonic development and pregnancy, but the developmental competence of oocytes derived from the small antral follicles seems not to be adversely affected by the presence of a dominant follicle. Priming with the use of FSH or hCG before immature oocyte retrieval improves oocyte maturation and pregnancy rates. The use of IVM technology can therefore be broadened to treat women suffering from all causes of infertility with results of acceptable pregnancy and live birth rates. Although IVM has resulted in several thousand healthy babies born, IVM technology is still considered to be experimental. It may be time to reconsider IVM technology as an effective clinical treatment.

As the development of IVM treatment continues, an attractive possibility for increasing the successful outcome rate is combining natural-cycle IVF treatment with immature oocyte retrieval followed by IVM of those immature oocytes. If the treatment processes can be simplified, especially for immature oocyte retrieval, more infertile women may be able to take

advantage of these treatments. Mild-stimulation IVF combined with IVM treatment may represent a viable alternative to standard treatment. As we accumulate more experience and outcome data, mild-stimulation IVF combined with IVM may prove to be not just an alternative to standard treatments, but potentially a first-line treatment choice.

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