



Protective Effects of Puerarin on Premature Ovarian Failure via Regulation of Wnt/ β -catenin Signaling Pathway and Oxidative Stress

Cheng Chen¹ · Song Li¹ · Cong Hu¹ · Weiwei Cao¹ · Qingfeng Fu¹ · Jia Li^{1,2} · Liping Zheng^{1,2} · Jian Huang^{1,2}

Received: 9 July 2020 / Accepted: 16 September 2020
© Society for Reproductive Investigation 2020

Abstract

This study was designed to investigate the protective effects of puerarin (PUE), which work via the Wnt/ β -catenin signaling pathway, and oxidative stress in the premature ovarian failure (POF) model. Two-month-old female mice were randomly divided into four groups. One group was used as the control, and the other three groups were injected with cyclophosphamide and busulfan to create POF models. Two POF treatment groups were gavaged with 100 or 200 mg/kg PUE for 28 days. Next, the ovaries were fixed, and the numbers of different stage follicles were measured, and the ovarian surface epithelium (OSE) was collected. Oct4 and Mvh expression, Wnt/ β -catenin signaling pathway activity, the oxidative stress factors SOD2 and Nrf2, and the apoptosis-related proteins Bcl-2 and Bax were detected by IHC, RT-QPCR, and western blotting. We found that the number of follicles, Oct4 and Mvh expression, and Wnt/ β -catenin-signaling activity were reduced in the POF groups ($p < 0.05$ or $p < 0.001$). After PUE treatment, the follicle number and the primordial follicle ratio increased ($p < 0.01$), while the atresia ratio decreased ($p < 0.01$). In addition, the expression levels of Oct4, Mvh, Wnt1, β -catenin, cyclin D1, SOD2, and Nrf2 showed obvious recovery compared with levels in the POF group ($p < 0.01$, $p < 0.05$, or $p < 0.001$). The Bcl-2/Bax ratio in the POF model had reduced by about 60% compared with the control group ($p < 0.001$) and improved by about 50% after PUE treatment ($p < 0.001$). In conclusion, PUE may improve the survival of female reproductive stem cells (FGSCs) and play a protective role against POF via a mechanism involving the Wnt/ β -catenin signaling pathway, as well as relieving oxidative stress. Further investigations should focus on the culture of oocytes and FGSCs in vitro in a PUE environment with inhibitors or agonists of the Wnt signaling pathway.

Keywords Premature ovarian failure · Female reproductive stem cells · Puerarin · Wnt/ β -catenin pathway · Oxidative stress

Introduction

Premature ovarian failure (POF) is defined as the cessation of ovarian function before the age of 40 and is characterized by a low level of estrogen and upregulated gonadotropin. The POF

can lead to a decline in follicle number and quality, menstrual disorders, and infertility [1–3]. Iatrogenic factors account for about 25% of POF cases. However, in the majority of cases, the cause remains undetermined [4, 5]. Chemotherapy, an important cause of POF, is a routine treatment for women of

✉ Jian Huang
598490492@qq.com

Cheng Chen
chengchenc@163.com

Song Li
1602439272@qq.com

Cong Hu
cmb1998@hotmail.com

Weiwei Cao
936538352@qq.com

Qingfeng Fu
15166308125@163.com

Jia Li
490780258@qq.com

Liping Zheng
zhengliping@ncu.edu.cn

¹ Jiangxi Medical College Nanchang University, Jiangxi Province 330006 Nanchang, China

² The Key Laboratory of Reproductive Physiology and Pathology of Jiangxi Provincial, Nanchang University, Nanchang 330031, Jiangxi Province, China

reproductive age with cancer [6, 7]. Therefore, it is urgent to seek effective treatments for chemotherapy-induced POF. The classic chemotherapy drugs cyclophosphamide and busulfan (CTX/BU), which are often used to cure cancer in the clinical setting, have serious reproductive toxicity and are used to induce POF in the animal model [8].

In 2004, Johnson et al. discovered a phenomenon that female mammals potentially produced oocytes from birth to adulthood, which was shocking and revolutionized the traditional academic theory [9]. Since then, researchers have confirmed the existence of female reproductive stem cells (FGSCs) in postnatal ovaries in a variety of mammals, including humans, by methods such as the *in vitro* culture and transplantation of stem cells, genetic modification and *in vivo* lineage tracking [10–14]. FGSCs are located in the superficial epithelium of human and mouse ovaries [15]. Specific markers for germ cells and stem cells, such as *Mvh*, *Oct4*, *Sox-2*, *Nanog* and *Vasa*, can be detected in FGSCs [16–19]. The mouse *vasa* homolog (*Mvh*) protein, which can be regarded as a germ-cell marker, only expresses in primordial germ cells after colonization of embryonic gonads and in germ cells after meiosis [20]. *Oct4*, a member of the POU transcription factor family, is mainly expressed in embryonic and reproductive stem cells and plays an important role in maintaining the pluripotency and self-renewal of embryonic stem cells [21]. Researchers have found that activating FGSCs produce oocytes to slow down ovarian aging and protect ovary function. Furthermore, FGSCs may repair damaged ovaries by differentiating into oocytes or inhibiting follicular apoptosis by paracrine signaling [22]. But the mechanism is rarely reported. Previous studies in our laboratory have found that the Hippo and Notch- signaling pathways are associated with this process [23, 24]. Increased levels of immune factors inhibit stem cell reproduction, indicating that the immune system also participates in the survival of ovarian germline stem cell niches to protect ovarian function and delay ovarian aging [25]. Under appropriate conditions, the resting follicles in the aging or damaged ovaries may produce new oocytes [26]. In this study, *Mvh* and *Oct4* were used as germ cell and stem cell markers, respectively, and were used to evaluate the levels of ovarian function and FGSCs survival.

Puerarin (PUE), the main active ingredient of puerarin isoflavone, has a similar chemical structure to phytoestrogen and has been widely used in the clinical setting. It has various pharmacological functions, such as vascular endothelium protection, antioxidant, anti-aging, anti-tumor, and estrogenic activities [27–30]. Huang et al. found that the combined application of vitamin D and PUE improved the anti-hepatic fibrosis effect and reduced CCl₄-induced liver fibrosis in rats through the Wnt1/ β -catenin pathway [31]. Lin et al. treated ischemia-reperfusion injury (IRI) mice with PUE and found there was downregulation of mast cell chymase, TGF- β , and TGF- α . The uterine fibrosis caused by IRI was improved by regulating the α -SMA and Wnt/ β -catenin pathway [32]. Zheng

et al. found that PUE stimulated osteoblast differentiation and bone formation via the Wnt/ β -catenin pathway [33]. These studies showed that PUE might affect tissues and organs via the Wnt/ β -catenin pathway. However, whether PUE has a similar influence on ovaries needs to be clarified.

The Wnt signaling pathway is a highly conservative pathway composed of the Wnt family secreted protein, the Frizzled (FZD) family, β -catenin, and others. Activating the Wnt/ β -catenin pathway may lead to oxidative damage of cellular DNA and upregulation of the P53/P21 pathway, which is involved in the senescence of hematopoietic stem/progenitor cells [34]. A study demonstrated that the Wnt pathway has significant effects on differentiation, homeostasis, and aging of the kidney, bone, and intestinal tissues [35]. More recent studies have shown that the Wnt/ β -catenin pathway is essential for the growth and development of the female reproductive system. Naillat et al. found that mice lacking *Wnt4* had poor ovarian development and fewer healthy follicles [36]. The Wnt/ β -catenin pathway is significantly involved in the production of ovarian steroid control by FSH and LH. The normal expression of β -catenin in ovaries can continually enhance FSH secretion to promote follicular development and reduce follicular granulosa cell apoptosis [37, 38]. The mechanisms of the Wnt/ β -catenin signaling pathway in the ovary have gradually become a focus in recent years.

Promoting the surrounding micro-environment is good for stem cell proliferation. Reactive oxygen species (ROS) produced by oxidative stress can induce cell damage and inhibit stem cell growth [39]. Superoxide dismutase (SOD), as an antioxidant enzyme, can effectively remove ROS [40]. Nuclear factor erythroid 2-related factor (Nrf2) is responsible for the regulation of the cellular response to oxidation [41]. The levels of SOD2 and Nrf2 indirectly indicate the presence of oxidative stress in the tissues. Furthermore, the Bcl-2 family of proteins is involved in regulating cell life and death [42]. Bcl-2 protein is a member of the cytoprotective Bcl-2 family, and Bax is another pro-apoptotic protein. The rheostat model suggests that the ratio of pro-apoptotic factor Bax to anti-apoptotic factor Bcl-2 determines cell fate [43], and the ratio is used to assess cell apoptosis in this study.

In this study, we intended to clarify the therapeutic effect of PUE on ovary function by modulating the Wnt/ β -catenin pathway and regulating the oxidative stress levels of the micro-environment surrounding the FGSCs. Hopefully, this research can provide new treatment avenues for POF.

Materials and Methods

Animals and Treatment

Two-month-old female mice (25–30 g, level SPF of the Kunming strain) were obtained from the Laboratory Animal

Center of Jiangxi University of Traditional Chinese Medicine. After 3 days of acclimatization, the POF models were made by intraperitoneal injection of CTX (120 mg/kg, Sigma, St. Louis, MO, USA) and BU (30 mg/kg, Sigma) for 4 weeks of observation. Then PUE was dissolved in physiological saline, and mice were gavaged for 28 days. These mice were divided into three groups, which received gavages of either saline, low-concentration PUE (100 mg/kg), or high-concentration PUE (200 mg/kg). The weights of the mice and ovaries were measured and recorded on the last day of treatment. All mice were housed in 12-h dark and 12-h light environments with suitable temperature and an adequate supply of food and water. The treatments of all mice conformed to the guidelines of the Animal Ethics Committee of Nanchang University (NO. SYXK 2015-0001).

Hematoxylin-Eosin Staining and Follicle Counting

The ovaries were isolated and fixed in 4% paraformaldehyde overnight and embedded in paraffin. The ovarian morphological characteristics were observed after staining with H&E, and the number of follicles at each developmental stage was calculated by referencing the standard follicular classification described by Chen et al. [44]. The atresia ratio (the proportion of atresia follicles to total follicles) and the primordial follicle ratio (the proportion of primordial follicles to total follicles) were also calculated.

Immunohistochemical Staining

Sections were dried in a constant temperature oven at 55 °C for 2 h to ensure the tissue adhered to the slides. Then, slices were dewaxed in xylene, rehydrated in graded ethanol solutions, placed in citrate buffer (pH 6.8), and repaired in a microwave oven at medium-high heat for 5 min. When the temperature was below 85 °C, they were repaired at medium-low heat for 2.5 min, and the above steps were repeated. Then, sections were incubated in 3% hydrogen peroxide to inactive endogenous peroxidase for 10 min and in 5% BSA to block non-specific reactions for 60 min. Sections were incubated in primary antibody (anti-mouse/anti-rabbit IgG) and diluent overnight at 4 °C and in streptomycin-labeled IgG followed by horseradish peroxidase for 15 min the following day. Finally, the sections were incubated with fresh DAB developer (ZSGB-BIO, China) for 6 min to detect the positive signal and then dyed with hematoxylin. The PBS solution was used as the negative control for the primary antibody.

Reverse Transcription-Quantitative PCR

The ovarian epithelial tissues and cells were collected as much as possible under the stereomicroscope by a cell brush, and total RNA was extracted by TRizol (ER501-01, TransGen

Biotech, China). We obtained cDNA by reverse transcription, according to the method of PrimeScript RT kit (R047A, TAKALA, Shiga, Japan), and conducted RT-QPCR according to the instructions of the TB Green Mix Kit (R820A, TAKALA). Table 1 lists the RT-QPCR primer sequences, and the internal reference used in this study was the house-keeping gene GAPDH.

Western Blotting

Total protein was extracted with RIPA lysis buffer (Applygen, Beijing, China), and concentrations were measured using the BCA kit (Applygen). The protein was subjected to polyacrylamide gel electrophoresis and transferred to a PVDF membrane. Membranes were blocked with 5% BSA for 1 h and then incubated overnight at 4 °C with the primary antibodies: Mvh (ab27591, Abcam, Cambridge, UK), Oct4 (ab18976, Abcam), β -catenin (WL0962a, Wanlei, China), SOD2 (WL02506, Wanlei), Bcl-2 (WL01556, Wanlei), Bax (WL01637, Wanlei), Nrf2 (WL02135, Wanlei), and GAPDH (ab181602, Abcam). After incubation of the secondary antibody (Affinity Biosciences, Cincinnati, USA), the blots were imaged using the Fluorescent Coloring Kit (DW101-01, TransGen Biotech). Then the images were scanned with the image analyzer AI600 and analyzed using ImageJ Software.

Table 1 Primer sequences used for quantitative real-time PCR

Genes	Primer Sequence (5'-3')
β -catenin	Forward: AACGGCTTTCGGTTGAGCTG Reverse: TGGCGATATCCAAGGGCTTC
CyclinD1	Forward: GATGAGGA AGAGTTGCTAGAAGAG Reverse: TCGTCAGCCAATCGGTAGTAG
Wnt1	Forward: GGTTTCTACTACGTTGCTACTGG Reverse: GGAATCCGTCAACAGGTTCTGT
Mvh	Forward: GTGTATTATTGTAGACCAACTCG Reverse: CACCTTGTACTATCTGTGCGAACT
Oct4	Forward: AGCTGCTGAAGCAGAAGAGG Reverse: GGTTCTCATTGTTGTCGGCT
Nrf2	Forward: CTTTAGTCAGCGACAGAAGGAC Reverse: AGGCATCTTGTTGGGAATGTG
SOD2	Forward: ACAACTCAGGTCGCTCTTCA Reverse: GAACCTTGGACTCCCACAGA
Bcl-2	Forward: GAACCTGGGGAGGATTGTGG Reverse: GCATGCTGGGGCCATATAGT
Bax	Forward: GAACCATCATGGGCTGGACA Reverse: AGCCACCCTGGTCTTGGAT
GAPDH	Forward: CGTGCCGCTGGAGAAACCTG Reverse: AGAGTGGGAGTTGCTGTTGAAGTCG

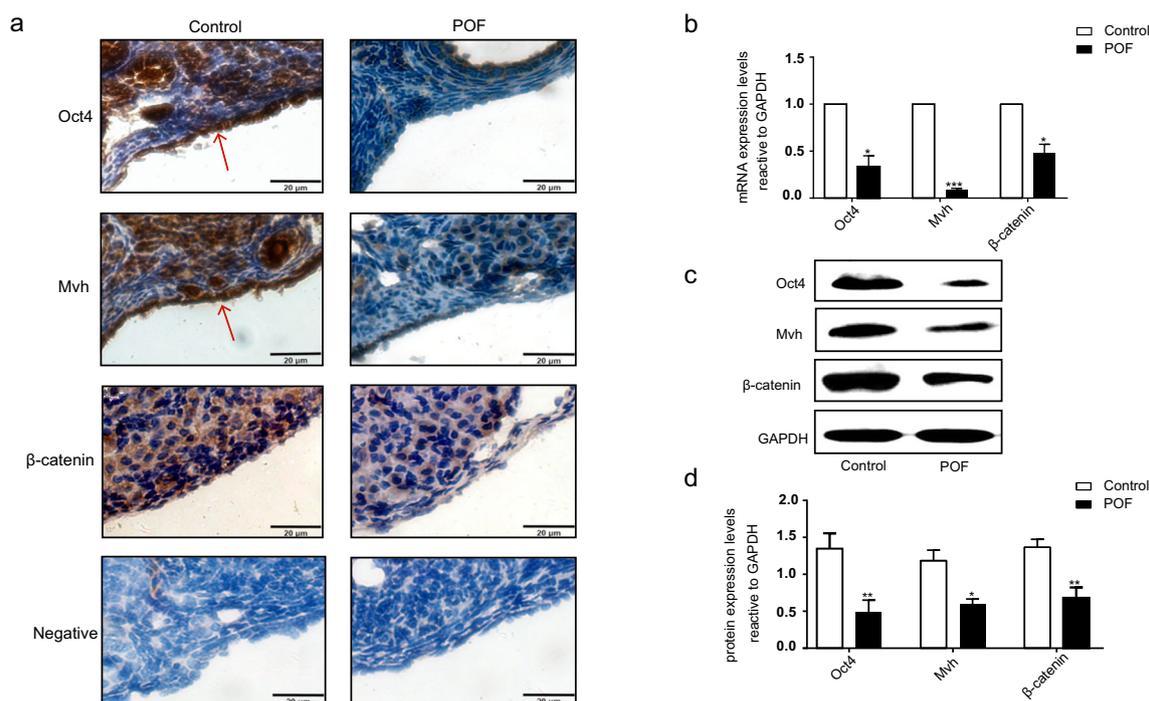


Fig. 1 Changes in Oct4, Mvh, and β-catenin expression in POF models. **a** Expression of Oct4, Mvh, and β-catenin in the ovaries visualized by IHC. FGSCs are indicated by red arrows; the scale bar represents 20 μm.

b The expression of Oct4, Mvh, and β-catenin mRNA in the ovaries. **c, d** The expression of Oct4, Mvh, and β-catenin proteins in the ovaries. (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. the control group)

Statistical Analysis

The experimental results are shown as mean \pm SEM, and we performed the statistical analysis on GraphPad Prism 7 software. A *t* test was used to detect the statistical difference between two groups, and one-way ANOVA was used for multiple groups of data. A *p* value of < 0.05 was considered statistically significant, and the experiments were repeated at least three times.

Results

Expression of Oct4, Mvh, and β-catenin in POF Models

The IHC images show that the brown reaction products of Oct4 and Mvh were distributed in the OSE, where the FGSCs are located. The marker of the Wnt/β-catenin pathway, β-catenin, was distributed in the cell membrane, cytoplasm, and nucleus of the ovary. The follicles and FGSCs in the POF groups were rarely detected. Compared with the control group, the expression of Oct4, Mvh, and β-catenin in the POF groups was obviously decreased (Fig. 1a). The Oct4, Mvh, and β-catenin mRNA levels were also decreased in the POF groups (Fig. 1b, $p < 0.05$ or $p < 0.001$). A similar trend was detected for the relative protein expression levels by western blotting (Fig. 1c–d, $p < 0.05$ or $p < 0.01$).

Effect of PUE on POF Models

Morphological comparisons were conducted between the control, POF, POF + PUE (100 mg/kg), and POF + PUE (200 mg/kg) groups after the intragastric administration of PUE for 28 days. The ovaries were significantly larger in the two POF + PUE groups compared with the POF group (Fig. 2a). H&E staining showed that all follicles stages were in the PUE-treated groups, but follicles were rare in the POF group. The number of follicles in each of the POF + PUE groups was lower than that in the control group but higher than that in the POF group (Fig. 2b). The numbers of primordial, primary, secondary, and antral follicles were elevated in the POF + PUE groups, while the number of atresia follicles was declined, especially in the PUE 200 mg/kg group (Fig. 2c–e, $p < 0.05$, $p < 0.01$, or $p < 0.001$). In addition, higher expression levels of Mvh and Oct4 were seen in the PUE groups compared with those in the POF group, especially the PUE 200 mg/kg group (Fig. 2f–h, $p < 0.05$, $p < 0.01$, or $p < 0.001$).

PUE Improved the Activity of the Wnt/β-catenin Pathway in POF Models

As shown in Fig. 3a, the positive β-catenin expression in the POF + PUE groups was higher than in the POF group. With the increase in the PUE dosage, the reaction products increased. After PUE treatment, the mRNA expression levels of

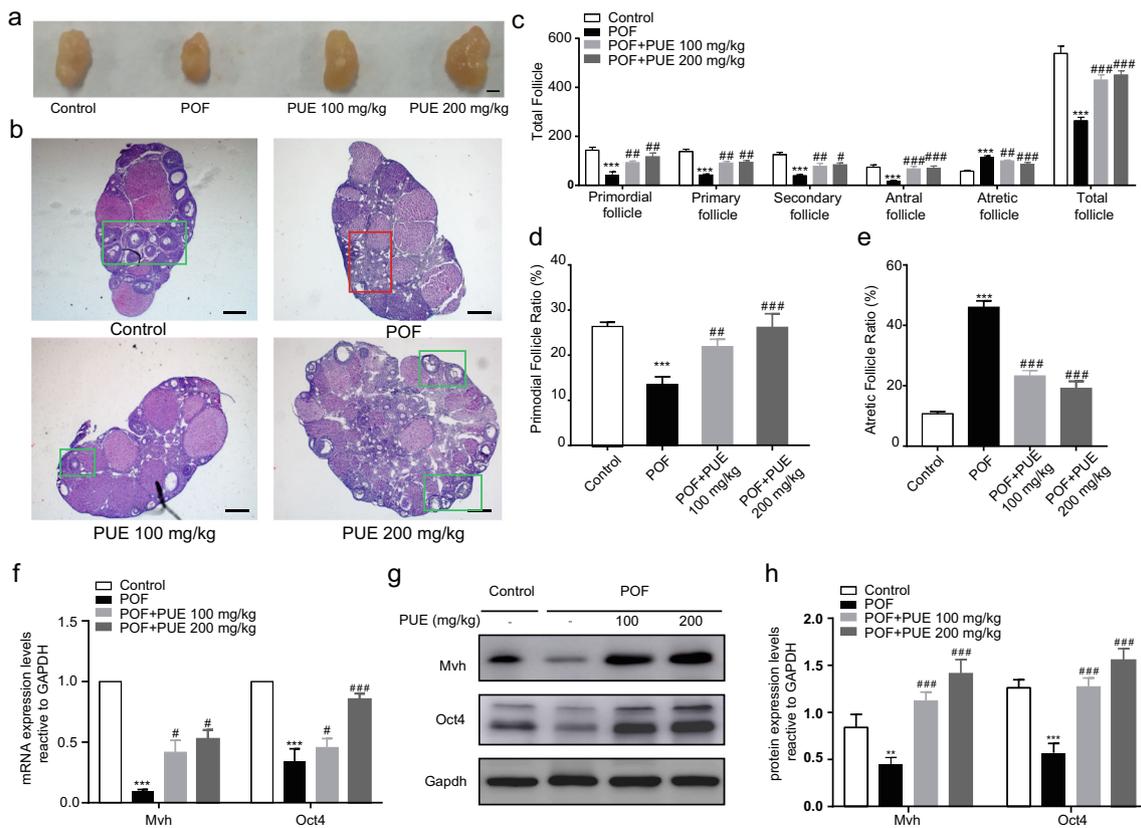


Fig. 2 Effect of PUE on POF models. **a** Morphological comparison of ovaries in control, POF, POF + PUE 100 mg/kg, and POF + PUE 200 mg/kg groups; the scale bar represents 1 mm. **b** H&E staining of the ovaries in the four groups; the scale bar represents 200 μ m. **c** Follicular counting for different follicle stages in the ovaries. **d**, **e** The

ratio of the primordial and atresia follicles in the ovaries. **f–h** mRNA and protein expression of Mvh and Oct4 in the four groups. (** $p < 0.01$ and *** $p < 0.001$ vs. the control group; # $p < 0.01$, ### $p < 0.001$ vs. the POF group)

cyclinD1, Wnt1, and β -catenin were higher than those in the POF group, especially for β -catenin in the POF + PUE 200 mg/kg group (Fig. 3b, $p < 0.05$, $p < 0.01$, or $p < 0.001$). Moreover, western blotting experiments showed that β -catenin protein expression was gradually upregulated as the PUE dosage increased (Fig. 3c–d, $p < 0.05$, $p < 0.01$, or $p < 0.001$).

PUE Reduced Oxidative Stress in the POF Models

RT-QPCR and western blotting showed that the expression of the antioxidative factors SOD2 and Nrf2 decreased in the POF groups compared with the control group. In addition, the anti-apoptotic protein Bcl-2 was downregulated in the POF model, while the apoptotic protein Bax was upregulated. After PUE treatment, the trend was reversed. PUE elevated SOD2, Nrf2, and Bcl-2 but reduced Bax. The treatment showed varying degrees of recovery, especially in the PUE 200 mg/kg group (Fig. 4a–c, $p < 0.05$, $p < 0.01$, or $p < 0.001$). Additionally, the mRNA- and protein-expression ratios of Bcl-2/Bax in the POF model were reduced by about 60% compared with those in the control group, and the ratios

improved by about 50% after PUE treatment (Fig. 4d, e, $p < 0.001$).

Discussion

In the POF model, we observed a reduction in the ovarian volume, a decrease in the number of dominant follicles, a significant increase in the number of atresia follicles, and a decline in the expression of Mvh and Oct4; thus, the POF model was successfully established. Recent studies have shown that female animals can replenish their follicles from FGSCs after birth [10, 45–47], and an increasing number of studies are focusing on effective methods to cure POF. Resveratrol has been proven to play a protective role against POF and improve FGSCs survival via antioxidative stress [48]. In this study, we explored the effect of PUE on the mouse POF model.

PUE’s main active ingredient is flavonoid glycoside. The latest research shows that PUE can significantly inhibit inflammation by downregulating the secretion of nuclear factor- κ B (NF- κ B) and pro-inflammatory mediators,

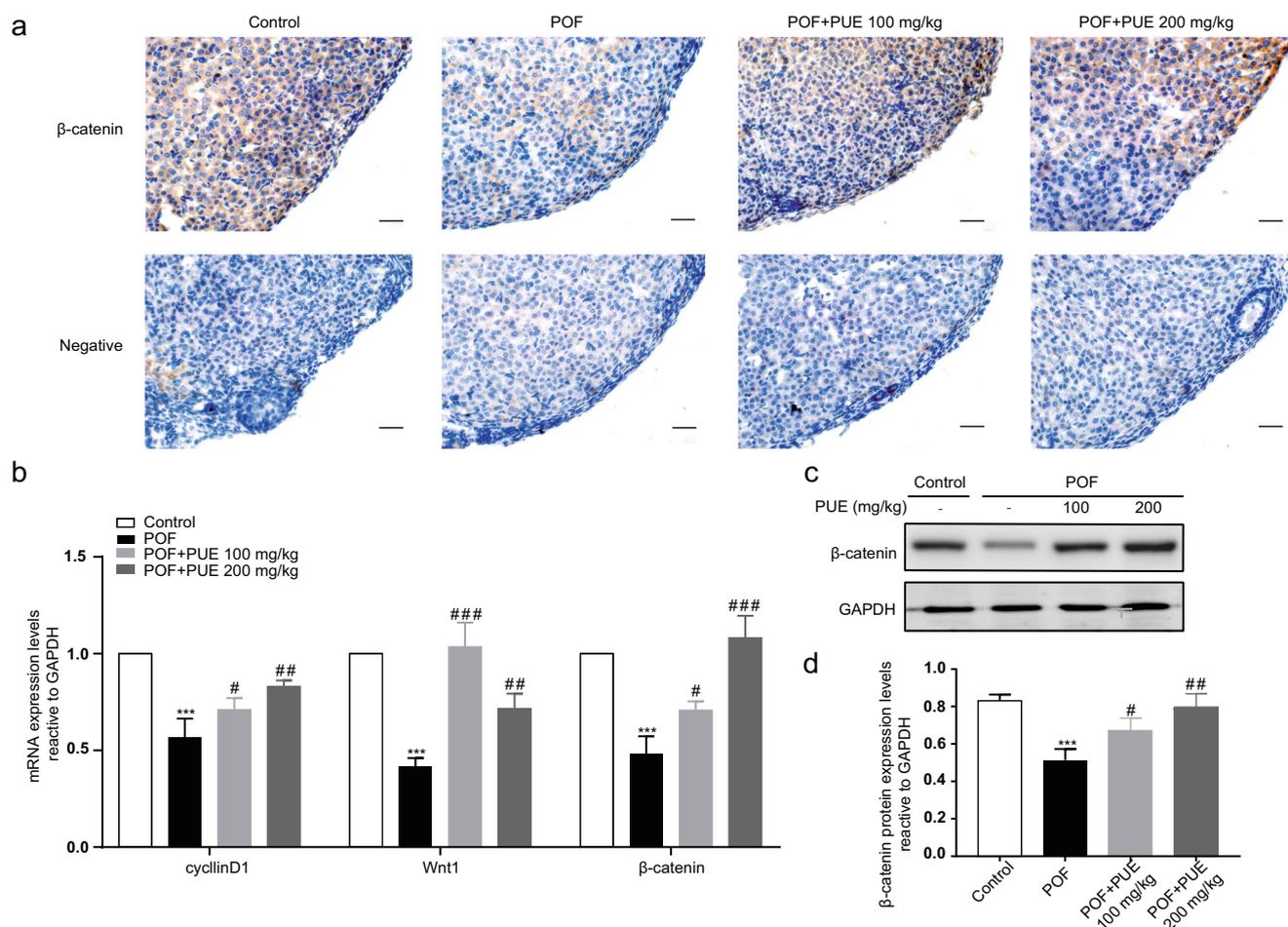


Fig. 3 Changes in Wnt/ β -catenin in the POF + PUE groups. **a** The expression of β -catenin in ovaries by IHC. The scale bar represents 20 μ m. **b** Expression of cyclinD1, Wnt1, and β -catenin mRNA in ovaries

as shown by RT-QPCR. **c, d** Expression of the β -catenin protein by western blotting. (***) $p < 0.001$ vs. the control group; # $p < 0.05$, ## $p < 0.01$, or ### $p < 0.001$ vs. the POF group)

regulating the NF-E2 p45-related factors 2 (Nrf2) pathway and the expression of antioxidant enzymes. Furthermore, PUE has been shown to have antioxidant effects [49]. PUE can eliminate the immune damage of POF and restore ovarian function, but the detailed mechanism is still undefined [50]. We used Mvh and Oct4 as indicators to evaluate the survival of FGSCs in mice. After PUE treatment, there was an obvious change in the ovarian morphology of the mice. The proportion of follicles in the POF + PUE groups was lower than those in the control group but was higher than those in the POF group. Besides, an upregulation of Mvh and Oct4 expression was detected at the genetic and protein level, indicating that PUE might maintain the survival of FGSCs to protect ovarian function. The Wnt/ β -catenin pathway plays an essential role in cell proliferation, differentiation and apoptosis. When the classic Wnt pathway is activated, Wnt ligands combine with the FZD receptor and low-density lipoprotein receptor-related proteins (LRP) 5/6 to form a trimer. LRP phosphorylates and activates Dsh, and glycogen synthase kinase-3 β is inhibited. β -catenin

is dephosphorylated, released, and converted into unbound β -catenin; it accumulates in the cytoplasm where it is transported to the nucleus to target regulatory genes (e.g., VEGF, survivin) [51]. Upregulation of Wnt transcription can activate the Wnt/ β -catenin signaling pathway to reduce inflammatory responses and cell apoptosis, relieve ischemia-reperfusion injury, and thus protect the liver [52]. Similarly, the activity of the Wnt/ β -catenin signaling pathway in the POF model was significantly reduced compared with the control group. β -catenin in the ovaries has been demonstrated to continuously enhance FSH secretion to promote follicular development [37]. We found that β -catenin expression was significantly lower in the POF group than in the control group, indicating that the Wnt/ β -catenin pathway was downregulated in the POF model and may have a certain effect on ovarian function. To further investigate, we detected the Wnt-pathway-related protein markers β -catenin, Wnt1, and the downstream protein cyclinD1 and found they were upregulated to different extents after PUE treat. Furthermore,

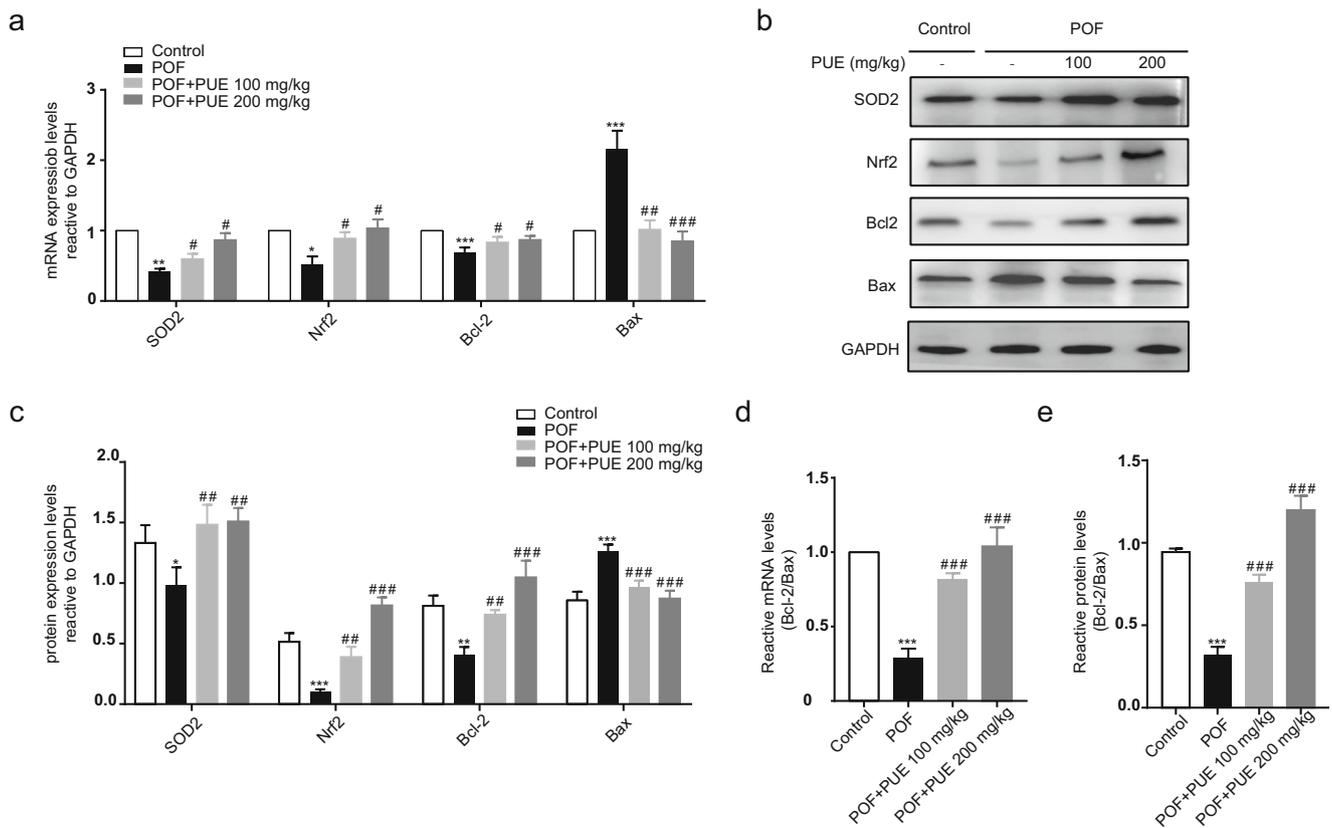


Fig. 4 Expression of oxidative stress and apoptosis-related factors in the POF models. **a–c** The mRNA and protein expression of SOD2, Nrf2, Bcl-2, and Bax in the control, POF, POF + PUE 100 mg/kg, and POF + PUE

200 mg/kg groups. **d–e** The ratios of Bcl-2/Bax mRNA and protein expression in the four groups (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. the control group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. the POF group)

the expression of β -catenin increased more in the PUE 200 mg/kg group than in the PUE 100 mg/kg group. These results emphasize that PUE may activate the Wnt/ β -catenin pathway to promote the recovery of ovarian function.

There have been many studies on isoflavones in the reproductive system. Phytoestrogens, especially soy isoflavones (ISOs), can reduce the generation of ROS, improve the state of cell oxidative stress, and reduce apoptosis to improve ovarian follicle survival [53]. It has also been shown that flavonoid naringenin significantly increases the levels of ROS scavenging enzymes, CAT, SOD, and GPX, in the rat polycystic ovary syndrome model [54]. To further study the protective effects of PUE on ovarian oxidative stress, we detected SOD2 and Nrf2. Both were decreased in the POF group, which could lead to the accumulation of ROS and damage to ovarian functions. After 28 days of PUE treatment, the expression of SOD2 and Nrf2 protein increased, suggesting that PUE promotes antioxidant enzymes to eliminate ROS and subsequently delay ovarian senescence. Bcl-2 protein can prevent the release of apoptogenic proteins from mitochondria to prevent apoptosis or necrosis. In contrast, Bax protein can induce harmful changes in cells and promote apoptosis [42]. We

detected increased expression of the anti-apoptotic gene Bcl-2 and decreased expression of the pro-apoptotic gene Bax in the PUE groups, and the ratio of Bcl-2/Bax increased significantly compared with the POF group. Taken together, the results suggest that PUE inhibits apoptosis and improves cell survival in the ovaries.

In conclusion, PUE maintains FGSC activity and improves ovarian function by regulating the Wnt/ β -catenin signaling pathway and antioxidative stress. These findings indicate that PUE has a certain beneficial effect on POF. However, a detailed clarification of the molecular mechanisms is needed. To observe the effect of PUE at the cellular level, we plan to separate and culture oocytes and FGSCs with different concentrations of PUE. Furthermore, Wnt/ β -catenin signaling pathway inhibitors and agonists can be added to the oocytes and FGSCs cultures, and the corresponding effects of the pathway on the growth and proliferation of FGSCs can be observed.

Acknowledgments This work was supported by the Project. The authors thank every member of our lab. We are grateful for the help from The Key Laboratory of Reproductive Physiology and Pathology of Jiangxi Provincial, Nanchang University.

Authors' Contributions Cheng Chen: Conception and design, data curation, and original draft writing. Song Li and Jia Li: Investigation, collection, and assembly of data and data analysis. Cong Hu, Weiwei Cao, and Qingfeng Fu: Data collection and original draft writing. Liping Zheng: Writing-reviewing and editing. Jian Huang: Conception and design, writing-reviewing, and editing.

Funding This study was funded by the National Natural Science Foundation of China (No. 81660245 and 81771583); the Natural Science Foundation of Jiangxi Province (No. 20192BAB215009); and Program to Cultivate Major Academic and Technical Leaders of Jiangxi Province (No. 20194BCJ22005).

Availability of Data and Material Available.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval This study was approved by the animal ethics committee of Nanchang University.

Consent to Participate Not applicable.

Code Availability Not applicable.

Abbreviations POF, premature ovarian failure; PUE, puerarin; FGSCs, female germline stem cells; CTX/BU, cyclophosphamide and busulfan; Mvh, the mouse vasa homolog; Oct-4, octamer-4; IRI, ischemia-reperfusion injury; FZD, Frizzled; DshD, disheveled; GSK3, glycogen synthesis kinase 3; ROS, reactive oxygen species; GSH-Px, glutathione peroxidase; SOD, superoxide dismutase; Nrf2, NF-E2 p45-related factors 2; ISOs, soy isoflavones

References

- Sullivan SD, Sarrel PM, Nelson LM, Oktay K, Turan V, Titus S, et al. Ovarian aging and premature ovarian failure. *Biol Reprod*. 2014;15(3):61–7.
- Sullivan SD, Sarrel PM, Nelson LM. Hormone replacement therapy in young women with primary ovarian insufficiency and early menopause. *Fertil Steril*. 2016;106(7):1588–99.
- Oktay K, Turan V, Titus S, Stobezki R, Liu L. BRCA mutations, DNA repair deficiency, and ovarian aging. *Biol Reprod*. 2015;93(3):61–7.
- Lai D, Wang F, Chen Y, Wang L, Wang Y, Cheng W. Human amniotic fluid stem cells have a potential to recover ovarian function in mice with chemotherapy-induced sterility. *BMC Dev Biol*. 2013;13(1):34–46.
- Ebrahimi M, Asbagh FA. Pathogenesis and causes of premature ovarian failure: an update. *Int J Fertil Steril*. 2011;5(2):54–65.
- Bryant H. Screening for cancer in children, adolescents, and young adults: questions—and more questions. *Cancer*. 2011;117(S10):2275–80.
- Sukur YE, Balik Kivancli I, Ozmen B. Ovarian aging and premature ovarian failure. *J Turk Ger Gynecol Assoc*. 2014;15(3):190–6.
- Melekoglu R, Ciftci O, Eraslan S, Cetin A, Basak N. Beneficial effects of curcumin and capsaicin on cyclophosphamide-induced premature ovarian failure in a rat model. *J Ovarian Res*. 2018;11(1):33–40.
- Johnson J, Canning J, Kaneko T, Pru JK, Tilly JL. Germline stem cells and follicular renewal in the postnatal mammalian ovary. *Nature*. 2004;428(6979):145–50.
- Gheorghisan-Galateanu AA, Hinescu ME, Enciu AM. Ovarian adult stem cells: hope or pitfall? *J Ovarian Res*. 2014;7(1):71.
- Volarevic V, Bojic S, Nurkovic J, Volarevic A, Ljubic B, Arsenijevic N, et al. Stem cells as new agents for the treatment of infertility: current and future perspectives and challenges. *Biomed Res Int*. 2014;2014:1–8. <https://doi.org/10.1155/2014/507234>.
- Terraciano P, Garcez T, Ayres L, Durli I, Baggio M, Kuhl CP, et al. Cell therapy for chemically induced ovarian failure in mice. *Stem Cells Int*. 2014;2014:1–8. <https://doi.org/10.1155/2014/720753>.
- Zhou L, Wang L, Kang JX, Xie W, Li X, Wu C, et al. Production of fat-1 transgenic rats using a post-natal female germline stem cell line. *Mol Hum Reprod*. 2014;20(3):271–81.
- Lu Z, Wu M, Zhang J, Xiong J, Cheng J, Shen W, et al. Improvement in isolation and identification of mouse oogonial stem cells. *Stem Cells Int*. 2016;2016:1–10. <https://doi.org/10.1155/2016/2749461>.
- Silva JRV, Van den Hurk R, Van Tol HTA, Roelen BAJ, Figueiredo JR. The kit ligand/c-kit receptor system in goat ovaries: gene expression and protein localization. *Zygote*. 2006;14(4):317–28.
- Gidekel S, Pizov G, Bergman Y, Pikarsky E. Oct-3/4 is a dose-dependent oncogenic fate determinant. *Cancer Cell*. 2003;4(5):361–70.
- Zhou HY, Katsman Y, Dhaliwal NK, Davidson S, Macpherson NN, Sakthidevi M, et al. A Sox2 distal enhancer cluster regulates embryonic stem cell differentiation potential. *Genes Dev*. 2014;28(24):2699–711.
- Abranches E, Bekman E, Henrique D. Generation and characterization of a novel mouse embryonic stem cell line with a dynamic reporter of Nanog expression. *PLoS One*. 2013;8(3):e59928.
- Hartung O, Forbes MM, Marlow FL. Zebrafish vasa is required for germ-cell differentiation and maintenance. *Mol Reprod Dev*. 2014;81(10):946–61.
- Toyooka Y, Tsunekawa N, Takahashi Y, Matsui Y, Satoh M, Noce T. Expression and intracellular localization of mouse vasa-homologue protein during germ cell development. *Mech Dev*. 2000;93(1–2):139–49.
- Nichols J, Zevnik B, Anastasiadis K, Niwa H, Klewe-Nebenius D, Chambers I, et al. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell*. 1998;95(3):379–91.
- Xia ZUO, Lei C. Research progress of stem cells in the treatment of premature ovarian failure. *J Int Obstet Gynecol*. 2014;41(1):25.
- Li J, Zhou F, Zheng T, Pan Z, Liang X, Huang J, et al. Ovarian germline stem cells (OGSCs) and the Hippo signaling pathway association with physiological and pathological ovarian aging in mice. *Cell Physiol Biochem*. 2015;36(5):1712–24.
- Pan Z, Sun M, Jia L, Zhou F, Zheng Y. The expression of markers related to ovarian germline stem cells in the mouse ovarian surface epithelium and the correlation with Notch signaling pathway. *Cell Physiol Biochem*. 2015;37(6):2311–22.
- Ye H, Zheng T, Li W, Li X, Fu X, Huang Y, et al. Ovarian stem cell nests in reproduction and ovarian aging. *Cell Physiol Biochem*. 2017;43(5):1917–25.
- Herraiz S, Pellicer N, Romeu M, Pellicer A. Treatment potential of bone marrow-derived stem cells in women with diminished ovarian reserves and premature ovarian failure. *Curr Opin Obstet Gynecol*. 2019;31(3):156–62.
- Zheng G, Zhang X, Zheng J, Meng Q, Zheng D. Estrogen-like effects of puerarin and total isoflavones from *Pueraria lobata*. *Zhong Yao Cai*. 2002;25(8):566–8.
- Gang C, Shi-Fen PAN, Xiang-Li CUI, Li-Hong LIU. Puerarin attenuates angiotensin II-induced cardiac fibroblast proliferation via the promotion of catalase activity and the inhibition of hydrogen peroxide-dependent Rac-1 activation. *Chin J Nat Med*. 2018;16(1):41–52.

29. Zhu J, Wang X, Shang Y, Xie X, Zhang F, Chen J, et al. Puerarin reduces endothelial progenitor cells senescence through augmentation of telomerase activity. *Vasc Pharmacol*. 2008;49(2–3):106–10.
30. Liu X, Zhao W, Wang W, Lin S, Yang L. Puerarin suppresses LPS-induced breast cancer cell migration, invasion and adhesion by blockage NF- κ B and Erk pathway. *Biomed Pharmacother*. 2017;92:429–36.
31. Huang G, Wei S, Huang Y, Xing W, Wang L, Liang L. Mechanism of combined use of vitamin D and puerarin in anti-hepatic fibrosis by regulating the Wnt/ β -catenin signalling pathway. *World J Gastroenterol*. 2018;24(36):4178–85.
32. Lin L, He Y, Zhang J, Liu Q, Wang L. The effects and possible mechanisms of Puerarin to treat uterine fibrosis induced by ischemia-reperfusion injury in rats. *Med Sci Monit*. 2017;23:3404–11.
33. Wang P-P, Zhu X-F, Yang L, Liang H, Feng S-W, Zhang R-H. Puerarin stimulates osteoblasts differentiation and bone formation through estrogen receptor, p38 MAPK, and Wnt/ β -catenin pathways. *J Asian Nat Prod Res*. 2012;14(9):897–905.
34. Zhang D, Wang H, Tan Y. Wnt/ β -catenin signaling induces the aging of mesenchymal stem cells through the DNA damage response and the p53/p21 pathway. *PLoS One*. 2011;6(6):e21397.
35. Ricken* A, Lochhead P, Kontogiannia M, Farookhi R. Wnt signaling in the ovary: identification and compartmentalized expression of wnt-2, wnt-2b, and frizzled-4 mRNAs. *Endocrinology*. 2002;143(7):2741–9.
36. Naillat F, Yan W, Karjalainen R, Liakhovitskaia A, Samoylenko A, Xu Q, et al. Identification of the genes regulated by Wnt-4, a critical signal for commitment of the ovary. *Exp Cell Res*. 2015;332(2):163–78.
37. Parakh TN, Hernandez JA, Grammer JC, Weck J, Hunzicker-Dunn M, Zeleznik AJ, et al. Follicle-stimulating hormone/cAMP regulation of aromatase gene expression requires beta-catenin. *Proc Natl Acad Sci U S A*. 2006;103(33):12435–40.
38. Fan H-Y, O'Connor A, Shitanaka M, Shimada M, Liu Z, Richards JS. β -Catenin (CTNNB1) promotes preovulatory follicular development but represses LH-mediated ovulation and Luteinization. *Mol Endocrinol*. 2010;24(8):1529–42.
39. Ighodaro OM, Akinloye OA. First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): their fundamental role in the entire antioxidant defence grid. *Alex J Med*. 2018;54(4):287–93.
40. Kensler TW, Wakabayashi N, Biswal S. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Annu Rev Pharmacol Toxicol*. 2007;47:89–116.
41. Zhao J, Cheng Y, Fan W, Yang C, Ye S, Cui W, et al. Botanical drug puerarin coordinates with nerve growth factor in the regulation of neuronal survival and neurite extension via activating ERK 1/2 and PI 3K/Akt signaling pathways in the neurite extension process. *CNS Neurosci Ther*. 2015;21(1):61–70.
42. Reed JC, Jurgensmeier JM, Matsuyama S. BCL-2 family proteins and mitochondria. *Biochim Biophys Acta*. 1998;1366(1):127–37.
43. Zamzami N, Brenner C, Marzo I, Susin SA, Kroemer G. Subcellular and submitochondrial mode of action of Bcl-2-like oncoproteins. *Oncogene*. 1998;16(17):2265–82.
44. Tilly JL. Ovarian follicle counts—not as simple as 1, 2, 3. *Reprod Biol Endocrinol*. 2003;1(1):11–4.
45. Cavo M, Bandini G, Benni M, Gozzetti A, Ronconi S, Rosti G, et al. High-dose busulfan and cyclophosphamide are an effective conditioning regimen for allogeneic bone marrow transplantation in chemosensitive multiple myeloma. *Bone Marrow Transplant*. 1998;22(1):27–32.
46. Zou K, Yuan Z, Yang Z, Luo H, Sun K, Zhou L, et al. Production of offspring from a germline stem cell line derived from neonatal ovaries. *Nat Cell Biol*. 2009;11(5):631–6.
47. White YAR, Woods DC, Takai Y, Ishihara O, Seki H, Tilly JL. Oocyte formation by mitotically active germ cells purified from ovaries of reproductive-age women. *Nat Med*. 2012;18(3):413–21.
48. Jiang Y, Zhang Z, Cha L, Li L, Zhu D, Fang Z, et al. Resveratrol plays a protective role against premature ovarian failure and prompts female germline stem cell survival. *Int J Mol Sci*. 2019;20(14):3605–20.
49. Jeon Y, Lee J, Lee Y, Kim D. Puerarin inhibits inflammation and oxidative stress in dextran sulfate sodium-induced colitis mice model. *Biomed Pharmacother*. 2020;124:109847. <https://doi.org/10.1016/j.biopha.2020.109847>.
50. Chen X, Yu J, Shi J. Management of diabetes mellitus with puerarin, a natural isoflavone from *Pueraria lobata*. *Am J Chin Med*. 2018;46(08):1771–89.
51. Hernandez Gifford JA. The role of WNT signaling in adult ovarian folliculogenesis. *Reproduction*. 2015;150(4):E137–R148.
52. Han Z, Li Y, Yang B, Tan R, Wang M, Zhang B, et al. Agmatine attenuates liver ischemia reperfusion injury by activating Wnt/ β -catenin signaling in mice. *Transplantation*. 2020;104:1906–16. <https://doi.org/10.1097/TP.0000000000003161>.
53. Teixeira CP, Florenciosilva R, Sasso GRDS, Carbonel AAF, Simoes RS, Simoes MJ. Soy isoflavones protect against oxidative stress and diminish apoptosis in ovary of middle-aged female rats. *Gynecol Endocrinol*. 2019;35(7):586–90.
54. Hong Y, Yin Y, Tan Y, Hong K, Zhou H. The flavanone, naringenin, modifies antioxidant and steroidogenic enzyme activity in a rat model of letrozole-induced polycystic ovary syndrome. *Med Sci Monit*. 2019;25:395–401.

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.