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Review Articles

# Cyclic nucleotide in oocyte In vitro maturation in Assisted Reproductive Technology

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**Abstract**

*In vitro* maturation (IVM) is a promising assisted reproductive technology (ART) for human infertility treatment. However, when cumulus oocyte complexes (COCs) are removed from their follicular environment when manipulated *in vitro*, it can lead to a decrease of intra-oocyte cyclic adenosine 3', 5'-monophosphare (cAMP) causing spontaneous nuclear maturation and an asynchrony with the oocytes' cytoplasmic maturation, resulting in poor embryo developmental outcomes. Nuclear and cytoplasmic synchrony is important during oocyte maturation within antral follicles. It is maintained partially by the actions of c-type natriuretic peptide (CNP) binding with natriuretic peptide receptor 2 (NPR2), supporting high cAMP levels thus holding the oocyte in meiotic arrest. Addition of CNP to pre-IVM media has the capacity of maintaining cAMP levels and thus improve synchrony. Moreover, in women with advanced maternal age, successful IVM of aging oocytes faces significant challenges due to the morphological and cellular changes. Inhibiting initiation of nuclear maturation by cAMP modulator, CNP during pre-IVM period and thus improve oocyte developmental competence regardless of oocyte age.

**Keywords:** Oocyte; maturation; infertility; Cyclic nucleotide
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## INTRODUCTION

Modern approaches to ART and the increasing demand from patients for a safe, less invasive and cheaper treatment, that also induce less side effects have created an important need to improve *In vitro* maturation (IVM). However, there are many debates on the efficacy of published IVM protocols.[1-3] *In vitro* maturation (IVM) is a fertility treatment in which immature cumulus-oocytes complex (COCs) are extracted from antral follicles and matured *in vitro* before being fertilized.[4] IVM of human oocytes is an attractive fertility treatment for women who otherwise have a contraindication to stimulated *in vitro* fertilization (IVF). Accordingly, IVM has been proposed as an alternative assisted reproductive technology (ART) to significantly eliminate or reduce the risk of ovarian hyperstimulation syndrome (OHSS) in patients with polycystic ovary syndrome (PCOS).[5,6]

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Moreover, IVM treatments have a reduced drug cost burden and more patient-friendly with less number of injections the patient need to have in the process compared with routine IVF.[5]

Another application of IVM is for fertility preservation in cancer patients who require potential oocyte-toxic cancer treatment.[6] Conventional hormonal stimulation is not appropriate for many of these patients due to the tumor estrogen sensitivity in which the tumor will be exacerbated by exogenous stimulation.[5] Additionally, there is an urgency associated with onco-fertility preservation and patient can face a very challenging decision regarding delaying cancer treatment in order to receive stimulation to preserve their fertility.[7] IVM eliminates both the waiting time and hormonal stimulation risks for cancer patients making it an ideal treatment option for these patients who are already going through a difficult time.

Despite its proven need in the ART industry, widespread uptake of IVM by the ART industry has not occurred. This is due to the relatively limited success of IVM protocols when compared to conventional hormonal

stimulation and the lack of knowledge surrounding maturation mechanisms. IVM derived oocytes have shown poorer developmental outcomes when compared to *in vivo* matured oocytes, particularly with PCOS patients.[8] Its use and further development as a fertility treatment have been relatively limited compared with that of classical IVF following hormonal stimulation of ovaries.[8] The biggest challenge of inhibiting IVM success is the spontaneous nuclear maturation that occurs when immature germinal vesicle (GV) oocytes are removed from their follicular environment, despite no hormonal exposure. [9-11] This spontaneous nuclear maturation is asynchronous with the oocyte's cytoplasmic maturation, which lags and negatively impacts further embryo development.

Aging oocytes are also associated with morphological and cellular changes that significantly lower the fertilization rates. Age causes a loss of mitochondrial membrane integrity in mouse oocytes as a result of increasing oxidative stress.[11, 12] Similarly, during IVM culture of bovine oocytes, aging oocytes increase the concentration of reactive oxygen species (ROS), changing mitochondrial activity and ATP content.[13] It is very important to find technologies to regulate oocyte aging and improve fertilization efficiency to further benefit ART which further studies are required.

## CLINICAL APPLICATION OF IVM

IVM has been proposed as a viable treatment option for certain patient groups who are seeking ART treatment, such as women with POCS[8] , normal ovulatory with PCO[14], fertility preservation[15], poor ovarian responders[16], patients with an unexplained condition such as poor embryo development[17] and rare situations such as nil mature oocytes in a stimulated cycle.[18] The IVM process involves aspiration of immature oocytes from antral follicles and *in vitro* culture to promote maturation.[18] Young women with PCOS still have a larger number of small follicles within the ovaries, which indicates the potential of pregnancy.

Polycystic ovary syndrome is the most common endocrine disorder causing ovarian dysfunction and anovulatory infertility.[19,20] Some PCOS patients respond well to the drugs that control ovarian hyperstimulation and IVF.[21] However, those drugs need to be injected daily and are related to a high cost burden. In addition, stimulation of ovulation in PCOS women may cause multiple pregnancies due to the development of numerous follicles as well as increasing the risk of OHSS.[21] Immature oocytes are aspirated without hormonal stimulation to avoid the related side effects in IVM treatment. Therefore, in the context of PCOS, IVM has been suggested to overcome these problems and achieved a successful pregnancy.[21,22]

Although patients with advanced maternal age (AMA) are believed to be more prone to chromosomal aneuploidies[23,24] and the associated higher abortion rates and reduced implantation rates[25-27], a few studies have shown that a higher risk of aneuploidy has been found in early stage IVF embryos[25,28,29] suggesting that stimulated IVF may not be considered the best ART treatment for advanced maternal age women. These risks have been estimated to rise from 1.9% aneuploidy embryos from 25-29 years old women to 19.1% in women aged 40 years or older.[30] These aneuploidies are believed to be the result of non-disjunction during the first meiotic division.[30,31]

IVM has been successfully applied to an extensive variety of infertile women. Positive ART outcomes have been reported in onco-fertility preservation patients, AMA patients and PCOS patients, demonstrating the high potential of this technology, such as women who want to avoid the hormone stimulation because of repeated cycles without success, or may undergo radiation or chemotherapy treatment and don't have enough time to undergo hormone stimulation. Overall, advanced maternal age women are also a good target group to consider the possible benefit of IVM.

## CURRENT CHALLENGES IN IVM

Pincus and Enzmann have first described the concept of IVM on rabbit oocytes.[9] Edwards then performed IVM of human oocytes and found that the immature oocytes reached metaphase II (MII) *in vitro*.[10] In 1970, Cross and Brinster applied IVM by administering gonadotropin priming of mice and successfully produced healthy offspring[32]. Later on, Cha *et al.* conducted a study of IVM on human oocytes and reported a successful pregnancy and live birth with immature oocytes collected from the unstimulated ovaries.[18] Since then, IVM has been widely accepted as a successful treatment for groups of infertile patients.

Although IVM has been considered a new ART technology since the first healthy baby was delivered from a PCOS patient[22], clinical pregnancy rates of IVM treatment correlated with the number of oocytes retrieved have not been efficient: 38.5%[5], 21.5% [8], and 22.5%.[33] Sanchez *et al.* revealed that retrieval of a large number of oocytes at once might not allow normal homogeneous development over the full cohort due to the variations of oocyte size, chromatin formation and mitochondrial formation.[34] Age-related reduction in oocyte quality is also a factor that leads to lower the maturation and implantation potential for aging women.[35,36] The studies showed that women with advance maternal age often have poorer oocyte developmental competence, resulting in a significant decrease in embryonic development following IVF.[37-39] The reduced developmental competences are also likely to contribute to poor implantation and pregnancy rates in human IVM.

Another major hurdle is the synchronization of nuclear and cytoplasmic maturation, what is required to achieve full oocyte competency. The reason behind this problem is due to the physical removal of immature mammalian oocytes from antral follicle that results in spontaneous meiotic resumption[9-11] This spontaneous nuclear maturation is without hormone stimulation and is thought to be triggered by low levels of cAMP *in vitro* [40,41], thereby significantly decrease the oocyte developmental competency.

A large number of studies have confirmed that physically aspirating COCs from their antral follicles may cause spontaneous meiotic resumption in mouse [42-44], bovine [45], goat [46] and pig [47,48] oocytes. This accumulating evidence suggests that cAMP concentration may be a factor in controlling the spontaneous nuclear maturation, the regulation of cAMP within the oocyte may be a solution to maintaining oocyte

meiotic arrest in order to synchronize cytoplasmic and nuclear maturation *in vitro*, thereby improving IVM oocyte developmental competency[1,42,43]

The strategies used in attempt to improve IVM, such as combining hormonal priming, while also adjusting the IVM culture media may have themselves resulted in low efficacy of true IVM. Some studies have focused on improving IVM culture media, such as a two-step culture protocol [1,3,42], and other studies have focused on optimizing the quality of the oocytes by giving human chorionic gonadotrophin (hCG) or follicle-stimulating hormone (FSH) priming [49,50] which has been named pseudo-IVM. *In vitro* maturation (IVM) treatment for women with PCOS has involved the use of hormonal priming, thus could be classed as pseudo IVM. According to Walls *et al*[14], IVM patients were first administered 100 to 150 IU of recombinant follicle-stimulating hormone (rFSH) for 3 to 6 days in order to stimulate antral follicles growth. In patients with PCOS, the administration of hCG has continued to be used and has also been adopted by other groups in the setting of fertility preservation for cancer patients.[51] The study showed that pregnancy rates obtained from unstimulated ovaries were similar to those of patients who had stimulated treatment (40%),[52] thus showing that hCG given *in vivo* is not necessary. Likewise, hCG in the *in vitro* system has been used for the culture of immature oocytes of women with PCOS. Ge *et al.* reported that the addition of hCG to culture medium did not improve oocyte maturation.[53]

Despite some improvement in oocyte maturation rates and pregnancy rates, immature oocytes may be at different stages of development and thus a standardized IVM protocol could not be established. There are still many controversial areas of debate regarding hormone stimulation and the optimal IVM protocol. Data from previous studies can assist in tailoring an IVM system that promotes synchronization of nuclear and cytoplasmic maturation of oocytes. Further research would focus on improving the two-step IVM protocol in order to control resumption of mammalian oocyte meiosis. The aim of future research is to establish an IVM culture system using unprimed young mice oocytes and determine if the same system can be applied to both young and aged oocytes.

## OOCYTE DURING FOLLICULAR DEVELOPMENT

Folliculogenesis is the complex progression of the resting primordial follicles through primary, preantral and finally to the antral follicle whereby the oocyte within gains the ability to mature, fertilize and achieve competency. Developing follicles regularly leave the non-growing primordial follicle pool to join the growing pool, and the undergo atresia before puberty. After puberty, there is a continuous recruitment of follicles from the primordial follicle pool to form primary follicles. The oocyte contained within a primary follicle has a single layer of granulosa cells, which become cuboidal and proliferative.[54]

The secondary follicles are now responsive to the gonadotrophins FSH and LH, and the growing oocytes are surrounded by several layers of cuboidal granulosa cells. [55,56] Oocytes are meiotically incompetent at this stage. During the early antral follicle stage, granulosa cell secretions result in the development of an antrum with follicular fluid. The transition from a preantral to an antral follicle is dependent upon the synthesis of steroid hormone gonadotrophins. Antral follicles are also known as tertiary follicles. The majority of these oocytes are meiotically competent. At this stage, appropriate gonadotrophins may sustain optimal follicular development. In addition, during this stage and with the influence of FSH, granulosa cells start differentiating and producing estrogen.[55,56]

During the pre-ovulatory stage, further proliferation of granulosa cells occurs and is comprised of cumulus cells and mural granulosa cells. Under the influence of the preovulatory gonadotrophins surge, oocytes continue to develop until they obtain competence to resume meiosis and progress to metaphase II before ovulation. During the follicular growth, granulosa cells play a crucial role in supporting oocytes to obtain developmental competence.[57,58] Cumulus cells also start producing hyaluronic acid, which is deposited into the intercellular space causing the space between the cumulus cells to expand.[59] In conjunction with the supporting cells, the growing oocyte also regulates the proliferation, differentiation, extracellular matrix and hormone activation of granulosa cells via gap junctions.[60]

## OOCYTE MATURATION

In most mammals, intact immature cumulus-oocyte complexes (COCs) are arrested at the prophase I stage.[61] These immature eggs undergo many dynamic events to ensure the maturation of oocytes, including the resumption of meiosis, germinal vesicle breakdown (GVBD) which results in chromosome condensation and polar body extrusion.[61] Oocyte maturation is comprised of nuclear maturation and cytoplasmic maturation. The nuclear maturation is the resumption of the first meiotic division, from prophase I to metaphase II of meiosis.[62] The nuclear maturation process is defined by morphological changes within the oocyte. Ovulated oocytes are halted in metaphase II until fertilization.[63]

## NUCLEAR MATURATION

The nuclear maturation process is mainly characterized by morphological chromosomal changes during meiosis, such as chromosomal segregation and alignment. Before meiotic resumption, the intact nuclear structure of the oocyte contains diffuse chromosomes known as a germinal vesicle (GV).[64] During meiotic resumption, GVBD occurs, chromosomes start migrating to the pole and beginning division. The nuclear membrane vanishes. GVBD leads to chromosomes condensing and RNA synthesis ceasing.[65,66]

The majority of mammalian oocytes are arrested in meiosis I in the antral follicles; the occurrence of nuclear maturation (also known as meiotic resumption) is associated with the presence of luteinizing hormone (LH). [67-69] GVBD is the only morphological change that can be easily observed during oocyte meiotic resumption.[70] However, physically removing the oocytes from the antral follicles can cause spontaneous meiotic resumption in the *in vitro* environment.[9-11] The completion of the nuclear maturation process during IVM does not assure that the cytoplasmic component of the

oocyte is completed.[71,72] Vivarelli *et al.* discovered that the maintenance of high levels of cAMP could be an effective means to inhibit GVBD.[40] In addition, this nuclear maturation process is regulated by the oscillating activation of a cytoplasmic maturation-promoting factor (MPF).[64] High levels of MPF initially induce not only GVBD, but also sustain chromosome condensation. The levels of MPF decrease when the first polar body is extruded.[61,64]

## CYTOPLASMIC MATURATION

Oocyte cytoplasmic maturation refers to a series of complicated molecular changes, including the accumulation of mRNA, proteins, nutrients and other substrates, which are required for the activation of the oocyte and formation of pronuclei.[73,74] These molecular changes enable oocytes to acquire developmental competence in order to support fertilization and preimplantation development.[64]

Some aspects of cytoplasmic maturation are associated with nuclear maturation. For example, oocytes undergo early stage of cytoplasmic activation by releasing low levels of intracellular calcium iron ($Ca^{2+}$) in response to microinjection of inositol triphosphate ($IP_3$), and when the intracellular $Ca^{2+}$ levels reach a threshold, nuclear maturation will occur.[75] Wang *et al.* found that heat stress had a detrimental effect on the cytoplasmic competency rather than the nuclear competency of mouse oocytes, thereby decreasing blastocyst rates.[72] The experiment of exchanged chromosomal spindles between metaphase II oocytes [76] and between aged and fresh oocytes [77] showed that the poor oocyte developmental competence was predominantly affected by cytoplasmic components. These findings indicate that activation of cytoplasmic maturation is more susceptible to environmental changes compare to nuclear maturation during oocyte development such as heat stress.

## RESUMPTION AND ARREST OF MEIOSIS

Meiotic resumption and arrest is greatly dependent on a delicate balance between the levels of factors maintaining the oocyte in arrest and the levels of factors stimulating oocyte maturation. The total number of oocytes is to be produced in a lifetime which are arrested at an early stage of the first meiotic division are present prior to birth in most newborn mammalian species.[78,79]

Before the LH surge, these immature oocytes obtain meiotic capability at the time of antrum construction completion (Erickson & Sorensen 1974), and this time is synchronized when the oocyte reaches a maximum level of cyclin-dependent kinase (CDK1) and cyclin protein.[79] These competent oocytes are then arrested at prophase I until the preovulatory surge of LH. A large number of studies have confirmed that cAMP can be produced by the oocyte or by the granulosa cells, and is transported via gap junctions to inhibit meiotic resumption.[80] Spontaneous nuclear maturation is due to oocytes being removed from their follicular environment can be prevented by a cAMP modulator, such as CNP[1,11] or 3-isobutyl-1-methylxanthine (IBMX) [47] in the pre-IVM culture medium. The surge of LH binds with its receptor, which is primarily expressed by granulosa cells that enable oocytes to undergo meiotic resumption. As cumulus cells and oocytes lack LH receptors, the LH signal indirectly triggers resumption of meiosis.[82,83]

Oocyte meiotic competence is also related to other factors. Oocytes retrieved from larger follicles had better development compared to smaller follicles.[84,85] In addition, the size of mammalian follicles can be used as a non-invasive marker to determine the developmental ability of oocytes and improve reproductive outcomes. Specific follicle with diameter of 300-350μm had significantly higher percentage of MII and the best oocyte development to blastocysts in mice[85] Other than that, maturation-promoting factor (MPF) normal activity depends on the same amount of kinase catalytic $p34^{cdc2}$ subunit protein[86] and B-type cyclin protein present[86,87] Pig oocytes isolated from preantral follicles less than 80μm indicated that the $p34^{cdc2}$ subunit of MPF levels is limited during the early stage of follicular growth.[88] A diameter of 90μm in pig oocytes indicated that $p34^{cdc2}$ catalytic subunit and B-type cyclin levels are relatively higher compared with those of fully-grown oocytes.[89] Furthermore, activation of MPF can also trigger meiotic resumption. The components of MPF, $p34^{cdc2}$ catalytic subunit and B-type cyclin are also present in meiotically dividing oocytes[89] MPF increases in the growing oocytes, and oocytes acquire meiotic competency when MPF reaches its threshold levels.[90]

The increasing estrogen levels trigger a significant spike in LH, causing release of the oocyte from the mature follicle and activation of oocyte maturation. The corpus luteum (ruptured follicle) starts secreting progesterone and estrogen in order to prepare the uterus for pregnancy.[91] The levels of cAMP in the oocyte and granulosa cells are reduced just before ovulation and thereby decreasing its inhibitory impact on meiosis. Following MPF activation, the breakdown of the germinal vesicle drives the oocyte towards meiosis.[92] After GVBD, the ovulated oocyte is halted at the metaphase II until fertilization. Sperm fusion with the oocyte causes an increase in intracellular $Ca^{2+}$ and initiates the breakdown damage of endogenous cyclin. The onset of the chromosome segregation during the transition from metaphase to anaphase enables the oocyte to complete meiosis. At this stage, chromosome segregation errors can lead to aneuploidy, and women with advanced maternal age have increased risk of aneuploidy that can result in miscarriage later in the pregnancy.[93]

## CYCLIC NUCLEOTIDE AND CONTROL OF OOCYTE MATURATION

The cyclic nucleotides, including cAMP and cyclic guanosine monophosphate (cGMP), play a critical role in regulation of mammalian oocyte meiotic arrest and resumption in vitro[42] (Li et al. 2016). The cAMP is an essential signaling molecule, which is synthesized from adenosine triphosphate (ATP) by active G-protein coupled receptors and membrane-bound adenylate cyclase within the cell.[94,95] It can also be produced from cumulus cells as the result of LH or FSH stimulation, and is continuously supplied to the oocyte through gap junctions.[58,96] Phosphodiesterases (PDE3A) is a second regulator for cAMP levels in oocytes and it is involved in the hydrolysis of the cyclic phosphate bond in cGMP and cAMP to produce 5-GMP and 5-AMP.[41,97-,99] The cGMP

is synthesized from the natriuretic peptides pathway. Those guanylin peptides bind transmembrane guanyl cyclase, which catalyses the conversion of guanosine triphosphate (GTP) into cGMP. Much like cAMP, the cGMP acts as a second messenger to active intracellular protein kinase phosphorylation and impacts the effect of different PDE3A. [41]

Before the LH surge, high levels of oocyte cAMP keep the oocyte meiotically arrested. The granulosa cells also supply cGMP to the oocyte which inhibits PDE3A activity. After the LH surge, cGMP levels decrease and induce a secondary cascade of epidermal growth factor (EGF-like protein) in the granulosa cells of the follicle, showing this cascade is required for oocyte maturation.[81] In response to LH, cAMP levels increases, and the high levels of cAMP affect the cumulus cells and interrupt communication in the COCs.[58] Under these conditions, the flow of cAMP to the oocyte decreases, PDE3A inhibition is relieved and meiosis is resumed.[81] (Figure 1).

It has been identified that meiotic arrest of oocytes in vitro is dependent on the preservation of high levels of cAMP within oocytes.[100] The cAMP is maintained within oocyte through two mechanisms. Firstly, oocytes can autonomously produce cAMP, the expression of G-protein coupled receptor 3 and 12 (GPR3 and GPR12) is responsible for the regulation of cAMP levels in the oocyte.[95] Secondly, FSH or LH triggers the production of cAMP in the cumulus cells and flows to the oocyte through gap junction.[58,96] This is the main source of cAMP supply to the oocyte to maintain meiotic arrest (Figure 1).

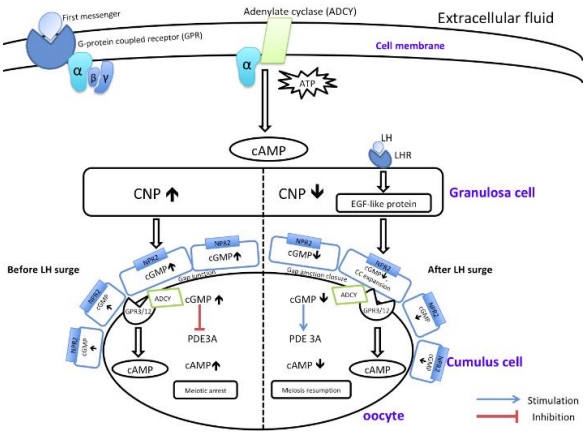


**Figure 1.** Proposed model depicting the role of CNP, cAMP and cGMP in maintenance of mammalian oocyte meiotic arrest before LH surge and the LH signaling pathway of mammalian oocyte resumption. First pathway of cAMP is synthesized from ATP by activation of G-protein coupled receptor (GPR) with adenylate cyclase (ADCY). It is the mainly source of cAMP supplies to the oocyte by cumulus cells via gap junction to maintain meiotic arrest. Secondary pathway of intra-oocyte cAMP is produced by the expression of G-protein coupled receptor 3 and 12 (GPR3 and GPR12) with ADCY. Before LH surge, binding of CNP with NPR2 increase the levels of cGMP, which inhibits PDE3A activity, thus maintain cAMP levels within oocyte. High levels of cAMP sustain meiotic arrest. After LH surge, the activation of EGF-like protein pathway interrupts the expression of CNP with NPR2, the levels of cAMP decrease enabling oocyte resumed[81].

The cAMP and cGMP production are self-regulated. Törnell et al. investigated the function of cGMP during rat oocyte maturation, and they reported that cyclic GMP is an effective inhibitor of cAMP degradation; thereby regulation of PDE3A and low levels of cGMP would contribute to spontaneous meiotic resumption in vitro.[41] In addition, Norris et al. showed that cGMP enzyme is generated in cumulus cells, and it permeates from the cumulus cells into the oocyte to inhibit cAMP hydrolysis by PDE3A through gap junctions.[81] Taken together, these promising results provide confirmation that the concentration of cAMP must be maintained at optimum levels to assure oocyte meiotic arrest.

## PROBLEM RELATED TO OCYTES FROM AGED MOTHERS

Women's fertility declines with advancing age from the mean age of 30 years-old and declines more significantly when ≥ 35 years-old which results in the progressive decreased oocyte number and poor oocyte quality.[16] Lower pregnancy rate was found in women with advanced maternal age largely as a result of the higher aneuploidy rate compared to younger women. The effect of aging also causes complex cellular and molecular changes in oocytes.[101] and the changes of which may affect normal embryo development and result in early pregnancy loss in humans. Recent studies on murine oocytes have shown that the aging of oocytes can cause a loss of mitochondrial membrane integrity as a result of increasing oxidative stress.[10,46] The study has identified that oxidative stress acts as a key mediator, which regulates the intrinsic apoptotic pathway in the aged oocytes, thus, the oxidative stress raised as oocyte age increased result in negative effect on oocyte development.[10]

Chromosomal segregation errors can occur during both mitotic and meiotic divisions. During the second meiotic cell division, chromosomal segregation errors can cause DNA damage and incorrect chromosome number. Aneuploidy is a direct consequence of chromosome segregation error in meiosis. As a result, aneuploidy of fertilized eggs and embryos can cause miscarriage or with trisomy such as Down syndrome.[93] This result was consistent with the study by Loane's team that the risk of children born with Down syndrome increased in women at advanced maternal age.[102] The risk of women conceiving a child with DS is 1 in 1400 births at age 24, 1 in 350 at age 35 and increases to 1 in 25 by age 45. Moreover, the two core subunits of meiotic cohesion complex SMC1B and REC8 have a critical role to ensure precise distribution of chromosomes during meiosis. In Yoon *et al.*'s study showed that the decrease in cohesion concentration is associated with age-related meiotic segregation errors in mammalian oocytes.[103] Compared with a 20-year-old, the levels of SMC1B and REC8 are significantly decreased in women who are 40 years old.[93]

## DETRIMENTAL EFFECTS OF OOCYTES FROM WOMEN WITH ADVANCED MATERNAL AGE IN AN IVM SYSTEM

The aging of oocytes is a key factor that compromises the overall quality of oocytes during IVM culture, which is associated with a decline in fertilization and embryonic

development. Increased ROS production in aging oocytes subsequently causes the release of cytochrome *c*, which is involved in a key apoptotic pathway.[104]

The reactive oxygen species (ROS) can diffuse through cell membranes and significantly damage biological molecular structures, including nucleic acids, lipids and protein, resulting in dysfunctional mitochondria and apoptosis.[61] Excessive levels of ROS are associated with meiotic arrest in human oocytes and cell death in mouse embryos.[105] In addition, an increased concentration of glucose increases the generation of ROS during oocyte maturation. When the levels of ROS exceed normal physiological conditions, the resultant oxidative stress can reduce oocyte quality in the mouse.[61]

Prolonged maturation culture could reduce the quality of mammalian oocytes and induce ageing of the oocytes during the MII arrest period.[106] The study has found that the percentage of mature bovine oocytes reached the highest level after being cultured for 22 hours, compared with oocytes cultured for 28 or 34 hours. The aged oocytes were more likely to undergo germinal vesicle breakdown (GVBD) from germinal vesicle (GV) stage during the maturation culture period.[106] Faster developing immature oocytes showed an increased activation, a higher spontaneous fragmentation rate and a decrease in maturation promoting factor (histone H1 kinase) activity.[107] Moreover, the activity of histoneH1 kinase is related to the competency of the cytoplasm of the oocyte.[107] It can be seen that cytoplasmic changes affect oocyte quality when the meiotic arrest period is prolonged. Additionally, one study has shown that *in vitro* aged oocytes lost the microfilament-rich area over the meiotic spindle first, then disrupted spindle location and subsequently misplaced chromatin organization. These abnormal morphological changes of *in vitro* aged oocytes are similar with that occurring with in vivo ageing.[108]

The development of a three-dimensional (3D) matrix has provided a valuable *in vitro* model to investigate the regulation of folliculogenesis in prepubertal, young and older adult monkey.[7] In this study, compared with follicles from older adult monkeys, there was a larger percentage of secondary follicle survival from prepubertal and young monkey during the culture period. The lower surviving follicle rate obtained from older monkeys might be due to the preantral follicles dying as a result of lack of apoptosis inhibiting factor.[109] Alternatively, the activation of other factors responsible for follicle atresia during early follicular development increased as a result of the aging process.[110]

Taken together, the maturation culture period is important for oocyte maturation and subsequent successful fertilization. The mechanisms of prolonged meiotic arrest resulting in poor survival rate of follicles may be relevant to the declining reproductive ability of older women. These features of ovarian aging, including poor oocyte quality or decreased survival rate are frequently observed clinically in patients with advanced maternal age[7]

## CONCLUSIONS AND NEW PERSPECTIVES

It is widely accepted that high levels of cAMP within mammalian oocytes during IVM could sustain oocyte meiotic arrest and substantially synchronize nuclear and cytoplasmic maturation, thus improve oocyte developmental competence. The communication between oocyte and the surrounding cumulus cells via gap junctions diffuses the levels of cAMP and cGMP in supporting oocyte meiotic arrest and resumption. The cAMP levels are produced from the ATP by the activation of GPR with ADCY, which is continually supplied to the oocyte by cumulus cells via gap junctions. A large number of studies have suggested that pre-IVM step medium supplemented with cAMP modulator, such as CNP, acts as the main defense factor against 'spontaneous' maturation, which can regulate the period of nuclear maturation. One of the cAMP modulators, CNP, binding with its cumulus cell receptor NPR2, effectively regulates the levels of cGMP in order to maintain ideal concentrations of cAMP within oocytes. A relative high level of cAMP extends the duration of nuclear maturation, which improves the synchronization of cytoplasmic and nuclear maturation and contributes to further sustained acquisition of developmental competence of oocytes. The duration of nuclear maturation plays a critical role in supporting oocyte maturation and is a crucial factor that can enhance IVM efficiency.

A two-step IVM system with different cAMP modulators to regulate levels of cAMP during pre-IVM phase is needed. However, enhancement of IVM culture conditions is complicated as it depends not only on the use of different cAMP modulators, but also many other factors. Firstly, cAMP modulators can differently influence the oocytes according to developmental stage in different mammalian species. For instance, IBMX may effectively inhibit some mammalian oocytes resumption, however, it is not able to maintain pig oocyte meiotic arrest during the pre-IVM phase. The duration of the pre-IVM phase and the concentrations of cAMP modulator also need to be considered. Secondly, even though a large number of studies have concluded that two-step IVM system improve oocyte developmental competency, these COCs were retrieved from hormone-stimulated ovaries.

In current IVM protocols, serum, hormone and growth factors have been identified as the key factors, however, synchronization of nuclear and cytoplasmic maturation during IVM is also important for the proportion of oocytes matured to MII and further oocyte developmental competence. Regulation of cAMP during the pre-IVM phase may be a solution to the current discrepancies in the literature and may promote oocytes to acquire developmental competency, with particular attention paid to the effect on oocytes from older mothers.

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