Animal Science Journal
Animal Science Journal (2016) **87,** 1340–1346 doi:10.1111/asj.12611

ORIGINAL ARTICLE

Post-ovulatory aging of mouse oocytes *in vivo* and *in vitro*: Effects of caffeine on exocytosis and translocation of cortical granules

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ABSTRACT

The developmental potential of post-ovulatory oocytes decreases with aging *in vivo* and *in vitro*. In this study, we aimed to investigate the effects of a potent antioxidant caffeine on cortical granules (CGs) distribution in mouse oocytes aging *in vivo* and *in vitro*. We found that *in vivo* administration of 150 mg/kg caffeine caused ovulation of some morphologically abnormal oocytes showing premature exocytosis or congregation of CGs, but significantly decreased abnormal distribution of CGs in oocytes aging for 6 h, 12 h and 18 h *in vivo* compared to those without caffeine treatment. Unexpectedly, supplementation of oocyte culture medium with 10 mmol/L caffeine accelerated CGs release of oocytes and the normal CG distribution rate dramatically decreased from 6 h in oocytes aging *in vitro*. It appeared that oocytes showed a high degree of abnormal CG distribution by aging for 18 h, and caffeine might delay oocyte CG exocytosis *in vivo*, but accelerates CG exocytosis *in vitro*. Our findings may have implications for improving assisted reproduction technologies.

Key words: *aging oocytes, caffeine, cortical granules.*

INTRODUCTION

After gonadotropin surge stimulation, prophase I oocytes resume meiosis and undergo a maturational process involving germinal vesicle breakdown (GVBD), peripheral migration of the metaphase spindle and extrusion of the first polar body (PBE). Following these events, mammalian oocytes are arrested at meiotic metaphase II (MII) until they are activated by penetrating spermatozoa or artificial activation. The health and integrity of oocytes can influence the fertilization and developmental competence of embryos (Prasad *et al.* 2015). Mammalian oocytes have a limited time for fertilization after ovulation. If not fertilized, the MII oocyte undergoes a process of deterioration *in vivo* and *in vitro*, referred to as oocyte aging, leading to a decrease in the potential for fertilization and embryo development. Additionally, aged oocytes are associated with many deleterious effects such as cellular fragmentation and cell death (McGinnis *et al.* 2014). What is more, these oocytes commonly exhibit spindle abnormalities and loss of chromosomal integrity and are increasingly susceptible to polyspermy. On the other hand, it is also reported that aging oocytes experience partial cortical granule (CG) exocytosis and zona hardening, making them less receptive to fertilization (Wang *et al.* 2014).

The intracellular signals involved in these aging events have not been well defined. In the mouse, the unfertilized mature oocytes have been viewed as a manifestation of apoptosis, or cell death, and many other defects occur, such as oxidative stress and specific gene mutations. Oxidative stress is purportedly a prominent mediator of aging oocytes and tissue types. Reactive oxygen species (ROS) are certainly potential orchestrators of this process. The level of ROS (particularly hydrogen peroxide) has been shown to significantly increase with

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Received 31 October 2015; accepted for publication 8 January 2016.

oocyte aging, with oocytes exhibiting a decreased fertilization rate and more likely entry into apoptosis.

The other prominent manifestations of aged oocytes include an increased susceptibility to activating stimuli, a decrease in maturation-promoting factor (MPF) and changes in CGs. In addition, the expression of the anti-apoptotic protein BCL2 was gradually reduced during oocyte aging (Ma *et al.* 2005; Liu *et al.* 2009). 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 & Kasibhatla 1997; Hsueh *et al.* 2000).

In mammalian oocytes, the CGs are found in Golgi-derived organelles during the early oocyte growth and maturation, which is located in the cortical region of the mature oocyte (Cran & Esper 1990). CGs contain enzymes including proteinases, glycosidases and also glycosylated materials. CG exocytosis takes place and the contents are subsequently released into the perivitelline space (PVS) after sperm-oocyte fusion (Ghetler *et al.* 1998), a process named cortical reaction (CR). At the moment, the enzymes are released to modify the zona pellucida (ZP), thus preventing polyspermy (Ben-Yosef *et al.* 1993). In fresh oocytes, the membrane-bound small CGs underneath the oocyte cortex will undergo a precisely coordinated CR triggered at fertilization and will release their contents into the PVS, which results in modification of the ZP and an extracellular block to ploypermy (Okada *et al.* 1993).

Based on the data from mice, caffeine can modify oocyte aging by controlling MPF activity (Ono *et al.* 2011; Zhang *et al.* 2011). Caffeine plays an important role in delaying oocyte aging. Although the effects of caffeine on aging have been examined in multiple tissues, its function on the distribution of CGs in oocyte aging *in vivo* and *in vitro* have not yet been well defined.

Numerous reports have illustrated beneficial effects of many reagents such as resveratrol, nicotinamide, β-mercaptoethanol, dithiothreitoland so on, on aging oocytes and embryonic development (Li *et al.* 2012; Lee *et al.* 2013; Lord *et al.* 2013). In this study, we set out to investigate the potential involvement of caffeine in improving CG distribution in oocyte aging *in vivo* and *in vitro*.

MATERIALS AND METHODS

Oocyte collection

The use of 5-7-week-old ICR female mice was reviewed and approved by the Ethics Committee of Qingdao Agricultural University. Mice were administrated intraperitoneal injections of 10 IU pregnant mare's serum gonadotropin (PMSG) followed 48 h later by human chorionic gonadotropin (hCG, 10 IU, intraperitoneally (i.p.)) (both from Ningbo Hormone Product Co. Ltd., Cixi, China). For the *in vivo* experiment, the superovulated mice were killed at different times after hCG injection, and the oviductal ampullae were broken to release cumulus-oocyte complexes (COCs). Cumulus-denuded oocytes (DOs) were removed to M2 medium containing 0.1% hyaluronidase. After dispersal and washing three times in M2 medium, the normal oocytes were used for immunostaining of CGs. For the *in vitro* experiment, the superovulated mice were killed at 13 h after hCG injection, and the oviductal ampullae were broken to release COCs. DOs were obtained by treating COCs in M2 medium containing 0.1% hyaluronidase. After washing three times in M2 medium, the oocytes were cultured *in vitro* in M2 for different times.

Caffeine treatment

In vivo

Caffeine was obtained from Sigma-Aldrich (St. Louis, MO, USA). Based on earlier studies, the lethal dose at 50% (LD50) for an i.p. injection to mice was 168 mg/kg body weight (Senda & Hirota 1974) and 250 mg/kg body weight of caffeine could block induced ovulation in mice (Jagiello *et al.* 1972). As reported Mailhes *et al.* (1996), we used 150 mg/kg body weight, which did not cause toxicity and lethality in any of the mice. A 15 mg/mL solution was prepared no more than 30 min before injection, which was given by i.p. injection 0 h relative to treatment with hCG. A group of mice without caffeine treatment was used as a control. Three mice were used per group and each group was repeated three times.

In vitro

For *in vitro* aging, DOs were cultured in M2 medium supplemented with or without 10 mmol/L caffeine (Miao *et al.* 2009; Ono *et al.* 2011). A total of 25-30 oocytes were placed in a 35 μL droplet of M2 medium, and cultured at 37°C under in 5% CO2 in humidified air for 6 h, 12 h, 18 h and 24 h. At different time intervals of *in vitro* aging, the oocytes were allocated for immunofluorescence staining.

Immunofluorescence microscopy

The manipulations were conducted at room temperature unless otherwise specified. Oocytes were washed in M2 medium between treatments. This experiment was performed by labelling the oocytes with the lectin lens culinaris (LCA) (Vector Laboratories, Burlingame, CA, USA) which binds specifically to CG content (Ghetler *et al.* 1998; Li *et al.* 2012).

The ZP of the studied oocytes was removed by a brief exposure to 0.25% pronase (Sigma-Aldrich, St. Louis, MO, USA) followed by three washes in M2 medium. Zona-free oocytes were fixed in 3.7% paraformaldehyde supplemented with M2 medium for 40 min at room temperature, washed three times in blocking fluid

supplemented with 10 mmol/L glycine (Solarbio, Beijing, China), then treated for 5 min in 0.1% Triton X-100 in M2 medium. Oocytes were blocked at 4°C overnight and washed twice, then CGs were stained for 1 h in 100 ug/mL of fluorescein isothiocyanate (FITC)-labeled lens culinaris agglutinin in M2, and chromosomes were stained for 15 min in M2 with 10 ug/mL Hoechst 33342. After washing twice, the stained oocytes were mounted on antifade medium (Vector Laboratories, Burlingame, CA, USA) and then observed with a laser scanning confocal microscope (Leica TCS SP5 II, Wetzlar, Germany). Blue diode (405-nm) and argon (488-nm) were used to excite Hoechst and FITC, respectively. Fluorescence was detected with bandpass emission filters: 420-480 nm for Hoechst, 505-540 nm for FITC, and the captured signals were recorded as blue and green, respectively.

Assessment migration of CGs

CGs in fresh oocytes were densely populated in a layer just beneath the oolemma, with a typical normal CG-free domain above the meiotic apparatus which we called normal CG distribution. During oocyte aging, CGs became displaced and underwent partial exocytosis, with disrupted, discontinuous distribution of CGs beneath the oolemma, which we called exocytosis; while formation of a cap of higher density CGs located above the chromosome was called CG congregation (Fig. 1) (Dodson *et al.* 1989; Miao *et al.* 2009). In this paper we classified distribution of CGs into three categories: normal CG distribution, exocytosis and CG congregation.

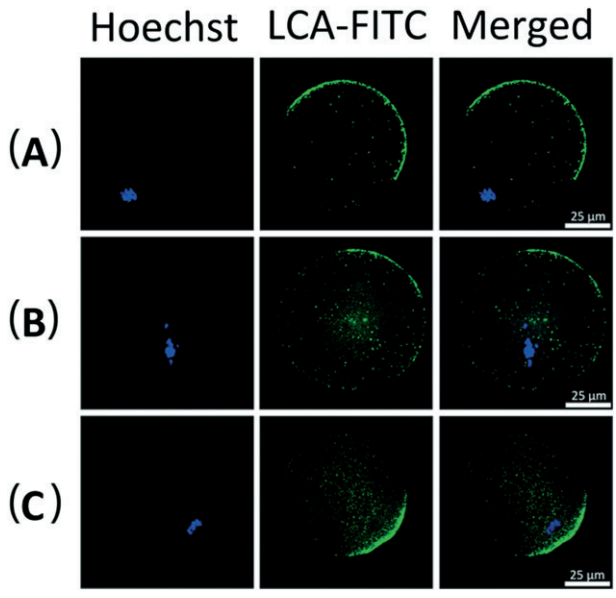

Figure 1 Confocal micrographs of fresh and aged mouse oocytes displaying distributions of cortical granules (CGs) with or without caffeine treatment. (A) Distribution of CGs in fresh oocytes; (B,C) distribution of CGs in aged oocytes.

Statistical analysis

All experiments were conducted at least three times on independent samples, and results were analyzed by GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). Data are presented as mean ± SEM; Student's two-tailed *t*-test was used to determine statistical significance for data generated from oocyte counts. A significant difference was considered at $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ for all tests.

RESULTS

Caffeine reversed the changes in redistribution of cortical granules *in vivo*

As we all know, oocyte aging causes numerous morphological and cellular alterations, including changes in structure and displacement of the spindle, misalignment of chromosomes, displacement of (first polar body) PBI and CGs as well as premature exocytosis of CGs. We first investigated the distribution of CGs in aging oocytes without caffeine treatment (Fig. 2A). Confocal microscopy showed that different patterns of CG distribution appeared during oocyte aging. We observed that 93.7% of oocytes showed a layer of CGs beneath the oolemma and an evident CG-free domain in the area of MII apparatus that we called normal distribution in freshly collected oocytes (0 h) while only 0.8% of oocytes showed normal distribution after aging for 24 h. With the oocyte aging, CGs became displaced and underwent exocytosis, with the typical CG-free domain above the meiotic apparatus gradually reduced to 51.8% and exocytosis increased to 47% at 12 h of *in vivo* aging (Fig. 2A–B). CG congregation referred to higher density CGs locating above the chromosome area, and during oocytes aging, CG congregation gradually appeared. When aged for 18 h, 18.5% of oocytes showed CG congregation, and CG congregation percentage increased to 46.2% at 24 h of aging (Fig. 2B).

After treatment with caffeine, the normal distribution rate of CGs increased, while the CG exocytosis rate and CG congregation rate decreased in early aging oocytes compared to untreated oocytes. For oocytes aging for 12 h, the normal CG distribution rate increased to 71.8% compared to 51.8% of untreated oocytes, while CG exocytosis rate decreased at 6 h and 12 h of aging *in vivo* ($P < 0.05$). After aging for 18 h, a large proportion of oocytes showed exocytosis, and after aging for 24 h, a large proportion of oocytes showed CG congregation, and there was no difference between the caffeine and control groups (Fig. 2B–C).

Unexpectedly, only 58.7% of freshly ovulated oocytes from caffeine-administered mice showed normal distribution of CGs, significantly lower than that observed in untreated oocytes ($P < 0.001$). After caffeine treatment the exocytosis dramatically increased to 38.8% compared to 8.9% of untreated oocytes ($P < 0.01$). We then examined the oocyte morphology of freshly ovulated

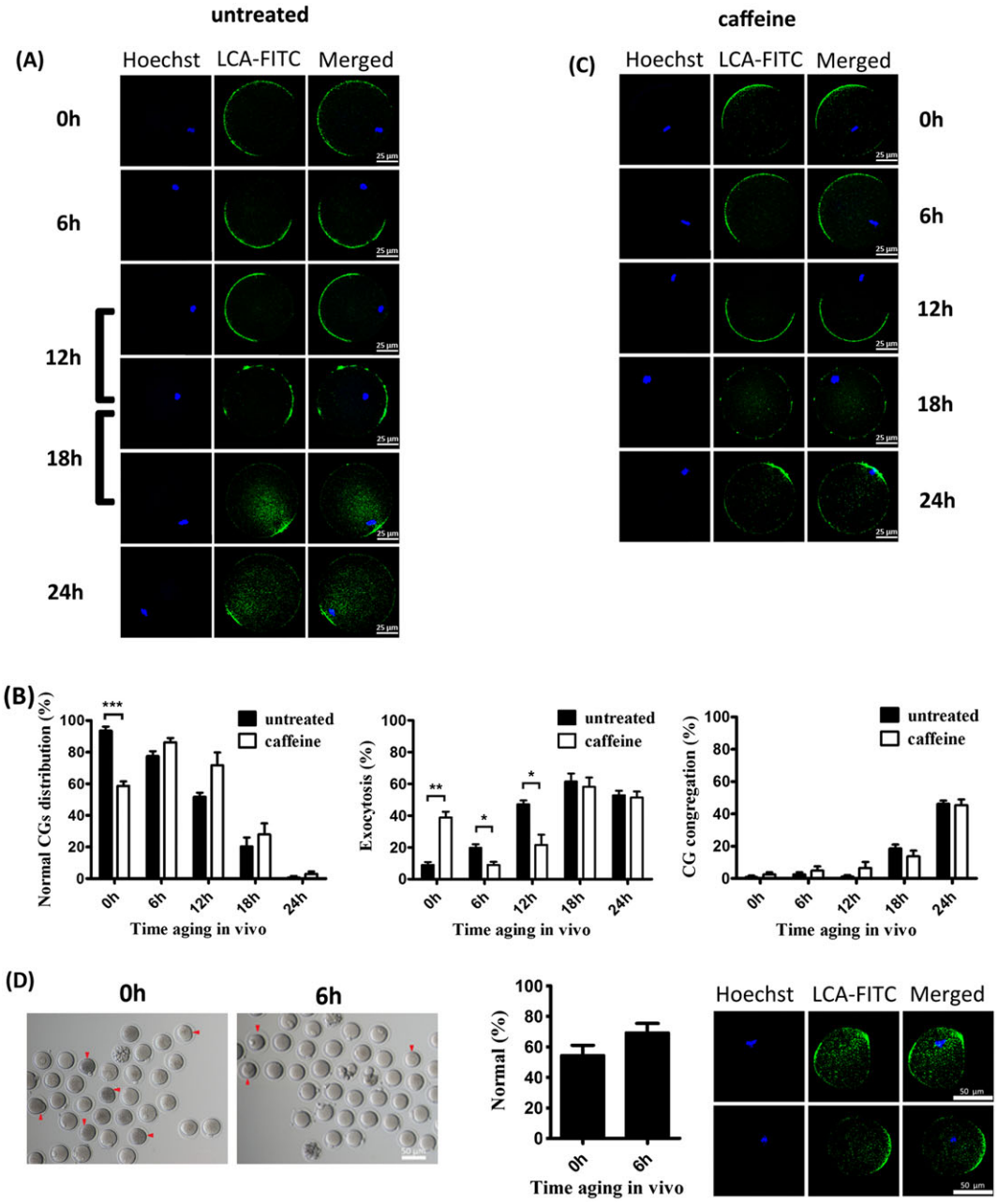

Figure 2 Confocal laser scanning microscopy for the distribution of cortical granules in oocytes aging *in vivo*, with or without 150 mg/kg caffeine administration. (A,B) Metaphase II oocytes were immunolabeled for cortical granules (CGs) with lens culinaris (LCA)-FITC (fluorescein isothiocyanate: green) and chromosomes were counterstained with Hoechst 33342 (blue), showing oocytes collected 13 h, 19 h, 25 h, 31 h, 37 h post-human chorionic gonadotropin and with or without caffeine administration, corresponding to 0 h (freshly collected oocytes), 6 h, 12 h, 18 h and 24 h of aging. (A) Confocal laser scanning micrographs of oocytes aging *in vivo* without caffeine treatment, and (C) confocal laser scanning micrographs of oocytes aging *in vivo* with caffeine treatment. (B) Percentages of oocytes with different patterns of CG distribution with or without caffeine treatment. The patterns were divided into three categories: normal CG distribution with a typical normal CG-free domain above the meiotic apparatus, for example, distribution of CGs at 0 h and 6 h without caffeine treatment and 6 h and 12 h with caffeine treatment *in vivo*; exocytosis with disrupted, discontinuous distribution of CGs beneath the oolemma, for example distribution of CGs at 12 h and 18 h without caffeine treatment and 0 h, 18 h with caffeine treatment; and CG congregation with a cap of higher-density CGs located above the chromosome area, for example distribution of CGs at 18 h and 24 h without caffeine treatment and 24 h with caffeine treatment. (D) Normal morphology rates of freshly collected oocytes and those aging for 6 h *in vivo* after 150 mg/kg caffeine treatment (red arrowheads:abnormal oocytes aging *in vivo*). The abnormal oocytes were immunolabeled with LCA-FITC, showing CG exocytosis or congregation. Independent replicates were conducted with a minimum of 25 oocytes/replicate. Data are presented as averages ± SEM; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

oocytes, and found that the percentage of normal oocytes was significantly decreased in the caffeine group, and the distribution of CGs in these abnormal oocytes showed exocytosis or congregation (Fig. 2D). This might be because the stress stimulation of caffeine on mice resulted in poor quality of oocytes to a certain extent, and these oocytes might either quickly degenerate or restore to normal morphology, depending on their quality. In a word, we concluded that *in vivo* caffeine administration may cause damage to low-quality oocytes, but delays CG exocytosis and congregation in good-quality oocytes, and thus delayed oocyte aging.

Caffeine supplementation increased exocytosis of oocytes aging *in vitro*

The above experiments showed that caffeine administration *in vivo* could improve the redistribution of CGs. Next, we examined effects of caffeine on distribution of CGs during *in vitro* aging of oocytes. Thus, we observed aging oocytes at different times in culture medium supplement with or without 10 mmol/L caffeine. First, we analyzed the aging oocytes without caffeine supplementation. Immuostaining and confocal microscopy showed that 93.7% of freshly collected oocytes (0 h) showed a layer of CGs beneath the oolemma (normal distribution). Additionally, 77.2% and 86.7% of oocytes aging for 6 h and 12 h also showed normal CG distribution. For oocytes aging for 18 h, the normal CG distribution rate decreased to 48.6% and CG exocytosis started to appear ($P < 0.05$). After aging for 24 h, 20.2% of oocytes showed CG congregation just like a cap of higher density CGs located above the chromosome area as previously reported (Lee *et al.* 2013) (Fig. 3A–B).

Unexpectedly, we observed that caffeine accelerated CG exocytosis in oocytes aging *in vitro*. After caffeine supplementation, 73.2% of the oocytes underwent CG migration and exocytosis, increasing by 50.4% when compared to oocytes without caffeine supplementation at 6 h *in vitro* aging ($P < 0.01$), and 18.7% of oocytes aging for 6 h showed CG congregation, significantly higher than that of the control group ($P < 0.05$). More than half of the oocytes showed CG exocytosis in the caffeine supplementation group after aging for 6 h and 12 h ($P < 0.01$), significantly higher than in the control group.

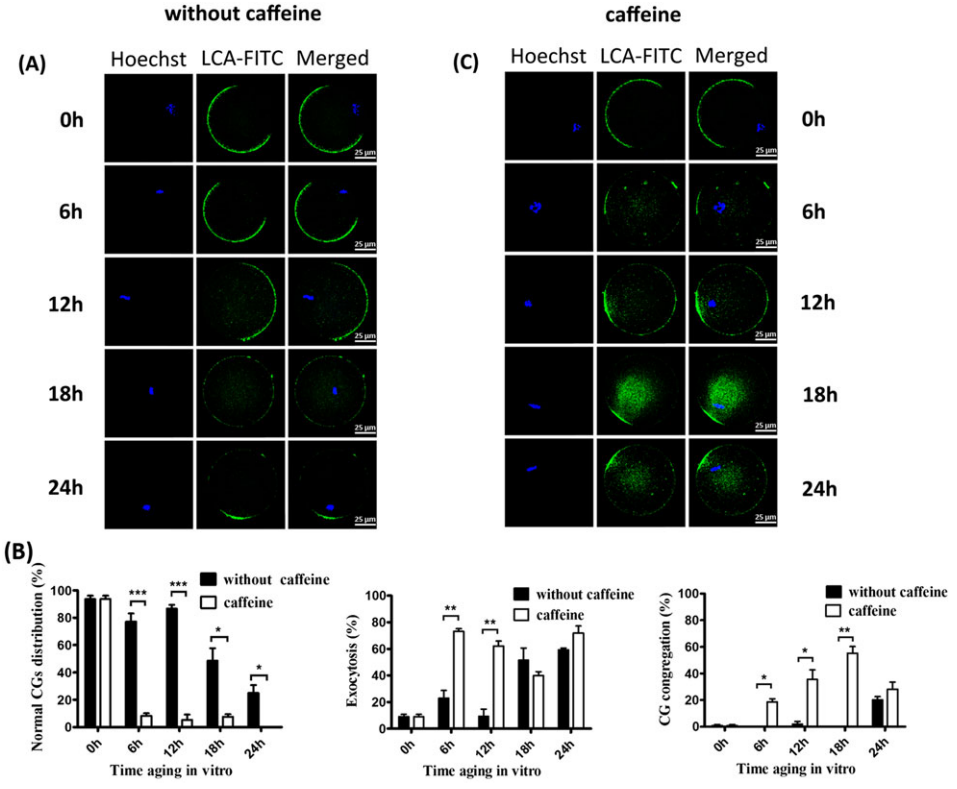

Figure 3 Confocal laser scanning microscopy for the distributions of cortical granules (CGs) of oocyte aging *in vitro* with or without supplementation of 10 mmol/L caffeine. (A,B) Metaphase II oocytes were immunolabeled for CGs with lens culinaris (LCA)-FITC (fluorescein isothiocyanate: green), and chromosomes were counterstained with Hoechst 33342 (blue), showing oocytes collected 0 h, 6 h, 12 h, 18 h, 24 h of *in vitro* culture with or without caffeine supplementation. (A) Confocal micrographs of aging oocytes *in vitro* without caffeine. (B) Percentages of oocytes with three patterns of distribution for CGs in oocytes cultured *in vitro* in medium supplemented with or without caffeine, normal CG distribution, exocytosis and CG congregation. (C) Confocal micrographs of aging oocytes with caffeine supplementation. Independent replicates were conducted with a minimum of 25 oocytes/replicate. Data are presented as averages ± SEM; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

The percentage of CG congregation also increased with time after aging for 6 h (Fig. 3B–C). The results indicated that caffeine could accelerate the abnormal distribution of CGs, and the abnormal rate increased significantly from 6 h *in vitro* aging. However, from these experiments we conclude that caffeine accelerates CG exocytosis in oocyte aging *in vitro*.

DISCUSSION

Cellular fragmentation commonly occurs in postovulatory aging oocytes, which is characterized by a sequence of molecular processes that deteriorate during aging, negatively impacting fertilization and development (Miao *et al.* 2009). The postovulatory aging oocytes *in vivo* or *in vitro* are dependent upon the functional expression of several conserved apoptotic cell death proteins (Senda & Hirota 1974; Morita *et al.* 1999). However, oocyte aging can be delayed or reversed by various reagents, preventing developmental failures or abnormalities (Miao *et al.* 2009). In this study, we treated aging oocytes with caffeine *in vivo* and *in vitro*, aiming to decrease the abnormal distribution of CGs. We found that oocytes aging *in vivo* and *in vitro* underwent time-dependent CG exocytosis and congregation. Premature exocytosis of CGs began to appear in a small proportion of oocytes aging *in vivo* for 6 h, while a large proportion of oocyte underwent CG exocytosis by *in vivo* aging for 12 h. By aging *in vivo* for 18 h and 24 h, evident CG congregation to the area above the spindle was observed. In oocytes aging *in vitro*, CG exocytosis and congregation occurred later than that in oocyte aging *in vivo*. These data suggest that oocyte aging occurs faster *in vivo* than *in vitro*.

Previous research has shown that oxidative stress is a key mediator of oocyte aging and appears to act as a trigger for induction of the intrinsic apoptotic pathway, just as has been observed for spermatozoa (Koppers *et al.* 2011). Oocytes aging *in vivo* expressed a regular elevation in ROS levels during the aging process. However, the onset of oxidative stress early in the oocyte aging process is not particularly surprising to us when taking into consideration the reported decline in alleviate glutathione (GSH) levels within the oocyte aging after postovulation as well as the absence of antioxidant-rich follicular fluid to provide protection (Yoshida *et al.* 1993). Additionally, the free-radical theory of aging proposes that ROS produced by the mitochondria as by-products of oxidative phosphorylation may result in the progressive accumulation of toxic oxidative metabolites in the oocytes during aging, initiating further ROS production in a damaging redox cycle (Barja 2004).

Kikuchi *et al.* (2000) first explored that aging oocytes can be reversed by controlling the activity of MPF. It has been established that caffeine can modify oocyte aging through different methods by controlling MPF activity. On the other hand, caffeine could inhibit Mytl/Weel activity (Smythe & Newport 1992). Lee & Campbell (2008) also showed that in aging bovine oocytes. 10 mmol/L caffeine could prevent the decline in MPF and mitogen-activated protein kinase (MAPK) activity. Additionally, caffeine increased fertilization and decreased fragmentation when spermatozoa were injected (Miao *et al.* 2009).

The distribution, the time and place of releasing of CGs from oocytes have important significance with respect to the functions in fertilization (Liu *et al.* 2003). Oocytes from sea urchin have at least two populations of CGs that differ biochemically (Brummett & Dumont 1981). After oocyte maturation, if fertilization does not occur in time, the distribution of CGs will change. We found that the percentages of exocytosis and disrupted cortical granule free-domain (CGFD) increased during aging compared to fresh oocytes. No matter whether aging *in vivo* or *in vitro*, exocytosis (disrupted CGs) and CG distribution abnormality increased (Ma *et al.* 2015). On the basis of previous reports that oocyte aging could be delayed for a longer time by supplementing caffeine to medium, it is supposed that caffeine could extend oocytes' competence by activating MPF in mice and human reproduction (Goud *et al.* 2014; Jiang *et al.* 2015). We thus supposed that caffeine might delay CG exocytosis in aging oocytes. We did observe positive effect of caffeine on delaying CG exocytosis and congregation in oocyte aging *in vivo*, but unexpectedly, caffeine accelerated CG exocytosis and congregation when added to *in vitro* culture. Additionally, caffeine administration *in vivo* also caused premature CG exocytosis in a small proportion of oocytes of lower quality. Thus, although caffeine could delay molecular oocyte aging and improve oocyte morphology, it is not an appropriate supplementation to *in vitro* culture medium for the purpose of delaying oocyte aging. However, reports have demonstrated that preventing oxidative stress using melatonin was better than caffeine. There is more evidence to support that melatonin may be more effective and safer than caffeine to delay oocyte aging during the cell metabolism process (Lord *et al.* 2013). Notably, beyond our expectations, our study also showed that fresh oocyte treatment with caffeine showed more fragmentation and decreased fertilization (data not shown). Further investigations are required to extend CR and delay oocyte aging process.

In summary, our study shows that CGs undergo time-dependent exocytosis, followed by congregation above the spindle area in oocyte aging *in vivo* and *in vitro*. Caffeine administration *in vivo*, on one hand delays CG exocytosis and congregation, but on the other hand, causes premature CG exocytosis in a small proportion of freshly ovulated low-quality oocytes. Unexpectedly, caffeine supplementation accelerates CG exocytosis and congregation in oocyte aging *in vitro*.

ACKNOWLEDGMENTS

This study was supported by National Basic Research Program of China (No. 2012CB944404) and Shenzhen R&D Basic Research Program (No. JCYJ20140415162338779).

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