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The Effect of Zuogui Pill on Autophagy and Apoptosis in Chemotherapy-damaged Granular Cells and Theca Cells*

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Abstract **Objective** To investigate the effect and mechanism of Zuogui pill-containing serum (ZGP-containing serum) on chemotherapy-damaged granular cells (GCs) and theca cells (TCs). **Methods** GCs and TCs were cultured respectively, the model group was established with phosphoramide mustard (PM), then treated by ZGP-containing serum. The survival rates of GCs and TCs was determined by CCK-8. Real-time fluorescent quantitative PCR (RT-PCR) and Western blot methods were used to detect the expression of Beclin-1, light chain 3 (LC3B), p62, Bax and Caspase3. **Results** 10% ZGP-containing serum had the best effect on the recovery of cell survival rate. Compared with the blank control group, Beclin-1, LC3B, Bax and Caspase3 have higher expression in the model group (P<0.05), and 10% ZGP-containing serum can down-regulate the expression of them (P<0.05). Moreover, the expression of p62 is lower in the model group than the blank control group (P<0.05), and 10% ZGP-containing serum can up-regulate it (P<0.05). In addition, in the groups of GCs, after activating or inhibiting the autophagy pathway, the expression of autophagy-related proteins and apoptosis-related proteins both changed correspondingly. **Conclusion** PM can damage GCs and TCs by promoting apoptosis and activating autophagy/lysosomal degradation pathways, 10% ZGP-containing serum can alleviate the damage. There is a cross-talk between autophagy and apoptosis in the process of PM damaging GCs which can be alleviated by 10% ZGP containing serum.

Key words Zuogui pills, chemotherapy, granular cells, theca cells, autophagy, apoptosis
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The disease caused by premature exhaustion of ovarian reserve before the age of 40 is called premature ovarian failure (POF). Patients have symptoms of oligomenorrhea or amenorrhea, often accompanied by genital atrophy, infertility, nervous system dysfunction, osteoporosis and cardiovascular disease, *etc*., which seriously affect the quality of life and reproductive function of them[1]. With the younger trend of female malignant tumors, the incidence of chemotherapeutic POF continues to increase through the years[2].

It is believed that the follicle is basic functional unit of female reproductive system. The basic pathogenesis of POF is related to the deficiency of the kidney essence in traditional Chinese medicine theory[3]. The follicle is composed of theca cells (TCs), granulosa cells (GCs) and oocytes, among

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them, GCs are of great significance to the maturation of oocytes. A number of experimental studies have shown that GCs are seriously damaged in chemotherapeutic POF animal models[4]. In addition, TCs play a vital role in the occurrence and development of follicles. It has been shown that follicular TCs can not only provide necessary androgens for follicular development[5], but also mediate the interaction between oocytes and GCs, participating in the regulation of apoptosis and follicular development[6]. However, in most follicle-related researches, researchers pay more attention to GCs instead of TCs. In *Jingyue Quanshu*[7], ZHANG Jing-Yue first proposed that Zuogui pill (ZGP) has the effect of kidney-tonifying, essence-generating, and marrow-benefiting, indicating it has a better therapeutic effect on POF. Results of our recent pharmacodynamic research also show that the apoptosis rate of ovarian GCs in POF mice induced by cyclophosphamide is increased, and ZGP can restore the ovarian function of mice and reduce the apoptosis of GCs to a certain extent[8]. And phosphoramide mustard (PM, the active ingredient of cyclophosphamide *in vitro*) can damage GCs by promoting apoptosis and activating autophagy/lysosomal degradation pathways, but 10% ZGP-containing serum can alleviate the damage and affect the autophagy and apoptosis of GCs[9].

In order to provide more theoretical basis for the clinical medication of traditional Chinese medicine for POF, in recent years, our research group have been committing to exploring the mechanism of the damage to GCs and TCs caused by PM and the effect of ZGP-containing serum on them. We already found that ZGP-containing serum can protect GCs by inhibiting apoptosis and autophagy. However, there are still some problems in the process, such as whether there is a cross-talk between autophagy and apoptosis in this process and what is the effect of ZGP-containing serum on TCs damaged by PM. So, in the current study, we further explored the mechanism of PM's damage to GCs and TCs and the effect of ZGP-containing serum on them.

1 Materials and methods

1.1 Materials

1.1.1 Animals and cells

Sixty specific pathogen free (SPF) female SD rats, 8 weeks old, with 250−270 g were provided by Hunan Center of Drug Safety Evaluation and Research of Drugs & Hunan Key Laboratory of Pharmacodynamics and Safety Evaluation of New Drugs, experimental animal production lot number: 1107271911005469. The animals were dedicated to serum preparation and raised in SPF environment, the license number of experimental animals is SCXK (Xiang) 2019-0004. All procedures were operated according with the guide for the Care and Use of Laboratory Animals (8th Edition) issued by The National Academics (Washington D. C.) and Animal Ethics Committee of Hunan Center of Drug Safety Evaluation and Research of Drugs & Hunan Key Laboratory of Pharmacodynamics and Safety Evaluation of New Drugs.

Primary rat ovarian GCs and primary rat ovarian TCs were purchased from iCell Bioscience Inc., Shanghai, the lot number of which are iCell201910027 and iCell201912025 separately. The cells were cultured in a 37℃, 5% CO_2 cell incubator.

1.1.2 Main reagents and instruments

ZGP is composed of 8 Chinese herbs, including Shudi (*Radix Rehmanniae Preparata*), Shanyao (*Rhizoma Dioscoreae*), Gouqizi (*Fructus Lycii*), Shanyurou (*Fructus Corni*), Niuxi (*Radix Achyranthis Bidentatae*), Tusizi (*Semen Cuscutae*), Guibanjiao (*Colla Plasti Testutinis*), and Lujiaojiao (*Colla Cornus Cervi*) and was purchased from the First Affiliated Hospital of Hunan University of Chinese Medicine. According to the ratio of 8∶4∶4∶3∶4∶4∶4∶4, 1 kg/L crude drug of Chinese medicinal extract is prepared for use. PM (synthesized by Jiangsu Beida Pharmaceutical Technology Co., Ltd., batch number: 20180908). Electrophoresis instrument (Beijing Liuyi Biotechnology Co., Ltd.); chemiluminescence instrument (Guangzhou Biolight Biotechnology Co., Ltd.); inverted microscope (Olympus IX71); real-time fluorescence quantitative PCR instrument (model: 7500, Thermo Fisher); spectrophotometer (model: NanoDrop Lite, Thermo Fisher); microplate reader (Biotek Co., Ltd.); special medium for GCs (Icell Bioscience Inc, Shanghai, lot number: PriMed-iCell-028); special medium for TCs (Icell Bioscience Inc, Shanghai, lot number: PriMed-iCell-042). CCK-8 kits (Beyotime Co., Ltd., lot number: C0039). BCA protein quantification kit (Beyotime Biotechnology Co., Ltd., lot number: P0012S); RIPA protein lysate (Beyotime Biotechnology Co., Ltd., lot number:

P0013B); secondary antibody: anti rabbit IgG/HRP (Abclonal Biotechnology Co., Ltd., lot number: AS014). Primary antibodies: rabbit anti-p62 (Abclonal Biotechnology Co., Ltd., lot number: A19700); rabbit anti-Beclin-1 (Abclonal Biotechnology Co., Ltd., lot number: A7353); rabbit anti-CASP3 (Abclonal Biotechnology Co., Ltd., lot number: A19654); rabbit anti-Bax (Abclonal Biotechnology Co., Ltd., lot number: A19684); rabbit anti-LC3B (Abclonal Biotechnology Co., Ltd., lot number: A19665); β-actin internal control (Abclonal Biotechnology Co., Ltd., lot number: AC026). High capacity cDNA reverse transcription kits (Thermo Fisher, lot number: EP0742); PowerUp SYBR green master mix (Thermo Fisher, lot number: A25742). Primers (Sangon Biotechnology Co., Ltd.); fast pure cell/tissue total RNA isolation kit (Vazyme Biotechnology Co., Ltd., lot number: RC101-01).

1.2 Methods

1.2.1 CCK-8

The GCs and TCs were seeded in a 96-well plate, and when growing to 75%−80% of the bottom wall, they were treated with 200 μl PM with a final concentration of 30 μmol/L and were cultured in a 37℃, 5% CO_2 cell incubator for 24 h. Then, different concentrations of ZGP-containing serum (2.5%, 5%, 10%, 20%) were added for treatment. After 24 h, they were added with CCK-8 solution, and cultured in cell incubator for 4 h, we use the microplate reader 450 nm for color rendering and read the A_{450} value. The test was repeated 3 times.

1.2.2 Modeling and grouping

The GCs were seeded in a 96-well plate, and when growing to 75%−80% of the bottom wall, they were divided into 11 groups: ① blank control group; ② normal serum group; ③ ZGP-containing serum group (ZGP group); ④ low phagocytic group; ⑤ high phagocytic group; ⑥ low phagocytic+ZGP group; ⑦ high phagocytic+ZGP group; ⑧ model group (M); ⑨ model+ ZGP group; ⑩ model+low phagocytic group+ ZGP group; ⑪ model+ high phagocytic+ZGP groups. In the ⑧⑨⑩⑪ groups, GCs were treated with 200 μl PM with a final the concentration of 30 μmol/L. In the ④ ⑥ ⑩ groups, GCs were treated with RNA interference silenced *Beclin-1* gene to inhibit autophagy activity, and in the ⑤⑦⑪ groups, GCs were simultaneously transfected with mRFP-GFP-LC3 to increase autophagy activity. All of groups were cultured in a 37℃, 5% CO_2 cell incubator for 24 h.

The TCs were seeded in a 96-well plate, and when growing to 75%−80% of the bottom wall, they were divided into 5 groups: blank control group (Control); normal serum group (Normal); model group (M); ZGP-containing serum group (ZGP); model+ZGP-containing serum group (M+ZGP). In M and M+ZGP group, GCs were treated with 200 μl of PM with a final concentration of 30 μmol/L in each well, all of groups were cultured in a 37℃, 5% CO_2 cell incubator for 24 h.

1.2.3 Preparation and administration of Zuogui pill-containing serum

One g/ml crude drug of ZGP extract is converted into the dosage of rats based on the body surface area of adults and rats, which is equivalent to 9 times the clinical equivalent dosage, that is, 245.7 g/(kg·d). The rats in the ZGP-containing serum group were administered with ZGP extract twice daily for 3 consecutive days and the rats in the normal serum group were administered with the same amount of purified water. On the third day, the rats were anesthetized with ether for 2−2.5 h after gavagy. Blood was collected from the aorta, the whole blood was allowed to stand for 30 min and then centrifuged. The serum was aseptically separated, stored at −20°C, inactivated at 56°C for 30 min before the experiment, and used after filtration with a 0.22 μm filter.

After all of groups were cultured for 24 h under different treatments (see **1.2.2**), normal rat serum of equal concentration was added in the ② group, the Normal group and the M group; ZGP-containing serum of optimum concentration was added in the ③⑥⑦⑨⑩⑪ group, the ZGP group and the M+ZGP group. At 37℃, 5% CO_2 cell incubator, GCs and TCs in all groups were cultured for another 24 h.

1.2.4 RT-PCR

Real-time qPCR was conducted to detect the expression of *Beclin-1*, *LC3B*, *p62*, *Bax* and *Caspase3* genes in the cells. The total RNA was extracted according to the requirements of the kit, then the purity and concentration of the RNA was checked. Next, the integrity of the RNA was checked by agarose electrophoresis. Then, referring to the kit operating instructions, 1 μg of total RNA was reverse-transcribed to synthesize cDNA. The cDNA was amplified using the following program: 95°C, 10 min;

95° C, 10 s, 60° C, 30 s, 72° C, 15 s, a total of 40 cycles. Referring to the literature method[10], we analyze the experimental data and use the $2^{-\Delta\Delta C_t}$ method (C_t represents the basic cycle value) to determine the relative expression of each target mRNA gene. The primer sequences and amplified fragment sizes of each group of target genes are shown in Table 1. The test was repeated 3 times.

Table 1 The sequence of each set of primers

Gene	Forward primer sequence (5'→3')	Reverse primer sequence (5'→3')
Beclin-1	CGACATCTGGCACAGTGGACAGTTTG	AGCATGGAGCAGCAACACAGTC
LC3B	GTCAGCGTCTCCACACCAATCTC	TCCTGGGAGGCATAGACCATGTAC
p62	TGATTGAGTCCCTCTCCCAGATGC	CCGCTCCGATGTCATAGTTCTTGG
Bax	GATGCGTCCACCAAGAAGCTGAG	CACGGCGGCAATCATCCTCTG
Caspase3	GTGGAGGCCGACTTCTTGTATGC	TGGCACAAAGCGACTGGATGAAC

1.2.5 Western blot

Western blot analysis was performed to assess the expression of Beclin-1, LC3B, p62, Bax, Caspase3 proteins in cells. The cells were rinsed twice with 1 ml PBS buffer after discarding the culture medium, after the PBS was aspirated, they are digested with trypsin, pipette repeatedly to make a cell suspension, appropriate amount of cell suspension was taken from each group and placed in the numbered EP tubes. After centrifugating in the tube at a speed of 1 000 r/min for 5 min, the supernatant was removed and transferred to a sterile 1.5 ml EP tube. Each tube was added with 200 μl of RIPA buffer, and fully dispersed, then putted in the refrigerator at −80℃. After freezing and thawing for 2 times, the cells were broken with an ultrasonic disruptor, and putted in a centrifuge pre-cooled to 4°C for 10 min at 12 000 r/min; then, we transfer the supernatant to a new 1.5 ml EP tube and mark it for use. Protein sample was placed at 105° C for high temperature denaturation for 10 min to make denatured histones. And they goes through the following process: SDS gel electrophoresis (2−3 h), membrane transfer (90 min), blocking (60 min), incubation with primary antibody (for 4−6 h or overnight at 4° C), membrane washing (10 min×3 times), incubation with the secondary antibody (60 min), and washing the membrane again (10 min×3 times). ECL developer solution was used for development. Then the membrane was placed in the Tanon-5200 system, and a chemiluminescent substrate was added to detect the fluorescent signal and perform imaging analysis. The expression of Beclin-1, LC3B, p62, Bax, Caspase3 proteins in each group was analyzed qualitatively and quantitatively. The test was repeated 3 times.

1.2.6 Statistical processing methods

Measurement data were expressed as mean± standard deviation, statistical analysis was performed using SPSS22.0. The Leven's test method was used to test normality and homogeneity of variance. If the data conforms to normality and homogeneity of variance, the means of each group is compared by one-way analysis of variance (ANOVA) and LSD test, otherwise, the Kruskal-Wallis rank sum test method is used to compare the means between groups. All P values of less than 0.05 were interpreted as showing significant difference.

2 Results

2.1 Results of groups in granulosa cells

2.1.1 Alleviative effect of different concentrations of ZGP-containing serum

As shown in Figure 1, the survival rate of GCs after PM treatment was significantly lower than that of the control group (P<0.05). 10% ZGP-containing serum had the best effect on the recovery of cell survival rate (P<0.05). When the concentration was increased to 20%, the cell growth was inhibited significantly (P<0.05), suggesting that the concentration was too high and had a killing effect on the GCs. Therefore, 10% ZGP-containing serum was used in the following experiments.

2.1.2 The expression of target mRNA gene

As shown in Table 2, compared with the blank group, the mRNA expression of Beclin-1, LC3B, Bax and Caspase3 were increased (P<0.05), and the mRNA expression of p62 is decreased in the model group (P<0.05). The mRNA expression of Beclin-1, LC3B, Bax, and Caspase3 in high phagocytic group,

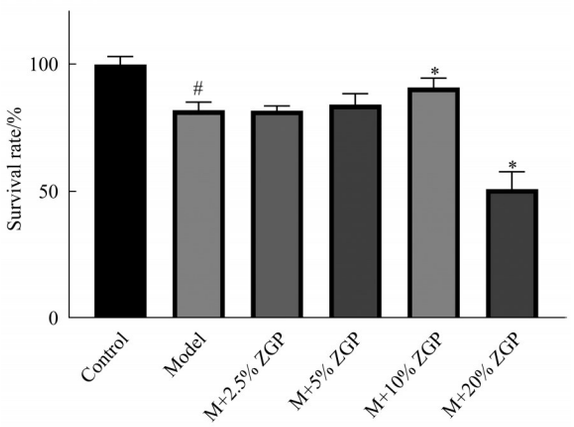

Fig. 1 Alleviative effect of different concentrations of ZGP−containing serum in GCs

$^{\#}P$<0.05 compared with the control group; $^{*}P$<0.05 compared with the Model group.

Table 2 The expression of target mRNA gene in granule cells

Group	Beclin-1	LC3B	p62	Bax	Caspase3
①	1.00±0.04	1.00±0.12	0.99±0.03	0.86±0.02	1.00±0.04
②	0.90±0.06	1.00±0.09	0.89±0.08	0.87±0.01	0.74±0.14
③	0.36±0.05	0.38±0.10	1.34±0.31	0.73±0.01	0.21±0.04
④	0.36±0.02	0.36±0.02	0.85±0.11	0.38±0.01	0.36±0.03
⑤	3.65±0.06	3.17±0.04	0.39±0.01	4.8±0.04	3.02±0.26
⑥	0.17±0.02	0.16±0.02	1.39±0.06	0.31±0.01	0.10±0.07
⑦	0.97±0.07	0.99±0.04	0.37±0.10	0.98±0.01	0.74±0.04
⑧	3.73±0.09#	3.80±0.73#	0.40±0.06#	3.24±0.04#	2.69±0.04#
⑨	1.85±0.05*	1.78±0.03*	0.86±0.09*	1.62±0.03*	1.67±0.17*
⑩	0.69±0.04^{+}	0.70±0.03^{+}	0.71±0.48	0.82±0.05^{+}	0.69±0.06^{+}
⑪	3.43±0.37^{+}	3.39±0.17^{+}	0.21±0.13^{+}	3.64±0.03^{+}	2.89±0.37^{+}

$^{\#}P$<0.05 compared with the blank control group; $^{*}P$<0.05 compared with the Model group; ^{+}P<0.05 compared with the model+ZGP group. ① blank control group; ② normal serum group; ③ ZGP-containing serum group (ZGP group) ④ low phagocytic group; ⑤ high phagocytic group; ⑥ low phagocytic+ZGP group; ⑦ high phagocytic+ZGP group; ⑧ model group; ⑨ mModel+ZGP group; ⑩ model+low phagocytic group+ZGP group; ⑪ model+high phagocytic+ZGP group.

high phagocytic+ZGP group, model+high phagocytic+ZGP group were higher than those in blank control group (P<0.05), ZGP group and model+ZGP group, respectively, while the mRNA expression of p62 was lower. The mRNA expression of Beclin-1, LC3B, Bax, and Caspase3 in low phagocytic group, low phagocytic+ZGP group, model+low phagocytic+ZGP group were lower than those in blank control group, ZGP group and model+ZGP group, respectively, while the mRNA expression of p62 was higher (P< 0.05). After treatment with ZGP-containing serum, the mRNA expression of Beclin-1, LC3B, Bax and Caspase3 is reduced, while the mRNA expression of p62 is increased in above groups. (P<0.05, ① *vs* ③; ④ *vs* ⑥; ⑤ *vs* ⑦; ⑧ *vs* ⑨).

2.1.3 The expression of target protein

As shown in Figure 2, 3, after PM was used to treat GCs, the protein expression of Beclin-1, LC3B, Bax and Caspase3 were increased (P<0.05), and the protein expression of p62 is decreased (P<0.05). The protein expression of Beclin-1, LC3B, Bax, and Caspase3 in high phagocytic group, high phagocytic+ ZGP group, model+high phagocytic+ZGP group were higher than those in blank control group, ZGP group and model+ZGP group, respectively, while the protein expression of p62 was lower (P<0.05). The protein expression of Beclin-1, LC3B, Bax, and Caspase3 in low phagocytic group, low phagocytic+ZGP group, model+low phagocytic+ZGP group were lower than those in blank control group, ZGP group and model+ ZGP group, respectively, while the protein expression of p62 was higher (P<0.05). After treatment with ZGP-containing serum, the protein expression of Beclin-1, LC3B, Bax and Caspase3 is reduced, while the expression of p62 is increased in above groups (P< 0.05, ① *vs* ③; ④ *vs* ⑥; ⑤ *vs* ⑦; ⑧ *vs* ⑨).

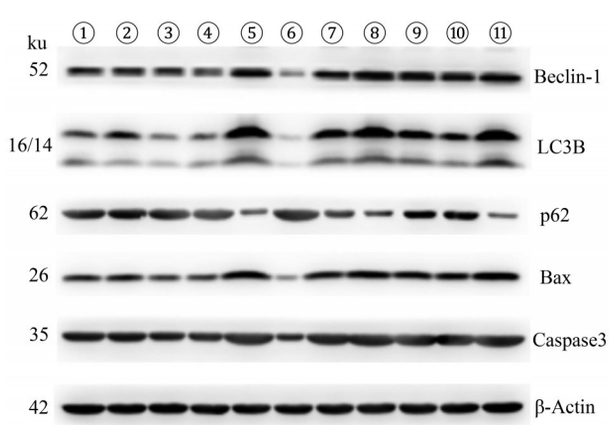

Fig. 2 The expression of target protein in granulosa cells

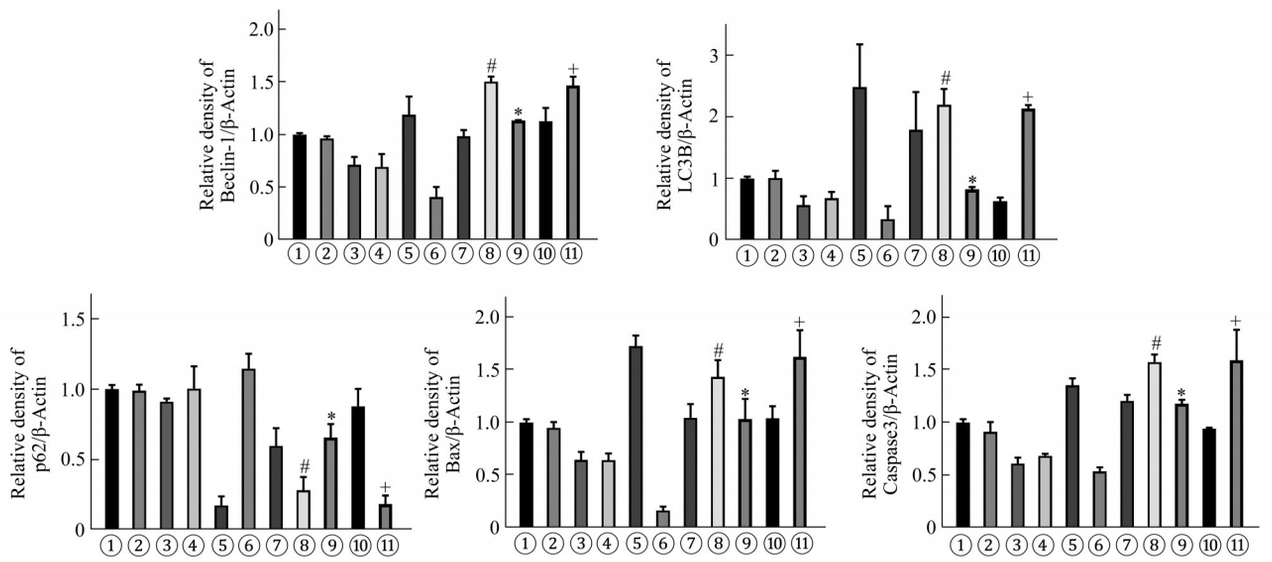

Fig. 3　The expression of target protein in granulosa cells (analysis of grey intensity)

$^{\#}P$<0.05 compared with the blank control group; $^{*}P$<0.05 compared with the model group; ^{+}P<0.05 compared with the model+ZGP group. ① blank control group; ② normal serum group; ③ ZGP-containing serum group (ZGP group) ④ low phagocytic group; ⑤ high phagocytic group; ⑥ low phagocytic+ZGP group; ⑦ high phagocytic+ZGP group; ⑧ model group; ⑨ model+ ZGP group; ⑩ model+low phagocytic group+ZGP group; ⑪ model+high phagocytic+ZGP group.

2.2　Results of groups in theca cells

2.2.1　Alleviative effect of different concentrations of ZGP-containing serum

As shown in Figure 4, the survival rate of TCs after PM treatment was significantly lower than that of the control group (P<0.05). 10% ZGP-containing serum had the best effect on the recovery of cell survival rate (P<0.05). When the concentration was increased to 20%, the cell growth was inhibited significantly (P<0.05), suggesting that the concentration was too high and had a killing effect on the TCs. Therefore, 10% ZGP-containing serum was used in the following experiments.

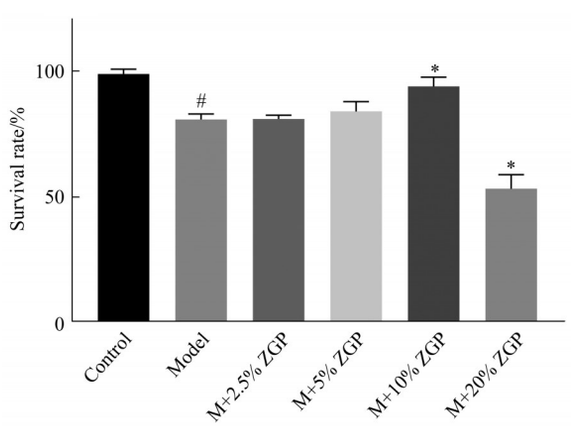

Fig. 4　Alleviative effect of different concentrations of ZGP−containing serum in TCs

$^{\#}P$<0.05 compared with the control group; $^{*}P$<0.05 compared with the M group.

2.2.2　The expression of target mRNA gene

As shown in Table 3, compared with the control group, the mRNA expression of Beclin1, LC3B, Bax and Caspase3 in the model group were increased, and the mRNA expression of p62 is decreased (P<0.05). After treatment with ZGP-containing serum, the mRNA expression of Beclin1, LC3B, Bax and Caspase3 were decreased, and the mRNA expression of p62 is increased (P<0.05).

2.2.3　The expression of target protein

As shown in Figure 5, 6, compared with the control group, the protein expressions of Beclin1, LC3B, Bax, and Caspase3 were increased, while the protein expression of p62 is decreased in the model group (P<0.05). ZGP-containing serum can significantly reduce the protein expression of Beclin1, LC3B, Bax and Caspase3, while increasing the protein expression of p62 (P<0.05).

Table 3　The expression of target mRNA gene in theca cells

Group	Beclin-1	LC3B	p62	Bax	Caspase3
Control	1.00±0.03	1.00±0.01	1.02±0.29	1.00±0.05	1.02±0.24
Normal	0.85±0.03	0.91±0.03	0.71±0.14	0.92±0.05	1.03±0.06
ZGP	0.51±0.02	0.50±0.02	2.13±0.22	0.52±0.03	0.57±0.13
M	2.36±0.65$^{\#}$	2.37±0.04$^{\#}$	0.63±0.02$^{\#}$	2.30±0.05$^{\#}$	2.66±0.29$^{\#}$
M+ZGP	0.75±0.03*	0.72±0.01*	1.00±0.09*	0.73±0.03*	1.00±0.07*

$^{\#}P$<0.05 compared with the control group; $^{*}P$<0.05 compared with the M group.

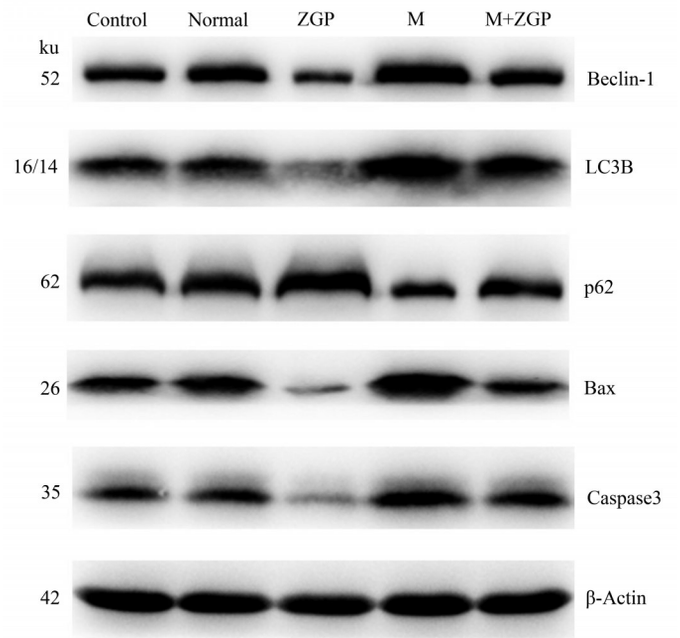

Fig. 5　The expression of target protein in theca cells

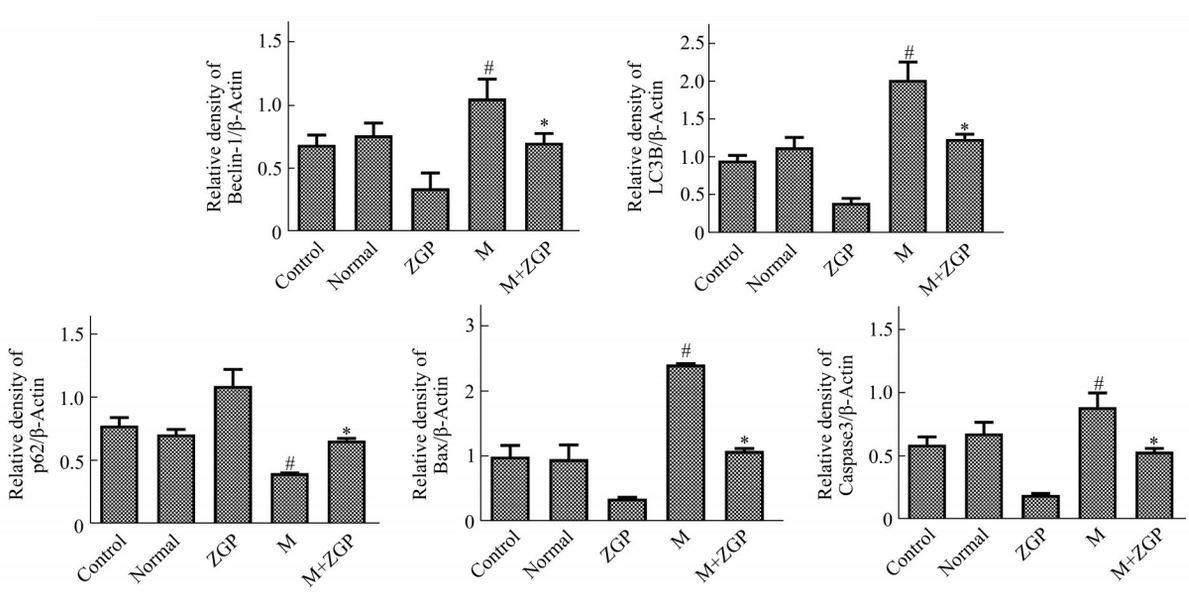

Fig. 6　The expression of target protein in theca cells (analysis of grey intensity)

$^{\#}P$<0.05 compared with the control group; $^{*}P$<0.05 compared with the M group.

3 Discussion

Different chemotherapeutic drugs have different mechanisms of action on ovarian injury and show varying degrees of ovarian toxicity. Among them, alkylating agents have the strongest ovarian toxicity. Since primordial follicles are static, they are more sensitive to non-specific drugs such as alkylating agents in the cell cycle. As an alkylating agent, cyclophosphamide can not only interfere with the process of cell division, but also induce cell apoptosis[11]. It has also been shown that there is a certain correlation between follicular atresia and apoptosis, the main target cells of which are GCs. In addition, the apoptosis of GCs is closely related to autophagy. For POF, kidney-tonifying Chinese medicine can promote follicular development, inhibit granular cell apoptosis, reduce follicular atresia, and improve ovarian function, so as to alleviate clinical symptoms[12].

We found that autophagy-related proteins such as Beclin-1, LC3B and apoptosis-related proteins such as Bax and Caspase3 were increased by PM in the GCs and TCs, while 10% ZGP-containing serum can down-regulate the expression of the above-mentioned proteins. And, in the model group, the expression of p62 is higher than that of the blank control group, 10% ZGP-containing serum can up-regulate the expression of p62 protein. In addition, in the group of GCs, after activating or inhibiting the autophagy pathway, the expression of autophagy-related proteins and apoptosis-related proteins changed correspondingly.

Autophagy is a lysosomal-dependent intracellular degradation system, which plays an important role in a variety of physiological processes and become a hot topic in medicine. It has been confirmed that autophagy is related to various diseases such as cardiovascular diseases and tumors[13-14]. Beclin-1 is the key factor of autophagy initiation, LC3B is a marker protein of autophagy. The degradation of autophagy substrate was confirmed by labeling the degradation of p62 protein, the expression level of which was inversely proportional to the activity of autophagy and could be used to monitor autophagy flow[15]. Also, it has been found that autophagy has a molecular relationship with apoptosis. Beclin-1 is the cleavage target of Caspase3, 7, 8 in the death receptor and mitochondria mediated apoptosis pathway. Bax is a member of Bcl-2 family, which can act as the upstream regulatory protein of Caspase3 and start the caspase cascade reaction[16].

All in all, we found that PM damaged GCs and TCs through promoting apoptosis and activating autophagy/lysosomal degradation pathways. 10%ZGP-containing serum alleviated the damage caused by PM, and affect the autophagy and apoptosis process of GCs and TCs. There was a cross-talk between autophagy and apoptosis in the process of PM damaging GCs and 10%-ZGP-containing serum alleviating it. We provide a new theoretical and experimental basis for the pharmacodynamic study of ZGP, and also provide a new theory for the prevention and treatment of POF. If we can selectively inhibit the process of autophagy and apoptosis of GCs and TCs, protect the ovary from the role of chemotherapy drugs or help restore ovarian function after chemotherapy, it can greatly benefit the patients with POF after chemotherapy, which is of great clinical significance. Next, we may further explore the effect of ZGP's effective fractions on chemotherapy-damaged GCs and TCs.

4 Conclusion

PM can damage GCs and TCs by promoting apoptosis and activating autophagy/lysosomal degradation pathways, 10% ZGP-containing serum can alleviate the damage. There is a cross-talk between autophagy and apoptosis in the process of PM damaging GCs which can be alleviated by 10% ZGP containing serum.

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基于自噬与凋亡机制研究左归丸对化疗损伤颗粒细胞及膜细胞的影响*

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摘要　目的　探讨左归丸含药血清对化疗损伤性颗粒细胞和膜细胞的影响及作用机制。**方法**　制备左归丸含药血清，培养大鼠卵巢颗粒细胞和膜细胞，使用磷酰胺氮芥造模分组后给药。CCK-8法测定颗粒细胞和膜细胞存活率，实时荧光定量PCR法（RT-PCR）及蛋白质免疫印迹法（Western blot）分别检测卵巢自噬启动因子Beclin-1、微管结合蛋白轻链3（LC3B）、自噬受体蛋白p62、凋亡蛋白Bax、Caspase3在转录水平和翻译水平上的表达。**结果**　10%左归丸含药血清对于细胞存活的挽救率最高。Beclin-1、LC3B、Bax、Caspase3在磷酰胺氮芥作用的颗粒细胞和膜细胞中，相对于空白对照组有高表达（P<0.05），10%左归丸含药血清可下调上述蛋白质在模型组中的表达（P<0.05）；然而受体蛋白p62较空白对照组升高（P<0.05），10%左归丸含药血清可上调模型组p62的表达（P<0.05）。此外，在颗粒细胞实验组中，激活或抑制自噬途径后，自噬相关蛋白的表达在发生相应改变的同时，凋亡相关蛋白的表达也会发生相应改变。**结论**　磷酰胺氮芥可通过促进凋亡、激活自噬/溶酶体降解途径的机制损伤颗粒细胞和膜细胞。10%左归丸含药血清能缓解由此带来的损伤，同时影响了颗粒细胞和膜细胞自噬和凋亡过程。在磷酰胺氮芥损伤颗粒细胞的过程和10%左归丸含药血清缓解其损伤过程中均存在自噬与凋亡串流（cross-talk）。

关键词　左归丸，化疗，颗粒细胞，膜细胞，自噬，凋亡
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