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Effect of Aging on Meiosis Progression, Developmental Competence and DNA Double-Strand Breaks in Mouse Oocytes

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Authors' Contribution
XF, GJ and LG designed and organized this research. GJ and LG performed the in vitro maturation, oocyte parthenogenetic activation (PA) and embryo *in vitro* culture. LG, GJ and ZH performed the measurement of intracellular reactive oxygen species (ROS) and glutathione (GSH) levels. LG, ZH and MY performed immunofluorescent microscopy experiment. LG, CZ and GJ analysed the obtained data. XF and SZ interpreted the results. XF, LG and GJ wrote the manuscript.

Key words
Aging, DNA double-strand breaks, Embryo, Mouse, Oocyte maturation.

ABSTRACT

This study investigated the effect of aging on meiosis progression, embryo developmental competence and DNA double-strand breaks (DSBs) in mouse oocytes and resultant early embryos. Germinal vesicle (GV) oocytes were first cultured to monitor the progression of germinal vesicle breakdown (GVBD) and polar body extrusion (PBE) during *in vitro* maturation (IVM), then the harvested metaphase II (MII) oocytes were parthenogenetically activated to evaluate pronuclear (PN) formation of parthenogenetic embryo and embryo development. The cytoplasmic maturation was examined by measuring the intracellular reactive oxygen species (ROS) and glutathione (GSH). DNA DSBs were examined by immunostaining of pi-H2AX, the marker of DNA DSBs. The results showed that the GVBD rates were similar in oocytes of young and aged mice. Polar body extrusion was significantly delayed in aged mice ($P < 0.05$), however the rate of polar body extrusion was similar to that of young mice at 16 h of IVM. Moreover, PN formation of parthenogenetic embryo was significantly delayed in aged mice ($P < 0.05$). Afterward the two groups obtained similar results with respect to the percentages of activated oocytes, 2-cell embryos and blastocysts. The cytoplasmic maturation of MII oocytes and blastocysts in aged mice were significantly compromised to those of young mice ($P < 0.05$). Furthermore, GV oocytes, 2-cell embryos and blastocysts showed significantly higher relative intensities of pi-H2AX in aged mice ($P <0.05$). Taken together, our result indicate that aging disturbed oocyte maturation and parthenogenetic embryo development, which could be related to insufficient cytoplasmic maturation and worsening DNA DSBs in oocytes and early embryos.

INTRODUCTION

Although both men and women suffering aging effect, fecundability has a stronger correlation with maternal age (Rothman *et al*., 2013). In aged women, 20–30% of ovulated oocytes are aneuploid (Nagaoka *et al*., 2012). Despite the rapid progress in assisted reproductive technology increasing the possibility to overcome the reproductive problems, the sterility of advanced maternal age is still an important question (Tarin *et al*., 2014).

There is significant reduction in the possibility of fertilization and pregnancy with advanced maternal age in human beings and mice (Schwartz and Mayaux, 1982; Cui *et al*., 2013). Even if pregnancy takes place, older females have greater risks of miscarriage and nondisjunction (Te and Pearson, 2002; Herbert *et al*., 2015). These phenomena are ascribed to a complex series of factors including inappropriate hormone (follicle-stimulating hormone) secretion (McTavish *et al*., 2007), deteriorative uterine apparatus (Schreuder *et al*., 2006) and shrinking oocyte pool (Fu *et al*., 2014). However, oocyte competency is thought to be the primary determinant of female reproductive aging (Krey and Grifo, 2001; Keefe *et al*., 2015), considering that oocytes donations from young

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to older women nearly completely abrogate the effects of aging on fertility (Cohen *et al*., 1999).

Compared to young female mice, there are substantially increased aneuploidy rates (Sebestova *et al*., 2012; Fu *et al*., 2014) and serious DNA damage in aged mice oocytes (Fujino *et al*., 1996). When chromosomes are broken, the DNA damage checkpoint is activated to allow extra time for repairing damaged DNA (Sancar *et al*., 2004; Carroll and Marangos, 2013; Rinaldi *et al*., 2017). Recent reports confirm that oocytes carrying DNA damage associated with DNA double-strand breaks (DSBs) arrest in meiosis I through activation of the spindle assembly checkpoint, which results in anaphase-promoting complex (APC) inhibition (Collins *et al*., 2015; Marangos *et al*., 2015; Lane *et al*., 2017). Furthermore, DSBs also disrupt oocyte spindle assembly (Wang *et al*., 2016) and microtubule-kinetochore attachment during metaphase of the meiosis I (MI) (Mayer *et al*., 2016), which is harmful to the segregation of homologous chromosomes and may result in aneuploidy (Lu *et al*., 2017).

Besides, studies have also revealed that accumulation of reactive oxygen species (ROS) could induce DNA damage which may give rise to dramatic decline in oocyte cytoplasmic quality (Menezo *et al*., 2010; Collins and Jones, 2016; Meldrum *et al*., 2016). Reactive oxygen species (ROS) increases with age (Kregel and Zhang, 2007), owing in part to lowered endogenous antioxidant defenses generating glutathione (GSH) levels (Suh *et al*., 2004; Noreen, 2018). GSH, an important indicator of oocyte cytoplasmic maturation (Curnow *et al*., 2010), plays an important role in protecting the cell against oxidative damage by eliminating the ROS (de Matos *et al*., 2000). Impaired GSH synthesis has been reported to compromise developmental potential of mouse oocytes (Jiao *et al*., 2013).

ROS and DNA damage effect accumulate with age (Garinis *et al*., 2008; Li *et al*., 2008; Meldrum *et al*., 2016). Oocytes are the special cells which arrested at G2/prophase in the ovary for the entire reproductive lifespan. Thus oocyte is particularly vulnerable to DNA damage. The histone H2A variant H2AX is phosphorylated on serine residue 139 at DNA damage sites, which is widely accepted as a marker for DNA DSBs (Rogakou *et al*., 1998). Hence, in this study we examined the dynamic changes of pi-H2AX during oocyte maturation and early embryo development, to investigate whether DNA DSBs were involved in the compromised oocyte meiosis progression and embryos developmental potential in aged mice.

MATERIALS AND METHODS

Unless otherwise stated, all chemicals and media were purchased from Sigma–Aldrich (MO, USA). All procedures contributing to this work were approved by the Animal Ethics Committee of the China Agricultural University and comply with the ethical principles of animal experimentation adopted by this committee.

Oocyte collection and in vitro *maturation (IVM)*

Swiss CD1 mice (Vital River Laboratory Animal Technology Co. Ltd., China) were housed in a room at 20°C to 22°C for 12 h (8AM-8PM) light and 12 h dark cycle. Mice were superovulated with 10 IU (intraperitoneal) equine chorionic gonadotropin (eCG; Ningbo Hormone Products Co., China). According to previous report, germinal vesicle (GV) oocytes were isolated from "young" (6-week-old) or "aged" (9-month-old) female Swiss CD1 mice at 48 h after eCG treatment and transferred into M2 medium supplemented with 3 mg/ml bovine serum albumin (Flurkey, 2007). Then oocytes were rinsed thoroughly and placed in M16 medium containing 10% fetal bovine serum (FBS), 10μg/mL FSH, 10μg/mL LH, 0.01μg/mL epidermal growth factor (EGF) and 0.23 mM sodium pyruvate for IVM, covered with mineral oil and maintained in an incubator (at 37°C in an atmosphere of 5% CO_2 and at maximum humidity). Germinal vesicle breakdown (GVBD) was observed every 0.5 h beginning at 0 h of IVM and polar body extrusion (PBE) was assessed every 1 h since 8 h of IVM, until the incidence rates did not increase further.

Oocyte parthenogenetic activation (PA) and embryo development

At 16 h of IVM, metaphase II (MII) oocytes with polar bodies were chosen and activated incalcium-free human tubal fluid (HTF) medium containing 10 mM $SrCl_2$ and 5 mg/ml cytochalasin D (CD) for 2.5 h. Then oocytes were transferred into regular HTF medium supplemented with 5 mg/ml CD for 3.5 h. Finally, activated oocytes were cultured in drops of KSOM medium (Millipore, MA, USA). The pronuclear (PN) formation of parthenogenetic embryo was evaluated every 1 h from 3 h post activation until rising to a peak. Thereafter, the percentages of activated oocytes, 2-cell embryos and blastocysts were assessed at 8 h, 24 h and 96 h post activation, respectively.

Measurement of intracellular reactive oxygen species (ROS) and glutathione (GSH) levels

To measure ROS level, cells were incubated in dark with M2 supplemented with 1 mM 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) for 20 min at 37°C, washed three times with phosphate-buffered saline (PBS) and placed into 50 μl droplets. The fluorescence was measured under an epifluorescence

microscope with a filter at 460 nm excitation. ImageJ software (National Institutes of Health, MA, USA) was used to analyse fluorescence images. The GSH level was measured by 10 μM 4-chloromethyl-6.8-difluoro-7-hydroxycoumarin (Cell-Tracker Blue) with a filter at 370 nm excitation, the experimental procedure was the same as the ROS measurement described above.

Immunofluorescent microscopy

Immunostaining analysis was carried out as described previously (Lin *et al*., 2014) with some modification. After brief washing, oocytes or embryos were fixed with 3.7% (w/v) paraformaldehyde in PBS for 30 min and permeabilized with 0.5% (v/v) Triton X-100 in PBS for 20 min. Subsequently, oocytes or embryos were blocked with 1% (w/v) BSA in PBS for 1 h before incubation with anti-phosphorylated H2AX at Ser139 (diluted 1:200; Bioworld, Beijing, China) overnight at 4°C. Following several washes, oocytes or embryos were incubated with fluorescein isothiocyanate (FITC) conjugated anti-rabbit secondary antibody (diluted 1:200; Beyotime, Shanghai, China) for 1h at room temperature. Finally, extensively rinsed oocytes or embryos were mounted on a glass slide in a drop of Vectashield anti-bleaching solutioncontaining 3 μg/ml 4', 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, CA, USA) and examined on a laser scanning confocal microscope (Nikon, Tokyo, Japan). The relative intensity of pi-H2AX was calculated using the ratio of antibody signal to DNA signal after subtraction of cytoplasm background fluorescence.

Statistical analysis

All experiments were repeated at least three times. Statistical analysis was conducted by one-way analysis of variance followed by Duncan's test using SPSS software (IBM, NY, USA). Data were expressed as the mean ± standard error and a value of $P< 0.05$ was considered significant.

RESULTS

Aging did not prevent GVBD but delayed PBE (poler body extrusion)

To investigate whether aging affected oocyte meiosis resumption and final maturation, the progression of GVBD and PBE were traced successively during IVM. As shown in Figure 1A and B, most oocytes had undergone GVBD before 3.5 h of IVM in the aged and young groups (86.22±2.38% *vs*. 87.50±3.71%). However, the PBE started after 10 h of IVM in the aged group, which was delayed about an hour than that of the young group. Furthermore, the PBE rate in the aged group remained significantly below that of the young group at 10, 11 and 12 h during

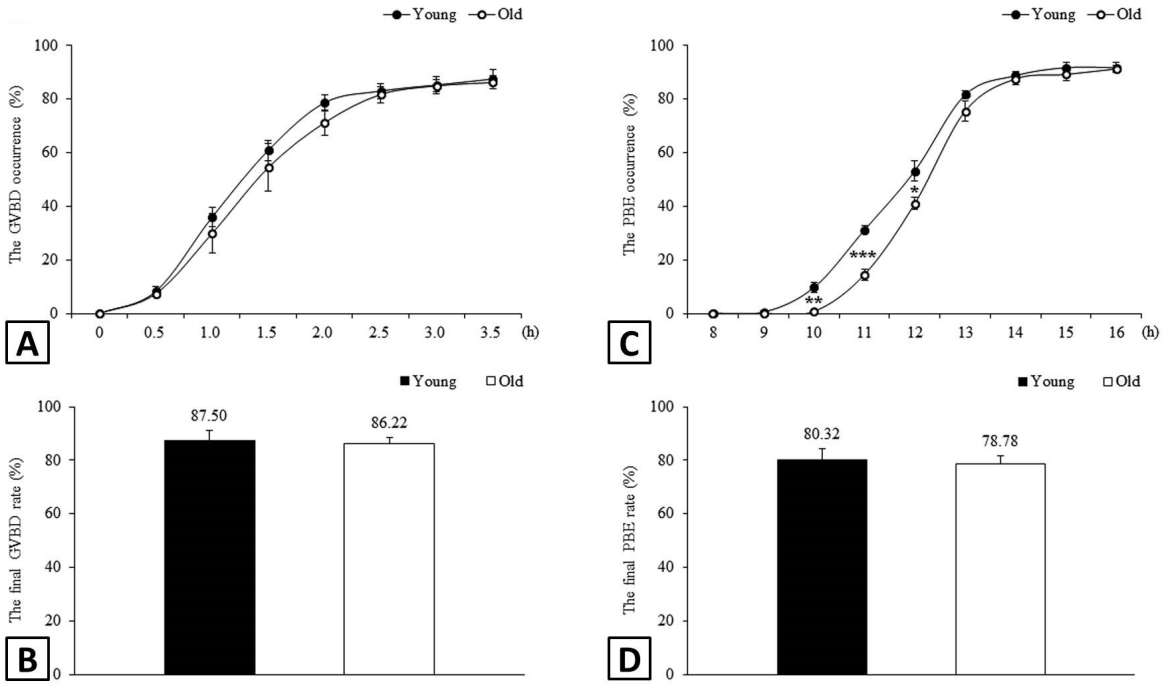

Fig. 1. Kinetics of GVBD and PBE during oocyte maturation. Shown are the temporal trends of GVBD (A) and PBE (C) occurrence during IVM and the final rates of GVBD (B) and PBE (D) in oocytes. Number of oocytes analysed in total: n = 150 in the young

group; n = 147 in the aged group. *P< 0.05, **P< 0.01, ***P< 0.001.

IVM (0.69±0.69%, 12.52±1.99% and 35.39±2.41% *vs.* 8.59±1.73%, 27.07±1.32% and 46.39±3.50%, P<0.05; Fig. 1C), although they finally achieved a similar incidence at 16 h of IVM (78.78±2.75% *vs.* 80.32±3.90%; Fig. 1D).

Aging delayed PN formation but did not affect embryo development

To further determine whether mature oocytes went through normal embryo development in aged mice, the developmental process of PA embryos was monitored. Similar to PBE, PN formation of parthenogenetic embryo also showed a significant decline at 3, 4, 5 and 6 h post activation in the aged group (13.79±1.26%, 29.24±2.19%, 57.45±2.50% and 78.72±1.26% *vs.* 36.30±1.48%, 59.77±4.63%, 75.79±2.74% and 85.71±2.71%, P < 0.05, Fig. 2A). Afterward the two groups obtained similar results with respect to the percentages of activated oocytes, 2-cell embryos and blastocysts (Fig. 2B).

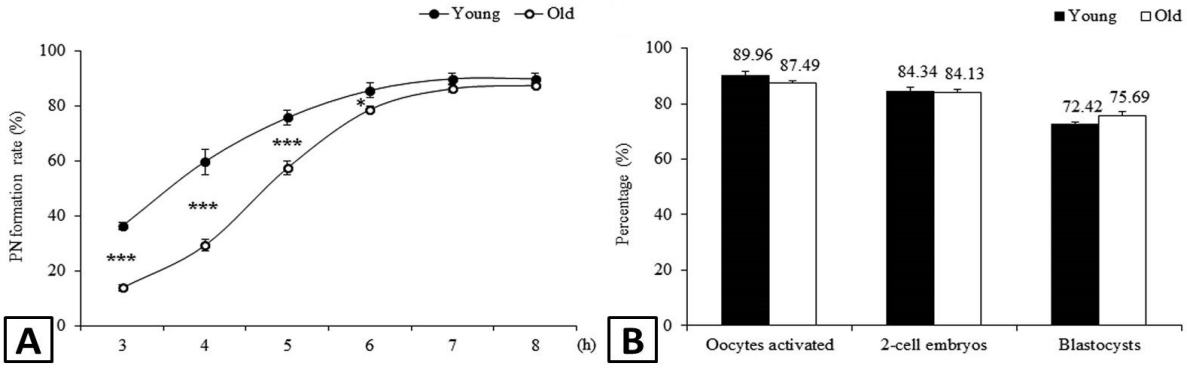

Fig. 2. Kinetics of PN formation in PA embryos. Shown were the temporal trends of PN formation (A) and the percentages of activated oocytes, 2-cell embryos and blastocysts (B). Number of oocytes analysed in total: n = 193 in the young group; n = 183 in the aged group.

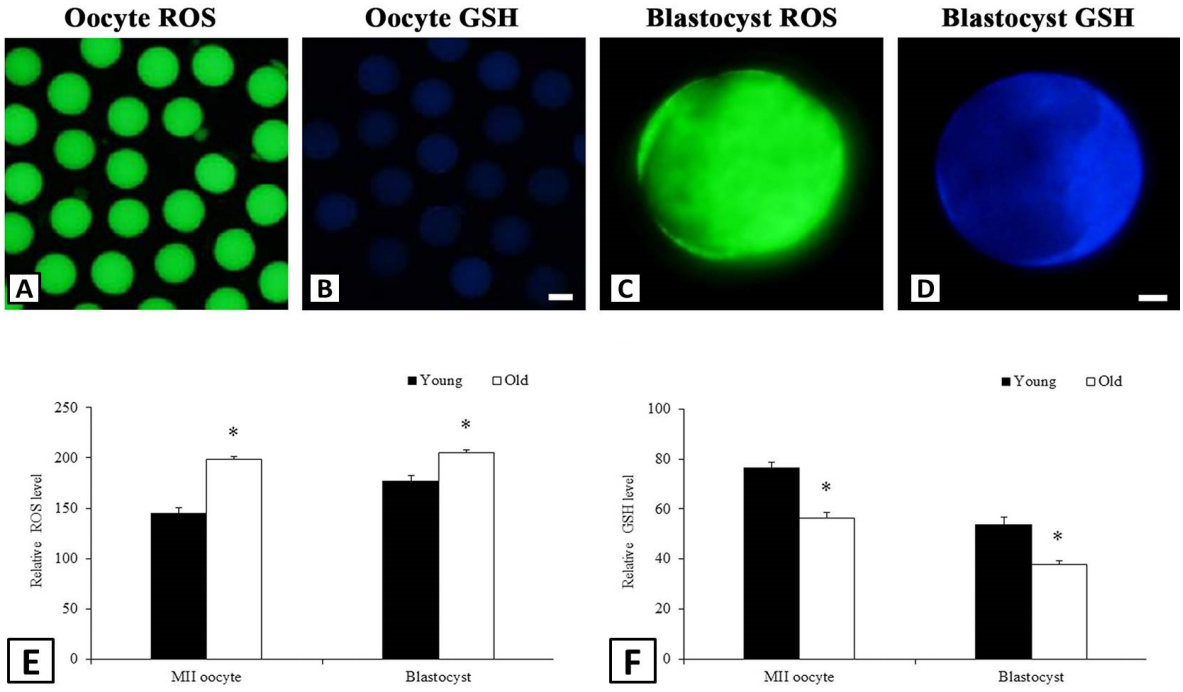

Fig. 3. Intracellular ROS and GSH levels in MII oocytes and blastocysts. Shown were representative images of ROS (A) and GSH (B) in MII oocytes. C and D were the representative images of ROS and GSH, respectively in blastocysts. The quantitative results from fluorescence intensities of ROS (E) and GSH (F) in MII oocytes and blastocysts, respectively. At least 25 MII oocytes or

blastocysts were analysed for each treatment per group. Scale bar is 50 μm in A and B and 20 μm in C and D.

Aging compromised cytoplasmic maturation in oocytes and early embryos

Although the percentage of GV oocytes developed to MII stage or blastocyst stage showed no significant difference between two groups, intracellular ROS and GSH levels were detected in MII oocytes and blastocysts. The aged group showed remarkably increased ROS levels and declined GSH levels both in MII oocytes and blastocysts (P<0.05, Fig. 3), meaning that their cytoplasmic maturation was substantially insufficient as compared with that of the young group.

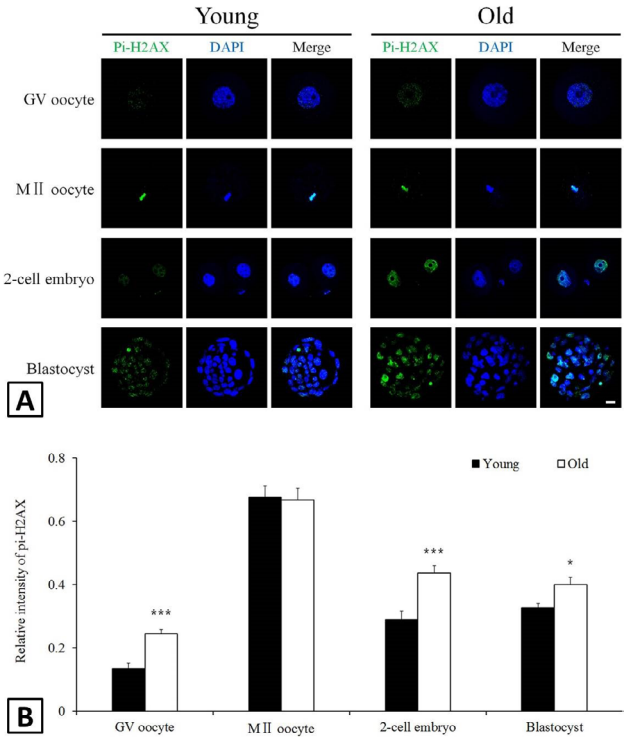

Fig. 4. Immunofluorescent staining of DNA DSBs in oocytes and early embryos. A, representative images of oocytes and early embryos stained with pi-H2AX antibody (green) and DAPI (blue). Scale bar = 20 μm. B, relative intensities of pi-H2AX signals normalized against DNA signals. At least 25 oocytes or early embryos were analysed at each stage per group.

Aging caused the worsening of DNA DSBs in oocytes and early embryos

Given that cell cycle was postponed during oocyte maturation and parthenogenetic embryo development in aged mice, it is doubtful whether the delay was related to DNA DSBs. To address this possibility, the signal of pi-H2AX was detectedin oocytes and early embryos (Fig. 4A). The aged group showed stronger relative intensities of pi-H2AX in GV oocytes, 2-cell embryos and blastocysts than the young group (P<0.05), while their relative intensities of pi-H2AX were similar in MII oocytes (Fig. 4B).

DISCUSSION

Our results indicated that the progression of PBE was delayed and the level of DSBs was significantly increased in aged mice oocytes (Figs. 1C, 4A). Previously, oocytes with high level of DSBs exhibit a delay in the time of emission of the first polar body (Ma *et al*., 2013; Lin *et al*., 2014). It had been observed that DNA damage induced before GVBD would lead to a MI arrest through activation of the spindle assembly checkpoint, which results in anaphase-promoting complex (APC) inhibition (Collins *et al*., 2015; Marangos *et al*., 2015; Lane *et al*., 2017). The oocytes with high level of DSBs also disrupts microtubule-kinetochore attachment (Mayer *et al*., 2016), which would raise the rate of oocyte aneuploidy (Lu *et al*., 2017). According, our previous research has observed the increased aneuploidy rates in aged mice oocytes (Fu *et al*., 2014).

DSBs existed in aged GV oocytes do not prevent meiotic maturation. In this study, there was no significant difference on the rates of PBE at 16 h of IVM (Fig. 1D). Some studies provide evidence that the SAC activity is compromised in old oocytes which maybe a likely explanation for the inability of aged oocytes to establish an efficient MI checkpoint in response to DNA damage (Riris *et al*., 2014; Marangos *et al*., 2015). Recent study shows that escaping DNA damage checkpoint in maternal aging may be one of the causes of increased chromosome anomalies in oocytes from older mothers (Marangos *et al*., 2015; Sakakibara *et al*., 2015).

Recent studies have revealed that the accumulation of ROS cause DNA damage in the aged oocytes during long periods of dictyate arrest and if it is not repaired, the DNA damage may give rise to dramatic declines in oocyte cytoplasmic quality (Menezo *et al*., 2010; Collins and Jones, 2016; Meldrum *et al*., 2016). Accordingly, our results showed oocytes cytoplasmic maturation was substantially compromised as remarkably increased ROS levels and declined GSH levels in aged mice MII oocytes (Fig. 3). ROS increases with age (Kregel and Zhang, 2007), owing in part to lowered endogenous antioxidant defenses generating glutathione (GSH) levels (Suh *et al*., 2004) which compromise the developmental potential of mouse oocytes (Jiao *et al*., 2013).

The compromise in oocyte cytoplasmic maturation could be harmful to embryo development (Krey and Grifo, 2001; Keefe *et al*., 2015). We observed that PN

formation of parthenogenetic embryo was delayed in aged mice (Fig. 2A). Similar result was also observed by other researchers in zygotes of middle-aged and aged mice (Cui *et al*., 2013). The cell cycle arrest found in zygotic development could be triggered by induced DNA DSBs (Carroll and Marangos, 2013). The arrest is mandatory to allow DNA repair activity in order to avoid mutation in all kinds of germ line (Goldmann *et al*., 2018). In this study, aged mice still had higher levels of DNA DSBs in 2-cell embryos and blastocysts in compared to the young mice (Fig. 4B), indicated the unrepaired DNA damage in oocytes could persist into embryo development stage (Derijck *et al*., 2008).

In addition, the DNA DSBs in early embryos do not influence the subsequently embryo development. We confirmed the degrees of DNA DSBs in 2-cell embryos were higher in aged than young mice, however, the two groups obtained similar results with respect to the percentages of blastocysts (Fig. 2B). This probably because the embryo development through cells mitosis with the same property as somatic cells, which do not halt mitosis in response to DNA damage, and instead respond in G1 by either repairing their DNA or undergoing apoptosis (Hustedt and Durocher, 2017).

CONCLUSION

In conclusion, our study demonstrated that oocyte maturation and embryo development could be compromised with advanced maternal age, and it could be related to insufficient cytoplasmic maturation and worsening DNA DSBs in oocytes and early embryos.

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Statement of conflict of interest

The authors declare no conflict of interest.

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