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Original Research

Effect of triptorelin on the protection from damage on mouse ovarian cells caused by tripterygium polyglycoside

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Objective: To investigate the effect of triptorelin on the protection of tripterygium polyglycoside-induced damage to ovarian function on mouse ovarian cells through the study of Bcl-2 and Smac. *Method*: Thirty female SD mice with normal estrous cycle were selected and randomLy divided into three groups of 10 mice each. *Group A* (blank control group); *Group B* (tripterygium glycoside group); *Group C* (tripterygium glycoside + triptorelin group). For all three groups, the drug was stopped for 3 weeks and all mice were then sacrificed. Ovaries were taken to detect the expression of Bcl-2 and Smac in ovarian tissue. *Results*: Compared with the blank control group, the expression of Bcl-2 in the tripterygium polyglycoside group was reduced and the expression of Smac was enhanced, while for the triptorelin group, increased expression of Bcl-2 and decreased expression of Smac were observed with a statistical significance ($P < 0.05$). *Conclusions*: Tripterygium polyglycoside can cause damage to ovarian function in female mice; Triptorelin has a protective effect on tripterygium polyglycoside-induced damage to ovarian function in female mice.

Keywords

Triptorelin; Tripterygium; Ovarian function; Bcl-2; Smac

1. Introduction

Tripterygium glycoside is a Chinese herbal extract with immunomodulatory, anti-tumor, anti-inflammatory and other effects. It is mainly used for the treatment of autoimmune diseases such as rheumatoid arthritis and lupus erythematosus. Long-term use can cause reproductive toxicity including menstrual disorders and amenorrhea [1, 2]. It can also induce apoptosis in lymphocytes and synovial fibroblasts and inhibit their proliferation [3]. Accordingly, many researchers have attempted to establish animal models of ovarian dysfunction using their reproductive toxicity, to seek ovarian protection agents through study of the pathological mechanism [4–7].

Gonadotropin releasing hormone-a (GnRH-a) is a decapeptide that is produced in the hypothalamus and acts upon GnRH receptors on the surface of gonadotropin cells in the pituitary gland. It stimulates the release of luteinizing hormone (LH) and follicular stimulating hormone (FSH) which then stimulate the production and release of testosterone by the male testes and estrogen by the female ovaries and placenta. It is widely recognized as an effective ovarian function protector during chemotherapy and has been widely used in clinical practice. Moore *et al.* [8] conducted a large (n = 218) randomized clinical trial study of GnRH-a in preventing chemotherapy-induced Premature Ovarian Failure (POF). They confirmed that the use of GnRH-a can reduce the incidence of POF. Through another randomized clinical trial, Leonard *et al.* [9] also confirmed that the intramuscular injection of the gosereline group in the 12–24 months after chemotherapy was significantly less than the chemotherapy alone group (22% vs 38%), and the incidence of POF was significantly lower (18.5% vs 34.8%). The difference was especially pronounced in patients younger than 40 years (2.6% vs 20%). In the early stage of the first administration of GnRH agonist, all the GnRH receptors are bound to the pituitary gland, which stimulates the transient rise of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which is the so-called "ignition" period. After half a month, the receptors were completely depleted and had no response to GnRH-a. FSH and LH began to drop sharply; ovarian sex hormones began to drop significantly to the menopausal level, and artificial menopause appeared, leaving the ovaries in a dormant state which could protect the ovaries. Triptorelin, as a synthetic analogue of GnRH, has a similar function. However, its performance to protect ovarian function is unknown and has attracted very limited research.

Apoptosis is one of the most important mechanisms of drug-induced destruction of ovarian structure and function [10]. Wang *et al.* [11] suggested that reduction of apoptosis may help reduce infertility and maintain the reproductive ability in women for the treatment of premature ovarian failure. There are two representative members of the apoptotic signal transduction pathway, Bcl-2 [12] and Smac [13–15], as an apoptosis inhibitor and promoter, respectively. The ratio between them determines the survival of the cell. Through observing and comparing three groups of Kunming mice using tripterygium polyglycoside, this study aims to explore the effect of triptorelin on tripterygium polyglycoside-induced ovarian dysfunction in female mice by measuring Bcl-2 and

Smac. Such a study is important to seek an effective ovarian function protection scheme when applying tripterygium polyglycoside.

2. Materials and methods

2.1 Experimental animals

Thirty female adult Sprague Dawley (SD) healthy mice of 7–8 weeks old were selected, weighing 18 ± 4 g, clean grade, provided by Shanghai Slark Laboratory Animal Co., Ltd. An outbred multipurpose breed of albino rat used extensively in medical and nutritional research, this breed of rat was first produced by the Sprague-Dawley farms (later to become the Sprague-Dawley Animal Company) in Madison, Wisconsin in 1925. Main advantages include its calmness, ease of handling, rapid growth and strong disease resistance. With a low rate of spontaneous tumor and high sensitivity to sex hormone, it is often used in nutrition, endocrinology and toxicology.

The animals were adaptively fed for 5 days after arriving at the animal experiment center. Vaginal exfoliated cell smears were then performed. The mice with normal estrous cycles were screened and included in the experiment. The animal ethics approval is provided in the Appendix.

2.2 Experimental drug

A tripterygium glycoside tablet (10 mg/tablet), commonly used for adult human 2–3 tablets/time, was orally ingested three times a day by each subject (Zhejiang Deende Pharmaceutical Co., Ltd. National Drug Standard Z33020422). The triptolide polyglycoside solution was produced by dissolving the tablets in physiological saline and formulating at 40 mg/kg. The justification of this concentration can be found in [16, 17], which investigated the influence of triptorelin on reproductive function of female mice [16] and premature ovarian insufficiency kidney deficiency and blood stasis pattern mouse model with tripterygium therapy [17].

The triptorelin acetate injection dosage for this study is 0.1 mg (based on C64H82N18O13). The usual dosage for adults is 0.5 mg subcutaneously once a day (Chengdu Tiantaishan Pharmaceutical Co., Ltd. National Drug Standard H20058648).

2.3 Experimental reagents and instruments

Main reagents used in this study include

(a) RIPA lysate (Servicebio, product number G2002);

(b) 50X Cocktail (Servicebio, product number G2006);

(c) PMSF (100 mM) (Servicebio, product number G2008);

(d) Phosphorylated protease inhibitor (Servicebio, product (Cat. No. G2007));

(e) BCA protein quantitative detection kit (manufacturer Servicebio, article number G2026);

(f) 5X protein loading buffer (manufacturer Servicebio, article number G2013);

(g) SDS-PAGE gel preparation kit (manufacturer Servicebio, article number G2003);

(h) Protein Marker (Producer Therm (Fermentas), Cat. No. 26616);

(i) PVDF membrane (Producer 0.45 μm, Cat. No. Millipore).

Primary instruments and software include

(1) Electronic balance (METTLER TOLEDO Instruments (Shanghai) Co., Ltd., model: PL-203);

(2) Microplate reader (Rayto, model: Rt2100c);

(3) Refrigerated centrifuge (heal force, model: neofuge 13R);

(4) Pure water meter (Chongqing Aikepu, model: AJC-0501-P);

(5) Paper trimmer (deli, model: NO.8014);

(6) Handheld centrifuge (Servicebio, model: D1008E);

(7) Vortex mixer (Servicebio, model: MX-F);

(8) Magnetic stirrer (Servicebio MS-PB);

(9) Decolorization shaker (Servicebio TSY-B);

(10) Power supply for electrophoresis instrument (Beijing Liuyi Instrument Factory, model: DYY-6C);

(11) Grayscale analysis software (Alpha Innotech, Model: alphaEaseFC);

(12) Image analysis software (Adobe PhotoShop);

(13) Image analysis software (Image-Pro Plus 6.0);

(14) Statistical software (SPSS 19.0).

2.4 Experimental method

For qualified, healthy SD female mice, the vaginal exfoliated cell method was used to select 30 mice with normal estrous cycle as test animals, which were randomly divided into 3 groups of 10 mice each:

- *Group A*: blank control group, where 0.35 mL of saline was administered to the stomach once daily for 11 weeks;
- *Group B*: tripterygium glycoside group, where 0.35 mL of tripterygium glycoside solution was administered to the stomach from the 8th day; once a day for 10 weeks;
- *Group C*: triptolide + triptorelin group: 0.1 mg/kg daily subcutaneous injection of triptorelin injection; once a day; continuous injection for 11 weeks; from the 8th day, the triptolide solution was administered to the stomach 0.35 mL, once daily for 10 weeks.

From the first day of the experiment, the general conditions of the mice were observed and recorded, including energy, activity, hair, food intake, water intake, stomach appetite, second stool, etc. The mice were weighed once a week to observe changes in body weight. The vagina exfoliation cell method, simple to operate, was used to observe the estrous cycle [18].

After 11 weeks of treatment, the drug was stopped for 3 weeks and all mice were sacrificed. The ovaries were then obtained by laparotomy, and the ovarian wet weight was measured using an electronic analytical balance. The ovarian index was calculated by ovarian wet weight (mg) / mouse weight (g) × 100%.

After weighing, the ovaries were fixed in a 4% paraformaldehyde solution for 3 days, and were routinely dehydrated, xylene-transparented, wax-impregnated,

Table 1. Comparison of body weight and ovary of mice in each group.

Group	Number of mice	Original weight (gram)	Weight after 13 weeks (gram)	Ovarian weight after 13 week (gram)	Ovarian index
Blank	10	18.418 ± 0.723^a	28.945 ± 3.314	0.086 ± 0.093	0.286 ± 0.297
Tripterygium	10	18.564 ± 0.941	26.264 ± 3.569	0.014 ± 0.004	0.055 ± 0.013
Tripterygium + Triptorelin	10	18.964 ± 0.410	27.909 ± 1.799	0.290 ± 0.053	1.020 ± 0.186
F^b	-	1.668	1.511	4.178	3.979
P^c	-	0.206	0.237	0.025	0.029

[a] Values are expressed as mean $\pm$ standard deviation. [b] F: F-test value. [c] P: P-value.

embedded, sectioned (4 μm), and operated according to the instructions of immunohistochemistry kit [18]. Immunohistochemical average optical density (average optical) analysis method: each slice in each group randomly selected at least three positions with a 200$\times$ field of view (FoV) for photographing. When taking pictures, FoV was selected to ensure that the testing tissue fully filled the view. In addition, the background illumination of each photo was kept as consistent as possible. Image-Pro Plus 6.0 software was used to select the same brown-yellow color as the uniform standard for determining the positives of all photos. Each photo was analyzed to obtain the Integrated Optical Density (IOD) and the pixel area (AREA). The average optical density (AO) was obtained by

$$\mathrm{AO} = \mathrm{IOD}/\mathrm{AREA}. \tag{1}$$

The larger the AO value, the higher the positive expression level.

The Western Blotting method was used to measure the Smac level in ovarian tissue [19]. The principle of Western blotting is based on the specific binding of antigens and antibodies. Cells or biological tissue samples that have been subjected to gel electrophoresis are stained by specific antibodies. The location and depth of staining are used to obtain specific proteins in the cells. This method can perform qualitative and semi-quantitative analysis of proteins, combined with chemiluminescence detection, and can simultaneously compare the differences in expression of the same protein in multiple samples. After the mice were sacrificed, the mouse ovarian protein was extracted, and the expression of Smac protein in ovarian tissue was detected. The specific operation process can be summarized as

(1) Collecting the sample;

(2) Determining the total protein concentration of the sample;

(3) Subjecting the sample protein to SDS-PAGE electrophoresis;

(4) Transferring membrane;

(5) Measuring immune response;

(6) Processing data.

With β-actin as the internal reference, the relative expression of Smac protein was expressed by the gray ratio of Smac and β-actin. Finally, this data was used for statistical analysis.

2.5 Statistical methods

The software, SPSS 19.0, was used for statistical analysis, and the results were expressed by ($\bar{x} \pm$ std), where $\bar{x}$ denotes the mean value and std denotes the standard deviation. Comparisons between groups were performed using one-way ANOVA to determine whether there are any statistically significant differences between the means of three or more independent (unrelated) groups. The data passed the normality test and the homogeneity of variance test, which conformed to the normal distribution. The level of statistical significance is often expressed as a P-value between 0 and 1. The null hypothesis states that there is no difference between the two groups being studied. The smaller the P-value, the stronger the evidence that the null hypothesis should be rejected. This study considers the groups have statistically significant difference if $P < 0.05$. It indicates strong evidence against the null hypothesis, as there is less than a 5% probability the null is correct (and the results are random). Therefore, we reject the null hypothesis, and accept the alternative hypothesis. However, this does not mean that there is a 95% probability that the research hypothesis is true. The P-value is conditional upon the null hypothesis being true is unrelated to the truth or falsity of the research hypothesis.

3. Results

3.1 Growth comparison of three groups of mice

Table 1 shows that there is no statistically significant difference among three groups for either the original body weight or the body weight after ten weeks ($P > 0.05$). However, the ovarian weight and ovarian index of the three groups have statistically significant differences ($P < 0.05$). Further pairwise comparison shows that for ovarian weight, there are statistically significant differences between each two groups ($P < 0.05$). The ovarian weight is ranked as tripterygium + triptorelin group $>$ blank group $>$ tripterygium group. As for the ovarian index, the pairwise comparison between the groups also shows statistically significant difference ($P < 0.05$). The ovarian index was ranked as tripterygium + triptorelin group $>$ blank group $>$ tripterygium group. This shows that tripterygium glycosides can directly damage the gonads of model mice and reduce their weight, while triptorelin can relieve the inhibitory effect of triptolide and increase ovarian weight.

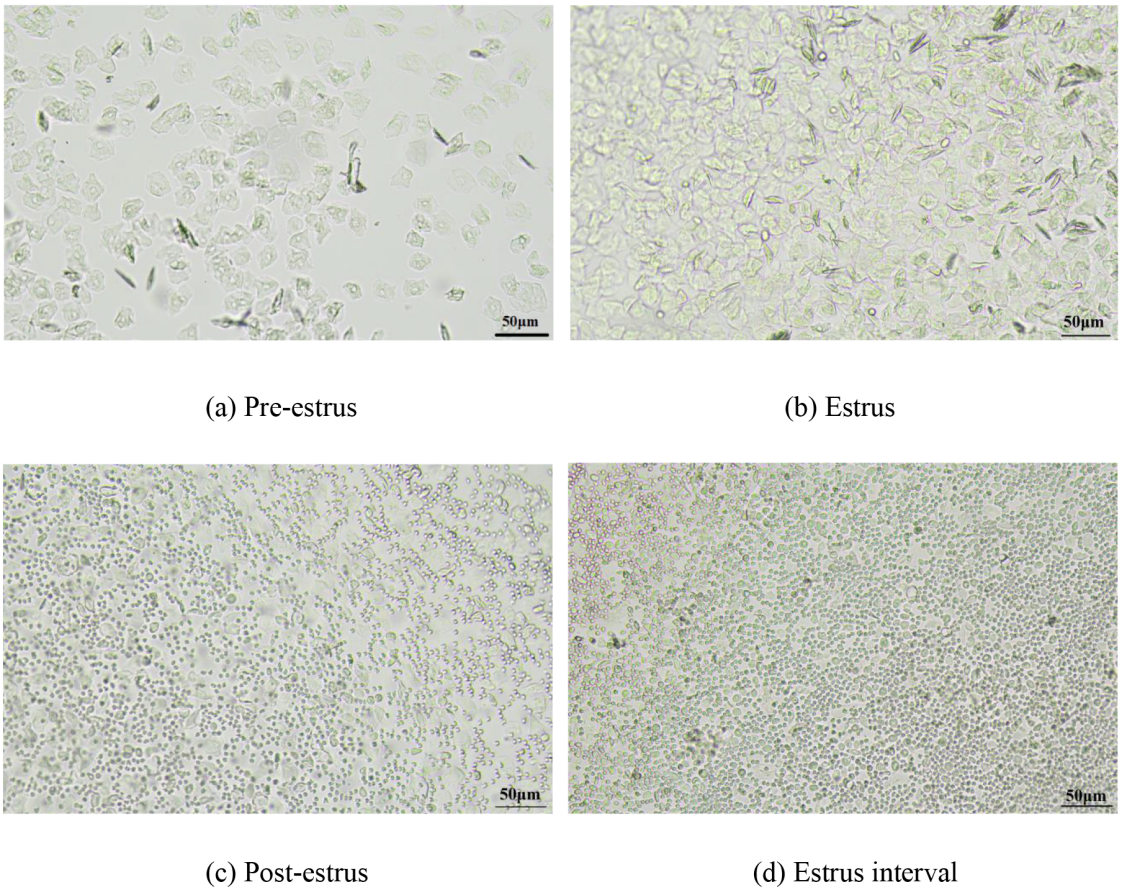


Fig. 1. Results of vaginal smear.

Table 2. Comparison of serum AMH, FSH, LH, and E2 among different groups.

Group	Cases	AMH[a] (ng/mL)	FSH[a] (mIU/mL)	LH[a] (mIU/mL)	E2[a] (pg/mL)
Blank (A)	10	45.34 ± 3.59 [b]	28.05 ± 2.72	8.36 ± 1.77	32.08 ± 3.89
Tripterygium (B)	10	43.24 ± 1.49	31.41 ± 4.35	11.05 ± 3.55	28.01 ± 1.79
Tripterygium + Triptorelin (C)	10	50.72 ± 5.14	23.45 ± 3.41	6.84 ± 0.51	39.1 ± 3.21
F [c]	-	7.069	17.173	10.551	13.923
P [d]	-	0.001	< 0.001	< 0.001	< 0.001

[a] AMH: Anti-Mullerian Hormone; FSH: follicle-stimulating hormone; LH: luteinizing hormone; E2: estradiol.

[b] Values are expressed as mean ± standard deviation.

[c] F: *F*-test value.

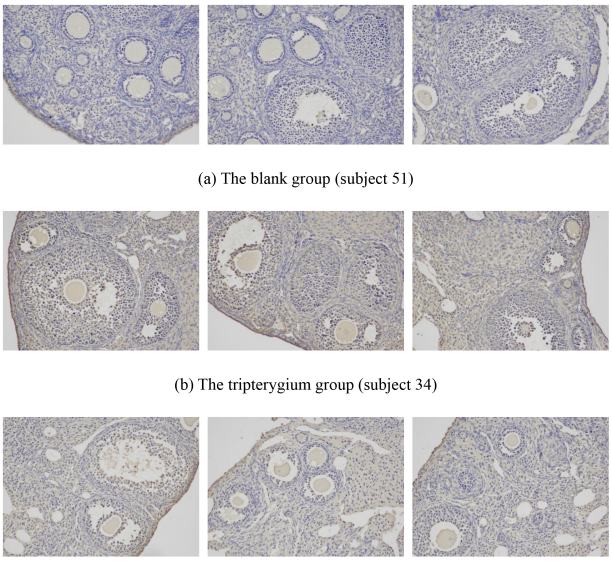
[d] P: *P*-value.

3.2 Vaginal exfoliated cell smear results

The estrous cycle is a manifestation of ovarian endocrine function. The ovarian endocrine function was normal, and the levels of estrogen and progesterone were coordinated. The estrous cycle under the action of estrogen and progesterone showed regular periodic changes. After the tripterygium group was modeled with tripterygium polyglycoside tablets, the vaginal exfoliated cell smear reflected the disorder of the estrous cycle in mice, indicating that long-term administration of tripterygium polyglycoside can significantly inhibit the endocrine function of the mouse ovaries. Triptorelin can release the inhibitory effect of triptolide, and then manifest as stable estrous cycle. Fig. 1 shows the results of vaginal smear for pre-estrus, estrus, post-estrus and estrus interval, respectively. The number of leukocytes increased significantly from pre-estrus, estrus, to post-estrus while the number of keratosic cells decreased. It can be observed that there is a large amount of leukocyte and scant mucus during the estrus interval.

Table 2 shows the results of ANOVA, revealing significant differences in the serum levels of AMH (Anti-Mullerian Hormone), FSH (follicle-stimulating hormone), LH (luteinizing hormone), and E2 (estradiol) in different groups ($P < 0.05$). Further pairwise comparisons showed that the sorting of AMH was as follows: Group C > Group A > Group B; the

sorting of FSH was Group B > Group A > Group C; the sorting of LH was Group B > Group A > Group C; the sorting of E2 was Group C > Group A > Group B.

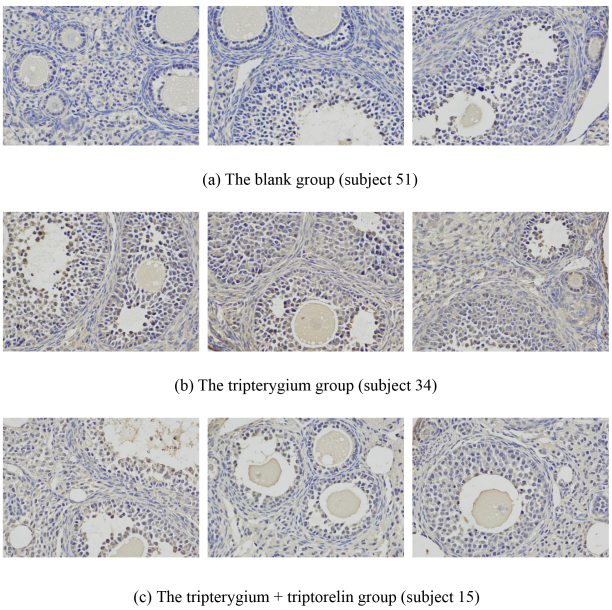


(a) The blank group (subject 51)

(b) The tripterygium group (subject 34)

(c) The tripterygium + triptorelin group (subject 15)

Fig. 2. Immunohistochemical images of Bcl-2 for the three groups (200×).



(a) The blank group (subject 51)

(b) The tripterygium group (subject 34)

(c) The tripterygium + triptorelin group (subject 15)

Fig. 3. Immunohistochemical images of Bcl-2 for the three groups (400×).

3.3 Bcl-2 expression in ovarian cells of each group of mice

As shown in Table 3, compared with the normal group, the expression of Bcl-2 in the triptorelin group was increased about 33% with a statistically significant difference ($P = 0.046 < 0.05$). The expression of Bcl-2 protein in the tripterygium group was decreased about 42% compared with the normal group, with a statistically significant difference ($P = 0.042 < 0.05$). The expression of Bcl-2 in the tripterygium + triptorelin group was about 3x higher than that in the tripterygium group, with a statistically significant difference ($P = 0.026 < 0.05$).

Table 3. Bcl-2 expression in ovaries of different groups.

Group	Bcl-2 (IOD)
Blank group	8470.30 ± 2445.16[a]
Tripterygium group	4937.11 ± 2015.11
Tripterygium + Triptorelin group	11251.46 ± 2905.11

[a] Values are expressed as mean ± standard deviation.

The results of this experimental study demonstrate that the anti-apoptotic factor Bcl-2 was expressed in the ovarian tissue of mice in each group, mainly via the cytoplasm of ovarian granulosa cells, mostly brown and brownish yellow. The results are judged by the average optical density. It can be seen from Figs. 2,3 that the Bcl-2 protein expression of ovarian granulosa cells in the triptorelin group was strongly positive, which was significantly higher than that in the triptolide group, and the difference was statistically significant ($P < 0.01$).

3.4 Western blotting to detect smac protein expression in each group

As shown in Fig. 4, compared with the blank group, the expression of Smac in the tripterygium group was increased in all five comparisons, suggestive of damage caused by Tripterygium. The expression of Smac in the triptorelin group was consistently decreased. Pairwise comparison, shown in Fig. 5, suggests the difference is significant (triptorelin vs tripterygium $P = 0.007$, triptorelin vs blank $P = 0.038$, blank vs tripterygium $P = 0.01$). Three members of triptorelin group (subjects 13, 14 and 15) had at least 3x lower Smac than the tripterygium group (subjects 33, 34 and 35). Two from the triptorelin group (subjects 11 and 12) had at least 2 times lower Smac than the tripterygium group (subjects 31 and 32). The results confirm that tripterygium polyglycoside can increase the expression of Smac in Group B, while the Smac expression is weakened in the group of tripterygium combined with triptorelin. These findings suggest that triptorelin can reduce the damaging effect of vine polyglycoside, thus prolonging the life span of ovarian cells in model mice. Therefore, it has a moderately protective effect.

4. Discussion and conclusion

Smac, also known as DIABLO, is a pro-apoptotic protein in the mitochondria. When a cell is stimulated by a signal to undergo apoptosis, it is released into the cytoplasm along with cytochrome C (cytc), which is achieved by releasing the inhibitory effects of apoptosis inhibitory proteins (IAPs) on caspase-3, caspase-7, and caspase-9. Bcl-2 is a type of protein

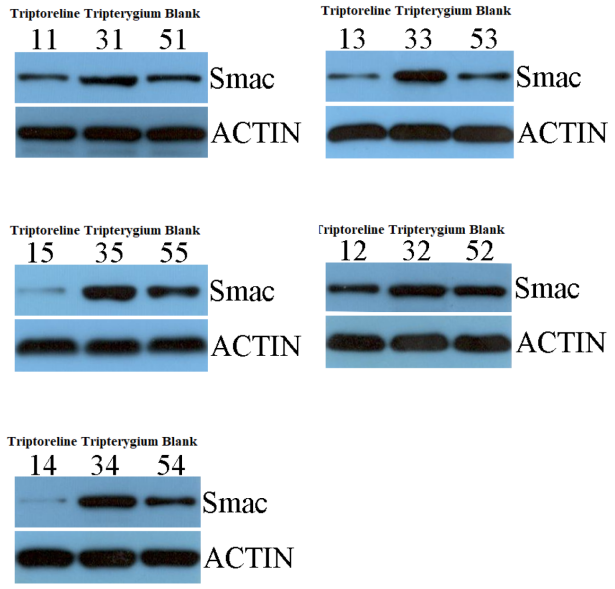


Fig. 4. Western Blotting to detect the expression of Smac in each group, where 5 comparisons are presented.

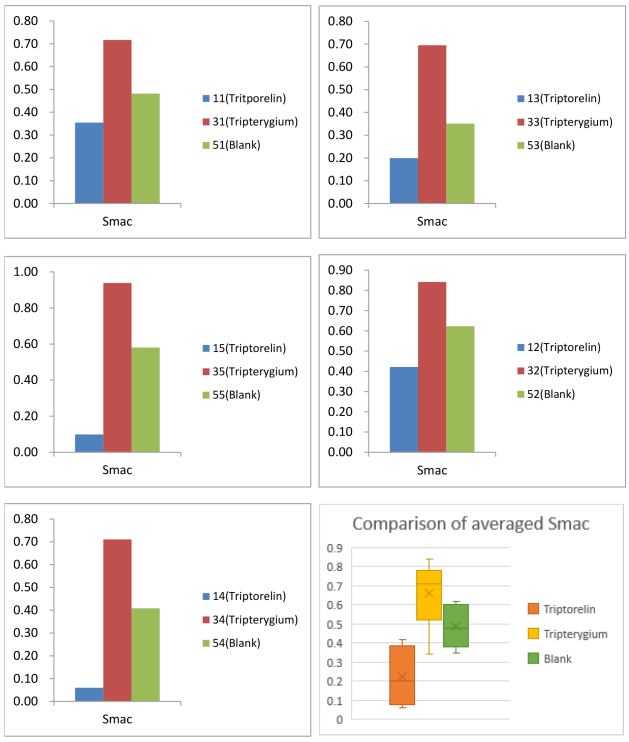


Fig. 5. The ratio of the gray value of the Smac band of each group of items to the gray value of the internal reference band β-actin.

that plays an important role in the process of apoptosis. Bcl-2 family proteins can be divided into two categories according to their functions, namely anti-apoptotic proteins (Bcl-2, etc.) and pro-apoptotic proteins [20]. Highly expressed Bcl-2 can inhibit the release of Smac from mitochondria, and at the same time inhibit the release of cytc as well as apoptosis [21]. Recent studies have shown that the Smac signaling pathway has a strong correlation with the Bcl-2 protein family in regulating apoptosis. Tian *et al.* [22] found that Bcl-2 and Smac expression are negatively correlated, suggesting that the two may play an antagonistic role and jointly regulate cell apoptosis.

In this experiment, considering the ignition effect of GnRH-a, the mouse's estrous cycle was 4–5 days, so triptorelin was used one week prior; after 1 week, it was used in combination with tripterygium. At the same time, the drug's inhibition of ovarian function was considered. Accordingly, the drug was discontinued for 3 weeks and the mice were executed after recovery of the ovary. The results show that the expression of Bcl-2 in the ovaries of the tripterygium group alone decreased, and the expression of Smac increased, while the expression of Bcl-2 in the tripterygium + triptorelin group increased, and the expression of Smac decreased. Triptorelin can increase the expression of Bcl-2 (ovarian apoptosis inhibitory gene) in mice and inhibit apoptosis, thereby prolonging the life span of ovarian cells. It can delay ovarian aging by helping to prevent follicular atresia or corpus luteum degradation, which in turn protects against ovarian damage caused by drugs.

Tripterygium glycosides can cause pathological conditions in the reproductive system of mice and cause significant damage to sex hormones and reproductive organs and ovaries in mice. Triptorelin has protective effects on the gonad and ovarian function of female mice induced by tripterygium. At present, there are few studies on the Smac signaling pathway and the related mechanisms of Bcl-2 family proteins. The development of Smac and Bcl-2 functional domain drugs has just begun. Further research on their mechanisms and forms of action is important to develop gene therapy and drug screening for drug-induced ovarian damage. However, this study also has shortcomings. The small sample size does not completely reflect the overall change of ovarian function. In addition, the time and dose of medication must be further explored. It should also be noted that different drug reactions among populations under the same group have been observed in Fig. 5, which could be caused by the varied weight of the mouse shown in Table 1 and the variation of absorption, metabolization and health condition. However, this difference is relatively small compared to that between the different groups. Lastly, in order to simplify the process, a group with triptorelin only was not selected, which may limit the understanding of the specific protection on Tripterygium.

In summary, tripterygium glycosides can weaken the expression of Bcl-2 and increase the expression of Smac in mouse ovaries, which can damage the reproductive system of mice. Triptorelin can up-regulate the expression of Bcl-2, a mouse ovarian apoptosis inhibitor, and thus attenuate the expression of Smac. It therefore can aid in the protection of ovaries from damage caused by drugs.

Author contributions

YCZ: Conceptualization; Data curation; Data analysis; Investigation; Methodology; Writing-original draft; Funding acquisition. YFZ: Resources; Data analysis; Writing-review & editing.

Ethics approval and consent to participate

The scheme of animal experiments in the study on the protective mechanism of GnRH-a on the damage of ovarian function caused by Tripterygium wilfordii polyglycoside was approved by Experimental Animal Ethics Committee of Hangzhou Medical College under the application No. 20200034.

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Conflict of interest

The authors declare no conflict of interest.

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