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Protective effects of quercetin against H_2O_2 induced KGN cells injury

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Author contributions

Hong JN and Ma XL conceived and designed the experiments. Zhang N and Zhang HX carried out the experiment. Hong JN and Zhang N contributed to reagents, materials, and analysis tools. Hong JN, Zhang N and Ma XL wrote the paper. All authors read and approval the final manuscript.

Competing interests

The authors declare no conflicts of interest.

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Abbreviations

H_2O_2, hydrogen peroxide; CCK-8, cell counting kit-8; LDH, lactate dehydrogenase; ROS, reactive oxygen species; GSH, glutathione; AO, acridine orange; EB, ethidium bromide; KGN, human ovarian granulosa tumor cell line; TEM, transmission electron microscope; GCs, granulosa cells; DMEM, Dulbecco's modified eagle medium; MMP, mitochondrial membrane potential.

Citation

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Abstract

Background: Oxidative stress is one of the key contributors to cellular senescence and ovarian aging. Quercetin has a variety of physiological activities such as antioxidant. Given that hydrogen peroxide can cause oxidative damage to cells, the present study is designed to verify the protective effect of quercetin on human ovarian granulosa tumor cell line under oxidative stress. **Methods:** Cell counting kit-8 and lactate dehydrogenase assays examined cell viability and toxicity. Flow cytometry detected reactive oxygen species accumulation. Glutathione level was measured to analyze the oxidation resistance. Cell apoptosis was evaluated by Hoechst 33258 staining, acridine orange/Ethidium Bromide staining and western blot. The mitochondrial structure was observed under a transmission electron microscope. Mitochondrial membrane integrity was detected by JC-1 staining and western blot. **Results:** Hydrogen peroxide could induce cell injury, promote reactive oxygen species accumulation, and lead to glutathione depletion. hydrogen peroxide also resulted in mitochondrial morphological damage and depolarization, which activate caspase3/9 subsequently. However, quercetin could mitigate these damages. **Conclusions:** Present study found that hydrogen peroxide induced oxidative stress and mitochondrial apoptosis of human ovarian granulosa tumor cell line cells, which could be attenuated by quercetin.

Keywords: oxidative stress; granulosa cells; apoptosis; mitochondria; quercetin

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Highlights

Increased apoptosis of granulosa cells is an important pathological process in ovarian aging. In this study, a series of experiments was conducted to investigate the protective effect of quercetin on granulosa cells under oxidative stress, and it was confirmed that this effect may be related to maintaining mitochondrial homeostasis.

Medical history of objective

Epimedium was first recorded in the "*Shennong Materia Medica Classic*" (during the Eastern Han Dynasty) and is a perennial herbaceous plant in the Berberidaceae family. Epimedium has important applications in the clinical treatment of perimenopausal syndrome, premature ovarian failure, polycystic ovary syndrome, threatened abortion, etc. Quercetin derived from Epimedium, recent studies have found that the antioxidant stress effect of Quercetin has unique advantages in treating ovarian aging.

Background

On account of postponing pregnancy resulted from economic and cultural changes over the past few decades, the aging problem of the female reproductive system and the ensuing age-related infertility have become more and more common [1, 2]. Ovary is one of the organs vulnerable to aging-associated dysfunction [3]. Women typically experience a significant decline in ovarian function after the age of 35, which mainly manifested as menstrual disorders, follicle quality decline and hormone levels changes [4]. Ovarian aging is irreversible and the effect of current therapeutic interventions is very limited [5, 6]. However, there is still a lack of sufficient understanding of ovarian aging [7, 8].

A number of theories have been put forward to explain the potential mechanism of ovarian aging, the most classic of which is the free radical theory, which maintains that oxidative stress caused by the increase of intracellular reactive oxygen species (ROS) is the main reason for mammalian cell senescence [9, 10]. In addition, many studies have shown that oxidative stress initiates other processes that promote ovarian aging, such as inflammation, mitochondrial dysfunction, mitochondrial depolarization, endogenous apoptosis and so on [11]. Based on the above theory, oxidative stress may be a critical mechanism leading to ovarian aging, and finding effective natural antioxidants may be a novel and safe way to prevent ovarian aging [12, 13].

Granulosa cells (GCs) plays an important role in follicle development, including follicle initiation, recruitment, selection, domination, ovulation and luteinization, which determine the fate of follicles partly [14]. GCs apoptosis can induce follicular atresia, which is the main cause of follicle loss and is involved in ovarian aging [15, 16]. A growing number of evidences demonstrate that oxidative stress is a key trigger of GCs apoptosis and follicles atresia [17, 18]. There are two main pathways through which oxidative stress induce GCs apoptosis. One is exogenous pathway, the other is endogenous pathway, also known as mitochondrial apoptosis [19]. Oxidative stress leads to mitochondrial depolarization, mitochondrial membrane permeability increases, and cytochrome C is released from mitochondria to cytoplasm. Cytochrome C activates capase-9/3 pathway, and capase-3 as effector plays a part in the executive phase of apoptosis [20, 21].

Natural products-originated antioxidants outperform synthetic antioxidants in the anti-oxidative stress capacity, safety and acceptability [22]. Quercetin is a bioactive flavonoid ubiquitously present in medicinal plants and foods. It has a variety of biological activities, such as anti-oxidation, anti-apoptosis, promoting mitochondrial synthesis and so on. In recent years, more and more attention has been paid to the application of quercetin in the treatment of ovarian aging, but there is limited research on the relevant mechanisms [23, 24].

In the present study, the hypothesis that quercetin mitigated GC damage induced by hydrogen peroxide (H_2O_2) was tested and the relative mechanism was explored. The results confirmed that H_2O_2 caused mitochondrial injury and mitochondrial apoptosis. Nevertheless, these effects of H_2O_2 could be partly eased by quercetin.

Materials and methods

Materials

Standard quercetin with purity > 98 (Cat No. 117-39-5) was purchased from the National Institute for the Control of Pharmaceutical and Biological Produces (Beijing, China). Cell Counting Kit-8 (CCK-8) used in the experiment was manufactured by Apexbio (Boston, MA, USA). Hoechst 33258, acridine orange (AO)/ethidium bromide (EB) and 2',7'-dichlorofluorescin diacetate were obtained from Sigma Chemical Co., Ltd. (St Louis, MO, USA). lactate dehydrogenase (LDH) assay kit, JC-1 kit and bicinchoninic acid assay (BCA protein kit assay) were bought from Beyotime Institute of Biotechnology (Nanjing, China). Glutathione (GSH) assay kit was purchased from Nanjing Jiancheng Bioengineering Research Institute (Nanjing, China). Fetal bovine serum, Dulbecco's modified eagle medium (DMEM) and penicillin/streptomycin were supplied by Gibco (Carlsbad, CA, USA). All the primary antibodies were purchased from Proteintech (Wuhan, China). Enhanced chemiluminescence and Trizol reagents were manufactured by Thermo Fisher Scientific (Sunnyvale, CA, USA). H_2O_2 was purchased from Boster (Wuhan, China).

Cell culture

Human ovarian granulosa tumor cell line (KGN) supplied by Peking Union Medical College, and cultured in DMEM containing 10% fetal bovine serum and 1% penicillin-streptomycin. The cells were divided into several groups receiving different treatments. Groups includes: control group (DMEM), model group (250 μM H_2O_2), low-concentration quercetin group (250 μM H_2O_2 and 2 μM quercetin), medium-concentration quercetin group (250 μM H_2O_2 and 5 μM quercetin) and high-concentration quercetin group (250 μM H_2O_2 and 10 μM quercetin). KGN were treated with quercetin (2, 5, 10 μM) for 24 h, and then exposed to H_2O_2 (250 μM) for 2 h.

CCK-8 assay and LDH assay

KGN cells were seeded in a density of 50,000 cells/mL in 96-well plates and cultured for 24 h. Then the cells were pre-treated with quercetin for 24 h and H_2O_2 for 2 h successively. After that, cell cultured supernatant were exchanged with mixture of CCK8 and DMEM (1:9), and then placed cells in the incubator for 1 h. The absorbance was detected by a microplate reader (Thermo) at 450 nm. LDH can be released from the cell in case of damaged cell plasma membranes. After the treatment of cells, the supernatant was collected and LDH-related reagents were added, then detected the absorbance (the wavelength is 450 nm).

Cellular level measurement for ROS

KGN cells were seeded in a density of 50,000 cells/mL in 6-well plates and cultured for 24 h. After the treatment of cells, the cells were stained with 10 μM 2',7'-DCFDA. Then cells were collected and the average fluorescence intensity of ROS in cells was measured by flow cytometry (BD).

Measurement of intracellular reduced GSH levels

KGN cells were seeded in a density of 50,000 cells/mL in 6-well plates and cultured for 24 h. Radio immunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology) was added into cells treated with H_2O_2 or quercetin. Supernatant was collected after centrifugation, and then tested with a commercial kit (Nanjing Jiancheng Bioengineering Institute).

Morphology of mitochondria observed under a transmission electron microscope (TEM)

KGN cells were seeded in a density of 50,000 cells/mL in 6-well plates and cultured for 24 h. After the treatment of cells, the cells were digested with trypsin and fixed with glutaraldehyde after centrifugation. Subsequently, the sample was dehydrated in absolute ethanol, embedded in Epon 812 and cut into ultra-thin section. Finally, the cells were observed under transmission electron microscope (Hitachi HT7700).

Mitochondrial membrane potential (MMP) analysis (by JC-1 staining)

KGN cells were seeded in a density of 50,000 cells/mL in 6-well plates and cultured for 24 h. The cells were stained with JC-1 dye after the treatment with H_2O_2 or quercetin, then observe the changes of mitochondrial membrane potential with fluorescence microscope (Olympus).

Hoechst 33258 staining and AO/EB staining

KGN cells were seeded in a density of 50,000 cells/mL in 24-well plates and cultured for 24 h. Then the cells were pre-treated with quercetin for 24 h and H_2O_2 for 2 h successively. Cells were fixed with 4% paraformaldehyde for 10min and drilled with 0.25% triton x-100, then added Hochest 33258 dye solution and incubated for 30 min. At last, the cells were observed under a fluorescence microscope (Olympus).

Mixed AO with EB in 1:1, then diluted with PBS (AO/EB: PBS = 1:20), added the diluent to the cells, leaved it at room temperature for 15 min, then observed cells under the fluorescence microscope (Olympus).

Western blot analysis

KGN cells were pre-treated with quercetin for 24 h and H_2O_2 for 2 h successively. Cells were scraped off after the treatment with drugs, added 1 mL Lysis Buffer, 10 μl phosphatase inhibitors, 1 μL protease inhibitors, 5 μL 100 mM Phenylmethanesulfonyl fluoride, centrifuged at 12,000 rmp for 20 min at 4 °C, then collected the supernatant and detected the concentration of each group. Proteins were loaded on SDS-PAGE after the denature and then transferred to the polyvinylidene fluoride membrane. After blocked with 5% nonfat powdered milk, the polyvinylidene fluoride membrane was incubated with primary antibody (caspase-3, Cat No. 19677-1-AP, dilution 1:1000; caspase-9, Cat No. 10380-1-AP, dilution 1:300; PARP, Cat No. 13371-1-AP, dilution 1:1000; bax, Cat No. 50599-2-lg, dilution 1:5000; bcl-2, Cat No. 68103-1-lg, dilution 1:5000) overnight at 4 °C and then incubated with peroxidase-conjugated secondary antibodies for 1 hour at room temperature. Finally, protein bands were visualized with enhanced chemiluminescence regents and quantified with Image J software (BIO-RAD).

Statistical analysis

Dates obtained from at least three independent experiments were expressed as mean ± SEM. All experiments were repeated at least three times, and fresh cell samples were used in repeated experiments. The data were compared between groups by one-way analysis of variance (ANOVA) and Tukey post-test. $P < 0.05$ indicated statistical significance.

Results

Quercetin prevented H_2O_2 from injuring KGN cells

Cells were treated with quercetin (2, 5, 10 μM) for 24 h first and then H_2O_2 (250 μM) for 2 h. Cell viabilities were detected by the CCK-8, and the results are summarized in Figure 1A. It was found that H_2O_2 could significantly decrease the cell viability compared with the control group ($P < 0.01$). However, the decline in cell viability caused by H_2O_2 was dose-dependently reversed by quercetin (2, 5, 10 μM). This finding was confirmed by the result of the cell toxicity test (Figure 1B), in which LDH leakage was used as the biomarker. Similarly, H_2O_2 markedly accelerated LDH release from human ovarian GCs ($P < 0.01$), but this process was arrested by quercetin in a dose-dependent manner. These results indicate that H_2O_2 can induce cytotoxicity and decreased cell viability, but quercetin can attenuate the effects.

Quercetin inhibited oxidative stress induced by H_2O_2

ROS production was assessed by DCFDA staining and flow cytometry (Figure 2A). It can be concluded from the figure that H_2O_2 promoted ROS generation while quercetin dose-dependently reduced the ROS level ($P < 0.01$). As one of the main natural antioxidants in cells, GSH can eliminate ROS in cells. As shown in Figure 2B, GSH was depleted but quercetin reverted it ($P < 0.05$). This indicates that oxidative stress induced by H_2O_2 can be partially arrested by quercetin.

Quercetin alleviated H_2O_2 induced KGN cells apoptosis via the caspase-9/3 pathway

To further investigate the role of apoptosis in KGN cells exposed to H_2O_2, cell apoptosis was detected by Hoechst 33258 staining. It was found that blue fluorescence was uniformly distributed in the nucleus of normal cells, while dense and hyperchromatic blue fluorescent granules were observed in a massive number of apoptotic cells. In Figure 3A, compared with the control group, cells treated with H_2O_2 had more dense and hyperchromatic blue fluorescent granules ($P < 0.001$). However, that difference dwindles under the effect of quercetin ($P < 0.001$).

Acridine orange/ethidium bromide (AO/EB) double staining

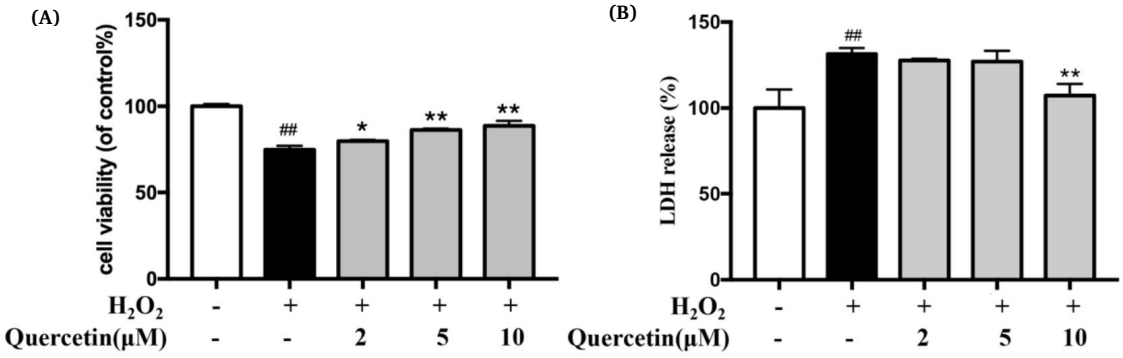

Figure 1 Quercetin prevents H_2O_2 from injuring KGN cells. After treating with quercetin (2, 5, 10 μM) for 24 h, KGN cells were exposed to H_2O_2 (250 μM) for 2 h. (A) Cell viability relative to untreated control cells was evaluated by CCK-8 assay. (B) LDH release of cells was assessed relative to untreated control cells. Data were presented as mean ± SEM from independent experiments performed in triplicate. Data were compared between groups by one-way ANOVA and Tukey post-test. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, relative to control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, relative to H_2O_2 group. KGN, human ovarian granulosa tumor cell line; H_2O_2, hydrogen peroxide; CCK-8, cell counting kit-8; LDH, lactate dehydrogenase; ANOVA, analysis of variance.

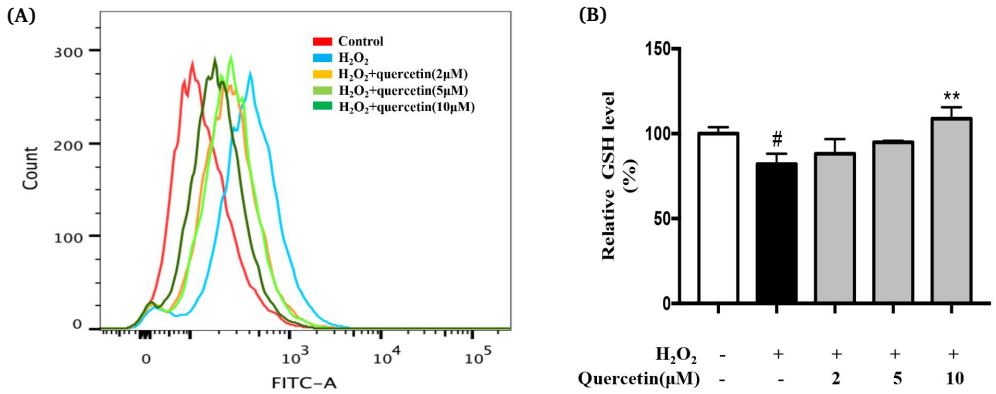

Figure 2 Quercetin inhibits oxidative stress induced by H_2O_2. KGN cells were treated with quercetin (2, 5, 10 μM) for 24 h before being exposed to H_2O_2 (250 μM) for 2 h. (A) After stained with 2',7'-DCFDA, the mean fluorescence intensity of ROS in cells was measured by flow cytometry. (B) Intracellular GSH levels were assessed compared with untreated control cells. Data were presented as mean ± SEM from independent experiments performed in triplicate. Data were compared between groups by one-way ANOVA and Tukey post-test. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, relative to control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, relative to H_2O_2 group. H_2O_2, hydrogen peroxide; KGN, human ovarian granulosa tumor cell line; GSH, glutathione; ROS, reactive oxygen species; ANOVA, analysis of variance.

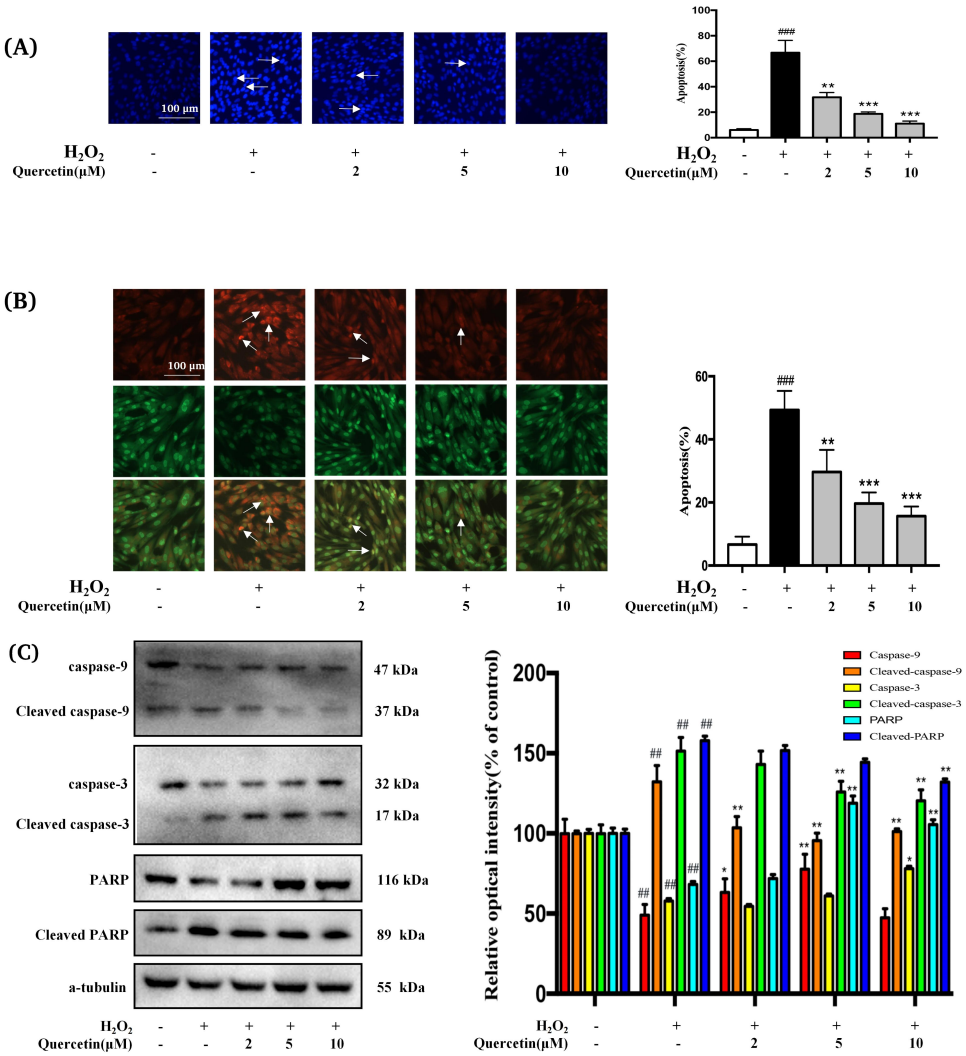

Figure 3 Quercetin alleviated H_2O_2 induced KGN cells apoptosis via the caspase-3/9 pathway. KGN cells were treated with quercetin (2, 5, 10 μM) for 24h before being exposed to H_2O_2 (250 μM) for 2 h. (A) Apoptotic cells were identified by staining with Hoechst 33258. (B) AO and EB double staining was used to identify the apoptotic cells. (C) Western blot was used to assess the protein expression of cleaved caspase-9, caspase-3, cleaved caspase-3, PARP and cleaved PARP. Data were presented as mean ± SEM from independent experiments performed in triplicate. Data were compared between groups by one-way ANOVA and Tukey post-test. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, relative to control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, relative to H_2O_2 group. H_2O_2, hydrogen peroxide; KGN, human ovarian granulosa tumor cell line; AO, acridine orange; EB, ethidium bromide; PARP, poly(ADP-ribose) polymerase; ANOVA, analysis of variance.

analysis further confirmed this result. AO can penetrate the integral cell membrane, embed into the nucleus DNA and show green fluorescence, while EB can only penetrate the impaired cell membrane (apoptotic cells) and display red fluorescence after binding with DNA. It can be seen from Figure 3B that H_2O_2 boosted co-localization of EB (red) and AO (green), while quercetin partly hampered this process. Taken above, H_2O_2 notably accelerated KGN cells apoptosis ($P < 0.001$), while quercetin effectively alleviated the apoptosis ($P < 0.001$). In order to investigate the mechanism underlying H_2O_2-induced apoptosis and the role of quercetin in this process, we further analyzed the caspase-9/3 pathway, which played a vital part in mitochondrial apoptosis. Western blot analysis results revealed that H_2O_2 encouraged caspase-9/3 pathway and PARP to cleave in KGN cells, while quercetin stopped the cleavage (Figure 3C). Hence, quercetin effectively protected KGN cells from H_2O_2 induced apoptosis through caspase-9/3 pathway.

Quercetin maintained mitochondrial homeostasis in the presence of H_2O_2

The mitochondrial structure of KGN cells was observed under a TEM. The results are shown in Figure 4A. It was revealed that H_2O_2 lead to mitochondrial swelling, vacuolization, and fragmentation, but quercetin deteriorated the changes. Mitochondrial depolarization is a marker event of early apoptosis, and changes in MMP alter mitochondrial membrane permeability, which results in the release of cytochrome C and initiates a cascade of apoptotic enzymes. MMP is commonly detected by JC-1 staining. In normal mitochondria, JC-1 forms a polymer in the mitochondrial matrix and emit red fluorescence, while when MMP decreases, JC-1appears as a monomer in the cytosol and emit green fluorescence. In Figure 4B, H_2O_2 promoted mitochondrial depolarization (green fluorescence), which, however, was relieved by quercetin in a dose-dependent manner. Bcl-2 family plays an important role in mitochondrial apoptosis, especially in maintaining MMP and inhibiting mitochondria related caspase apoptosis pathway. We further examined the changes of pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2, western blot indicated that H_2O_2 upregulated the expression of Bax and downregulated the expression of Bcl-2, but quercetin weakened these effects in a dose dependent manner (Figure 4C).

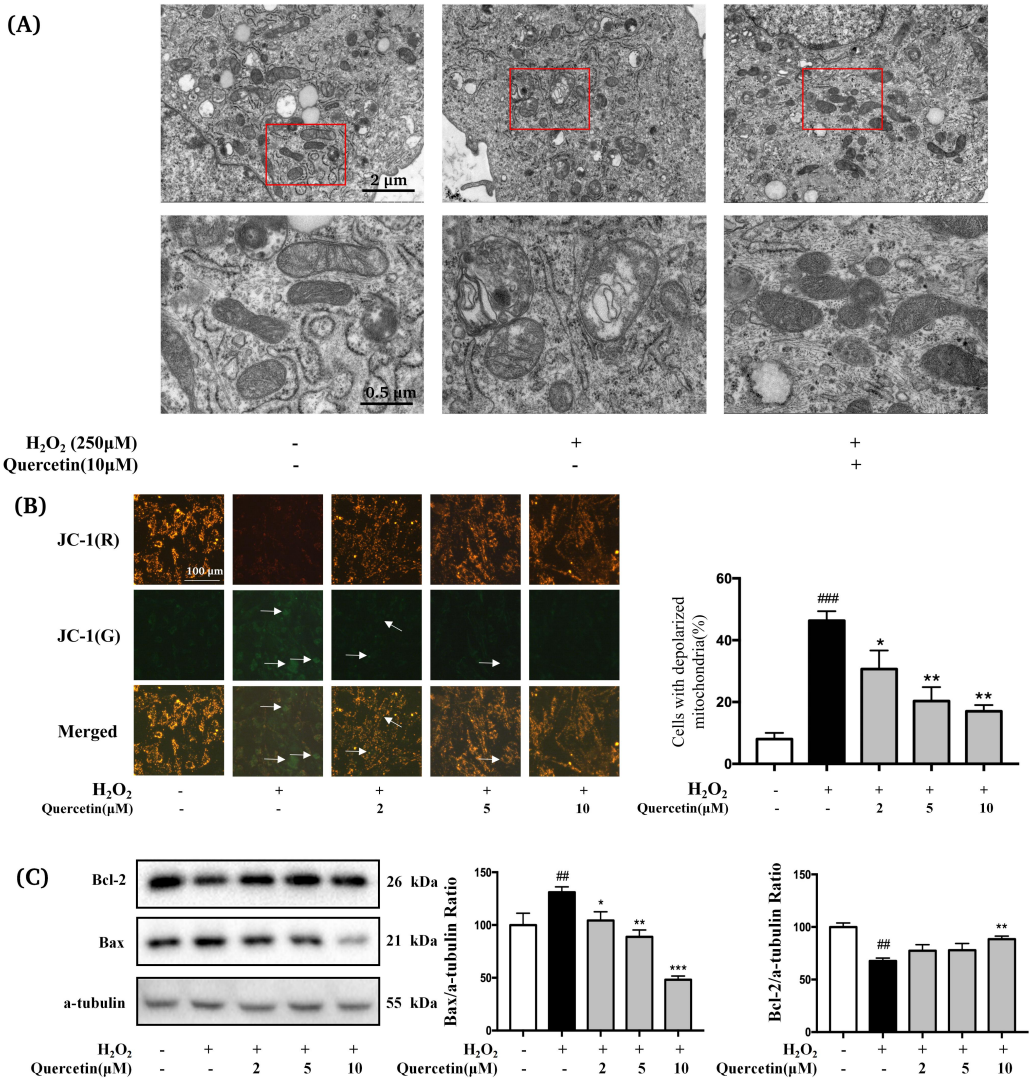

Figure 4 Quercetin maintained mitochondrial homeostasis in the presence of H_2O_2. Human ovarian GCs were treated with quercetin (2, 5, 10 μM) for 24 h, and then exposed to H_2O_2 (250 μM) for 2 h. (A) The mitochondrial structure of KGN cells was observed under a TEM. (B) Mitochondrial depolarization was tested by JC-1 staining. (C) The expression of Bax and Bcl-2 proteins was examined by western blot. Data were presented as mean ± SEM from independent experiments performed in triplicate. Data were compared between groups by one-way ANOVA and Tukey post-test. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, relative to control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, relative to H_2O_2 group. H_2O_2, hydrogen peroxide; KGN, human ovarian granulosa tumor cell line; GCs, Granulosa cells; TEM, transmission electron microscope; ANOVA, analysis of variance.

Discussion

Remarkably prolonged human life renders aging-related problems more prominent [25]. Ovary tends to age earlier and faster than other body organs, which is also considered a trigger for female aging [26].

The aging process of the ovary is complex and influenced by many factors [27]. The free radical theory has been a classic theory in aging research, and it is also the most influential theory [28]. H_2O_2 easily diffuse through the cell membrane and cause oxidative damage to cells, which is often be used as a strong oxidant and conduct relevant research. As shown in Figure 2, our study demonstrated that H_2O_2 upregulated the ROS level and caused GSH depletion, which reflected that H_2O_2 induced oxidative stress in cells. However, quercetin as a common antioxidant could effectively curb the responses, which suggested that quercetin might be an effective medicine for ovarian aging.

Ovarian aging is often accompanied by an increase in granulosa cells apoptosis, and reducing the apoptosis may delay ovarian aging [28]. In our study, as shown in Figure 1, we first demonstrated that H_2O_2 lowers the cell viability and promotes the release of LDH in KGN cells, which could reflect that H_2O_2 induced cells injury, and this is partially consistent with the other research, but quercetin could attenuate the cells injury induced by H_2O_2 [29]. In order to demonstrate the protective effect of quercetin, as shown in Figure 3A and Figure 3B we further detected the cells apoptosis by Hoechst 33258 and AO/EB staining analysis, and the results suggested that quercetin could effectively reduce apoptosis induced by H_2O_2. We also detected the caspase9/3 pathway, and the results showed that quercetin alleviated H_2O_2 induced apoptosis via the caspase-3/9 pathway (Figure 3C). These results implied that quercetin could block granulosa cells apoptosis via the caspase-9/3 pathway, through which quercetin might achieve protective effect on ovarian function.

Mitochondria play an essential role in ovarian follicle development and early embryonic development. Decreased ovarian functions also has severe adverse effects on mitochondrial homeostasis as well as the state of oocytes and peripheral granulosa cells, and mitochondria damage can also lead to oxidative stress and cell injury [30]. To further explore the cellular protective mechanism of quercetin, we first observed the mitochondrial structure of KGN cells under TEM, the results showed that H_2O_2 induced mitochondrial swelling, vacuolization, and fragmentation, but quercetin could alleviate the damage (Figure 4A). We also observed the mitochondrial membrane potential and found that H_2O_2 could lead to mitochondrial depolarization, similar result has been seen in other study, but quercetin could reverse it effectively (Figure 4B) [31]. The Bcl-2 family is the central regulator of mitochondrial membrane permeability. The ratio of Bax/Bcl-2 changes mitochondrial membrane permeability and affects the mitochondrial membrane potential [32]. Our further study demonstrated that quercetin markedly upregulates Bcl-2 expression and downregulates Bax expression under the action of H_2O_2. These results indicated that quercetin maintained mitochondrial homeostasis under oxidative stress, and the cellular protective mechanism of quercetin might be related to this.

Conclusion

Quercetin, as a bioactive flavonoid present in medicinal plants and food, can effectively protect KGN cells from H_2O_2 induced apoptosis. This protective effect may be related to maintaining mitochondrial homeostasis and blocking mitochondrial dependent cell apoptosis, but the specific mechanism needs further research. The findings of this study suggest that quercetin may be a potential protective agent against ovarian aging.

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