

Oocyte quality and aging

Ali Reza Eftekhari Moghadam^{1,2}, Mahin Taheri Moghadam², Masoud Hemadi², Ghasem Saki²

¹Cellular & Molecular Research Center, Medical Basic Sciences Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

²Department of Anatomical Science, Faculty of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

ABSTRACT

It is well known that female reproduction ability decreases during the fourth decade of life due to age-related changes in oocyte quality and quantity; although the number of women trying to conceive has today increased remarkably between the ages of 36 to 44. The causes of reproductive aging and physiological aspects of this phenomenon are still elusive. With increase in the women's age, during Assisted Reproductive Technologies (ART) we have perceived a significant decline in the number and quality of retrieved oocytes, as well as in ovarian follicle reserves. This is because of increased aneuploidy due to factors such as spindle apparatus disruption; oxidative stress and mitochondrial damage. The aim of this review paper is to study data on the potential role of the aging process impacting oocyte quality and female reproductive ability. We present the current evidence that show the decreased oocyte quality with age, related to reductions in female reproductive outcome. The aging process is complicated and it is caused by many factors that control cellular and organism life span. Although the factors responsible for reduced oocyte quality remain unknown, the present review focuses on the potential role of ovarian follicle environment, oocyte structure and its organelles. To find a way to optimize oocyte quality and ameliorate clinical outcomes for women with aging-related causes of infertility.

Keywords: oocyte quality, aging, ovary, female infertility

INTRODUCTION

The human female reproductive system has a faster rate in aging than any other system of the body. According to population studies and reports the biological ability of a woman to fertilize decreases with age, similar to other species (Wood, 1989). The number of women delivering their first child after the age of 35 increased from 1/100 to 1/12 from 1970 to 2006 (Cil *et al.*, 2015). The fecundity capacity of females peaks in their 20s, it fails in late 30s, in defiance of regular menstrual cycles, ending in menopause at the mean age of 50 - 51 years (te Velde & Pearson, 2002). The *in vitro* fertilization (IVF) study shows that the mother's age is an essential cause that leads to impressing clinical results (Tan *et al.*, 2014). While, between 1970 and 2002, the percentage of first births in women over the age of 30 years increased 6-fold (Crawford & Steiner, 2015). According to an American report based on >120,000 assisted reproduction technologies (ART), successful delivery rate per embryo transfer dropped from 43.2% in women <35 years old to 15.1% in women aged 41-42 years and to 5.9% in women >42 years old (Wright *et al.*, 2005). Today, in developed countries, because of the women's decision to delay their conception, families faced female aging-related infertility (Balasch, 2010). Despite neuroendocrine and uterine factors, it is well defined that ovaries and ovarian follicles are prominent regulators of reproductive aging. In humans, changes to the ovarian follicle pool and diminishing of ovarian reserve are the chief causes

of decline in female reproduction ability (Faddy, 2000). This claim is confirmed by oocyte donation from younger women, in overcoming age-related infertility (Sauer *et al.*, 1990; te Velde & Pearson, 2002). By the fifth month of prenatal development, the size of ovarian follicle pool is estimated at 7 million oogonia (Sadler, 2011). At this time large numbers of oogonia, as well as primary oocytes become atretic, except for a few near the ovarian surface. The surviving oogonia enter the prophase of first meiotic division and transform into primary oocytes, and are surrounded by flat epithelial cells to forming the pool of primary follicles (Pepling, 2006). Primary oocytes stay arrested in prophase in the so-called diplotene stage and do not complete their first meiotic division before puberty is reached. At birth, one million of primordial follicles are present, a number which will disappear by atresia. During childhood, this number reaches the amount of approximately 300,000 at the time of menarche (Faddy *et al.*, 1992). Over the past decades there has been controversy about the origin of ovarian germ cells and oogenesis during one's life span, which indicates the neo-oogenesis in mice ovaries and adult humans' (Bukovsky, 2011; de Souza *et al.*, 2017). During the female reproductive life span, under the influence of intra and extra ovarian factors, such as cytokines, growth factors and gonadal steroid follicles leave the resting pool to enter the growing pool on a regular manner (Pangas, 2007). Therefore, follicles behave differently in response to such stimulatory and regulatory signals (Craig *et al.*, 2007). As a result of the ovarian follicular pool diminishing exponentially with age, decline is more prominent at the age of 38-39 and above (Faddy *et al.*, 1992).

Therefore, according to the observations mentioned above, assessment of cellular and molecular aspects of follicles/oocytes need precise attention to some factors. First, some oocytes and their surrounding cells remain in quiescent phase (diplotene stage) for ~40 years or more, it is unknown how this stage can retain the cells against environmental influences (Absalan *et al.*, 2017; Sadler, 2011). The fact that the increase in maternal age is directly related to chromosomal abnormalities in the fetus confirms that primary oocytes are susceptible to damage as they age (Sadler, 2011). Second, the growth of a mature oocyte depends on fine cross-talk signalling between all ovarian compartments. Age-related infertility is a multifactorial process and understanding factors affecting follicle/oocyte aging requires the comparison of the theories on aging mechanisms based on studies on tissues and organs other than the ovary (Hamet & Tremblay, 2003). Ultrastructural changes in oocytes, such as meiotic spindle disruption and zona pellucida hardening are some of the factors on age-related declined fecundity (Eftekhari Moghadam *et al.*, 2018). In addition, higher tendency for acquired conditions such as uterine problems, hormonal changes, endometriosis, fibroids and pelvic infections occur in aged women (Schwartz & Mayaux, 1982). The most relevant theory for ovarian aging, conveys a reduction in oocytes and its surrounding cells' capacity to nullify reactive oxygen species (Sousa *et al.*, 2015), which is the most essential cellular

damage inducer with aging (Harman, 2006). Loss of active DNA repair apparatus, bioenergetics status and mitochondrial damage of the oocytes are possibilities for follicle/oocyte aging phenomenon (Titus *et al.*, 2013). Other feasible mechanisms, like molecular pathways that could lead to loss of germ cells in aged ovaries, are poorly defined (Ben-Meir *et al.*, 2015).

In this paper, we review studies on human and mice models about the basic age-related aspects of follicles/oocytes, ovarian microenvironment and involvement of oxidative stress in progressive reproductive aging.

OVARY AGING

The ovarian tissue, like every organ of the body undergoes aging with no exclusion. Every day, in an ovary, a certain number of primordial follicles are engaged from resting follicular pool (ovarian reserve) for more growth, a process called folliculogenesis. This action, takes approximately 16 – 24 weeks, during which time the oocyte and its encompassing somatic cells endure a string of changes that finally result in an antral follicle to release a mature ovum (Baird *et al.*, 2005). Primordial follicle recruitment ratio raises with age and it is reciprocally related to ovarian reserve (Orisaka *et al.*, 2009). Ovarian follicles are essential for keeping endocrine function of female gonads, as well as fertility accordingly, the number of follicles present in the tissue control ovarian lifespan (Tilly & Sinclair, 2013). In older women, it appears that follicles enter precociously the growing phase in contrast with their younger counterparts. This condition may illustrate the quantitative and qualitative features of follicle aging (Westergaard *et al.*, 2007). Based on research, the increased FSH serum level, reported to occur in the early follicular phase during reproductive aging, increases the final reduction in ovarian follicular reserve (Richardson & Nelson, 1990; te Velde & Pearson, 2002). However, studies on transgenic mice models with rising FSH serum levels show that elevation of this hormone decreases the proportion of good fertilized oocytes without significant alteration in ovarian follicle pool (McTavish *et al.*, 2007), therewith indicating the intricate role of this hormone in ovarian aging-related changes. Anti-Müllerian hormone (AMH) is a 140kDa glycoprotein that does not belong to the pituitary-gonadal axis, secreted by granulosa cells of growing non-selected follicles. The serum levels accurately reflect the size of antral follicles' pool, representing the quantity of the remaining primordial follicles. AMH is the best currently available and effective assay for ovarian reserve in several clinical cases. In addition, AMH has a role in predicting reproductive lifespan, ovarian dysfunction and the impact of gonadotoxic cancer treatment, or ovarian surgery (Broer *et al.*, 2014). There is a positive relation between follicular fluid AMH concentrations and embryo implantation rates, but its correlation with the qualitative aspects of follicle aging remains to be elucidated (Fanchin *et al.*, 2007).

Based on a central dogma, irreversible age-alteration in ovarian reserve in mammals depends on the lack of proliferative germ cells in adult ovarian tissues. However, there are new studies in rodents and humans offering the evidence of germline stem cells (GSCs), and consequently generating oocytes to form new follicles in adults -a process called neo-oogenesis (White *et al.*, 2012). Therefore, these paradigm shifting evidences support GSCs as arguments on how female fertility and ovarian lifespan might be regulated in mammals.

OOCYTE CHARACTERISTICS IN AGING

Here, we provide a comprehensive discussion on morphological criteria related to maturation competence of oocytes for understanding their role in the age-related

pathophysiology of human oocytes, including the oocytes and their extracellular compartments and cells.

Cumulus Cells

The cells immediately encompassing oocyte are called Cumulus Cells. These cells accompany the oocyte throughout development from an immature to a fully mature ovulated oocyte, and play a central role in supporting the oocyte, whether *in vivo* or *in vitro*. There is a bilateral dependency between the two-cell compartments of the cumulus-oocyte complex (COC). Indeed, the cumulus cells rely on the oocyte for their normal differentiation, regulation, and functions through intercellular interaction gap junctions and paracrine signaling (Gilchrist *et al.*, 2004; 2008). In addition, the direct interaction with COCs appears to depend on members of transforming growth factor- β (TGF- β) – a family of signaling molecules (Knight & Glister, 2006). The cumulus-oocyte complex is embedded in a hyaluronic acid-rich extracellular matrix (ECM) which are secreted by them. This matrix is essential not only in ovulation but also for the fertilization process (Knight & Glister, 2006). In the pre-ovulatory follicle, the cumulus cells are firmly packed around the oocyte, because the follicle is stimulated by luteinizing hormone (Hemadi *et al.*, 2009) before ovulation, the cumulus cells producing ECM become much less tightly packed by the time of ovulation. The ovulated oocyte is still restricted by a number of cumulus cells called corona radiata. In the ampullary portion of the uterine tube ala, the place where the spermatozoa first encounter the oocyte, they are confronted by the corona radiata, and some of cumulus cells. Recent studies suggest that mammalian spermatozoa have odorant receptors as similar to olfactory receptors in the nose, so they can respond to chemically specific odorants (Spehr *et al.*, 2006).

The COCs collected from mature ovarian follicles are categorized based on compactness of the cumulus and appearance of oocyte cytoplasm (de Wit *et al.*, 2000). For instance, the quality of bovine COCs are classified in three groups:

1. Class A COCs. Five layers of dense and compact cumulus cells enclose the oocyte and the cytoplasm of oocytes, it is homogeneous, transparent with a dark ring encircling the cytoplasm.
2. Class B COCs. Less compact cumulus cells along with an oocyte that has a dark and moderately granular cytoplasm.
3. Class C COCs. Expanded cumulus cells and oocyte with a dark and granular cytoplasm (Mayes & Sirard, 2001) (Figure 1). The cumulus cells' layers are a remarkable factor in determining oocyte quality (Hemadi *et al.*, 2009), and the oocytes which belong to the group B of COCs classification showed a better developmental ability when compared to other groups (B > A > C) (Boni *et al.*, 2002).

An aged follicular microenvironment could highly affect oocytes and put a specific transcriptional footprint in the surrounding CCs (Al-Edani *et al.*, 2014). New evidence shows that female age may have influence on the gene expression profiles of CCs that are crucial for oocyte quality and competence (McReynolds *et al.*, 2012). For the first time as Al-Edani *et al.* (2014) showed that the angiogenic genes were notably overexpressed compared to patients of the younger groups. In contrast, the genes involved in TGF- β signaling pathway such as AMH, TGF- β 1, inhibin, and activin receptor were under-expressed (Al-Edani *et al.*, 2014). Miao *et al.* (2005), hypothesis suggests that CCs would accelerate the progression of *in vitro* aging of mice oocytes. Also, they assessed the effects of CCs on the susceptibility to activating stimuli, maturation promoting factor (MPF) activity, exocytosis of cortical granules (CGs), and anaphase onset during aging process of both *in vivo* and *in vitro* matured mice oocytes. They concluded that

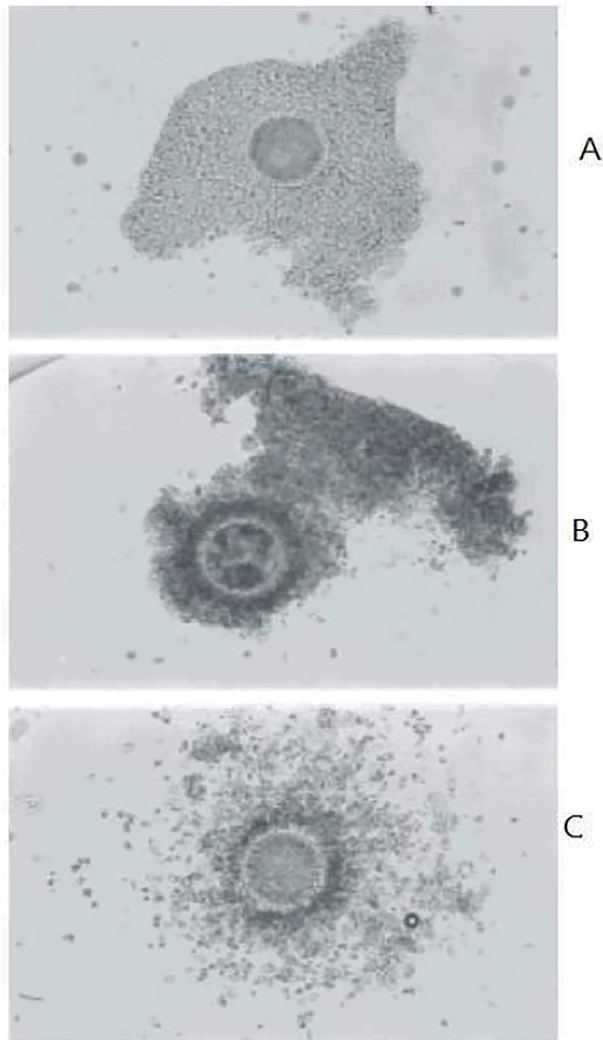


Figure 1. Different classes of COCs (de Wit et al., 2000). Class A. Oocyte is surrounded by a compact cumulus with a homogeneous cytoplasm. Class B. Oocyte is surrounded by a less compact and darker cumulus. The cytoplasm is dark and moderately granular. Class C. Oocyte is surrounded by dispersed cumulus cells. Oocyte has a granular cytoplasm. Reproduced from Lasienne et al. (2009) with permission.

CCs accelerated the aging progression of both *in vivo* and *in vitro*-matured mice oocytes (Miao et al., 2005).

Zona Pellucida

Zona pellucida (ZP) is a glycoprotein interface among oocytes and their surrounding cells that coat and protect the mammalian ovum. In humans, it comprises four glycoproteins-hZP1 to hZP4 (Lefièvre et al., 2004). ZP2 and ZP3 combine to form basic units that polymerize into long filaments. These filaments are periodically linked by cross-bridges of ZP1 and ZP4 molecules. But, the mice ZP is composed of three major glycoproteins: mZP1, mZP2, and mZP3 (Wassarman et al., 2005). In the mice, the acrosome reaction inductor and the sperm receptor is the glycoprotein ZP3, a component of all mammalian oocyte ZPs (Wassarman, 1990). Because of difficulties with direct observation of the human oocyte, there has been a large number of investigations exploring the molecular mechanism of fertilization in mice. It is estimated that the ZP of

unfertilized mice oocyte include more than 1 billion copies of the ZP3 protein (Carlson, 2018). In mammals, there is less species alteration in ZP3 composition; this may illustrate why penetration of the zona pellucida by spermatozoa of nearly related mammalian species is sometimes feasible, whilst it is rare amongst lower beasts (Carlson, 2018). The normal maturation of follicles and oocytes rely on presence of the ZP (Rawe & Combelles, 2009). The zona pellucida plays an essential role in the sperm-oocyte interaction, acrosomal reaction, sufficient block to polyspermy, preimplantation embryos and following troubles in developmental progression (Ebner et al., 2008). After penetrating the corona radiata, the spermatozoa attach firmly to the ZP via plasma membrane of the sperm head. Spermatozoa binds in a specific manner to a sialic acid molecule, which is the terminal part of a sequence of four sugars at the end of an O-linked oligosaccharides that are attached to the polypeptide core of the ZP3 molecule (Carlson, 2018). Ultrastructural study of mice ZP by conventional scanning electron microscopy (SEM) (Figures 2 and 3) shows various patterns in its outer and inner surfaces. The external surface is defined by the presence of abundant fenestrations, giving it a somewhat spongy configuration, whilst inner surface is smooth and condensed (Phillips et al., 1985). Two main forms of zona pellucida are identified as a mesh-like (spongy structure) ZP in mature oocytes and a more compact (smooth surface) one in atretic or immature oocytes (Familiari et al., 2006). Studies have revealed a direct association between the ZP surface structure and the grade of oocyte development and (Sousa et al., 2015), hence it can be a valid predictor of oocyte quality (Calafell et al., 1992). Familiari et al. (2006) postulated that the condensation of the outer side of the ZP causes sperm-binding site confusion, which would cause decreased sperm binding and reduced penetration ability, so it goes to *in vitro* oocyte fertilization disability. Calafell et al. (1992) reported four types of ZP in mice oocytes, aged *in vivo* or *in vitro* from immature, young, and elderly females, termed A, B, C, and D. Type-A oocytes have a structure observed in prepuberal females that may be related to immature oocytes. Type B was the usual type of the ZP between the groups reported by these authors and its rate decreases with advancing age. The A/B type ZP was specified by abundant large pores with fully cytoplasmic and matured nuclear and freshly ovulated oocytes. Type C was observed before degeneration and raise conspicuously with increasing female age (both *in vivo* and *in vitro*). This concept is suggesting that there is a direct relation among the properties of the ZP fertilization ability of the oocyte. Type D with amorphous appearance, seemingly corresponded to degenerated oocytes, because the surface was similar to that seen in fragmented eggs (Familiari et al., 2006). In routine clinical intracytoplasmic sperm injection (ICSI) trials, it is possible to recognize the ZP alterations. In spite of IVF, removal of cumulus cells enables clinicians to assess the ZP variants such as thickness, appearance, irregularities and composition (Kilani et al., 2006). We know that the thicker ZPs are related to fertilization, implantation and fecundity rate (Figure 4) (Bertrand et al., 1995; Rama Raju et al., 2007). The zona pellucida of fresh oocytes consists of granulofibrillar, interrelated reticulum with pores; whereas the zona pellucida of aged oocytes have 'cobblestone-like' cover, which is composed of narrow clusters of granulofibrillar material, apart from one another by an obvious space (0.3mm in width). Zona pellucida hardening (explained increased time to enzymatically digest the ZP and decreased sperm binding and penetration) evaluations have shown that the time for chymotrypsin-mediated dissolution of the zona pellucida increases notably in aged oocytes, compared with fresh oocytes (Miao et al.,

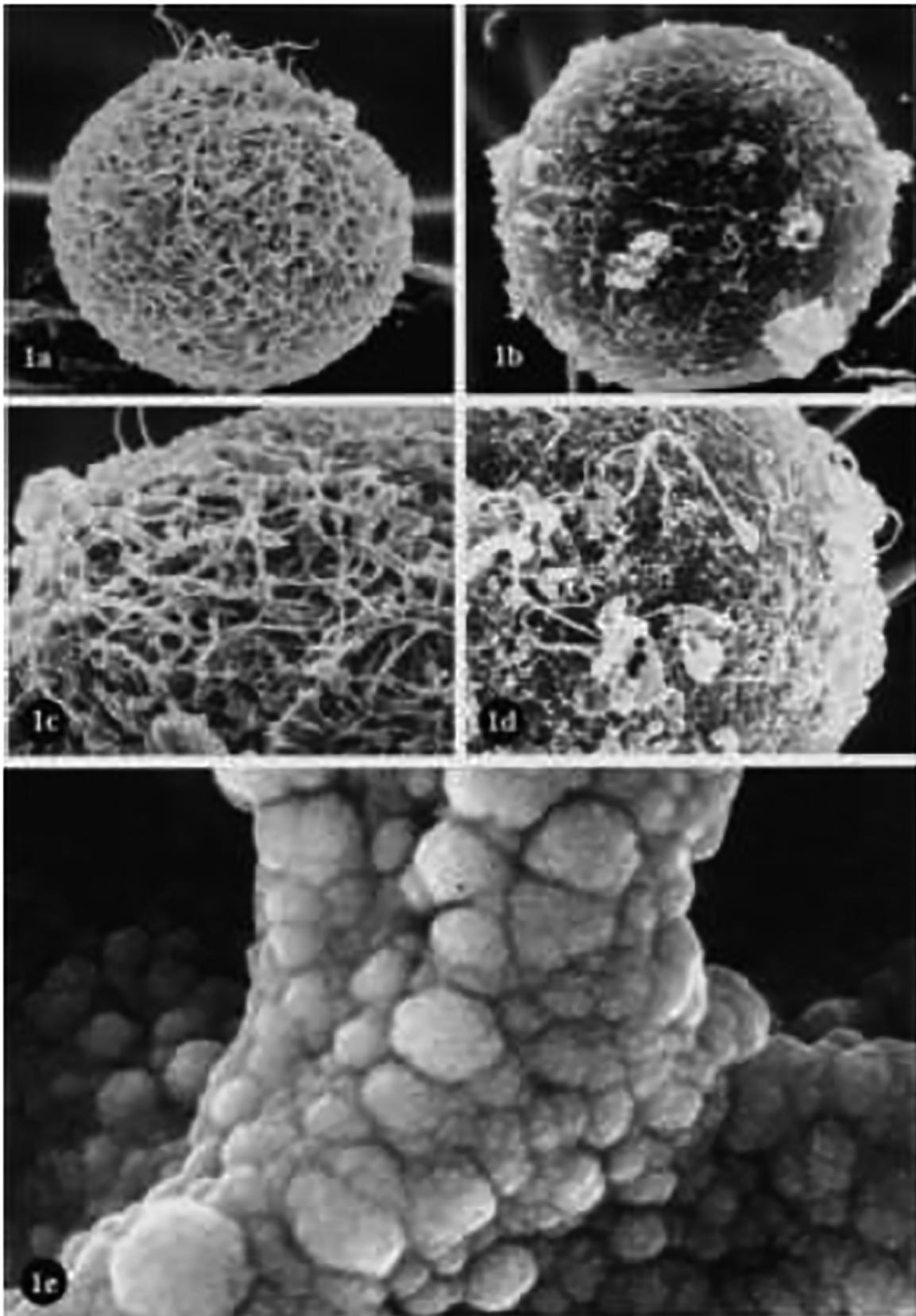


Figure 2. Conventional SEM. (a): Unfertilized human oocyte. Porousnet appearance of ZP (X 2,000). (b): Unfertilized human oocyte. Compact and smooth surfaced ZP (X 2,000). (c): Higher magnification of (a). The spongy ZP structure is evident (X 4,000). (d): Higher magnification of (b). It shows a dense and compact ZP structure (X 4,000). (e): Unfertilized mouse oocyte with very high magnification showing a branch of the spongy structure of the ZP (X 350,000). Familiari *et al.* (2006) with permission.

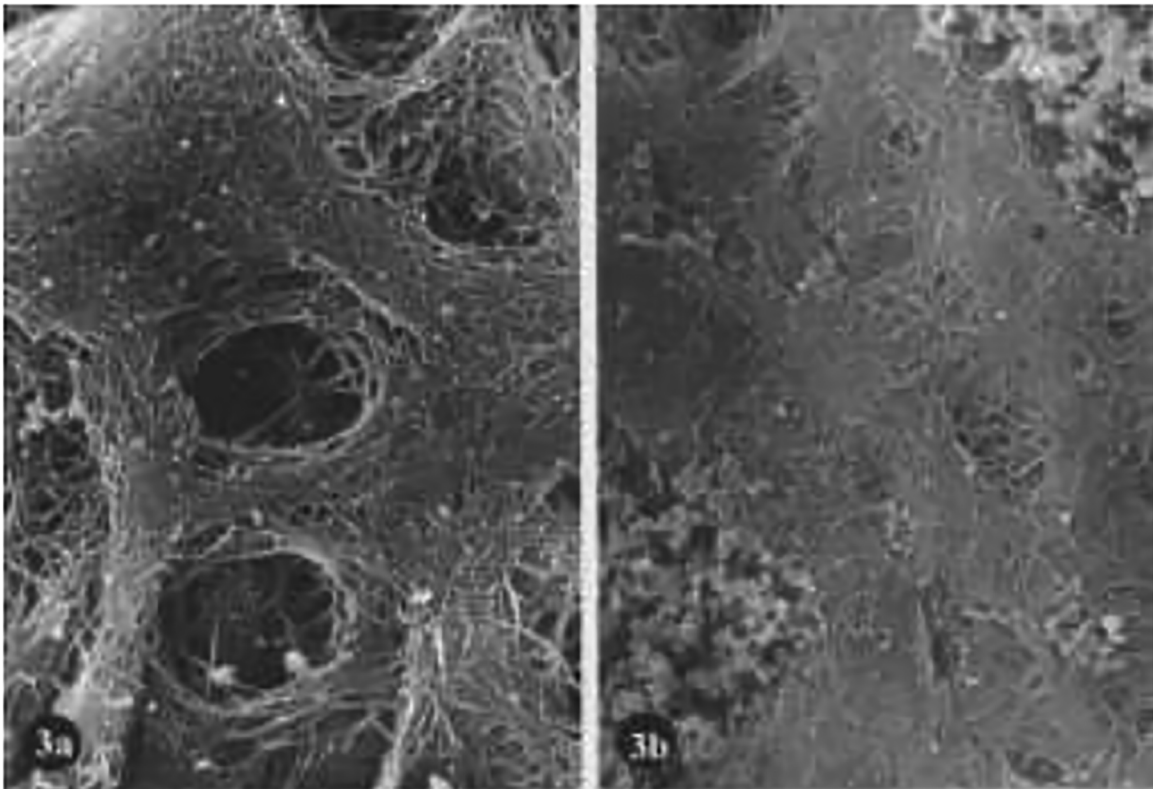


Figure 3. (a): The outer surface of the ZP of a human mature oocyte. Many fenestrations are present in which the filaments form a large meshed network (X 9,000). (b): The outer surface of the ZP of a human atretic oocyte. The filaments form a tight meshed network (X 9,000). Familiari et al. (2006) with permission.

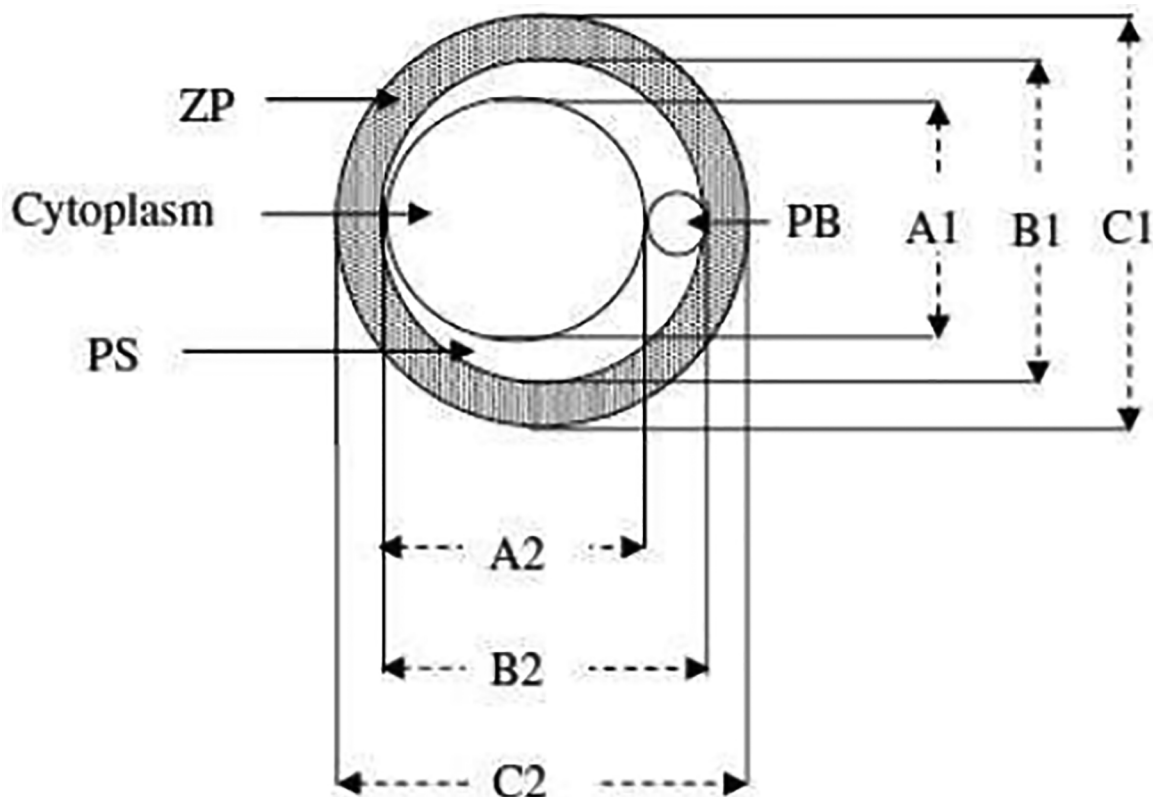


Figure 4. An ideal oocyte which displaying good spindle retardance and length, trilaminar structure of zona with good retardance of inner layer. Magnification $\times 200$. Reproduced from Rama Raju *et al.* (2007) with permission.

2005). In the clinic, assisted hatching (AH) of the ZP is sometimes applied to help the embryo escape its ZP. Some environmental agents such as non-physiological and prolonged culture conditions can lead to irreversible chemical changes or Zona hardening. Despite such knowledge, we need to increase our understanding about age-related abnormalities in zona morphology and their possible clinical importance (De Vos & Van Steirteghem, 2000).

Perivitelline Space

The perivitelline space (PVS) demonstrates that the acellular portion between the plasma membrane of the oocyte (oolema) and the ZP PVS contains a hyaluronan-rich extracellular matrix that is not visible under light microscopy. In a mature human oocyte, it is fluctuating in size, and evident with the expelled polar body located in its most eminent part (Figure 5). PVS is one of the most significant parts of extracytoplasmic maturation. There are three kinds of PVS classifications that are possible: immediately opposed to the plasma membrane, apart and recognizable, exaggerated (Rawe & Combelles, 2009; Talbot & Dan-dekar, 2003). Despite these differences, there is a clear disagreement that still remains over PVS arrangement regarding what may be typical or deviant from healthy. PVS-size is important as it affects the prevalence of the polyspermy, fertilization rate and pronuclear morphology (Yoshida & Niimura, 2010). A retrospective study shows a direct relationship between PVS width and reduction in fertilization and embryo quality, while others reported no similar relevance (Plachot *et al.*, 2002). Ten *et al.* showed no PVS effect on fertilization, but oocytes with large PVS were associated with higher likelihoods of yielding good-quality embryos (Ten *et al.*, 2007). Anyway, there is no agreement as to whether PVS evaluation alone may influence oocyte

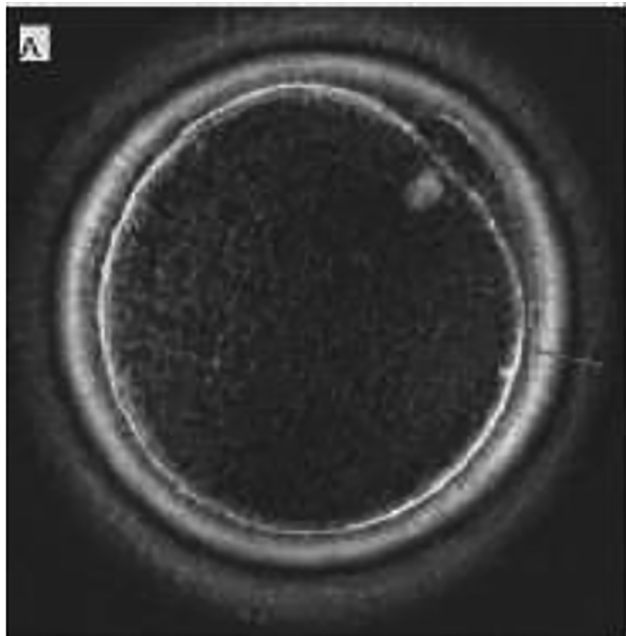


Figure 5. Calculation method for measuring the size of each part of the oocyte. ZP: Zona pellucida. PS: perivitelline space. PB: 1st polar body. Diameter of cytoplasm (A) $(A1+A2) / 2$. Inner diameter of zona pellucida (B) $(B1+B2) / 2$. Outer diameter of zona pellucida (C) $(C1+C2) / 2$. Thickness of zona pellucida $(C B) / 2$. Size of perivitelline space $(B A) / 2$. Yoshida & Niimura (2010) with permission.

quality. In another study on hamsters by Li *et al.* (2017), they reported that old hamsters produced a meaningfully lower percentage of healthy oocytes with minimal PVS, compared with those in younger hamsters (Figure 6). They also gathered that a large PVS has been related to lower fertilization rates in humans (Rienzi *et al.*, 2008). The reason for the high occurrence of polyspermy after insemination in oocytes with small perivitelline space is vague. The precise source of the PVS chemicals remain unknown, although the cumulus cells and oocytes maybe candidates. Hyaluronic acid (HA) has been shown to have an inhibitory impact on oocyte plasma membrane fusion. Therefore, it is believed that large amounts of HA in the expanded PVS may hinder the membrane fusion of sperm and oocytes, resulting in a lower rate of polyspermy in oocytes with larger PVS (Yoshida & Niimura, 2010). Finally, Yoshida & Niimura (2010), in rodents, reported that, oocytes with smaller perivitelline space show higher incidence of polyspermy after insemination, and suggested the size of PVS, whereas, evidence confirmed the presence of cortical granule material in the PVS, in addition to creation of cortical granule envelope inside the PVS. In this manner PVS can impact sperm penetration and appropriate block to polyspermy. However, Yoshida & Niimura (2010) showed that the diffusion of cortical granules in some rodents with smaller PVS is similar to those with larger PVS. They discussed the higher frequency of polyspermy in oocytes with small PVS, which is not caused by the lack of cortical granules in those oocytes. Because of the lack of knowledge, we can understand that a large PVS may break the connection between the cumulus cells and the oocyte, specifically by means of transzonal projections and gap junctions. On the other hand, it may be useful to disconnect some ways of cell communication at the suitable time, and there may be a mechanism by which PVS controls oocyte competence (Rawe & Combelles, 2009). Overall, morphological assessments of the three extracellular parts of the oocyte yields a definite measure of oocyte evaluation while not freely rendering predictors of oocyte quality. According to the intricacy in morphological diversity, we still require much research to detect and recognize these aspects, and then in determining their clinical importance. Randomized controlled experiments may then test the impact of aging on extracytoplasmic evaluation on fecundity yields.

INTRACYTOPLASMIC COMPONENTS

Polar Body

Oocytes from mammalian species is arrested in the prophase meiosis I (MI). At puberty, a surge in luteinizing hormone [LH] impels a preovulatory growth phase and MI is complete, resulting in the formation of two unequal size cells with 23 chromosomes. One cell receives most of the cytoplasm (secondary oocyte), and the other cell, that extruded between the ZP and the plasma membrane of the secondary oocyte in the form of a first polar body (PBI), has no function. Then the oocyte enters the metaphase meiosis II (MII) stage, almost 3 hours before ovulation (Rawe & Combelles, 2009). Eichenlaub-Ritter *et al.* (1995) reported that the PBI morphology revealed the postovulatory age of the oocyte. Several studies have revealed that the PB shape, size and contour can help anticipate oocyte quality, but other documents show that there is no association between PB properties and oocyte developmental ability or genetic form for the embryo (Ciotti *et al.*, 2004; De Santis *et al.*, 2005). In a study by De Santis *et al.* (2005), they suggested that PBI morphology cannot give provide embryos with high developmental competency, and they proposed alternative parameters for oocyte selection. Halvaei *et al.* (2011) analyzed the role of PBI morphology on rates of fertilization and embryo development in

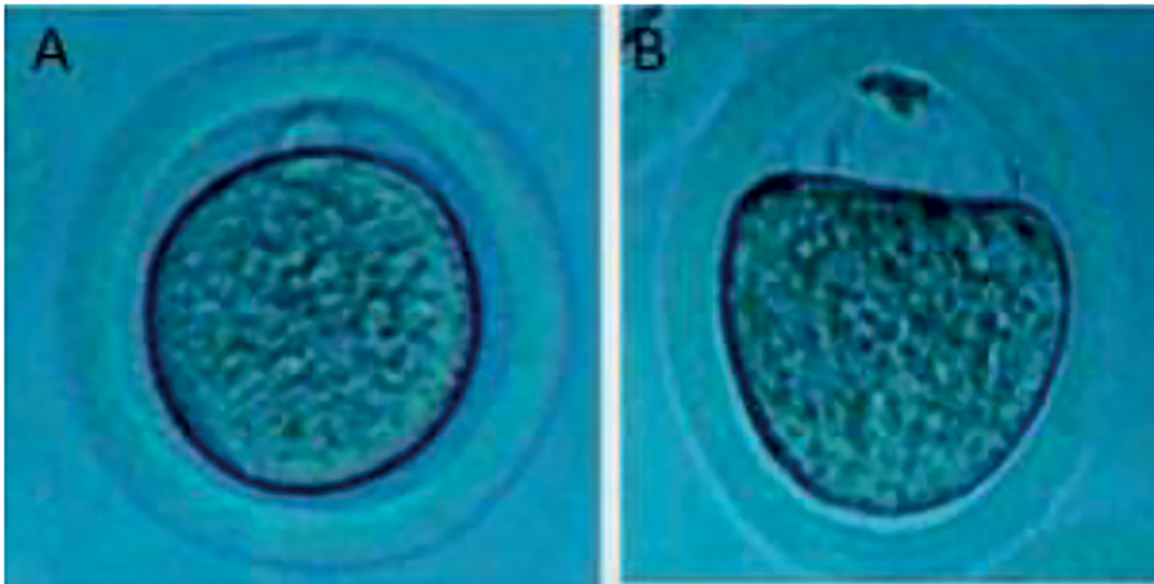


Figure 6. Young oocytes (A) expanded against the zona pellucida with a minimal perivitelline space (PVS) and a clear indentation at the polar body, whereas old oocytes (B) had wide PVS with no indentation from the polar body. Reproduced from Li *et al.* (2017) with permission.

ICSI cycles. They concluded that PBI morphology does not seem to be a prognostic factor for rates of fertilization and embryo development in ICSI cycles (Figure 7) (Halvaei *et al.*, 2011). Another study conducted by Zhou *et al.* (2016), on the relationship of polar body-morphology to embryo quality and pregnancy outcome at 16–18 hours after insemination. They reported that no remarkable differences in pregnancy rate (PR) or implantation rate (IR) were seen between the intact and fragmented groups of PBI. Although they reported little effects of PR or IR in fresh embryo transfer cycles, and concluded that better embryo quality can be attained in the PB-intact group, which is important for embryo selection (Zhou *et al.*, 2016). Other studies show that oocytes with intact and good shaped PBI yield higher fertilization rates and higher embryo quality (Ebner *et al.*, 2000). Evidence on the relationship between PBI and mother's age show that the PBI from young individuals is intact and adjacent to the MII spindle, while aged oocytes contain degenerated and deviated PBI from the MII spindle, and space between PBI and the spindle raises with time throughout the oocyte aging (Miao *et al.*, 2004). PBI displacement can be due to increased PVS with time and facilitated moving from its usual place (Miao *et al.*, 2004). Polar bodies due to their amount of cytoplasm, organelles and chromatids, can yield applicable data (by cytogenetic studies like fluorescent in situ hybridization) about the genetic condition of oocytes, without possibly endangering it. Does aging-related changes cause molecular alterations in the PBI in spite of apparent morphological modification? This question was the aim of a study by Jiao *et al.* (2012). In a prospective mice model survey, they assessed the oocyte-specific mRNAs detection in the PB of MII oocytes and define the effects of age on oocyte-specific transcript levels. They showed that transcript levels were lower in aged PBs compared with young PBs. Finally, they concluded that there is a remarkable difference in the transcript levels of oocyte-specific genes in aged vs. young PB, that correlates with age-related reduction in oocyte capability (Jiao *et al.*, 2012). This gene expression alteration in PB may be a possible biomarker of MII oocyte competence (Jiao *et al.*, 2012). Therefore, the assessment of PBI could work as a strong genetic diagnostic device

during pre-fertilization screening without the requirement of embryo biopsy (Gitlin *et al.*, 2003), but some limitations should also be understood when PB analysis is taken into account (Rawe & Combelles, 2009). These restrictions include: first, the maternal genetic portion, which can be studied alone. In second, possible nondisjunction of sister chromatids during MII can happen, which requires second polar body analysis. In third, PB chromosomes are shorter, which makes the technique more difficult (Rawe & Combelles, 2009).

Ooplasm

Cytoplasmic and nuclear factors are important criteria in oocyte quality, which directly influence the developmental competence of embryos in ART. For this, nuclear maturity indicates meiosis continuation and the progression to metaphase II, the arrested stage at the time of ovulation. MPF can be created by cyclin B and p34cdc2, which supports the transition from G₂ to M phase. Active MPF starts nuclear maturation (e.g., germinal vesicle breakdown) and condensation of metaphase I (MI) chromosomes, then a reduction in MPF causes entry into anaphase I, and a second peak in MPF activity drives the oocyte to metaphase II (Eppig, 1996). Even if oocytes get nuclear competence, they still may be lacking cytoplasmic maturation, which indicates all processes construct the oocytes for activation, fertilization, and embryo development. Completion of both kinds of maturation are highly sensitive to changes in follicular hormonal environment and/or *in vitro* culture status (e.g., pH, temperature, oxygen) that may lead to alterations in oocyte morphology. Some of these oocyte cytoplasmic aberrations are visible at the light-microscopic level (Hu *et al.*, 2001). Elimination of cumulus cells with the ICSI method enables the clinician to directly evaluate the morphological attributes of oocytes under light microscopy. Ooplasmic morphological characteristics can be categorized by the presence of vacuoles, inclusion, debris, fragmented corpuscles, dark cytoplasm, and organelle clustering (Kahraman *et al.*, 2000; Moussa *et al.*, 2015). In fact, more than 50% of human oocytes show at least one of these morphological abnormal features in the center or in the periphery of the Ooplasm (Ebner *et al.*, 2003).

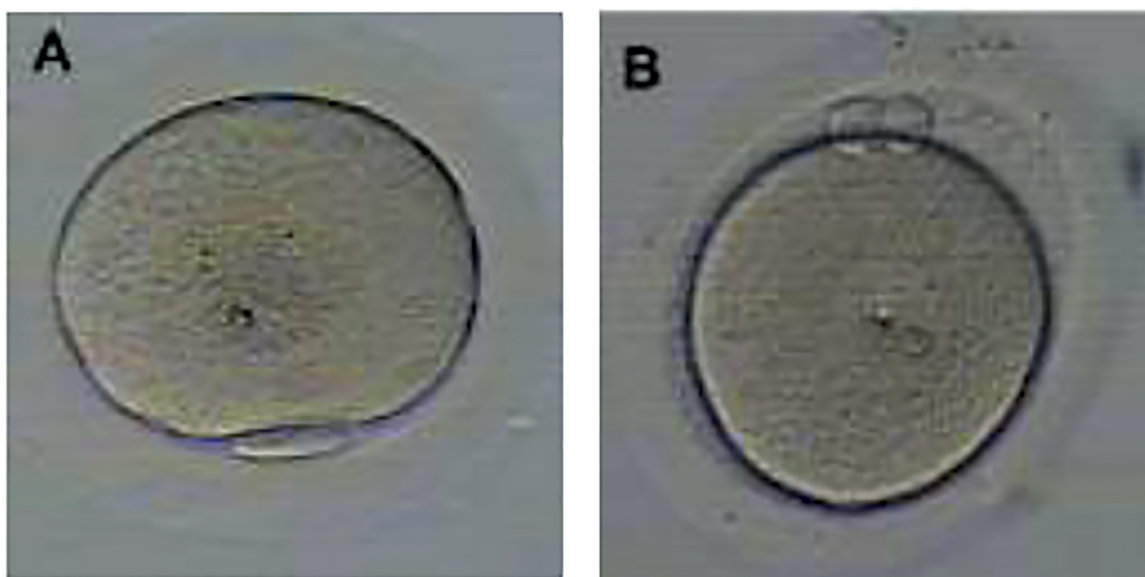


Figure 7. MII oocytes after denudation of cumulus cells before microinjection. (A) normal PBI and (B) fragmented PBI. Halvaei *et al.* (2011) with permission.

Nagano *et al.* (2006) in an animal model study revealed that the dark cytoplasm has an accumulation of lipids and good developmental potential of oocytes after *in vitro* fertilization. They found that a light-coloured cytoplasm indicated a low density of organelles and poor developmental potential. An oocyte with a dark ooplasm declines by 83% in its likelihood of yielding good-quality embryos (Ten *et al.*, 2007). The most evident cytoplasmic characteristics that hinders developmental ability are aggregations of smooth endoplasmic reticulum (sER) and vacuoles (Figure 8). Vacuoles originate spontaneously by fusion of pre-existing vesicles derived from the sER or Golgi apparatus (Shafie *et al.*, 2000; Van Blerkom, 1990). Few studies have been carried out on the effects of oocyte vacuoles on human fertility, but a large number of reports noted a negative influence on fertilization outcomes (Loutradis *et al.*, 1999). Otsuki *et al.* (2004) for the first time evaluated the origin of smooth Endoplasmic Reticulum Clusters (ERCs) and defined their effect on embryonic development and clinical fecundity rates. They reported that pregnancy rates remain low with sERC-negative cohort oocytes in sERC-positive cycles (Otsuki *et al.*, 2004). Another physiological consequence of women's aging is FSH rise, that can be correlated with morphometric parameters. In a study by Santos *et al.* (2015) they concluded that patients with high FSH levels displayed low-quality oocytes when compared with controls. This can be suggestive of ovarian aging, which negatively affects oocyte development into viable embryos. These findings were confirmed by other studies carried out by Kdous *et al.* (2016). Although, Valeri *et al.* (2011) showed that there is no significant relationship between FSH levels and morphometric oocyte parameters, they concluded that it maybe because other factors have an effect on this relationship. Despite several studies on the relationship between oocyte morphology and embryo quality, it is clear that microscopic observation alone cannot determine fecundity pathology or its relative effect in ART, and we need more research and studies in this field.

Meiotic Spindle

The spindle is an important barrel-shaped cellular structure, primarily composed of microtubules and centrosomes, that are responsible for the precise segregation

of homologous chromosomes (meiosis I) or two sets of chromatids (meiosis II) within germ cell division (Karsenti & Vernos, 2001; Wang & Sun, 2006). Failure of equal separation will cause aneuploidy, which is thought to be a source of a lot of genetic problems and aneuploid embryos (Hassold & Hunt, 2001; Wang & Sun, 2006). Some aneuploid embryos implant in the uterus undergo spontaneous abortion, while others develop to full term and bear genetic disorders (Bruyère *et al.*, 2000; Robinson *et al.*, 2001). Aneuploidy is indeed a main problem in getting reproductive success. It is estimated that 20% of all human oocytes are aneuploid, although this can fluctuate from 10% to as high as 40–60% (Kuliev *et al.*, 2005; Pacchierotti *et al.*, 2007). The meiotic spindle organization and morphology is essential for ensuring accurate chromosome separation during MI and MII. The structural features of the meiotic spindle, such as length and location, are usually used for assessing oocyte quality. In somatic cells, there is a spindle check point mechanism that controls chromosome segregation (Eichenlaub-Ritter, 2012), while in mammalian oocytes these monitoring apparatus are not exactly engaged during the meiotic procedure, which may cause chromosome mis-segregation (Jones, 2008). An intact meiotic spindle is critically significant for precise segregation of chromosomes to the dividing blastomeres, thus ensuring accurate embryo development (Miao *et al.*, 2009). Spindle size is approximately 11.2 ± 3.4 μm . Fresh human oocytes carry compact anastral spindles (spindles without centrosomes) placed orthogonally to the oolemma, with the pole close to the oolemma being smaller than that related to the center of the oocyte (Miao *et al.*, 2009). The human meiotic spindle size in the first day is shorter (8.08 ± 0.84 μm) than the spindle in fresh oocytes. In 2-day oocytes, the spindles are smaller and can be bi- or multipolar, which will have serious aftermath for chromosome segregation and, therefore, result in aneuploidies (Figure 9) (Miao *et al.*, 2009). Microtubules of meiotic spindles in 2-day-aged oocytes radiate to cell periphery and make additional microtubule asters in the cytoplasm. Immunocytochemical of tubulin staining in aged oocyte assessments shows increased staining through the meiotic spindle, compared with that of fresh oocytes (Wang *et al.*, 2001). This staining shape exhibits a loss of centrosome

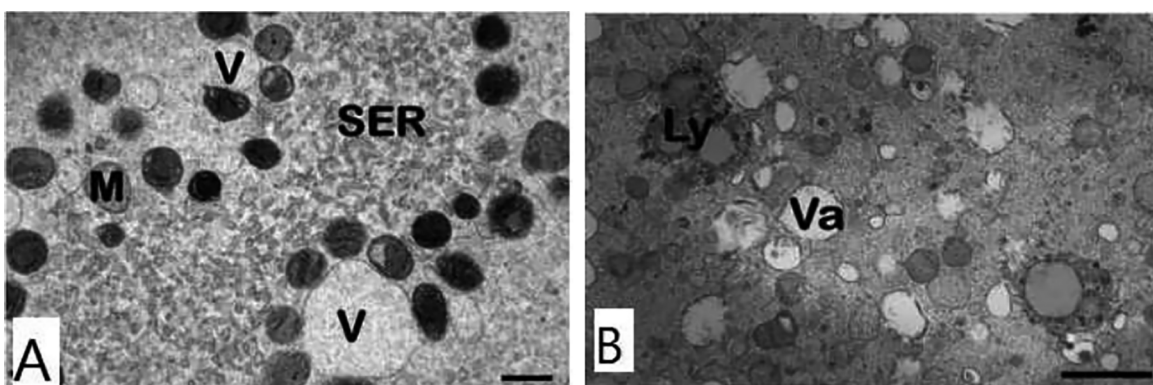


Figure 8. (A) Mature oocyte. Tubules (SER) and vesicles (V) of smooth endoplasmic reticulum associated with mitochondria (M) to form M-SER aggregates and MV complexes. TEM, bar is 0,5 μm . (B) Early degenerating mature oocyte. Numerous vacuoles (Va) associated with lysosomes (Ly) are found in the ooplasm. TEM bar is 1,2 μm . Nottola *et al.* (2007) with permission.

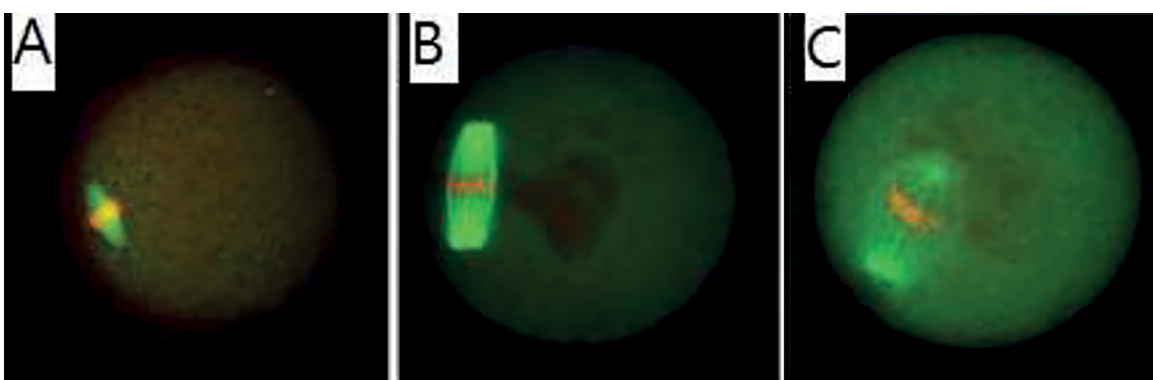


Figure 9. Confocal micrographs of fresh and aged mice oocytes. Configuration of meiotic spindle and chromosomes in (A) Normal spindle morphology of fresh oocytes, microtubules traversing between both poles and chromosomes aligned in a compact group on the equatorial plane (B) and (C) abnormal spindle morphologies of aged oocytes. Miao *et al.* (2009) with permission.

structure in the meiotic poles, which accompanies the loss of microtubule integrity (Schatten, 2008). Studies show that different patterns of spindle disruption can be seen in aged, failed-to-fertilize oocytes. This spindle disorganization includes: tetrapolar spindles, abnormal expression of the nuclear mitotic apparatus (NuMA) protein, which gives spindle stability to fresh oocytes, and alters the microtubule kinesin motor protein EG5 (Hall *et al.*, 2007). Because of ethical issues and limitations of human oocytes for research, mice and pigs have been used for many studies with the purpose to infer data to humans (Prather, 2007). These studies indicate that microtubules in fresh mice oocytes are obviously detected in the meiotic spindle, additional small microtubule asters are detected in the cytoplasm, which are arranged by cytoplasmic centrosomes. Microtubules in aged mice oocytes become slowly misplaced from the spindle, with preferential loss in the central spindle area, close to the chromosomes. Astral fibers radiate out from the polar centrosomes into the cytoplasm, whereas the mean pole-to-pole distance becomes remarkably decreased (Miao *et al.*, 2009). Concurrently, astral microtubules in the cytoplasm become steadily depolymerized (Segers *et al.*, 2008). Studies revealed that the expression of the spindle checkpoint protein MAD2 (mitotic arrest deficient protein) is gradually decreased in pig and mice oocyte aging (Steuerwald *et al.*, 2005). Increases in premature chromosome segregation is an oocyte aging-related change that causes embryo aneuploidy (Steuerwald

et al., 2005). Oocyte from older women grown in sub-optimal environments are prone to a poorly-controlled mechanism of chromosome segregation. Rama Raju *et al.* (2007) investigated the meiotic spindle and other oocyte structure characteristics using the PolScope microscope, and analyzed their relationship to the embryonic development potential. They showed that delays and the meiotic spindle length have a positive predictive value in relation to progression to blastocysts. In addition, they reported a significant change in retardance and spindle length in aged patients, and deduced that retardance reduction and spindle length is correlated with advanced age (Rama Raju *et al.*, 2007). Eichenlaub-Ritter *et al.* (2002) found that human oocytes without birefringent spindles may still be at the telophase or prometaphase I stage, and as a result, have worse prognosis after IVF/ICSI. It has been indicated, that's why meiotic spindle assessment is an important structure for evaluating oocyte quality (Moon *et al.*, 2003; Rienzi *et al.*, 2003).

Mitochondria

Mitochondrias are double membrane-bound organelles present in cytoplasm of eukaryotic cells, with a highly specialized function and morphology that generate necessary energy for different cellular purposes (Kelly & Scarpulla, 2004). They are vital for metabolism, signalling, and apoptosis (Danial & Korsmeyer, 2004). Oocyte mitochondria produce ATP via oxidative phosphorylation (OXPHOS),

which renders the energy demanded from fertilization through the blastocyst stage (Dumollard *et al.*, 2007). In response to extra and intra cellular requirements, mitochondrial function is improved by complexed mechanisms that regulate various morphologies and distributions. In most types of cells, mitochondrial injury and oxidative stress represent the principle of cellular aging (Liochev, 2013). Each mitochondrion consists of 1 to 15 mitochondrial DNA (mtDNA) molecules and developing oocyte competence is associated with a copy of mtDNA per oocyte (Wai *et al.*, 2010). The mitochondrial genome, which contains approximately 16k base pairs (bp) and encodes core complexes for cellular respiration like ATP synthase, cytochrome c-oxidase, cytochrome b and so on (Kogo *et al.*, 2011). Mitochondrial DNA has no histone safeguarding, antioxidant system and impression correction apparatus (Zhang *et al.*, 2017). Thus, oxidative factors such Reactive Oxygen Species (ROS) can simply lead to mtDNA and cellular damage, when there is oxidative/antioxidant imbalance (Zhang *et al.*, 2017). In accordance to oocyte ATP demands for energy consumption in cytoplasmic and nuclear maturation, meiotic spindle formation, the mitochondria are able to spread and concentrate in ooplasmic regions (Figure 10). In addition to this, it appears that the arrangement of mitochondrial distribution is different in the mature oocyte of various species (Yu *et al.*, 2010). Mitochondria, which are the main factors of oocyte quality, may be directly affected throughout ovarian aging. Studies revealed that there is a relationship between the mtDNA content, copy number, maternal age and ovarian reserve indexes (Duran *et al.*, 2011). Studies have also indicated that mtDNA copy number in human oocytes reduces with advancing maternal age (Chan *et al.*, 2005). It is possible that mitochondrial abnormalities may also play an essential role in age-related oocyte competence and aneuploidy rates (Barritt *et al.*, 2000). Large numbers of evidence have revealed that mtDNA levels are remarkably higher in aneuploid embryos, compared with those in chromosomally-normal embryos (Fragouli *et al.*, 2015). In humans and rodents, ultrastructural studies of aged mitochondria (Figure 11) show morphological and functional abnormalities such as mitochondrial swelling, vacuolization and cristae alterations in comparison with young individuals (Kushnir *et al.*, 2012; Simsek-Duran *et al.*, 2013). Mitochondrial membrane potential ($\Delta\psi$), as is ATP production, which indicates mitochondrial activity is another age-related change in human and mice (Ben-Meir *et al.*, 2015). Mitochondrial ATP production requires the operation of the electron transport chain, situated on the inner mitochondrial membrane. Complexes I and II oxidize the tricarboxylic acid (TCA) cycle products and transfers the electrons to ubiquinone, also the well-known coenzyme Q (CoQ). CoQ has an important antioxidant characteristic, controls cellular redox and impacts transcriptional activity, necessary to the electron transport chain (Crane, 2001). CoQ proteins make a big mitochondrial complex and the existence of all protein elements is essential for steadiness (Wang *et al.*, 2009). Evidence has shown that coenzyme Q10 supplementation in aged animal models (but not in younger animals), can be used to postpone the reduction of ovarian reserve, fortify the expression of mitochondrial genes in oocytes and ameliorate the mitochondrial activity. Ben-Meir *et al.* (2015) concluded that the reduction of mitochondrial activity produced by suboptimal CoQ10 availability plays a key role in age-related oocytes, and its lack causes infertility. Different studies on animal models have shown that injection of mitochondria or cytoplasm from young donor oocytes into an aged oocyte enhances oocyte competence, embryo quality, decreased fragmentation, and finally enabling successful implantation (May-Panloup *et al.*, 2016). With this definition, cytoplasmic mtDNA, ATP

content and mitochondrial distribution may be considered as prognostic factors of oocyte developmental competence. However, these procedures are invasive and therefore have no value as prediction implements in the clinical IVF laboratory.

EPIGENETICS

Waddington (1942) was the first who presented the word of epigenetics in the early 1940s. He explained epigenetics as "the branch of biology which studies the causal interactions among genes and their products, which cause the phenotype into being (Waddington, 1968). In recent years, with the rapid growth of genetics, the meaning of this term has gradually changed. Epigenetics defined the process that regulates gene function and does not affect DNA sequence, and is heritable through cell division (Baird *et al.*, 2005). Gene activation in the zygote and early embryonic development are controlled by both genetic and epigenetic mechanisms. Epigenetic mechanisms are not DNA sequence-based, while the genetic mechanism relies on DNA sequence and codes. Epigenetic mechanisms establish inheritable alterations that play an important role in regulating gene expression (Lucifero *et al.*, 2004). The main epigenetic alterations include DNA methylation, modification of histones and chromatin remodelling; they are intimately related and operated at the transcriptional level. Epigenetic reprogramming takes place at gametogenesis and the accurate establishment of epigenetic modifications are crucial for normal embryo growth and viability (Baird *et al.*, 2005). The DNA methylation process is mostly catalysed by DNA methyltransferase 3s (DMT3s) (Tomizawa *et al.*, 2012). Studies imply that the epigenetic modifications in oocytes may be influenced by advanced maternal age, owing to the expression of DNMTs and histone acetyltransferases that varies with aging (Hamatani *et al.*, 2004). In women older than 38 years, the expression pattern of TAP73 in oocytes is lower, and down regulated when compared with oocytes from younger women (Guglielmino *et al.*, 2011). However, there is still no clear evidence that the DNA methylation in human oocytes is influenced by aging. Immunocytochemical studies report histone deacetylation at the MI and MII stages by histone deacetylase (HDAC) activity in mammalian oocytes. Akiyama *et al.* (2004) revealed that if meiotic histone deacetylation is restrained, aneuploidy raised in fertilized mice oocytes, and this culminated in embryonic death in the uterus at an early stage of development. Hamatani *et al.* (2004) showed that HDAC is down-regulated at transcript level in aging mice oocytes older than 42 weeks, while histone remains acetylated in young female mice. Evidence revealed the relationship between histone acetylation and maternal age. The results show advanced maternal age negatively affects the deacetylation of some histone proteins (H4K12) in human MII oocytes (Steuerwald *et al.*, 2007). Studies indicate that alterations in enzymes comprising methyltransferases (DNMTs) and demethylases (TETs), are possibly the direct cause of epigenetic changes in aged oocytes. However, whether/how aging impacts the modifications in their expression requires more explanation (Ge *et al.*, 2015). Investigations recommend that age-related epigenetic changes on oocyte might be inhibited by diets, medicine or other methods.

NEW INSIGHTS IN OVARIAN STEM CELLS

Recent evidence indicate that components of adult mammalian ovaries in primary follicles; that is, primitive granulosa and germ cells may differentiate from mesenchymal progenitor cells (MPC) resting in the ovarian tunica albuginea (Bukovsky, 2015). Jonathan Tilly's team for the first time explained stem cells in adult mice ovary (Johnson

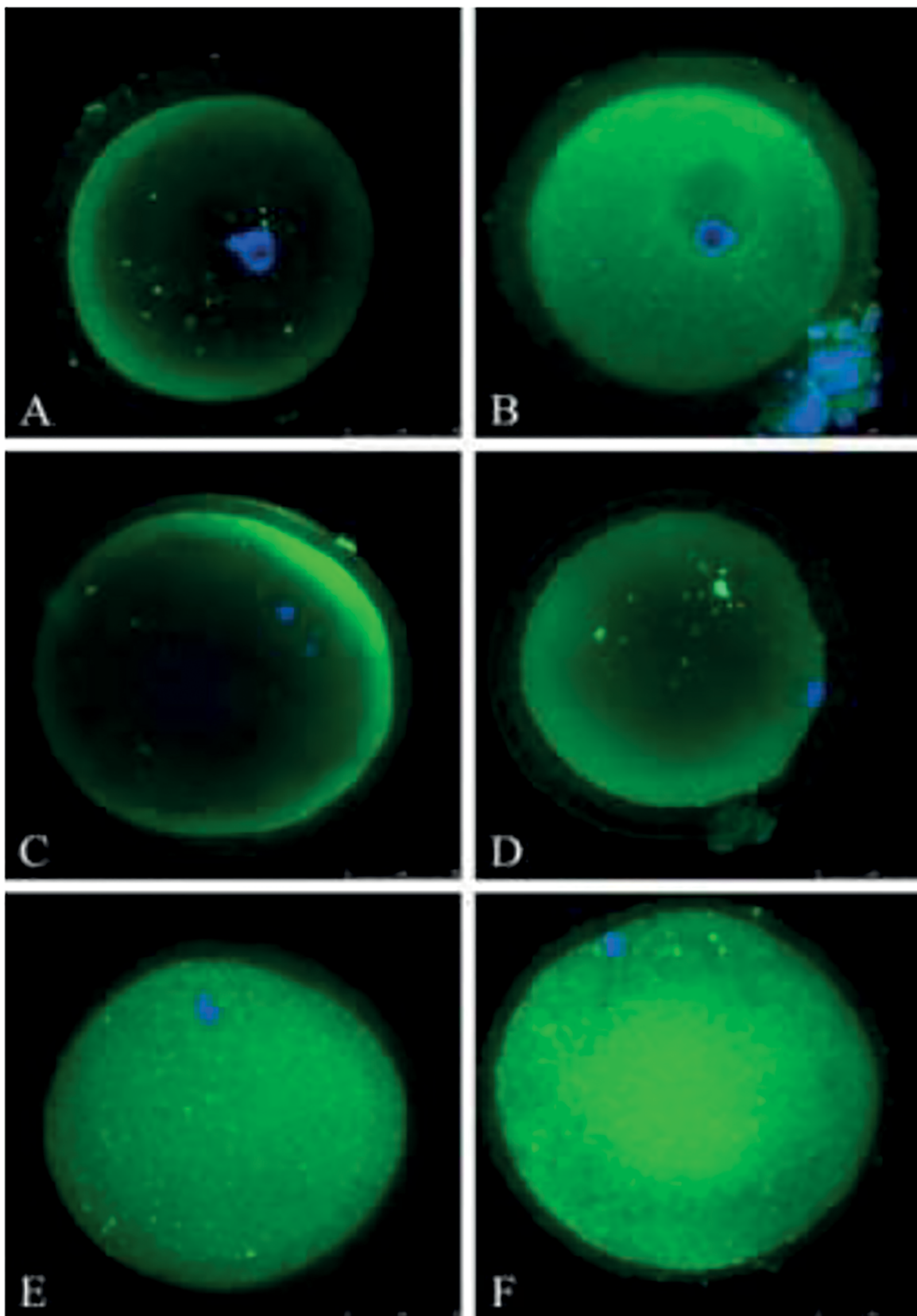


Figure 10. Confocal microscopy images demonstrating mitochondria distribution during maturation of human oocytes. (A) GV oocyte displays the distribution of mitochondria in the peripheral zone. (B) GV oocyte with mitochondria diffused fairly in the cytoplasm. (C) MI oocyte with mitochondria displaying a peripheral type of distribution. (D) In vitro matured oocyte indicating the semiperipheral distribution of mitochondria in the oocyte. (E) In vitro matured oocyte showing the distribution of mitochondria throughout the cytoplasm. (F) In vivo matured oocyte: mitochondria are disseminated throughout the cytoplasm, and there are mitochondria in the central region. Scale bar represents 50 μ m. Liu *et al.* (2010) with permission.

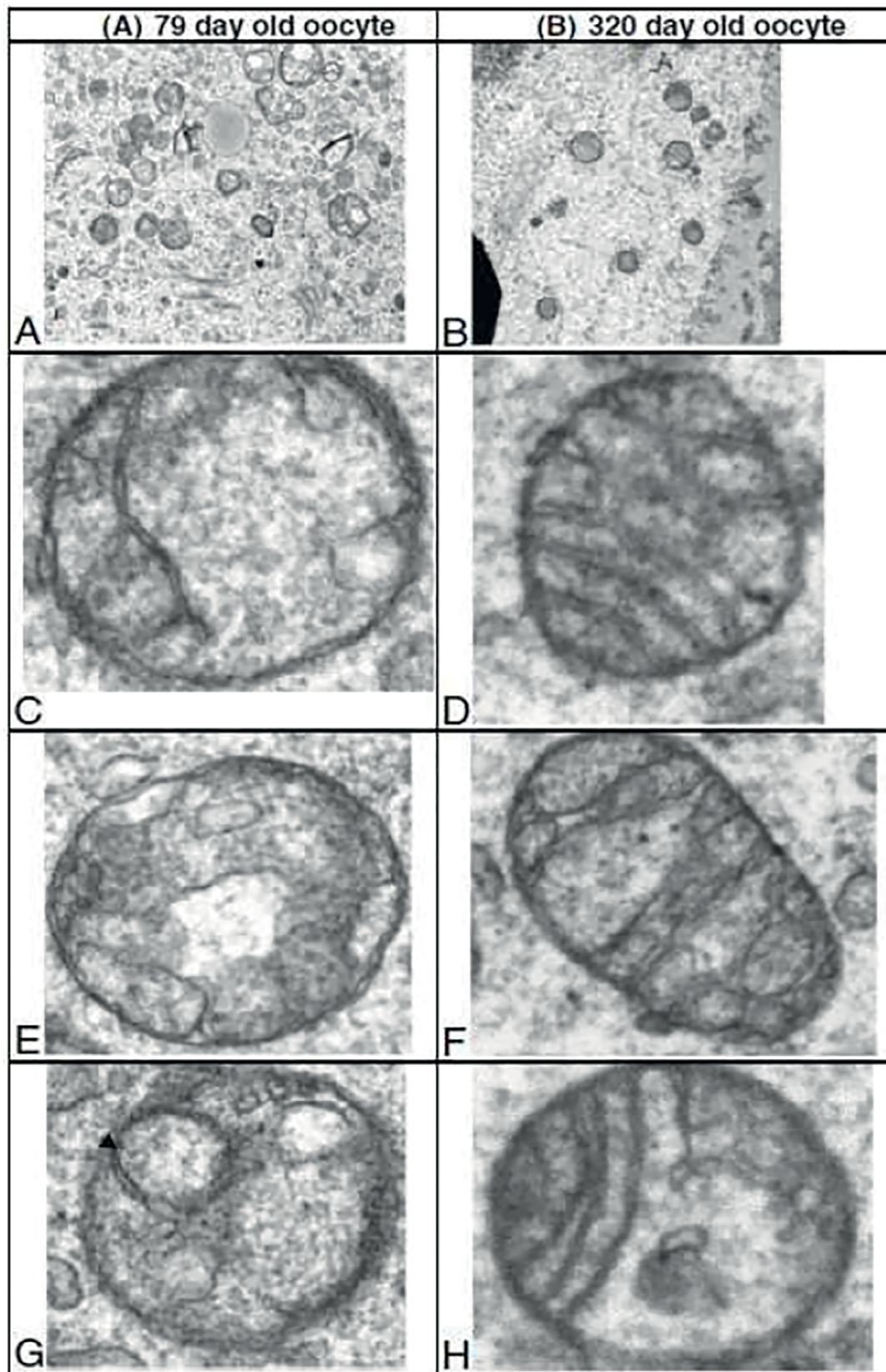


Figure 11. TEM images of mitochondria from young and old mice oocytes. MII oocytes from hyperstimulated young and old mice were evaluated and the mitochondrial structures were compared. Representative electron micrographs of ooplasm at 11,000x magnification are shown; higher magnification views of individual mitochondria are also presented. (A) abundant mitochondria per field, also notice different size mitochondria compared to B. (B) relatively few mitochondria of uniform size per field in ooplasm of an aged animal. (C & E) undifferentiated round mitochondria with an electron dense matrix vs D, F & H. More differentiated mitochondria with an elongated shape and distinct cristae G. arrow indicating vacuoles within mitochondria, Kushnir *et al.* (2012) with permission.

et al., 2004). Their results challenged old central dogmas that ovaries have a finite number of follicles that get reduced with age, resulting in menopause. Ovarian stem cells have opened a new landscape for a further understanding of human oogenesis, infertility preservation, and therapy. Recent findings reported Ovarian Stem cells (OSCs) isolated from rabbits, marmosets, sheep, and human ovaries. Researchers characterized two distinct populations of stem cells based on their size, which comprises quiescent Very Small Embryonic-Like (VSEL) stem cells that expresses nuclear OCT-4 and slightly bigger cells expressing cytoplasmic OCT-4 (Bhartiya *et al.*, 2019; Kumar *et al.*, 2009; Sriraman *et al.*, 2015). Evidence shows that ovarian stem cells on culture medium developed spontaneously to oocyte-like cells (OLCs) and expresses oocyte and germ cell specific markers (Kumar *et al.*, 2009). Our recent findings showed that ovarian derived SCs could differentiate to OLCs in BMP15 conditioned medium, and expresses ZP genes and proteins, which confirm earlier reports (Taheri Moghadam *et al.*, 2021). Increasing our knowledge about ovarian stem cells and their differentiation into OLCs can help us better understand this phenomenon and retard the aging process.

CONCLUSION(S) AND PERSPECTIVES

The main role of oocyte competence and aging in relation to an embryo's development has encouraged wide research for reliable predictors of oocyte quality. A series of age-related molecular, cellular and morphological alterations take place during the process of oocyte aging and fertilization. These changes not only influence pre- and post-implantation of embryo development quality but also the later life of the offspring. Essays to describe morphological features related to oocyte quality and aging have attained little results. A number of chemicals and physical facilities have been used to characterize a good oocyte quality and postpone the oocyte aging process, to provide hopeful chances for intervention in the aging phenomenon. These specific steps are essential for ART procedures to elevate the rate of successful delivery, which leads to optimal production of healthy babies. With modern ART, the successful rate of this technology is affected by oocyte aging; therefore, control of oocyte aging would provide an important advantage in allowing adequate manipulation and selection of high quality oocytes. Hence, creating methods to control aging might increase progress in assisted reproduction technologies. Because in the lack of a good oocyte grading plan, the ability of morphological monitoring to aid oocyte/embryo selection is reduced.

In this review, we summarized the oocyte quality, and mechanisms of oocyte aging, and delay or reversibility of the aging process. Then, we showed briefly the importance of procedures to control oocyte quality and age-related changes in oocyte competence. There are still many unanswered questions, but several paths have now been paved unto much awaited advances and the detection of oocytes with compromised quality.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests.

Corresponding author

Ghasem Saki

Department of Anatomical Science

Faculty of Medicine

Ahvaz Jundishapur University of Medical Sciences

Ahvaz, Iran

E-mail: ghasemsaki@yahoo.com

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