Resveratrol Can Revert Adverse Effects of Oocyte aging in Pig

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Abstract

This study was aimed to evaluate the potential of resveratrol (RES) to alleviate the adverse effects of aging in porcine oocytes through alleviating oxidative stress and up-regulating anti-apoptotic genes. Porcine oocytes were cultured in an *in vitro* medium supplemented with four levels (0, 1, 2, and 4 $\mu\text{mol/L}$) of RES. Based on initial screening; optimum RES level (2 $\mu\text{mol/L}$) was used to evaluate its potential effects on oocytes aged for 24 and 48 hours. Our results revealed that aged oocytes (24 h and 48 h) treated with RES showed higher ($P < 0.05$) ROS fluorescence intensity than the control group, but lower ($P < 0.05$) than untreated aged groups. The GSH content in untreated aged groups (24 h and 48 h) was lower ($P < 0.05$) than RES treated groups but both groups showed higher levels than the control. Similarly, the relative expression of antioxidant genes (CAT, GSH-Px, and SOD1) in RES treated groups was lower ($P < 0.05$) than the control group, but higher than untreated aged groups. Moreover, the relative mRNA expression of caspase-3 and Bax in RES treated groups was higher ($P < 0.05$) than the control group but lower than untreated groups. Furthermore, the expression of Bcl-2 in the RES treated group was significantly lower than control but higher than untreated aged groups. Our findings revealed that the RES can increase the activity of antioxidant enzymes while decreasing the level of ROS in porcine aged oocytes. Moreover, RES showed its potent anti-apoptotic effects and potentially resisted against the decline in mitochondrial membrane potential in aged oocytes.

Introduction

The quality of oocyte is the key factor which controls the viability of embryos for successful reproductive technologies like somatic cell nuclear transfer (SCNT), intra-cytoplasmic sperm injection (ICSI), and *in vitro* fertilization (IVF) (Ma et al., 2005a; Mukherjee et al., 2014). The quality of an oocyte is affected by structural and functional changes induced by aging such as chromosome and spindle anomalies (Saito et al., 1993), cortical granule exocytosis (Díaz and Esponda, 2004), lower fertilization rates (Goud et al., 1999), zona pellucida (ZP) hardening (Díaz and Esponda, 2004), and abnormal or retarded development of embryos/fetuses (Kosubek et al., 2010; Tarín et al., 1999). The exact molecular mechanism of reduced oocyte competence due to postovulatory aging is not fully understood yet. However, major factors that mediate time-dependent degradation in oocyte competence include oxidative stress (Lord and Aitken, 2013), chromosomal abnormalities (Mailhes et al., 1998), modification of poly(A) tails (deadenylation) of genes responsible for maternal effects (Dankert et al., 2014) as well as epigenetic alteration (Huang et al., 2007; Trapphoff et al., 2016). Therefore, it is imperative to better understand the various molecular pathways responsible for the postovulatory aging process to devise effective strategies to delay oocyte aging and increase the time required for performing normal reproductive functions (Lord and Aitken, 2013; Wang et al., 2017).

Oxidative stress is strongly associated with a declined in oocyte quality because it significantly reduces the glutathione (GSH) contents and enhances the accumulation of reactive oxygen species (ROS). The ROS such as superoxide anions (O^{-2}), hydroxyl radicals ($\text{OH}^{\cdot}$), and hydrogen peroxide (H_2O_2) are released during normal metabolic (intermediate steps of oxygen reduction) processes (Arain et al., 2018;

Kim et al., 2019). The mitochondrion is the major cell organelle responsible for ROS production (Inoue et al., 2003; Liu et al., 2002). A dynamic balance is required between ROS production and antioxidant enzymes to ensure proper cellular homeostasis including cell proliferation, host defense, signal transduction, and gene expression (Droge, 2002). The oxidative stress disrupts this delicate balance through the overproduction of ROS which cannot be scavenged by the antioxidant defense system. Excessive load of ROS results in pro-apoptotic signaling subsequently leading to the activation of cell apoptosis (Redza-Dutordoir and Averill-Bates, 2016). Post-ovulatory aging is associated with excessive accumulation of ROS leading to oxidative stress, which exposes aged oocytes to the risk of apoptosis or improper embryonic development after fertilization (Liang et al., 2017; Lord and Aitken, 2013). The mitochondria as the major “energy generators” have a significant role in regulating proper function and survival of oocytes, following ovulation. However, being a chief source of ROS production, mitochondria are susceptible to ROS-induced damage (Ramalho-Santos et al., 2009), which results in decreased ATP synthesis, altered mitochondrial membrane potential, oxidative stress and early onset of apoptosis (Babayev et al., 2016; Wang et al., 2016). The excessive accumulation of ROS can affect the permeability of mitochondrial membranes to open mPTP and promote the flow of calcium ions (Zheng et al., 2004) which subsequently induces the release of cytochrome C and caspase 3 activation leading to apoptosis (Suzuki et al., 2001; Verhagen et al., 2000).

The apoptotic activation is mainly induced by the glutathione efflux (Fico et al., 2008), and leads to several morphological changes including cell shrinkage and progressive DNA and cell membrane damage, ultimately leading to cell death (Papaliagkas et al., 2007). Therefore, one of the major challenges in reproductive embryology is to prevent oocytes' degeneration to maintain their developmental competences (Hoffmann et al., 2001). To avoid oxidative damage by maintaining a robust antioxidant defense system in the oocyte, supplementation of exogenous antioxidants is envisaged as the most effective strategy. Resveratrol (RES) is a natural polyphenolic compound with excellent antioxidant and free radical scavenging capacity. It is associated with reduced ROS accumulation, scavenge superoxide radicals, inhibit lipid peroxidation, and regulate the expression of antioxidant cofactors and enzymes (Pervaiz and Holme, 2009). Moreover, RES has shown to prevent mitochondrial damage in cardio-myocytes through the up-regulation of the deacetylation of apoptotic proteins. Studies have revealed that treatment of porcine oocytes with 2 μ M Resveratrol significantly reduced the levels of intracellular ROS while increasing GSH contents during *in vitro* maturation (Kim et al., 2018; Kwak et al., 2012).

Despite the exceptional antioxidant ability of RES, no study is available on the potential effects of RES on aged oocytes. Moreover, the mechanism of action of RES to protect oocytes against oxidative damage for optimal *in vitro* fertilization is still unclear. Therefore, this study was conducted to evaluate the effect of RES on pig oocytes during aging and to provide mechanistic insights regarding its potential protection of oocytes against ROS attack. We hypothesized that the resveratrol can maintain the quality of oocytes by scavenging free radicals produced during oocyte aging.

Materials And Methods

Oocytes collection and maturation

Pig ovaries were collected from a local slaughterhouse and immediately placed in sterile physiological saline at 37°C and delivered to the laboratory within 2 h. The ovaries were washed twice in the sterile condition under pre-warmed PBS at 37°C, and the cumulus-oocyte complexes (COCs) having 3 to 6 mm diameter follicles were collected by aspiration. Under the stereo-microscope, the COC containing ≥ 3 layers of cumulus cells were selected for the experiment and randomly divided into 4 treatment groups by adding 0, 1, 2, and 4 $\mu\text{mol/L}$ RES and transferred to TCM-199 culture medium. The cells were cultured in a CO₂ incubator at 38.5°C with 5% CO₂ gas phase and saturated humidity for 44 h, and the optimum concentration of resveratrol was screened out. According to the experiment, the culture was carried out for 44 h, and after aging, continued for further 24 and 48 h. As 2 $\mu\text{mol/L}$ RES was found as an optimum level for porcine oocytes, so further treatment was carried out by using only this level.

In vitro aging and RES treatment of porcine oocytes

Resveratrol was diluted with dimethyl sulfoxide (DMSO) to a 10 mmol/L concentrated stock solution and stored at -20 °C. The resveratrol concentrated stock solution was added to the TCM199 culture solution to adjust the concentration to 2 $\mu\text{mol/L}$. The COCs were cultured for 44 h as a control group, aged 24 and 48 h as *in vitro* aging groups, placed in a mature medium containing 2 $\mu\text{mol/L}$ RES, and then aged for 24 and 48 h, as a RES treatment group. The first polar body (pbl) of the oocyte was observed by microscopy and the change of the perivitelline space of the oocyte was detected.

RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

Fresh COCs were collected and washed twice with DPBS solution and stored at -80 °C until the RNA was extracted. The total RNA was isolated from the oocytes by using the Trizol RNA extraction kit (Takara, Bio Inc., Tokyo, Japan) according to the manufacturer's instructions. The RNA thus extracted was quantified using nano-Drop and stored at -80 °C until further use. After that, cDNA was synthesized from 2 μg of total RNA using the Revert Aid™ First Strand cDNA synthesis kit (Fermentas, St. Leon Rot, Germany) according to the manufacturer's protocol.

The levels of relevant mRNA, products of antioxidant-related genes, and apoptosis-related genes were determined by qRT-PCR using a One-Step SYBR Prime Script RT-PCR kit (TaKaRa Bio Inc., Tokyo, Japan) in a Light Cycler instrument (Roche, Mannheim, Germany). The levels of accumulated fluorescence were analyzed using the second derivative method after the melting-curve analysis and the expression levels of the target genes were normalized using GAPDH as a control. The primer pairs for *GAPDH*, *Caspase-3*, *Bcl-2*, *Bax*, *CAT*, *GSH-PX*, and *SOD1* were synthesized by Nanjing Qingke Biotechnology Co., Ltd. The details of primers are presented in Table1. The results of quantitative real-time PCR (qPCR) were analyzed using

the $2^{-\Delta\Delta C_t}$ method.

Oocyte reactive oxygen species (ROS) assay

The intracellular ROS level was determined by 2,7 dichlorofluorescein (DCF) fluorescence assay as previously described (Gupta et al., 2010). Briefly, oocytes were incubated with 10 mM DCF for 20 min at 37°C followed by washing thrice in TCM199. It took 1 h or less to reflect the fluorescence intensity of ROS (Excitation wavelength: 450- 490 nm and Emission wavelength: 515-565 nm). The results were immediately observed and photographed using an epifluorescence microscope. The mean values of fluorescent oocytes were further processed by using Image J 6.0 software. Background fluorescent values were subtracted from the final values before analyzing for the statistical difference between the groups. The experiment was replicated 3 times with 20–25 oocytes each time.

Determination of intracellular GSH contents

The contents of total glutathione (T-GSH) were examined through an enzymatic method by using a GSH/GSSG assay kit (Beyotime Institute of Biotechnology, China) according to the manufacturer's instructions. A total of 50 oocytes from each group were mixed with 30 µl of protein scavenger M solution supplied with the kit and vortexed thoroughly for 5 min, then the mixture was frozen at liquid nitrogen for 2 min and thawed in a water bath at 37°C repeatedly for 3 times. Then the mixture was centrifuged at 10,000 rpm for 10 min at 4°C and placed on ice for 5 min using a 96-well plate, the samples or standard in the sequence were added and mixed accordingly. Immediately absorbance was observed at 405nm with a microplate reader, for 25 min, with a reading recorded for every 5 min. A standard curve was developed for the determination of the GSH content of each sample. The GSH concentration was calculated by dividing the total concentration of each sample by the total number of oocytes present in the sample (pmol/oocyte).

Mitochondrial membrane potential assay

The mitochondrial membrane potential assay kit with JC-1 (Beyotime Institute of Biotechnology, China) was used for the evaluation of mitochondrial membrane potential of the oocytes. The oocytes were exposed to 10µl MJC-1 in 100µL working solution at 38.5°C in 5% CO₂ for 20 min. To remove surface fluorescence, oocytes were treated with washing buffer and then mounted on glass slides using D-PBS for microscopy. Laser excitation was set at 488nm for green and 525nm for red fluorescence, respectively. The fluorescence microscope (Olympus, Tokyo, Japan) with the same scan settings for each sample was used to measure the fluorescence intensity of each oocyte. Image J software was used to analyze the normal fluorescence pixel intensities of each oocyte. The ratio of green to red fluorescence pixels was used to analyze mitochondrial membrane potential.

Statistical analysis

Each treatment group had a minimum of 3 replicates and the data thus obtained were analyzed by the Statistical Package for Social Sciences (SPSS) software (version 18.0) by using One-Way analysis of variance (ANOVA). The treatment means were compared by the least significant difference (LSD) test at 1% and 5% probability levels. The P-value of <0.05 was considered as a significant difference while, P <0.01 was considered as highly significant whereas, P <0.001 was considered as extremely significant.

Results

Effect of RES on aged oocytes

The effect of treatment of RES (0, 1, 2, and 4 $\mu\text{mol/L}$) on the proliferation of cumulus cells was observed after 44 h under a microscope. The results revealed that cumulus diffusion in 1 and 2 $\mu\text{mol/L}$ RES treated group was better than the control and 4 $\mu\text{mol/L}$ RES treated groups (Fig. 1). Moreover, we observed a significant difference in the first polar body extrusion rate (pbl) between the 2 $\mu\text{mol/L}$ RES and the control group. However, no significant difference in first polar body extrusion rate (pbl) in 1 and 4 $\mu\text{mol/L}$ RES treated groups was observed as compared to control.

Effect of RES on perivitelline space of aged oocytes

Treatment with resveratrol significantly increased the perivitelline spaces in oocytes aged for 24h and 48h group compared with the control group and resveratrol treated groups (Fig 2). Furthermore, oocytes treated with RES showed a significant decrease in perivitelline spaces after 24h and 48h of aging. However, differences were non-significant as compared to the control group (Fig 2B).

Effect of RES on the expression of apoptotic genes in aged oocytes

Our results revealed that the relative mRNA expression of *caspase-3* and *Bax* in aged oocyte groups was significantly higher than the control group; while the expression of *Bcl-2* was significantly lower (Figure 3). However, the expression of *caspase-3* and *Bax* in 24 and 48 h RES treated groups was significantly decreased than untreated aged groups. Moreover, the expression of *Bcl-2* in the RES treated group was significantly lower than the control group but significantly higher than untreated aged groups.

Effect of RES on the expression of antioxidant enzymes

Our results showed that RES could significantly increase the relative expression of different antioxidant genes in aged oocytes. In this experiment, the expression of CAT and SOD-1 gene in RES treated groups was significantly higher than untreated aged groups, although the expression of CAT and SOD-1 gene was lower than that of the control group (Fig 4). Likewise, the expression of the GSH-Px gene in RES treated oocytes (aged for 24 and 48h) was significantly increased, but there was no significant difference as compared with the control group.

Effect of RES on GSH enzyme activity of aged oocytes

Results showed that the activity of GSH was significantly decreased in 24 and 48 h aged groups whereas RES treatment significantly improved the GSH activity in 24 and 48 h aged groups (Fig 5). However, this difference was non-significant as compared to the control group.

Effect of RES on ROS in aged oocytes

Our results revealed that ROS levels significantly increased in 24 and 48 h aged groups compared with control, while the oocytes treated with resveratrol (both at 24 and 48 h) showed significantly higher ROS levels than those of the control group, but significantly lower than those of untreated aged groups (Fig 6).

Effect of RES on mitochondrial membrane potential of oocytes

To evaluate the mitochondrial membrane potential, we analyzed the ratio of green/red fluorescence. Compared to the oocytes treated with RES, the oocytes aged for 24 and 48h showed high ratios while fresh oocytes displayed the lowest values. These results suggested that RES possess a significant tendency of keeping mitochondrial membrane potential in its normal state during the oocyte aging process (Fig: 7 and 8).

Discussion

Effect of RES on GSH enzyme activity, antioxidant genes, and ROS

Our study revealed that under *in vitro* conditions, RES could significantly increase the GSH contents in 24 h aged oocytes as compared with the control group ($P < 0.001$). Although, RES at 48 h of aging significantly lowered the GSH contents than the control group ($P < 0.05$), but still it was significantly higher than its untreated counterparts. Our findings are in agreement with earlier studies that RES can induce progesterone secretion in bovine oocytes and reduce intracellular ROS levels while increasing GSH contents (Wang et al., 2014). Furthermore, (Boerjan and De Boer, 1990) also reported that *in vivo* aging of mouse oocytes was associated with the decreased cytoplasmic concentration of total glutathione (GSH plus GSSG) contents suggesting a concomitant decline in GSH/GSSG ratio. The GSH maintains the dynamic balance of the cell membranes by protecting the proteins from oxidative damage. During the *in vitro* maturation of bovine oocytes, the addition of cysteamine increases GSH content and blastocyst rate (Izumi et al., 2013) indicating its potential to regulate GSH to improve *in vitro* oocyte maturation (Matos et al., 1995). Similarly, our study revealed the potential of RES to significantly increase the GSH contents in aged oocytes with remarkable effects on the 48h aging group indicating alleviation of oxidative damage to facilitate proper oocytes development.

The apoptosis is a very complex process being affected by many factors such as inflammatory response and oxidative stress etc. The ROS induced oxidative stress can adversely affect a variety of biological processes including sperm capacitation, ovulation, and corpus luteum production, and can also trigger oocyte apoptosis. The accumulation of ROS has serious manifestation regarding the quality and aging of oocytes. Also, uncontrolled and excessive production of free radicals may harm DNA, proteins, and lipids,

which can severely compromise cell health and contribute to disease development (Birben et al., 2012; Mccord, 2000; Therond, 2006). Our results showed that RES significantly reduced the ROS level in aged oocytes (24 and 48 h) which is consistent with earlier findings (Liang et al., 2018). Outcomes in this study indicate the potential of RES to inhibit oocyte aging by reducing ROS levels owing to its reported antioxidant (De?La?Lastra and Villegas, 2007), anti-apoptosis (Jong-Wook et al., 2001), and anti-aging (Liang et al., 2018) activities. Moreover, RES has also shown to reduce lipid peroxidation by eliminating free radicals, and thus achieve the effect of protecting cells (Wenzel et al., 2005).

The primary antioxidant enzymes are SOD, catalase (CAT), and glutathione peroxidase (GSH-Px). Moreover, O_2 is converted by SOD to H_2O_2 , which is decomposed to water and oxygen by CAT, preventing the production of hydroxyl radicals. Additionally, GSH-Px converts peroxides and hydroxyl radicals into nontoxic forms by oxidizing reduced glutathione (GSH) into glutathione disulfide and triggers reduction to GSH by glutathione reductase (Wu, 2013). When Cu^{+2} or Fe^{+2} are available, H_2O_2 reacts with these ions to form unstable hydroxyl radicals. Previous studies have shown that RES can increase the activity of various antioxidant enzymes such as CAT, SOD, and GSH-Px in cells (Meng et al., 2018; Wu et al., 2015). When low-dose of RES was used to treat cardio-myocytes, the catalytic activity of CAT and SOD increased significantly with no effect on glutathione activity. Moreover, SOD can reduce intracellular superoxide levels and potentially resist against cell apoptosis, membrane permeability changes, and mitochondrial dysfunction (Movahed et al., 2012).

Previous studies in our laboratory have demonstrated that RES can eliminate mitochondrial injury while relieving oocyte aging and improving the expression of sirtuin-1 (sirt1) and quality of aged porcine oocytes (Ma et al., 2005b). Similarly, in the present study RES treatment increased the expression of GSH-Px gene in 24 h aged oocytes as compared to the control and untreated aged groups ($P < 0.01$). However, expression of CAT and SOD1 genes was lower than that of the control group ($P < 0.05$), but still higher than their untreated counterparts. Moreover, RES significantly increased the expression CAT, SOD1, and GSH Px in 48h aged group than the untreated aging group. Our findings revealed that RES can effectively mediate oxidative stress induced by the aging oocytes during *in vitro* culture through increasing the antioxidant enzyme activity. Moreover, our study provides mechanistic insights into the potential antioxidant effects of RES to alleviate the adverse effects of oocyte aging and regulation of aging in porcine oocytes.

Effect of resveratrol on perivitelline space in aged oocyte

RES can accelerate the formation of bovine oocyte polar bodies and increases the maturation rate of bovine oocytes (Wang et al., 2014). Progesterone causes elongation of the Mos poly-A tail via cytoplasmic polyadenylation, and this polyadenylation increases the rate of Mos translation leading to the accumulation of Mos protein (Gebauer et al., 1994). Mos protein is essentially required for the initiation of oocyte germinal vesicle breakdown (Yew et al., 1992). In our study, we observed an increase in cumulus spread after treatment of oocytes with different concentrations of RES (1 $\mu\text{mol/L}$ and 2 $\mu\text{mol/L}$) which is consistent with earlier findings as mentioned above. Furthermore, the first polar body

extrusion rate of the 2 $\mu\text{mol/L}$ RES group was also significantly higher than other groups, indicating the potential of RES to increase oocyte maturation rate (Fig 1B). During this study, we found that oocytes treated with RES showed a significant decrease in perivitelline spaces during 24h and 48h. However, differences were non-significant compared with the control group (Fig 2). A full expansion of cumulus cells is mandatory for the proper maturation of the oocyte. Moreover, feeding RES to young mice for a longer period has shown to enhance the number and quality of oocytes (Liu et al., 2013) which is consistent with our findings. Similarly, RES supplementation into the *in vitro* maturation medium improved the quality of bovine embryos and increased their resistance against oxidative damage during cryopreservation (Abdel-Wahab et al., 2012). The beneficial effects of RES might depend upon its ability to improve oocyte quality therefore, it can be concluded that RES (2 $\mu\text{mol/L}$) can inhibit the increase of perivitelline space of oocytes aged for 24h and 48h, indicating its ability to alleviate the adverse effects of oocyte aging by improving the quality which is necessary for its development during fertilization.

Effect of resveratrol on the expression of apoptotic genes of aged oocyte

Apoptosis is one of the exogenous (mediated by death receptors) and mitochondria guided endogenous pathways. Both of these pathways participate in the activation of certain members of the Caspase family to trigger apoptosis. The proteins involved in the mitochondria-mediated endogenous pathway are mainly the Bcl-2 family, which includes both anti-apoptotic and pro-apoptotic proteins. The anti-apoptotic proteins (Bcl-XL, Bcl-2, and Mcl-1) potentially inhibit the activation of the Caspase family and block the transduction of apoptotic signals while pro-apoptotic proteins (Bcl XS, Bak, Bax, and Bad) promote and initiate an apoptotic response. Caspase 3 acts as a key effector in the process of apoptosis and directly hydrolyzes specific substrates. It can damage cellular DNA and promote apoptosis (Wang et al., 2015). When there is apoptosis, Bax acts on the outer membrane mitochondrial of cell causing the release of mitochondrial cytochrome C (CytC) that activates Caspase 3 and triggers Caspase cascade. The nucleated cytoskeleton recombines and degrades cytoskeletal structure (Nutt et al., 2005). However, Bcl 2 inhibits CytC and Caspase causing an anti-apoptotic effect. Previous studies have shown that activated Caspase 3 promotes the further release of Ca^{+2} which may lead to oocyte fragmentation and accelerates aging (Ma et al., 2005a).

Furthermore, oocyte aging can lead to a series of morphological changes, such as increased perivitelline space, loose chromosome distribution, premature separation of sister chromatids, spindle abnormalities, cortical granule exocytosis, zona pellucida sclerosis, and cytoplasmic fragmentation (Zhang et al., 2017). Although many molecules, such as Bcl-2 (Hsu and Hsueh, 2000), Bcl-X (Parborell and F., 2002), Bax (Tilly et al., 1995) and caspases (Flaws et al., 1995; L. and K., 1998), have been implicated in the regulation of ovarian apoptosis. Bax has been confirmed to be involved in follicles. The recruitment of Bax, which is knocked out, limits the expression of Bax and leads to an increase in the number of ovarian oocytes (Pascuali et al., 2015). Deacetylated Sirt1 and Sirt 3 inhibit the apoptotic pathway by affecting mPTP pores of the mitochondrial membrane (Tong et al., 2017). Furthermore, down-regulation of caspase 3 up-regulates the expression of the anti-apoptotic protein such as Bcl-2 that subsequently inhibits apoptosis. We observed lower expression of Bax and Caspase 3 in RES treated aged oocytes in the present study.

Moreover, relative mRNA expression of Bcl-2 was significantly lower in the RES treated group than the control but was significantly higher as compared to untreated aged groups. Our findings suggested that RES can effectively inhibit mitochondrial apoptotic pathway through down-regulation of the Bax and Caspase 3 while up-regulating Bcl 2 in aged oocytes, which subsequently alleviates adverse effects of aging in porcine oocytes.

Mitochondrial membrane potential

Mitochondria are responsible to maintain cellular metabolic functions and its physiological efficiency can be assessed by examining the mitochondrial membrane potential state. In this regard, fluorescence probes like JC-1 tend to accumulate in the mitochondrial matrix (by forming IJ-aggregates) and produce red excitation light when the mitochondrial membrane potential is maintained high. However, if mitochondrial membrane potential is maintained low, JC-1 cannot accumulate in the mitochondrial matrix, hence forms monomers and generates green excitation light. During the present study, inclusion of RES in the oocyte culture medium maintained the mitochondrial membrane potential of aged oocytes in a state consistent with non-aged counterparts. Furthermore, RES significantly increased the expression of BCL-2 in 24 and 48h aged oocytes which subsequently can regulate the mitochondrial apoptotic pathway by controlling the permeability of the outer mitochondrial membrane (Brunelle and Letai, 2009). Consequently, the RES regulated follicular development primarily through increased expression of mitochondrial related *Bcl-2* gene which might have played its role in the maintenance of mitochondrial membrane potential in its normal position in 24 and 48h aged oocytes. However, further *in vivo* studies are required to elucidate its potential mechanism of action.

Our study concluded that RES can effectively alleviate the adverse effects of oocyte aging by increasing the expression of antioxidant enzymes while decreasing the ROS level. Moreover, the RES treatment resisted against the decline in mitochondrial membrane potential in aged oocytes. Additionally, RES showed potent anti-apoptotic effects by potentially up-regulating the *Bcl 2* while down-regulating the mRNA expression of *Bax* and *Caspase 3*.

Declarations

Ethics approval and consent to participate

This animal study (short title: Resveratrol effect on oocyte aging) was carried out in strict accordance with the recommendation of the National Ethical commission (Nanjing, Jiangsu P.R. China). All experiments and procedures compile with the guideline and were approved by the local ethical committee of the Nanjing Agricultural University (Jiangsu Province P.R. China) with respect to animal experimentation and care of animal under study.

Consent for publication

Not applicable.

Availability of data and materials

Corresponding author will provide the data on request.

Competing Interest

The authors declare that they have no competing interests.

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Author Contributions

B. A., conceived the idea and perform experiment and wrote the draft of the manuscript. D. Y., perform statistical analysis of the data. R. R., supervise the experiment and improve the final draft of the manuscript.

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Tables

Table1. The details of RT-PCR primer pairs used for analysis of pro-apoptotic genes

Target gene	Sequence	Accession Number	Size (bp)
<i>GAPDH</i>	F: 5'-GTCGGTTGTGGATCTGACCT-3'	NM_001206359	207
	R: 5'-TTGACGAAGTGGTCGTTGAG-3'		
<i>Caspase-3</i>	F: 5'-CGTGCTTCTAAGCCATGGTG-3'	NM_214131	186
	R: 5'-GTCCCACTGTCCGTCTCAAT-3'		
<i>Bcl-2</i>	F:5'-AGGGCATTTCAGTGACCTGAC-3'	NM_214285	193
	R: 5'-CGATCCGACTCACCAATACC-3'		
<i>Bax</i>	F:5'-TGCCTCAGGATGCATCTACC-3'	XM_003127290	199
	R: 5'-AAGTAGAAAAGCGCGACCAC-3'		
<i>CAT</i>	F-5'-AACTGTCCCTTCCGTGCTA-3'	XM_021081498	195
	R-5'-CCTGGGTGACATTATCTTCG-3'		
<i>GSH-PX</i>	F-5'-CAAGTCCTTCTACGACCTCA-3'	AJ010340	210
	R-5'-GAAGCCAAGAACCACCAG-3'		
<i>SOD1</i>	F-5'-ACCTGGGCAATGTGACTG-3'	NM_001190422	197
	R-5'-TCCAGCATTTCCCGTCT-3'		

Figures

Figure 1

Effect of RES treatment on cumulus spread and first polar body (pbl) extrusion rate in porcine oocytes A. The degree of cumulus spread in oocytes; B. First polar body extrusion rate (pbl) of oocytes (** $P < 0.001$).

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Figure 2

The effect of RES treatment on the perivitaline space of oocyte; A: The perivitaline space of the oocyte under the microscope, scale bar=180 μm; B: Increased perivitaline space after oocyte treatment.

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Figure 3

Effects of RES on the relative expression of Caspase-3, Bcl-2 and Bax genes in porcine oocytes at different aging time (* indicates $P < 0.05$; ** indicates $P < 0.01$ and ***)

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Figure 4

Relative mRNA expression of antioxidant enzymes in aged oocyte treated with RES, * means significant difference ($P<0.05$).

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Relative mRNA expression of antioxidant enzymes in aged oocyte treated with RES, * means significant difference ($P<0.05$).

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Relative mRNA expression of antioxidant enzymes in aged oocyte treated with RES, * means significant difference ($P<0.05$).

Figure 5

The effect of RES on glutathione (GSH) activity in aging oocytes

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Figure 6

A: The effect of RES on ROS levels in aging oocytes, bar=280 μ m, B: Relative ROS fluorescence intensity* means significant difference ($P<0.05$).

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Figure 7

Mitochondrial membrane potentials in porcine oocyte cultured in the absence or presence of Resveratrol. (A) control (B) aged 24h (C) aged 24h+RES (D) aged 48h (E) aged 48h+RES. Membrane potential was calculated as the ratio of red fluorescence, which corresponds to activated mitochondria (J-aggregates), to green fluorescence, which corresponds to less-activated mitochondria (J-monomers). Fluorescence emitted from each oocyte was analyzed using the Image J software.

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Figure 8

Effect of RES on mitochondrial membrane potential in aged oocytes (* $P < 0.05$ Values represents the means $\pm$ SEM from at least three separate experiments).

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