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REVIEW ARTICLE

What is the optimal condition for fertilization of IVM oocytes?

Hiroaki Funahashi

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Abstract Application of in vitro maturation (IVM) is recently increasing for human infertility, especially to rescue patients of polycystic ovarian syndrome and ovarian hyperstimulation syndrome. To increase the application of IVM oocytes for embryo production and the efficiency of successful production of babies using IVM oocytes, quality control of oocytes and achievement of fertilization in the most suitable condition may be very important. In this paper, suitable conditions for fertilization of IVM oocytes will be discussed with recent knowledge about IVM and in vitro fertilization of oocytes in domestic animals. Currently, human oocytes are collected mainly from patients' ovaries 36 h following mild gonadotropin stimulation and used for IVM for 24–26 h. However, asynchronous progression of those oocytes to reach the metaphase-II stage may have occurred during the IVM culture. In the oocytes that have already progressed to the metaphase-II stage, sudden aging such as reduction in maturation promoting factor and MAP kinases will start to occur. Application of specific inhibitors of phosphodiesterase to control intracellular cAMP (cyclic adenosine monophosphate) level may be effective to synchronize timings of the germinal vesicle breakdown and consequently the meiotic progression of oocytes, and to improve the developmental competence. Furthermore, treatment of aging oocytes with caffeine appears to rescue them from reductions in maturation promoting factor and MAP kinases and to improve the developmental competence. Assessment methods to select oocytes with good quality may also be important to improve the successful rates.

Keywords Oocytes · IVF · IVM · Developmental competence · Aging

H. Funahashi (✉)
Department of Animal Science, Okayama University, Tsushima-Naka, Kita-Ku, Okayama 700-8530, Japan
e-mail: hirofun@cc.okayama-u.ac.jp; hirofun@okayama-u.ac.jp

Introduction

In human assisted reproductive technology (ART), application of the in vitro maturation (IVM) technology has been attempted [1] with oocytes collected from dominant follicles following gonadotropic stimulation [2–4] and is effective for patients who have polycystic ovarian syndrome and ovarian hyperstimulation syndrome. Although both the incidence of mature oocytes following IVM culture and the results of early development after fertilization have been improved [5], there are still many questions concerning IVM especially about cytoplasmic maturation to improve the efficiency of successful production of live babies by using IVM oocytes. Since the efficiency of IVM oocytes to develop to term following in vitro fertilization (IVF) or intracellular sperm injection (ICSI) appears to be lower than that of mature oocytes collected from gonadotropin-stimulated follicles, IVM has not become the main technology of assisted reproduction for infertile couples. In order to achieve successful fertilization and to acquire positive birth results using IVM oocytes, the oocytes are required to be fertilized in the most ideal state. The development of markers to select cumulus-oocyte complexes (COCs) with good quality at the time of collection as well as the culture of COCs in a suitable IVM condition may be useful to increase the efficiency. Furthermore, conditions to maintain the quality of IVM oocytes for IVF or ICSI is also important to improve the efficiency. In

domestic and laboratory animals, on the other hand, IVM of COCs is very common for in vitro embryo production. Information about IVM of oocytes has been integrated well, and IVM of COCs derived from non-stimulated middle size follicles of about 3–6 mm in diameter has been developed successfully [6, 7]. Therefore, the objective of the current paper is to provide a general review of possible methodologies for improving the quality of IVM oocytes and also about optimal fertilization conditions for obtaining the high quality of embryos, based on the knowledge mainly obtained by research on IVM–IVF of mammalian oocytes.

Oocytes as a material for IVM

The reproductive activity of women declines with age, rapidly beyond the mid-30s [8, 9]. However, the number of women attempting to have babies through ART beyond the mid-30s has increased drastically [10]. This problem is especially serious in Japan since donation of oocytes from others (younger women) is not permitted. Decline of oocyte quality with age has been pointed out to be associated with aneuploidy due to the age-dependent increase of meiotic errors [11, 12]. Recent microarray research in mice has shown that not only genes involved in chromatin structure, DNA methylation, genome stability and RNA helicases but also those involved in mitochondrial function and oxidative stress were altered between mature oocytes derived from young (5–6 week old) and old mice (42–45 week old) [13]. Mature oocytes have the largest number of mitochondria of any cells (approximately 2.56 to 7.95 $\times$ 10^5 mitochondria DNA copies in human [14, 15], 1.19 to 1.59 $\times$ 10^5 mitochondria DNA copies in mice [16, 17] and 2.60 $\times$ 10^5 mitochondria DNA copies in cattle [18]). Mitochondrial abnormalities and mutations are believed to contribute to reproductive aging [19]. Mitochondria injection into porcine oocytes has significantly improved fertilization rates following both IVF and ICSI [20]. Improvement or replacement of mitochondrial functions in oocytes derived from aging women could be required to obtain a good result in human ART. As a result, interest in age-related changes in mitochondrial function in oocytes is increasing [21, 22].

In contrast with human oocytes that are collected for IVM from dominant follicles [4] of patients who are relatively mature and have various backgrounds following gonadotropic stimulation [2, 3], those of domestic animals have been obtained as COCs from follicles of 3–6 mm in diameter on the surface of ovaries of females slaughtered just before puberty [6]. In domestic animals, a majority of follicles on the surface of ovaries are small, such as less than 2 mm in diameter in pigs [23], and oocytes derived from them are not fully grown yet so the incidence of mature oocytes following IVM culture is known to be lower than those from middle follicles [24, 25]. Developmental competence of oocytes also varies among the source, i.e., prepubertal, adult or aged donor females. Oocytes from prepubertal gilts appeared to be less meiotic and have lower developmental competences as compared with their adult counterparts [26–28]. Differences in the morphologies of oocytes from gilts and sows, such as the diameter of oocytes, thickness of the zona pellucida or the perivitelline space, are known to coincide with the oocyte developmental competence [29]. Integration of information about IVM of oocytes derived from various origins (e.g., size of follicles and the donor status) may make it possible to develop a new IVM methodology of oocytes from non-stimulated follicles of patients.

A possible marker to select oocytes with good quality

Brilliant cresyl blue (BCB) has been used to detect the activity of glucose-6-phosphate dehydrogenase (G6PD), a key enzyme in the pentose phosphate pathway which regulates the reaction from glucose-6-phosphate to 6-phosphogluconate. This blue dye is digested and the color disappears in the cells with a high G6PD activity. Application of BCB assessment to COCs as demonstrated is used to select oocytes with developmental competence [30–32], since there are variations in the G6PD activity among oocytes collected from middle follicles for IVM and the G6PD activity is negatively correlated with competence following IVM and IVF [33]. Furthermore, it has recently been reported that BCB activity of oocytes also appears to be negatively reflected in the mitochondrial DNA copy number of the oocytes [20]. Since injection of mitochondria into the BCB− (G6PD active) oocytes improves fertilization rate similar to the rates of BCB+ (relatively G6PD inactive) oocytes following IVF or ICSI [20], oocyte mitochondria content appears to be a direct indicator of the developmental competence of oocytes, as well as viability [34–36]. In fact, it has been reported that embryos containing relatively lower mitochondrial DNA copy numbers fail to develop during embryogenesis after embryo transfer [37]. Recently, we have found that higher RNA contents in cumulus cells of COCs positively reflected the result (BCB+) of staining selection of oocytes (Fig. 1) [38]. Since application of BCB staining selection for human ART may be avoided due to fear of potential toxicity threat to the oocytes, this selection method may be altered by another suitable method to reflect the selection results. RNA content in the cumulus cells bisected from COCs may be a good candidate for the alternative method. Therefore,

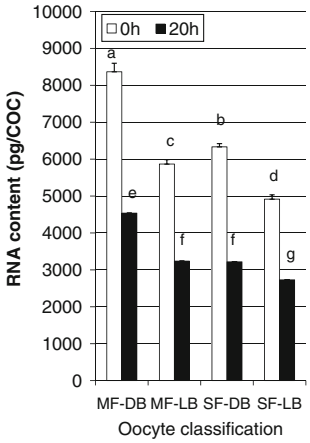

Fig. 1 Total RNA contents of cumulus cell mass from COCs classified by BCB assay at 0 and 20 h of IVM culture. COCs were collected from middle or small follicles (MF and SF, respectively) and then classified by BCB assay (*DB* dark blue showing a low G6PD activity, *LB* light blue showing a high G6PD activity). RNA contents were assessed before or after IVM culture with gonadotropins and dibutyryl cAMP for 20 h. $^{a-g}P < 0.05$

this methodology may valuable in selecting the COCs that have ability to achieve meiotic maturation and developmental competence.

Oocyte-secreted factors associated with developmental competence of oocytes

Recently, it has been reported that two oocyte-secreted paracrine factors, growth-differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15), are promoting their own cytoplasmic maturation and developmental competence through *Sma*- and *Mad*-related (SMAD) signaling pathways to regulate the function of cumulus and granulosa cells [39–42]. Although the apoptosis of cumulus cells was increased when the cumulus cell mass was cultured in the absence of oocytes, this was reversed by exposing the cumulus cell mass to denuded oocytes [43]. Oocyte-secretion factors also appear to stimulate growth [44], and prevent luteinization of their cumulus cells [45, 46]. Furthermore, oocyte-derived factors appear to be associated with the promotion of glycolysis [47], EGF receptor expression [48] and sterol biosynthesis [49] in cumulus cells. Thus, cumulus cells stimulated by gonadotropins appear to be maintained in a healthy state by oocyte secretions, and sufficient materials from the cumulus cells could be also essential for successful cytoplasmic maturation of oocytes [45]. Healthy auto-regulatory loop mechanisms between oocytes and cumulus cells [42] and clarification of the detailed mechanism to control cytoplasmic maturation will permit the improvement of the developmental competence of oocytes.

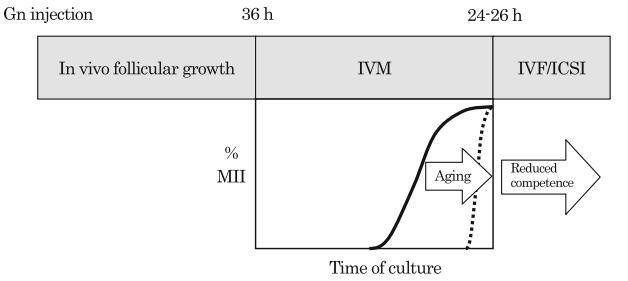

Fig. 2 Schematic illustration of the relation between asynchronous meiotic progression and the aging of oocytes. Asynchronous maturation of oocytes produces aged oocytes with reduced developmental competence at the time of IVF/ICSI (*black line*). A suitable IVM culture system to achieve synchronous meiotic progression (*dotted line*) will increase the quality of oocytes

Table 1 Effect of cAMP modulating agents during the first half of IVM

Agents	Species	Effect on embryo development	References
cAMP analogues	Porcine	Improved	[51]
Invasive adenylate cyclase	Bovine	Improved	[53]
	Bovine	Improved	[54]
PDE inhibitors	Murine	Improved	[55]
	Human	Modestly improved	[56, 57]

Asynchronous meiotic progression of oocytes during IVM

Commonly, for commercial IVM in human ART clinics, COCs are aspirated from never or gently stimulated ovaries and then cultured for 24–48 h in vitro [50]. In human, oocytes have usually been collected from patients around 36 h following mild stimulation with gonadotropins and then cultured for IVM during 24–26 h before IVF or ICSI. However, the efficiency of IVM, in terms of embryo development, implantation and live birth rates is lower than that of conventional IVF with oocytes matured in vivo [42]. In the conventional IVM culture system of domestic animals, the timing of these oocytes to reach the metaphase-II stage is known to be asynchronous [51]. Oocytes that have already reached the metaphase-II stage will suddenly experience aging effects, such as reduction in maturation promoting factor and MAP kinases [52]. Therefore, an IVM culture system to achieve synchronous meiotic progression of oocytes will reduce the incidence of aged oocytes at the time of IVF/ICSI (Fig. 2). Since both the synchronous meiotic progression of oocytes and developmental competence have been improved in biphasic IVM such that a cAMP analog, dibutyryl cAMP, was

supplemented with gonadotropins during a first half-period of IVM culture only [51], various agents modulating intracellular cAMP level [53–57] have been applied to IVM systems and were found to be effective (Table 1). Supplementation of IVM medium with FSH plus milrinone, a specific inhibitor of type 3 PDE, or rolipram, a specific inhibitor of type 4 PDE, maintained the relative intracellular gap junctional communication and significantly improved the early development of the oocytes to the blastocyst stage following IVF [55]. In human oocytes, the presence of a specific inhibitor of type 3 PDE, cilostamide, in IVM medium synchronized GV morphology [58] to the similar phase, which was observed in porcine oocytes following dibutyryl cAMP [51]. Since type 3 PDE is principally expressed in the oocytes but not in the cumulus cells, use of a type-3 PDE inhibitor may target intracellular cAMP of oocytes [42]. In fact, the presence of cilostamide maintained gap junctional communication between cumulus cells and oocytes 6 h longer than controls [59]. Although supplementation of IVM medium with cilostamide and forskolin did not improve the incidence of blastocyst formation following IVF of the IVM oocytes (17.6 vs. 5 % in control, $p < 0.066$), the developmental competence has been significantly improved by pre-IVM culture for 1–2 h with forskolin and IBMX before extended IVM in the presence of cilostamide and FSH in cattle [60]. Less successful response of COCs against type 3 specific PDE inhibitor and gonadotropins in humans may be overcome by using pre-IVM culture for 1–2 h in the presence of regulator(s) of intracellular cAMP and extended IVM, as it has in cattle.

Aging of IVM oocytes

Mature oocytes are usually penetrated by sperm within 12 h in various species and within 12–14 h in humans after ovulation [52, 61]. As described above, oocytes that have reached the metaphase-II stage will soon begin aging and decrease in quality over time. The morphological changes during aging have been well reviewed [52, 61]. In aging oocytes, failures in plasma membrane reaction to sperm penetration and in the second metaphase spindle morphology have been observed [61]. Oocyte aging is one of the largest obstacles to overcoming ART failures. Application of specific inhibitors of phosphodiesterase to control intracellular cAMP level may be effective in synchronizing the timing of the germinal vesicle breakdown, and consequently the meiotic progression, of oocytes and in improving the developmental competence. Furthermore, treatment of aging ovine oocytes with caffeine appears to rescue them from reductions in maturation promoting factor and MAP kinases and improves developmental competence. In addition, when denuded mature oocytes were exposed to about 10 mM caffeine, the loss of oocyte quality due to spontaneous aging was prevented for 6 h, and consequently improved the incidences of normal fertilization and development to the blastocyst stage. Therefore, exposure of mature oocytes to caffeine may be effective in preventing aging of oocytes following collection.

IVF conditions affecting the quality of oocytes

To obtain IVF embryos of the highest success potential, the most ideal conditions for fertilization with good quality of oocytes and sperm are required. Failures in male pronuclear formation, polyspermy and early development have been observed in domestic animals when oocytes of less than ideal quality were used [6, 62, 63]. Since glutathione content in IVM porcine oocytes decreased drastically if cumulus cells were removed, the oocytes may not be strong against oxidative stress. In fact, intracellular glutathione content was lower in denuded oocytes than cumulus-enclosed ones.

In conventional IVF system, in which 10–50 oocytes were co-cultured with a number of sperm (1 to 10 $\times$ 10^5 cells/ml) in a small-volume droplet (about 50–100 μl), a relatively large number of sperm flock around the oocyte at one time and try to enter into the oocyte simultaneously. It is possible that the efficiency of successful penetration is reduced by oxidative stress from near dead and dying sperm. Efforts to reduce the oxidative stress during IVF may be one of the important factors for achieving the best fertilization conditions.

Higher rates of normal fertilization and blastocyst formation were obtained after IVF in the new system using the microfluidic sperm sorter than in a conventional system. Supplementation of IVF medium with cysteine for 3 h after ICSI improved both incidences of blastocyst formation and litter size from the embryos. The regulation of the number of sperm around the oocyte during IVF and the reduction of oxidative stress at the time of fertilization may be important conditions for obtaining better results of early development of IVM oocytes following IVF/ICSI.

In IVF and IVC involving porcine oocytes, we have studied the effect of a strong reducing agent, beta-mercaptoethanol, on sperm penetration and early development [64]. Interestingly, in the presence of beta-mercaptoethanol, sperm capacitation and the spontaneous acrosome reaction were partially inhibited even in an IVF medium containing caffeine [64]. If sperm were co-cultured with oocytes in beta-mercaptoethanol-free IVF medium containing caffeine for 30 min and then the oocytes binding with sperm on the zona pellucida were transferred to caffeine-free IVF medium containing beta-mercaptoethanol (biphasic IVF method), the statement of cortical reaction, the incidence of monospermic

penetration and the quality of blastocyst formation were significantly improved [65]. Therefore, the reduction of oxidative stress at the time of fertilization, except during sperm capacitation, should be very important to maintain the quality of the oocytes, to normalize both the reaction of oocytes at sperm penetration and, consequently, to result in a high production of normal embryos and babies.

In conclusion, use of oocytes with a high degree of development competence should be essential for obtaining a good result in human ART. For effective selection of the oocytes, the amount of total RNA in the surrounding cumulus cells may be a useful marker since the content reflects the result of BCB assay, which has been shown to relate with the mitochondrial DNA copy number. Analogues of cAMP or PDE inhibitors will be effective in synchronizing the meiotic progression of oocytes. To prevent aging of mature oocytes, caffeine may be effective in the maintenance of MPF and MAPK activities. Biphasic IVF methods may also be beneficial for reduction of oxidative stress during fertilization.

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