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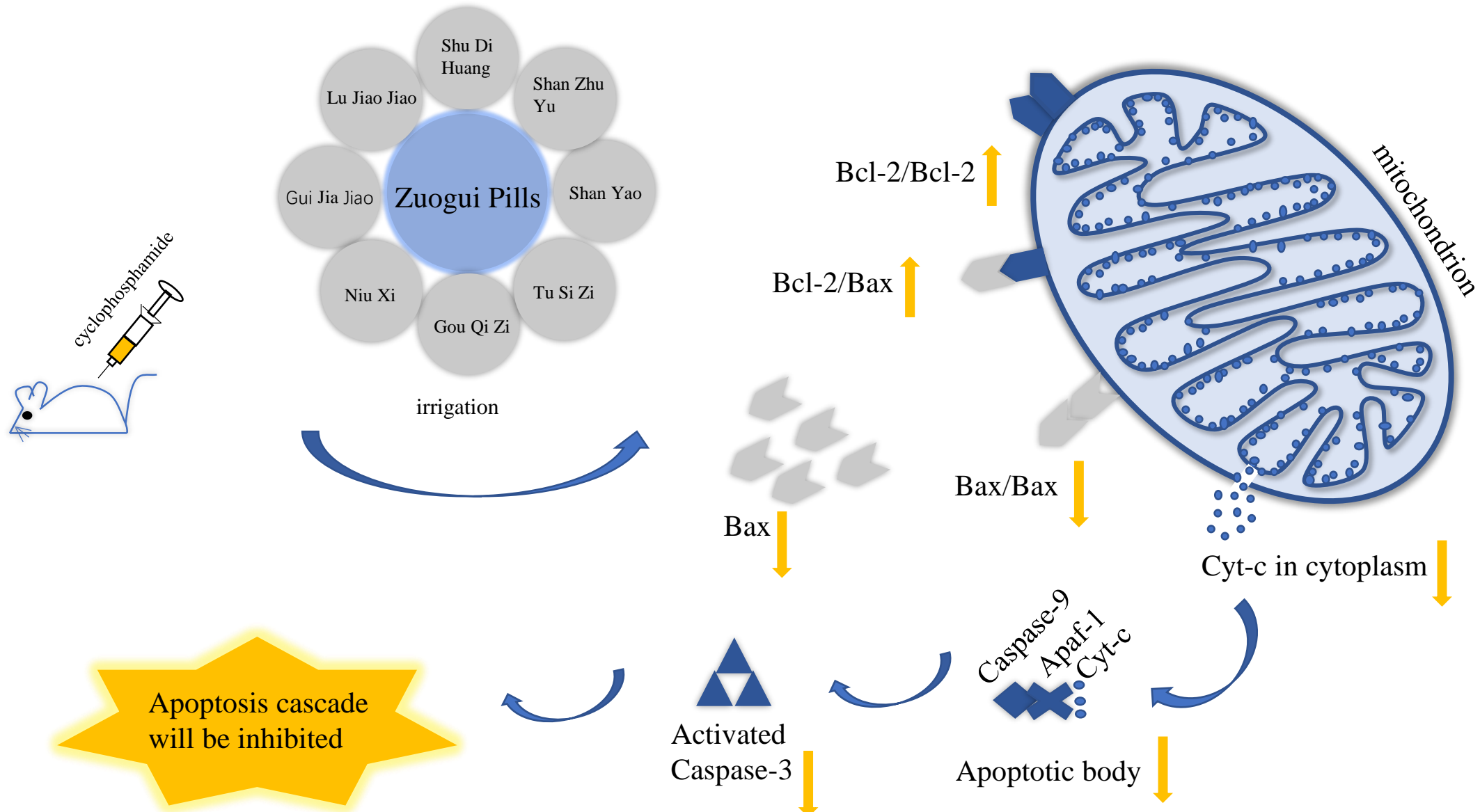
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Apoptosis cascade will be inhibited

Article Category: Research paper

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Abstract:

Ethnopharmacological relevance: Zuogui Pills (ZGP), which is a classical prescription of Traditional Chinese Medicine (TCM), has been reported to be widely used in the treatment of premature ovarian failure (POF).

Aim of the study: To investigate the therapeutic effects of ZGP on the treatment of POF induced by chemotherapy, and elucidate the potential molecular mechanism.

Materials and methods: Female 8-week-old Sprague-Dawley rats (N=54) were randomized to six groups, containing the Control group, Model group, three ZGP groups and Triptorelin group which was served as a positive control. The Triptorelin group received triptorelin injection ten days before model establishment by cyclophosphamide. The three ZGP groups (high dose group, medium dose group and low dose group) were given a daily intragastric administration of ZGP at doses of 3.2, 1.6 and 0.8 g/kg for sixty days. We observed the general growth of rats and examined the estrous cycle and the rate of pregnancy, ovarian ultrastructures, follicles and corpora lutea numbers. The serum hormone concentrations were measured by Enzyme-linked immunosorbent assay (ELISA). To explore the molecular mechanism of the effect, gene and protein expression

levels of Bax, Bcl-2 and Cyt-c related to apoptosis were determined by quantitative PCR (qPCR), Western Blot and Immunohistochemistry, respectively.

Results: After treating with ZGP, though the rate of pregnancy showed no significant difference, the estrous cycle, ovarian ultrastructures, numbers of follicles and corpora lutea were improved substantially. And ZGP led to a significant lower concentration of follicle stimulating hormone (FSH) in the serum, and the level of oestradiol (E2) was increased. Furthermore, a substantial downregulation of Bax, cytochrome c (Cyt-c), and upregulation of B cell lymphoma/leukemia-2 (Bcl-2) both on gene and protein levels were observed after the administration with ZGP. And effects showed a positive correlation with the dosages.

Conclusions: Our study suggested that ZGP exerted significant effect on POF, which was mediated by inhibiting mitochondria-dependent apoptosis in the follicles.

Keywords: Premature ovarian failure; Chemotherapy; Zuogui pills; mitochondria-dependent apoptosis;

Abbreviations: ANOVA, analysis of variance; Bcl-2, B cell lymphoma/leukemia-2; Cyt-c, cytochrome c; DAB, 3,3'-diaminobenzidine; ELISA, Enzyme-linked immunosorbent assay; ET, embryo transfer; E2, oestradiol; ECL, enhanced chemiluminescence; ESI, electrospray ionization; FSH, follicle stimulating hormone; GnRH-a, gonadotropin releasing hormone agonist; GSH-Px, glutathione peroxidase; H&E, hematoxylin and eosin; HRP, horseradish peroxidase; i.m., intramuscular injection; i.p., intraperitoneal injection; i.g., irrigation; IL-2, interleukin-2; IL-4, interleukin-4; IL-10, interleukin-10; OD, optical densities; POF, Premature ovarian failure; PVDF, polyvinylidene difluoride; qPCR, quantitative Real time-Polymerase Chain Reaction; SD, standard deviation; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SOD, superoxide dismutase; CAT, catalase; TCM, traditional Chinese medicine; Th1, helper T cell 1; Th17, helper T cell 17; UPLC-Q-TOF-MSultra, performance liquid chromatography with quadrupole orthogonal time-to-flight mass spectrometry; ZGP, Zuogui Pills.

1. Introduction

Premature ovarian failure (POF) is a disease due to oocyte depletion under the age of forty. Patients may appear oligomenorrhea or amenorrhoea, perimenopausal syndrome such as depression and irritation related to decreased sex steroid, elevated level of gonadotropins (The ESHRE Guideline Group on POI, 2016). Women with POF are usually accompanied with atrophy of reproductive organs, infertility, dysfunction of urogenital and neurological system, osteoporosis,

cardiovascular risk etc., which all compromise the quality of life greatly. POF can be caused by factors including chromosome abnormality, Fragile-X premutation, autosome mutation (like growth differentiation factor 9, follicle stimulating hormone receptor etc.), autoimmune disorder, iatrogenic injury like chemotherapy and radiotherapy, absence of related enzymes (like 17 α -hydroxylase, 1-uridine-phosphate galactose acyl transferase etc.), and some unknown reasons.

With the increased prevalence of cancers in young women, occurrence of POF caused by chemotherapy also raised. Different chemotherapeutic drugs have diverse mechanisms on ovarian damage. Cyclophosphamide, as an alkylating agent, carries a high risk of ovarian toxicity. Cyclophosphamide not only interferes cell division by cross-linking of DNA in granulosa cells, but also up-regulates the expression of Bax and down-regulates the expression of Bcl-2, which could reduce the transmembrane potential of mitochondria. Subsequently lots of Cyt-c will remove from mitochondria to cytoplasm, and then apoptosis will be triggered (Morgan et al., 2012; Yuan and Akey, 2013; Würstle et al., 2012).

Nowadays, methods of protecting ovarian reserve for patients with the reproductive requirements are limited, such as applying gonadotropin releasing hormone agonist (GnRH-a), oocyte, embryo and ovarian cortex cryopreservation. Technology of oocyte and ovarian cortex cryopreservation in China is immature at present. Meanwhile, successful pregnant rate of embryo transfer (ET) is only 30% (Siristatidis et al., 2018), live birth rate after ET is only about 23% (Laokirkkiat et al., 2018). So GnRH-a has been popular in recent years. Cyclophosphamide is prone to attack cells with high mitotic index. And GnRH-a could alleviate ovarian injury by preventing antral follicles from recruitment. It can also decrease the concentration of chemotherapeutic agents by reducing ovarian vascularity (Hickman et al., 2018). But protective effects and mechanism are still controversial because a longterm follow-up suggested that girls received chemotherapy in prepuberty also experienced ovarian damage in adulthood (Green et al., 2009). And whether it can keep primordial follicles silent is disputed because of no gonadotropin receptors on primordial follicles (Oktay et al, 1997).

According to traditional Chinese medicine (TCM) theories, oocyte derived from kidney essence, so the basic pathogenesis of POF is related to deficiency of kidney essence. ZGP, effective in tonifying kidney essence, was firstly prescribed by Zhang Jingyue in *Jing Yue Quan Shu* about 400 years ago. The whole prescription shows a great effect of kidney-tonifying, essence-generating, and marrow-benefiting (Chai and Fan, 2018). It is suggested that herbs which could tonify kidney have a better effect on curing POF (Shang et al., 2018). And studies also indicated that ZGP could inhibit apoptosis, as well as promote proliferation and differentiation of cells (Yao et al., 2015). In this study, we investigated the protective effects of ZGP at different doses in a rat model of POF induced by chemotherapy. And we further combined the therapeutic effect of ZGP and mechanism of mitochondria-dependent anti-apoptosis to confirm ZGP as a available option for treating POF.

2. Materials and methods

2.1 Details about ZGP

The ZGP used for this experiment was purchased from Henan Wanxi pharmaceutical Co.,Ltd. (China, 150211). The voucher specimen was deposited at Zhongjing wanxi pharmaceutical Co., Ltd.(Henan, China) ZGP (total 45g) contained *Rehmanniae Radix Praeparata* (*Shu di Huang*), *Corni Fructus* (*Shan Zhu Yu*), *Dioscoreae Rhizoma* (*Shan Yao*), *Cuscuta chinensis* (*Tu Si Zi*), *Lycium barbarum* (*Gou Qi Zi*), *Achyranthes bidentata* (*Niu Xi*), *Testudinis Carapax et Plastic Collar* (*Gui Jia Jiao*), *Cervi Conrnus Colla* (*Lu Jiao Jiao*) with a 8:4:4:4:4:3:4:4 ratio (Table 1.) The extraction process of pills was as follow: All herbs were crushed to powder and filter with 150-180 mesh. Powder and honey (10:3) formed to 0.5-1.0 mm pills in the molding machine. The small pills gradually grew to 0.5 cm when adding herbs' powder. After smoothing the surface with water, the pills were dried at 60 °C. If the pills passed the quality test, they would be packed. The testing standards were provided by the National Institutes for Food and Drug Control (Beijing, China).

Table 1. Components of Zuogui Pills

	Chinese name	Botanic family	Botanical nomenclature	Pharmacognostic nomenclature
Components from plants	<i>Shu Di Huang</i> (熟地黄)	scrophulariaceae	<i>Rehmannia glutinosa</i> <i>Libosch.</i>	<i>Rehmanniae Radix</i> <i>Praeparata</i>
	<i>Shan Zhu Yu</i> (山茱萸)	cornaceae	<i>Cornus officinalis</i> Sieb. <i>et Zucc.</i>	<i>Corni Fructus</i>
	<i>Shan Yao</i> (山药)	dioscoreaceae	<i>Dioscorea opposita</i> <i>Thunb.</i>	<i>Dioscoreae Rhizoma</i>
	<i>Tu Si Zi</i> (菟丝子)	convolvulaceae	<i>Cuscuta chinensis</i> <i>Lam.</i>	<i>Cuscuta chinensis</i>
	<i>Gou Qi Zi</i> (枸杞子)	solanaceae	<i>Lycium barbarum</i> L.	<i>Lycium barbarum</i>

	Chinese name	Origination	Processing methods	Pharmacognostic nomenclature
	<i>Niu Xi</i> (牛膝)	amaranthaceae	<i>Achyranthes bidentata</i> <i>Bl.</i>	<i>Achyranthes bidentata</i>
Components from animals	<i>Gui Jia Jiao</i> (龟甲胶)	chinemys reevesii	decocted carapace of <i>Chinemys reevesii</i> with water and then concentrated	<i>Testudinis Carapax et Plastic Collar</i>
	<i>Lu Jiao Jiao</i> (鹿角胶)	cervus elaphus Linnaeus/ cervus nippon Temminck	decocted Ossified cornu cervi or fallen cornu cervi after sawing with water and then concentrated	<i>Cervi Conrnus Colla</i>

2.2 Analysis of ZGP by ultra performance liquid chromatography with quadrupole orthogonal time-to-flight mass spectrometry (UPLC-Q-TOF-MS)

LC-MS analysis was conducted on a Waters ACQUITY UPLC system connected to an Waters Xevo G2-S Q-TOF mass spectrometry (Waters Co., Milford, MA, USA) by an electrospray ionization (ESI) interface. Liquid chromatographic separation was performed on an Waters HSS T3 column (1.8 μm , 2.1 mm \times 100 mm) maintained at 35 $^{\circ}\text{C}$ and eluted with gradient water (A) and acetonitrile (B), both with 0.1% (v/v) formic acid at a flow rate of 0.3 ml/min. The following gradient elution was performed as follows: 5% B from 0 to 1 min, 5-70% B from 1 to 15 min, 70-100% B from 15 to 17 min, 100% B from 17 to 18 min, 100-5 % B from 18 to 20 min, 5% B from 20 to 22 min. The injection volume was 3 μl .

Mass spectra were operated in negative-ion mode. The source ionization condition were as follow: drying and sheath gas temperature, 100 $^{\circ}\text{C}$; cone gas flow rate, 50 L/h, desolvation gas flow rate, 550 L/h; capillary voltage, 2800 V. Data were acquired over a range of m/z 100-1200 for MS with an acquisition rate of 0.2 spectra/second, and analyzed by MassLynx V4.1.

Powder of ZGP was dissolved into 50% methanol to 20 mg/ml for use. The mixture containing six reference components were used for the qualitative analysis: loganic acid, morroniside, sweroside, hyperoside, beta-ecdysterone and kaempferol. The compounds were verified by comparing the individual peak retention times with those of the reference substances.

(Fig. 1)

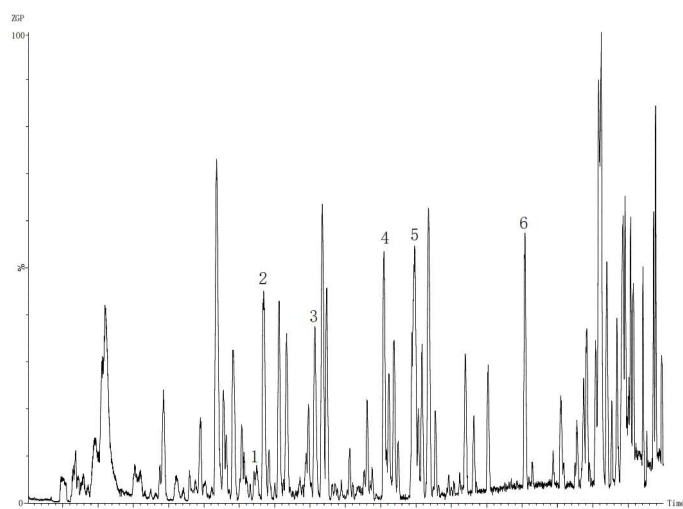


Figure 1. The total ion chromatogram in negative model of ZGP.

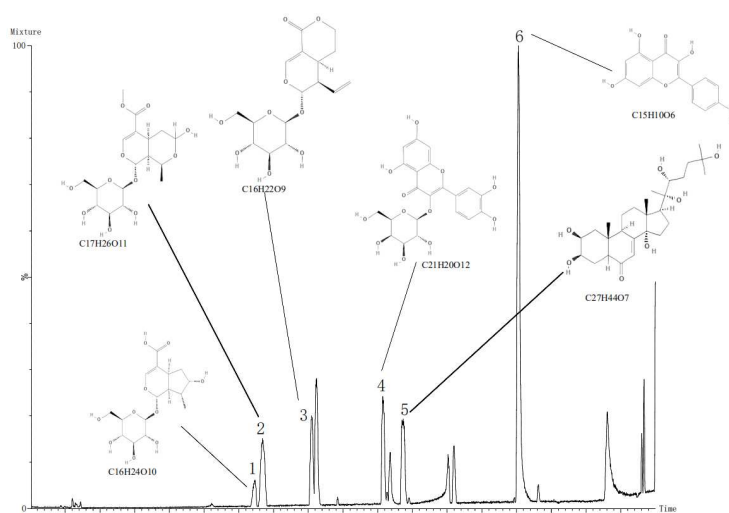


Figure 2. The total ion chromatogram in negative model of the mixture containing six reference components: 1. loganic acid, 2. morroniside, 3. sweroside, 4. hyperoside, 5. beta-ecdysterone, 6. kaempferol.

Table 2. Informations about the six components

Peak no	t_R (min)	Formula	Selected ion	Extraction mass (Da)	Found at mass (Da)	Error (ppm)	Origination
1	6.47	C ₁₆ H ₂₄ O ₁₀	[M-H] ⁻	375.1291	376.1369	0.28	<i>Corni Fructus</i>
2	6.68	C ₁₇ H ₂₆ O ₁₁	[M-H] ⁻	405.1397	406.1475	0.26	<i>Corni Fructus</i>
3	8.10	C ₁₆ H ₂₂ O ₉	[M-H] ⁻	375.1186	358.1264	0.30	<i>Corni Fructus</i>

4	10.15	C21H20O12	[M-H] ⁻	463.0877	464.0995	0.23	<i>Cuscuta chinensis</i>
5	10.80	C27H44O7	[M-H] ⁻	479.3009	480.3087	0.22	<i>Corni Fructus</i>
6	14.06	C15H10O6	[M-H] ⁻	285.0399	286.0477	0.37	<i>Cuscuta chinensis/ Dioscoreae Rhizoma</i>

2.3 Animals

Seventy 8-week-old Sprague-Dawley rats (fifty four female rats and sixteen male rats. Permit: No.44007200026331) were obtained from the Guangdong Medical Lab Animal Center (China). The rats were kept in a cage under the conditions of light (12-hour light and 12-hour dark regime), temperature (22 °C), humidity (65%), and fed ad libitum on rat cubes and tap water. All protocols were approved by Institutional Animal Care and Ethics Committee of Guangzhou University of Chinese Medicine.

2.4 Experimental protocol

After adapting for a week, fifty four 8-week-old female rats (weight 180-220 g) were equally randomized to six groups: the Control group, Model group, three ZGP groups (high dose group, medium dose group and low dose group) and Triptorelin group. The detailed process was as follow: On the first day, rats in the Triptorelin group were intramuscular injected (i.m.) with 1.5 mg/kg triptorelin (Ipsen Pharma Biotech, France, H16290), which was dissolved into the appendant solution to a concentration of 0.75 mg/ml. Each rat in other groups were received 0.5 ml saline. All groups were established POF model except the Control group which served as a negative control. Cyclophosphamide was dissolved into saline to concentrations of 5 mg/ml and 0.5 mg/ml. On the 11th day, all groups except the Control group were intraperitoneally injected (i.p.) with 50 mg/kg cyclophosphamide (5 mg/ml, Shanxi Powerdone Pharmaceutical Co.,Ltd. China, 04150403). And from the 12th to 25th day, all groups except the Control group were intraperitoneally injected with 5 mg/kg cyclophosphamide (0.5 mg/ml), each rat in Control group received 1 ml saline at the same time. According to the dose conversion between human and rat related to surface area, approximately 6.17, we designed high/middle/low dose for intragastric administration (equivalent to two-times/ full/ half dose for human). Powders ground from pills were dissolved into distilled water to a concentration of 0.2 g/ml and stored at 4 °C for further use. During the 26th to 85th day, three ZGP groups were intragastric administrated (i.g.) with 0.2 g/ml daily at doses of 3.2 g/kg, 1.6 g/kg and 0.8 g/kg respectively; Meanwhile each rat in other groups received 2 ml saline. Details of treatment schedule are shown in the table 3.

Vaginal smears were taken in every morning. One to five days after the last irrigation, blood from orbital venous plexus of five rats in each group was collected on non-estrus period under anesthesia, by intraperitoneally administering 10% chlorali hydras (3 ml/kg), as well as oophorectomy. Then rats were euthanized by cervical dislocation. The blood samples was allowed to coagulate for approximately 1 h at room temperature and serum was obtained by centrifugation at 3000 rpm/min for 15 min and stored at -80 °C for future use. Several ovarian tissues were immersed in 10% formaldehyde for histopathology, and the rest were frozen and preserved at -80 °C for genes and proteins expression analysis. Additionally, the other four rats in each groups were sent to mate with male rats at the ratio of 1:1 for 12 h. The mixture of sperm and vaginal smears seen on the next morning indicated the success of pregnancy, and this was considered as the 0.5th day of the gestation. The pregnant rats were euthanized on the 13.5th day of the gestation.

Table 3. Details of treatment schedule

Day	Control	Model	ZGP(3.2 g/kg)	ZGP(1.6 g/kg)	ZGP(0.8 g/kg)	Triptorelin
1 st day	0.5 ml saline, i.m.	0.5 ml saline, i.m.	0.5 ml saline, i.m.	0.5 ml saline, i.m.	0.5 ml saline, i.m.	1.5 mg/kg triptorelin (0.75 mg/ml), i.m.
11 th day	1 ml saline, i.p.	50 mg/kg cyclophosphamide (5 mg/ml), i.p.	50 mg/kg cyclophosphamide (5 mg/ml), i.p.	50 mg/kg cyclophosphamide (5 mg/ml), i.p.	50 mg/kg cyclophosphamide (5 mg/ml), i.p.	50 mg/kg cyclophosphamide (5 mg/ml), i.p.
15 th -26 th day	1 ml saline, i.p.	5 mg/kg cyclophosphamide (0.5 mg/ml), i.p.	5 mg/kg cyclophosphamide (0.5 mg/ml), i.p.	5 mg/kg cyclophosphamide (0.5 mg/ml), i.p.	5 mg/kg cyclophosphamide (0.5 mg/ml), i.p.	5 mg/kg cyclophosphamide (0.5 mg/ml), i.p.
26 th -85 th day	2 ml saline, i.g.	2 ml saline, i.g.	3.2 g/kg ZGP (0.2 g/ml), i.g.	1.6 g/kg ZGP (0.2 g/ml), i.g.	0.8 g/kg ZGP (0.2 g/ml), i.g.	2 ml saline, i.g.

2.5 Histopathological Evaluation of Ovarian Tissue

After oophorectomy, ovary tissues were fixed in 10% formaldehyde for more than 24 h, and dehydrated in standard graded alcohol (70%, 85%, 95% and 100%) and then embedded in paraffin. Those tissues were serially sectioned latitudinally at 3 µm for next hematoxylin and eosin (H&E) staining. The slides were covered with neutral balsam (Beijing Zhongshan Jinqiao Biotechnology

Inc., China), all phases of follicles and corpora lutea were count under the microscope (Leica, DM500, Germany).

2.6 Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed to test the concentrations of FSH and oestradiol E2 in blood samples of all groups. Blood samples were obtained from orbital venous plexus and the serum was isolated through centrifugation for ELISA analyses using kits from Cusabio Biotech Co.,Ltd (Wuhan, China, CSB-E06869r, CSB-E05110r) according to the instruction of the manufacturers. Optical densities (OD) were read at 450 nm, and concentrations of FSH and E2 were determined by comparison with standard curves.

2.7 quantitative Real time-Polymerase Chain Reaction (qPCR)

Expression of Bax, Bcl-2 and Cyt-c in ovarian tissues was determined by RNA preparation and qPCR. Briefly, total cellular RNA was isolated from ovary tissue homogenate using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Imprinted gene regions were amplified using specific primers (Table 4) synthesized by Sangon Biotech Co.,Ltd (Shanghai, China). The qPCR analysis was carried out using the Prime ScriptTM RT reagent Kit SYBR (Takara, DRR047A) followed by Premix EX Taq[□] Kit (TaKaRa, Japan, RR820A). The reaction run at one cycle of 95[□] for 3 minutes, followed by 40 cycles of 95[□] for 5 seconds, 60[□] for 30 seconds. We used GAPDH expression as an internal control. All the experiments described above were performed in triplicate. Relative gene expression was quantified by using the $2^{-\Delta\Delta Ct}$ method.

Table 4. Sequences of the primers

Gene	Accession number	Product size (bp)	Primer sequence (5'-3')
Bax	NM 017059.2	88	F:CCCACCAGCTCTGAACAGATC
			R:TCTCCCAGCCATCCTCTCT
Bcl-2	NM 016993.1	86	F:GGCATCTGCACACCTGGAT
			R:ATCAAACAGAGGTCGCATGCT
Cyt-c	NM 012839.2	102	F:GGGAGAGGATACCCTGATGGA

R:TCTGCCCTTTCTCCCTTCTTCT

GAPDH

NM 017008.4

110

F:AGGGCTGCCTTCTCTTGTGA

R:AACTTGCCGTGGGTAGAGTCA

2.8 Western Blot Analysis

Ovary tissues were fully split by protein lysis buffer. Then supernatant was obtained after centrifugation at $12000\times g$ for 3-5 min. Equal amount of proteins were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically to polyvinylidene difluoride (PVDF) membranes at 300 mA for 20-30 minutes. Then it was blocked in 5% skim milk at room temperature for 2 h. The membranes were incubated at 4 °C overnight with anti-Bax (1:1000, Abcam, USA, Ab32503), Bcl-2 (1:1000, Abcam, USA, Ab59348), Cyt-c (1:1000, Cell Signaling Technology, USA, #11940) and immunoblotted with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (Santa Cruz, sc-2004, 1:5000) at room temperature for 2 hours. The membranes were then developed with enhanced chemiluminescence (ECL) substrate (Beyotime, China, P0018M) and exposed to X-ray film. The mean optical density (OD) of image was analyzed by Langgi image analysis software.

2.9 Immunohistochemistry

The paraffin sections of ovaries were deparaffinized, rehydrated, and then incubated in the citrate antigen retrieval solution (Beijing Solarbio Science & Technology Co.,Ltd., C1032, China) for 2.5 min in a pressure cooker. After quenching endogenous peroxidase activity with 3% H₂O₂ for 8 min, the slides were incubated with anti-Bax (1:250, Abcam, USA, Ab32503), Bcl-2 (1:200, Abcam, USA, Ab59348), Cyt-c (1:200, Cell Signaling Technology, USA, #11940) at 4 °C overnight. On the next day, the slides were incubated at 37 °C for 20 min with HRP-conjugated goat anti-rabbit IgG (free of dilution, GeneTech Company Ltd., GK5007, China). After 3,3'-diaminobenzidine (DAB) (Gene Tech Company Ltd., GK5007, China) staining, the slides were counterstained with hematoxylin for 3 minutes at room temperature, dehydrated in a series of 70% to 100% alcohol baths and cleared in a xylene bath. The slides were mounted with neutral balsam and observed using a microscope (Leica, DM500, Germany). The mean optical densities (OD) were semi-quantitatively analyzed with Image J software.

2.10 Statistical Analysis

Enumeration data in this study were expressed as values and analyzed by Fisher exact

probability method. Measurement data were expressed as means \pm standard deviation (SD). The mean values and standard deviations were calculated from three independent experiments. Statistical analysis of measurement data was carried out by SPSS 22.0 for Windows. Analysis of variance (ANOVA) was adopted for comparison among groups, and Student's t-test was used for inter-group comparison. $P < 0.05$ was considered statistically significant.

3.Result

3.1 ZGP reduced the abnormality of estrous cycle caused by cyclophosphamide

In our study, we found that rats in the Model group were depressed or irritable, intensive, and prone to alopecia comparing with other groups. Although rats in groups treating with ZGP or Triptorelin were less excited and less vivid than those in the Control groups, which was still better than those in the Model group. Estrous cycles after treatments also showed a significant difference (Figure 3. B). Sixty days after model establishment, no rats in Model group showed a normal estrous cycle. Comparing with the Model group, estrous cycles were recovered significantly in the ZGP groups (3.2 g/kg and 1.6 g/kg) and Triptorelin group ($p < 0.05$). To further assess the recovery of ovarian function, we selected four rats in each groups to mate with the male rats, although results (Figure 3. C) showed no significant difference, even the rate of pregnancy in the ZGP group (1.6 g/kg) seemed more than those in the ZGP group (3.2 g/kg). However, numbers of embryos (Figure 3. A) indicated that only the ZGP group (3.2 g/kg) had the same numbers (fourteen) with the Control group, embryo resorption may occur in other groups. And the small numbers of the rats in each group may also lead to the no statistically significant result.

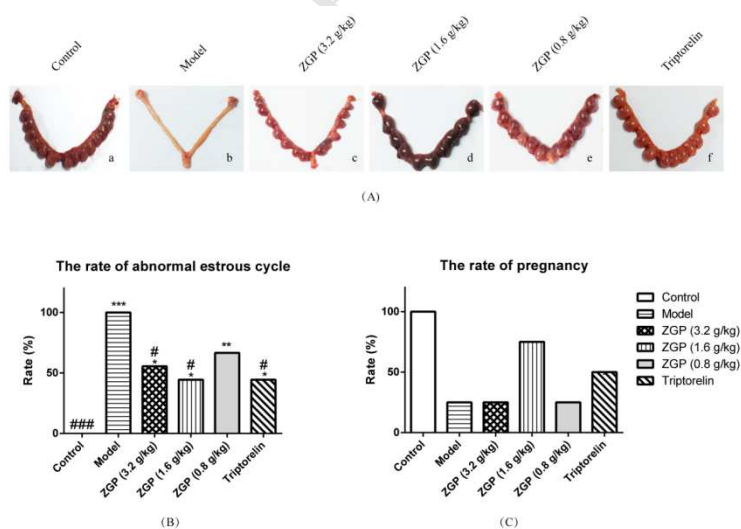


Figure 3. A. (a) and (c) showed 14 embryos in the Control group and ZGP group (3.2 g/kg), (b) showed an uterus without pregnancy in the

Model group. (d) showed 10 embryos in the ZGP group (1.6 g/kg), (e) showed 11 embryos in the ZGP group (0.8 g/kg), and (f) showed 12 embryos in the Triptorelin group. B. 60 days after molding, no rats in the Model group showed a normal estrous cycle, ZGP and triptorelin reduced the abnormality of estrous cycle caused by cyclophosphamide. $N=9$ /group; * $p<0.05$, ** $p<0.01$, *** $p<0.001$ versus the Control group. # $p<0.05$, ## $p<0.01$, ### $p<0.001$ versus the Model group. C. The rate of pregnancy indicated no significant difference among groups; $N=4$ /group.

3.2 ZGP reduced the follicle apoptosis induced by cyclophosphamide

After administration with cyclophosphamide, the ovarian volumes and weight in the Model group were less than those in Control group. Inflammatory cells and fiberization occurred in the interstitial portion. Primary follicles, second follicles and corpora lutea decreased, atretic follicles increased (Figure 4. A. b). Meanwhile, after treating with ZGP and triptorelin, the ovarian volumes and weight were similar to those in the Control group (Figure 4. A. c, d, e, f). Only a few of inflammatory cells and mild fiberization occurred in the stroma. Decrease of primary follicles, second follicles and corpora lutea, and increase of atretic follicles could be alleviated significantly comparing with those in the Model group (Primary follicles: $p<0.01$ for the ZGP group (3.2 g/kg) and the Triptorelin group; Secondary follicles: $p<0.05$ for the ZGP group (0.8 g/kg), $p<0.01$ for the ZGP groups (3.2 g/kg and 1.6 g/kg) and the Triptorelin group; Corpora lutea: $p<0.05$ for the ZGP group (3.2 g/kg) and the Triptorelin group; Atretic follicles: $p<0.01$ for the ZGP group (0.8 g/kg), $p<0.001$ for the ZGP groups (3.2 g/kg and 1.6 g/kg) and the Triptorelin group. As is shown in Figure 3. B).

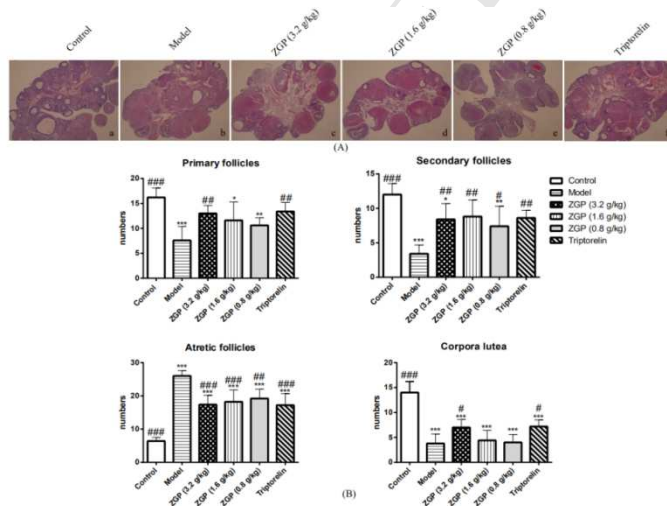


Figure 4. ZGP reduced the follicle apoptosis induced by cyclophosphamide. A. Ovarian tissue slides with H&E staining (40 ×): (a) Ovarian section in the Control group expressed a clear ultrastructure, many follicles and fresh corpora lutea but no inflammatory cells or fiberization could be found; (b) Ovarian section in Model group expressed many inflammatory cells, atretic follicles and fiberization in stroma; (c-f) small number of inflammatory cells and mild fiberization in stroma could be found in the ZGP groups and Triptorelin group. And primary, secondary follicles and corpora lutea in the ZGP groups (3.2g/kg and 1.6g/kg), as well as the Triptorelin group were a little

more than those in the ZGP group (0.8g/kg) , contrary to the atretic follicles. B. Numers of primary follicles, secondary follicles, atretic follicles and corpora lutea in groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the Control group. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ versus the Model group.

3.3 ZGP regulated the concentrations of serum hormone

In order to investigate the changes of serum hormone concentrations, we obtained blood on non-estrous period. As the figure 4 showed, the serum FSH concentration increased and the E2 concentration decreased significantly in the Model group comparing with the Control group ($p < 0.001$). And treating with ZGP and triptorelin could reduce the serum FSH concentration comparing with the Model group ($p < 0.001$), however, which were still higher than that in the Control group (the Triptorelin group: $p < 0.05$ versus the Control group; the ZGP groups: $p < 0.001$ versus the Control group). Effect of The ZGP groups (3.2 g/kg) showed an advantage than the rest ZGP groups ($p < 0.001$).

The serum E2 levels in the ZGP group (0.8 g/kg) was as low as those in the Model group, the serum E2 levels in the ZGP groups (3.2 g/kg and 1.6 g/kg), and the Triptorelin group showed no difference with the Control group ($p > 0.05$). And the effects of the ZGP groups (3.2 g/kg and 1.6 g/kg) were better than that of the the ZGP group (0.8 g/kg) ($p < 0.01$), but no significant difference showed in the first two ZGP groups ($p > 0.05$).

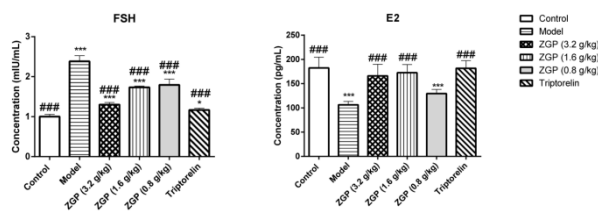


Figure 5. The concentrations of FSH and E₂ among groups 60 days after administration with cyclophosphamide. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the Control group. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ versus the Model group.

3.4 ZGP regulated the balance of Bax/Bcl-2 in ovaries.

To identify the molecular mechanism of ZGP responsible for inhibiting the ovarian toxicity induced by cyclophosphamide. We investigated the expression of Bax/Bcl-2, which were related to apoptosis, both on genes and proteins through qPCR, western blot and immunohistochemical analysis. The expression of Bax genes and proteins in Model group were overexpressed, and the expression of Bcl-2 genes and proteins were less expressed comparing with the Control group ($p < 0.001$ in qPCR and immunohistochemical analysis, $p < 0.01$ in western blot analysis).

After treating with ZGP or triptorelin, the expressions of Bax gene (Figure 6.) were higher

than that in the Control group ($p < 0.001$) but obviously lower than that in the Model group ($p < 0.001$), which were contrary to the expressions of Bcl-2 genes ($p < 0.001$). On the expressions of the Bax genes, the ZGP groups (3.2 g/kg and 1.6 g/kg) showed a lower levels than that showed in the ZGP group (0.8 g/kg) ($p < 0.001$). And on the expression of the Bcl-2 gene, the ZGP group (3.2 g/kg) showed an advantage than the rest ZGP groups ($p < 0.001$).

Analysis in western Blot (Figure 7.) also showed the consistent results that the expressions of Bax in the ZGP groups and the Triptorelin group were significantly decreased than that in the Model group (the ZP groups (3.2 g/kg and 1.6 g/kg): $p < 0.01$, the ZGP groups (0.8 g/kg) and the Triptorelin group: $p < 0.001$). Additionally, results revealed that only in the ZGP group (3.2 g/kg), the expression of Bcl-2 had been upregulated significantly ($p < 0.01$ versus the Model group, $p > 0.05$ versus the Control group).

It was also convicted in the immunochemical analysis that after ZGP or triptorelin administration, the expressions of Bax were significantly lower than those in the Model group ($p < 0.001$), and the expressions of Bcl-2 were obviously higher than those in the Model group ($p < 0.001$) (Figure 8). And the ZGP group (3.2 g/kg and 1.6 g/kg) showed a better effect comparing with the ZGP group (0.8 g/kg) ($p < 0.001$).

3.5 ZGP inhibited the release of Cyt-c from mitochondria to cytoplasm induced by cyclophosphamide.

Cyt-c, a channel protein in mitochondria, had also been investigated to reveal the effect of mitochondria on the cell apoptosis. Results in the expression of gene and protein (Figure 6, 7, 8) suggested that after exposing to cyclophosphamide, Cyt-c in mitochondria was released to cytoplasm substantially ($p < 0.001$). After treating with ZGP or triptorelin, the expression of gene Cyt-c was markedly downregulated ($p < 0.001$), and the ZGP groups (3.2 g/kg and 1.6 g/kg) showed a better effect than the ZGP groups (0.8 g/kg). Both in the western blot and immunohistochemical analysis, the expressions of Cyt-c were decreased in the ZGP groups and the Triptorelin group versus that in the Model group (the ZGP group (0.8 g/kg): $p < 0.05$; the ZGP group (1.6 g/kg): $p < 0.01$; the ZGP groups (3.2 g/kg) and the Triptorelin group: $p < 0.001$).

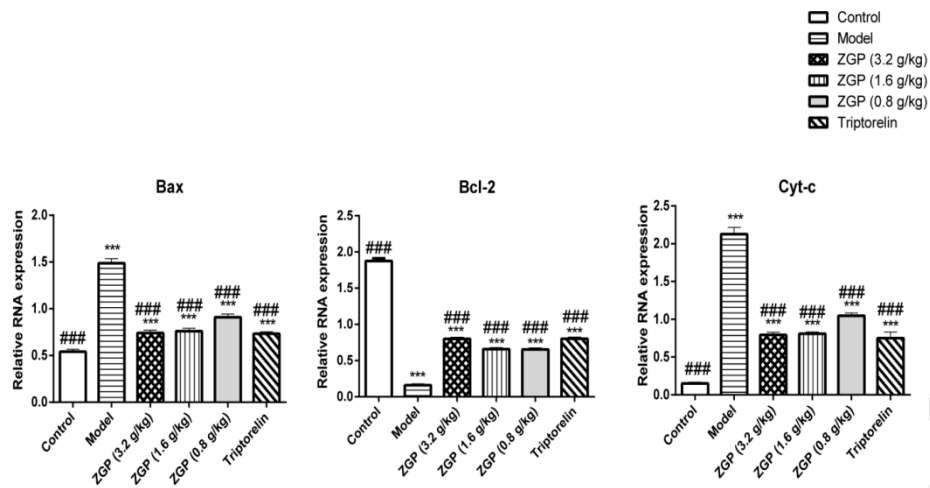


Figure 6. ZGP downregulated the expression of Bax and Cyt-c gene, as well as upregulated the expression of Bcl-2 gene. $N = 5$ /group. Each value was presented as the mean \pm SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the Control group. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ versus the Model group.

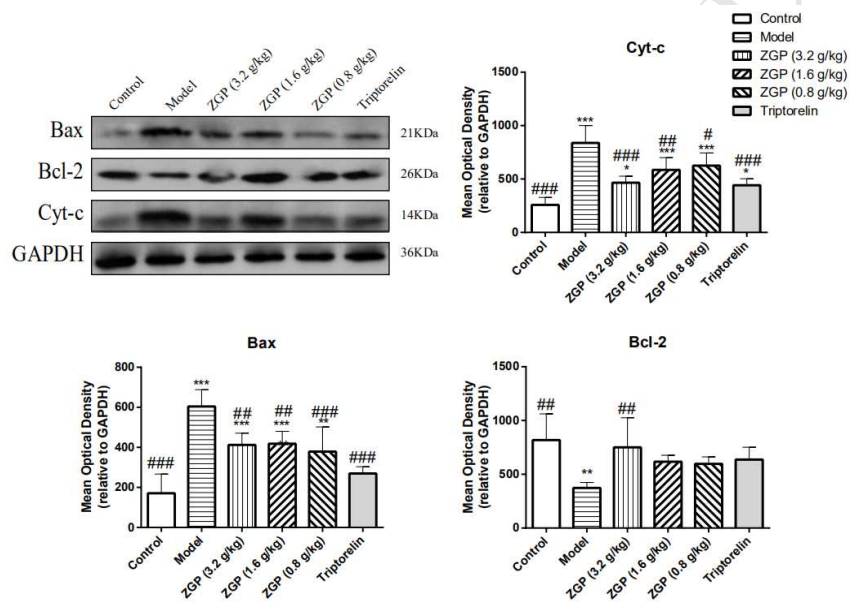


Figure 7. ZGP downregulated the expression of Bax and Cyt-c protein, as well as upregulated the expression of Bcl-2 protein. $N = 5$ /group. Each value was presented as the mean \pm SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the Control group. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ versus the Model group.

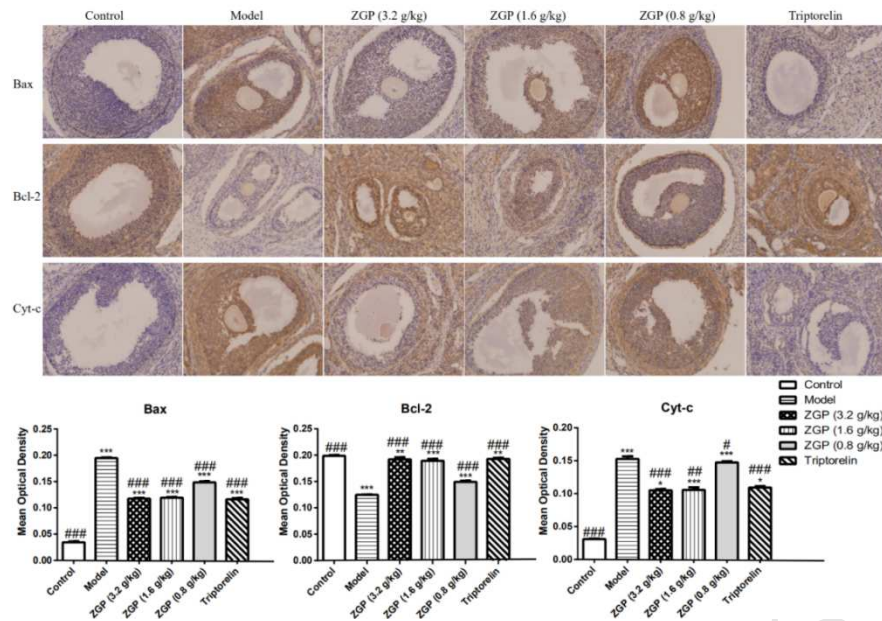


Figure 8. Immunohistochemistry was performed to evaluate the protein expression levels of Bax, Bcl-2 and Cyt-c in ovaries (200 ×). N = 5 /group. Each value is presented as the mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the Control group. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ versus the Model group.

4. Discussion

Based on the present theories, the essence of POF is thought to be the depletion of follicles which would not regenerate, although it has been reported that oocytes could regenerate from stem cells (Zou et al., 2009; Wu et al., 2018). A recent study reported that exposure to chemotherapy counts for about 1.9% among the risk factors of POF (Jiao et al., 2017). And chemotherapeutic drugs cause an ovarian damage by activating mitochondria-dependent apoptotic cascade in follicles. Our study focused on utilizing ZGP from TCM as a novel therapeutic agent for treating POF and we found that ZGP prevented the loss of follicles and ameliorated estrous cycle after exposure to chemotherapeutic drugs. In addition, administration of ZGP could decrease the serum level of FSH, and increase the serum level of E2. Further investigation of mechanism suggested that the protective effect of ZGP was associated with inhibiting the activation of mitochondria-dependent apoptotic cascade at both gene and protein levels. These findings established an experimental foundation for the use of ZGP as a promising treatment for POF.

In order to detect the injury mechanism of chemotherapy and protective mechanism of ZGP, we adopted cyclophosphamide to establish the rat model. Based on previous study (Wang et al., 2011), we explored a more appropriate method for model establishment. Results showed that no animal died after exposure to cyclophosphamide with first dose of 50mg/kg and maintenance dose of 5mg/kg. Constant abnormality of estrous cycle, low fertility rate, high concentration of FSH, low concentration of E2, the loss of follicles, and high expression of protein related to apoptosis

showed the reliability and stability of the model. As is known to all, cyclophosphamide is not only widely used in clinics, but also showed a higher risk of POF prevalence than other chemotherapeutic drugs (Lambouras et al., 2018; Nishi et al., 2018; Chun et al., 2014). Apoptosis induced by cyclophosphamide is prone to occur in cells with high mitosis index such as granulosa cells in growing follicles. After cross-linking of DNA and upregulating the expression of Bax, massive Bax/Bax homodimer formation makes the mitochondrial membrane depolarized and Cyt-c will be released to cytoplasm and combines with Apaf-1 and caspase-9 to form the apoptotic body. Activated caspase-9 further activates caspase-3 etc., then apoptotic cascade will be activated (Morgan et al., 2012; Yuan and Akey, 2013; Würstle et al., 2012; Zhao et al., 2010). Cyt-c is thought to be the trigger of the apoptosis occurrence. And loss of Cyt-c will cause the dysfunction of chondriosomal respiratory chain and accumulation of reactive oxygen species, which could promote the apoptosis in both granulosa cells and oocytes (Matsuda et al., 2012). Previous studies also indicated that dormant primordial follicles could also be activated by cyclophosphamide, then quiescence rather than apoptosis occurred by activating PI3K/PTEN/Akt signaling pathway (Kalichphilosoph et al., 2013).

ZGP is a well known prescription for its effect of tonifying kidney yin and essence, and has been widely applied clinically by TCM practitioners over the past four hundred years, although the exact pharmacological and toxicological mechanism remain unclear. Recipes in TCM usually have many components and obtain effects from multiple targets. In this study, recovered morphology and estrous cycle, variously increased primary and secondary follicles, as well as decreased atretic follicles all suggested a good protective effect of ZGP. Furthermore, our previous studies indicated that ZGP could not only shorten anestrus which was elongated in the autoimmune model of POF, but also increase the ratio of growing follicles and decrease the ratio of atretic follicles (Zhu et al., 2017). And atresia of follicles was the manifestation of apoptosis. Follicles that had received direct damage caused by cyclophosphamide could release an abundance of ovarian antigens to arouse immunological imbalance (Notarianni, 2011). Recipe tonifying kidney could alleviate this excessive immunoreaction by downregulate the ratio of $CD4^+/CD8^+$ and the numbers of Th1, Th17 and Treg cells (Huang et al., 2017).

In the study, we also examined the effect of ZGP for regulating the endocrinological function by testing the concentrations of serum FSH and E2. FSH originated from pituitary acts on the ovary, and then combines with FSHR along with aromatase activation to promote the production of E2 in the granulosa cells which is essential for the follicle growth. While E2 could suppress the secretion of FSH via a negative feedback loop (Dewailly et al., 2016). In the model of POF, apoptosis of follicles caused a low level of E2, which is sent to the pituitary and stimulates more secretion of FSH via a negative feedback loop. In our work, we observed a significant decreased level of serum FSH and an increased level of serum E2 in both ZGP groups and the Triptorelin group, as well as the gradually docile temperament of rats. And high dose of ZGP group showed a

better effect. It is suggested that FSH along with AMH and INHB reflected the ovarian reserve (Riggs et al., 2008). And the rate of pregnancy also indicated that ZGP could improve the ovarian reserve, although differences were not significant.

To further investigate the anti-apoptotic mechanism of ZGP, expression of Bax, Bcl-2 and Cyt-c were detected on the levels of both genes and proteins. Results suggested that ZGP exerted its anti-apoptotic effect through inhibiting the expression of Bax (pro-apoptotic molecule), and improving the expression of Bcl-2 (anti-apoptotic molecule). Decreased Bax moved to mitochondria and mostly combined with increased Bcl-2 to form the Bax/Bcl-2 heterodimer on the membrane of mitochondria, which showed an anti-apoptosis effect. The mitochondria membrane potential would be stable and little Cyt-c would be released to cytoplasm. Bcl-2 family contains pro-apoptosis molecules (Bid, Bax, BAD, etc.) and anti-apoptosis molecules (Bcl-2, Bcl-xL, Bel-w, etc.) (Benito et al., 2015). Bcl-2 is a proto-oncogene located at chromosome 18a21 and closely associated with inhibiting apoptosis of cancer cells. While Bax is a pro-apoptotic gene located at chromosome 19q13. Studies revealed that the Bax/Bcl-2 heterodimer and Bcl-2/Bcl-2 homodimer are beneficial to maintain the stability of mitochondria membrane, and the Bax/Bax homodimer is devoted to reducing the mitochondria membrane potential. So the ratio of Bax and Bcl-2 decides the fate of the cells, and Cyt-c is the key to open the gate of fate (Wallgren et al., 2013).

TCM is profound not only for the unique system of syndrome differentiation, but also for the exquisite composing principles of the recipes. While complex and unidentified mechanism is a great barrier preventing the wide application of TCM. The mainly effective component of *Rehmanniae Radix*, which is the principal drug of ZGP, is rehmanniae radix polysaccharides. And it seemed to be a good antioxidant by increasing the level of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px), as well as an immunomodulator by regulating the serum interleukin-2 (IL-2), interleukin-4 (IL-4) and interleukin-10 (IL-10) levels (Sui et al., 2013). Previous pharmacological studies have revealed that lots of bioactive components existed in the *Corni Fructus*. Morroniside extracted from *Corni Fructus* could inhibit the neuron apoptosis by decreasing caspase-3 and increasing the ratio of Bcl-2/Bax (Zeng et al., 2018). And beta-ecdysterone derived from *Corni Fructus* could not only reduce the production of reactive oxygen species but also promote a Bcl-2/Bax ratio (Xu et al., 2018). It was also reported that Tannin derived from *Corni Fructus* and Chinese yam polysaccharide derived from *Dioscoreae Rhizoma* (the ministerial drug of ZGP) had the capacity of antioxidation (Sobeh et al., 2017; Liu et al., 2016). Besides, total flavones and hyperoside derived from *Cuscuta chinensis* (adjuvant drug of ZGP) had the ability of anti-apoptosis through regulating the ratio of Bax and Bcl-2 (Miao et al., 2018; Hao et al., 2016). Limitations also existed in these studies about components of ZGP, and absorbable bioactive components of ZGP need further research.

5. Conclusion

In summary, this study illustrated the therapeutic efficiency of ZGP for POF induced by chemotherapy and the potential mechanism might involve in suppressing the mitochondria-dependent apoptosis, which sets up an experimental basis for ZGP as a sensible therapeutic option for the treatment of POF.

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Author contributions

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Wrote the manuscript: Lihua Zeng, Huijuan Peng, Ling Zhu.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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