Protective mechanisms of triptorelin against tripterygium glycoside-induced ovarian dysfunction.

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Abstract

The aim of this study was to investigate the protective effects of Triptorelin (TRI) on Tripterygium Glycoside-induced Ovarian Dysfunction (TG-OD). Thirty mice with normal estrous cycles were randomly divided into three groups (n=10): the control group (group A) was orally administered 0.35 ml of saline daily; the TH group (group B) was orally administered 0.35 ml of TG solution from the 8th d for 10 w continuously; the TG+TRI group (group C) was subcutaneously injected with 0.1 mg/kg TRI daily, as well as orally administered 0.35 ml of TG solution from the 8th d for 10 w continuously. All mice were sacrificed during the 11th week to detect blood anti-Mullerian hormone, follicle-stimulating hormone, luteinizing hormone, and estradiol; calculate the ovarian index; and observe the morphologies of ovarian tissues. Mice in group C were more flexible, and had increased weight, reduced follicle-stimulating hormone and luteinizing hormone, and increased anti-Mullerian hormone and estradiol; additionally, ovarian follicles and mature follicles increased. TG caused gonad and ovarian dysfunction in female mice; TRI exhibited protective effects against TG-induced gonad and ovarian dysfunction in female mice.

Introduction

Tripterygium Glycosides (TG) are extracts obtained from a traditional Chinese medicine with immunomodulatory and anti-tumor effects; because the incidence and severity of side effects following TG administration are much lower than those of immunosuppressive agents, TG has been widely used in the clinical treatment of kidney diseases or rheumatism. Rational application of TG may significantly reduce the amount of immunosuppressive drugs required for therapy, thus greatly reducing medical costs and side effects as well as increasing patient compliance. Based on extensive applications and studies of TG, TG was observed to cause damage to the female gonad and ovarian functions [1]; therefore, its application in female patients before and in childbearing age has been extremely limited. It is important to minimize the side effects of TG, which would have important clinical and social effects.

Gonadotropin-releasing hormone agonist (GnRH-a) [2,3] is a synthesized gonadotropin-releasing hormone and is a preferred ovarian function-protecting agent in chemotherapy [4-6], and thus has been widely used in clinical practice [7]. The synthesized analog of GnRH, Triptorelin (TRI), has the same effects as GnRH [8]. Early in the first administration, GnRH agonists completely bind GnRH receptors on the surface of the pituitary gland, stimulating the transient increase of Follicle-Stimulating Hormone (FSH) and Luteinizing Hormone (LH) (the "ignition" period) [9]. Approximately 15 d later, the receptors become exhausted and are no longer sensitive to GnRH-a; thus, FSH and LH begin to decrease significantly and ovarian hormones begin to significantly decrease to postmenopausal levels. Therefore, artificial menopause can occur and protect the ovaries.

This study was performed to observe and compare the changes in the estrous cycle and morphologies of ovarian tissues in Kunming mice after administration of TRI and TG; additionally, changes in serum anti-Mullerian hormone (AMH) [10], FSH, LH, and estradiol (E2) were detected to investigate the protective effects of GnRH against TG-induced ovarian dysfunction in female mice and develop ovarian function-protective protocols for effective application of TG.

Materials and Methods

Animals and reagents

Thirty healthy, female Kunming (KM) mice, 7-8 w old, weighing 32-36 g, and clean-grade, were provided by Shanghai Slaccas Experimental Animal Co., Ltd. (Shanghai, China).

After 1-2 d adaptive feeding, all mice were subjected to the smear method to collect vaginal exfoliated cells, and those with a normal estrous cycle were selected for the study. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Zhejiang Chinese Medical University.

An Enzyme-Linked Immunosorbent Assay (ELISA) kit (Hangzhou Chengwei Biotechnology Co., Ltd., Zhejiang, China) was used to detect AMH, FSH, LH, and E2. Pathological detection reagents included xylene, formaldehyde, 95% ethanol, hematoxylin and eosin stain, wax, and were provided by the Animal Experimental Center of Zhejiang Chinese Medical University. TG tablets containing 10 mg/ tablet, which are typically administered at 2-3 tablets/dose to adults, were orally administered three times per day (Zhejiang DND Pharmaceutical Co., Ltd., Zhejiang, China; State medical Approval No: Z33020422); 1 ml achieve 0.1 mg TRI acetate was injected (calculated as $C_{64}H_{82}N_{18}O_{13}$). The common dose in adults is 0.5 mg subcutaneous injection once per day (Chengdu Tiantaishan Pharmaceutical Co., Ltd., Chengdu, China; State medical Approval No: H20058648); to prepare TG solution, TG tablets were dissolved in saline to achieve a concentration of 40 mg/kg.

Experimental design

Thirty qualified KM mice were randomly divided into three groups (n=10). The control group was orally administered 0.35 ml of saline daily for 11 w. The TH group was orally administered 0.35 ml of TG solution on the 8th d for 10 w continuously. The TG+TRI group was subcutaneously injected with 0.1 mg/kg TRI daily for 11 w, as well as orally administered 0.35 ml of TG solution on the 8th d for 10 w continuously.

All mice were sacrificed during w 11 to detect blood AMH, FSH, LH, and E2, calculate the ovarian index, and observe the morphologies of ovarian tissues. The best protocol was then screened.

General information

From the first day of the experiment, the general conditions of mice in each group were observed and recorded, including spirit, activity, fur, food intake, water intake, appetite, urine, and stool; their body weights were determined weekly.

The estrous cycle in mouse is approximately 4-5 d, including diestrus, proestrus, estrus, and metestrus. This study used a relatively simple smear method of vaginal exfoliated cells to observe the estrous cycle.

ELISA

All mice were sacrificed during w 11 and blood samples were obtained using the eyeball enucleation method to detect the serum levels of AMH, FSH, LH, and E2 by ELISA.

Tissue section

The abdomen of each mouse was then cut open to obtain the ovaries, which were first weighed to determine the wet weight using an electronic analytical balance, followed by calculation of the ovary index=ovary wet weight (mg)/mouse body weight (g) × 100%. Ovaries were then fixed with 4% paraformaldehyde, followed by paraffin-embedding, serial slicing, drying, and hematoxylin and eosin staining; the numbers of different-stage ovarian follicles were observed and analysed under a microscope.

Figure 1 shows ovarian tissue slices under the light microscope, including the primordial follicle, primary follicle, secondary follicle, mature follicle, and corpus luteum. To simplify the experiment, the original, primary, and secondary follicles were referred to as follicles.
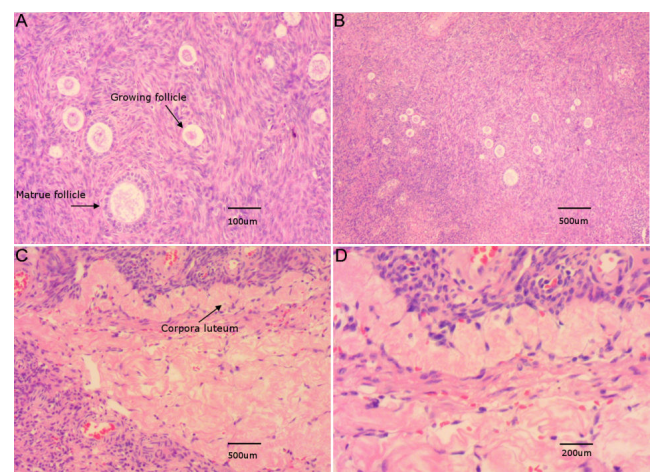

Figure 1. *Ovarian tissue sections. A. Under the microscope of mature and growing follicles (field diameter 1mm, the eyepiece magnification 10, lens magnification 20). B. Under the microscope of mature and growing follicles (field diameter 5 mm, the eyepiece magnification 10, lens magnification 4). C. Under the microscope of corpora luteum (field diameter 5 mm, the eyepiece magnification 10, lens magnification 4). D. Under the microscope of corpora luteum (field diameter 2 mm, the eyepiece magnification 10, lens magnification 10).*

Statistical analysis

All data were analysed with SPSS statistical software (SPSS, Inc., Chicago, IL, USA). Differences among groups were detected using the Student-Newman-Keuls method. Categorical data were analysed using variance analysis, and the homogeneity test of variance was performed. All results are expressed as the mean ± standard error of the mean. Statistical significance was accepted at P<0.05.

Results

General data

After one week of feeding, mice in group B showed significantly reduced activities and the amounts of food and water intake gradually reduced; after continuous feeding, the mice exhibited overall edema. However, mice in groups A and C exhibited better performance with lustrous fur, flexible activities, and increased food and water intake. The performance sorting of the three groups was as follows: group C>group A>group B.

Table 1 shows the results of Analysis of Variance (ANOVA), which revealed no significant difference in the original body weight and body weight 10 w later among different groups (P>0.05); however, differences in the ovarian weight and ovarian index among different groups were significant (P<0.05). Further pairwise comparison showed that the differences in ovarian weight between any two groups were significant (P<0.05). Ovarian weights differed between groups as follows: group C>group A>group B; pairwise comparisons of ovarian index between any two groups also revealed significant differences (P<0.05), with ovarian index values in the order of group C>group A>group B.

Effect of GnRH treatment on estrous cycle

The estrous cycle is the foundation of ovarian endocrine functions, which may exhibits regular differences under normal ovarian functions and coordination of estrogen and progesterone. After administration of TG tablets, vaginal smears showed a disturbance in the estrous cycle of mice in group B, indicating that TG significantly inhibits mouse ovarian endocrine functions. However, mice in group C exhibited stable estrous cycles, which were extended to 6-7 d.

Table 2 shows the results of ANOVA, which revealed significant differences in the serum levels of AMH, FSH, LH, and E2 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.

Effect of GnRH treatment on ovarian follicles

Table 3 shows the results of ANOVA, which revealed significant differences in growing ovarian follicles, mature ovarian follicles, total ovarian follicles, ratio of mature ovarian follicles to total ovarian follicles, and corpora lutea among different groups (P<0.001), and further pairwise comparisons showed that the sorting of growing ovarian follicles was group C>group A>group B. The sorting of mature ovarian follicles was group C>group A>group B; the sorting of total ovarian follicles was group C>group A>group B; the sorting of the ratio of mature ovarian follicles/total follicles was group C>group A>group B; the sorting of the corpora lutea was group C>group A>group B.

Table 1. Comparison of body weights among the three groups.

Group	Cases	Original body weight	Body weight 10 w later	Weight of ovaries	Ovarian index
A	10	34.7 ± 1.16	50.4 ± 3.47	0.0432 ± 0.0078	0.0009 ± 0.0002
C	10	34.78 ± 0.96	55.95 ± 11.11	0.063 ± 0.0112*	0.0012 ± 0.0002*
B	10	34.68 ± 1.45	48.91 ± 3.05	0.0302 ± 0.0059*Δ	0.0006 ± 0.0001*Δ
F	-	0.06	2.145	46.767	23.217
P	-	0.98	0.112	<0.001	<0.001

Note: *Compared with group A, P<0.05; Δcompared with group C, P<0.05.

Table 2. Comparison of serum AMH, FSH, LH, and E24 among different groups.

Group	Cases	AMH	FSH	LH	E2
A	10	45.34 ± 3.59	28.05 ± 2.72	8.36 ± 1.77	32.08 ± 3.89
C	10	50.72 ± 5.14*	23.45 ± 3.41*	6.84 ± 0.51	39.1 ± 3.21*
B	10	43.24 ± 1.49Δ	31.41 ± 4.35*Δ	11.05 ± 3.55*Δ	28.01 ± 1.79*Δ
F	-	7.069	17.173*	10.551	13.923
p	-	0.001	<0.001	<0.001	<0.001

Note: *Compared with group A, P<0.05; Δcompared with group B, P<0.05.

Table 3. Comparison of ovarian follicles among different groups.

Group	Cases	Growing ovarian follicles	Mature ovarian follicles	Total ovarian follicles	Mature ovarian follicles/ total ovarian follicles	Corpus lutea
A	10	11.9 ± 1.91	1.8 ± 0.79	19.3 ± 1.42	0.09 ± 0.04	6.7 ± 0.82

C	10	15 ± 0.82[*]	3 ± 0.67[*]	20.3 ± 1.06	0.15 ± 0.03[*]	7.7 ± 0.82
B	10	6.9 ± 1.2[*Δ]	1 ± 0.82[*Δ]	14.1 ± 2.38[*Δ]	0.07 ± 0.06[Δ]	3.2 ± 1.69[*Δ]
F	-	85.616	17.633	31.45	8.244	31.646
p	-	<0.001	<0.001	<0.001	<0.001	<0.001

Note: [*]Compared with group A, P<0.05; [Δ]compared with group C, P<0.05.

Discussion

Tripterygium wilfordii is a woody climber with a bitter taste, cold nature, and an effective poison, but can promote blood circulation and remove meridian obstruction, dispel wind, eliminate dampness, kill worms and detoxify, and relieve swelling and pain. It has multiple bioactivities such as anti-tumor and anti-immune effects. TG is a group of bioactive substances extracted from *T. wilfordii* root fragments and is commonly used clinically to treat toxin and rheumatism-induced autoimmune diseases such as nephrotic syndrome and rheumatoid arthritis. However, its side effects have gained increasing attention, particularly those on the reproductive system. Short-term usage can cause menstrual cycle disorders in young women, and long-term usage may cause complete amenorrhea. According to previous studies, at doses greater than 800 mg, the incidence of amenorrhea was as high as 95%.

Based on the above toxicities of TG towards the reproductive system, we selected this drug for modeling in this study to build a TG-OD model in mice [11] to further investigate the reproductive toxicities and mechanisms of TG, as well as TG-induced reproductive organ and ovarian damage and changes in sex hormones.

The dosage and medication timing when using TG to establish mouse models with follicular developmental disorder and ovarian dysfunctions are not standardized; in this study, we orally administered 0.35 mL of TG solution at 50 mg/(kg•d) per day.

The results showed that after oral administration of TG solution, the mice exhibited poor general conditions and their estrous cycles were disturbed; the levels of FSH and LH increased, while the levels of AMH and E2 decreased. Mouse body weights and ovarian indices significantly decreased; the numbers of ovarian follicles, mature ovarian follicles, and corpus lutea were reduced, clearly indicating the reproductive toxicity of TG in mice. When endocrine disorders and ovarian follicular development disorders occur and the numbers of follicles and mature ovarian follicles are reduced, normal ovulation is affected. Thus, TG significantly disrupted mouse sex hormones and reproductive organs (ovaries).

TG brings new challenges to gynecological clinical practice for treating diseases. Therefore, predicting women's ovarian functions during treatment is an important research topic [12]. Currently, commonly used clinical methods include using estrogen and progestin for intervention during TG treatment to maintain the efficacies of TG, while also protecting ovary and other reproductive organ functions, thus avoiding or reducing TG-induced premature ovarian failure and other side effects. However, long-term use of estrogen and progesterone also has some risks, such as migraine or frequent and abnormally severe headache, increased incidences of arterial and venous thrombosis, dementia, or breast cancer. Therefore, new, effective, and safe ovarian protective agents are urgently needed.

Gonadotropin-Releasing Hormone (GnRH) is a 10-peptide hormone secreted by hypothalamic neurons. It stimulates the release of FSH and LH in the pituitary gland, promotes the secretion of estrogen and progesterone in the ovary, and promotes the development and maturation of follicles. GnRH analogs include gonadotropin-releasing hormone antagonists and GnRH agonists (GnRH-a) [13]. GnRH-a has been widely used in the clinic in recent years. GnRH agonist administration after all receptors, the pituitary is not sensitive to GnRH, FSH, LH secretion decreased significantly, ovarian hormone levels also decreased significantly, to appear artificial menopause, menopause, and to protect the ovarian function.

GnRH-a significantly protects against the toxic effects of chemotherapy drugs in the ovaries [14]. In a long-term clinical follow-up study of 240 children younger than 15 years who received chemotherapy to treat Hodgkin disease, only 13% of girls showed premature ovarian failure, while the percentage of boys with aspermia was as high as 83%. These results indicate that ovarian functions before puberty are less affected by chemotherapeutic drugs; studies attempted to create a temporary preadolescence environment for women of childbearing age before chemotherapy to protect ovarian functions. As early as 1985, Ataya observed that long-acting GnRH-a TRI could protect against cyclophosphamide-induced ovarian damage in a rat model [15]. Subsequently, Yuan and Meirow confirmed the above conclusions in animal experiments [16], showing that GnRH-a could protect rat ovarian functions, save most of the primary follicles, and thus prevent quiescent follicles from damage caused by chemotherapeutic drugs [17,18]. Numerous clinical studies have confirmed that GnRH-a protects ovarian functions in patients from chemotherapy-induced damage [19,20].

Based on the above findings, TG-induced reproductive toxicities can be prevented by GnRH agonists to protect ovarian functions. The TRI used in this study was a synthetic GnRH analog, the structure of which was modified by replacing the sixth L-amino acid (glycine) in the natural molecule with D-tryptophan. TRI has the same effects as GnRH; however, because of its longer plasma half-life, it exhibits stronger affinity to GnRH receptors.

In this study, considering the effects of GnRH-a and that the mouse estrous cycle is 4-5 d, TRI was administered one week before this cycle and then co-administered with TG one week later. The results showed that the mice had lustrous fur, flexible activities, increased food and water intake, and body weight increase. FSH and LH were reduced, while AMH and E2 increased; the numbers of ovarian follicles and mature ovarian follicles increased, indicating that TRI significantly inhibited TG-induced reproductive toxicity, thus better protecting the development and maturation of growing follicles inside the ovaries.

TG can cause pathological conditions in the reproductive system of mice and significantly damage mouse hormone and reproductive organs. TRI protects against TG-induced gonad and ovarian dysfunctions in female mice. However, this study had a small sample size, and thus may not reflect overall changes; in addition, the administration timing and dosage require further analysis. Therefore, prospective, multi-center, and large-sample size studies are needed.

Conflicts of Interest

None

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