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Protective effect of onion peel extract on ageing mouse oocytes

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Research Article

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Summary

Mammalian oocytes not fertilized immediately after ovulation can undergo ageing and a rapid decline in quality. The addition of antioxidants can be an efficient approach to delaying the oocyte ageing process. Onion peel extract (OPE) contains quercetin and other flavonoids with natural antioxidant activities. In this study, we investigated the effect of OPE on mouse oocyte ageing and its mechanism of action. The oocytes were aged *in vitro* in M16 medium for 16 h after adding OPE at different concentrations (0, 50, 100, 200, and 500 μg/ml). The addition of 100 μg/ml OPE reduced the oocyte fragmentation rate, decreased the reactive oxygen species (ROS) level, increased the glutathione (GSH) level, and improved the mitochondrial membrane potential compared with the control group. The addition of OPE also increased the expression of *SOD1*, *CAT*, and *GPX3* genes, and the caspase-3 activity in OPE-treated aged oocytes was significantly lower than that in untreated aged oocytes and similar to that in fresh oocytes. These results indicated that OPE delayed mouse oocyte ageing by reducing oxidative stress and apoptosis and enhancing mitochondrial function.

Introduction

Oocyte ageing induces several functional changes that affect oocyte quality and subsequent embryo development after fertilization (Zhang *et al.*, 2017). Post-ovulatory ageing is associated with a range of oocyte defects, including abnormalities in the structures of the zona pellucida, oolemma, cortical granules, mitochondria, and meiotic spindles, in addition to chromosomal structural abnormalities (Cheng *et al.*, 2017). It is also accompanied by various biochemical and molecular changes, such as the generation of reactive oxygen species (ROS), decrease in the activity of maturation promoting factor (MPF), decrease in the expression of anti-apoptotic factor BCL-2, and activation of caspase-3, in addition to epigenetic modifications (Shimoi *et al.*, 2019; Xu *et al.*, 2019; Kasapoğlu and Seli, 2020; Kim *et al.*, 2020).

The addition of antioxidants during oocyte ageing has been shown to delay the process and improve the oocyte quality (Lord *et al.*, 2013). Plant polyphenolic compounds, including flavonoids, are natural antioxidants. Supplementation with quercetin and the phenolics from *Kaempferia galanga* rhizome to maturation medium during oocyte ageing has been reported to reduce oxidative stress in cells and help maintain the spindle shape and function, consequently delaying oocyte ageing and improving the quality of the aged oocytes (Wang *et al.*, 2017; Liang *et al.*, 2018; Yao *et al.*, 2019).

Onion peel extract (OPE) contains notable amounts of quercetin and other flavonoids, displaying better antioxidant activity than onion flesh extract (Kim *et al.*, 2014). In women with obesity, the antioxidant properties of OPE inhibited the decrease in SOD activity and the production of ROS associated with obesity (Kim and Yim, 2015). Such findings suggest that OPE may help to prevent the development of various common chronic diseases associated with oxidative stress (Masood *et al.*, 2021). It has also been shown that OPE exerts a hypocholesterolemic effect. In particular, OPE increased the faecal excretion of serum and hepatobiliary sterols in mice fed a high cholesterol diet (Kang *et al.*, 2016). In addition, OPE has been recognized as a potential candidate for human infertility treatment because of its ability to improve the flagellar voltage-gated proton channel Hv1 in sperm and increase sperm motility (Chae *et al.*, 2017).

However, despite these advantages, research on the effect of OPE in oocytes is still scarce. In this study, we examined the influence of OPE on the ageing process of mouse oocytes, including its effects on mitochondrial function, antioxidant activity, and apoptosis-related processes, to provide a theoretical basis for determining the mechanism by which OPE improves the quality of ageing oocytes.

Materials and methods

All chemicals and reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

Preparation of OPE

The onion peels were washed in distilled water to remove the silt from the skin and dried in a drying oven with an air blast at 60°C. The onion peels dried to a constant weight were crushed into powder with a high-speed pulverizer, and the powder was screened through a 40-mesh sieve for later use. The prepared onion peel powder was wrapped tightly in a non-woven impregnated cloth and extracted using 60% ethanol at a 1:40 solid-to-liquid ratio for 2 h at 60°C. The extraction was repeated two more times, and the pooled extract was concentrated using rotary evaporation at 60°C. The concentrated extract was dried using a freeze dryer to produce OPE for later use.

OPE was dissolved in dimethyl sulphoxide (DMSO) to prepare a concentrated stock solution, and different concentrations of OPE (0, 50, 100, and 200 μg/ml) were dissolved in M16 (keeping the DMSO content <0.5%).

Oocyte collection, culture, and ageing

Kunming mice aged 6–8 weeks were selected. For the ageing group and OPE treatment group, 10 IU of pregnant mare serum gonadotropin (PMSG) was injected intraperitoneally (i.p.) at 15:00 h on the first day, and 10 IU of human chorionic gonadotropin (hCG) was injected at 15:00 h on the second day. At 7:00 h on the third day, the stage II (MII) oocytes were collected from the ampulla of the fallopian tube. The ageing group was placed in M16 for direct culture, and the OPE treatment group was placed in M16 with different concentrations of OPE for culture. For the control group (fresh oocytes), PMSG 10 IU was injected i.p. at 24:00 h on the first day, hCG 10 IU was injected i.p. at 24:00 h on the second day, and the MII phase oocytes were collected at 16:00 h on the third day, and cultured in M16. The correlation test was conducted at 21:00 h on the third day.

Measurement of mitochondrial membrane potential (ΔΨm), ROS, and GSH generation

To assess ΔΨm, denuded MII-stage oocytes were incubated with 2 μM JC-1 (Invitrogen, Waltham, MA, USA) for 1 h at 37.5°C in the dark. The average ΔΨm of oocytes was then calculated as the ratio of red fluorescence intensity (J-aggregates; corresponding to activated mitochondria) to green fluorescence intensity (J-monomers; corresponding to inactive mitochondria) using ImageJ software. The fluorescence intensity of the resulting oocytes was analyzed using a fluorescence microscope (Nikon Corp., Tokyo, Japan). ROS levels were measured using a 2′,7′-dichlorofluorescein (H2DCFDA; Thermo Fisher Scientific, Waltham, MA, USA) assay. Briefly, denuded MII-stage oocytes were cultured in 0.1% bovine serum albumin (BSA)-PBS containing 10 μM H2DCFDA for 15 min at 37.5°C in the dark and then visualized with excitation at 485 nm and emission at 535 nm. GSH levels were quantified using CellTracker™ Blue dye (4-chloromethyl-6,8-difluoro-7-hydroxycoumarin, CMF2HC; Invitrogen). Briefly, denuded MII-stage oocytes were incubated in 0.1% BSA-PBS medium containing 10 μM CMF2HC for 15 min at 37.5°C in the dark and visualized with excitation at 371 nm and emission at 464 nm. The fluorescence intensity (1 s after shutter opening with 10 ms exposure for H2DCFDA; 3 s after shutter opening with 100 ms exposure for CMF2HC) of the resulting oocytes was analyzed using fluorescence microscopy (Nikon Corp.) using ImageJ software.

Table 1. Primer sequences used for RT-qPCR

Gene	Primer sequences (5′–3′)
GAPDH	F: GGAGCCAAAAGGGTCATCAT
	R: GTGATGGCATGGACTGTGGT
GPX3	F: TTGAGAAAGGAGATGTGAACGG
	R: CAAAGTTCCAGCGGATGTCA
CAT	F: GTGTTGAACGAGGAGGAGAG
	R: CTGCGTGTAGGTGTGAATTG
SOD1	F: AACAATCTCAACGCCACCG
	R: AGTCACGCTTGATAGCCTCCA

RT-PCR

MII oocytes were harvested, and mRNA was extracted from each of the 15 oocyte pools using the DynaBeads mRNA Direct Kit (catalogue #61012; Dynal Asa, Oslo, Norway) according to the manufacturer's instructions. cDNA was obtained by reverse transcription of mRNA using oligo(dT)12–18 primers and SuperScript III reverse transcriptase (Invitrogen). qRT-PCR was performed using a KAPA SYBR® FAST kit (#KK4601; Kapa Biosystem Ltd, Cape Town, South Africa), in which each reaction contained 10 μl SYBR Green, 1 μl each of forward and reverse primers, and 2 μl cDNA template (10 ng/μl), in a final reaction volume of 20 μl. The amplification run was programmed as follows: 95°C for 3 min, followed by 40 cycles of 95°C for 3 s, 60°C for 30 s, and 72°C for 20 s. The primers used to amplify each gene are shown in Table 1. The target genes were *SOD1*, *CAT*, and *GPX3*, and *GAPDH* was used as a reference gene. The mRNA quantitation data were analyzed using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by the least significant difference (LSD) test using SPSS software (version 19.0; SPSS, Inc., Chicago, IL). Figures were generated using the GraphPad Prism software package (version 6.01; GraphPad, La Jolla, CA). Data are expressed as the mean ± standard deviation (SD), and statistical significance was set at $P < 0.05$. The total number (N) of oocytes/embryos used in each group is shown in the data columns. Each experiment was repeated three times.

Results

OPE reduces the fragmentation rate in aged oocytes

To explore the influence of OPE on the morphological changes in ageing oocytes, the oocytes were treated with OPE at different concentrations, and the fragmentation of oocytes was analyzed after 16 h of *in vitro* ageing. Compared with the ageing control group, the rate of oocyte fragmentation increased significantly to 18.78 ± 2.65% upon treatment with 200 μg/ml OPE (Figure 1b)

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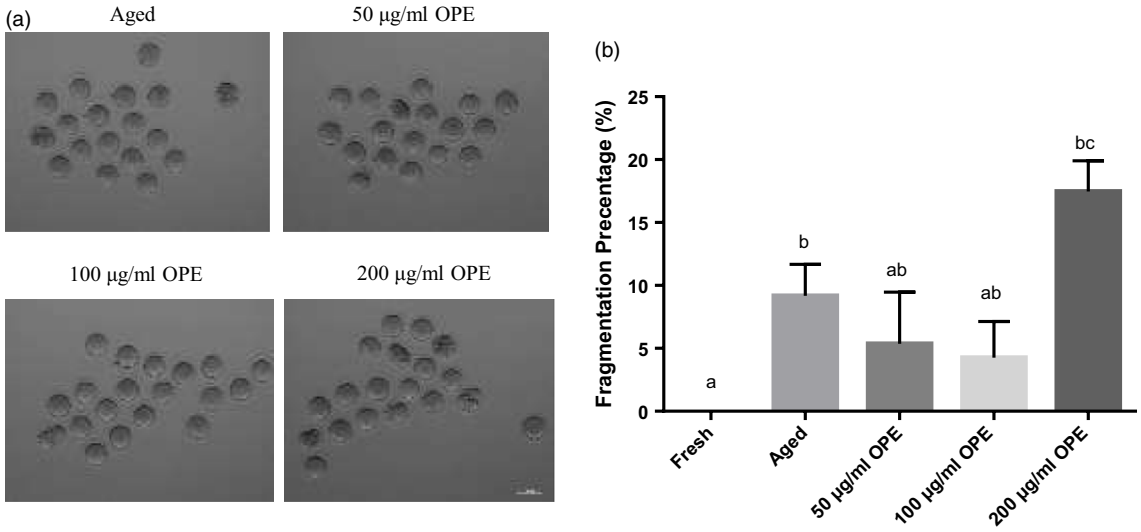

Figure 1. Effect of OPE on cellular fragmentation in aged oocytes. (a) Representative images of oocytes matured under different culture conditions. Oocytes were cultured in M16 medium for 16 h for ageing. (b) Oocyte fragmentation rate. Significant differences are represented with different letters ($P < 0.05$).

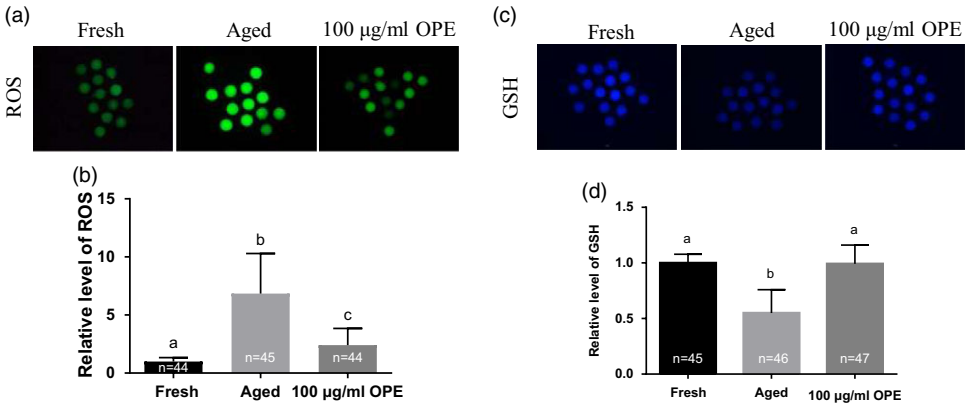

Figure 2. Effects of OPE on oxidative stress in mouse aged oocytes. (a, c) Representative images of ROS and GSH fluorescence in MII oocytes. Oocytes were cultured in M16 medium for 16 h for ageing. (b) Quantitative analysis of ROS fluorescence intensity. (d) Quantitative analysis of GSH fluorescence intensity. Significant differences are represented with different letters ($P < 0.05$).

and decreased, albeit not significantly, to 5.36 ± 4.11% and 4.27 ± 2.87% when treated with OPE at 50 and 100 μg/ml, respectively (Figure 1b). These results indicated that OPE exhibits a certain protective effect on the morphological changes in ageing oocytes. Therefore, a concentration of 100 μg/ml OPE was used in subsequent experiments.

OPE reduces oxidative stress in aged oocytes

Oxidative stress is a key factor that affects oocyte senescence (Jiang *et al.*, 2019; Sasaki *et al.*, 2019). Therefore, we examined the effects of OPE on oxidative stress levels in ageing mouse oocytes by determining the relative levels of ROS and GSH. The addition of 100 μg/ml OPE significantly reduced the level of ROS (2.41 ± 1.43 vs. 6.84 ± 3.45; Figure 2b) and increased the level of GSH (0.99 ± 0.17 vs. 0.55 ± 0.21; Figure 2b) in ageing oocytes compared with the control, indicating that OPE can reduce oxidative stress in ageing oocytes and therefore protect then against the ravages of ageing.

OPE increases ΔΨm in aged oocytes

The generation of oxidative stress is closely related to mitochondrial function (Babayev and Seli, 2015; Babayev *et al.*, 2016). Therefore, we determined the ΔΨm of oocytes using a JC-1 dye-based assay in which the ΔΨm activity is proportional to the red/green fluorescence ratio. Compared with the ageing group, OPE treatment significantly increased the ΔΨm activity of ageing oocytes (0.94 ± 0.23 vs. 0.62 ± 0.17; Figure 3b), although the potential did not reach that of the control (fresh oocyte) group. This result shows that OPE can increase the mitochondrial activity of oocytes to protect them against the ravages of ageing.

OPE reduces caspase-3 activity in aged oocytes

As caspase-3 is an important apoptosis marker, we measured the activity of caspase-3 in oocytes (Porter and Jänicke, 1999). Aged oocytes showed significantly higher caspase-3 activity than fresh oocytes, indicating the induction of apoptosis. By contrast, the caspase-3 activity in OPE-treated aged oocytes was significantly lower than that in untreated aged oocytes and similar to that in fresh oocytes (1.21 ± 0.54 vs. 1.73 ± 0.37; Figure 4b).

OPE increases the expression of oxidative stress-related genes in aged oocytes

Next, to evaluate the effect of OPE on the expression of oxidative stress-related genes in mouse oocytes, we determined the

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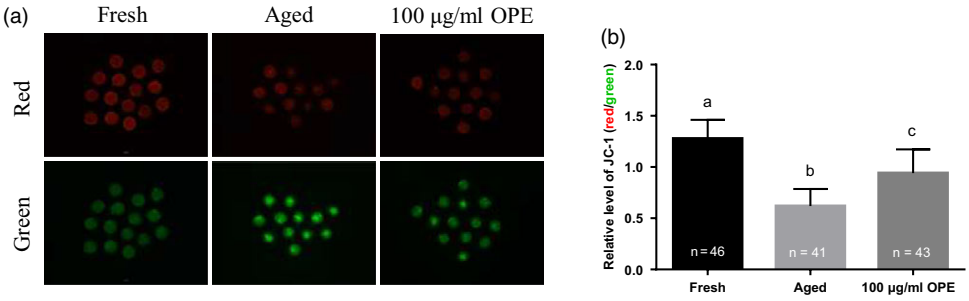

Figure 3. Effect of OPE on mitochondrial membrane potential in aged oocytes. (a) JC-1 staining for mitochondrial membrane potential activity. Green fluorescence indicates the JC-1 monomeric form (low intensity) and red fluorescence indicates the JC-1-aggregated form (high intensity). Oocytes were cultured in M16 medium for 16 h for ageing. (b) Quantitative analysis of JC-1 fluorescence intensity (red/green). Significant differences are represented with different letters ($P < 0.05$).

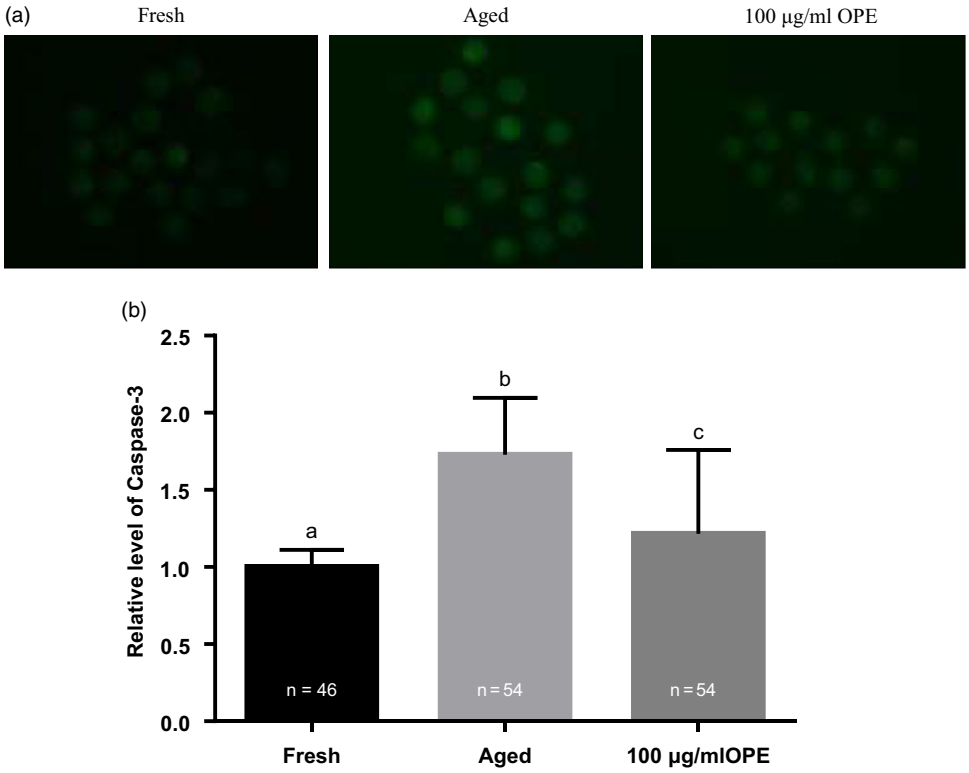

Figure 4. Effects of OPE on caspase-3 activity in mouse aged oocytes. (a) Representative images showing caspase-3 activity in fresh, aged, and OPE-treated aged MII oocytes. (b) Quantified fluorescence intensity for caspase-3 in oocytes. Significant differences are represented with different letters ($P < 0.05$).

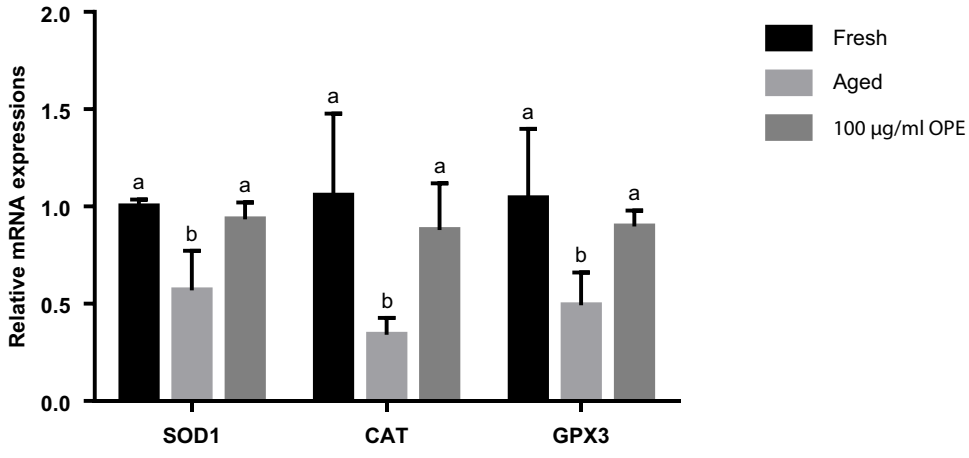

Figure 5. Effects of OPE on expression of oxidative stress-related genes in mouse aged oocytes. Significant differences are represented with different letters ($P < 0.05$).

mRNA levels of *GPX3*, *SOD1*, and *CAT* genes. The results showed that the mRNA expression levels of *SOD1* (0.93 ± 0.09 vs. 0.57 ± 0.20; Figure 5), *CAT* (0.84 ± 0.23 vs. 0.33 ± 0.08; Figure 5), and *GPX3* (1.06 ± 0.10 vs. 0.53 ± 0.11; Figure 5) in oocytes from the OPE-treated group were significantly higher than those from the ageing group but did not show any significant difference compared with the control (fresh oocyte) group.

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Discussion

If the oocyte is not fertilized, it will rapidly age and lose its development ability. Adding antioxidants before ageing can alleviate this process and ensure its developmental ability. As mentioned above, OPE is rich in flavonoids and other substances with antioxidant activity, but its effect on oocytes has not yet been studied. In this study, the *in vitro* culture medium for mouse oocytes was supplemented with OPE at different concentrations. The results showed that treatment with 100 μg/ml OPE reduced the oocyte fragmentation rate during ageing, thereby maintaining the quality of oocytes. Additionally, OPE treatment reduced oxidative stress and apoptosis and improved the mitochondrial activity and expression of antioxidative stress response genes in oocytes. These results suggest that OPE protects ageing oocytes from oxidative stress-induced apoptosis.

Senescent oocytes typically exhibit cellular fragmentation, and the fragmented oocytes have decreased developmental potential (Zhang *et al.*, 2019). To examine the effect of OPE on oocyte ageing, we first examined its effect on the fragmentation rate. The results showed that high concentrations (200 and 500 μg/ml) of OPE increased the fragmentation rate, and all oocytes died after treatment with 500 μg/ml OPE. This may be attributed to the openness being a mixture that causes a change in the osmotic pressure of the medium, causing irreparable damage to the oocytes (Goto *et al.*, 2019). However, there was no significant difference in oocyte fragmentation rate between the ageing group and the low OPE concentration (50 and 100 μg/ml) groups. Therefore, we chose 100 μg/ml OPE for subsequent experiments.

Oxidative stress caused by the generation of ROS is a key mediator of oocyte ageing (Yao *et al.*, 2018; Soto-Heras and Paramio, 2020), and oxidative stress has been closely associated with oocyte fragmentation (Lin *et al.*, 2018; Jia *et al.*, 2019). An overabundance of ROS can compromise the molecular and structural defence mechanisms of the ageing oocyte, leaving it vulnerable to oxidative insults (Mihalas *et al.*, 2017). The present study shows that OPE, rich in antioxidants, can reduce oxidative stress during oocyte ageing by significantly decreasing ROS in cells, increasing the GSH level, and increasing the expression of antioxidant genes, which is similar to the study that OPE can reduce the ROS generated during obesity (Kim and Yim, 2015). In summary, OPE has beneficial effects on ageing oocytes by alleviating oxidative stress.

Oocyte ageing is accompanied by decreased activity of the mitochondrial respiratory complex and ΔΨm (Miao *et al.*, 2020; Soares *et al.*, 2020), and ΔΨm affects ATP synthesis during oxidative phosphorylation (Babayev and Seli, 2015; Al-Zubaidi *et al.*, 2019). Quercetin can promote the expression of PVT1 to inhibit the oxidative stress and apoptosis of H9C2 cells and alleviate the structural and functional dysfunction of mitochondria (Li *et al.*, 2021). Quercetin can also restore the damaged ΔΨm of ageing porcine oocytes and reduce the intracellular ROS level and cell apoptosis (Jiao *et al.*, 2022). Our results showed that ΔΨm of ageing oocytes decreased significantly, indicating that ATP synthesis and the energy supply to cells were decreased. However, the addition of OPE assuaged this downward trend. This is similar to the previous observation that quercetin can alleviate mitochondrial function damage (Li *et al.*, 2021). Combined with the results of oxidative stress, OPE can inhibit the decrease in ΔΨm caused by oxidative stress.

Caspase-3 is a frequently activated death protease that catalyzes the specific cleavage of many key cellular proteins (Papandile *et al.*, 2004; Nicholas *et al.*, 2005). It has been suggested that caspase-3 activity is related to oocyte quality, and caspase-3 activity was significantly increased in aged oocytes compared with young oocytes (Papandile *et al.*, 2004). Quercetin can inhibit chrome-induced cell apoptosis in the kidney and decrease the caspase-9 and caspase-3 protein and mRNA expression levels (Huang *et al.*, 2022). Consistent with these observations, caspase-3 activity increased significantly in ageing oocytes in the present study, but its activity decreased significantly after adding OPE. It shows that OPE can improve the quality of ageing oocytes.

SOD1, CAT, and GPX3 are antioxidant factors (Jia *et al.*, 2019). The increase in the ratio of SOD1 activity to GPX/CAT activity correlates with an increase in lipid damage, cell ageing, and/or cell death (Lin *et al.*, 2018). A recent study has shown that when the expression of GDF8 was inhibited, the maturation rate of buffalo oocytes and the activities of SOD, CAT, and GPX enzymes were significantly decreased (El-Magd *et al.*, 2019). Another study found that quercetin could upregulate the expression level of SOD2 and CAT in porcine ageing oocytes (Jiao *et al.*, 2022). In the present study, we found that the mRNA expression levels of *SOD1*, *CAT*, and *GPX3* in ageing oocytes were significantly decreased, whereas the expression levels of these three genes were significantly increased after OPE treatment, which is similar to the reported effects of quercetin (Jiao *et al.*, 2022). This indicates that OPE can promote gene expression for antioxidant enzymes and delay the ageing of oocytes. In conclusion, OPE delayed mouse oocyte ageing by reducing oxidative stress and apoptosis and enhancing mitochondrial function.

Author contribution. Yong-Nan Xu conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, and reviewed drafts of the paper. Guo-Bo Han performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, and reviewed drafts of the paper. Ying-Hua Li analyzed the data, reviewed drafts of the paper, and contributed reagents/materials/analysis tools. Guan-Hao Li and Nam-Hyung Kim conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared visual material, and reviewed drafts of the paper. All authors read and approved the final manuscript.

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Competing interests. The authors declare none.

Ethical standard. All mouse care and protocols were used in accordance with the guidelines of the Animal Research Committee of Yanbian University, China (SYXK2020–0009).

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