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Oocyte aging: cellular and molecular changes, developmental potential and reversal possibility

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BACKGROUND: In humans, normal healthy children are regularly produced through fertilization of fresh oocytes with fresh spermatozoa. However, asynchrony between oocytes and spermatozoa, especially when aged oocytes are fertilized by fresh or senescent spermatozoa, will not only affect the rate of fertilization and pre- and post-implantation embryo development but also the life of the offspring. As many failures in assisted reproduction technologies (ART) are related to oocyte aging, new methods are needed to control oocyte aging to benefit modern ART.

METHODS: We review changes associated with decreased fertilization rates and developmental potential of aged oocytes, and we present methods and approaches that prevent or delay oocyte aging.

RESULTS: Cellular and molecular abnormalities occur during oocyte aging, but prevention, delay or reversal is possible to various extents. Modifying existing culture conditions, or treatment of oocytes with agents such as caffeine, DL-dithiothreitol, nitric oxide or trichostatin A may correct molecular pathways that are affected by aging, and thus benefit and improve success rates in modern ART.

CONCLUSIONS: Aging of oocytes is characterized by a sequence of molecular processes that deteriorate during aging and negatively impact fertilization and development. However, oocyte aging can be delayed or reversed by various treatments to increase success rates and produce increased numbers of healthy embryos, preventing failures or abnormalities that are frequently associated with ART using aged oocytes.

Key words: oocyte aging / ART / delay and prevention of oocyte aging

Introduction

In most mammals, oocyte meiosis is initiated during fetal development and arrested at the diplotene (germinal vesicle (GV)) stage of the first meiotic prophase around the time of birth. Oocytes that have almost reached their full sizes are stimulated to resume meiosis after an endogenous LH surge at puberty to reach the second meiotic division, metaphase II—division metaphase (MII) with the first polar body (PB1) extruded into the perivitelline space (PVS) when they are released from the ovary and exhibit a second block at MII stage (McGee and

Abbreviation	Definition
APF	aging-promoting factor
CC	cumulus cells
CG	cortical granules
COC	cumulus-ooctye complexes
DO	denuded oocytes
DTT	DL-dithiothreitol
ER	endoplasmic reticulum
GSH	glutathione
GV	germinal vesicle
hCG	human chorionic gonadotrophin
ICM	inner cell mass
IP3	inositol 1,4,5-triphosphate
MAD2	mitotic arrest deficient protein
MAPK	mitogen-activating protein kinase
MF	microfilaments
MII	metaphase II
MPF	maturation-promoting factor
MV	microvilli
NO	nitric oxide
NT	nuclear transfer
NuMA	nuclear mitotic apparatus protein
OMD	ooplasm microtubule dynamics
PB1	polar body 1
PCS	premature chromosome separation
PLCζ	sperm-specific phospholipase C
PM	plasma membrane
PVS	perivitelline space
ROS	reactive oxygen species
TSA	trichostatin A
ZP	zona pellucida

Hsueh, 2000; Fan and Sun, 2004; Liang *et al*., 2007). The process from GV to MII is called 'oocyte maturation', which includes a complex sequence of nuclear and cytoplasmic events that prepare the oocyte for fertilization and initiation of embryo development. The molecular biology of oocyte meiosis, and predisposition to aneuploidy as well as its increased incidence with age have recently been well reviewed (Jones, 2008), and are not addressed here.

It is well established that oocyte quality determines the embryo's developmental potential after fertilization (Wang and Sun, 2007). Oocytes arrested at the MII stage are normally fertilized soon after ovulation; the window for optimal fertilization differs in different species and has been determined for the mouse (8–12 h), rat (12–14 h), rabbit (6–8 h), rhesus monkey and human (<24 h) (Austin, 1974). If fertilization does not occur within that time, unfertilized oocytes remaining in the oviduct (*in vivo* aging) or culture (*in vitro* aging) will undergo a time-dependent deterioration in quality, a process called 'oocyte aging'. 'Aged oocytes' are those oocytes in which aging already has occurred. Ooocyte aging occurs in the ovaries of females who show a progressive decline in fecundity as they pass through their reproductive age, a transition which is directly associated with a poor developmental potential of aged oocytes. The physiological process is called 'ovary aging', which has been well reviewed (Tatone *et al*., 2008) and is not addressed here. The present review is focused on the cellular and molecular processes that occur in oocytes during *in vivo* and *in vitro* aging. We discuss mechanisms underlying oocyte aging and possibilities to reverse oocyte aging which would have significance for assisted reproduction technologies (ART).

Functional changes associated with oocyte aging include decreased fertilization rates (Lanman, 1968; Ben-Rafael *et al*., 1986; Badenas *et al*., 1989; Winston *et al*., 1993; Goud *et al*., 1999b), polyspermy (Badenas *et al*., 1989; Pool *et al*., 1990), digyny (Marston and Chang, 1964; Lanman, 1968; Szollosi, 1971; Meyer and Longo, 1979; Juetten and Bavister, 1983; Webb *et al*., 1986; O'Neill and Kaufman, 1988; Zackowski and Martin-Deleon, 1988), parthenogenesis (Blandau, 1952; Marston and Chang, 1964), chromosomal anomalies (Szollosi, 1971; Butcher, 1976; Plachot *et al*., 1988; Mailhes *et al*., 1998), apoptosis (Fujino *et al*., 1996; Perez and Tilly, 1997), increased susceptibility to activating stimuli (Szollosi, 1971; Edirisinghe *et al*., 1986; Ware *et al*., 1989; Bergere *et al*., 1992; Presicce and Yang, 1994; Kikuchi *et al*., 1995; Kim *et al*., 1996; Xu *et al*., 1997; Goud *et al*., 1999a, b), onset of anaphase II (Xu *et al*., 1997; Abbott *et al*., 1998), partial exocytosis of cortical granules (CG) (Szollosi, 1971; Gulyas, 1979; Dodson *et al*., 1989; Ducibella *et al*., 1990; Xu *et al*., 1997), structural alteration and hardening of the zona pellucida (Longo, 1981; Nogues *et al*., 1988; Dodson *et al*., 1989; Xu *et al*., 1997), a decrease in maturation-promoting factor (MPF) and mitogen-activated protein kinase (MAPK) activity (Kikuchi *et al*., 1995, 2000, 2002; Xu *et al*., 1997), epigenetic changes (Liang *et al*., 2008) and abnormal and/or retarded development of embryos/fetuses (Lanman, 1968; Szollosi, 1971; Winston *et al*., 1993; Tarin *et al*., 1999). These changes are either cascades of changes following an initial event or separate processes for which causes have not yet been fully determined.

In humans, most children are produced through *in vivo* fertilization but 1% of all babies in the Western world are born through ART (Giritharan *et al*., 2007). In some countries the rate is even higher and in Australia IVF accounts for 2.7% (1/37) of all born babies (Australian Bureau of Statistics. 'Births', 3301.0, 2007). Significant progress has been made to improve ART technologies; however, oocyte aging is one of the problems associated with ART failures and so methodological advances are in critical demand to overcome the oocyte aging process during ART procedures. In human ART, the average fertilization rate after standard IVF is about 60–70% (Kruger *et al*., 1986). Reinsemination of aged unfertilized oocytes failing in IVF cycles has been achieved with ICSI; however, problems persist. Studies in which a total of 115 MII 1-day-old unfertilized oocytes collected from 23 patients and injected with spermatozoa from their husbands showed that only 38% displayed two distinct pronuclei at 16–18 h or 42–44 h after ICSI, which was much lower than the fertilization rate of fresh oocytes after standard ICSI (64.2%). The resulting embryos showed low implantation rates and low developmental potential after transfer, which was related to changes in oocyte quality as a result of aging before ICSI (Nagy *et al*., 1993; Van Steirteghem *et al*., 1993; Liu *et al*., 1995). Subsequent studies by Wilcox *et al*. on 221 healthy women (ages 21–42 years, mean 30) with no history of fertility problems showed ovulation and early pregnancy losses. The causes were analyzed by immunoassays of urinary human chorionic gonadotrophin (hCG) and steroid metabolites to determine whether the risk of early pregnancy loss was higher with post-ovulatory aging of the oocyte. The results showed that 192 pregnancies, ranked by the probability that the oocytes may have aged before fertilization, displayed a statistically significant increase in the risk of early loss as the probability of oocyte aging increased. These

studies concluded that oocyte aging prior to fertilization may cause early pregnancy failure in humans, as it does in several other mammalian species (Wilcox *et al.*, 1998). The studies clearly indicate that improved procedures are needed in ART methods to overcome oocyte aging. Importantly, a variety of factors play a role in oocyte aging including environmental conditions, oocyte interactions with cumulus cells (CC) and various chemical components. New and encouraging data have clearly shown that a variety of chemicals can reversibly regulate oocyte aging. Managing oocyte aging by supplying such chemicals to culture medium may be particularly critical to improve modern ART technologies.

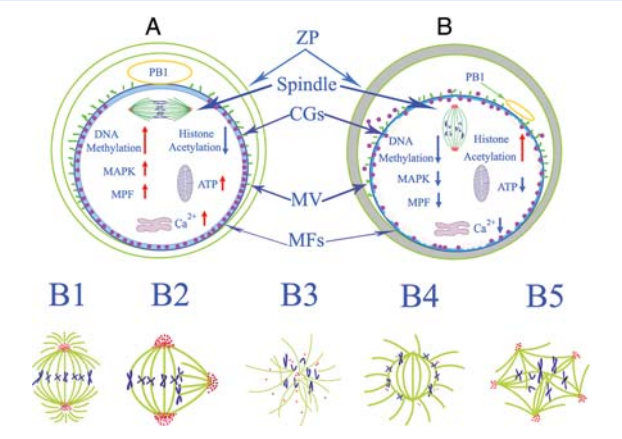

Figure 1 Differences between fresh and aged oocytes in morphological, cellular, biochemical, molecular and epigenetic aspects. (**A**) Fresh oocytes. (**B**) Aged oocytes. (**B1**–**B5**) Show abnormal spindles in aged oocytes. (B1) Large rounded spindle with microtubules emanating from most of its surface. (B2) Tripolar spindle. (B3) Highly disorganized spindle with scattered centrosomes and chromosomes. (B4) Large irregular spindle in transverse view exhibiting dense staining for tubulin with chromatin attached to the outer edges of the spindle in a rosette formation. (B5) Multipolar spindle.

In the present report, we have summarized (i) abnormal morphological and cellular changes that take place during oocyte aging (Fig. 1; Table I) and their effects on embryo/fetus development; (ii) mechanisms underlying oocyte aging and factors affecting the aging process (Tables II and III) and (iii) methods to reverse and control oocyte aging.

Methods

For this review, we included data and relevant information obtained through a PubMed database search for all articles published in English from 1952 though 2008 which included the term 'MPF', 'Ca^{2+} elevation', 'oocyte aging' and 'aged oocytes'. We further included published and unpublished data from our own laboratory and an 'in-house' library of relevant publications.

Changes in aged oocytes

Fresh matured oocytes with intact PB1 are encased in a thick glycoprotein shell called the zona pellucida. Major oocyte components and organelles include regularly aligned CGs underneath the

Table I Changes in morphology and cell biology during mammalian oocyte aging

	Fresh oocytes	Aged oocytes	Reference
PM	Microvilli extensions display intact structure	Microvilli extensions display structural alterations and are budded off into the PVS	Kim *et al.* (1996); Longo (1974); Pickering *et al.* (1988); Szollosi (1971); Webb *et al.* (1986)
Zona pellucida	Zona pellucida appears as a granulofibrillar, interconnected reticulum with pores	Zona pellucida displays a 'cobblestone' appearance and becomes harden	Goud *et al.* (2005b); Longo (1981); Miao *et al.* (2005); Xu *et al.* (1997)
PVS	Small	Large	Miao *et al.* (2004)
CG	CGs are densely populated in a line just beneath the oolemma, with a typical normal CG-free domain above the meiotic apparatus	CGs undergo migration and partial exocytosis	Dodson *et al.* (1989); Goud *et al.* (2005b); Gulyas (1979); Longo (1974); Szollosi (1971); Xu *et al.* (1997)
Microfilament	A thick microfilament domain exists in the oocyte cortex	Disrupted or lost	Kim *et al.* (1996)
Spindle	Spindles display vertical orientation to the oolemma and each pole is associated with a ring of centrosome proteins	Spindles become elongated and/or smaller and few microtubular foci are detectable at the cortex	Eichenlaub-Ritter *et al.* (1986); Eichenlaub-Ritter *et al.* (1988); Goud *et al.* (2004); Longo (1974); Meyer and Longo (1979); Segers *et al.* (2008); Slozina *et al.* (1990); Wang *et al.* (2001)
Chromosomes	Chromosomes are intact and arranged symmetrically on the metaphase plate	Chromosomes display PCS and are scattered throughout the degenerating spindle and some chromosomes show centripetal migration, dispersion, decondensation and formation of a single chromatin mass	Eichenlaub-Ritter *et al.* (1988); Mailhes *et al.* (1998); Rodman (1971); Steuerwald *et al.* (2005); Szollosi (1971); Van Wissen *et al.* (1991); Zenzes and Casper (1992)
Mitochondria	Mitochondria are intact	Membrane potential decrease and mitochondrial matrix swell	Wilding *et al.* (2001)
PB1	PB1 is intact and adjacent to the MII spindle	PB1 degenerates and deviates from the MII spindle	Miao *et al.* (2004)

Table II Effects of various environmental factors on oocyte aging

Aging environment	Effects	References
Temperature	Fertilization of room-temperature-aged (27°C) oocytes results in mouse full-term births. Oocytes aged in a refrigerator (4°C) or incubator (37°C) loses the developmental potential	Lei *et al.* (2008a); Lei *et al.* (2008b); Wakayama *et al.* (2004)
In vivo and *in vitro*	NO: similar morphological alterations and cytoskeletal organization	Longo (1980); Miao *et al.* (2005); Webb *et al.* (1986)
	YES: oocytes aged *in vivo* display a larger spindle and microtubule asters. Spindles in oocytes aged *in vitro* are close to the PM and display different orientations. *In vitro* culture retards oocyte aging	Abbott *et al.* (1998); Adenot *et al.* (1997)
CC	Accelerate oocyte aging by secreting a soluble APF into the medium	Miao *et al.* (2005); Qiao *et al.* (2008)
ROS	Superoxide induces oocyte zona pellucida hardening, ooplasmic microtubule dynamics increase and major CGs losses. H_2O_2 renders fresh oocytes resistant to aging but enhances the further aging in aged oocytes. Low levels of HOCl induce the aging of fresh and aged oocytes, while higher concentrations of HOCl compromise oocyte viability	Goud *et al.* (2008)

Table III Effects of various chemicals on oocyte aging

Chemicals	Effects	References
L-cystine	Decreases oocyte development to blastocyst stage	Tarin *et al.* (1998)
β-mercaptoethanol	Decreases development to blastocyst stage and decrease cellular fragmentation	
Vanadate	Accelerates oocyte aging	Kikuchi *et al.* (2002); Kikuchi *et al.* (2000)
Caffeine	Inhibits oocyte aging	
NO	Delays oocyte aging	Goud *et al.* (2005a, b)
DTT	Increases fertilization rate and number of embryo cells. Prevents fragmentations and increases blastocyst stage development. Decreases percentage of ICM cell nuclei with DNA fragmentation	Rausell *et al.* (2007); Tarin *et al.* (1998)
TSA	Porcine: reduces oocyte fragmentation and increases developmental capacity; Mouse: accelerate oocytes aging	Jeseta *et al.* (2008); Huang *et al.* (2007)

oocyte cortex, microfilaments localized underneath the plasma membrane (PM) and within microvilli, the MII spindle with chromosomes aligned by microtubules at the metaphase plate and microtubule organizing centers (centrosomes) located at the two opposite spindle poles (Eichenlaub-Ritter *et al.*, 1988; Sun and Schatten, 2007; Schatten, 2008), mitochondria, and other organelles that are typical for mammalian cells. Oocyte aging causes numerous morphological and cellular alterations, including changes in structure of the PM, zona pellucida, cytoskeleton, mitochondria, displacement of the spindle, misalignment of chromosomes, dispersion of centrosomal material, displacement of PB1 and CGs, as well as premature exocytosis of CGs.

During aging, microvillar extensions of the PM display structural alterations and may be budded off into the PVS as material with a flocculent appearance. In fresh oocytes, thick and thin microfilament domains are distributed along the oocyte cortex with chromosomes located beneath the thick microfilament domain. In contrast, the thick microfilament domain underlining the PM is disrupted or lost in aged oocytes (Szollosi, 1971; Longo, 1974; Webb *et al.*, 1986; Pickering *et al.*, 1988; Kim *et al.*, 1996). The zona pellucida of fresh oocytes consists of granulofibrillar, interconnected reticulum with pores, whereas the zona pellucida of aged oocytes displays a 'cobblestone' appearance, which is composed of tight aggregations of granulofibrillar material, separated from one another by a distinct space (0.3 μm in width) (Longo, 1981). Zona pellucida hardening assays reveal that the time for chymotrypsin-mediated dissolution of the zona pellucida increases significantly in aged oocytes compared with fresh oocytes (Longo, 1981; Xu *et al.*, 1997; Goud *et al.*, 2005b; Miao *et al.*, 2005). Oocyte aging also results in increases in the number of lysosomes and aggregates of tubuli from smooth endoplasmic reticulum (ER) and small mitochondria–vesicle complexes (Longo, 1974; Sundstrom *et al.*, 1985).

In fresh oocytes, the membrane-bound small CGs underneath the oocyte cortex will undergo a precisely coordinated cortical reaction triggered at fertilization and will release their contents into the PVS which results in modification of the zona pellucida and an extracellular block to polyspermy (Okada *et al.*, 1993). CGs are densely aligned as a layer just beneath the oolemma in fresh oocytes, with a typical CG-free domain above the meiotic apparatus (Fig. 2A). Although fresh oocytes will typically only undergo the CG reaction when fertilized with sperm, resulting in extrusion of the contents of all CGs into the extracellular space and fusion of CG membranes with the oocyte PM, cortical reactions in aged oocytes are easily triggered spontaneously without fertilization. CGs become displaced and undergo partial exocytosis (Szollosi, 1971; Longo, 1974; Gulyas, 1979; Dodson *et al.*, 1989; Xu *et al.*, 1997; Goud *et al.*, 2005b).

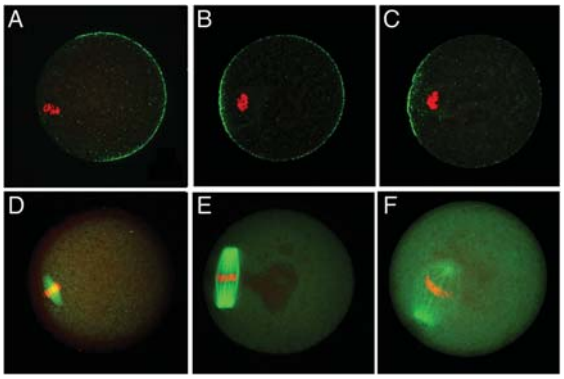

Figure 2 Confocal micrographs of fresh and aged mouse oocytes displaying distributions of cortical granules (green), spindle morphology (green) and chromosomes (red).
(**A**) Distribution of CG in fresh oocytes; (**B, C**) Distribution of CG in aged oocytes; (**D**) Spindle morphology of fresh oocytes; (**E, F**) Abnormal spindle morphologies of aged oocytes.

CG distribution in aged oocytes falls into two morphologically distinct groups: (i) a ring of CGs beneath the oolemma (Fig. 2B); (ii) a cap of higher density CGs located above the chromosome area (Fig. 2C).

Further aging effects are seen at the oocyte's surface with the displacement of the PB1. PB1 morphology is frequently used as for a criterion of embryo quality when ICSI is performed. Oocytes with intact well-shaped PB1 yield higher fertilization rates and higher quality of embryos (Ebner *et al*., 2000) whereas PB1 displacement and degeneration is associated with oocyte aging (Hardarson *et al*., 2000; Miao *et al*., 2004). It has been shown that the PB1 frequently begins to degenerate before ovulation, and around 70% became degenerated within 6 h after maximal nuclear maturation. Some PB1 showed displacement from the MII spindle at maximal nuclear maturation and the distance between PB1 and the spindle increases with time during oocyte aging. PVS increases with time and facilitated lateral displacement of the degenerating PB1 (Miao *et al*., 2004).

The meiotic spindle contains the oocyte's genetic information and an intact meiotic spindle is critically important for accurate distribution of chromosomes to the dividing blastomeres, thus ensuring accurate embryo development. New data are now available on molecular mechanisms underlying meiotic spindle functions in fresh oocytes that have clearly shown the critical importance of meiotic spindles in fertilization and embryo development (reviewed in Sun and Schatten, 2007; Schatten, 2008). The meiotic spindle is an essential cellular structure responsible for the accurate separation of homologous chromosomes (MI) or two sets of chromatids (MII) during germ cell division (Wang and Sun, 2006). Fresh human oocytes contain compact anastral spindles oriented orthogonal to the oolemma, with the pole adjacent to the oolemma being smaller than that directed towards the center of the oocyte; the spindle size is 11.2 ± 3.4 μm. Each pole contains centrosomes characterized by a ring of particulate γ-tubulin staining that extends a short distance into the spindle body. No α- and γ-tubulin staining is found elsewhere in the ooplasm. In contrast, spindles in human oocytes aged for 1 day are shorter (8.08 ± 0.84 μm) than spindles in fresh oocytes. The spindle of oocytes aged for 2 days is smaller still, and can be bi- or multipolar, which will have severe consequences for chromosome segregation and hence result in abnormalities. In spindles of 2-day-aged oocytes microtubules radiate towards the cell periphery and form additional microtubule asters in the cytoplasm. The γ-tubulin staining pattern in aged human oocytes reveals increased staining over the entire spindle compared with fresh oocytes (George *et al*., 1996; Wang *et al*., 2001), indicating a loss of centrosome structure at the meiotic poles which is associated with loss of microtubule integrity (Sun and Schatten, 2007; Schatten, 2008) and loss of chromosome maintenance at the metaphase plate. As shown in Fig. 1B1–B5 various patterns of spindle disorganization can be seen in aged oocytes. Furthermore, immunocytochemical studies show that aged, failed-to-fertilize human oocytes showed disrupted spindles, tetrapolar spindles, aberrant expression of the nuclear mitotic apparatus (NuMA) protein, which provides spindle stability in fresh oocytes, and changes in the microtubule kinesin motor protein EG5 (Hall *et al*., 2007).

As it is difficult to perform extensive studies on human oocytes because of ethical concerns and limitations to obtain sufficient numbers of human oocytes for research, the mouse and pig have been used for many studies with the intent to extrapolate data to humans (Prather, 2007). These studies show that in fresh mouse oocytes, microtubules are clearly detected in the metaphase spindle; additional small microtubule asters are detected in the cytoplasms which are organized by cytoplasmic centrosomes. The mouse oocyte spindle is barrel-shaped or slightly pointed and its poles do not display astral fibers (Schatten and Schatten, 1986; Adenot *et al*., 1997; Goud *et al*., 2004, 2005b) (Fig. 2D). However, in aged mouse oocytes, microtubules become gradually lost from the spindle, with preferential loss in the central spindle area near the chromosomes. Astral fibers radiate out from the polar centrosomes into the cytoplasm whereas the mean pole-to-pole distance becomes significantly reduced. At the same time astral microtubules in the cytoplasm become gradually depolymerized (Longo, 1974; Meyer and Longo, 1979; Eichenlaub-Ritter *et al*., 1986, 1988; Slozina *et al*., 1990; Wang *et al*., 2001; Goud *et al*., 2004; Segers *et al*., 2008) (Fig. 2E, F). More detailed studies on specific centrosome proteins have been performed in porcine oocytes by Lee *et al*. (2000), who showed that the centrosome proteins γ-tubulin and NuMA were in uniform distribution across the nucleus in pig oocytes. Nocodazole treatment, an inhibitor of microtubule polymerization, induced disappearance of the polar NuMA staining in porcine MII oocytes, whereas the mouse meiotic spindle pole was resistant to the treatment (Lee *et al*., 2000). Recent studies have shown that the expression of spindle checkpoint protein MAD2 (mitotic arrest deficient protein) is gradually reduced during pig and mouse oocyte aging (Ma *et al*., 2005; Steuerwald *et al*., 2005).

It has been established that oocyte aging results in significant increases in premature chromosome separation, that is strongly associated with aneuploidy (Mailhes *et al*., 1998; Steuerwald *et al*., 2005). Chromosomes in human oocytes aged for 2 days are no longer aligned at the spindle equator but are scattered within the degenerating spindle. In oocytes aged for 3–4 days, chromosomes become more decondensed and display nuclear alterations. Chromosomes show centripetal migration, dispersion, decondensation and formation of a single chromatin mass. Chromosome loss, fragmentation or clumping of chromosomes and chromatid separation have also been observed (Rodman, 1971; Szollosi, 1971; Eichenlaub-Ritter *et al*., 1988; Zenzes and Casper, 1992; Van Wissen *et al*., 1991). In

aged oocytes, mitochondrial membrane potential is decreased and swelling of the mitochondrial matrix takes place (Wilding *et al*., 2001).

Oocyte aging affects embryo/fetus development

It has been well established that reproductive success decreases if fertilization occurs at a prolonged interval after ovulation. Problems associated with delayed fertilization include decreased litter size in animals, low pregnancy rate and an increased risk of spontaneous miscarriage in humans and animals (Huhtinen *et al*., 1996; Wilcox *et al*., 1998; Tarin *et al*., 2000). After fertilization of aged oocytes, F_0 females exhibit lower pregnancy rates, shortened gestation length, decreased litter size and higher perinatal death of their pups compared with normal fertilization. Postovulatory aging of oocytes is also associated with increased numbers of growth-retarded pups, delayed development of the righting reflex, higher spontaneous motor activity and higher emotional distress in F_1 offspring; however, postovulatory aging of F_0 oocytes does not affect birthweight, weight gain during pre-weaning development, and both passive and active conditioned learning ability of F_1 offspring (Tarin *et al*., 1999). F_1 females exhibit longer in-between-labor intervals, decreased frequency of litters, and lower total numbers of litters and born offspring. F_2 pups display teratogenic defects, higher pre-weaning mortality, and decreased body weight at weaning. Incidence of infertility is higher in F_1 males, which translates into lower total number of born offspring. Life expectancy of F_1 offspring is decreased. These results clearly show that postovulatory aging of mouse oocytes decreases reproductive fitness and longevity of offspring (Tarin *et al*., 2002). Aged human oocytes showed low developmental potential when they were used for nuclear transfer (NT), as indicated by significantly lower fusion rates, cleavage rates and subsequent development (Hall *et al*., 2007).

Only inconclusive results are available on the sex ratios after delayed artificial insemination. Although it has been reported that delayed artificial insemination increases the percentage of male offspring in the mouse (Tarin *et al*., 1999), other reports do not show an effect of delayed insemination on the sex ratio of offspring in humans (Gray *et al*., 1998). Species-specific differences may exist; in some mammals, aged oocytes may be able to select for a specific sperm genotype based on intrinsic differences in viability, motility, capacitation and/or capability to undergo acrosome reaction of X- or Y-bearing spermatozoa before fertilization. In bovine MII oocytes fertilized *in vitro* immediately after PB1 extrusion, the male-to-female sex ratio of the resulting embryos was 0.71 ± 1.49, but increased to 1.67 ± 2.52 in MII oocytes aged *in vitro* for a period of 8 h prior to fertilization (Dominko and First, 1997; Gray *et al*., 1998; Gutierrez-Adan *et al*., 1999).

Mechanisms of oocyte aging

Oocyte aging: a prelude to apoptosis induced by abnormal Ca^{2+} elevation

In mammals, at fertilization the sperm activates oocyte development by initiating a prolonged series of elevations in intracellular Ca^{2+} ($[Ca^{2+}]i$), generated by increased production of inositol 1,4,5-triphosphate (IP3). The sperm initiates IP3 generation via the introduction of a sperm factor into the oocyte after sperm-oocyte fusion, and sperm-specific phospholipase C, is the primary candidate for the Ca^{2+} elevation-inducing factor (Bos-Mikich *et al*., 1995; Kurokawa *et al*., 2004, 2007; Igarashi *et al*., 2007; Yoon and Fissore, 2007). During fertilization of aged mouse oocytes, the frequency of Ca^{2+} elevations is significantly higher than in fresh oocytes. However, the amplitude of the oscillations in aged oocytes is significantly smaller than that in fresh oocytes (Igarashi *et al*., 1997). When extracellular Ca^{2+} ($[Ca^{2+}]e$) is raised, aged oocytes show significant increases in the duration of individual Ca^{2+} elevations, resulting in a sustained elevation of $[Ca^{2+}]i$ which may result in dysfunctions of $[Ca^{2+}]i$ regulation, presumably of the Ca^{2+} pump of the ER (Vincent *et al*., 1992; Jones and Whittingham, 1996; Igarashi *et al*., 1997, 2005; Takahashi *et al*., 2000, 2003). Others reported a decline in IP3-induced Ca^{2+} release in MII mouse oocytes at 24 h post hCG or later, although repetitive Ca^{2+} transients were induced in aged oocytes after IVF (Jones and Whittingham, 1996). The reasons for the contradictory reports are unclear. Abnormal Ca^{2+} elevations cause fragmentation and other indications of programmed cell death because one or several critical cytosolic molecules involved in the regulation of Ca^{2+} homeostasis and in maintaining the equilibrium between anti- and proapoptotic proteins are either lost or inactivated during postovulatory oocyte aging, turning the fertilizing Ca^{2+} signal into an apoptosis-inducing signal (Fissore *et al*., 2002; Gordo *et al*., 2002). Verbert *et al*. (2008) reported that the IP3 receptor is a tetrameric channel that accounts for a large part of the $[Ca^{2+}]i$ release, and the $[Ca^{2+}]i$ leak acts as a feed-forward mechanism to divert the aging oocytes into apoptosis when the cellular metabolism is compromised (Verbert *et al*., 2008).

Mitochondria can create energy for cellular activity by the process of aerobic respiration. Moreover, mitochondria contain anti-apoptotic and apoptogenic components, and its dysfunctions are likely to play an important role in the predisposition of aged oocytes to spontaneous and fertilization- or activation-induced fragmentation (Green and Reed, 1998; Wang, 2001). DNA fragmentation promotes the release of cytochrome c from the respiratory chain into the cytosol, which in turn results in activation of the caspase cascade. Caspase activity is regulated by the Bcl-2 family of proteins. The Bcl-2 family is comprised of proapoptotic (e.g. Bax and Bak) and antiapoptotic (e.g. Bcl-2 and Bcl-xL) regulators that block or stimulate cytochrome c release in response to multiple death-inducing stimuli (Kluck *et al*., 1997; Vander Heiden and Thompson, 1999; Hsu and Hsueh, 2000). The amounts of Bcl-2 mRNA and protein, and the level of ATP, decrease in aged oocytes and may negatively impact the function of the Ca^{2+} pump and induce abnormal Ca^{2+} elevation (Perez and Tilly, 1997; Gordo *et al*., 2002; Ma *et al*., 2005; Tatone *et al*., 2006; Verbert *et al*., 2008).

Abnormal epigenetic changes are associated with oocyte aging

Establishment of the epigenetic configuration of the genome is part of the maturation process of gametes, and is essential for normal development after fertilization which includes methylation of CpG islands and methylation/acetylation of histones among others (Li, 2002; Shiota and Yanagimachi, 2002). *Snrpn* and *Peg1/Mest* are two maternally imprinted genes which are expressed from the paternal allele. *Snrpn* is fully methylated in fresh oocytes, but clearly demethylated

in aged oocytes. In contrast, *Peg1/Mest* is fully methylated in fresh oocytes and does not show any demethylation at 29 h post-hCG both in *in vivo* aged oocytes and *in vitro* aged oocytes (Liang *et al*., 2008). Imamura *et al*. (2005) reported that *Peg1/Mest* showed demethylation at 42 h post-hCG under *in vitro* conditions (Imamura *et al*., 2005). These results show that imprinted genes in oocytes will undergo time-dependent demethylation during the process of postovulatory aging. Decreased *Dnmt1* (*Dnmt1o* and *Dnmt1s*) expression and increased *Dnmt3b* are also observed in oocytes during aging (Hamatani *et al*., 2004).

Acetylation of nuclear core histones is thought to play an important role in various cellular functions (Kurdistani and Grunstein, 2003). The acetylation levels of lysine 14 on histone H3 (H3K14) and lysines 8 and 12 on histone H4 (H4K8/K12) in mouse oocytes are gradually increased during oocyte aging. Furthermore, raising the level of histone acetylation in fresh oocytes by treatment with trichostatin A (TSA) for 5 h can accelerate the progression of oocyte aging, suggesting that alteration of acetylation on histones H3 and H4 can affect the aging process (Huang *et al*., 2007). However, these findings are different from the results reported by Jeseta *et al*. (2008) who showed that TSA could significantly reduce the percentage of fragmented pig oocytes from 30% in untreated oocytes to 9% in oocytes aged in 100 nM TSA-supplemented medium for 3 days (Jeseta *et al*., 2008). Building on the results above, we found that porcine oocytes maintained the fresh status longer than mouse oocytes. Most aged porcine oocytes underwent spontaneous parthenogenetic activation and fragmentation; however, few aged mouse oocytes underwent this process (our unpublished data). These studies suggest that TSA may have different effects on oocyte aging in different species.

Factors affecting oocyte aging (Table II)

Temperature. Negative effects of temperature changes on mouse oocyte aging have clearly been shown. When diploid parthenotes were aged at room temperature (27°C) development proceeded to blastocyst stages (10–57%, with different fertilization methods and embryo culture media). Fertilization of room-temperature-aged oocytes with fresh spermatozoa or round spermatids by ICSI or IVF resulted in full-term births (Wakayama *et al*., 2004; Lei *et al*., 2008a, b), which indicates that we may be able to extrapolate these data to humans and select appropriate temperatures to store human oocytes during ART for a short time. However, oocytes aged at 4°C almost entirely lost developmental potential and most oocytes developed abnormalities including water bubbles in the cytoplasm, and none were considered normal; only some of these oocytes (<13%) were able to develop to the morula/blastocyst stages. Cold treatment may have irreversibly destroyed vital oocyte components, perhaps by destroying cytoskeletal components in the microtubule-rich spindle that is highly responsive to environmental changes.

Aging in vivo *and* in vitro. Activation rates and MPF activity of mouse oocytes aged *in vitro* are similar to those aged *in vivo* (Miao *et al*., 2005). Mouse oocytes showed similar morphological alterations and cytoskeletal organization during aging *in vivo* and *in vitro* (Longo, 1980; Webb *et al*., 1986) although previous studies by Adenot *et al*. (1997) showed that microtubular organization in MII oocytes aged *in vivo* differed from oocytes aged *in vitro*. Rabbit oocytes aged *in vivo* generally contain a larger spindle and display microtubule asters. In contrast, the spindle of oocytes aged *in vitro* is generally localized close to the PM and displays altered orientation (Adenot *et al*., 1997). Abbott *et al*. (1998) also reported that *in vitro* culture of mouse oocytes retarded the spontaneous activation of cell cycle progression that normally occurs in *in vivo* aged mouse oocytes (Abbott *et al*., 1998). The liability of the MII spindle to aging may be species-specific; it has been shown by Mullen *et al*., that different species display different sensitivities to environmental factors including osmotic changes and that recovery also differs in different species (Mullen *et al*., 2004, 2007, 2008).

Cumulus cells. CC are critical for oocyte maturation, ovulation and fertilization (Tanghe *et al*., 2002). We found that CCs accelerate the aging process of mouse oocytes. During *in vitro* aging of both *in vivo* matured and *in vitro* matured mouse oocytes with CCs, activation rates increased, whereas MPF activity decreased significantly compared with *in vivo* aging of ovulated oocytes. However, during aging following removal of CCs, activation rates of both *in vivo* matured and *in vitro* matured oocytes remained low and the MPF activity decreased at a much slower rate compared with that of oocytes aged with CCs. Many oocytes aged *in vivo* and *in vitro* with CCs showed a partial CG release, but few oocytes without CCs released their CGs during *in vitro* aging (Miao *et al*., 2005). When denuded oocytes (DO) were cultured with cumulus–oocyte-complexes (COC) at a 1:2 ratio or on a CC monolayer, activation rates increased whereas MPF activity decreased significantly, which indicates that a soluble factor is responsible for the aging-promoting effect. The *in vivo* and *in vitro* matured DOs did not differ in responsiveness to aging-promoting factor (APF). Heat shock did not accelerate oocyte aging unless in the presence of CCs. The production of APF was not affected by age or by the maturation system of COCs but increased with density and duration of culture. These results strongly suggest that CCs accelerate oocyte aging by secreting a soluble APF into the medium. Further analysis showed that the APF was heat labile but stable to freezing. It had a threshold effective concentration and can be depleted by DOs (Qiao *et al*., 2008). Additionally, CCs that surround the oocytes, as well as follicular fluid, may protect the oocytes from the damaging effects of reactive oxygen species (ROS) (Tatemoto *et al*., 2004).

Reactive oxygen species. The free radical theory of aging highlights peroxidative damage to nuclear DNA or cell membranes as the main mechanism for the decay of cellular function (Harman, 1956). ROS such as superoxide, H_2O_2 and HOCl are produced continuously in mitochondria because of the 'leakage' of high energy electrons along the electron transport chain. Although mitochondrial proteins and lipids can be damaged, mitochondrial DNA is a major target for oxidative attack, because of its location near the inner mitochondrial membrane sites where oxidants are formed, as well as its lack of both protective histones and of DNA repair activity (Shigenaga *et al*., 1994). When fresh oocytes are exposed to superoxide, the zona pellucida dissolution time of these oocytes increases significantly. Further, superoxide exposure of fresh oocytes exhibited increased ooplasm microtubule dynamics (OMD) and major CG loss. But interestingly, fresh oocytes exposed to H_2O_2 resist 'aging', although the same concentrations of H_2O_2 enhanced the aging phenomena in relatively aged oocytes, which demonstrates that aged oocytes exhibit increased sensitivity to low concentrations of H_2O_2 and undergo the induction of the aging phenomena. Fresh oocytes exposed to low concentrations of HOCl (1–10 μM) accelerate the aging phenomena, although higher concentrations of

HOCl (0.1–1 mM) caused lysis of the cell membrane and death of the oocyte. In contrast, aged oocytes underwent lysis even when exposed to 1 μM HOCl. These data indicate that superoxide, H_2O_2, and HOCl each augment oocyte aging, more so in relatively old oocytes, suggesting compromised antioxidant capacity in aged oocytes (Goud *et al*., 2008).

Reversibility of oocyte aging: restoring aging-controlled factors

Mechanisms and practical applications of oocyte aging control

The reversibility of oocyte aging was first explored by Kikuchi *et al*., who found that oocyte aging can be reversed by controlling the activity of MPF (Kikuchi *et al*., 2000, 2002). Other investigators found that both MPF and MAPK are important for maintaining oocytes arrested at MII, as their activities gradually decreased during oocyte aging (Xu *et al*., 1997; Tian *et al*., 2002; Fan and Sun, 2004; Tatone *et al*., 2006; Liang *et al*., 2007). The inactivation of MPF and MAPK occurs earlier in aged oocytes in comparison to fresh oocytes, and the responses to oocyte activation by the two kinases are faster in aged than in fresh oocytes. When different stimuli were employed, aged oocytes were easily activated, with evident MAPK inactivation when compared with fresh oocytes. MPF comprises two molecules, a catalytic subunit, $p34^{cdc2}$, and a regulatory subunit, cyclin B (Murray and Kirschner, 1989). However, Kikuchi *et al*. (1999) found that aged porcine oocytes contained abundant MPF that is inactivated by phosphorylation (called pre-MPF, a phosphorylated MPF at threonine 14 and/or tyrosine 15) and suggested that the decrease in MPF activity in porcine aged oocytes could be attributed in part to the accumulation of pre-MPF via the imbalance of kinase and phosphatase activities. The reverse pathway from dephosphorylated to phosphorylated $p34^{cdc2}$ results in accumulation of inactive MPF and a decrease in the activity of MPF (Kikuchi *et al*., 1995, 1999, 2000, 2002). Controlling MPF activity by altering $p34^{cdc2}$ phosphorylation may enable *in vitro* oocyte aging delay.

Vanadate and caffeine can modify oocyte aging differently by controlling MPF activity. Previous reports have shown that vanadate is a potent inhibitor of tyrosine phosphatases, including cdc25 (Leis and Kaplan, 1982) and that caffeine could inhibit Myt1/Wee1 activity (Smythe and Newport, 1992). Fresh oocytes treated with vanadate have increased $p34^{cdc2}$ phosphorylation and reduced MPF activity as well as increased parthenogenetic activation and fragmentation; conversely, oocytes incubated with caffeine have reduced $p34^{cdc2}$ phosphorylation and increased MPF activity as well as decreased parthenogenetic activation and fragmentation. These results demonstrate that vanadate can accelerate oocyte aging, whereas caffeine can delay oocyte aging (Kikuchi *et al*., 2000, 2002) (Fig. 3).

In NT studies, high-level of MPF activity causes nuclear envelope breakdown and subsequent premature chromosome condensation when a donor nucleus is transferred into a non-activated MII oocyte, (Collas and Robl, 1991; Tani *et al*., 2001). In contrast, a donor nucleus transferred into an activated recipient cytoplasm forms a pronucleus-like structure (PN) because of the lower MPF activity (Szollosi *et al*., 1998; Tani *et al*., 2001). Kwon *et al*. (2008) found that caffeine-treated NT embryos induced premature chromosome condensation at a high rate, whereas most vanadate-treated NT embryos formed a pronucleus-like structure (Kwon *et al*., 2008), which demonstrates that caffeine could cause oocytes to maintain high MPF activity whereas vanadate decreased the MPF activity in oocytes. Lee and Campbell (2008) also found that 10 mM caffeine could prevent the decline in MPF and MAPK activity in aging bovine oocytes, however, caffeine could not reverse oocytes to acquire the activation competence. When aged oocytes treated with caffeine were used to reconstruct NT embryos, a significant increase in NT blastocyst cell numbers were obtained (Lee and Campbell, 2008).

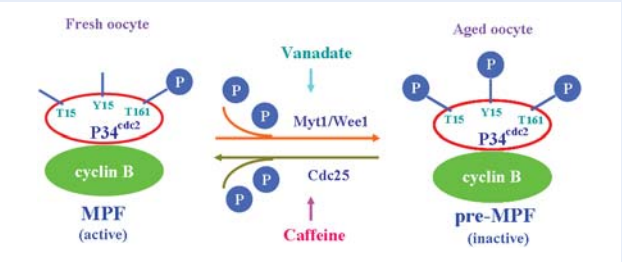

Figure 3 Schematic diagram of mechanisms involved in decreases in maturation promotion factor (MPF) activity in aged porcine oocytes and the effects of vanadate and caffeine.The balance of pre-MPF and MPF in matured oocytes is controlled by Myt1/Wee1 and cdc25 activities. In aged oocytes, MPF activity decreases because of pre-MPF accumulation caused by imbalance of Myt1/Wee1 and cdc25 activities. Vanadate suppresses cdc25 activity in fresh oocytes, resulting in accumulation of pre-MPF and decreased MPF activity; conversely, caffeine suppresses Myt1/Wee1 activity in aged oocytes, resulting in a shift of pre-MPF to active MPF and increased MPF activity.

Our recent unpublished results confirmed and supported their findings in mice. Continuous application of caffeine is necessary, as oocytes treated with 5 mM caffeine will age if they are subsequently cultured *in vitro* without caffeine. Spindles in oocytes treated with 5 mM caffeine maintain their normal morphology just like fresh oocytes. Although a cap of higher density CGs was often observed above the chromosome area in aged oocytes (Fig. 2B, C), oocytes treated with 5 mM caffeine showed decreased distribution of CGs over the chromosome area. Furthermore, oocytes treated with 5 mM caffeine showed increased fertilization and decreased fragmentation when spermatozoa were injected (our unpublished data). These results may indicate that caffeine could stabilize organelles in oocytes, which in turn results in positive development of oocytes after fertilization.

In human reproduction, sexual behavior can be controlled to avoid oocyte aging before fertilization. However, it is difficult to perform extensive studies on human oocytes because of ethical concerns and limited availability of sufficient human oocytes for research. Most research on oocyte aging is focused on animal models and numerous experiments have been performed on animal oocytes (Table III). Whether or not the results can be applied to human oocytes for clinical applications remains to be seen.

Except for vanadate and caffeine, there are other agents that can be used to control oocyte aging and have been tested in various animal species. The incorporation of L-cystine into the cells is the major

limiting step in the synthesis of glutathione (GSH), which has a major role in protecting cells from ROS and electrophiles. Abnormal level of GSH may cause impairment of Ca^{2+} transport and subsequent perturbation of intracellular Ca^{2+} homeostasis (Christensen, 1990). Dithiothreitol (DTT) and β-mercaptoethanol are chelate divalent cations (Cornell and Crivaro, 1972) that affect oocyte aging through different mechanisms. DTT is a dithiol with two end sulphydryl groups, and β-mercaptoethanol is a monothiol bearing one end sulphydryl residue. Tarin *et al*. (1998) showed that the probability of an oocyte reaching the blastocyst stage is decreased when incubated in the presence of L-cystine and β-mercaptoethanol. However, age-associated cellular fragmentation is partially prevented by incubation in the presence of β-mercaptoethanol, and both age-associated fragmentations at 24 h post-insemination and decreased potential of oocytes for development to the blastocyst stage are prevented, at least in part, by culturing oocytes in the presence of DTT (Tarin *et al*., 1998). DTT prevents membrane fusion during the first cell cycles in sea urchins (Schatten, 1994), and it may be speculated that it prevents membrane fusion of CGs with the PM, therefore preventing premature CG discharge. However, no detailed studies have been performed on this aspect and the mechanisms remain unclear. It is also possible that DTT may prevent dispersion of centrosomes in MII spindles and therefore prevent disorganization of the MII spindle; in sea urchin oocytes, centrosome dispersion was prevented by DTT treatment during first cell division whereas centrosome functions were not affected (Schatten, 1994). However, the precise mechanism(s) by which DTT exerts its beneficial effects on aged oocytes remains to be elucidated; it may protect oocytes by preventing oxidation of free thiol groups and/or altering a redox-independent signaling pathway that mediates cellular fragmentation and death. Rausell *et al*. (2007) reported that fresh oocytes exhibited higher relative levels of glutathione S-transferase activity and thiols. After oocytes were aged *in vitro* in the presence of 0, 5, 50 or 500 μM DTT for 6 h prior to insemination, day 5 blastocysts from the 5, 50 and 500 μM DTT groups exhibit higher total numbers of cells, including higher numbers of inner cell mass (ICM) cells, and ICM/ trophectoderm cell ratios but a lower percentage of numbers of nuclei with DNA fragmentation/number of ICM cells when compared with blastocysts from the 0 μM DTT group (Rausell *et al*., 2007). These results clearly show that DTT can positively affect oocyte aging and increase developmental potential, which may provide the basis of clinical applications. Although the effects are clear, the underlying mechanisms appear to be complex and the molecular pathways leading to the effects have not yet been thoroughly analyzed and elucidated.

Nitric oxide (NO) has also been positively implicated in preventing oocyte aging. NO is a ubiquitous molecule and forms a vital component in the oocyte's microenvironment from folliculogenesis through early embryo development (Jablonka-Shariff and Olson, 1998). Exposure of both fresh and aged oocytes to NO results in a significant reduction in OMD and the zona pellucida dissolution time; spontaneous CG loss is decreased in aged NO-exposed oocytes. Furthermore, NO exposure decreases the rate of spindle abnormalities. NO may serve as an atypical antioxidant, which could be attributed to its ability to scavenge cytotoxic ROS and peroxyl lipid radicals. Another mechanism by which NO could participate in delaying oocyte aging is through its activation of guanylate cyclase, which leads to an increased production of cyclic guanosine monophosphate and it could significantly diminish zona pellucida dissolution time and OMD. Collectively, NO delays oocyte aging and improves the integrity of the microtubular spindle apparatus in aged oocytes (Goud *et al*., 2005a).

Aging of oocytes can also be reversed by transferring cytoplasm from fresh oocytes containing fresh mitochondria and other organelles in order to replenish essential cellular components lost during aging, such as Bcl-2 mRNA and protein involved in the regulation of the apoptotic program. After transfer of cytoplasm, reconstructed oocytes with fresh cytoplasm have higher rates of blastocyst formation as seen after parthenogenetic activation (Bai *et al*., 2006; Harvey *et al*., 2007). Addition of fresh mitochondria into aged oocytes has positively overcome cases of infertility, which is consistent with data on mitochondrial dysfunctions in aged oocytes (Cohen *et al*., 1998; Perez *et al*., 2000). These observations demonstrate that fresh cytoplasm could partly rescue nuclear susceptibility to apoptosis resulting from *in vitro* aging.

Concluding remarks

A series of morphological and cellular changes occur during the process of oocyte aging and fertilization of aged oocytes not only affects pre- and post-implantation embryo development but also the later life of the offspring. A number of chemicals have been employed to delay oocyte aging and provide promising new possibilities for intervention in the aging process, which is particularly important for ART procedures to increase the rates of successful outcomes resulting in optimal production of normal and healthy babies.

With the development of modern ART, *in vitro* matured oocytes are widely used in advanced reproductive technologies such as IVF and ICSI. As the success rates of ART technologies are frequently impacted by oocyte aging, control of oocyte aging would offer a significant advantage in allowing sufficient manipulation time and selection of oocytes of the highest quality. Therefore, establishment of methods for aging control might enhance progress in ART technologies. In this review, we summarized the mechanisms of oocyte aging and delay or reversibility of the aging process and we emphasized methods to control oocyte aging.

The present report is the first to propose caffeine as a most promising treatment to significantly decrease aging of oocytes cultured for a long period of time; furthermore, caffeine treatment is easy to apply. We further emphasized NO and DTT, both of which provide promising avenues to increase the time for maintaining oocyte freshness which will be useful for ART technologies.

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References

Abbott AL, Xu Z, Kopf GS, Ducibella T, Schultz RM. In vitro culture retards spontaneous activation of cell cycle progression and cortical granule exocytosis that normally occur in in vivo unfertilized mouse eggs. *Biol Reprod* 1998;**59**:1515–1521.

Adenot PG, Szollosi MS, Chesne P, Chastant S, Renard JP. In vivo aging of oocytes influences the behavior of nuclei transferred to enucleated rabbit oocytes. *Mol Reprod Dev* 1997;**46**:325–336.

Austin CR. In: Whittaher JR (ed). *Concepts of Development*. Sinauer Associates Inc., Publisher, 1974.

Badenas J, Santalo J, Calafell JM, Estop AM, Egozcue J. Effect of the degree of maturation of mouse oocytes at fertilization: a source of chromosome imbalance. *Gamete Res* 1989;**24**:205–218.

Bai ZD, Liu K, Wang XY. Developmental potential of aged oocyte rescued by nuclear transfer following parthenogenetic activation and in vitro fertilization. *Mol Reprod Dev* 2006;**73**:1448–1453.

Ben-Rafael Z, Kopf GS, Blasco L, Tureck RW, Mastroianni L Jr. Fertilization and cleavage after reinsemination of human oocytes in vitro. *Fertil Steril* 1986;**45**:58–62.

Bergere M, Selva J, Dulioust E, Rince P, Auroux M. Influence of male sexual rest and oocyte aging on parthenogenesis frequency in mice: cytogenetic analysis after in vitro fertilization. *Arch Androl* 1992;**28**:115–120.

Blandau RJ. The female factor in fertility and infertility. I. Effects of delayed fertilization on the development of the pronuclei in rat ova. *Fertil Steril* 1952;**3**:349–365.

Bos-Mikich A, Swann K, Whittingham DG. Calcium oscillations and protein synthesis inhibition synergistically activate mouse oocytes. *Mol Reprod Dev* 1995;**41**:84–90.

Butcher RL. Pre-ovulatory and post-ovulatory overripeness. *Int J Gynaecol Obstet* 1976;**14**:105–110.

Christensen HN. Role of amino acid transport and countertransport in nutrition and metabolism. *Physiol Rev* 1990;**70**:43–77.

Cohen J, Scott R, Alikani M, Schimmel T, Munne S, Levron J, Wu L, Brenner C, Warner C, Willadsen S. Ooplasmic transfer in mature human oocytes. *Mol Hum Reprod* 1998;**4**:269–280.

Collas P, Robl JM. Relationship between nuclear remodeling and development in nuclear transplant rabbit embryos. *Biol Reprod* 1991; **45**:455–465.

Cornell NW, Crivaro KE. Stability constant for the zinc-dithiothreitol complex. *Anal Biochem* 1972;**47**:203–208.

Dodson MG, Minhas BS, Curtis SK, Palmer TV, Robertson JL. Spontaneous zona reaction in the mouse as a limiting factor for the time in which an oocyte may be fertilized. *J In Vitro Fert Embryo Transf* 1989;**6**:101–106.

Dominko T, First NL. Relationship between the maturational state of oocytes at the time of insemination and sex ratio of subsequent early bovine embryos. *Theriogenology* 1997;**47**:1041–1050.

Ducibella T, Duffy P, Reindollar R, Su B. Changes in the distribution of mouse oocyte cortical granules and ability to undergo the cortical reaction during gonadotropin-stimulated meiotic maturation and aging in vivo. *Biol Reprod* 1990;**43**:870–876.

Ebner T, Yaman C, Moser M, Sommergruber M, Feichtinger O, Tews G. Prognostic value of first polar body morphology on fertilization rate and embryo quality in intracytoplasmic sperm injection. *Hum Reprod* 2000; **15**:427–430.

Edirisinghe WR, Law HY, Ng SC, Chia CM, Ratnam SS. Superovulation of mice with human menopausal gonadotropin or pure follicle-stimulating hormone in combination with human chorionic gonadotropin and the effects of oocyte aging on in vitro fertilization. *J In Vitro Fert Embryo Transf* 1986;**3**:314–318.

Eichenlaub-Ritter U, Chandley AC, Gosden RG. Alterations to the microtubular cytoskeleton and increased disorder of chromosome alignment in spontaneously ovulated mouse oocytes aged in vivo: an immunofluorescence study. *Chromosoma* 1986;**94**:337–345.

Eichenlaub-Ritter U, Stahl A, Luciani JM. The microtubular cytoskeleton and chromosomes of unfertilized human oocytes aged in vitro. *Hum Genet* 1988;**80**:259–264.

Fan HY, Sun QY. Involvement of mitogen-activated protein kinase cascade during oocyte maturation and fertilization in mammals. *Biol Reprod* 2004; **70**:535–547.

Fissore RA, Kurokawa M, Knott J, Zhang M, Smyth J. Mechanisms underlying oocyte activation and postovulatory ageing. *Reproduction* 2002;**124**:745–754.

Fujino Y, Ozaki K, Yamamasu S, Ito F, Matsuoka I, Hayashi E, Nakamura H, Ogita S, Sato E, Inoue M. DNA fragmentation of oocytes in aged mice. *Hum Reprod* 1996;**11**:1480–1483.

George MA, Pickering SJ, Braude PR, Johnson MH. The distribution of alpha- and gamma-tubulin in fresh and aged human and mouse oocytes exposed to cryoprotectant. *Mol Hum Reprod* 1996;**2**:445–456.

Giritharan G, Talbi S, Donjacour A, Di Sebastiano F, Dobson AT, Rinaudo PF. Effect of in vitro fertilization on gene expression and development of mouse preimplantation embryos. *Reproduction* 2007;**134**:63–72.

Gordo AC, Rodrigues P, Kurokawa M, Jellerette T, Exley GE, Warner C, Fissore R. Intracellular calcium oscillations signal apoptosis rather than activation in in vitro aged mouse eggs. *Biol Reprod* 2002;**66**: 1828–1837.

Goud P, Goud A, Van Oostveldt P, Van der Elst J, Dhont M. Fertilization abnormalities and pronucleus size asynchrony after intracytoplasmic sperm injection are related to oocyte postmaturity. *Fertil Steril* 1999a; **72**:245–252.

Goud PT, Goud AP, Laverge H, De Sutter P, Dhont M. Effect of post-ovulatory age and calcium in the injection medium on the male pronucleus formation and metaphase entry following injection of human spermatozoa into golden hamster oocytes. *Mol Hum Reprod* 1999b;**5**:227–233.

Goud AP, Goud PT, Van Oostveldt P, Diamond MP, Dhont M. Dynamic changes in microtubular cytoskeleton of human postmature oocytes revert after ooplasm transfer. *Fertil Steril* 2004;**81**:323–331.

Goud AP, Goud PT, Diamond MP, Abu-Soud HM. Nitric oxide delays oocyte aging. *Biochemistry* 2005a;**44**:11361–11368.

Goud AP, Goud PT, Diamond MP, Van Oostveldt P, Hughes MR. Microtubule turnover in ooplasm biopsy reflects ageing phenomena in the parent oocyte. *Reprod Biomed Online* 2005b;**11**:43–52.

Goud AP, Goud PT, Diamond MP, Gonik B, Abu-Soud HM. Reactive oxygen species and oocyte aging: role of superoxide, hydrogen peroxide, and hypochlorous acid. *Free Radic Biol Med* 2008;**44**:1295–1304.

Gray RH, Simpson JL, Bitto AC, Queenan JT, Li C, Kambic RT, Perez A, Mena P, Barbato M, Stevenson W *et al*. Sex ratio associated with timing of insemination and length of the follicular phase in planned and unplanned pregnancies during use of natural family planning. *Hum Reprod* 1998;**13**:1397–1400.

Green DR, Reed JC. Mitochondria and apoptosis. *Science* 1998; **281**:1309–1312.

Gulyas BJ. Cortical granules of mammalian eggs. *Int Rev Cytol* 1979; **63**:357–392.

Gutierrez-Adan A, Perez G, Granados J, Garde JJ, Perez-Guzman M, Pintado B, De La Fuente J. Relationship between sex ratio and time of insemination according to both time of ovulation and maturational state of oocyte. *Zygote* 1999;**7**:37–43.

Hall VJ, Compton D, Stojkovic P, Nesbitt M, Herbert M, Murdoch A, Stojkovic M. Developmental competence of human in vitro aged oocytes as host cells for nuclear transfer. *Hum Reprod* 2007;**22**:52–62.

Hamatani T, Falco G, Carter MG, Akutsu H, Stagg CA, Sharov AA, Dudekula DB, VanBuren V, Ko MS. Age-associated alteration of gene

expression patterns in mouse oocytes. *Hum Mol Genet* 2004; **13**:2263–2278.

Hardarson T, Lundin K, Hamberger L. The position of the metaphase II spindle cannot be predicted by the location of the first polar body in the human oocyte. *Hum Reprod* 2000;**15**:1372–1376.

Harman D. Aging: a theory based on free radical and radiation chemistry. *J Gerontol* 1956;**11**:298–300.

Harvey AJ, Gibson TC, Quebedeaux TM, Brenner CA. Impact of assisted reproductive technologies: a mitochondrial perspective of cytoplasmic transplantation. *Curr Top Dev Biol* 2007;**77**:229–249.

Hsu SY, Hsueh AJ. Tissue-specific Bcl-2 protein partners in apoptosis: an ovarian paradigm. *Physiol Rev* 2000;**80**:593–614.

Huang JC, Yan LY, Lei ZL, Miao YL, Shi LH, Yang JW, Wang Q, Ouyang YC, Sun QY, Chen DY. Changes in histone acetylation during postovulatory aging of mouse oocyte. *Biol Reprod* 2007; **77**:666–670.

Huhtinen M, Koskinen E, Skidmore JA, Allen WR. Recovery rate and quality of embryos from mares inseminated after ovulation. *Theriogenology* 1996;**45**:719–726.

Igarashi H, Takahashi E, Hiroi M, Doi K. Aging-related changes in calcium oscillations in fertilized mouse oocytes. *Mol Reprod Dev* 1997; **48**:383–390.

Igarashi H, Takahashi T, Takahashi E, Tezuka N, Nakahara K, Takahashi K, Kurachi H. Aged mouse oocytes fail to readjust intracellular adenosine triphosphates at fertilization. *Biol Reprod* 2005;**72**:1256–1261.

Igarashi H, Knott JG, Schultz RM, Williams CJ. Alterations of PLCbeta1 in mouse eggs change calcium oscillatory behavior following fertilization. *Dev Biol* 2007;**312**:321–330.

Imamura T, Kerjean A, Heams T, Kupiec JJ, Thenevin C, Paldi A. Dynamic CpG and non-CpG methylation of the Peg1/Mest gene in the mouse oocyte and preimplantation embryo. *J Biol Chem* 2005; **280**:20171–20175.

Jablonka-Shariff A, Olson LM. The role of nitric oxide in oocyte meiotic maturation and ovulation: meiotic abnormalities of endothelial nitric oxide synthase knock-out mouse oocytes. *Endocrinology* 1998; **139**:2944–2954.

Jeseta M, Petr J, Krejcova T, Chmelikova E, Jilek F. In vitro ageing of pig oocytes: effects of the histone deacetylase inhibitor trichostatin A. *Zygote* 2008;**16**:145–152.

Jones KT. Meiosis in oocytes: predisposition to aneuploidy and its increased incidence with age. *Hum Reprod Update* 2008;**14**:143–158.

Jones KT, Whittingham DG. A comparison of sperm- and IP3-induced Ca^{2+} release in activated and aging mouse oocytes. *Dev Biol* 1996; **178**:229–237.

Juetten J, Bavister BD. The effects of amino acids, cumulus cells, and bovine serum albumin on in vitro fertilization and first cleavage of hamster eggs. *J Exp Zool* 1983;**227**:487–490.

Kikuchi K, Izaike Y, Noguchi J, Furukawa T, Daen FP, Naito K, Toyoda Y. Decrease of histone H1 kinase activity in relation to parthenogenetic activation of pig follicular oocytes matured and aged in vitro. *J Reprod Fertil* 1995;**105**:325–330.

Kikuchi K, Naito K, Noguchi J, Shimada A, Kaneko H, Yamashita M, Tojo H, Toyoda Y. Inactivation of p34cdc2 kinase by the accumulation of its phosphorylated forms in porcine oocytes matured and aged in vitro. *Zygote* 1999;**7**:173–179.

Kikuchi K, Naito K, Noguchi J, Shimada A, Kaneko H, Yamashita M, Aoki F, Tojo H, Toyoda Y. Maturation/M-phase promoting factor: a regulator of aging in porcine oocytes. *Biol Reprod* 2000;**63**:715–722.

Kikuchi K, Naito K, Noguchi J, Kaneko H, Tojo H. Maturation/M-phase promoting factor regulates aging of porcine oocytes matured in vitro. *Cloning Stem Cells* 2002;**4**:211–222.

Kim NH, Moon SJ, Prather RS, Day BN. Cytoskeletal alteration in aged porcine oocytes and parthenogenesis. *Mol Reprod Dev* 1996; **43**:513–518.

Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* 1997;**275**:1132–1136.

Kruger TF, Menkveld R, Stander FS, Lombard CJ, Van der Merwe JP, van Zyl JA, Smith K. Sperm morphologic features as a prognostic factor in in vitro fertilization. *Fertil Steril* 1986;**46**:1118–1123.

Kurdistani SK, Grunstein M. Histone acetylation and deacetylation in yeast. *Nat Rev Mol Cell Biol* 2003;**4**:276–284.

Kurokawa M, Sato K, Fissore RA. Mammalian fertilization: from sperm factor to phospholipase Czeta. *Biol Cell* 2004;**96**:37–45.

Kurokawa M, Yoon SY, Alfandari D, Fukami K, Sato K, Fissore RA. Proteolytic processing of phospholipase Czeta and $[Ca^{2+}]$i oscillations during mammalian fertilization. *Dev Biol* 2007;**312**:407–418.

Kwon DJ, Park CK, Yang BK, Cheong HT. Control of nuclear remodelling and subsequent in vitro development and methylation status of porcine nuclear transfer embryos. *Reproduction* 2008;**135**:649–656.

Lanman JT. Delays during reproduction and their effects on the embryo and fetus. 2. Aging of eggs. *N Engl J Med* 1968;**278**:1047–1054.

Lee JH, Campbell KH. Caffeine treatment prevents age-related changes in ovine oocytes and increases cell numbers in blastocysts produced by somatic cell nuclear transfer. *Cloning Stem Cells* 2008;**10**:381–390.

Lee J, Miyano T, Moor RM. Spindle formation and dynamics of gamma-tubulin and nuclear mitotic apparatus protein distribution during meiosis in pig and mouse oocytes. *Biol Reprod* 2000; **62**:1184–1192.

Lei ZL, Huang JC, Shi LH, Miao YL, Nan CL, Ouyang YC, Sun QY, Chen DY. Development of normal mice after microinjection of round spermatids into oocytes stored at room temperature for one day. *Mol Reprod Dev* 2008a;**75**:795–800.

Lei ZL, Huang JC, Shi LH, Miao YL, Nan CL, Yang JW, OuYang YC, Sun QY, Chen DY. Full term development of normal mice after transfer of IVF embryos derived from oocytes stored at room temperature for 1 day. *Zygote* 2008b;**16**:21–27.

Leis JF, Kaplan NO. An acid phosphatase in the plasma membranes of human astrocytoma showing marked specificity toward phosphotyrosine protein. *Proc Natl Acad Sci USA* 1982;**79**:6507–6511.

Li E. Chromatin modification and epigenetic reprogramming in mammalian development. *Nat Rev Genet* 2002;**3**:662–673.

Liang CG, Su YQ, Fan HY, Schatten H, Sun QY. Mechanisms regulating oocyte meiotic resumption: roles of mitogen-activated protein kinase. *Mol Endocrinol* 2007;**21**:2037–2055.

Liang XW, Zhu JQ, Miao YL, Liu JH, Wei L, Lu SS, Hou Y, Schatten H, Lu KH, Sun QY. Loss of methylation imprint of Snrpn in postovulatory aging mouse oocyte. *Biochem Biophys Res Commun* 2008;**371**:16–21.

Liu J, Nagy Z, Joris H, Tournaye H, Smitz J, Camus M, Devroey P, Van Steirteghem A. Analysis of 76 total fertilization failure cycles out of 2732 intracytoplasmic sperm injection cycles. *Hum Reprod* 1995; **10**:2630–2636.

Longo FJ. Ultrastructural changes in rabbit eggs aged in vivo. *Biol Reprod* 1974;**11**:22–39.

Longo FJ. Aging of mouse eggs in vivo and in vitro. *Gamete Res* 1980; **3**:379–393.

Longo FJ. Changes in the zones pellucidae and plasmalemma of aging mouse eggs. *Biol Reprod* 1981;**25**:399–411.

Ma W, Zhang D, Hou Y, Li YH, Sun QY, Sun XF, Wang WH. Reduced expression of MAD2, BCL2, and MAP kinase activity in pig oocytes after in vitro aging are associated with defects in sister chromatid

segregation during meiosis II and embryo fragmentation after activation. *Biol Reprod* 2005;**72**:373–383.

Mailhes JB, Young D, London SN. Postovulatory ageing of mouse oocytes in vivo and premature centromere separation and aneuploidy. *Biol Reprod* 1998;**58**:1206–1210.

Marston JH, Chang MC. The fertilizable life of ova and their morphology following delayed insemination in mature and immature mice. *J Exp Zool* 1964;**155**:237–251.

McGee EA, Hsueh AJ. Initial and cyclic recruitment of ovarian follicles. *Endocr Rev* 2000;**21**:200–214.

Meyer NL, Longo FJ. Cytological events associated with in vitro aged and fertilized rabbit eggs. *Anat Rec* 1979;**195**:357–374.

Miao Y, Ma S, Liu X, Miao D, Chang Z, Luo M, Tan J. Fate of the first polar bodies in mouse oocytes. *Mol Reprod Dev* 2004;**69**:66–76.

Miao YL, Liu XY, Qiao TW, Miao DQ, Luo MJ, Tan JH. Cumulus cells accelerate aging of mouse oocytes. *Biol Reprod* 2005;**73**: 1025–1031.

Mullen SF, Agca Y, Broermann DC, Jenkins CL, Johnson CA, Critser JK. The effect of osmotic stress on the metaphase II spindle of human oocytes, and the relevance to cryopreservation. *Hum Reprod* 2004; **19**:1148–1154.

Mullen SF, Rosenbaum M, Critser JK. The effect of osmotic stress on the cell volume, metaphase II spindle and developmental potential of in vitro matured porcine oocytes. *Cryobiology* 2007;**54**:281–289.

Mullen SF, Li M, Li Y, Chen ZJ, Critser JK. Human oocyte vitrification: the permeability of metaphase II oocytes to water and ethylene glycol and the appliance toward vitrification. *Fertil Steril* 2008; **89**:1812–1825.

Murray AW, Kirschner MW. Cyclin synthesis drives the early embryonic cell cycle. *Nature* 1989;**339**:275–280.

Nagy ZP, Joris H, Liu J, Staessen C, Devroey P, Van Steirteghem AC. Intracytoplasmic single sperm injection of 1-day-old unfertilized human oocytes. *Hum Reprod* 1993;**8**:2180–2184.

Nogues C, Ponsa M, Vidal F, Boada M, Egozcue J. Effects of aging on the zona pellucida surface of mouse oocytes. *J In Vitro Fert Embryo Transf* 1988;**5**:225–229.

O'Neill GT, Kaufman MH. Influence of postovulatory aging on chromosome segregation during the second meiotic division in mouse oocytes: a parthenogenetic analysis. *J Exp Zool* 1988;**248**:125–131.

Okada A, Inomata K, Nagae T. Spontaneous cortical granule release and alteration of zona pellucida properties during and after meiotic maturation of mouse oocytes. *Anat Rec* 1993;**237**:518–526.

Perez GI, Tilly JL. Cumulus cells are required for the increased apoptotic potential in oocytes of aged mice. *Hum Reprod* 1997;**12**:2781–2783.

Perez GI, Trbovich AM, Gosden RG, Tilly JL. Mitochondria and the death of oocytes. *Nature* 2000;**403**:500–501.

Pickering SJ, Johnson MH, Braude PR, Houliston E. Cytoskeletal organization in fresh, aged and spontaneously activated human oocytes. *Hum Reprod* 1988;**3**:978–989.

Plachot M, de Grouchy J, Junca AM, Mandelbaum J, Salat-Baroux J, Cohen J. Chromosome analysis of human oocytes and embryos: does delayed fertilization increase chromosome imbalance? *Hum Reprod* 1988;**3**:125–127.

Pool TB, Martin JE, Ellsworth LR, Perez JB, Atiee SH. Zygote intrafallopian transfer with 'donor rescue': a new option for severe male factor infertility. *Fertil Steril* 1990;**54**:166–168.

Prather RS. Targeted genetic modification: xenotransplantation and beyond. *Cloning Stem Cells* 2007;**9**:17–20.

Presicce GA, Yang X. Nuclear dynamics of parthenogenesis of bovine oocytes matured in vitro for 20 and 40 hours and activated with combined ethanol and cycloheximide treatment. *Mol Reprod Dev* 1994;**37**:61–68.

Qiao TW, Liu N, Miao DQ, Zhang X, Han D, Ge L, Tan JH. Cumulus cells accelerate aging of mouse oocytes by secreting a soluble factor(s). *Mol Reprod Dev* 2008;**75**:521–528.

Rausell F, Pertusa JF, Gomez-Piquer V, Hermenegildo C, Garcia-Perez MA, Cano A, Tarin JJ. Beneficial effects of dithiothreitol on relative levels of glutathione S-transferase activity and thiols in oocytes, and cell number, DNA fragmentation and allocation at the blastocyst stage in the mouse. *Mol Reprod Dev* 2007;**74**:860–869.

Rodman TC. Chromatid disjunction in unfertilized ageing oocytes. *Nature* 1971;**233**:191–193.

Schatten H. Dithiothreitol prevents membrane fusion but not centrosome or microtubule organization during the first cell cycles in sea urchins. *Cell Motil Cytoskeleton* 1994;**27**:59–68.

Schatten H. The mammalian centrosome and its functional significance. *Histochem Cell Biol* 2008;**129**:667–686.

Schatten H, Schatten G. Motility and centrosomal organization during sea urchin and mouse fertilization. *Cell Motil Cytoskeleton* 1986;**6**:163–175.

Segers I, Adriaenssens T, Coucke W, Cortvrindt R, Smitz J. Timing of nuclear maturation and post-ovulatory aging in oocytes of in vitro-grown mouse follicles with or without oil overlay. *Biol Reprod* 2008;**78**:859–868.

Shigenaga MK, Hagen TM, Ames BN. Oxidative damage and mitochondrial decay in aging. *Proc Natl Acad Sci USA* 1994;**91**:10771–10778.

Shiota K, Yanagimachi R. Epigenetics by DNA methylation for development of normal and cloned animals. *Differentiation* 2002;**69**:162–166.

Slozina NM, Ponomareva NA, Linskaia MN, Neronova EG, Nikitin AI. Variants of chromosome compactization and the estimation of the capacity of mammalian oocytes for further development. *Tsitologiia* 1990;**32**:1187–1192.

Smythe C, Newport JW. Coupling of mitosis to the completion of S phase in Xenopus occurs via modulation of the tyrosine kinase that phosphorylates p34cdc2. *Cell* 1992;**68**:787–797.

Steuerwald NM, Steuerwald MD, Mailhes JB. Post-ovulatory aging of mouse oocytes leads to decreased MAD2 transcripts and increased frequencies of premature centromere separation and anaphase. *Mol Hum Reprod* 2005;**11**:623–630.

Sun QY, Schatten H. Centrosome inheritance after fertilization and nuclear transfer in mammals. *Adv Exp Med Biol* 2007;**591**:58–71.

Sundstrom P, Nilsson BO, Liedholm P, Larsson E. Ultrastructural characteristics of human oocytes fixed at follicular puncture or after culture. *J In Vitro Fert Embryo Transf* 1985;**2**:195–206.

Szollosi D. Morphological changes in mouse eggs due to aging in the fallopian tube. *Am J Anat* 1971;**130**:209–225.

Szollosi D, Czolowska R, Borsuk E, Szollosi MS, Debey P. Nuclear envelope removal/maintenance determines the structural and functional remodelling of embryonic red blood cell nuclei in activated mouse oocytes. *Zygote* 1998;**6**:65–73.

Takahashi T, Saito H, Hiroi M, Doi K, Takahashi E. Effects of aging on inositol 1,4,5-triphosphate-induced Ca(2+) release in unfertilized mouse oocytes. *Mol Reprod Dev* 2000;**55**:299–306.

Takahashi T, Takahashi E, Igarashi H, Tezuka N, Kurachi H. Impact of oxidative stress in aged mouse oocytes on calcium oscillations at fertilization. *Mol Reprod Dev* 2003;**66**:143–152.

Tanghe S, Van Soom A, Nauwynck H, Coryn M, de Kruif A. Minireview: functions of the cumulus oophorus during oocyte maturation, ovulation, and fertilization. *Mol Reprod Dev* 2002;**61**:414–424.

Tani T, Kato Y, Tsunoda Y. Direct exposure of chromosomes to nonactivated ovum cytoplasm is effective for bovine somatic cell nucleus reprogramming. *Biol Reprod* 2001;**64**:324–330.

Tarin JJ, Ten J, Vendrell FJ, Cano A. Dithiothreitol prevents age-associated decrease in oocyte/conceptus viability in vitro. *Hum Reprod* 1998; **13**:381–386.

Tarin JJ, Perez-Albala S, Aguilar A, Minarro J, Hermenegildo C, Cano A. Long-term effects of postovulatory aging of mouse oocytes on offspring: a two-generational study. *Biol Reprod* 1999;**61**:1347–1355.

Tarin JJ, Perez-Albala S, Cano A. Consequences on offspring of abnormal function in ageing gametes. *Hum Reprod Update* 2000;**6**:532–549.

Tarin JJ, Perez-Albala S, Perez-Hoyos S, Cano A. Postovulatory aging of oocytes decreases reproductive fitness and longevity of offspring. *Biol Reprod* 2002;**66**:495–499.

Tatemoto H, Muto N, Sunagawa I, Shinjo A, Nakada T. Protection of porcine oocytes against cell damage caused by oxidative stress during in vitro maturation: role of superoxide dismutase activity in porcine follicular fluid. *Biol Reprod* 2004;**71**:1150–1157.

Tatone C, Carbone MC, Gallo R, Delle Monache S, Di Cola M, Alesse E, Amicarelli F. Age-associated changes in mouse oocytes during postovulatory in vitro culture: possible role for meiotic kinases and survival factor BCL2. *Biol Reprod* 2006;**74**:395–402.

Tatone C, Amicarelli F, Carbone MC, Monteleone P, Caserta D, Marci R, Artini PG, Piomboni P, Focarelli R. Cellular and molecular aspects of ovarian follicle ageing. *Hum Reprod Update* 2008;**14**:131–142.

Tian XC, Lonergan P, Jeong BS, Evans AC, Yang X. Association of MPF, MAPK, and nuclear progression dynamics during activation of young and aged bovine oocytes. *Mol Reprod Dev* 2002;**62**:132–138.

Van Steirteghem AC, Nagy Z, Joris H, Liu J, Staessen C, Smitz J, Wisanto A, Devroey P. High fertilization and implantation rates after intracytoplasmic sperm injection. *Hum Reprod* 1993;**8**:1061–1066.

Van Wissen B, Bomsel-Helmreich O, Debey P, Eisenberg C, Vautier D, Pennehouat G. Fertilization and ageing processes in non-divided human oocytes after GnRHa treatment: an analysis of individual oocytes. *Hum Reprod* 1991;**6**:879–884.

Vander Heiden MG, Thompson CB. Bcl-2 proteins: regulators of apoptosis or of mitochondrial homeostasis? *Nat Cell Biol* 1999;**1**:E209–E216.

Verbert L, Lee B, Kocks SL, Assefa Z, Parys JB, Missiaen L, Callewaert G, Fissore RA, De Smedt H, Bultynck G. Caspase-3-truncated type 1 inositol 1,4,5-trisphosphate receptor enhances intracellular Ca^{2+} leak and disturbs Ca^{2+} signalling. *Biol Cell* 2008;**100**:39–49.

Vincent C, Cheek TR, Johnson MH. Cell cycle progression of parthenogenetically activated mouse oocytes to interphase is dependent on the level of internal calcium. *J Cell Sci* 1992;**103**:389–396.

Wakayama S, Thuan NV, Kishigami S, Ohta H, Mizutani E, Hikichi T, Miyake M, Wakayama T. Production of offspring from one-day-old oocytes stored at room temperature. *J Reprod Dev* 2004; **50**:627–637.

Wang X. The expanding role of mitochondria in apoptosis. *Genes Dev* 2001;**15**:2922–2933.

Wang WH, Sun QY. Meiotic spindle, spindle checkpoint and embryonic aneuploidy. *Front Biosci* 2006;**11**:620–636.

Wang Q, Sun QY. Evaluation of oocyte quality: morphological, cellular and molecular predictors. *Reprod Fertil Dev* 2007;**19**:1–12.

Wang WH, Meng L, Hackett RJ, Odenbourg R, Keefe DL. The spindle observation and its relationship with fertilization after intracytoplasmic sperm injection in living human oocytes. *Fertil Steril* 2001;**75**:348–353.

Ware CB, Barnes FL, Maiki-Laurila M, First NL. Age dependence of bovine oocyte activation. *Gamete Res* 1989;**22**:265–275.

Webb M, Howlett SK, Maro B. Parthenogenesis and cytoskeletal organization in ageing mouse eggs. *J Embryol Exp Morphol* 1986; **95**:131–145.

Wilcox AJ, Weinberg CR, Baird DD. Post-ovulatory ageing of the human oocyte and embryo failure. *Hum Reprod* 1998;**13**:394–397.

Wilding M, Dale B, Marino M, di Matteo L, Alviggi C, Pisaturo ML, Lombardi L, De Placido G. Mitochondrial aggregation patterns and activity in human oocytes and preimplantation embryos. *Hum Reprod* 2001;**16**:909–917.

Winston NJ, Braude PR, Johnson MH. Are failed-fertilized human oocytes useful? *Hum Reprod* 1993;**8**:503–507.

Xu Z, Abbott A, Kopf GS, Schultz RM, Ducibella T. Spontaneous activation of ovulated mouse eggs: time-dependent effects on M-phase exit, cortical granule exocytosis, maternal messenger ribonucleic acid recruitment, and inositol 1,4,5-trisphosphate sensitivity. *Biol Reprod* 1997;**57**:743–750.

Yoon SY, Fissore RA. Release of phospholipase C zetaand $[Ca^{2+}]$i oscillation-inducing activity during mammalian fertilization. *Reproduction* 2007;**134**:695–704.

Zackowski JL, Martin-Deleon PA. Second meiotic nondisjunction is not increased in postovulatory aged murine oocytes fertilized in vitro. *In Vitro Cell Dev Biol* 1988;**24**:133–137.

Zenzes MT, Casper RF. Cytogenetics of human oocytes, zygotes, and embryos after in vitro fertilization. *Hum Genet* 1992;**88**:367–375.

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