Effects of postovulatory oviduct changes and female restraint stress on aging of mouse oocytes

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Abstract

Postovulatory oocyte aging is one of the major causes for human early pregnancy loss and for a decline in the population of some mammalian species. Thus, the mechanisms for oocyte aging are worth exploring. While it is known that ovulated oocytes age within the oviduct and that female stresses impair embryo development by inducing apoptosis of oviductal cells, it is unknown whether the oviduct and/or female stress would affect postovulatory oocyte aging. By comparing aging characteristics, including activation susceptibility, maturation-promoting factor activity, developmental potential, cytoplasmic fragmentation, spindle/chromosome morphology, gene expression, and cumulus cell apoptosis, this study showed that oocytes aged faster in vivo in restraint-stressed mice than in unstressed mice than in vitro. Our further analysis demonstrated that oviductal cells underwent apoptosis with decreased production of growth factors with increasing time after ovulation, and female restraint facilitated apoptosis of oviductal cells. Furthermore, mating prevented apoptosis of oviductal cells and alleviated oocyte aging after ovulation. In conclusion, the results demonstrated that mouse oviducts underwent apoptosis and facilitated oocyte aging after ovulation; female restraint facilitated oocyte aging while enhancing apoptosis of oviductal cells; and copulation ameliorated oviductal apoptosis and oocyte aging.

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Introduction

After ovulation, oocytes of mammals, including humans, undergo a time-dependent process of aging, if they are not fertilized or activated in time (Yanagimachi & Chang 1961, Whittingham & Siracusa 1978, Miao et al. 2005). It has been reported that fertilization of the postovulatory aged oocytes can impair embryo development (Tarín et al. 1998, Liu et al. 2009, Zhu et al. 2015) and cause anomalies in offspring (Tarín et al. 1999, 2002). Humans and some animals potentially undertake sexual activity on any day of the estrous cycle because they do not have a mechanism to synchronize sexual activity with ovulation. In this case, fertilization may take place between aged oocytes and freshly ejaculated spermatozoa. In fact, Wilcox et al. (1998) have observed in human beings that a statistically significant increase in the risk of early pregnancy loss was associated with an increased likelihood of postovulatory oocyte aging. Furthermore, Prasad et al. (2017) have proposed that postovulatory oocyte aging is one of the major causes for the gradual decline in population size of several threatened mammalian species. However, although these data suggested an urgent need for control over postovulatory oocyte aging, the mechanisms for oocyte aging are not fully understood.

Ovulated oocytes undergo the aging process during their residence in the oviduct. Studies have shown that the oviductal epithelium is one of the most critical maternal tissues for the establishment of a healthy pregnancy (Leese et al. 2008), and that the oviduct contains growth factors that facilitate cell proliferation and differentiation of preimplantation embryos (Heyner 1997). In contrast, it has been reported that restraint stress of female mice during the preimplantation stage impaired embryo development by inducing apoptosis in oviductal cells via activating the Fas signaling (Zheng et al. 2016, Tan et al. 2017). Thus, the oviduct may affect the postovulatory oocytes as well as embryos it harbors, and the restraint stress applied on females after ovulation may trigger apoptosis of oviductal cells and enhances oocyte aging.

It is known that oocytes show typical morphological/molecular/biochemical characteristics during...
postovulatory aging, such as changes in susceptibility to activating stimulus (STAS), maturation-promoting factor (MPF) activity, developmental potential, cytoplasmic fragmentation, spindle/chromosome morphology, gene expression, and cumulus cell apoptosis. Both an increase in STAS (Kubiak 1989, Lan et al. 2004) and a decrease in MPF activity (Xu et al. 1997, Miao et al. 2005) and developmental potential (Liu et al. 2009) have been used as markers for early oocyte aging, whereas cytoplasmic fragmentation (Lord & Aitken 2013, Zhu et al. 2015) and spindle/chromosome morphology (Li et al. 2012, Lord & Aitken 2013) have been used as markers for late oocyte aging. It was reported that expression of the antiapoptotic protein BCL2 was gradually declined during postovulatory oocyte aging (Ma et al. 2005, Liu et al. 2009, Takahashi et al. 2009). Zhang et al. (2016) observed that SIRT1, 2, and 3 suppressed oxidative stress and inhibited postovulatory aging of mouse oocytes. Cumulus cells underwent degradation with pyknotic nuclei and a vacuolated cytoplasm after aging of mouse oocytes in vivo in oviducts or in vitro in culture medium (Longo 1980). Apoptosis of rat cumulus cells began shortly after ovulation (Szoltys et al. 2000). Furthermore, apoptotic cumulus cells promoted oocyte aging in vitro by producing Fasl (Zhu et al. 2015) and TNF-α (Kong et al. 2018).

The objective of the present study was to compare postovulatory aging of mouse oocytes in vivo in oviducts and in vitro in culture medium. Three regimens of oocyte aging were adopted (Fig. 1). For in vitro aging (ITA), freshly ovulated (FO) oocytes were cultured for 12 h, and for regular in vivo aging (IVA) and restraint-stressed in vivo aging (RSA), oocytes were recovered from oviducts of unstressed and restraint-stressed females, respectively, at 12 h after ovulation. While the aging characteristics in the ITA oocytes were examined immediately at the end of culture, those in the IVA or RSA oocytes were observed immediately after recovery from the oviducts. Aging characteristics of the oviducts were also examined immediately after recovery from IVA or RSA mice at 12 h after ovulation. Furthermore, although oviducts support healthy preimplantation embryo development during normal pregnancy after mating, oocyte aging (degeneration) is reported in oviducts of unstimulated animals. Mating (Mat) was therefore performed to study its effects on oviductal apoptosis and oocyte aging.

**Materials and methods**

The study was carried out in accordance with the relevant guidelines and regulations. Animal care and handling were conducted strictly according to the guidelines issued by the Animal Care and Use Committee of the Shandong Agricultural University, P.R. China (Permit number: SDAUA-2001-001). All the chemicals and reagents used were purchased from Sigma Chemical Co., if not specified otherwise.

**Oocyte recovery and aging treatment**

For ITA, the superovulated mice were sacrificed 13 h after hCG injection, and cumulus-oocyte complexes (COCs) were recovered by rupturing the oviduct ampullae. After being washed in M2 and CZB medium, the COCs were aged in the CZB medium for 12 h before further experiment. For IVA, COCs were recovered from oviducts of the unstressed mice at 25 h after hCG injection. For RSA, the mice were subjected to a 12-h restraint treatment at 13 h after hCG injection.

**Mice and treatment**

Mice of the Kunming strain, originally derived from ICR (CD-1), were kept in a room under a 14 h light:10 h darkness photoperiod, with lights-off at 20:00 h. Female mice (6–8 weeks of age) were superovulated with 10 IU equine chorionic gonadotropin (eCG, i.p.) and 10 IU human chorionic gonadotropin (hCG, i.p.) at a 48-h interval. Both eCG and hCG were purchased from Ningbo Hormone Product Co., Ltd. (China). For the restraint treatment, an individual mouse was put in a micro-cage we constructed by ourselves, and the micro-cage was placed in an ordinary home cage. While in the micro-cage, mice could move back and forth to some extent, but they could not turn around. Our previous studies indicated that this restraint treatment did not affect the food and water intake of mice (Zhang et al. 2011).
COCs were recovered from the oviducts at the end of the restraint. To recover oocytes from mated mice, female mice were caged with vasectomized male mice immediately after hCG injection, and mice that showed vaginal plugs at 08:00 h the next day were sacrificed to recover COCs at 25 h after hCG injection. The COCs recovered from the above four treatments were denuded of cumulus cells by pipetting with a thin pipette in a drop of M2 medium containing 0.1% hyaluronidase.

**Oocyte activation and embryo culture**

To observe oocyte susceptibility to activating stimulus (STAS), cumulus-free oocytes were treated with 3% ethanol in M2 medium for 5 min at room temperature, and then cultured in CZB medium for 6 h at 37.5°C in a humidified atmosphere containing 5% CO2 in air. To observe oocyte developmental potential, oocytes were activated by SrCl2 treatment for 6 h, using a calcium-free CZB medium containing 10 mM SrCl2 and 5 mg/mL cytochalasin B. At the end of the activation culture, oocytes were observed under an inverted microscope for activation. Oocytes showing one or two pronuclei, or showing two cells each having a nucleolus, were judged as activated. The Sr2+-activated oocytes were cultured in a regular CZB medium (about 30 oocytes per well containing 100 µL medium) at 37.5°C in humidified air with 5% CO2. On day 2 of culture, oocytes were examined for four-cell development and were transferred to CZB medium containing 5.55 mM glucose for further culture. On day 4.5 of the culture, oocytes were examined for blastocyst rates.

**Assessment of oocyte cytoplasmic fragmentation**

Cytoplasmic fragmentation was observed at different times during the post-treatment culture after different aging treatments. Briefly, cumulus-free oocytes were examined under a phase-contrast microscope. While oocytes with a clear moderately granulate cytoplasm, and an intact first polar body, were considered unfragmented, oocytes with more than two asymmetric cells were considered fragmented.

**Immunofluorescence microscopy for detection of tubulin and calcium-sensing receptor (CaSR)**

All the procedures were carried out at room temperatures unless otherwise specified. Cumulus-free oocytes were always washed three times in M2 between procedures. The oocytes were (a) fixed for 30 min in PBS with 3.7% paraformaldehyde; (b) treated with 0.5% protease in M2 for 10 seconds to remove zona pellucida; (c) permeabilized for 10 min at 37.5°C with 0.1% Triton X-100 in PBS; and (d) blocked for 30 min at 37.5°C with 3% BSA in PBS. For tubulin staining, the blocked oocytes were first incubated at 37°C for 1 h in PBS containing FITC-conjugated anti-α-tubulin monoclonal antibodies (1:50), and then incubated for 10 min with 10 µg/mL Hoechst 33342 in M2 to stain chromatin. For CaSR detection, the blocked oocytes were first incubated at 4°C overnight with rabbit polyclonal anti-CaSR (IgG, 1:100, Immunoway) in 3% BSA in M2 medium, and then, with Cy3-conjugated goat-anti-rabbit IgG (1:800, Jackson ImmunoResearch) in 3% BSA in M2 for 1 h. Finally, the oocytes were incubated for 10 min with 10 µg/mL Hoechst 33342 in M2 to stain chromatin. Samples in which the primary antibody was omitted were also processed to serve as negative controls. The stained oocytes were mounted on glass slides and observed with a Leica laser scanning confocal microscope (TCS SP2). Blue diode (405 nm), argon (488 nm), and helium/neon (543 nm) lasers were used to excite Hoechst, FITC, and Cy3, respectively. Fluorescence was detected with bandpass emission filters: 420–480 nm for Hoechst, 505–540 nm for FITC, and 560–605 nm for Cy3, and the captured signals were recorded as blue, green and red, respectively.

**Measurement for MPF (CDK1) activity in oocytes**

The CDK1 kinase activity was assayed using a CDK1 kinase activity assay kit (50145.1.3.v.A, Haling Biotechnology). Briefly, about 50 cumulus-free oocytes were placed in 10 µL of cell lysis buffer. The mixture was then frozen at −80°C and thawed three times at room temperature. The resultant cell extracts were stored at −80°C until use. For reaction, 130 µL buffer, 20 µL enzyme catalase reagent, 20 µL reaction reagent and 20 µL substrate reagent were added to wells of a 96-well plate and incubated for 3 min at 30°C after shaking gently. Then, 10 µL negative reagent and 10 µL lysis buffer containing 50 oocytes were added to each well and the optical density at 340 nm was measured at 0 and 5 min immediately after shaking gently. The value of MPF activity in the in vitro group was set as 100% and the other values were expressed relative to this value.

**Measurement of oocyte cytoplasmic calcium**

Intracellular Ca2+ was measured using the Ca2+-sensitive dye fluo-2. To load the Ca2+ probe, cumulus-free oocytes were cultured for 30 min at room temperature in a Hepes-buffered CZB medium with 1 µM Fluo-2 AM and 0.02% pluronic F-127. Then, the oocytes were transferred into the Hepes-buffered CZB drops made in a Fluoro dish (FD35-100, World Precision Instruments) and observed at 37°C with a Leica DMI 6000 inverted microscope. A Fluo-2 fluorescence module was used for excitation, and a Leica LAS-AF calcium imaging module was used to calculate the F490/F520 ratio. The oocytes were monitored for 10 min to record the F488/515 ratio, which stand for the concentration of cytoplasmic calcium.

**Preparation and apoptosis assessment of cumulus cells**

The cumulus cells released from COCs were cultured in CZB medium for 16 h before assessment for apoptosis. After culture, cells were separated by centrifugation (200 g) for 5 min at room temperature. Annexin and Hoechst double staining was performed using an Annexin V-FITC apoptosis detection kit (KGA105-KGA108, KeyGEN BioTECH). Briefly, cumulus cell pellets were resuspended in a mixture of 5 µL Annexin V–FITC and 500 µL binding buffer, and stained in the dark for 20 min. After centrifugation for 5 min at 200 g, the cumulus cell pellets were resuspended in 50 µL of M2 medium containing 10 µg/mL of Hoechst 33342 and stained in the darkness for 5 min. The stained cells were centrifuged...
for 5 min at 200 g, and after about half of the supernatant was removed, a 5 µL drop of suspension was smeared on a slide. The smears were observed under a Leica DMLB fluorescence microscope at a 400x magnification. The apoptotic cumulus cells showed annexin-positive plasma membrane, which gave green (FITC) fluorescence, and pyknotic nuclei, which were full of heterochromatin and gave bright Hoechst fluorescence, whereas healthy cumulus cells displayed normal nuclei with sparse heterochromatin spots and annexin-negative plasma membrane. On each smear, six to eight fields were randomly examined and the percentages of apoptotic cells were calculated from 60 to 80 cumulus cells in each field. All images were reviewed by two investigators in a double-blind manner.

Preparation of serum, oviduct homogenates and oviduct epithelia

Mice were killed by decollation, and trunk blood (about 1 mL) was collected into ice-cooled centrifugal tubes, and serum was separated by centrifugation (1700 g, 10 min, 4°C). The serum collected was stored at −80°C until use. For homogenization, the oviducts collected were first washed thoroughly with ice-cold PBS. Then, oviducts from each mouse were transferred into a homogenizer containing 300 µL PBS. Homogenization was conducted by hand at room temperature. The homogenates produced were centrifuged (1500 g, 10 min, 4°C), and the supernatant was collected for immunoblotting. Homogenization was performed using a pair of forceps.

Quantitative real-time PCR

One-step qRT-PCR was used for oocytes and cumulus cells and conventional qRT-PCR for oviduct epithelia. For one-step qRT-PCR, cumulus-free oocytes or cumulus cells were lysed to extract RNA using the CellAmp Direct Prep Kit for real-time PCR and Protein Analysis (3733Q, Takara). The lysate obtained was preserved at −80°C before use. Quantification of mRNA was performed using a One-Step TB Green PrimeScript PLUS RT-PCR Kit (RR096A, Takara). A 10 µL reaction volume was used for the amplification reaction (1 µL template, 2.2 µL RNase-Free dH₂O, 5 µL 2×One Step TB Green RT-PCR Buffer 4, 0.6 µL TaKaRa Ex Taq HS Mix, 0.2 µL PrimeScript PLUS RTase Mix, 0.2 µL ROX Reference Dye II, 0.4 µL PCR forward primers (10 µM) and 0.4 µL PCR reverse primers (10 µM). The cycle amplification facilities: (a) reverse transcription reaction of one cycle at 42°C for 5 min and 95°C for 10 s; (b) 40 cycles at 95°C for 5 s and at 59°C (Bcl2 and Bax) or 64°C (Sirt1) for 30 s; and (c) dissociation protocol at 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s.

For conventional qRT-PCR, after being washed three times in cooled PBS, oviduct epithelia homogenization was performed using Trizol reagent (0.5 mL for oviduct epithelia from one mouse). Isolated RNAs were resuspended in diethyl pyrocarbonate-treated MilliQ water (DEPC-dH₂O). Reverse transcription was performed in a total volume of 20 µL using Superscript III™ Reverse Transcriptase (Invitrogen Australia Pty. Ltd). Briefly, 2 µL of each RNA sample were mixed with 4 µL of dNTP, 1.5 µL Oligo dt₁₈ (Takara) and 6 µL of DEPC-dH₂O, and the mixture was incubated at 65°C for 5 min followed by treatment on ice for 2 min. Then, 4 µL of 5× RT buffer, 0.5 µL RNase inhibitor and 0.5 µL Superscript III Reverse Transcriptase were added. The mixture was then incubated at 50°C for 1 h, followed by incubation at 70°C for 15 min. Quantification of mRNA was conducted using the Mx3005P real-time PCR instrument (Stratagene, Valencia, CA). Amplification reactions were performed in a 10-µL reaction volume containing 1 µL of cDNA, 5 µL of 2× SYBR Green Master Mix (Stratagene), 0.15 µL of ROX (reference dye), 3.05 µL of RNase-free water, and 0.4 µL each of forward and reverse gene-specific primers (10 µM). Cycle amplification conditions comprised a step at 95°C for 10 min followed by 40 cycles at 95°C for 5 s and at 60°C for 20 s.

Gene expression was normalized to the reference genes. H2azf and Gapdh were used as reference genes when q-PCR was performed for oocytes, and Gapdh and Actb were used for cumulus cells or oviduct epithelia. Geometrical means calculated from CT values of the double reference genes were used for data normalization. All values were then expressed relative to calibrator samples using the 2⁻³ΔΔCT method. Sequences of gene-specific primers can be found in Table 1.

Enzyme-linked immunosorbent assay (ELISA)

Concentrations of sTNF-α, sFasL, IGF1, BDNF, corticosterone and CRH in oviduct homogenates, and corticosterone and CRH in blood serum, and CDK1 in oocyte lysates (50 oocytes were lysed in 10 µL RIPA lysis buffer) were assayed using the relevant ELISA kit (jinma, Shanghai, China). Briefly, 50 µL standards or samples (10 µL samples mixed with 40 µL dilution buffer) were added in duplicate to wells of a micro-titer plate pre-coated with mouse monoclonal antibodies and incubated at 37°C for 30 min. After the micro-titer plate was washed using a washing buffer, 50 µL conjugate reagent was added to each well and incubated again for 30 min at 37°C. After another washing procedure, 50 µL substrate A and 50 µL substrate B were added to each well and incubated for 10 min at 37°C. The optical density was measured at 450 nm.

Table 1 Primer sequences used for qRT-PCR in this study.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward (5’–3’)</th>
<th>Reverse (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl2</td>
<td>TTCGGGATGGAGGATGAAACTGG</td>
<td>TGGATCACCAGGCTCTAGGGT</td>
</tr>
<tr>
<td>Bax</td>
<td>TGCAGAGGATGATTGCTGAC</td>
<td>GATCCAGCTCGGGGACCTTGA</td>
</tr>
<tr>
<td>Sirt1</td>
<td>TATCTATGCTCCGCTGGCCG</td>
<td>CCGGATATTTTCTTGTGAAACCT</td>
</tr>
<tr>
<td>Gapdh</td>
<td>AAACRCCGACAGTATGGATGA</td>
<td>GTGTCGCCAGGTGGTTCTTAC</td>
</tr>
<tr>
<td>H2azf</td>
<td>ACAGCGCAGAATCCGGAGTA</td>
<td>TTCCCGGATACGGATTTGAGTA</td>
</tr>
<tr>
<td>Actb</td>
<td>CTACCTCATGAGATCCCTGACC</td>
<td>CACGCTTCTCGTTGAC</td>
</tr>
</tbody>
</table>
nm using a plate reader (BioTek-ELx808, BioTek Instruments, Inc.) within 15 min after the reaction was terminated by 50 µL of stop solution. The concentrations of relative protein were calculated against the respective standard curves. The total protein in oviduct homogenates was assayed using BCA protein concentration assay kit (Beyotime, Shanghai, China). The final concentration shown in the ‘Results’ section was the ratio of a protein and total protein from each sample.

Flow cytometry assay

Cell apoptosis in oviduct epithelia was measured by flow cytometry using an Annexin V-FITC/propidium iodide (PI) staining kit (C1062, Beyotime) according to the manufacturer’s protocol. After being washed in PBS, the oviduct epithelial tissue from two animals was digested at 37°C with 0.25% trypsin for 30 min, and then DMEM/F12 medium containing 10% fetal bovine serum was added to neutralize the residual trypsin. Cell blocks were filtered using a 200-mesh sieve and washed twice in PBS. Pellets were resuspended in a 195 µL binding buffer containing 5 µL Annexin V–FITC. After incubating at room temperature in the dark for 10 min, 10 µL PI was added to the cells. The fluorescence of 10,000 cells per sample was analyzed using a Guava easyCyte (Millipore).

Statistical analysis

Each treatment was repeated at least three times. Percentage data were arc sine-transformed before analysis. Data were analyzed using an independent t-test when each measure contained only two groups and was analyzed using ANOVA when each measure had more than two groups. To locate differences during ANOVA, a Duncan multiple comparison test was performed. The Statistics Package for Social Sciences (SPSS 11.5, SPSS, Inc.) was used. Data are expressed as means ± SEM, and \( P < 0.05 \) is defined as significant.

Results

Measurement of stress hormones in serum and oviducts confirmed that our restraint system stressed animals effectively

To confirm that our restraint system stressed mice effectively, CRH and corticosterone were measured by ELISA in serum and oviduct homogenates recovered at 12 h after ovulation of unstressed (IVA), restraint-stressed (RSA) or mated (Mat) mice. The results showed that levels of both CRH and corticosterone in both serum and oviducts were significantly higher in RSA mice than in IVA or Mat mice, and the difference in all these parameters did not differ significantly between the IVA and Mat mice (Fig. 2A, B, C and D). The results suggested that our restraint system stressed mice effectively and that mating did not cause any stress on female mice.

Effects of different aging regimens on the susceptibility to activating stimuli (STAS) and fragmentation of aging oocytes

Immediately at the end of different aging treatments, oocytes were treated with ethanol for activation or cultured for different times before examination for fragmentation. The activation rate of the ITA oocytes (25%) was significantly lower than that (58%) in the IVA oocytes (Fig. 3A). Unexpectedly, however, the activation rate in the RSA oocytes from restraint-stressed mice (27%) was as low as that observed in the ITA oocytes. Starting from 36 h of post-aging culture, differences in fragmentation rates became significant (\( P < 0.05 \)) between the three aging protocols. Thus, percentages of fragmented oocytes were the highest in RSA oocytes and lowest in the ITA oocytes, with those in the IVA oocytes in between (Fig. 3B).
Effects of different aging regimens on spindle/chromosome morphology of aging oocytes

Spindle/chromosome morphologies were classified into a focused pole (F), barrel-shaped (B) or disintegrated (D) spindles with congressed (C) or misaligned (M) chromosomes (Fig. 3D, E, F and G). While percentages of oocytes with the F/C spindle/chromosome morphology decreased, those of oocytes with the D/C spindle/chromosome morphology increased significantly from ITA to IVA to RSA treatments (Fig. 3C), suggesting that oocytes aged faster in vivo in stressed animals than in unstressed animals than in vitro.

Effects of different aging regimens on MPF activity, developmental potential and expression of related genes in aging oocytes

By measuring CDK1 activity, we showed that the MPF activity was significantly lower in RSA than IVA than ITA oocytes (Fig. 4A). Although the percentages of activated oocytes and four-cell embryos did not differ, the percentages of blastocysts were significantly lower in RSA than IVA than ITA oocytes (Fig. 4B). Furthermore, while mRNA levels of the antiapoptotic genes, Bcl2 and Sirt1, were lower, that of the proapoptotic Bax were higher significantly in RSA than IVA than ITA oocytes (Fig. 4C). Together, the results suggested that mouse...
Effects of different aging regimens on apoptosis of cumulus cells in aging oocytes

Cumulus cells recovered after different aging treatments were cultured for 16 h in CZB medium before assessment of apoptosis by both annexin V/Hoechst 33342 staining and one-step RT-PCR assay for Bcl2 and Bax mRNA levels. While the apoptotic percentage was higher (Fig. 5A, B, C and D), the Bcl2/Bax ratio was lower significantly (Fig. 5E) in cumulus cells after RSA than IVA than ITA, confirming that mouse oocytes aged faster in vivo in stressed animals than in unstressed animals than in vitro, as our previous studies indicated that mouse cumulus cells underwent apoptosis and released soluble FasL and TNF-α, which facilitated oocyte aging after ovulation (Zhu et al. 2015. Kong et al. 2018).

Shortening or extending the time of different aging regimens confirmed that over-aged oocytes lost the responsiveness to activating stimuli

To verify that the unexpected loss of responsiveness to activating stimuli in over-aged oocytes was due really to over-aging, aging time was shortened to 6 h or extended to 18 h before ethanol activation rates of oocytes were observed. When aged for 6 h, activation rates were significantly higher in RSA than IVA oocytes (Fig. 5F). When aged for 18 h, however, the activation rates were very low and did not differ among the three aging protocols. Thus, the results confirmed that over-aging of oocytes led to a loss of their responsiveness to activating stimuli.

Effects of different aging protocols on oocyte levels of cytoplasmic calcium and expression of CaSR and CDK1 protein

While the level of cytoplasmic calcium was significantly higher in IVA oocytes than in ITA oocytes; it did not differ between RSA and ITA oocytes (Fig. 6A). While the level of CaSR did not differ between ITA and IVA oocytes, it decreased significantly in RSA oocytes (Fig. 6B, C, D, E and F). Furthermore, the CDK1 protein level was significantly lower in RSA than IVA than ITA oocytes (Fig. 6G). Thus, the results suggested that (a) the measurement of cytoplasmic calcium was correlated with oocyte STAS; (b) over-aged oocytes lost the ability to increase the cytoplasmic calcium due to a significant decrease in CaSR; and (c) the MPF inactivation we observed in RSA oocytes in spite of the extremely low level of cytoplasmic calcium was due to a protein degradation associated with over-aging.

Apoptosis of oviductal epithelial cells (OECs) in FO, IVA, RSA or mat mice

While OECs from FO mice were recovered immediately following ovulation, those from IVA, RSA or Mat mice were recovered 12 h after ovulation. Our flow cytometry analysis showed that percentages of healthy OECs were lower significantly in IVA and RSA than in FO mice due to increases in early and/or late apoptotic and necrotic cells (Fig. 7A and C). Percentages of healthy and apoptotic OECs did not differ between FO and MAT mice. Our RT-PCR analysis on the Bcl2/Bax ratio further confirmed that OECs from RSA mice suffered severer apoptosis than did OECs from IVA than those from FO and Mat mice (Fig. 7B). Together, the results suggested that (a) OECs underwent apoptosis with time after ovulation; (b) stress on females facilitated apoptotic OECs; and (c) mating prevented apoptosis of OECs after ovulation.

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Levels of growth factors and apoptosis-related cytokines in oviductal homogenates in FO, IVA, RSA or Mat mice

While OECs from FO mice were recovered immediately following ovulation, those from IVA, RSA or Mat mice were recovered 12 h after ovulation. While levels of growth factors IGF-1 and BDNF (Fig. 8A and B) were lower, that of TNF-α was higher significantly (Fig. 8D) in RSA than IVA or FO oviducts. The level of FasL was also significantly higher in RSA than FO and IVA oviducts, but it did not differ between FO and IVA oviducts (Fig. 8C). Levels of all the four proteins did not differ significantly between oviducts from FO and Mat mice. The results suggested that oviductal cells underwent apoptosis after ovulation with decreased production of growth factors and that mating prevented apoptosis and improved growth factor secretion.

Oocytes aged slower in Mat than in IVA oviducts

To verify that mating ameliorates oocyte aging, we compared oocyte rates of activation and fragmentation at 12 h after ovulation between IVA and Mat mice. The...
results showed that both the ethanol activation rate and the fragmentation rate were significantly lower in Mat than IVA mice (Fig. 9), confirming that mating ameliorates oocyte aging.

Discussion

In this study, while other aging characteristics indicated that the RSA oocytes aged faster than oocytes in other treatments, their activation rate was as low as that observed in ITA oocytes. To explain this loss of responsiveness to activating stimuli in the RSA oocytes, we observed a significant decline in cytoplasmic calcium along with a significant decrease in CaSR expression and MPF activity in RSA oocytes compared to those in IVA or ITA oocytes. The decrease in CaSR expression would explain the decline in cytoplasmic calcium observed in the RSA oocytes, as it was reported that CaSR was responsible for the elevation of cytoplasmic calcium during spontaneous activation of rat oocytes after ovulation (Yang et al. 2018). However, further explanation is needed for our results that the MPF activity and the cytoplasmic calcium declined simultaneously in the RSA oocytes, as it is known that MPF deactivation requires significant increases in cytoplasmic calcium during oocyte activation (Heytens et al. 2008). Our ELISA measurement, which revealed a significantly lower level of CDK1 contents in RSA than in ITA or IVA oocytes, suggested that the loss of responsiveness to activating stimuli in the RSA oocytes was due mainly to a global degradation of proteins that occurred in these over-aged apoptotic cells. In apoptotic cells, proteins are cleaved into fragments through a caspase-mediated proteolysis (Taylor et al. 2008).

This study demonstrated that OECs underwent a spontaneous apoptosis with increasing time after ovulation, and restraint stress on female mice facilitated this apoptosis of OECs. Furthermore, the apoptotic oviductal cells produced more FasL and TNF-α but less growth factors such as IGF-1 and BDNF. A massive apoptosis was observed in OECs in the oviduct ampulla of the rat after ovulation (Szołtys et al. 2000). In the bitch oviduct, a significant increase in the number of apoptotic cells was found 4 days after ovulation (Urhausen et al. 2011). Furthermore, Zheng et al. (2016) reported that restraint of female mice during the preimplantation stage induced apoptosis of oviduct cells with increased production of FasL but decreased expression of Igf-1 and Bdnf mRNAs. Because it was shown that the postovulatory aging mouse oocytes expressed both Fas receptors (Zhu et al. 2015) and TNF-receptor 1 (Kong et al. 2018), the current results suggested that the apoptotic oviductal cells might facilitate postovulatory oocyte aging by releasing proapoptotic FasL and TNF-α and by reducing the production of antiapoptotic growth factors.
It is known that the oviduct epithelium is critical for the establishment of a healthy pregnancy, as it secretes growth factors that stimulate the development of preimplantation embryos (Heyner 1997, Leese et al. 2020). The present results showed, however, that OECs underwent apoptosis and facilitate oocyte aging after ovulation. We thus tested whether copulation would have any effect on the oviduct physiology and oocyte aging. The results indicated that mating prevented apoptosis of OECs and alleviated oocyte aging after ovulation. Researches in insects showed that stimuli received during courtship and copulation, spermatooza, and seminal fluid were essential for the transition of a female from unmated to the mated state that ensures reproductive success (Carmel et al. 2016). In the llama, the oviductal environment changed in response to the copulation stimulus with increased production of matrix metalloproteinase-2 (Zampini et al. 2018), and enhanced expression of β-nerve growth factor and tropomyosin receptor kinase A (Sari et al. 2020). Because we observed the copulation effect on OEC apoptosis and oocyte aging using pseudopregnant mice that had been mated with vasectomized males, our results suggested that spermatooza may not be involved in the beneficial effect of mating on OEC apoptosis and oocyte aging. However, although the data suggest that copulation renders the oviduct environment beneficial to embryo development and oocyte survival, the underlying mechanisms are unclear and worth exploring.

In summary, because humans and some mammals potentially undertake sexual activity on any day of the estrous cycle, fertilization of aged oocytes may take place, which can impair embryo development and cause anomalies in offspring. Furthermore, while it is known that ovulated oocytes age within the oviduct and that maternal stresses impair embryo development by inducing apoptosis of oviductal cells, it is unknown if the oviduct and/or maternal stress would affect postovulatory oocyte aging. Thus, we have studied the effects of the oviduct, female restraint stress and copulation on postovulatory aging of mouse oocytes. The results demonstrated that (a) the oviduct underwent apoptosis and facilitated oocyte aging after ovulation; (b) female restraint stress increased apoptosis of oviductal cells and facilitated oocyte aging; and (c) mating ameliorated OEC apoptosis and oocyte aging. The data are important for the healthy reproduction of both humans and animals as they provide fundamental information for control over postovulatory oocyte aging.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Q Q K, G L W, J S A, J W H, H C, and T L conducted the experiments. Q Q K and J H T analyzed the data. J H T designed the experiments and wrote the manuscript. All authors reviewed the manuscript.

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