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Original Article

Quercetin and tanshinone prevent mitochondria from oxidation and autophagy to inhibit KGN cell apoptosis through the SIRT1/SIRT3-FOXO3a axis

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Abstract

Granulosa cells are somatic cells located inside follicles that play a crucial role in the growth and development of follicles. Quercetin and tanshinone are two key monomers in traditional Chinese medicine that have antioxidant and anti-aging properties. The KGN cell apoptosis model caused by triptolide (TP) was employed in this work to investigate granulosa cell death and medication rescue. Quercetin and tanshinone therapy suppressed KGN cell death and oxidation while also regulating the expression of critical apoptosis and oxidation-related markers such as B-cell lymphoma-2 (Bcl-2) and Bcl-2-associated X protein (Bax). Further research revealed that the effects of Quercetin and Tanshinone were accomplished via deacetylation of FOXO3A in the cytoplasm and mitochondria via the SIRT1/SIRT3-FOXO3a axis. In summary, Quercetin and tanshinone protect KGN cells from apoptosis by reducing mitochondrial apoptosis and oxidation via the SIRT1/SIRT3-FOXO3a axis.

Keywords: Quercetin, Tanshinone, Anti-oxidant, Apoptosis, SIRT1/SIRT3-FOXO3a

1. Introduction

Quercetin is a flavonoid compound that occurs naturally in various fruits, vegetables, and grains. It is also one of the active ingredients in the traditional Chinese medicine Cuscuta chinensis [1]. There have been many studies on its medicinal effects, including anti-cancer [2], anti-inflammatory [3], anti-viral activity [4], antioxidant [5], anti-apoptosis [6], anti-aging [7], and protective functions on the cardiovascular system [8], skin system [9] and nervous system [10]. In recent years, with the improvement of living standards and medical standards, research on the medicinal properties of quercetin has become increasingly concentrated [11]. These studies mainly focus on the antioxidant, anti-apoptotic and anti-inflammatory properties of quercetin.

Salvia miltiorrhiza is a traditional Chinese medicine. Salvia miltiorrhiza is widely used to promote blood circulation and remove blood stasis in the treatment of heart diseases [12]. Tan-IIA is a phenanthro [1, 2-b] furan-10, 11-dione, 6, 7, 8, 9-tetrahydro-1, 6, 6-trimethyl, which is a fat-soluble ingredient [13]. Its main component, Tanshinone IIA (tan-IIA), has anti-inflammatory and antioxidant activities [14], enhances angiogenesis [15], and induces significant cardioprotective [16], immunomodulatory [17], and neuroprotective effects [18].

The mechanism by which quercetin or tanshinone exert their anti-aging functions is complex—apoptosis, inflammatory response, mitochondrial function, autophagy, apoptosis and other signaling pathways are intertwined [19]. Therefore, two tasks are very important, studying the key players in the key pathways that exert their pharmacological activity and studying the pharmacological effects of co-administration of two compound monomers. This research work focuses on studying the pathways related to apoptosis by using triptolide to induce apoptosis model on KGN cells to explore the anti-aging pharmacological effects of quercetin and tanshinone administered separately or together.

2. Materials and Methods

2.1. Reagents and drug solution

Triptolide, quercetin, and tanshinone were obtained from MCE (HY-32735, HY-18085), the purity of which is above 98%. DCFH-DA was obtained from Sigma-Aldrich

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(St. Louis, MO, USA).

2.2. KGN cell experiment

KGN is a tumour that resembles human granulosa cells and produces steroids [20]. This study used Procell Life Technologies Co., Ltd. KGN cells. Short tandem repeat (STR) investigation discovered them. The cells were cultured under standard conditions in an incubator set to 37°C with 5% CO_2 humidity to promote cell growth. The cell surface was rinsed with PBS before testing. In experiments, triptolide killed KGN cells. After 24 hours of 10 μM TP treatment, KGN cells were washed with PBS. The cells were co-cultured with 10 and 20 μM Que or Tan for 24 hours.

2.3. ELISA detection of endocrine function of cells

Cell culture medium (5×10^4 cells) was collected, and centrifuged, and the supernatant was extracted. The hormone levels of estradiol (E2), progesterone (P) and Inhibin B were determined with commercial ELISA kits (Wuhan Saipei Biotechnology, Wuhan, China).

2.4. Fluorescent staining and quantification

In our experiments, we used a fluorescent stain DCFH-DA (Aladdin, Shanghai, China) to detect ROS. The cell-permeable probe DCFH-DA generates dichlorofluorescein (DCF) in live cells to make non-fluorescent materials fluorescent. The DCF excitation and scattering wavelengths are 480 and 525 nm.

2.5. Biochemical measurement by ELISA

We measured apoptotic and antioxidant markers in KGN culture supernatants using commercial ELISA and following directions. MDA, SOD, and GSH-Px are these indicators.

2.6. Cell viability assay

The CCK-8 experiment assessed GC survival after Que, Tan, or both treatments. Post-incubation, each well was incubated for 4 hours at 37°C with CCK-8 solution. Using a Bio-Tek microplate reader (Winooski, VT, USA), the optical density (OD) of each group was determined at 450 nm.

2.7. Annexin V/PI double dyeing flow cytometry

The apoptosis rate was detected using flow cytometry. Specifically, twice-washed treated cells (2 × 105/well) were removed and resuspended in 100 μl of binding buffer. They were then incubated with 5 μl of Annexin V-fluorescein isothiocyanate (FITC; Sigma, St. Louis, MO, USA) and 5 μl of propidium iodide (PI; Sigma, St. Louis, MO, USA) for 15 minutes. Finally, the samples were analysed on a flow cytometer with the addition of 400 μL of binding buffer (FACS Calibur, BD Biosciences, San Jose, CA, USA). The cells that are FITC-positive and PI-negative are in the early stages of apoptosis. These cells are classified as early apoptotic cells. On the other hand, FITC- and PI-positive cells are in the late stages of apoptosis. The apoptosis rate is calculated as the percentage of early and late apoptotic cells.

2.8. Mitochondrial membrane potential measurement with JC-1

After the KGN cells were resuspended, they were transferred to the confocal small dish. When the adherent KGN cells filled 50% of the small dish, they were processed according to the requirements. Afterwards, the original culture medium was disposed, and after washing twice with PBS, blank culture medium containing Jc-1 probe (1 μg/mL) was added. After incubation in the incubator for 15 minutes, adjust the excitation wavelength to 488 nm and 543 nm, and observe the imaging under a 40x objective lens. The fluorescence of the image was used to calculate the relative membrane potential of the mitochondria. By dividing the red fluorescence intensity by the green fluorescence intensity, the relative membrane potential of the mitochondria is calculated. The calculated relative membrane potential of mitochondria was expressed as fluorescence intensity ratio.

2.9. Mitochondrial Permeability Transition Pore (mPTP) measurement

KGN cells were inoculated into petri dishes and treated according to the experimental design. The culture medium was aspirated and the cells were rinsed with PBS 1-2 times. Then, 100 μl of Calcein AM Stain was added to each well of a 96-well plate. Fluorescence Quenching Solution was used as a control. The plate was gently shaken to ensure even coverage of the dye on all cells. The cells were incubated in the dark at 37°C for 30-45 minutes. The optimal incubation time varies for different cells. The culture was incubated in the dark at 37°C for 30 minutes. After that, the medium was replaced with fresh medium pre-warmed to 37°C. The culture medium was aspirated and rinsed 2-3 times with PBS. Then, Mitochondrial Permeability Transformation Reagent (Beyotime, C2009S, Shanghai, China) was added, and the culture was observed under a fluorescence microscope. The entire process was carried out in the dark.

2.10. Mitochondrial Superoxide (Mito-SOX) measurement

The content of reactive oxygen species (ROS) was determined using DCFH-DA stained ROS assay kit (Beyotime, Shanghai, China). A total of 2×10^5 KGN cells were seeded in a 6-well plate and exposed to the indicated concentration of TP for 24 hours. The culture medium was then discarded, and cells were treated with Que and/or Tan for 24 hours. Then, discard the culture medium and wash twice with 2 ml PBS. Cells were incubated with 10 μM DCFH-DA for 20 min in fresh serum-free medium. The excitation and emission wavelengths of the fluorescence spectrophotometer (FLx800; BioTek, Winooski, VT, USA) were 485 nm and 530 nm, respectively. Observe the stained cells under a fluorescence microscope and take pictures. ImageJ software was used for fluorescence intensity quantification.

2.11. Western Blot

KGN cells were killed by a cell-killing solution made in Chinese Nanjing KeyGen Biotech. It was cells that were used. Bichromoninic acid was used to test the sample protein. SDS-PAGE was used to sort equal amounts of proteins, which were then moved to PVDF membranes (Millipore, Burlington, MA, USA). For two hours, 5% nonfat milk was put on PVDF membranes to stop them from sticking to other things. Next, they were mixed with primary antibodies (GAPDH Abcam, Cambridge, MA,

Table 1. The primer sequence of Q-PCR.

Name	5'-3'
SIRT1	F: TAGCCTTGTCAGATAAGGAAGGA
	R: ACAGCTTCACAGTCAACTTTGT
SIRT3	F: ACCCAGTGGCATTCCAGAC
	R: GGCTTGGGGTTGTGAAAGAAG
FOXO3	F: CGGACAAACGGCTCACTCT
	R: GGACCCGCATGAATCGACTAT
Bax	F: CCCGAGAGGTCTTTTTCCGAG
	R: CCAGCCCATGATGGTTCTGA
bcl-2	F: GGTGGGGTCATGTGTGTGG
	R: CGGTTCAGGTACTCAGTCATCC
SOD2	F: GCTCCGGTTTTGGGGTATCTG
	R: GCGTTGATGTGAGGTTCCAG
CAT	F: TGGAGCTGGTAACCCAGTAGG
	R: CCTTTGCCTTGGAGTATTTGGTA

USA, ab8245, 1:500) and left overnight at 4°C. Finally, horseradish peroxidase membranes and goat anti-rabbit secondary antibodies worked well. Wanting to find more chemiluminescence.

2.12. RT-qPCR

Real-time RT-qPCR was used to measure oxidation, apoptosis, and mitochondrial oxidation genes. The cells' total RNA was extracted using TRIzol. HiScript II qRT SuperMix was used for reverse transcription and cDNA synthesis. qPCR was done using a Step One Plus real-time PCR apparatus (Applied Biosystems, Foster City, CA, USA) using ChamQTM Universal SYBR qPCR Master Mix (Vazyme, China), which meets the RT-qPCR standard. The Step One Plus real-time PCR apparatus (Applied Biosystems, Foster City, CA, USA) was used to perform the qPCR assay. Universal Biotech (Shanghai, China) generated the primer sequences utilized in this work (Table 1). Target mRNA expression levels of each gene were measured and normalized using 2-Ct.

2.13. Statistical analysis

The data is presented as mean ± SD. We used Graph Pad PRISM (Graph Pad Software, La Jolla, CA, USA) to compare the treatment group to the control group using One-way ANOVA and then Tukey's test to see if there was a significant difference. A P-value of less than 0.05 meant that the differences between the groups were statistically important.

3. Results

3.1. Effects of quercetin and tanshinone on cellular landscape

KGN cells are oocyte granulosa cells and their important cellular functions are endocrine and cell proliferation [21]. Inhibin B, estradiol, and progesterone are important indicators of the endocrine function of granulosa cells [22]. The findings of INDI indicated that the concentrations of progesterone, estradiol, and inhibin B were comparatively less in the group treated with TP than in the vehicle (Figure 1A). Under TP treatment, the cell secretion function of granulosa cells was inhibited, which meant that the apoptosis model induced by TP was successfully established. Compared with the TP group, the quercetin, tanshinone and quercetin-tanshinone co-treatment group showed significant improvements in hormone secretion (Figure 1A& 1B & 1C). The compound 3-TYP is the inhibitor of Sirtuin 3 (SIRT3) that plays an important role in inhibiting mitochondrial apoptosis and oxidation [32]. After the addition of 3-TYP, the hormone secretion of KGN cells was significantly reduced. In particular, progesterone was reduced to a degree comparable to that of the TP group (Figure 1A & 1B & 1C).

In terms of cell division and proliferation function, CCK-8 test was used. In Figure 1 D, the TP-treated group had the lowest cell survival rate. Compared with the TP group, the Que group, the Tan group and the Que+Tan group all showed better cell survival rates, which proved that separate administration and co-administration of the two groups restored cell division and proliferation activity. The two groups adding 3-TYP also showed a partial decrease, although this decrease was not significant, further illustrating the pharmacological activity of the two drugs.

Here, we used the TP-induced KGN cell model to verify the effects of quercetin and tanshinone on endocrine function. The positive effects of quercetin and tanshinone administered separately and together on the cellular landscape are described from both intracellular endocrine and

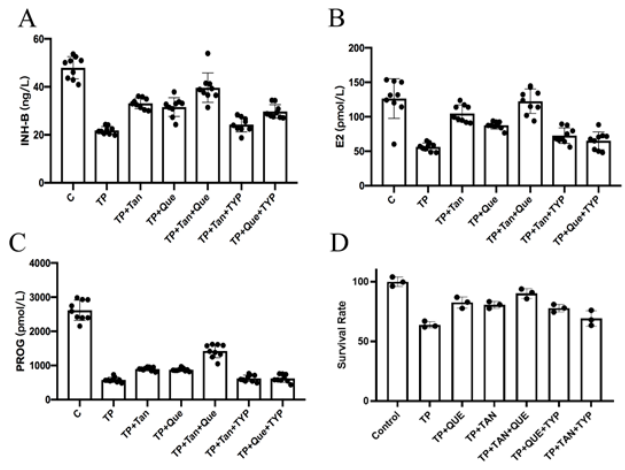

Fig. 1. Effects of quercetin and tanshinone on KGN cellular landscape.

cell division perspectives.

3.2. Effects of quercetin and tanshinone on mitochondrial function

To further explore the specific influence of the two drugs on the rescue of KGN cell apoptosis, the morphology and function of mitochondria were first focused on. The reason is that current research reveals that the main causes of cell apoptosis include the extracellular death receptor pathway, mitochondrial apoptosis pathway and endoplasmic reticulum pathway [23], and the cell apoptosis model is achieved through TP-induced inhibition of NF-κB activation [24,25], stimulating the first apoptotic pathway [26]. Therefore, mitochondrial permeability (Figure 2A) was first tested in subsequent studies and quantitatively analyzed by fluorescence (Figure 2B). The fluorescence intensity of the TP group was lowered when compared to the control group, but the three treatment groups showed the opposite tendency to the TP group. Following that, mitochondrial membrane potential was determined by labeling cells with JC-1 (Figure 2C). Similar to the mitochondrial permeability results, the fluorescence intensity of the TP group was significantly reduced compared with the control group. It is worth noting that the two compounds in the Que group and Tan group did not exert pharmacological effects when administered alone, but the Que+Tan group in which Que and Tan worked together showed a significant improvement.

Among the apoptotic pathways, more detailed mechanistic verification on the intracellular mitochondrial pathway was performed. QPCR (Figure 3A) and WB (Figure 3B) experiments on cellular mitochondrial SIRT1, SIRT3, FOXO3a, Bax, and bcl-2 were performed to verify the content changes of these genes and corresponding translated proteins.

Bax and Bcl-2 proteins are two proteins that are important in the regulation of apoptosis [27]. Their interactions and regulation are critical for the balance of cell survival and death [28]. Bax protein activation and aggregation can induce alterations in the permeability of the mitochondrial membrane, thereby instigating apoptosis [29]. Bacterial outer membrane Bcl-2 protein functions as an anti-apoptotic protein by impeding the activity of Bax protein [30], thereby preventing alterations in mitochondrial membrane permeability. In the experiment, we found that Bcl-2 and Bax protein expression in the treatment group was lower

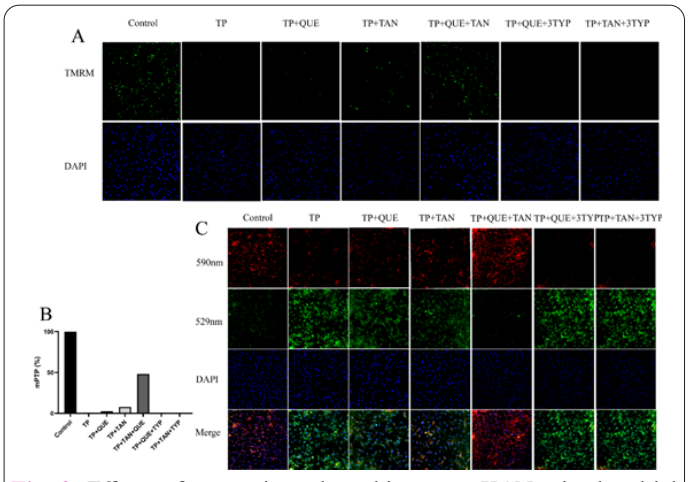

Fig. 2. Effects of quercetin and tanshinone on KGN mitochondrial function.

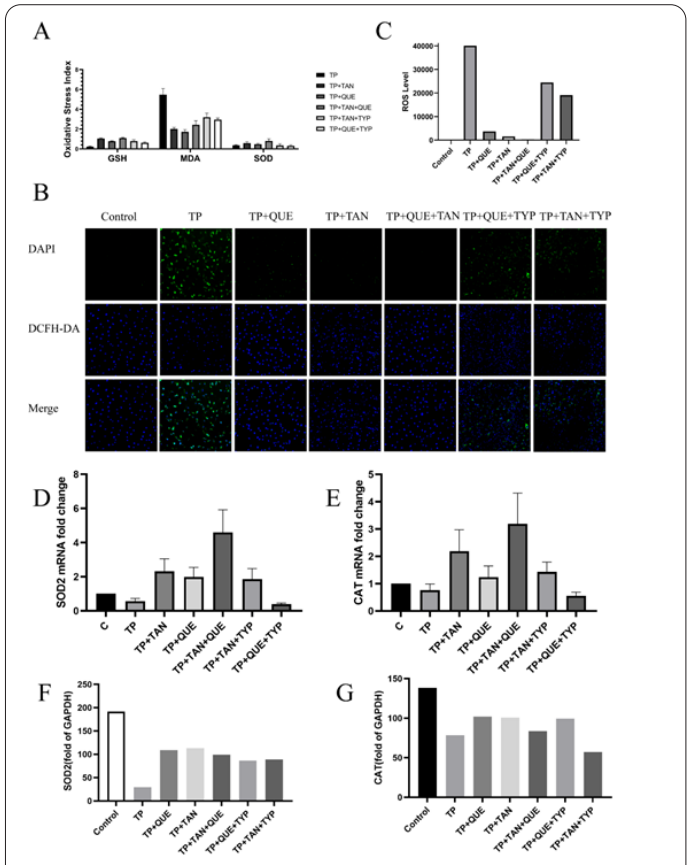

Fig. 3. Mechanism of quercetin and tanshinone on KGN cellular mitochondrial function.

than in the control group, and their corresponding transcripts showed an increasing and decreasing trend, respectively. This suggests that pharmacological stimulation induces cells to enhance Bcl-2 transcription while inhibiting Bax transcription, acting as a "brake" on cell death.

SIRT1 is a nicotinamide adenine dinucleotide-dependent deacetylase involved in mitochondrial biogenesis [31]. SIRT3 is a mitochondrial protein deacetylase that maintains redox balance [32]. FOXO3 is a member of the FOXO family that has an effect in mitophagy induction as well as mitochondrial function and biogenesis regulation [33]. The expression of proteins corresponding to these three genes was reduced in the treatment group compared with the TP group, which indicates the potential pathway through which the drug works.

3.3. Effects of quercetin and tanshinone on cellular oxidative pathways

Cellular oxidation is an early signal of apoptosis from the reason that subsequent generation of oxygen-free radicals from oxidation prompts cells to undergo apoptosis [34]. Antioxidant genes GSH, MDA and SOD have anti-apoptotic effects in ovarian aging. Therefore, examining the expression levels of GSH, MDA, and SOD can be used as key indicators of cellular oxidative pathways in the early process of apoptosis [35]. In order to explore the oxidation-reduction balance of KGN cells under drug treatment, the protein MDA SOD GSH related to cell oxidation in the cell culture medium was detected (Figure 4A). The level of the oxidation product MDA in the drug-treated group was much lower than in the TP group, while the levels of antioxidant enzymes SOD and glutathione GSH were raised, demonstrating the protective impact of drug thera-

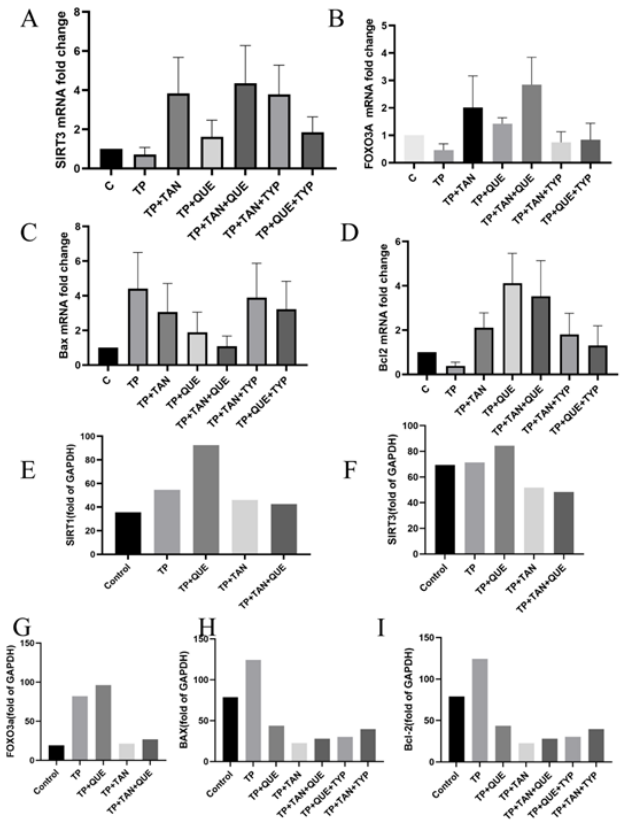

Fig. 4. Effects of quercetin and tanshinone on KGN cellular oxidative pathways.

py on cell oxidation and subsequent cell apoptosis.

Further, a ROS assay kit based on DCFH-DA dye was used to stain KGN cells (Figure 4B), and a fluorescence spectrophotometer was used to analyze the ROS-related fluorescence intensity (Figure 4C). With TP stimulation, the reactive oxygen species (ROS) content of cells increased significantly, but this phenomenon was attenuated by Que, Tan, and Que+Tan treatments, and even the ROS level of the Que+Tan group was reduced to approximately the same level as the control group.

Finally, in order to further confirm the effect of drugs on cellular oxidation, especially the changes in key indicators of the oxidative pathway in the cytoplasm, the mRNA and protein expression levels of SOD and CAT were examined (Figure 4D & 4E & 4F). The results demonstrated that TP stimulation decreased SOD and CAT mRNA and protein expression levels in KGN cells, which was restored by Que, Tan, and Que+Tan therapy. These results indicate that Que, Tan and Que+Tan exert antioxidant effects in TP-induced KGN cells, thereby alleviating cell apoptosis. Interestingly, the combination of Que and Tan exerts a better antioxidant effect than Que or Tan administered alone.

4. Discussion

Internal and external events trigger a cell's pre-existing death program, causing apoptosis. [34]. Apoptosis of granulosa cells is the main cause of reduced female fertility [36]. We explored the conditions under which TP induces apoptosis in KGN cells, and used this model to conduct anti-apoptotic drug research. This modeling is successful, and multiple experimental results can prove this conclusion, such as the decrease in GSH content in the TP group, an early signal of apoptosis.

Both quercetin and tanshinone are natural compounds with antioxidant and anti-apoptotic effects [37]. Previous research focused on the separate drug effects of the two and believed that the drug effect of the two co-work is equivalent to the sum of the drug effects of the two alone, that is, the drug action mode of 1+1=2 [37]. However, in the KGN cell apoptosis model induced by TP, co-administration of quercetin and tanshinone was found to be more effective than separate administration, such as mitochondrial Changes in permeability (Figure 2B), changes in mitochondrial membrane potential (Figure 2D), changes in ROS content (Figure 3C), and the expression of various genes and proteins related to cellular oxidation and apoptosis (Figure 3&4). Such results remind us that when the two compounds, quercetin and tanshinone, are administered together, their efficacy is greater than the sum of their efficacy when administered alone, that is, the drug action mode is 1+1>2.

FOXO3a (forkhead box O 3a) is a transcription factor [33]. Sirtuins are responsible for FOXO3a deacetylation [32]. SIRT1 deacetylates and activates FOXO3a, which can influence the activity of several downstream transcription factors, regulating autophagy and reducing oxidative stress in cells [31]. After treatment with Tan and Que, there were obvious expression changes of SIRT1 and SIRT3, which suggested that Tan and Que may activate the SIRT1/SIRT3-FOXO3a pathway and cause deacetylation of FOXO3a. The change of downstream cell signaling of FOXO3a also suggests this. The expression of Bcl-2 is regulated by FOXO3a [38]. Under normal conditions, the Bcl-2 protein binds to Bax in an inactive state and inhibits the activity of the latter, thereby maintaining cell survival [39]. When cells undergo apoptosis, the Bcl-2 protein is activated, causing Bcl-2 to release Bax [39]. Activated Bax protein will aggregate on the surface of mitochondria to form pores, causing changes in mitochondrial membrane permeability, and ultimately triggering cell apoptosis [40,41]. Under the treatment of Tan and Que, we observed an increase in the expression of Bcl-2, which inhibited the activation of Bax and subsequently inhibited cell apoptosis. In addition, deacetylation of FOXO3a will also enhance the expression of downstream antioxidant genes. Similarly, enhanced expression of SOD, CAT, and GSH was observed under treatment with Tan and Que. Therefore, one conclusion that can be drawn is that quercetin and tanshinone work together on the SIRT1/SIRT3-FOXO3a axis to relieve mitochondrial oxidation and autophagy and thereby inhibit KGN cell apoptosis.

This study still has several limitations. First, the effects of quercetin and tanshinone on other pathways in inhibiting apoptosis need to be further verified, including but not limited to the endoplasmic reticulum stress pathway and extracellular signal-mediated apoptosis. Secondly, we only evaluated the effects of quercetin and tanshinone on the KGN cell apoptosis model in vitro, and in vivo studies of them need to be performed.

5. Conclusions

Based on the results, it was concluded that quercetin and tanshinone play a protective role in mitochondrial oxidation and mitophagy in the TP-induced KGN cell apoptosis model to rescue cell apoptosis, mediated by activating the SIRT1/SIRT3-FOXO3a axis. The results indicate that quercetin and tanshinone exert pharmaceutical effects by

alleviating oocyte apoptosis and are promising therapeutic agents for reduced female fertility caused by granulosa cell apoptosis.

Conflict of Interest
Author(s) disclose no conflict of interest regarding article publication.

Consent for publications
The author read and approved the final manuscript for publication.

Ethics approval and consent to participate
No human or animals were used in the present research.

Informed Consent
The authors declare not used any patients in this research.

Availability of data and material
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions
QW and XX designed the study and performed the experiments, LT and KJ collected the data, XC and YL analyzed the data, QW and XX prepared the manuscript. All authors read and approved the final manuscript.

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