

Cryptotanshinone decreases granulosa cell apoptosis and restores ovarian function in mice with premature ovarian failure

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Abstract. With the increasing incidence of premature ovarian failure (POF) seriously threaten the women's health. Whether cryptotanshinone decreased the granulosa cell apoptosis to improve the POF would be explored. POF mice were conducted with intraperitoneal injection of cyclophosphamide and then treated with cryptotanshinone. The body weight and ovarian weight were recorded. The estrus was detected by vaginal smears. The pathological changes of ovarian were observed with hematoxylin and eosin staining. ELISA assay analyzed the levels of LH, FSH, AMH, E2 and AfpAB in mice serum. The expression of Bcl-2, Bax, KI67 and PCNA in ovarian tissues was detected by Western blot analysis and KI67 expression was also determined by immunohistochemistry. The body weight and ovarian weight were decreased and the pathological results of ovarian were worsen in POF mice. The estrus was decreased in POF mice. The levels of LH, FSH and AfpAB were increased and the levels of AMH and E2 were decreased in POF mice serum. The expression of Bcl-2, KI67 and PCNA was decreased and Bax expression was increased in ovarian tissues of POF mice. Those changes affected by cyclophosphamide could be reversed by cryptotanshinone. Cryptotanshinone could decrease the granulosa cell apoptosis to restore ovarian function.

Key words: Cryptotanshinone — Granulosa cell — Apoptosis — Premature ovarian failure

Introduction

Premature ovarian failure (POF) is a disease in which ovarian dysfunction is happened in women under 40 years old who are accompanied by high serum levels of follicle-stimulating hormone (FSH) and low estradiol (E2). Menstrual disorders, hot flashes, night sweats and other low estrogen symptoms and infertility are the main clinical manifestations (Podfigurna-Stopa et al. 2016). Studies indicated that the pathogenesis of POF was caused by the rapid depletion of oocytes (Ren et al. 2015; D'Ignazio et al. 2018; Miao et al. 2018). Activation and sustained growth of oocytes depend on the nutrition and paracrine functions of

the granulosa cells surrounding them. POF is a polygenic disease caused by a variety of causes and complex mechanism, but the main reason is the acceleration of follicular atresia (Chen et al. 2004; Herraiz et al. 2019; Zhang et al. 2019). Follicular atresia is actually the apoptosis of ovarian granulosa cells and oocytes, and the apoptosis of granulosa cells may be the main reason of follicular atresia. Therefore, the research on granulosa cell apoptosis is paid more and more attention.

Cryptotanshinone is a monomer isolated from tanshinone and has antibacterial, anti-inflammatory and antioxidant effects. At present, the role of cryptotanshinone in ovarian-related diseases has been reported. Yang et al. (2018) have demonstrated that cryptotanshinone inhibited the proliferation of ovarian cancer cells. Ma et al. (2011) have found that the Chinese medical herb *Salvia miltiorrhiza* could decrease the high levels of serum androgens to improve fertility of polycystic ovary syndrome (PCOS) patients. Huang et al. (2014) have indicated that

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cryptotanshinone inhibited the increase of androgen and relieve the insulin resistance in ovarian. Furthermore, cryptotanshinone was demonstrated to alleviate the hypoxia/reoxygenation injury and decrease apoptosis of renal tubular epithelial cells (Bai et al. 2019; Zhu et al. 2019). Cryptotanshinone could also reduce the apoptosis of ischemic myocardial cells and nerve cells (Yong et al. 2009; Lu et al. 2017). Therefore, cryptotanshinone could alleviate cell apoptosis and regulate ovarian function. However, its role in premature ovarian failure is unknown.

Therefore, we speculated that cryptotanshinone could decrease the granulosa cell apoptosis to restore the ovarian function in POF mice, which was investigated in this study.

Materials and Methods

Establishment of premature ovarian failure in mice

Twenty female mice (SPF grade, 6–8 weeks, 18~20 g) were obtained from Shanghai Jiesijie Experimental Animal Co. LTD. After one week of adaptive feeding, mice were randomly divided into four groups with five mice in each group. Mice in model group (POF) were intraperitoneally injected with 50 mg/kg cyclophosphamide at first, then continuously received intraperitoneal injection of 8 mg/kg cyclophosphamide for 14 days and administered by gavage equal volume of saline for 4 weeks. Mice in cryptotanshinone-treated group were the POF mice, continuously receiving 50 mg/day or 100 mg/day cryptotanshinone for 4 weeks. The Control group was not imposed with any treatment factors. The body mass and ovary weight of the twenty mice were weighed every day. The experimental period lasted fifty days.

Vaginal smears

Mice were labeled with marker pen at first, then grabbed and fixed in the palm. One fine cotton swab was wetting in saline, then gently inserted into the vagina to about 0.5 cm and slowly turned. Then, the swab was taken out and the vagina inclusion on the cotton swab was evenly coated on the glass slide. Slides were numbered, naturally dried, fixed in 95% alcohol solution for 10 min and dyed in 0.23% alkaline methylene blue for 5 min. Finally, slides were taken out, slowly rinsed with tap water along the edge of glass slide to remove the dyeing liquid and observed after air drying.

Hematoxylin and eosin (H&E) staining

The mice ovary tissues were fixed in 4% paraformaldehyde for 24 h. After the paraffin was completely immersed in the

tissue block, it was embedded, then cooled and solidified into the block. Mice ovary sections were obtained through slicing up the block into 5–8 μm sections, then stained with hematoxylin solution and counterstained with eosin solution. Finally, pathological changes of mice ovary tissues were observed by an inverted fluorescence microscope (MF53; Micro-shot Technology Co., Ltd., Guangzhou, China).

Enzyme-linked immunosorbent assay (ELISA)

The levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH), anti mullerian hormone (AMH), E2 and anti zona pellucida antibody (AzpAB) in mice serum were determined with commercially-available ELISA kits (Boster, China). The results were calculated according to the standard curves established from standard proteins.

Immunohistochemistry

Paraffin sections were dewaxed, hydrated and washed with phosphate buffered solution (PBS) (pH 7.4) for 3 times, 5 min each time. The sections were placed in the antigen repairing solution (0.01 mol/l citrate buffer, pH 6.0) with 95°C water bath for 10 min and naturally cooled to room temperature. Then, sections were placed in a 3% methanol peroxide solution to incubate at room temperature for 5–10 min to eliminate the activity of endogenous peroxidase. Sections were washed with PBS for 3 times, 5 min each time. Each section was added with 500 μl 10% donkey serum and incubated at room temperature for 20 min. Subsequently, each section was incubated against 400 μl primary antibody Ki-67 (ab16667; dilution, 1:1000; Abcam) at room temperature for 2 h and 400 μl IgG-HRP (ab6721; dilution, 1:1000; Abcam) at room temperature for 1 h. After three times PBS washing, sections were developing in DAB solution for 1–2 min, redyed with hematoxylin, dehydrated, sealed, observed and photographed.

Western blot analysis

Adequate amount of ovarian tissues were placed in the lysis solution containing PMSF and RIPA to completely lysed cells, which were centrifuged. The supernatant was the solution of total protein. The protein sample buffer was degenerated in boiling water for 15 min. Proteins were separated by 12% SDS-PAGE gels and transferred to polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Billerica, MA, USA). The membranes were sealed in 5% skim milk for 1 h and incubated against with primary antibodies including Bcl-2 (ab182858; dilution, 1:2000; Abcam), Bax (ab32503; dilution, 1:2000; Abcam), Ki67 (ab16667; dilution, 1:2000; Abcam), PCNA (ab92552; dilution, 1:2000; Abcam) and

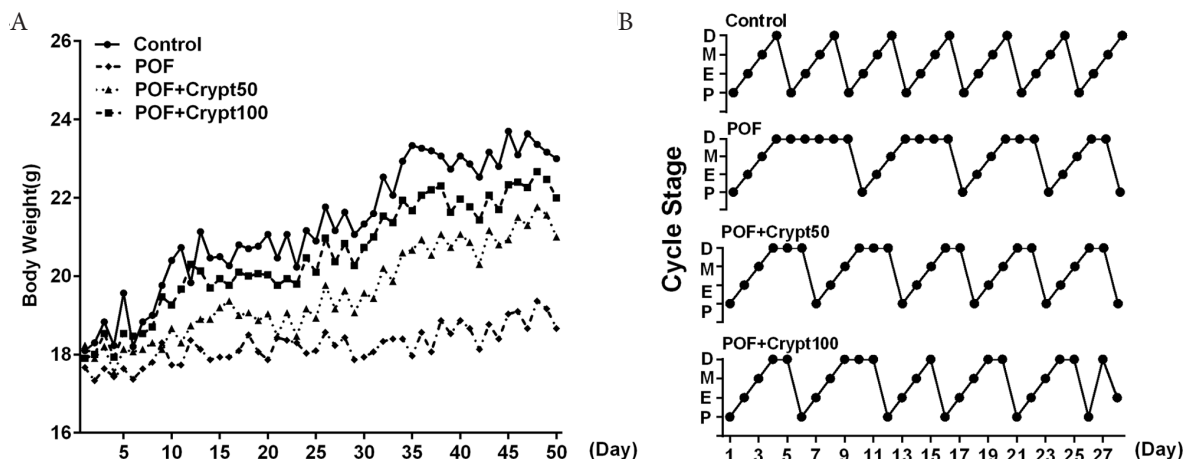


Figure 1. Cryptotanshinone increased the body weight and estrus of POF mice. **A.** Body weight growth curve in various groups. **B.** Mouse estrus cycle stage. D, diestrus; M, metaestrus; E, estrus; P, proestrus.

GAPDH (ab181602; dilution, 1:10000; Abcam) overnight at 4°C. After washing, membranes were incubated against secondary antibody for 30 min at room temperature, followed by the decoloring with TBST in shaking bed at room temperature 3 times, 5 min each time. The exposure of proteins on the membranes was performed in a dark room, which developed with ECL solution. The gray analysis was conducted with ImageLab4.0 software.

Statistical analysis

The experimental data were presented as mean ± standard deviation, which analyzed by SPSS 20.0 software. One way

ANOVA was used for comparison between multiple groups with LSD-t method. $p < 0.05$ showed a statistical meaning of difference.

Results

Cryptotanshinone increased the body weight and estrus of POF mice

The mice body weights in four groups were detected every day and the experiment period lasted for fifty days. Figure 1A showed that the body weights of POF mice were

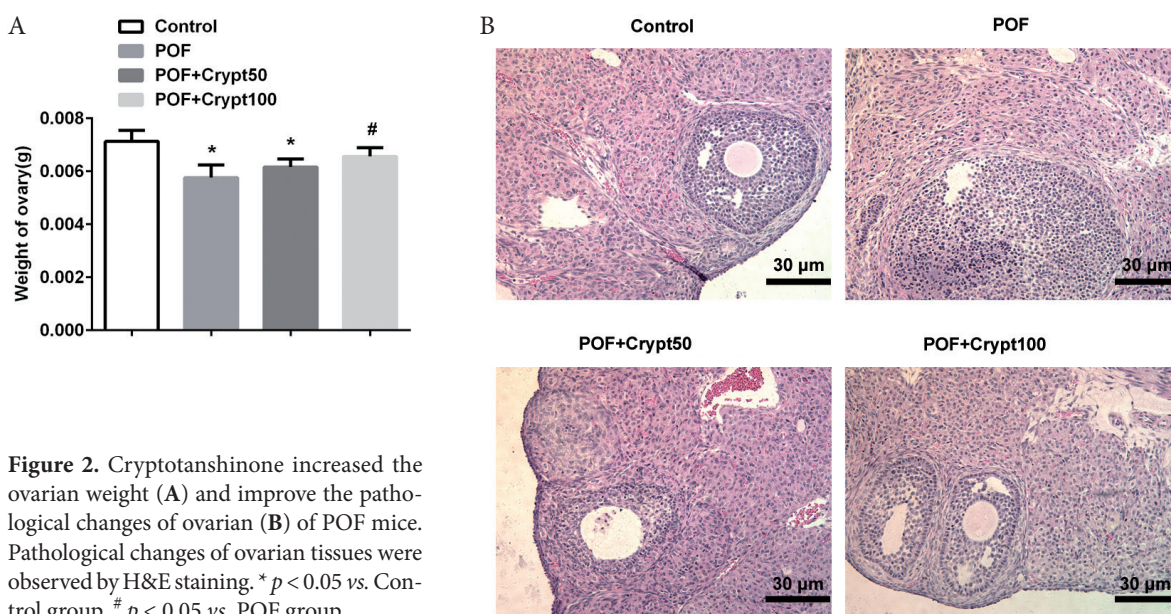


Figure 2. Cryptotanshinone increased the ovarian weight (A) and improve the pathological changes of ovarian (B) of POF mice. Pathological changes of ovarian tissues were observed by H&E staining. * $p < 0.05$ vs. Control group. # $p < 0.05$ vs. POF group.

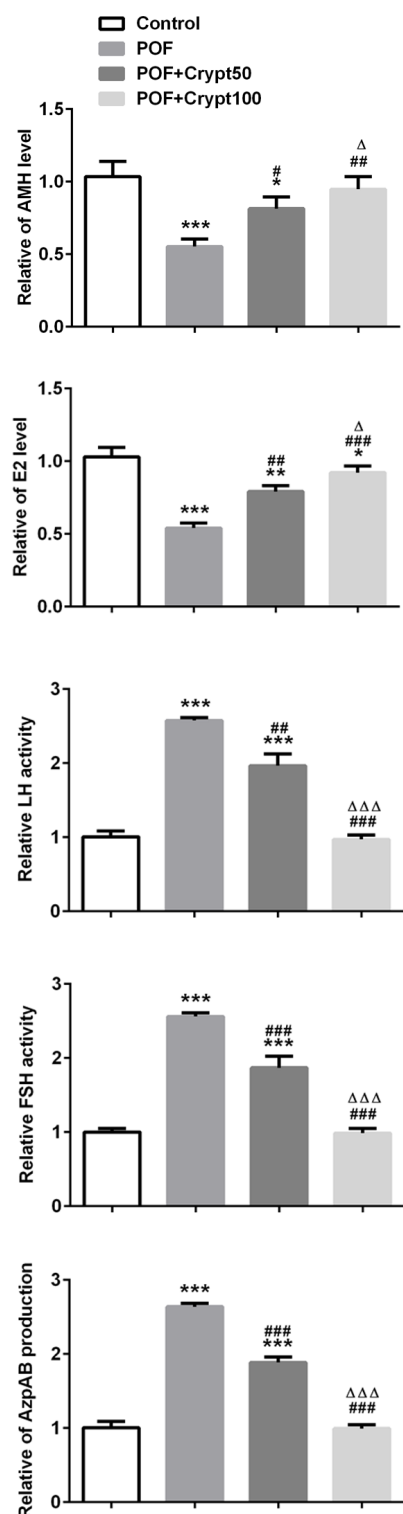


Figure 3. Cryptotanshinone regulates the levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH), anti mullerian hormone (AMH), E2 and anti zona pellucida antibody (AzpAB) in serum of POF mice detected by ELISA assay. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. Control group. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. POF group. Δ $p < 0.05$, $\Delta\Delta\Delta$ $p < 0.001$ vs. POF+Crypt50 group.

obviously decreased compared with the Control group. The cryptotanshinone treatment for 4 weeks improved the body weights of POF mice and the improvement of body weights was better in the relative high-dose group. The analysis of vaginal smear showed that estrus was obviously decreased and diestrus was obviously increased in POF mice and mice in Control group showed one estrus every 4 days regularly. Mice treated with cryptotanshinone showed more estrus compared with POF group, which also related to the dose changes of cryptotanshinone (Fig. 1B). Therefore, cryptotanshinone effectively reversed the decrease of the body weight and estrus of POF mice.

Cryptotanshinone increased the ovarian weight and improve the pathological changes of ovarian of POF mice

At the end of the experiment, whole ovaries were isolated from mice. The ovarian weights of POF mice were notably decreased compared with Control group. The treatment of 100 mg/day cryptotanshinone for POF mice effectively increased the weight of ovary compared with POF group (Fig. 2A). In Control group, granulosa cells are arranged regularly in many levels. A few atresia follicle and rich follicle fluid is observed. The granulosa cells of the follicle are extremely disorganized in POF group. Follicular granulosa cells have more layers and slightly disordered arrangement in cryptotanshinone-treated group (Fig. 2B).

Cryptotanshinone regulates the levels of LH, FSH, AMH, E2 and AzpAB in serum of POF mice

The levels of LH, FSH, AMH, E2 and AzpAB in mice serum were changed when there have been a damage of ovarian function. In serum of POF mice, the levels of LH, FSH and AzpAB were increased and the levels of AMH and E2 were decreased compared with Control group. The cryptotanshinone treatment effectively reversed those changes caused by POF and relative high-dose group showed a better reversal effect (Fig. 3). Therefore, cryptotanshinone increased the levels of LH, FSH and AzpAB and decreased the levels of AMH and E2 in serum of POF mice.

Cryptotanshinone promoted the proliferation of ovarian granulosa cells

The expression of proliferation-related proteins and apoptosis-related proteins in ovarian tissues was detected. As shown in Figure 4A, the expression of KI-67 was decreased in ovarian tissues of POF mice compared with Control group and cryptotanshinone treatment significantly increased the expression of KI-67 in ovarian tissues of POF mice. The results of Figure 4B showed that the expression of Bcl-2, KI67 and PCNA was decreased and Bax expression was increased

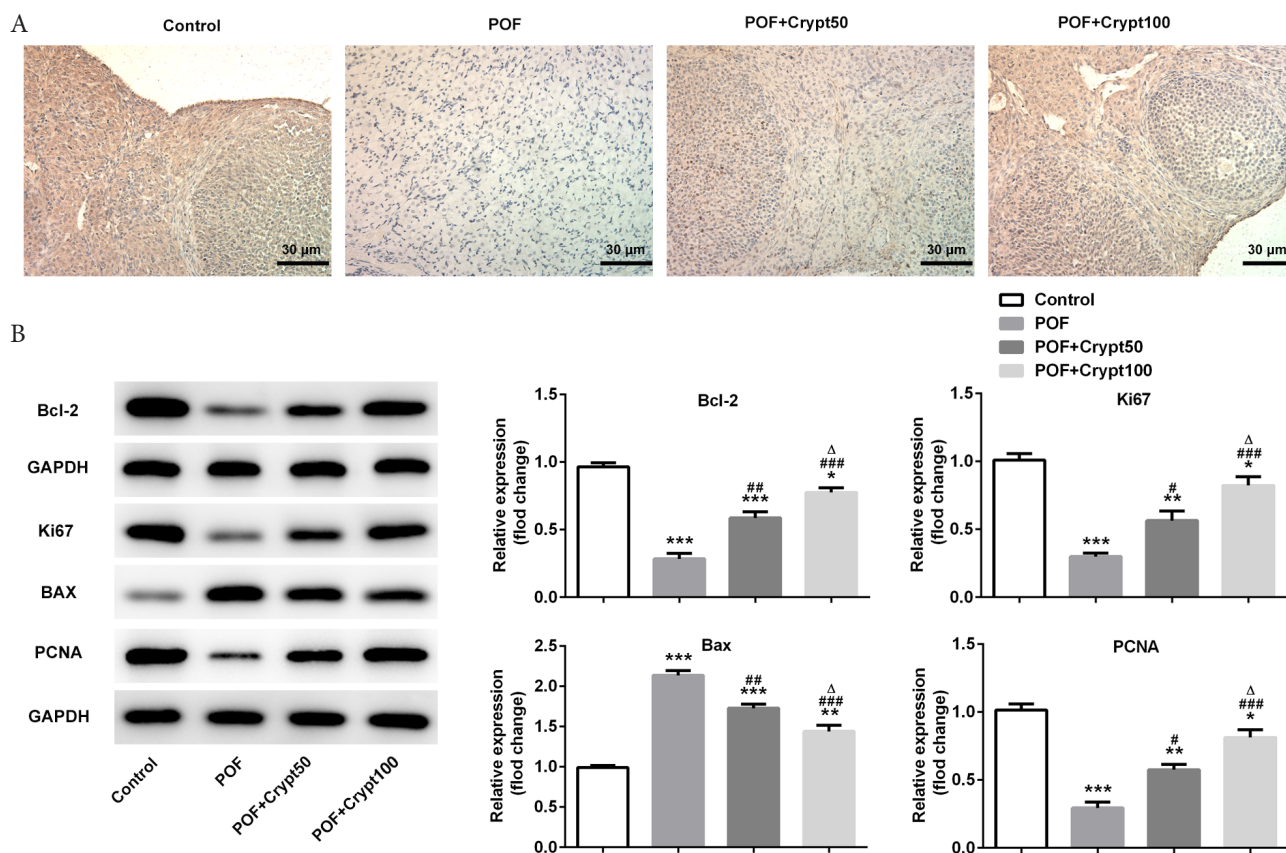


Figure 4. Cryptotanshinone proliferation of ovarian granulosa cells in ovarian tissues of POF mice. **A.** The KI67 expression in ovarian tissues detected by immunohistochemistry. **B.** The expression of Bcl-2, Bax, KI67 and PCNA in ovarian tissues was analyzed by Western blot analysis. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. Control group. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. POF group. Δ $p < 0.05$ vs. POF+Crypt50 group.

in ovarian tissues of POF mice, which could be reversed by cryptotanshinone treatment. Therefore, cryptotanshinone effectively increased the proliferation of ovarian granulosa cells.

Discussion

Recently, the incidence of cancer/tumors is on rise in population at younger age. With the continuous development of tumor treatment, the prognosis of patients is obviously improved. Chemotherapy has become an important reason for POF (Kort et al. 2014). Apoptosis of ovarian granulosa cells was detected *in vivo* and *in vitro* (Morgan et al. 2012). Granulosa cells are the largest cell masses that make up follicle, the main source of estrogen and progesterone, and play an important role in maintaining the normal structure and function of follicles. The apoptosis of granulosa cells is closely related to the atresia of follicles. Therefore, it is necessary to

find effective methods to inhibit the apoptosis of granulosa cells, thereby restoring the ovarian function.

The Chinese traditional medicine compounds including cryptotanshinone have been studied in many tumors and reproductive system diseases in recent years because of its effective treatments and small side effects. Cryptotanshinone can effectively inhibit the proliferation of cancer cells in various cancers, such as breast cancer, liver cancer, prostate cancer, and ovarian cancer (Xu et al. 2012; Jiang et al. 2017; Pan et al. 2017; Shen et al. 2017). Furthermore, cryptotanshinone can reverse the ovarian insulin resistance by regulating the expression of glucose transporters and hormone-synthesizing enzymes and reverse the reproductive and metabolic functions of progeny by regulating ovarian signaling mechanisms and androgen synthesis in female rats (Yang et al. 2011; Huang et al. 2014). Study indicated that E2 level was decreased in POF model, which lead to the positive feedback of the pituitary-gonadal axis for promoting the expression of FSH and LH in different degrees (Liu 2009).

We also found that the levels of FSH and LH were increased when E2 level was decreased in serum of POF mice in this study while cryptotanshinone treatment could effectively down-regulate the levels of FSH and LH and up-regulate the E2 level. AMH is a dimer glycoprotein secreted by female follicular granulosa cells which inhibits recruitment and growth of female follicles. AMH mainly plays a biological role through AMH receptor (di Clemente et al. 1994). At birth, AMH can hardly be detected in the serum. The serum concentration of AMH starts to rise slowly in the first few weeks after birth, reaches a peak in late adolescence, and persists throughout the whole reproductive age. However, with the increase of age and the consumption of follicles, the serum concentration gradually drops, which cannot be detected after menopause (Sowers et al. 2008). There was a significant positive correlation between AMH and the number of primordial follicles (Hansen et al. 2011). Smith et al. (1994) detected that the AzpAB was positive in serum of POF patients with immunofluorescence technique and concluded that the rupture and absorption of the zona pellucidum during ovulation could stimulate the immune system and sensitize the body to produce AzpAB. The changes of AMH and AzpAB in serum of POF mice at present study were consistent with the previous study. Cryptotanshinone treatment restored the ovarian function by changing the levels of LH, FSH, AMH, E2 and AzpAB in serum of POF mice.

In conclusion, cryptotanshinone could reverse the decrease of body weight and ovarian weight and improve the estrus and pathological injury of POF mice. And, cyclophosphamide decreased the levels of LH, FSH and AzpAB were increased and the levels of AMH and E2 were decreased in POF mice serum. Furthermore, cyclophosphamide also increased the expression of Bcl-2, KI67 and PCNA and decreased the Bax expression, thereby inhibiting the granulosa cell apoptosis.

Funding. Not applicable.

Conflicts of interest. The authors declare they have no competing interests.

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Received: September 30, 2019

Final version accepted: December 3, 2019