

REVIEW

The quality of human eggs and its pre-IVF incubation

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Abstract

Background: Multi-factors influence the success rate of infertility treatments, and one of the important points is to obtain good quality eggs.

Methods: Based on the literatures and unpublished data, the factors affecting egg quality were summarized.

Main Findings (Results): Egg quality is an important determinant in successful infertility treatment. In addition to maternal age, controlled ovarian hyperstimulation (COH) protocols also play a key role in affecting the quality of the egg. After egg retrieval, the insemination occurs 3–6 h after collection, with a pre-IVF incubation time by in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) (39–42 h post-HCG injection). The pre-IVF incubation refers to the short period time of 3 to 6 h after oocyte retrieval and before the insemination by IVF or ICSI. The pre-IVF incubation of collected eggs in the designed culture medium improves egg quality in terms of maturation and early embryonic development.

Conclusions: Pre-IVF incubation of the collected eggs contributes to the improvement of the quality of eggs; therefore, it may increase subsequent pregnancy and implantation rates following embryo transfer.

KEYWORDS

egg, human, oocyte, pre-IVF incubation, quality

1 | INTRODUCTION

Despite the relatively high success rates of assisted reproductive technologies (ARTs) in infertility treatment, many aspects of the procedures still need improvement. Each step in ART procedures is

essential to improving the rate of success in delivering healthy babies. However, an important challenge with modern ARTs is egg quality.

In mammalian ovaries, oocytes, or maturing eggs, are housed within follicles.¹ Oocytes perform essential functions in reproductive and inter-generational inheritance. Oocyte meiotic maturation

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relies on the utilization of cytoplasmic mRNA and ribosomes as well as protein translation that were previously accumulated during the extended period of oocyte growth. Regulation of protein translation and mitochondrial activity is vital in maintaining oocyte quality. The storage of RNA in arrested condensates and proteins in cytoplasmic lattices in mature oocytes ensures reliable information transfer to the offspring. Once fully grown and developed, oocytes are ovulated as eggs and are ready for fertilization.

As women age, the number and quality of oocytes decline. To clarify the terminology, it is important to distinguish between oocytes and eggs.² Oocytes can be either mature or immature, while eggs are specifically referred to as matured oocytes. Therefore, in this paper, the term “eggs” denotes only mature oocytes, whether matured in vivo or in vitro, as the concept of immature eggs is scientifically inaccurate.

Egg quality, typically assessed based on normal fertilization, subsequent embryonic development and implantation, and live births following embryo transfer, remains a critical factor in the success rate of ARTs. Currently, the quality of eggs is assessed by morphological system under microscopic observation,³ though this method is deemed largely primitive.⁴ Despite the methods using a number of potential genes (biomarkers), molecules, and physical characteristics to predict a good quality egg,⁵ the selection of high quality eggs has its limitation.⁶ A series of molecular, cellular, and morphological alterations occur during oocyte growth and maturation (meiosis), and those changes influence subsequent fertilization and embryonic development and affect pre- and post-implantation.^{7,8} Therefore, egg quality is influenced by many factors, with the source of eggs being one of the most important factors.²

The knowledge surrounding the processes of female reproductive aging remains limited. Fertility declines sharply around age 35, corresponding to the decline in egg quality, which impacts the reproductive outcomes. Notably, the uterus can adequately support pregnancies even in women in their late 40s,⁹ indicating that age-related fertility decline is due to poor egg quality rather than implantation failure. Ovarian aging, which is the process involving the changes of cellular and organelles within the ovaries, is the key factor driving the decline in egg quality with age.^{10,11}

Recent mouse studies have found improvements in the quality of old oocytes when grown in young follicles in comparison with those grown in old follicles.¹² Specifically, this treatment demonstrated that oocytes from older mice, when grown in young follicles, had fewer chromosomal abnormalities, improved mitochondrial function, and more favorable gene expression as well as metabolite-production profiles. Meanwhile, young oocytes grown in old follicles showed increased signs of aging. While promising, the application of this technology to human infertility treatment is still in its early stages.

In addition to maternal age, ovarian stimulation protocols may also be related directly to the quality of eggs. The number of eggs retrieved and the availability of embryos for transfer are key determinants of the success of in vitro fertilization (IVF) treatments.¹³ Ovarian stimulation protocols for IVF are designed to maximize egg

yield.¹⁴ However, different protocols have been shown to impact egg quality differently.^{13,15,16}

Interestingly, recent studies have reported that the yield of good-quality blastocysts progressively increases with follicle size, reaching up to approximately 19mm in diameter from the stimulated cycles.¹⁷ Recently, a multi-center study ($n=19082$ treatment of infertile women), including 11 European IVF centers, indicated that intermediately sized follicles are most important to the number of mature oocytes subsequently retrieved and that maximizing this proportion of follicles by the end of ovarian stimulation is associated with improved live birth rates, suggesting that the larger mean follicle sizes, especially those >18 mm, are associated with premature progesterone elevation by the end of ovarian stimulation and a negative impact on live birth rates with fresh embryo transfer.¹⁸

Follicle size at the time of oocyte retrieval from stimulated cycles varies due to the asynchronous follicular development in the ovary following ovarian stimulation protocols. Despite this, the majority of retrieved oocytes are at the mature stage 36h after human chorionic gonadotropin (HCG) injection. At the end of the oocyte retrieval procedure, the collected cumulus-oocyte complexes (COCs) are inseminated 3–6h after pre-IVF incubation (39–42h post-HCG injection) using fertilization medium by IVF or intracytoplasmic sperm injection (ICSI) procedures.^{19–21} Evidence suggests that a short pre-IVF incubation time in the designed culture medium, as opposed to regular fertilization medium, promotes oocyte maturation and subsequent embryonic development.²² Therefore, this review will aim to explore the factors influencing egg quality, such as maternal age, different controlled ovarian hyperstimulation (COH) protocols, and the role of pre-IVF incubation (Figure 1).

2 | THE QUALITY OF EGGS IN REPRODUCTIVE AGING WOMEN

Female reproductive aging is a process influenced both by biological and genetic factors.

It is well established that neuroendocrine processes, such as implantation, placentation, and delivery, may contribute to a reduced female reproductive performance with age. However, the primary determinant of reproductive aging is the decline in ovarian function, which is the main regulator of this process. Therefore, ovarian aging is classified as the main cause of female reproductive aging, which is significantly associated with the changes in the quantity and quality of oocytes.²³

The two main functions of the mammalian ovary are: (1) the formation of fertilizable eggs capable of developing into an embryo and, (2) the production of hormones regulating various biological processes. However, the ovary still remains a complex and mysterious organ, and ovarian aging has been the subject of scientific inquiry for decades. The failure of this organ represents one of the earliest phenomena characterizing natural female aging; thus, it raises intriguing questions on the relationship between reproductive and organismal aging. Reproductive aging is multifactorial and

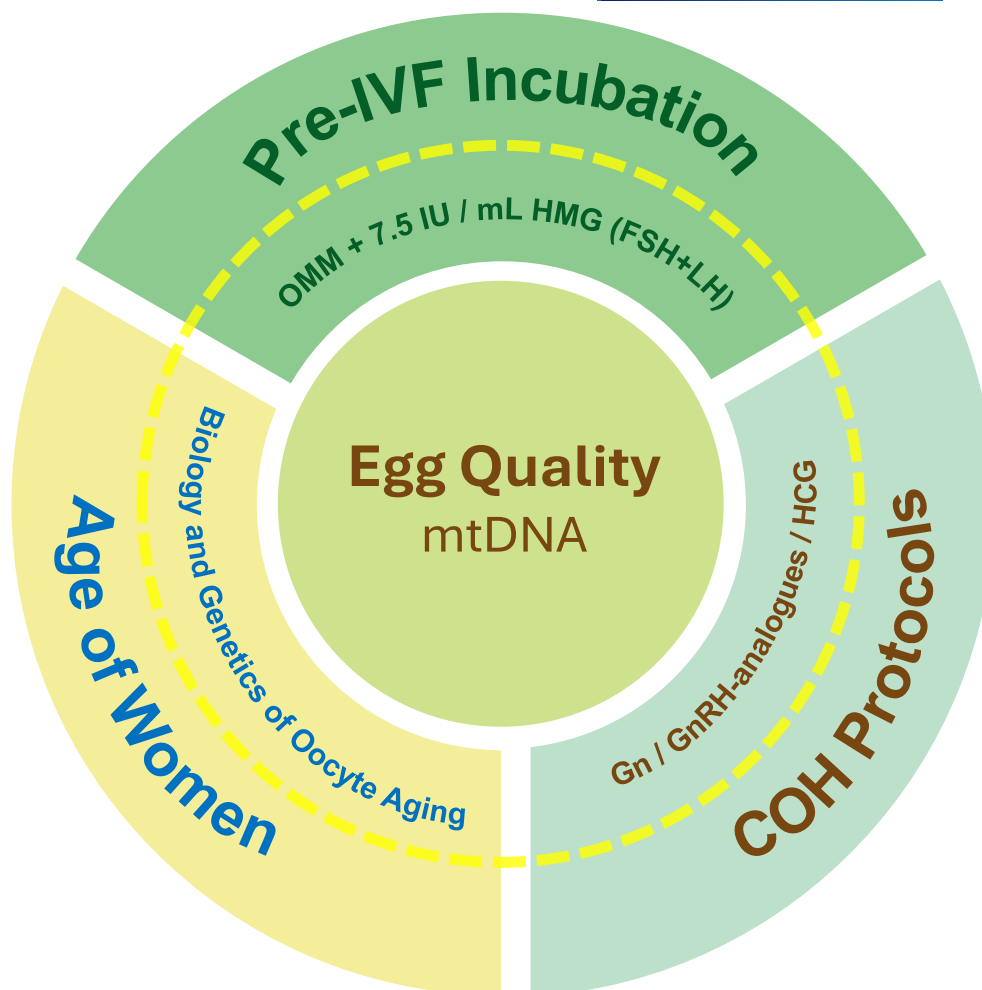


FIGURE 1 A multitude of factors can diminish the quality of eggs. Those mentioned factors are explained respectively further in the text.

involves many unknown factors, suggesting that it is important to adopt cutting-edge technologies for identifying new biomarkers and conducting thorough validations in population-based studies before clinical applications.¹² Therefore, this review will briefly focus on the impact of ovarian aging in relation to the quality of eggs.

2.1 | Biology of oocyte aging

The biology of reproductive aging is driven by a gradual decrease in both the quantity and the quality of oocytes in the ovaries. At the fourth month of fetal development, the ovaries contain approximately 6–7 million primordial follicles, and through a rapid transition of the majority of primordial follicles via apoptosis at birth, only 1–2 million primordial follicles remain. After birth, the rate of primordial follicles loss accelerates, and by puberty, approximately 300 000 to 400 000 follicles remain in the ovaries. During a woman's reproductive years, the number of primordial follicles declines steadily at a rate of approximately 1000 follicles per month, with this rate accelerating after age 37. By the time of menopause, fewer than 1000 follicles typically remain in the ovaries.²⁴ Therefore, it is of common

belief that ovarian aging correlates with progressive loss in both the quantity and quality of oocytes. This is due to a central dogma; the irreversible age-alteration in ovarian reserve in mammals depends on the lack of proliferative germ cells in the adult ovarian tissue.²⁵

Although there are reports indicating that germ stem cells (GSCs) are consequently generating oocytes to form new follicles in mice and human,^{26–29} the evidence of ovarian stem cells (OSCs) was isolated from adult ovaries,^{30,31} but the notion of this definition remains unclear.³² Oocytes remain dormant in the ovaries for long periods of time and must store genetic materials and maternal factors stably to support offspring development and health, while simultaneously managing cellular damage in a reliable manner. The notion of the gradual loss of resting follicles and the reduced ability to produce competent eggs for fertilization and embryo development is related to the ovarian functional decline with age.

Ovary aging is influenced by both intra-ovarian and extra-ovarian factors, with recent studies providing deeper understanding of the mechanisms of ovarian aging. These factors include chromosomal cohesion deterioration, DNA damage, and mitochondrial dysfunction, telomere shortening, genetic mutation, and alterations in protein metabolism and the stromal

microenvironment, all of which influence both the quantity and quality of the ovarian reserve.³³ Notably, egg quality is the most important measure of reproductive potential than follicle quantity, as follicle count does not accurately represent a woman's reproductive potential. Ovarian aging is also associated with an increase in aneuploidy, up to 60%, miscarriages, and birth defects.²³ This increased aneuploidy is attributed to many factors, including disruption of the spindle apparatus, oxidative stress, and mitochondrial damage.⁷ However, some of these factors not only increase the aneuploidy, but are also related to molecular mechanisms including follicular dynamics, granulosa cell, and oocyte apoptosis, which in turn affect the egg quality. It is much understood that meiotic errors of relatively small chromosomes in the eggs result in aneuploidies, which cause miscarriages and congenital diseases. By live chromosome identifying and tracking, it reveals that in the inner region, chromosomes were pulled by stronger bipolar microtubule force, which facilitates premature chromosome separation, a major cause of segregation errors in aged eggs.³⁴

Mammalian oocytes undergo a prolonged meiotic arrest which can last for much of the entire reproductive lifespan. This meiotic arrest, which occurs after DNA replication and is prolonged with age, poses a challenge to oocytes in maintaining the replication-dependent chromosomal proteins required for the completion of meiosis. In mouse models, it has been reported that the levels of chromosomal histones decrease with age,³⁵ particularly in both types of histone H3 variants, replication-dependent H3.1/H3.2 and replication-independent H3.3. Aging-associated histone reduction is associated with transcriptomic features that are caused by genetic depletion of histone H3.3. Neither the genetic reduction of chromosomal H3.1/H3.2 nor H3.3 accelerates the aging-associated increase in premature chromosome separation that causes meiotic segregation errors. The authors indicate that aging-associated reduction of chromosomal histones is linked to several transcriptomic abnormalities but does not significantly contribute to errors in meiotic chromosome segregation during the reproductive lifespan of mice.³⁵

In addition, recent studies indicate that oxidative stress, mitochondria, and changes in cellular and extracellular compartments of the ovarian stroma are directly related to ovarian aging. Interestingly, single-cell RNA sequencing on ovarian tissue from young and reproductively aged mice revealed a doubling of immune cells in the aged ovary, with lymphocyte proportions increasing the most.³⁶ These changes indicate that follicular cells displayed stress-response, immunogenetic, and fibroidal signaling pathway inductions with ovarian aging. Oxidative stress affects mitochondrial dysfunction, leading to apoptosis of ovarian cells and subsequently resulting in a decline in the quantity and quality of oocytes in the ovaries. Ovarian aging is mainly due to oxidative stress,^{37–39} because mitochondrial dysfunction can result in failure of cell organelles, apoptosis, and cellular senescence,⁴⁰ suggesting that clinical manifestations can be mitigated with treatments using antioxidants directly, agents affecting the cell response against oxidative stress, or combining both of these activities.⁴¹ Pharmacological agents targeting mitochondrial function have shown promising improvement in oocyte quality.

These include antioxidant coenzyme Q10 and mitoquinone,^{42,43} mammalian target of rapamycin signaling pathway inhibitor rapamycin, and nicotinamide mononucleotide.⁴⁴

Interestingly, recent reports have indicated that oxidative damage in oocytes from women of advanced maternal age occurs even at the primordial follicle stage. These mitochondria-dysfunctional oocytes may use alternative energy sources, such as glycolysis and the adenosine salvage pathway, rather than avoiding oxidative phosphorylation to prevent oxidative damage in dictyate arrest oocytes.⁴⁵ Therefore, when oxidative damage in oocytes of advanced maternal age occurs, even at the primordial follicle stage, the mitochondrial dysfunction in the oocytes is likely the result of the use of alternative energy sources. This suggests that oxidative damage and³⁷ mitochondrial dysfunction in oocytes are key factors contributing to the poor quality of oocytes in women with advanced maternal age.⁴⁶

The ooplasm provides essential nutrients required for fertilization and early embryonic development, with the mitochondria playing a major role in maintaining the quality of cytoplasm in the eggs.^{38,47} It is well established that the quantity of mitochondria can vary between oocytes, even within the same individual.^{38,48} Oocyte mitochondrial DNA (mtDNA) not only regulates cellular metabolism but also regulates cell signaling and apoptosis, with mitochondrial abnormalities contributing significantly to the decline in egg quality.^{38,49} mtDNA damage further blocks the function of other organelles, such as the spindle assembly. Proper spindle organization by microtubules of meiosis and mitosis requires sufficient ATP supply to maintain the dynamic function.⁵⁰ Otherwise, without sufficient energy, spindle dysfunction can lead to aneuploidy and cell cycle arrest.^{51,52}

Egg quality can be affected by many adverse microenvironment factors, such as ovarian aging, obesity, diabetes, and ART alternatives. Mitochondria, essential organelles in the oocyte cytoplasm, play a crucial role for oocyte maturation and embryonic development.³⁸ As mtDNA in each cell of offspring is inherited maternally, any abnormalities in the mtDNA in oocytes may cause mitochondrial diseases in the offspring.⁵³ Therefore, aging affects the quantity of mitochondria in the oocytes, resulting in a decrease in the mtDNA content coupled with an increase in age-related mtDNA mutations. This aggravates the reduction of mtDNA copy numbers in the ooplasm, in turn diminishing the oocyte viability or quality. Thus, mitochondrial dysfunction and mtDNA mutations affect the normal oocyte development, and decreased number of mitochondria contributes to the egg quality.³⁸ Recent reports indicate that the accumulation of mtDNA mutations, coupled with the decline in repair mechanisms, contributes to a reduced ovarian reserve and developmental competence, heightening the risk of aneuploidy. This suggests that there is an urgent need for clinically viable and practical approaches to assess the degree of mtDNA mutations and repair capacity in oocytes.⁵⁴

It has been reported that the mitochondrial function in cumulus cells undergoes dynamic changes and age-related modifications negatively affect egg quality,⁵⁵ indicating that a pattern of

mitochondrial abnormalities in human cumulus cells may compromise egg quality, mainly associated with ATP synthase.⁵⁶ Indeed, the cumulus cells that are in contact oocyte with gap-junction to form the cumulus-oocyte complex (COC). This complex facilitates communication between the oocyte and cumulus cells by directly affecting gene expression and protein synthesis, leading to differentiation and expansion of the cumulus cells, as well as oocyte maturation. The cumulus cells connect to the oocyte cytoplasm and penetrate the zona pellucida (ZP) with gap junctions. Therefore, following the development from antral follicle to pre-ovulatory follicles, cumulus cells that undergo proliferation and gap junctions are gradually released from the ooplasm with meiotic maturation of the oocyte.⁵⁷

In addition to mtDNA, both apoptotic and anti-apoptotic genes in cumulus cells are important predictors of egg quality and early embryo development.⁵⁸ Therefore, human ovarian aging is clearly characterized by oxidative damage and mitochondrial dysfunction.⁴⁵ Within oocytes, the quantity of mitochondria can significantly fluctuate during growth, maturation, and post-fertilization. Impaired mitochondrial dynamics in oocytes have been associated with female reproductive aging, particularly in women of advanced reproductive age.⁵⁹ Mitochondrial dysfunction may result in fertility problems and may also impact the success of treatment.⁵⁹

Interestingly, recent studies have discussed that, during ovarian aging, follicle development may be delayed by a number of factors, including increased stiffness of the ovary and a reduced rate of granulosa cell proliferation. These changes indicate the potential contribution of defects in protein synthesis (translation) to the incidence of oocyte meiotic failure.⁶⁰ The reduced rate of development increases the duration for which the components of the translational apparatus must survive in oocytes, ultimately diminishing the functionality of these molecules. As a result, the reduced ability of oocytes to sustain sufficient levels of translation may lead to defects observed during meiotic maturation, including the loss of spindle bipolarity during maturation. This suggests that the reduced translational capacity of oocytes could be a significant factor contributing to age-related female infertility.

2.2 | Genetics of oocyte aging

As mentioned above, the mechanisms involved in ovarian aging are not completely understood. In general, the factors that contribute to the variation in the age at menopause are unknown, though many factors, such as environment and lifestyle, have been largely considered to affect reproductive aging at natural menopause.⁶¹ Therefore, ovarian aging has a genetic basis that controls the functions of different types of cells in the ovaries. Notably, the association between menopausal age in mothers and daughters, and among sisters, has been convincingly demonstrated, implying that genetic factors are greatly involved in the reproductive aging process.^{62–64} Recently, increasing attention has been given to the role of genes as key determinants of menopausal age.⁶⁵ For instance, homozygosity

for *CCD201* loss-of-function has been shown to have a substantial impact on female reproductive health, especially for a stop-gain variant in *CCD201* that causes primary ovarian insufficiency (POI).⁶⁶ Using single-cell RNA sequencing technique to analyze young and reproductive-aged mouse ovarian tissues indicated that follicular cells display stress-response, immunogenetic, and fibrotic signaling pathway inductions with aging.³⁶

Genomic approaches, including next-generation sequencing (NGS) and cytogenetic arrays, have been used to identify relationships between maternal gene variants and clinical infertility phenotypes. As maternal age increases, egg quality decreases, suggesting that other causes of poor quality of eggs likely exist.⁶⁷ Pathologic genetic variants dysregulate meiotic processes that occur during prophase-I, meiotic resumption, chromosome segregation, and cell cycle regulation.⁶⁸ In addition to the pathologic genetic variant dysregulation of meiosis, as discussed above, other genetic drivers contribute to altered ovarian reserve, ovarian function, defective follicle activation, and growth⁶⁹ as well as syndromic causes of infertility.^{70,71} Recent reports have indicated that DNA repair dysregulation may cause female infertility.⁷²

Biological processes and genetic causes are implicated in female infertility. Ovarian function depends on the level and timing of transcription of many genes and is controlled by non-coding regulatory DNA sequences,⁷³ microRNAs⁷⁴ and small non-coding RNA molecules.⁷⁴ Large X chromosome alterations are the frequent cause of ovarian dysgenesis, with causative genes displayed beneath the key biological processes, and they can be involved in multiple pathways, affecting several stages of oocyte growth and quality.⁶⁹ With the advances of exome and genome sequencing and careful phenotyping, substantial progress has been made in the discovery of genes causing isolated and syndromic forms of POI, preimplantation development, and germline-limited alterations associated with lethal X-linked and autosomal phenotypes.^{75,76}

Gene activation in the zygote and early embryo is controlled by both genetic and epigenetic mechanisms, with epigenetic alteration also affecting egg quality. Epigenetics is defined as the process that regulates gene function, which does not affect DNA sequence and is heritable through cell division.⁷⁷ Epigenetic mechanisms set inheritable alterations that play an important role in regulating gene expression.⁷⁸ The main epigenetic alterations include DNA methylation, modification of histones, and chromatin remodeling. However, it remains unclear whether DNA methylation in human oocytes is affected by maternal aging.^{79,80} Nevertheless, it has been recommended that the advanced maternal age-related epigenetic changes may be corrected by antioxidants, melatonin, growth hormones, and mitochondrial replacements.⁴¹

Gene mutations in ovaries and oocytes can impair mitochondrial function, in turn affecting oocyte maturation and embryogenesis.^{37,81} Therefore, mitochondrial function must be governed by genes located both in the nucleus and within the mitochondria themselves.⁸² Oocyte maturation arrest, encountered occasionally in women undergoing ART treatment, is often associated with genetic factors that still remain largely unknown. It has been reported

that *TUBB8* gene mutations have dominant-negative effects that disrupt microtubule behavior and oocyte meiotic spindle assembly and maturation.^{83,84} Mutations in *PATL2* lead to decreased amounts of protein, resulting in human oocyte maturation arrest,⁸⁵ while newly identified mutations in *TUBB8* are associated with oocyte maturation, fertilization, and developmental arrest.^{86,87} Pathogenic variants in *TRP13* are responsible for oocyte meiotic arrest,⁸⁸ and meiotic progression requires coordinated assembly and disassembly of protein complexes involved in chromosome synapsis and meiotic recombination.⁸⁹ In addition, the *PABPC1L* gene has been reported to play a key role in translational activation of maternal mRNA prior to zygotic genome activation in eggs and embryos,⁹⁰ and *MAD2L1BP* gene was identified and characterized with novel biallelic variants for human oocyte arrested at the M-I stage.⁹¹

3 | THE QUALITY OF EGGS FROM DIFFERENT CYCLES

The first treatment of IVF used the spontaneous natural cycle of women, with the retrieval of only one mature egg.⁹² However, this procedure was gradually replaced by COH, as the number of eggs retrieved determined the embryos available for transfer, which in turn directly influencing chance of successful treatment. Initially, clomiphene citrate (CC) was used as a single ovarian stimulation agent, and subsequently it was utilized in combination with human menopausal gonadotropin (HMG) to generate multiple follicle developments and to increase the yield of more than one egg.⁹³⁻⁹⁵ Although many approaches were proposed in the field,⁹⁶⁻¹⁰³ COH remains the fundamental practice in IVF treatment.

Many stimulation protocols have been developed for COH in IVF treatments. In treatment cycles, growth and maturation of multiple follicles is induced by using exogenous and supra-physiological dosages of gonadotropins to stimulate follicles to obtain a desired number of eggs. Different COH protocols with different combinations of hormones may result in follicular asynchrony and variations in terms of quantity and quality of mature oocytes. Thus, the COH protocols are extremely important to the quantity and quality of eggs obtained.

3.1 | The quantity and quality of eggs from the stimulated cycles

Gonadotropins are essential in COH protocols to achieve the desired number of eggs. Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are the main hormones in ovulation induction. Urinary HMG (uHMG), which contains FSH and LH of urinary origin, is extracted from the urine of menopausal women. Additionally, highly purified urinary FSH, which contains pre-dominantly urinary FSH (uFSH) and LH (uLH), is also extracted from the urine of menopausal women. Recombinant FSH (rFSH) and LH (rLH) are available from a laboratory, which contains only FSH or LH, respectively.

In today's golden standard stimulated cycles, gonadotropin-releasing hormone (GnRH)-analogues are also used. GnRH analogues prevent a premature LH surge and avoid cycle cancellation due to early ovulation. Several studies have shown that GnRH antagonists are more potent suppressors than GnRH-agonist.^{21,104} However, when comparing GnRH antagonist and GnRH-agonist protocols, similar cumulative live birth rates (CLBRs) have been reported, despite more eggs being retrieved in the GnRH-agonist protocol.¹⁰⁵

Stimulation protocols are commonly using FSH in combination with either GnRH-agonist or antagonist, along with oral supplementations and ovarian triggering. One of the most effective stimulation protocols is the combined use of human-derived urinary FSH (uFSH) and rFSH. This combined protocol has resulted in a significant increase in the proportion of mature metaphase II oocytes and grade 1 embryos compared to the use of either rFSH or uFSH alone.¹⁰⁴ Women undertaking COH can be broadly categorized as poor, normal, or high responders, based on their ovarian reserve. As noted above, besides maternal age, the ovarian reserve may contribute to determining the category, with high responders generally being younger and having higher ovarian reserve.^{106,107} Interestingly, it has been reported that a significantly higher delivery rate was achieved in the protocol using combined rFSH+uFSH, compared to other protocols in poor and normal responders.¹³

The LH surge at the end of COH plays an important role in activating oocyte meiosis. In COH cycles where the pituitary is blocked by GnRH-agonists or antagonists, a spontaneous LH surge does not occur. Therefore, the LH surge must be triggered using human chorionic gonadotropin (HCG) when the size of follicles reaches 18–20 mm in diameter. HCG, which is the LH surge inducer, is administered to trigger oocyte maturation 36 h after injection for oocyte retrieval. HCG can be of urinary origin (uHCG) or recombinant (rHCG). In cycles where GnRH antagonist is used, it prevents complications of ovarian hyperstimulation syndrome (OHSS). GnRH-agonists are also used as a trigger for the endogenous release of LH and for the final maturation of oocytes,^{108,109} in which it helps to block the hypothalamic-pituitary axis.

Although high-dose gonadotropin COH cycles yield a higher number of eggs collected, this approach is associated with a number of adverse short- and long-term side effects, including great risks of OHSS.¹¹⁰ GnRH-antagonist protocols have been demonstrated to significantly improve clinical pregnancy rates in both expected poor and high responders, and in women at high risk of developing OHSS. The use of GnRH-agonist trigger in combination with GnRH-antagonist cycles has become a widely adopted method aimed to prevent severe early OHSS.^{111,112} OHSS is one of the most frequent and life-threatening complications of COH.¹¹³ Women at risk of developing OHSS can be predicted prior to ovarian stimulation to employ strategies to decrease the likelihood of developing the syndrome. A history of prior OHSS, young age and low body weight, and polycystic ovary syndrome (PCOS) can predict the risk of OHSS. Therefore, the fine-tuning of ovarian stimulation for the purpose of pregnancy with fewer complications is the main goal of the treatment.

With the development of COH protocols, especially in methods of triggers for oocyte maturation and ovulation by inducing LH surge, current strategies can be categorized as HCG alone, GnRH-agonist trigger alone, and dual trigger, as well as double trigger, respectively.¹⁰⁹ The dual trigger involves using a combination of GnRH-agonist and low-dose HCG to trigger oocyte maturation and ovulation.^{114,115} The double trigger extends the interval between ovulation triggering and oocyte pick-up and the use of GnRH-agonist trigger to induce a simultaneous FSH surge.¹¹⁶ Studies have shown that using the dual trigger for final follicular maturation increases the number of oocytes, mature oocytes, and blastocysts when compared to triggering with HCG alone.¹¹⁷ However, there is insufficient evidence to support using GnRH-agonist alone, dual trigger, or double trigger protocols over HCG trigger alone in terms of improving clinical pregnancy rates (CPRs).¹⁰⁹ Similar results reported that for poor and normal ovarian responders, the dual trigger, when compared with the HCG alone trigger, did not improve the blastocyst quality and frozen embryo transfer cumulative pregnancy outcomes.¹¹⁸

Therefore, there is no single ovarian stimulation protocol suitable for all women; rather, each woman requires an individualized approach to optimize pregnancy outcomes while ensuring maximum safety. In addition to age, ovarian reserve, which is described as the functional potential of the ovary, is an important indicator of the quantity and possibly also the quality of eggs. Therefore, antral follicle count (AFC) and anti-Müllerian hormone (AMH) level are important parameters to predict response to COH and to identify good responders, poor responders, and those at higher risk of developing OHSS.

A recent report suggests that the stimulation protocols and timing of oocyte retrieval can be adjusted to a patient's age and ovarian function, indicating that oocyte maturity grades change with advancing female age, which can impact the ability to produce good quality embryos.¹¹⁹ However, the findings in this report require further confirmation. Existing evidence suggests that the quantity and quality of eggs obtained correlate directly with COH protocols, including the length of stimulation and dosage and the trigger times and methods.¹²⁰ Additionally, recent studies have also reported that excessive exogenous gonadotropin administration is associated with increased embryonic mosaicism and decreased CLBR after euploid embryo transfer in couples with good prognosis, suggesting that consideration should be given to minimizing exogenous gonadotropin dosage and limiting treatment duration to improve embryo outcomes and increase the LBR.¹²¹

3.2 | The efficacy of treatment with quantity and quality of eggs

In recent years, the protocols for COH with IVF treatment have undergone considerable changes, especially following the introduction of GnRH-antagonist, which blocks the LH surge for a few days in the woman's natural cycle, permitting milder forms of stimulation

(mild IVF) with the aim of reducing complications and focusing on the quality rather than quantity of the eggs. This suggests that mild stimulation is as effective as regular COH¹²² while being safer and less expensive. Mild ovarian stimulation could replace conventional stimulation, thus making IVF safer and more accessible worldwide.¹²³ Nevertheless, the current standard or conventional COH still is the mainstream method in the field of ART for infertility treatment. Therefore, the question to be asked is often how many oocytes retrieved per IVF cycle can impact the LBR and the rate of multiple gestation pregnancy (MGP).

It is difficult to evaluate the relationship between the number of eggs retrieved in non-donor IVF cycles and LBR and the risk such as MGP rates. This means that the number of eggs needed to achieve optimal LBR remains difficult to determine. Retrieving more than 15 eggs significantly increases the risk of OHSS without improving LBR in fresh autologous IVF cycles.¹²⁴ This suggests that less aggressive stimulation protocols should be considered, especially in high responders, to optimize outcomes.¹²⁵ Therefore, most studies have indicated that an optimal LBR can be obtained during fresh cycles when 8 to 18 eggs are retrieved.^{14,126,127} Interestingly, it has been emphasized that the number of eggs needed to reach a plateau in pregnancy rates for fresh cycles is 7 to 8 eggs, and there is no need to stimulate beyond this number.¹²⁸ At the same time, a high pregnancy rate can be maintained while OHSS is minimized. In a large cohort of 172 541 fresh oocyte retrieval cycles in the United Kingdom, it was reported that there was only limited improvement in LBR when comparing the 6–15 oocyte group to the 16–25 oocyte group, with a significant decline in LBR beyond 25 oocytes.¹⁰⁶ Similar results have been reported that the numbers of eggs between 6 and 15 oocytes can achieve the highest chances of positive IVF outcomes in terms of embryo quality and fresh embryo transfers with lower risks of OHSS.¹²⁹

It is known that, to some extent, a higher egg yield leads to a higher cumulative LBR (CLBR) with remaining frozen embryos.^{14,127,130} However, this trend has been observed only in women under the age of 35 with the remaining vitrified embryos, where LBR was 11.33% per egg retrieved.¹³¹ The greatest decline in clinical outcomes is seen in the group of women over the age of 42.^{131,132} A recent report indicated that the LBR and CLBR are optimized when 8 eggs are retrieved, and at most 14 eggs are recommended to avoid freezing surplus blastocysts. In this context, 13 autologous eggs were ideal for optimization, while CLBR was optimized after three blastocysts in donor eggs and four for autologous egg patients.¹³³ Similarly, another report found a positive association between the number of oocytes and the CLBR, but this association varies according to the woman's age.¹³⁴ While in women under 35, little benefit is derived from increasing the number of eggs above 25–30, while in women over 35, the number of eggs seems to improve the CLBR until the extreme of reproductive age is reached. In women aged 44 or older, the CLBR remains consistently low, independent of the number of eggs retrieved. Therefore, it has been suggested that women up to 38 years can achieve sustainable CLBR while limiting the number of inseminated eggs and the resulting blastocysts remaining unused.¹³⁵

To date, most studies published on the topic have examined the approximate number of eggs needed to achieve a LBR. However, a recent study introduced a concept of using LBR per egg, indicating that this standard may be more informative of the true biological efficacy of treatment and a better tool to measure ART success.¹³¹ The results indicated that the entire women population (including those with remaining frozen embryos) underwent a total of 48 259 cycles, yielding 130 111 oocytes retrieved, and 6313 LBs. Therefore, a higher LBR of 4.85% per egg, with the results being similar to their previous report.¹²⁴ When analyzing the entire population (women with and without remaining frozen embryos), women under 35 had an overall higher LBR of 4.73% per egg, and it progressively decreased with age, reaching 0.71% per egg in women over 42 years of age. Thus, it can also be used as a prediction tool for practitioners and women to calculate LBR per egg retrieved. They concluded that despite clinical and scientific advances in ART, with current COH protocols, the LBR per egg remains low, reflecting a biological barrier that has yet to be overcome. Overall, the addition of the specific technology of preimplantation genetic test for aneuploidy (PGT-A) using NGS did not demonstrate improved outcomes.¹³¹

The success of treatment with LB outcome is profoundly influenced by the quality of eggs retrieved during different cycles. Indeed, the quality of eggs is affected by different COH protocols. Although many COH protocols have been developed and tested to improve the quality of eggs and to increase the efficiency of treatment in terms of LBR over the years, those attempts of COH protocols with supra-physiological and exogenous hormones are not able to fully compensate for the innate biology of eggs, which remains the key factor in achieving a LB.

4 | THE QUALITY OF EGGS FROM PRE-IVF INCUBATION

As previously discussed, the quality of egg can be significantly influenced by both the woman's age as well as COH protocols, especially the timing of HCG triggers to induce the LH surge in vivo. Without an LH surge, oocytes collected from leading or dominant follicles are all at immature stages, regardless of whether they are obtained from natural or COH cycles.¹³⁶ In a normal physiological ovulatory cycle, a mid-cycle LH surge is typically induced by a rise in endogenous estrogen levels from the pre-ovulatory follicles. Pioneer work on ovulation induced by HCG remains very important because the timing of the LH surge is difficult to predict for the time of ovulation in women.^{137,138} HCG shares a common α -subunit with LH and 85% of amino acid residues within the β -subunit.^{139,140} As a result, both HCG and LH have similar structural and biological activities, resulting in both HCG and LH to be able to bind to induce and activate the same LH/HCG receptor and triggering LH surge with the onwards cascade of events required for oocyte maturation and ovulation.¹⁴¹

In clinical practice, oocyte retrieval is performed immediately before ovulation to obtain mature oocytes. Oocyte maturation or ovulation induction is generally triggered by HCG when one leading follicle reaches 18 mm in diameter, or when two leading follicles reach 16 mm in diameter during COH cycles.^{19,120,142} Following the oocyte retrieval procedure, the collected COCs are inseminated 3 to 6 h post oocyte retrieval or 39–42 h after trigger with HCG and/or GnRH-agonists following an assessment of oocyte maturity of the morphological form of COCs to optimize

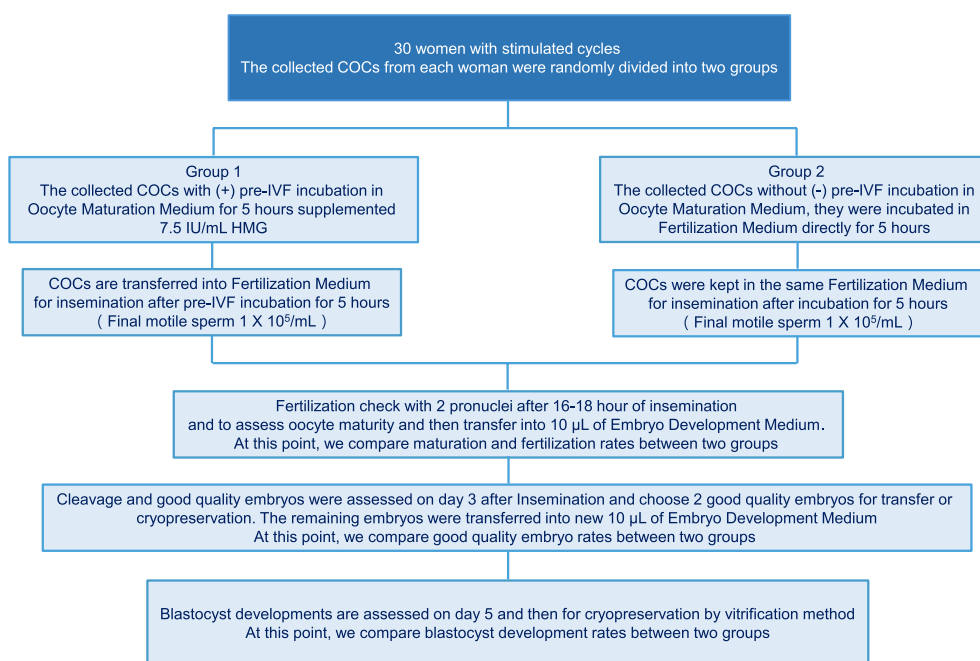


FIGURE 2 Flow chart of two groups in each woman with (+) and without (-) pre-IVF incubation for 5 h in the designed oocyte maturation medium (OMM) after oocyte retrieval and before insemination. Comparison of oocyte maturation, fertilization, good embryo quality, and blastocyst development rates. Reproduced with permission [Chian et al., Unpublished data].

time by IVF or ICSI.^{20,111} For clarity, we define pre-IVF incubation as the short period of 3 to 6 h after oocyte collection and before insemination by IVF or ICSI.

The diameters of punctured follicles can vary in range from the different sizes at retrieval in COH cycles. It is well known that oocyte maturation and developmental competence are acquired progressively with increasing follicle size. However, the development of embryos in cohort follicles from COH cycles appears to be independent of the diameter of the leading follicle at the time of HCG injection.¹⁷ Numerous studies have shown that oocyte maturity, fertilization, and cleavage-stage embryo morphology are correlated with the size of follicles from which the egg originated.^{19,143,144}

COH cycles often result in asynchronous follicular development in the ovary, although most oocytes collected at oocyte retrieval 36 h after HCG injection are typically mature.^{136,145} However, some oocytes may not be fully mature despite extruding the first polar bodies observed morphologically.^{146–148} After retrieval, morphologically mature oocytes may be required in vitro culture before insemination to achieve complete maturation for normal fertilization and early embryonic development.^{145,149–151} An interesting study investigating the relationship between the size of punctured ovarian follicles and subsequent embryonic development found that follicles less than 12.5 mm in diameter rarely result in good-quality blastocysts. In contrast, the yield of good-quality blastocysts progressively increases with follicle size up to approximately 19 mm in diameter, with no substantial decline beyond that size.¹⁷ Additionally, the ploidy of the blastocysts appears to be unaffected by follicle size.^{17,152}

Alternatively, attempts have been made to prolong the time from HCG trigger to oocyte retrieval to obtain more oocytes. Studies investigating the effect of ovulation trigger to oocyte retrieval interval time of different COH protocols on oocyte maturation and clinical outcomes have shown that a longer trigger to oocyte retrieval interval does not result in more mature oocytes or improved clinical outcomes.^{153–155} Conversely, other reports indicate that the HCG trigger to oocyte retrieval time from different COH protocols should be gradually prolonged to obtain more mature oocytes as well as a better and cumulative LBRs.^{156–159}

As part of the standard operating procedure (SOP) of oocyte retrieval, collected COCs are inseminated after 3–6 h pre-IVF incubation in fertilization medium (FM). However, the composition of FM was designed initially for sperm capacitation and fertilization and is not optimal for final oocyte maturation,^{160–162} although some commercial FMs have modified its components later.¹⁶³ We hypothesize that the quality of eggs may be improved by pre-IVF incubation in a designed culture medium in terms of oocyte maturation, fertilization, and embryonic development as well as subsequent implantation and clinical outcomes following embryo transfer.

To test this hypothesis, COCs were collected from the same women and divided into two groups (Figure 2). As shown in Figure 2, Group 1 was pre-IVF incubated in a specially designed

TABLE 1 Composition of Oocyte Maturation Medium (OMM) supplemented with 7.5 IU/mL FSH and LH pre-IVF incubation for 3–6 hours before insemination by IVF or ICSI. Reproduced with permission (Li et al., 2022).

Composition of oocyte maturation medium (OMM)	Component check mark with (✓)
Sodium chloride	✓
Potassium chloride	✓
Magnesium sulfate heptahydrate	✓
Magnesium chloride hexahydrate	✓
Sodium phosphate monobasic monohydrate	✓
Sodium bicarbonate	✓
Calcium chloride dihydrate	✓
D-(+)-glucose	✓
Sodium pyruvate	✓
Sodium-DL-Lactate	✓
Alanyl-glutamine	✓
EDTA tetrasodium salt dihydrate	✓
L-Asparagine	✓
L-Aspartic acid	✓
Glycine	✓
L-Proline	✓
L-Serine	✓
L-Arginine· HCl	✓
L-Cystine dihydrochloride	✓
L-Cysteine	✓
L-Histidine hydrochloride monohydrate	✓
L-Isoleucine	✓
L-Leucine	✓
L-Lysine hydrochloride	✓
L-Methionine	✓
L-Phenylalanine	✓
L-Threonine	✓
L-Tryptophan	✓
L-Tyrosine	✓
L-Valine	✓
D-Calcium pantothenate	✓
Choline chloride	✓
Folic acid	✓
i-Inositol	✓
Nicotinamide	✓
Pyridoxine· HCl	✓
Riboflavin	✓
Thiamine· HCl	✓
Gentamicin	✓
Human serum albumin (HSA)	✓
H ₂ O	✓

oocyte maturation medium (OMM) (Table 1), while Group 2 underwent pre-IVF incubation in the standard FM. The results indicated that the good quality embryos on day 3 and blastocyst

TABLE 2 Effect of pre-IVF incubation in the designed medium on maturation, fertilization, and embryonic development^a (Chian et al., Unpublished data).

	Pre-IVF incubation with (+) Oocyte maturation medium	Pre-IVF incubation without (-) Oocyte maturation medium	P value
No. of patients	30	30	-
Age of women (Mean ± SD)	30.2 ± 3.5	30.2 ± 3.5	-
No. of oocytes divided (Mean ± SD)	299	305	-
No. of oocytes matured (%)	273 (90.3 ± 2.1)	238 (86.3 ± 4.1)	>0.05
No. of oocytes fertilized (%)	227 (83.1 ± 8.2)	238 (85.3 ± 9.7)	>0.05
No. of oocytes polyspermied (%)	20 (7.9 ± 4.6)	19 (7.2 ± 3.5)	>0.05
No. of zygotes cleaved (%)	223 (98.2 ± 2.1)	232 (97.4 ± 2.5)	>0.05
No. of good quality embryos developed (%) ^b	175 (78.2 ± 10.3)	161 (69.1 ± 13.7)	<0.05
No. of blastocysts formed (%) ^b	154 (69.0 ± 13.6)	138 (58.5 ± 15.4)	<0.05

^aPercentage also appeared with Mean ± SD from each woman.

^bIndicate significantly different between two groups. There were 2 good quality embryos transferred or frozen on day 3; therefore, the base numbers of continuously cultured embryos to day 5 were different from the numbers of fertilized oocytes or cleaved zygotes.

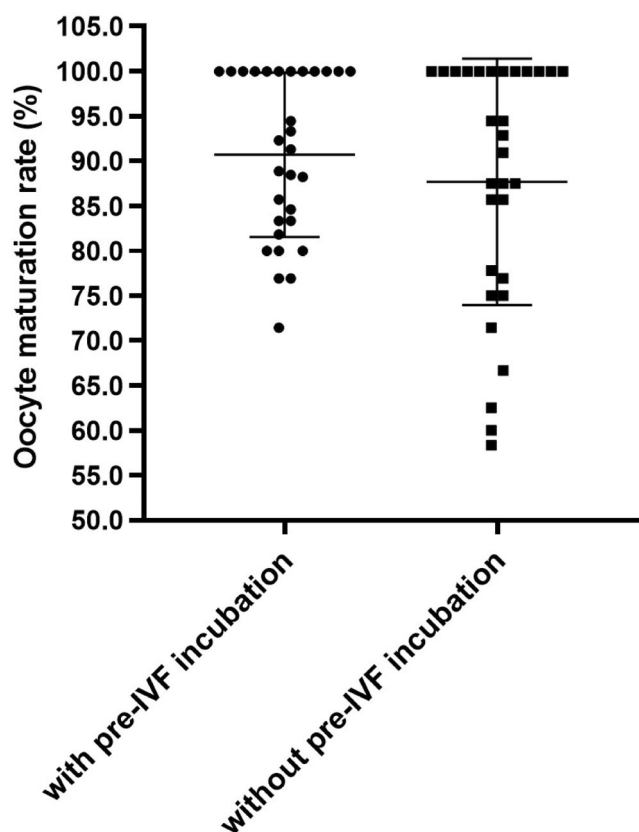


FIGURE 3 Comparison of oocyte maturation rates following with (+) and without (-) pre-IVF incubation in the designed oocyte maturation medium (OMM) evaluated at 16–18h after insemination in 60 women. The dots indicate the oocyte maturation rate of each woman. Although there is no statistical difference between the two groups, the range of maturation rates for each woman is obviously bigger in the group without (-) pre-IVF incubation compared to the group with (+) pre-IVF incubation. Reproduced with permission.²²

development rates on day 5 were significantly higher in pre-IVF incubation with OMM than in the standard FM (Table 2). This rationale was proven by a study that a short pre-IVF incubation

time in the designed OMM promotes oocyte maturation and embryonic development, indicating that pre-IVF incubation of COCs after retrieval in the designed OMM may be important for subsequent final oocyte maturation (Figure 3) and early embryonic development.²² In addition to the designed OMM formulation itself, the medium was also supplemented with 7.5 IU/mL of HMG. This supplementation suggests that gonadotropins and other factors may play a critical role in supporting oocyte maturation. Overall, the results of the study indicate that pre-IVF incubation of COCs after oocyte retrieval improves oocyte maturation and early embryonic development. However, the impact of this approach on clinical outcomes, in terms of implantation and clinical pregnancy, requires further investigation through multi-center clinical trials to confirm these findings.

5 | CONCLUSIONS

Many factors affect the success of infertility treatments, with the source of human eggs being directly associated with treatment outcomes. Maternal age is considered a key factor correlated with the quality of eggs. Female reproductive aging is involved in the process of biological and genetic factors, with ovarian aging being the main cause of female reproductive aging. Reproductive aging is significantly associated with changes in both the quantity and quality of oocytes. The quality of eggs is an important factor for successful infertility treatment. In addition to maternal age, the quantity and quality of eggs are also affected by COH protocols. While the number of eggs obtained may be correlated with live birth rates, the quality of eggs does not positively correlate with the number of eggs retrieved. Different COH protocols with different combinations of hormones may result in follicular asynchrony and variations in terms of the quantity and quality of mature oocytes. Thus, COH protocols are also extremely important in determining both the quantity and quality of eggs obtained.

Finally, the egg quality retrieved from COH cycles may be improved by the short period of 3–6 h pre-IVF incubation in the specially

designed OMM before insemination by IVF/ICSI. The maturity of eggs collected from COH cycles is different because they originate from follicles of different sizes, even if those eggs are at the M-II stage. Evidence proves that the quality of obtained eggs can be improved by pre-IVF incubation in terms of maturation and fertilization, as well as embryonic development. Therefore, pre-IVF incubation of the obtained COCs before insemination by IVF/ICSI may increase subsequent pregnancy and implantation rates following embryo transfer.

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CONFLICT OF INTEREST STATEMENT

The author declared that there are no conflicts of interest with the contents of this article.

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