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Quercetin ameliorates cyclophosphamide-induced premature ovarian insufficiency by modulating SIRT1/HIF-1α pathway

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Abstract

Background

Premature ovarian insufficiency (POI) is characterized by complex etiologies and currently lacks effective treatments, posing significant threats to women's physical and psychological health. Quercetin (QUE) , a bioactive flavonoid inherent in traditional herbs such as Flos Sophorae and Cuscuta chinensis, demonstrates potent redox-modulating and anti-senescence capacities. This investigation validates its therapeutic efficacy against POI, specifically elucidating granulosa cell-centric mechanisms.

Methods

POI mouse models and human ovarian granulosa cells KGN injury models were induced through the administration of cyclophosphamide (CTX). Subsequently, these models were treated with drugs such as QUE. Morphological changes in the ovaries of the mice were observed, and vaginal

smears were conducted to monitor the estrus cycles and patterns of the mice. ELISA was employed to detect sex hormone levels in various mouse groups, while Hematoxylin-eosin staining Staining(H&E) staining was utilized to assess the status of ovarian follicles at different stages. Moving forward, immunohistochemical analysis and Western blot were conducted to detect the expression levels of Sirtuin 1 (SIRT1), Hypoxia-Inducible Factor 1 Alpha (HIF-1α), and other apoptosis-related proteins. Ultimately, methods such as Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling (TUNEL) assay, *β*-galactosidase staining, flow cytometry for apoptosis detection, and 5-Ethynyl-2′-deoxyuridine (EdU) Assay assay were utilized to evaluate the apoptosis, senescence, and proliferation of mouse ovarian tissues and human ovarian granulosa cells KGN.

Results

QUE ameliorated CTX-induced ovarian atrophy and follicular atresia by upregulating SIRT1 expression. Furthermore, QUE partially restored estrous cyclicity, normalized sex hormone levels, and improved follicular counts through SIRT1/HIF-1α modulation. Concurrently, QUE suppressed apoptosis in both murine and human granulosa cells via the SIRT1/HIF-1α pathway.

Conclusion

Our findings demonstrate that QUE enhances ovarian reserve by inhibiting granulosa cells apoptosis via SIRT1/HIF-1α signaling, highlighting its therapeutic potential for POI management.

Keywords

Premature ovarian insufficiency， quercetin， SIRT1, HIF-1α

Clinical trial number

Not applicable.

Introduction

POI refers to a condition affecting women under the age of 40, characterized by the cessation of ovulation and abnormal serum sex hormone levels, with an incidence rate as high as 3.7% [1]. According to the most recent guidelines (updated in 2023) of the European Society of Human Reproduction and Embryology, the diagnostic criteria for POI are as follows: the presence of amenorrhea or severe oligomenorrhea for four consecutive months or longer, accompanied by two sex hormone tests, conducted with an interval of more than four weeks, showing an follicle-stimulating hormone (FSH) level exceeding 25 IU/L [2]. POI, being the later stage of ovarian function decline, often imposes dual pressures on women, both physiologically and psychologically, in clinical settings. Physiologically, the deterioration of ovarian function can lead to menstrual irregularities and even amenorrhea [3], while also potentially decreasing egg quality, thereby elevating the risks of miscarriage and fetal malformations [4] . Psychologically, numerous women experience anxiety and depression when confronted with reduced fertility, impacting their quality of life and mental wellbeing [5]. POI is a significant threat to women's health globally and a major public health concern. Meanwhile, POI can also be attributed to iatrogenic factors. CTX, a

widely administered chemotherapy drug, although effective in inhibiting breast tumors, may adversely affect ovarian function, leading to POI. Currently, hormone replacement therapy (HRT) stands as one of the primary treatments for POI patients, effectively mitigating symptoms arising from estrogen deficiency, such as hot flashes and vaginal dryness. However, the implementation of HRT in POI patients encounters numerous challenges, including a lower-than-anticipated adoption rate among patients, varying efficacy among individuals, and potential side effects [6, 7]. Therefore, finding effective POI treatments with minimal side effects is crucial.

QUE, a flavonoid derived from Traditional Chinese Medicines, is ubiquitous in fruits and vegetables, demonstrating multiple bioactivities, including antioxidant properties [8, 9]. QUE's unique chemical structure confers strong pharmacological properties, making it valuable for preventing and treating various diseases, particularly in enhancing ovarian reserve. QUE significantly increases serum levels of Anti-Müllerian Hormone (AMH), estradiol (E_2), and progesterone in mice. It also reduces FSH and luteinizing hormone (LH) levels, improves ovarian pathology, and reduces chemotherapy-induced ovarian damage [10]. Our prior study revealed QUE as the predominant bioactive constituent of Zuo Gui Pill for managing diminished ovarian reserve DOR through data mining approaches. Thus, QUE may become an effective POI treatment.

Ovarian granulosa cells are indispensable in protecting ovarian function and maintaining fertility. Each step of follicle development, oocyte maturation, cumulus expansion, and ovulation relies on continuous and precise interactions with surrounding granulosa cells [11]. Previous research has indicated that QUE has the capacity to restore damaged ovarian granulosa cells to a certain degree

[12], but its mechanism of action remains inadequately explored. SIRT1 is a NAD+-dependent deacetylase that plays a role in diverse cellular processes, encompassing the cell cycle, metabolism, and stress responses. By deacetylating multiple proteins, SIRT1 regulates gene expression [13] and participates in biological processes such as cellular senescence and apoptosis, making it an important regulator in protecting cells against oxidative damage [14] . Therefore, SIRT1 may serve as an effective therapeutic target for treating POI.

This investigation seeks to delineate the therapeutic efficacy and molecular basis of QUE for POI, thereby establishing an experimental foundation for its clinical translation.

Materials and methods

Animals

Eight-week-old female BALB/c mice (SPF grade) were obtained from Guangdong PharmaGene Biotechnology Co., Ltd. and housed at the Laboratory Animal Center of Jinan University. The mice were maintained under standard conditions (12h/12h light-dark cycle) with ad libitum access to food and water. The study protocol was approved by the Animal Welfare and Ethics Committee of Jinan University (Approval No. IACUC-20241101-11).

Animal Grouping and Treatment

Animal experiments were divided into two parts: therapeutic experiments and reversal experiments. The therapeutic experiment was divided into three groups: the Control group, the model group (CTX group), and the QUE group, with six mice in each group. A singular

intraperitoneal administration of cyclophosphamide (CTX; 120 mg/kg, MedChemExpress，New Jersey, USA, Cat# HY-17420) was administered to induce POI in model (CTX) and therapeutic (QUE) cohorts, whereas control subjects received PBS in equivalent volumes. Vaginal cytology smears were continuously examined starting from day 1 of modeling. By day 14, most mice exhibited disordered estrous cycles, indicating successful model establishment. From day 14 onward, mice in the QUE group received QUE via gavage (25 mg/kg, Selleck, Houston, USA, Cat# S2391), while mice in the Control and CTX groups received an equal volume of PBS via gavage, for a duration of 21 days. The reversal experiment was divided into four groups: the Control group, CTX group, QUE group, and QUE+EX527 group, with nine mice in each group. The administration methods and doses for the Control, CTX, and QUE groups were the same as above. The QUE+EX527 group received QUE (25 mg/kg) by gavage along with intraperitoneal injections of the SIRT1 inhibitor EX527 (2.5 mg/kg, Aladdin, China, Cat# E129892) starting on day 14 post-CTX administration. Body weights were recorded weekly. Mice were anesthetized with isoflurane. All samples (including body weight measurements, blood, and ovarian tissues) were collected one day after the final treatment.

Estrous Cycle

Observation of the estrous cycle began on day 1 of drug administration for all animals and continued for 28 days. Vaginal secretions were collected daily in the morning. Using a pipette, 10 μL of PBS buffer was aspirated and inserted 2–3 mm into the mouse vagina, followed by repeated pipetting. The collected exfoliated cell fluid was spread evenly on a glass slide, dried, and then

subjected to H&E staining. The slide was sealed and observed under a microscope (AXIO, model: Vert-A1) to determine stage of the estrous cycle.

H&E Staining

After dissection, the mouse ovaries were fixed by immersion in formaldehyde for 24 hours, embedded in paraffin, and sectioned at a thickness of 5 μm. The sections were dewaxed and stained with hematoxylin followed by eosin counterstaining, then dehydrated and sealed. Vaginal secretions from the mice were fixed in formaldehyde for 10 minutes and directly stained with hematoxylin and eosin, then dehydrated and sealed.

Observation and Counting of Ovarian Follicles in Mice

Follicles were classified into developmental stages according to the method described by Pedersen and Peters (1968), the number of follicles at each stage (primordial follicles, preantral follicles, antral follicles, and atretic follicles) was recorded for each section, and the results were expressed as mean ± SEM [15].

Enzyme-linked immunosorbent assay (ELISA)

The SIRT1 levels in the mice were quantified according to the Experimental Procedure Guide of the ELISA kit (ELK Biotechnology, Wuhan, China; Cat# ELK6397). Additionally, the levels of AMH, FSH, and E_2 were determined according to the manufacturer's protocol (Wellbio, Shanghai, China; Cat# EM30583; EM30584; EM3066).

Western blot Analysis

Protein lysates from mouse ovaries and KGN granulosa cells were prepared through 30-minute incubation with lysis buffer on ice. The lysates were centrifuged at 12,000 ×g for 10 minutes at 4°C, and the supernatants were collected. Protein concentrations were determined using a BCA kit (Wuhan Sevier Biotechnology, China, Cat# G2026) and equalized before adding 5× loading buffer. Samples were heat-denatured at 100°C for 10 minutes. Proteins (10 μL per sample) were separated by electrophoresis and transferred to PVDF membranes for 40 minutes at room temperature. Membranes were blocked for 10 minutes using rapid blocking buffer, then incubated overnight at 4°C with primary antibodies against Sirt1, HIF-1α (Abcam, UK, Cat# ab189494, ab179483), Bcl-2, Bax, Caspase-3, and β-Tubulin (Abmart, China, Cat# T40056S, T40051M, TD6879S, P60039S). After incubation with HRP-conjugated secondary antibody (Abmart, China, Cat# M21002S) for 1 hour at room temperature, protein bands were visualized using a gel imaging system and analyzed with ImageJ.

Identification of POI Targets and Construction of Protein-Protein Interaction (PPI) Networks

Through OMIM (http://www.omim.org/) [16] and GeneCards (https://www.genecards.org/） [17] , using "Premature Ovarian Failure" (a previous name for POI) as the keyword, a total of 6,872 POI targets were identified. On the SwissTargetPrediction (http://www.swisstargetprediction.ch/) and Super-PRED (https://prediction.charite.de/) websites, 198 targets of QUE were obtained. The

intersection of these two datasets yielded 145 targets of QUE associated with POI. Finally, using the STRING database [18]（https://string-db.org/）with a minimum interaction coefficient greater than 0.700, a PPI network was constructed. This network was visualized using Cytoscape software [19] , and the top 10 relevant targets were confirmed.

Gene Ontology (GO) functional annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis

GO analysis [20] annotates differentially expressed genes between different groups, revealing their Biological Process (BP), Cell Component (CC), and Molecular Function (MF). KEGG [21] is a frequently used database that provides detailed information on genomes, diseases, biological pathways, and drugs. We performed GO and KEGG pathway enrichment analysis on 145 targets of QUE associated with POI using the R package clusterProfiler29.

Immunohistochemistry

Ovarian paraffin sections were dewaxed and subjected to antigen retrieval. Endogenous peroxidase activity was quenched with 3% H_2O_2 for 25 minutes at room temperature in the dark. After blocking with 3% BSA for 30 minutes, sections were incubated overnight at 4°C with primary antibodies (Sirt1, HIF-1α, Caspase-3; 1:500 dilution). HRP-conjugated secondary antibody was applied for 50 minutes at room temperature. Staining was developed using DAB substrate, counterstained with hematoxylin, and mounted for microscopic examination. Brown staining indicated positive immunoreactivity, which was quantified using Image J.

TUNEL

Ovarian apoptosis was detected using the CF488 TUNEL Kit (Servicebio, China, Cat# G1504) according to the manufacturer's protocol. Paraffin sections underwent antigen retrieval, membrane permeabilization, equilibration, and labeling before nuclear counterstaining. Apoptotic cells were visualized by fluorescence microscopy at 488 nm excitation wavelength.

Cell Culture and Treatment

KGN cells were purchased from Shanghai Bide Pharmaceutical Technology Co., Ltd. (Cat# BD02215448) and authenticated by STR profiling at Bide Technology. The cells were cultured at 37°C with 5% CO_2 in DMEM/F-12 medium (Gibco, California, USA, Cat# C11330500BT) supplemented with 10% fetal bovine serum (Servicebio, Wuhan, China, Cat# G8003) and 1% penicillin-streptomycin (Gibco, California, USA, Cat# 15140122). Treatments were administered when cell confluency reached 70–80%. Based on preliminary results, the cells were divided into four groups: the Control, CTX (200 μmol/L, 24 h), QUE (200 μmol/L CTX + 10 μmol/L QUE, 24 h), and QUE+EX527 (200 μmol/L CTX + 10 μmol/L QUE + 10 μmol/L EX527, 24 h) groups.

EdU Detection

Prepare a cell suspension at 1×10^4 cells/well in a 96-well plate. After treatment, follow the kit instructions (Servicebio, Wuhan, Cat# G1601) to perform EDU labeling, fixation, and permeabilization of KGN cells. Add the EDU click reaction mix and incubate at room temperature

in the dark for 30 minutes, followed by nuclear staining. Visualize under an inverted fluorescence microscope and analyze using Image J.

Annexin V-FITC/PI Double Staining for Apoptosis Detection

Harvest KGN cell pellets from each group. Resuspend the pellet in 500 μL Binding Buffer according to the kit instructions (Biosharp, Beijing, Cat# BL110A), then add 5 μL Annexin V-FITC and 5 μL PI, and mix well. Incubate at room temperature in the dark for 10 minutes, then analyze using a flow cytometer (BioTek, Model: Synergy H1).

β-Galactosidase Staining

Cells were incubated with 1 mL β-galactosidase staining fixative for 15 min at room temperature. After fixative removal, three consecutive PBS washes were performed (3 min/wash). After aspirating PBS, add 1 mL staining working solution (Beyotime, Shanghai, Cat# C0602) to each well. Cover the 6-well plate with plastic wrap and incubate overnight at 37°C. Examine under a microscope.

Statistical Analysis

Data were analyzed using GraphPad Prism 9.1 (San Diego, CA, USA). Experimental data are presented as mean ± SD. One-way ANOVA was used for statistical analysis. Statistical significance was set at $P < 0.05$, with $P \geq 0.05$ indicating non-significant differences.

Results

QUE exerts protective effects on ovarian function in POI mice

To investigate how QUE affects mouse body weight and ovaries, we established a POI model by administering a single intraperitoneal injection of 120 mg/kg CTX. Within seven days post-modeling, the mice experienced temporary weight loss, which gradually recovered by the eighth day but remained. The CTX group exhibited significantly lower body weight than the control group (Fig. 1B). QUE treatment partially reversed CTX-induced weight loss (Fig. 1B). Ovarian morphology, ovarian index, and the number of atretic follicles are key indicators for assessing ovarian reserve function, and we evaluated these three parameters. The results demonstrated that relative to control group, CTX-exposed ovaries exhibited significant atrophy, enhanced fragility, and diminished ovarian indices (Fig. 1C-D). Increased atretic follicle burden (Fig. 1E-F) collectively validated successful POI modeling. Following QUE treatment, both ovarian size and ovarian index showed partial recovery, while the number of atretic follicles was significantly reduced. Collectively, these data establish QUE-mediated protection against ovarian reserve impairment in POI mice.

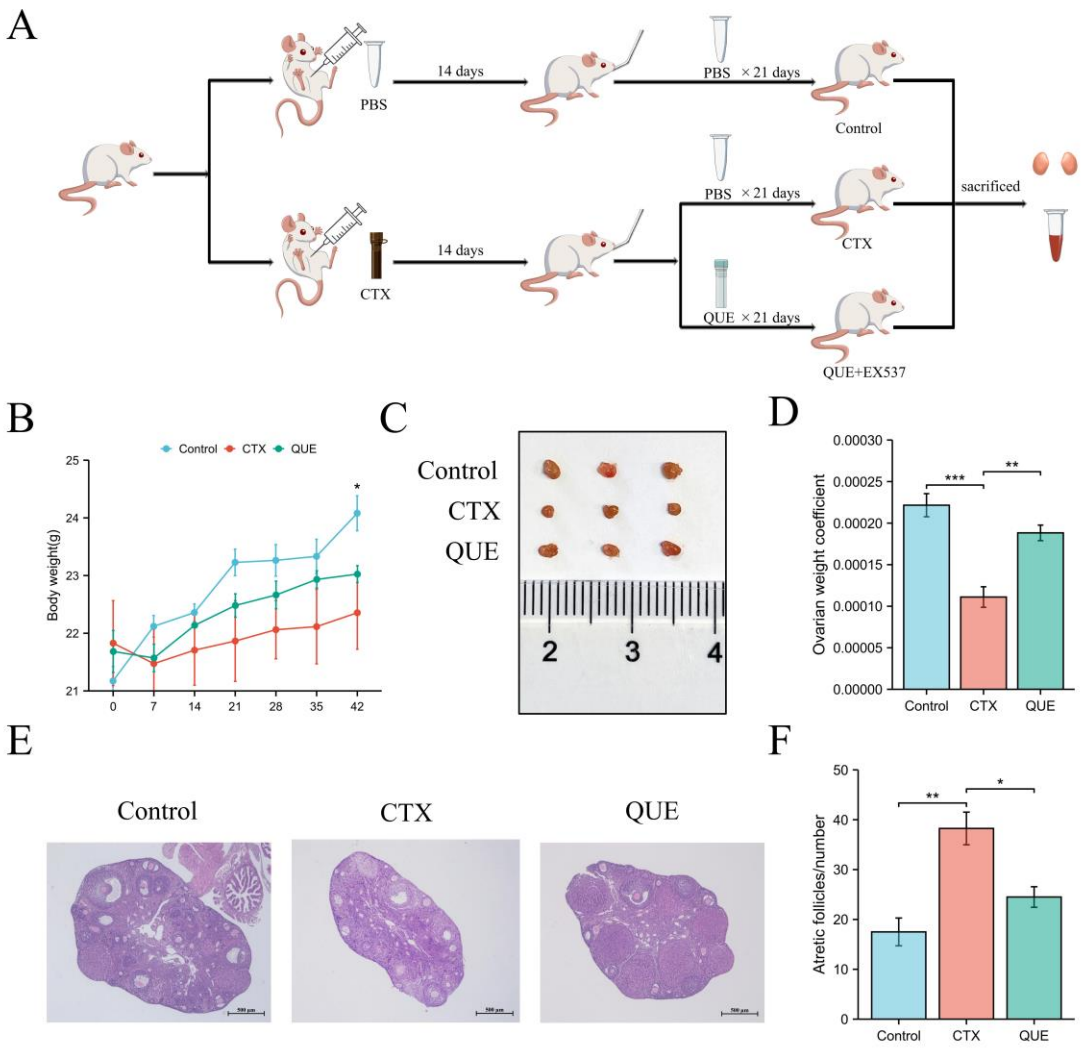


Figure 1. QUE exerts protective effects on ovarian function in POI mice. **A** Schematic diagram of experimental groups and drug administration. **B** Changes in body weight during the experiment ($n = 6$), * $P < 0.05$ vs. CTX. **C** Macroscopic morphology of ovaries from different groups at endpoint ($n = 3$). **D** Ovarian organ index (ovary weight/body weight ratio, $n = 6$). **E** Representative H&E staining images of ovarian sections. **F** Quantification of atretic follicles across groups ($n = 6$). * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

QUE protects ovaries in POI mice by activating SIRT1

To investigate the SIRT1's role in QUE 's protective mechanism in POI mice, we performed

Western blot analysis of ovarian tissues and used SIRT1 ELISA kit assays on serum. The CTX group exhibited a significantly reduced SIRT1expression in Western blot analysis (Fig. 2A, B). Similarly, SIRT1 concentration was significantly lower in the CTX group as measured by ELISA (Fig. 2C). Interestingly, the QUE group showed increased SIRT1 protein expression and serum concentration. Therefore, SIRT1 activation potentially underlies QUE-mediated ovarian reserve preservation in POI models.

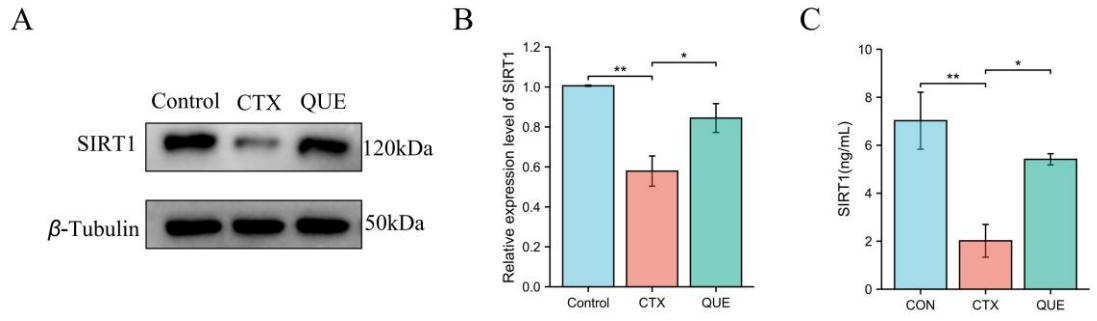


Figure 2. QUE exerts protective effects in POI mice through SIRT1 upregulation. Sample collection was performed to detect the expression level of SIRT1 protein in mouse ovaries and the concentration of SIRT1 in mouse serum (n=4) for each group. **A** Representative Western blot images of SIRT1 protein expression across groups. **B** Quantification of protein expression levels of SIRT1. **C** Serum SIRT1 concentrations in experimental groups. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

QUE ameliorates CTX-induced POI by upregulating SIRT1

To further confirm the role of SIRT1 in QUE's prevention and treatment of POI, we added a SIRT1 inhibitor treatment group (QUE+EX527 group) for a reversal experiment. The animals were divided into the control group, CTX group, QUE group, and QUE+EX527 group for

observation of various aspects of ovarian function. The estrus cycle in mice is an important indicator of periodic changes in the reproductive system and is regulated by sex hormones secreted by the ovaries.

Therefore, we monitored the estrus cycle and sex hormones FSH, E_2, and AMH to assess ovarian function. First, we evaluated the estrus cycle of mice by performing vaginal smears and H&E staining on vaginal secretions. The four different estrus cycles exhibited specific morphological changes in vaginal epithelial cells (Fig. 3A). The control group mice had an estrus cycle duration of approximately 5 days, with all cycles regular. Following CTX intervention, the estrus cycle duration was significantly prolonged and the cycle became irregular. However, after QUE treatment, the estrus cycle duration in POI mice was shortened and tended to become regular. Interestingly, the QUE+EX527 group could not restore the estrus cycle duration and rhythm to the same level as the QUE group (Fig. 3B, C).

FSH, AMH, and E2 are essential for ovarian follicular development and key ovarian function marker. Similar to the hormonal characteristics of ovarian aging, the CTX group and QUE+EX527 group mice exhibited significantly elevated FSH levels in their serum. After QUE intervention, the QUE group showed low FSH levels (Fig. 3D). In the ELISA monitoring of E_2 and AMH, the CTX group and QUE+EX527 group mice also exhibited low hormone levels. However, the QUE group, while unable to fully reach the high hormone levels of the control group, did show relatively high hormone levels (Fig. 3E, F). In summary, QUE can alleviate the adverse effects of CTX on the ovaries. However, when SIRT1 is inhibited, QUE cannot effectively exert its protective function. This indicates that the protective effect of QUE on POI mice is achieved through the upregulation

of SIRT1.

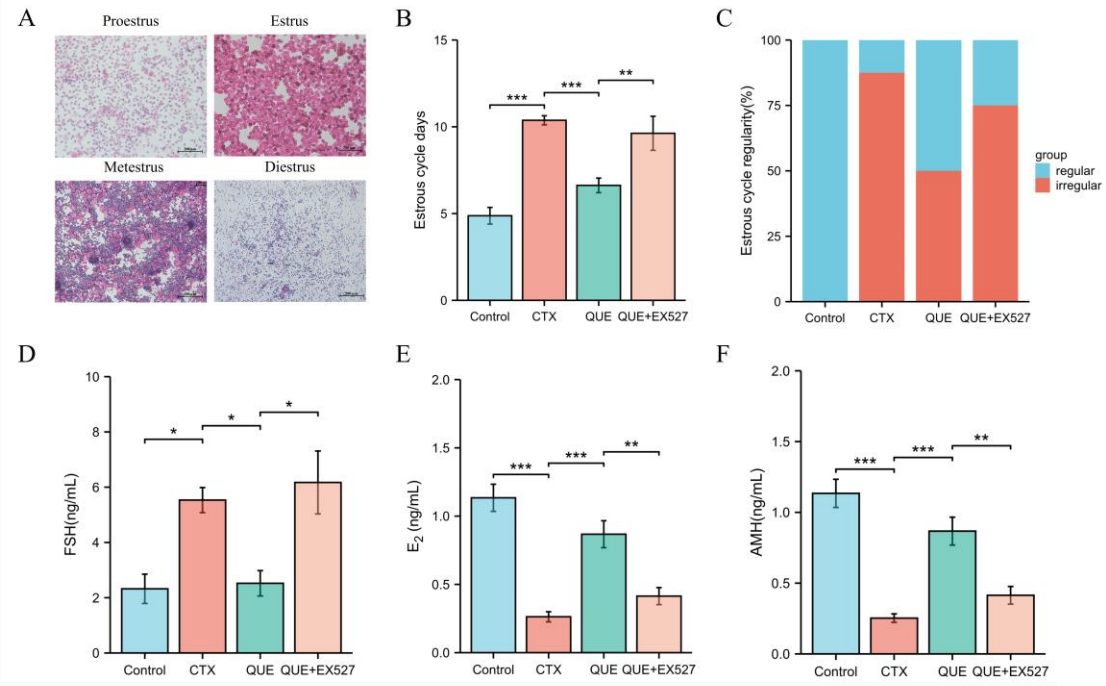


Figure 3. QUE ameliorates CTX-induced POI by upregulating SIRT1. A Representative diagrams of estrous cycles at different stages: Proestrus, Estrus, Metestrus, and Diestrus. Proestrus is characterized by a large number of nucleated epithelial cells and a small number of keratinized cells; Estrus consists of a large number of flattened keratinized epithelial cells; Metestrus shows coexistence of keratinized epithelial cells and white blood cells; Diestrus is dominated by a large number of white blood cells and a small amount of mucus (scale bar = 200 μm). **B** Number of estrous cycle days in each group (n=8). **C** Regularity of estrous cycles in each group (n=8). **D-F** ELISA-based serum levels of FSH, E_2, and AMH in each group of mice (n=5). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

QUE improves follicle quality in POI mice by upregulating SIRT1

The aforementioned experimental approaches confirmed QUE's regulation of sex hormones.

Nevertheless, given the ovary's dual functionality in endocrine secretion and gamete generation, follicular ontogeny was comparatively quantified for all developmental stages: primordial, preantral (primary/secondary), antral, and atretic follicles. After H&E staining, we compared the follicle conditions of each group under three magnifications (40x, 100x, and 200x) under a microscope (Fig. 4A).

Compared to the control group, the ovarian slices of the CTX and QUE+EX527 groups showed smaller ovarian volume, more atretic follicles, and significantly reduced numbers of primordial, preantral, and antral follicles, with loose granulosa cells arrangements. Compared to the CTX group, the QUE group exhibited increased numbers of growing follicles, reduced atretic follicles, and a denser granulosa cells arrangement. No statistically significant intergroup variations in corpus luteum numbers were detected. (Fig. S1A). These results indicate that QUE activates SIRT1 to improve follicle quality in POI mice.

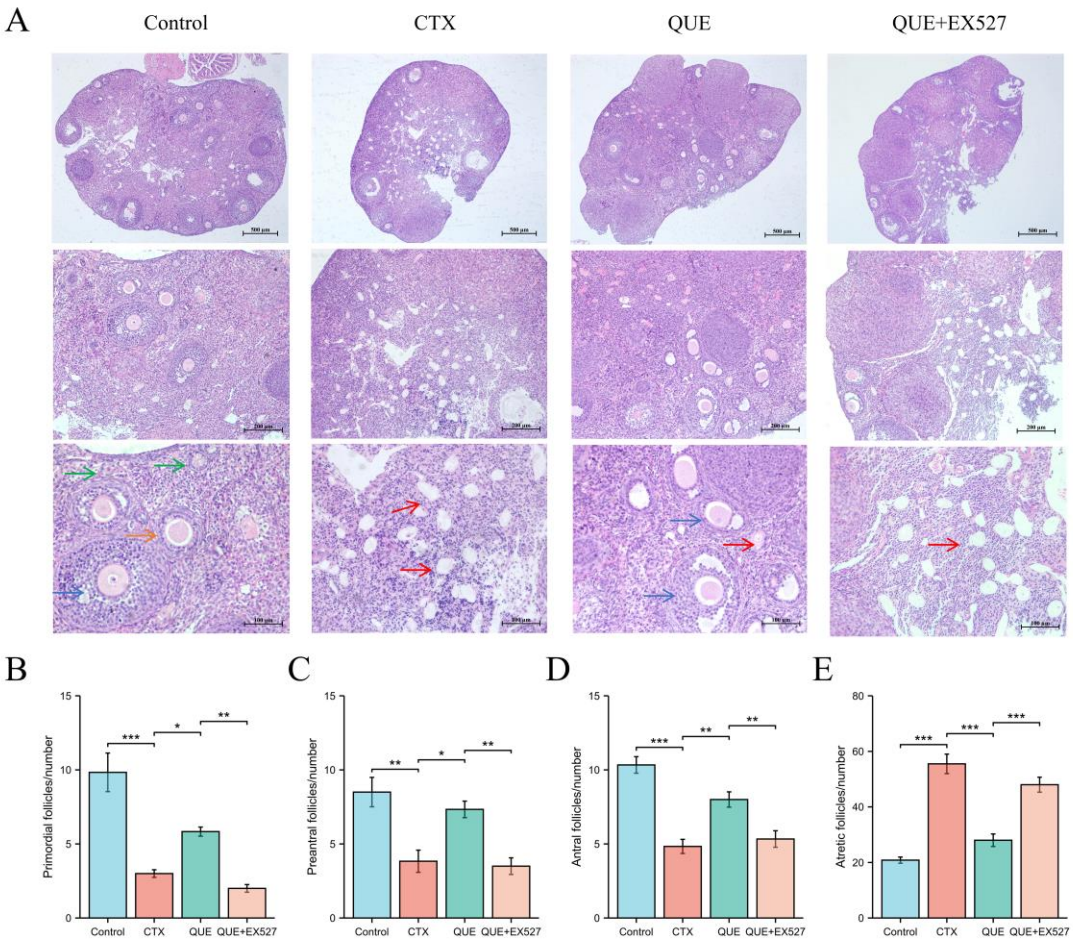


Figure 4 QUE improves follicle quality in POI mice by upregulating SIRT1. **A** Ovarian sections at magnifications of 40x, 100x, and 200x; **B-E** Numbers of primordial follicles, preantral follicles, antral follicles, and atretic follicles in groups of mice (n=6). Green arrows indicate primordial follicles; orange arrows indicate preantral follicles; blue arrows indicate antral follicles; red arrows indicate atretic follicles. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Network pharmacological analysis of QUE-improved POI-related targets and pathways

To further investigate the downstream pathways mediated by SIRT1 upregulation induced by QUE, a network pharmacology analysis was conducted. The study intersected the target points of

QUE and POI and presented the results using a Venn diagram. The results showed that QUE and POI shared 145 common gene targets (Fig. 5A). Subsequently, "Cytoscape " was used for PPI interaction network analysis to demonstrate the interconnections among the targets (Fig. 5B) and identify the Top 10 associated targets (Fig. 5C), including AKT, SRC, mTOR, NFκB, EGFR, GSK3B, and others.

To gain a more comprehensive understanding of the biological functions of these key targets, GO and KEGG pathway enrichment analyses were performed, and the results were visualized using bubble charts (Fig. 5D). GO analysis results indicate that QUE primarily exerts a protective effect on the ovaries through aspects such as protein serine/threonine kinase activity, positive regulation of phosphorylation, and the cellular secretory granule lumen. Additionally, the KEGG pathway enrichment analysis (Fig. 5E) reveals enrichment in the PI3K/AKT and HIF-1 pathways. The top 10 associated targets are closely related to the HIF-1α pathway, with AKT, mTOR, EGFR, PTK2, and GSK3B regulating the synthesis or stability of HIF-1α [22-25]. HIF-1α regulates the expression of NFκB and SRC [26, 27], while MMP2/9 are key effector molecules mediating HIF-1α-induced invasion and angiogenesis [28]. Interestingly, SIRT1, an upstream factor of HIF-1α, can influence the expression and activity of HIF-1α by regulating the AMPK and mTOR signaling pathways [29, 30]. Therefore, we hypothesize that QUE may alleviate POI in mice through the SIRT1/HIF-1α pathway.

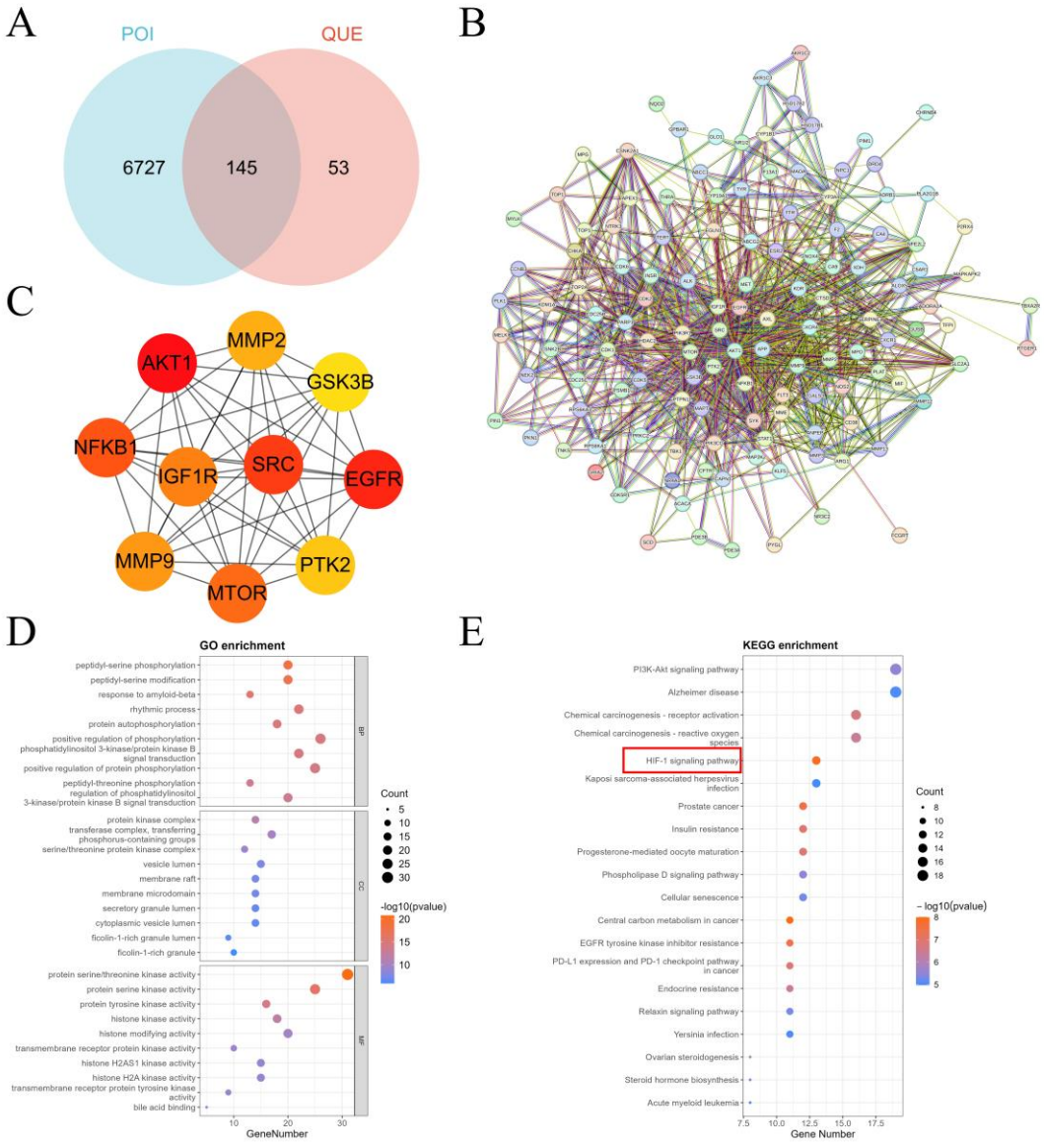


Figure 5 Network pharmacological analysis of QUE ameliorating POI-related targets and signaling pathways. **A** Venn diagram of QUE drug targets and POI disease targets. **B** Construct a PPI network using the STRING database and use Cytoscape for visualization. **C** The top 10 targets for QUE treatment of POI obtained from the MCC algorithm. **D** GO analysis of biological processes. **F** KEGG pathway analysis of signaling pathways.

QUE alleviates POI in mice via the SIRT1/HIF-α pathway

We performed immunohistochemistry on ovarian paraffin sections and Western blot analysis of mouse ovarian tissues to validate the roles of SIRT1 and HIF-1α in QUE treatment for POI. We found that SIRT1 is expressed not only in the ovarian cortex and medulla but also in granulosa cells and oocytes (Fig. 6A). After CTX treatment, SIRT1 expression was significantly reduced, while HIF-1α expression increased. The QUE+EX527 group showed a similar trend, whereas the QUE group showed the opposite trend (Fig. 6A, B, and C). Additionally, Western blot analysis of SIRT1 and HIF-1α expression levels confirmed the immunohistochemistry results (Fig. 6D, E). It was worth noting that, given the role of ovarian granulosa cells in nutrient supply and information transfer to oocytes, we observed the expression of SIRT1 and HIF-1α in granulosa cells of each group and found that their expression levels also showed differences. Therefore, we concluded that the protective effect of QUE on POI mice is mediated through the influence on granulosa cells via the SIRT1/HIF-1α pathway.

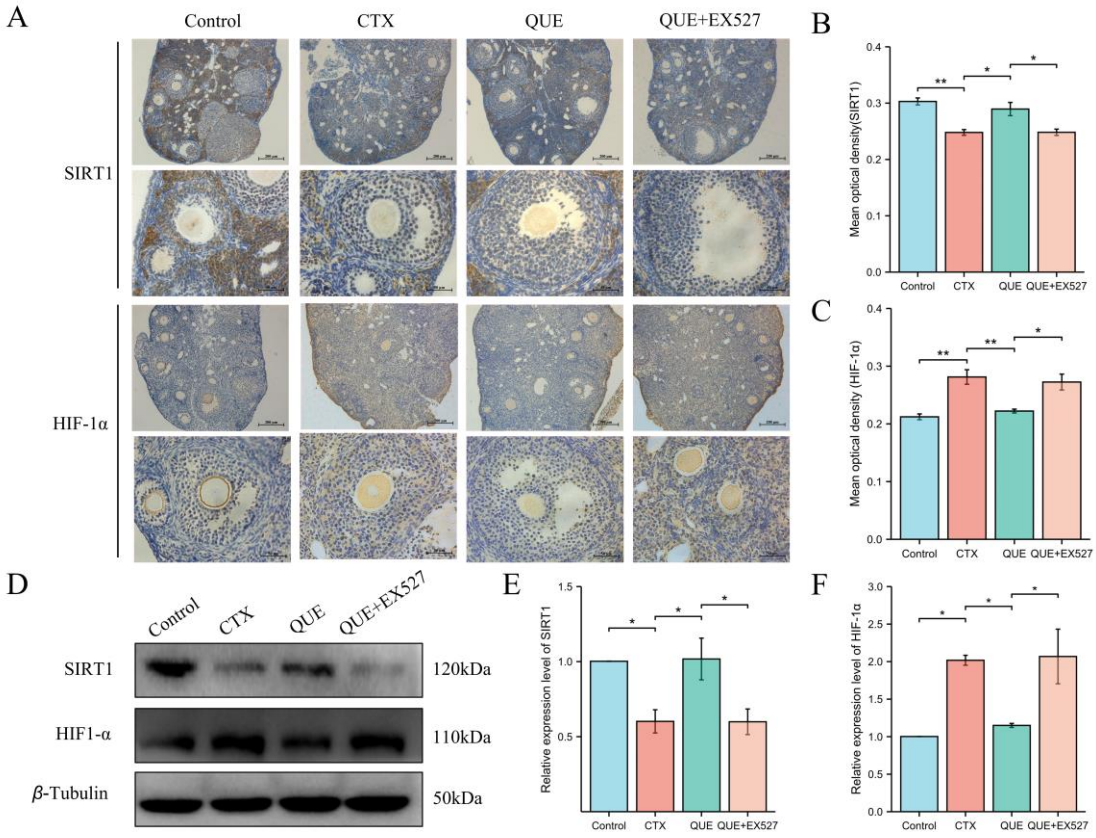


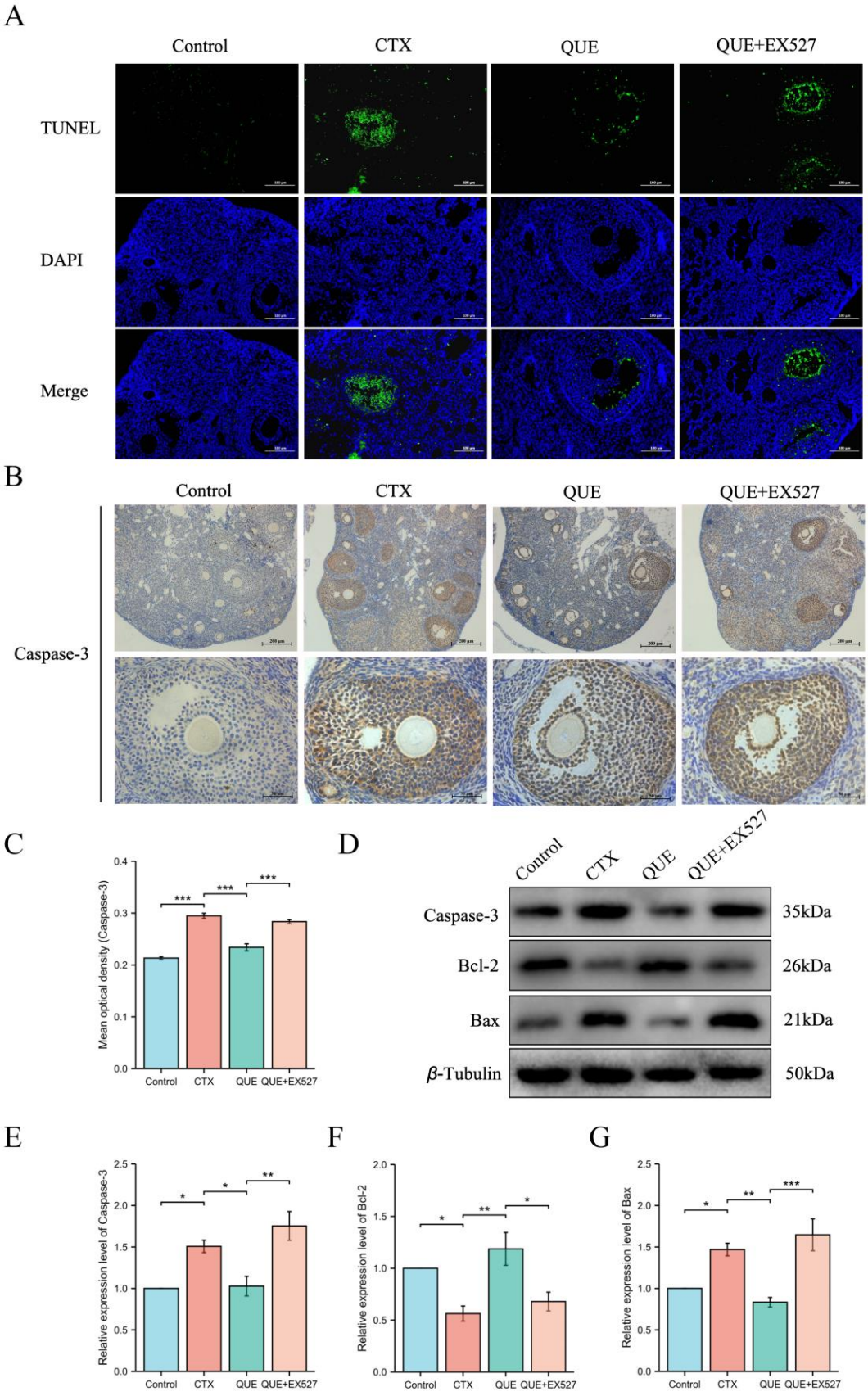
Figure 6 QUE alleviates POI through the SIRT1/HIF-1α pathway. A Representative immunohistochemical images of ovarian SIRT1 and HIF-1α expression at different magnifications (scale bar = 200 μm; scale bar = 50 μm). **B-C** Quantitative analysis of SIRT1 and HIF-1α expression ($n = 4$). **D** Representative Western blot images of SIRT1 and HIF-1α in each group of mice. **E-F** Quantification of protein expression levels of SIRT1 and HIF-1α ($n = 4$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

QUE protects against granulosa cells apoptosis in POI mice by modulating the SIRT1/HIF-1α pathway

Initially, we conducted a TUNEL assay to label apoptotic cells in ovarian tissue with CF488-dUTP. The results indicated that CTX promotes apoptosis, predominantly in ovarian granulosa

cells, whereas QUE can partially ameliorate this apoptosis in ovarian granulosa cells (Fig. 7A). Consistent with the TUNEL findings, the pro-apoptotic protein Caspase-3 is predominantly expressed in ovarian granulosa cells (Fig. 7B), with notable increases observed in both the CTX group and the QUE+EX527 group.

Furthermore, in addition to assessing the protein levels of Caspase-3 through Western blot experiments, we also examined the classic pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-2. The results indicated that QUE could mitigate the apoptotic effects of CTX on ovarian tissue to a certain extent. Nevertheless, the QUE+EX527 group, where SIRT1 was inhibited, was unable to fully exert the protective role of QUE against ovarian senescence (Fig. 7D-G). In summary, CTX primarily triggers apoptosis in ovarian granulosa cells, resulting in POI, whereas QUE alleviates granulosa cells apoptosis and exerts a protective effect on the ovaries.


A
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CTX
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Control
CTX
QUE
QUE+EX527
D
Control
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QUE
QUE+EX527
Caspase-3
35kDa
Bcl-2
26kDa
Bax
21kDa
β-Tubulin
50kDa
E
Relative expression level of Caspase-3
F
Relative expression level of Bcl-2
G
Relative expression level of Bax

395

Figure 7 QUE protects against granulosa cells apoptosis in POI Mice by Modulating the SIRT1/HIF-1α Pathway. **A** TUNEL staining of ovarian apoptosis (Scale bar=100 μm; green fluorescence indicates apoptotic cells). **B** Immunofluorescence images of Caspase-3 expression at different magnifications (Scale bar = 200 μm; scale bar = 50 μm). **C** Quantitative analysis of Caspase-3 expression ($n = 4$). **D** Representative Western blot images of Caspase-3, Bax, and Bcl-2 in each group of mice. **E-G** Quantification of protein expression levels of Caspase-3, Bcl-2 and Bax ($n = 4$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

QUE ameliorates senescence and apoptosis in human granulosa KGN cells

To further clarify QUE's functional mechanisms, we performed in vitro cultures of human ovarian granulosa cells, establishing CTX-induced cellular injury models. Initially, we investigated the impact of various CTX concentrations (0， 50, 100, 200, and 400 μmol/L) on KGN cell apoptosis (Fig. S1B) and selected a CTX concentration of 200 μmol/L with a 24-hour exposure period to establish the KGN cell injury model. Subsequently, based on the results of the preliminary assessment (Fig. S1C), we administered 10 μmol/L QUE and 10 μmol/L EX527 for 24 hours for subsequent experiments. Flow cytometry apoptosis assays and β-galactosidase staining results (Fig. 8A, B) demonstrated that CTX induced senescence and apoptosis in KGN cells, whereas a 24-hour QUE intervention markedly suppressed these effects. Similarly, EDU assay results (Fig. 8C) revealed that the CTX group exhibited notable proliferation defects with decreased cell proliferation rates. In contrast, the QUE group exhibited higher proliferation rates compared to the CTX group. Therefore, QUE inhibits CTX-induced senescence and apoptosis in KGN cells and

ameliorates proliferation defects. However, when SIRT1 was inhibited by EX527, the beneficial effects of QUE on KGN cells were significantly attenuated.

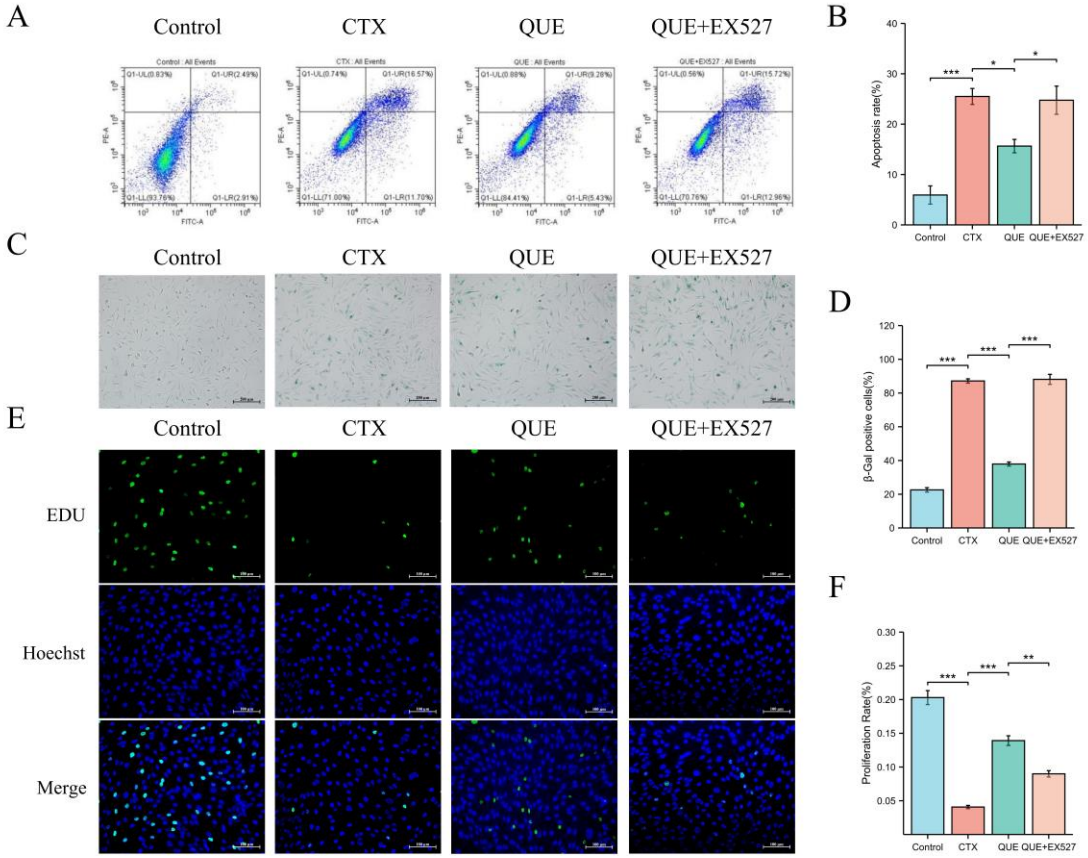

Figure 8 QUE ameliorates Senescence and Apoptosis in KGN Cells. **A** Flow cytometry analysis of apoptosis in KGN cells under different treatments. **B** Apoptosis rate quantification (n=4). **C** β-galactosidase staining of senescent KGN cells (blue indicates senescent cells, , scale bar = 200 μm). **D** Senescence rate quantification (n=4). **E** EdU proliferation assay (green fluorescence indicates proliferating cells, scale bar = 100 μm). **F** Proliferation rate quantification (n=3). *P < 0.05, **P < 0.01, ***P < 0.001.

QUE ameliorates senescence and apoptosis in human granulosa KGN cells via the SIRT1/HIF-1α pathway

In this study, we used Western blot to analyze differences in the expression levels of key pathway proteins (SIRT1 and HIF-1α) and apoptosis regulators (Caspase-3, Bax, and Bcl-2) in KGN cells (Fig. 9A). Mirroring the in vivo findings, QUE significantly counteracted CTX-induced effects, restoring SIRT1 levels while suppressing HIF-1α expression. Concurrently, QUE antagonistically regulated apoptosis-related proteins: elevating Bcl-2 expression while suppressing Caspase-3 and Bax levels. Notably, the QUE+EX527 group showed opposing protein expression trends compared to the QUE group. In summary, QUE ameliorate CTX-induced senescence and apoptosis in KGN cells by upregulating SIRT1 while downregulating HIF-1α, thereby correcting the dysregulation of apoptosis-related proteins.

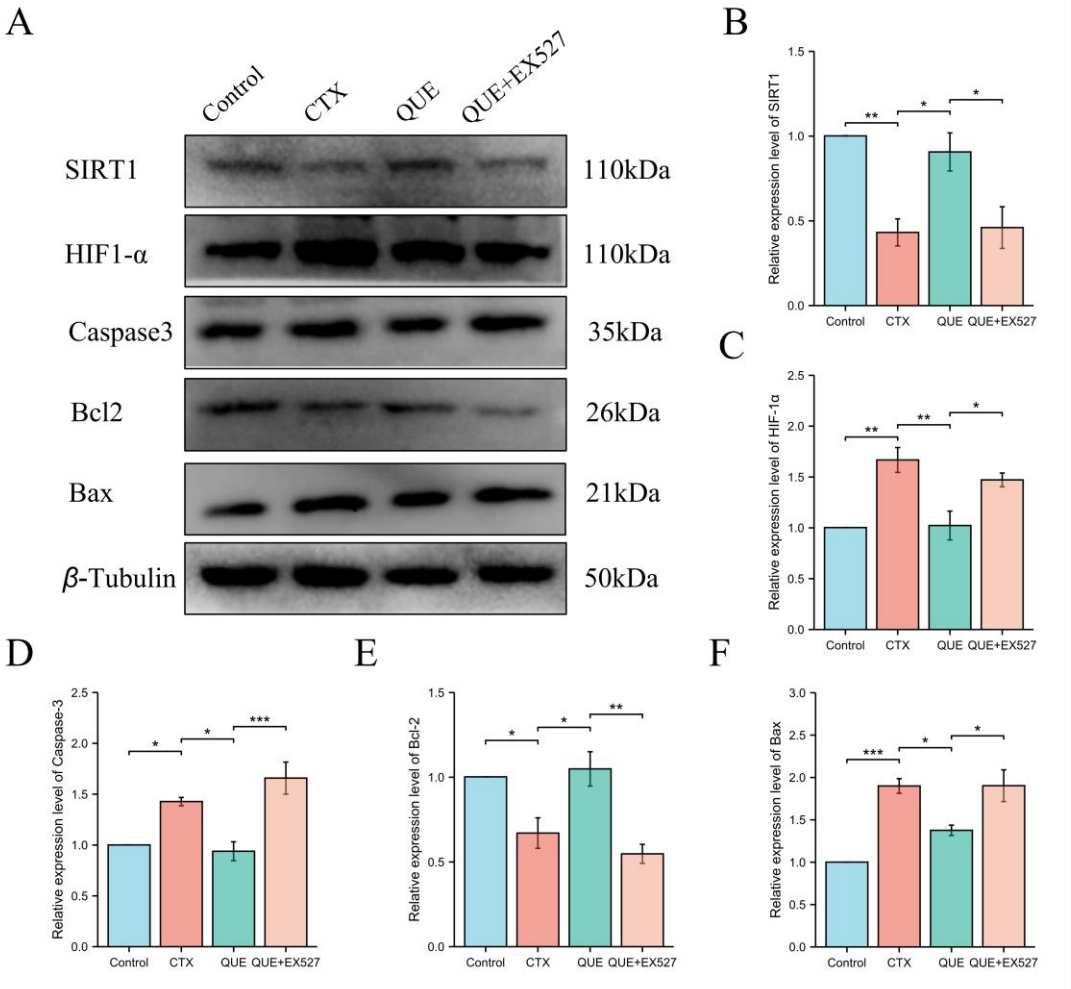

Figure 9 QUE alleviates senescence and apoptosis in KGN cells through the SIRT1/HIF-1α pathway. **A** Representative Western blot images of pathway proteins SIRT1, HIF-1α, and apoptosis-associated proteins Caspase-3, Bcl-2, and Bax in KGN cells. **B-F** Quantification of protein expression levels of SIRT1, HIF-1α, Caspase-3, Bcl-2, and Bax (n=4). *P < 0.05, **P < 0.01, ***P < 0.001.

Graphical Abstract

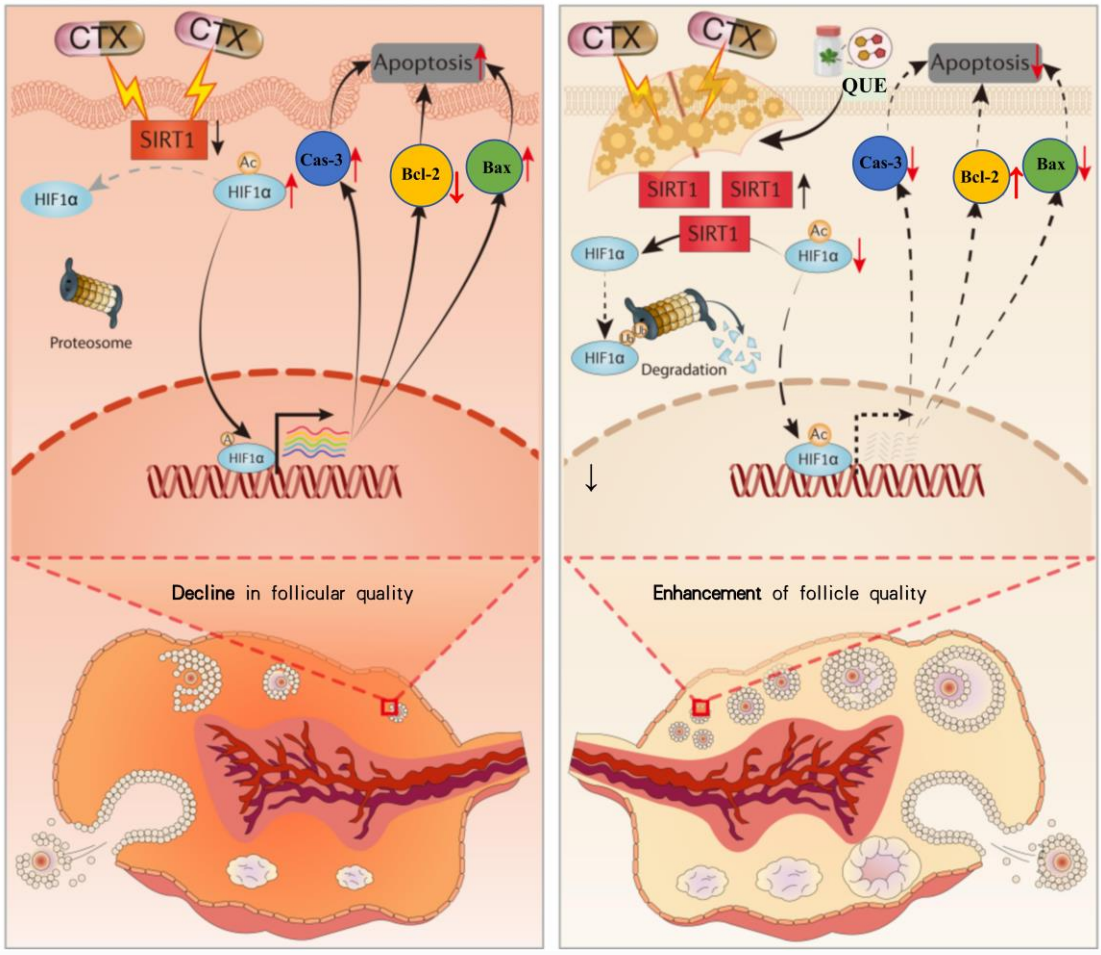

Figure 10 The mechanism of QUE in treating POI. Under the influence of CTX, the SIRT1 protein undergoes downregulation, which in turn activates the downstream factor HIF-1α. This activation promotes the expression of pro-apoptotic proteins

Caspase-3 and Bax, while simultaneously inhibiting the expression of the anti-apoptotic protein Bcl-2. Consequently, this cascade leads to apoptotic damage in ovarian granulosa cells, manifesting as decreased follicle quality (characterized by a reduction in normal ovaries and an increase in atretic follicles) and abnormal ovulation (left panel). In stark contrast, QUE, an active ingredient derived from traditional Chinese medicine, effectively upregulates SIRT1 and inhibits HIF-1α, thereby diminishing the expression of Caspase-3 and Bax while enhancing the expression of Bcl-2. This action mitigates apoptosis in ovarian granulosa cells, safeguards follicles, and enhances ovarian function (right panel).

Discussion

POI poses considerable risks to women and warrants utmost attention. It is influenced by a multitude of internal and external factors, encompassing genetics, autoimmune disorders, lifestyle habits, and cancer treatments like chemotherapy [31, 32]. CTX, a widely utilized alkylating agent in cancer therapy, is renowned for its potent antitumor effects. Its pharmacological mechanism primarily entails the formation of DNA crosslinks, which inhibits cell division and proliferation, ultimately leading to the demise of tumor cells. Nevertheless, CTX can directly impair ovarian tissue, inducing follicular apoptosis, particularly impacting primordial follicles [33]. Furthermore, metabolites of CTX, such as aldophosphamide, are regarded as primary sources of its toxic effects. These metabolites further exacerbate ovarian damage through oxidative stress and inflammatory responses [34]. In this experiment, we successfully established a mouse model of

POI by administering CTX through intraperitoneal injection. We observed a series of notable physiological alterations, including prolonged and disrupted estrous cycles, a significant elevation in FSH levels, a marked decrease in E_2 and AMH levels, an increased count of atretic follicles, and a corresponding decrease in the number of primordial, preantral, and antral follicles. These observations clearly indicate a substantial decline in ovarian function due to CTX.

QUE, one of the effective components of traditional Chinese medicines such as Flos Sophorae, dodder, mulberry leaves, and scutellaria, is a flavonoid compound widely present in plants and exhibits various biological activities. The antioxidant, anti-inflammatory, and antibacterial properties of QUE have garnered widespread attention in the fields of medicine and nutrition. Numerous studies have demonstrated that QUE can improve ovarian damage induced by CTX in mice and rats [35], and have verified its potential role in delaying aging and apoptosis to some extent [36]. Our research results also confirm the positive effects of QUE on the ovaries. After 21 days of oral treatment with QUE, we observed that the estrus cycle in the QUE group was significantly shorter than that in the CTX group, gradually becoming more regular. The level of the sex hormone FSH also decreased, while the levels of E_2 and AMH significantly increased. The number of atretic follicles decreased, while the numbers of primordial follicles, preantral follicles, and antral follicles increased. These results indicate that QUE can improve ovarian damage caused by CTX to some extent, demonstrating its potential therapeutic effect.

SIRT1, as a deacetylase, is involved in various cellular processes, including cell cycle regulation, metabolic regulation, and stress responses. HIF-1α is a key transcription factor for cellular responses to hypoxia, and it is regulated by SIRT1 through its deacetylation activity, controlling

the expression of genes related to cell metabolism, proliferation, and survival. SIRT1 activators, such as resveratrol, have been found to inhibit the overexpression of HIF-1α by enhancing SIRT1 activity, thereby alleviating hypoxia-induced cellular damage [37, 38]. Additionally, numerous studies have shown that the interaction between SIRT1 and HIF-1α extends beyond deacetylation, involving cross-talk among multiple signaling pathways. For instance, SIRT1 regulates the HIF-1α signaling pathway through Phd3, effectively reducing apoptosis and enhancing cardiomyocyte viability [39]；SIRT1 downregulates the phosphorylated extracellular signal-regulated kinase (p-ERK)/ERK pathway to modulate HIF-1α levels, increasing cerebral blood flow, improving neurological function, and reducing neuronal apoptosis [40]. In addition, the efficacy of QUE in ameliorating hypoxia-related disorders has been established, enhancing cellular adaptation to hypoxic conditions through modulation of SIRT1 expression [41, 42]. Despite numerous studies focusing individually on QUE's impact on SIRT1 and HIF-1α, the precise mechanism by which QUE influences these two factors to safeguard ovarian reserve function remains elusive. Consequently, this study pioneers the investigation into the relationship between QUE, the SIRT1/HIF-1α pathway, and premature ovarian aging. Initially, we evaluated serum SIRT1 levels across diverse groups, observing a marked decrease in SIRT1 levels in both serum and ovarian tissue of mice in the CTX group, whereas the QUE group exhibited a notable increase in SIRT1 concentrations and protein expression. This allows us to logically deduce that QUE exerts beneficial effects on ovarian function by upregulating SIRT1 expression. Further validation of QUE's mechanism was conducted using the SIRT1 inhibitor EX527, revealing that mice in the QUE+EX527 group failed to exhibit the same improvements in ovarian function as those in the

QUE-only group. Additionally, immunohistochemical and Western blot analyses confirmed that QUE upregulates SIRT1 while inhibiting HIF-1α, thereby conferring protective effects on the ovaries. This finding partially fills the research gap in the field of ovarian premature aging concerning SIRT1 and HIF-1α, offering new perspectives and directions for future studies.

Granulosa cells play a pivotal role in ovarian development and the sustenance of ovarian function. They not only facilitate the growth of oocytes but also modulate the physiological state of the ovaries by secreting hormones and cytokines. Research has demonstrated that the proliferation and differentiation of granulosa cells are essential for normal follicular development, whereas their dysfunction may result in ovarian endocrine abnormalities and a decline in ovarian reserve [43]. In patients with diminished ovarian reserve, granulosa cells exhibit an elevated apoptosis rate compared to those with normal ovarian reserve [44]. Numerous studies have indicated that QUE can markedly inhibit granulosa cells apoptosis, potentially paving the way for novel approaches and therapies in the treatment of related reproductive disorders [45]. The mechanism of action of QUE primarily revolves around its regulation of cellular signaling pathways, particularly exerting a significant impact on pathways associated with oxidative stress and apoptosis [46]. For instance, QUE can notably upregulate anti-apoptotic genes (such as Bcl-2) and downregulate pro-apoptotic genes (such as Bax and Caspase-3), thereby mitigating the incidence of apoptosis [47]; furthermore, by activating the autophagy pathway, QUE can afford additional protection to granulosa cells against oxidative damage and apoptosis [48]. These mechanisms offer a theoretical foundation for the application of QUE in safeguarding ovarian granulosa cells. Our experimental findings align well with these observations. The TUNEL assay conducted in this study revealed

that CTX triggers apoptosis, predominantly in granulosa cells, whereas apoptosis in the granulosa cells of the QUE group was markedly suppressed, hinting that QUE's protective effect on mice could be mediated through inhibiting granulosa cells apoptosis. Caspase-3, serving as the pivotal executor of apoptosis (programmed cell death), is responsible for the ultimate dismantling of cellular structures, propelling the apoptosis process into its irreversible phase [49]. In vivo experiments in this study employed immunohistochemical staining and Western blot analysis to detect alterations in Caspase-3 expression, revealing notable differences in Caspase-3 expression across groups, predominantly in ovarian granulosa cells. Intriguingly, apoptosis-associated proteins Bax and Bcl2 also exhibited corresponding expression variations. To delve deeper into the effects of QUE on ovarian granulosa cells, an in vitro model was established using KGN cells and CTX. Analogous to the outcomes from the in vivo animal model, QUE exhibited a pronounced ameliorative effect on granulosa cells senescence, apoptosis, and proliferation impairments. In summary, QUE inhibits granulosa cells senescence and apoptosis, thereby enhancing ovarian function and mitigating POI. These mechanisms provide a theoretical foundation for the utilization of QUE in protecting ovarian granulosa cells.

Although this study has demonstrated that QUE inhibits senescence and apoptosis in ovarian granulosa cells via the SIRT1/HIF-1α pathway, it still possesses certain limitations. KEGG analysis reveals that the PI3K pathway is similarly enriched in the context of QUE treatment for POI; however, it was not included in this study. Our research team aims to delve deeper into the relationships among SIRT1, HIF-1α, and PI3K in future endeavors. Furthermore, there is a notable absence of research on the mechanisms underlying HIF-1α activation or inhibition, necessitating

further expansion and refinement of the current data. Finally, QUE exhibits promising therapeutic effects in both in vivo and in vitro models, yet its clinical efficacy and safety await further validation.

Conclusion

QUE alleviates CTX-induced POI in mice and damage to the human ovarian granulosa cells line KGN. Mechanistic studies indicate that QUE regulates the expression of apoptosis-related proteins Caspase-3, Bax, and Bcl-2 by upregulating SIRT1 and inhibiting HIF-1α expression, thereby exerting anti-senescence and anti-apoptosis effects on ovarian granulosa cells and enhancing ovarian function.

Data Availability

Data and materials from this study are available on request.

Abbreviations

AMH: Anti-Müllerian Hormone

Bax: Bcl-2-associated X protein

Bcl-2: B-cell lymphoma 2

BP： Biological Process

Caspase-3: Cysteine-containing Aspartate Specific Protease-3

CC： Cell Component

CTX: Cyclophosphamide

EdU： 5-Ethynyl-2′-deoxyuridine

ELISA： Enzyme-linked immunosorbent assay

E_2： Estradiol：

FSH: Follicle-Stimulating Hormone

GO： **Gene Ontology**

H&E： Hematoxylin-eosin staining Staining()

HIF-1α: Hypoxia-Inducible Factor 1 Alpha

HRT： Hormone replacement therapy

KEGG： Kyoto Encyclopedia of Genes and Genomes

LH: Luteinizing Hormone

MF： Molecular Function

POI: Premature ovarian insufficiency

PPI： Protein-Protein Interaction

QUE： Quercetin

SIRT1: Sirtuin 1

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Contributions

Mei Wang: writing—review & editing, validation, writing—original draft. Hongling Chen: writing—review & editing, data curation. Shaozi Lin: writing—original draft, methodology. Zuyu Meng, Yiyao Zhang, Fang Liu, Menghai Li: data curation, validation. Chengjin Sun, Minxi Guan, Lingliang Song, Guoping Zhao: data curation, formal analysis. Di Zhang and Jiajia Qin: writing—review & editing, writing—original draft, funding acquisition. All authors read and approved the final manuscript.

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Ethics declarations

Ethics declarations and consent to participate

The study protocol was approved by the Animal Welfare and Ethics Committee of Jinan University (Approval No. IACUC-20241101-11).

Consent for publication

Not applicable.

Competing interests

780 The authors declare no competing interests.

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