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Original Article

Biochemical changes of oxidative stress in premature ovarian insufficiency induced by tripterygium glycosides

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Abstract: Premature ovarian insufficiency (POI) is one of clinical manifestations of ovarian damage. This study is to evaluate biochemical changes of oxidative stress in POI induced by tripterygium glycosides (TG) via subcutaneous injection. 24 female KM mice were assigned to two groups: control group and TG group. The mice in TG group were subjected to 50 $mg.kg^{-1}.d^{-1}$ TG for 35 days, while these in control group were fed with parallel volume of sterile water. Blood samples were separately obtained in day 15, 22, 29, 36 and 43. Ovarian histopathological changes were determined when finished the administration and observed under optical microscope. Serum levels of malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and anti-mullerian hormone (AMH) and ovarian homogenates levels of MDA, SOD, GSH-Px and AMH were assessed by ELISA. AMH expression in the ovaries was analyzed by immunohistochemistry. Compared with control group, the results in TG group showed a significant reduction of serum levels of SOD and GSH-Px in day 15, 22, 29, 43 and increase of MDA in day 22, 36. They also presented decreased SOD and GSH-Px levels and increased MDA level in ovarian homogenates. Our data suggested that oxidative stress was involved in POI and might be the potential pathogenesis of POI induced by TG.

Keywords: Premature ovarian insufficiency, tripterygium glycosides, oxidative stress, malondialdehyde, superoxide dismutase, glutathione peroxidase

Introduction

Premature ovarian insufficiency, generally irreversible, is a hypergonadotropic ovarian deficiency with primary or secondary amenorrhea affecting about 1% women before the age of 40 years [1-5]. It is one of clinical manifestations of ovarian damage. However, the etiologies of POI are complex: genetic disorders; enzyme deficiencies; autoimmune disease; iatrogenic causes including radiotherapy, chemotherapy and surgeries; infection; exposure of environment toxins; metabolic factors; social psychological factors and so on [6-10]. In fact, the cause of most POI patients is not clear yet [11]. Therefore, it lacks effective therapy methods. So far, the mechanism, early diagnose and therapy of POI have still been an important problem, which should be of more concern.

According to the past researches, the major mechanisms of ovarian injuries include follicle cell apoptosis, oxidative stress, ovarian atrophy, cortical fibrosis and blood-vessels damage [12-15]. Precisely, POI is a kind of ovarian injuries with rare corpus luteum, reduced developing follicles and increased cell apoptosis [16]. In our previous study, a POI mouse model induced by TG via subcutaneous injection has been successfully established [16]. It isthe same that follicle cell apoptosis and serious ovarian fibrosis appeared in POI mouse model. However, no studies have reported the relationship between oxidative stress and POI caused by TG and that whether oxidative stress was involved in POI. Based on the above observations, several oxidative stress related substances were measured. In this article, we compared the biochemical changes of MDA, SOD and GSH-Px levels of serum and ovarian homogenates in two groups to evaluate the relationship between oxidative stress and POI.

Materials and methods

Chemicals

Tripterygium glycoside (TG) was bought from Shanghai Fudan Fuhua Pharmaceutical CO., Ltd. (10 mg/tab, batch number Z31020415, China). Dimethyl sulfoxide (DMSO) was obtained from Sigma Corporation of USA. 60 mg TG was dissolved in 1 ml DMSO, and then the mixture was dissolved in 11 ml normal saline to be 5 $mg{\cdot}ml^{-1}$ of final concentration. At the same time, 1 ml DMSO was added into 11 ml normal saline as placebo.

Animals

A total of 24 female KM mice (6 weeks old) were purchased from Department of Laboratory Animal Science of Fudan University, Shanghai, China. They were randomly assigned to two groups: control group and TG group. All experimental animals, housed in groups of 4 of per wire wage, were kept on an equal light and dark cycle, constant environmental conditions and maintained on a proper diet chow and water ad libitum.

Treatment

All of the experimental animals were adaptive to be fed for one week under these standard laboratory conditions. From the 8^{th} day, the mice in TG group were treated with TG 50 $mg{\cdot}ml^{-1}$ per day for 35 days, while these in control group with placebo. Body weight of mice was recorded per week. Blood samples were collected by the angular vein in day 15, 22, 29, 36 and 43. In day 43, all the mice were anesthetized by intraperitoneal injection with 10% chloral hydrate solution (0.3 $ml{\cdot}100\ g^{-1}$). The ovaries were immediately weighted, then the right ones were fixed with 4% paraformaldehyde for 48 hours and the left ones were used for the preparation of ovarian homogenates.

Histopathology

The right ovaries were fixed in 4% paraformaldehyde for histopathological examination. After fixation, each tissue sample was routinely processed and embedded in paraffin. Then, they were sectioned at 4 μm thickness and stained with hematoxylin and eosin for observation. After HE staining, developing follicles and corpus luteum were counted according to the reference [16].

Immunohistochemistry

Sections were incubated at 60°C for 1 hour, and then routinely deparaffinized in xylene and rehydrated in a graded series of ethanol. After that, antigen retrieval was performed by incubating the sections in 0.01 M citrate buffer (pH 6.0) and applying high microwave irradiation for 30 min. Following, sections were incubated with 10% H_2O_2 for 30 min to eliminate endogenous peroxidase activity and then blocked with 10% normal goat serum for 1 hour at room temperature. Afterwards, all sections were incubated with primary antibodies against AMH (1:100, Abcam, UK) for overnight at 4°C and then were placed for 45 min at room temperature. The sections were rinsed with PBS, and then biotinylated secondary antibodies were added for 10 min. When washed in PBS, all sections were incubated with horseradish peroxidase (HRP) for 10 min at room temperature. Following washing sections were incubated with 3,3-diaminobenzidine (DAB) to visualize the final product, and finally stained with hematoxylin, dehydrated and mounted. Meanwhile, positive control was selected according to the protocol and negative control was treated by substituting PBS for primary antibodies.

ELISA

The left ovaries were homogenized in ice-cold PBS to be 10% ovarian homogenates of final concentration. Ovarian homogenates and blood samples were centrifuged at 3,000 rpm for 10 min to separate the supernatants and serum, respectively. Then, the supernatants and serum were separately collected and stored at -80°C until analysis. The supernatants and serum levels of AMH, MDA, SOD and GSH-Px were measured by ELISA. All the procedures were strictly done according to the instructions. These ELISA kits were bought from Shanghai Huayi Biotechnology Co., Ltd., China.

Statistical analysis

Data analysis was performed using SPSS version 16.0 for windows (SPSS Inc, Chicago, IL). The differences in two groups were analyzed by independent sample t-test. The data was presented as mean ± standard deviation ($\overline{X} \pm S$). A *P*-value of < 0.05 was considered to indicate a statistically significant difference.

Table 1. The main characteristics in two groups ($\bar{X}$ ± S, n = 12)

	Control	TG
Body weight into groups (g)	23.44 ± 1.34	23.23 ± 1.65
Body weight before administration (g)	29.76 ± 2.41	28.61 ± 2.05
Body weight after administration (g)	38.51 ± 4.41	34.40 ± 3.85**
Ovarian index (%)	0.062 ± 0.011	0.042 ± 0.007*
Uterus index (%)	0.364 ± 0.118	0.199 ± 0.042*
Developing follicles number	42.83 ± 10.18	19.83 ± 3.04*
Corpus luteum number	6.00 ± 2.56	0.83 ± 0.83*
Serum AMH levels (ng/ml)	7.281 ± 1.405	6.734 ± 0.661
Ovarian homogenates AMH levels (ng/ml)	11.027 ± 0.349	7.898 ± 0.592*

**$P < 0.05$, *$P < 0.01$, compared with control group. Control = control group, TG = TG group.

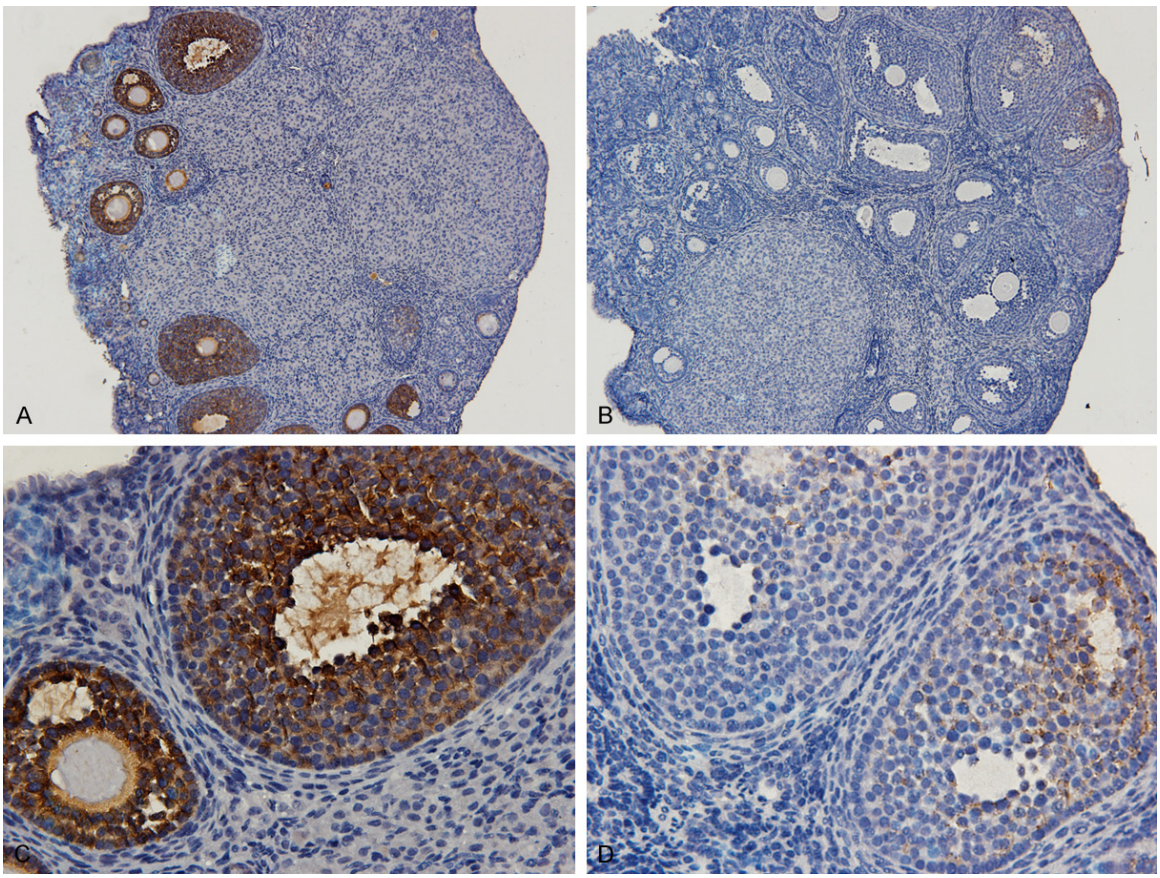

Figure 1. Location of AMH expression in the ovaries by immunohistochemistry in two groups. A and C. Strong AMH expression in normal ovaries in control group. B and D. Weak AMH expression in ovaries in TG group. Original magnification: A and B. × 100; C and D. × 400.

Results

Reevaluation of POI mouse model

Previously, we have established a POI mouse model induced by TG via subcutaneous injection [16]. **Table 1** depicted the main features in two groups, which was consistent with the results in our previous studies. The findings showed that mice in TG group presented the critical characteristics with significantly decreased body weight, reduced ovarian and uterus index, dropped developing follicles and corpus luteum, and low AMH levels when com-

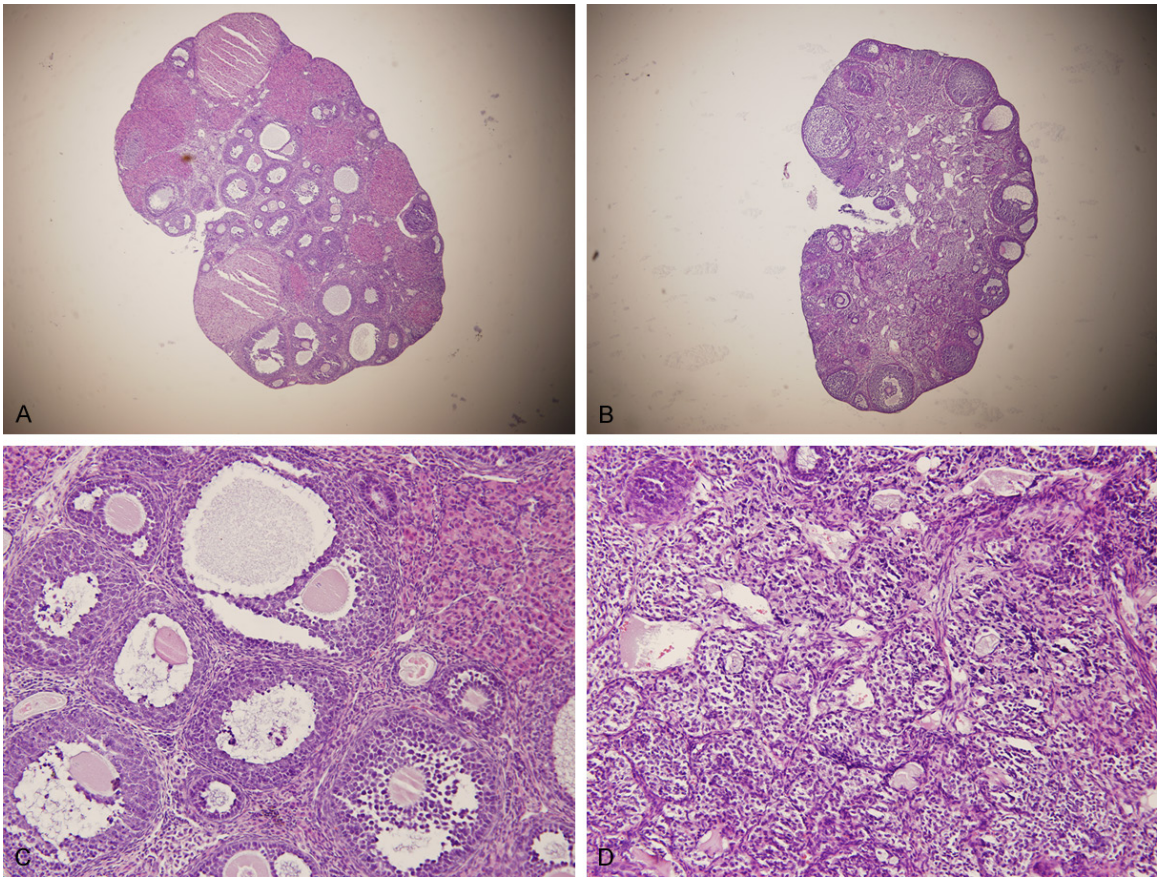

Figure 2. The histopathological changes of the ovaries by HE staining in two groups. A and C. Normal ovarian histomorphology with normal stroma, the appearance of all types of follicles in the cortex (primordial, primary, secondary and tertiary) and the presence of corpus luteum in control group. B and D. Abnormal ovarian histomorphology with rare corpus luteum, reduced developing follicles, increased atretic follicles and thinner layers of granulosa cells in TG group. Original magnification: A and B. × 40; C and D. × 200.

pared with control group. Meanwhile, **Figure 1** presented weaker staining of AMH in the ovarian sections by immunohistochemistry in TG group (**Figure 1B** and **1D**) than that in control group (**Figure 1A** and **1C**), which indicated AMH secretion was inhibited by TG. Overall, the POI mouse model was considered to be fluky.

Histopathological changes

Figure 2A-D illustrated the histopathological changes of the ovaries in two groups from an optical microscopic view. The control group demonstrated normal ovarian histomorphology with normal stroma, the appearance of all types of follicles in the cortex (primordial, primary, secondary and tertiary) and the presence of corpus luteum (**Figure 2A** and **2C**). But the ovarian sections in TG group displayed other phenomenon: rare corpus luteum, reduced developing follicles and increased atretic follicles, thinner layers of granulosa cells (**Figure 2B** and **2D**). It also proved a successful POI mouse model.

Serum levels of MDA, SOD and GSH-Px

As shown in **Table 2**, serum levels of MDA, SOD and GSH-Px, some related substances of oxidative stress, were clearly seen in two groups. Compared to control group, the results showed a significant reduction of serum levels of SOD and GSH-Px in day 15, 22, 29 and 43 and a significant increase of serum level of MDA in day 22 and 36 in TG group. The most obvious changes of oxidative stress in serum were in day 22.

Ovarian homogenates levels of MDA, SOD and GSH-Px

Ovarian homogenates levels of MDA, SOD and GSH-Px were demonstrated in **Table 3**. In accor-

Table 2. Serum levels of MDA, SOD and GSH-Px in different days in two groups ($\overline{X} \pm S$, n = 12)

Groups	MDA (nmol/L)				
	Day 15	Day 22	Day 29	Day 36	Day 43
Control	1.528 ± 0.050	1.695 ± 0.095	2.107 ± 0.217	1.498 ± 0.228	1.785 ± 0.113
TG	1.527 ± 0.035	1.961 ± 0.035**	2.237 ± 0.161	2.000 ± 0.297*	1.822 ± 0.074
	SOD (U/L)				
	Day 15	Day 22	Day 29	Day 36	Day 43
Control	62.002 ± 9.732	63.009 ± 8.490	67.506 ± 6.480	66.430 ± 3.249	71.523 ± 7.167
TG	41.173 ± 5.503*	52.094 ± 0.035*	55.694 ± 6.870*	61.668 ± 4.479	54.358 ± 4.415*
	GSH-Px (pmol/ml)				
	Day 15	Day 22	Day 29	Day 36	Day 43
Control	61.991 ± 5.679	60.312 ± 8.182	65.301 ± 6.388	65.328 ± 3.479	69.989 ± 4.757
TG	41.273 ± 2.205*	48.958 ± 5.987*	53.489 ± 5.887**	60.100 ± 4.99	52.048 ± 1.572*

**$P < 0.05$, *$P < 0.01$, compared with control group. Control = control group, TG = TG group.

Table 3. Ovarian homogenates levels of MDA, SOD and GSH-Px in two groups ($\overline{X} \pm S$, n = 12)

Groups	MDA (nmol/L)	SOD (U/L)	GSH-Px (pmol/ml)
Control	2.039 ± 0.091	79.136 ± 1.93	73.879 ± 2.244
TG	2.148 ± 0.148*	64.415 ± 1.620*	61.058 ± 1.336*

**$P < 0.05$, *$P < 0.01$, compared with control group. Control = control group, TG = TG group.

dance with the changes in serum, they also showed decreased SOD and GSH-Px levels and increased MDA level in ovarian homogenates.

Discussion

Premature ovarian insufficiency (POI) is a distressing condition that is a cause of infertility. It is well known that long-term deprivation of estrogen in POI patients will bring about some complications including infertility, osteoporosis, cardiovascular diseases, sexual dysfunction, cognitive dysfunction, associated endocrine and autoimmune conditions and so on [6, 17-19]. Therefore, more attention should be paid to POI patients.

Recent researches demonstrated that oxidative stress was closely associated with POI [20-21]. However, the relationship between oxidative stress and POI caused by TG still remains unknown. TG, a traditional Chinese patent medicine, is widely used to treat autoimmune and inflammatory diseases such as rheumatoid arthritis and chronic glomerulonephritis. In this study, we measured the biochemical changes of serum and ovarian homogenates levels of MDA, SOD and GSH-Px to evaluate their relationship. MDA, a marker of lipid peroxidation, was estimated to indicate oxidative stress damage to membrane [22]. SOD and GSH-Px, primary antioxidant enzyme contained in mammalian cells, were believed to be necessary for life in all oxygen-metabolizing cells [23]. SOD convert superoxide radical into hydrogen peroxide and molecular oxygen, whereas GSH-Px convert hydrogen peroxide into water. In this way, two toxic species, superoxide radical and hydrogen peroxide, are converted to the harmless product water. Therefore, these enzymes are very important for maintaining cell survival.

As predicted, we found a significant decrease of serum and ovarian homogenates levels of SOD and GSH-Px and a significant increase of serum and ovarian homogenates levels of MDA. And the most obvious biochemical changes of serum oxidative stress were in day 22. However, not every indicator presented a statistically significant difference such as serum levels of SOD and GSH-Px in day 36 and MDA in day 15, 29 and 43, which was not clear and needed further study. To sum up, oxidative stress was involved in POI induced by TG via subcutaneous injection and might be one potential pathogenesis of POI. But the special molecular mechanisms of POI caused by oxidative stress still needed to be elucidated. Therefore, we came up with a scientific hypothesis that whether Vitamin E, Vitamin C and melatonin, as antioxidant substance, could protect or therapy POI by eliminating or relieving oxidative stress to ameliorate ovarian function. It still requires in-depth study.

In conclusion, this study is the first to evaluate the relationship between oxidative stress and POI caused by TG. Our findings showed that oxidative stress was involved in POI induced by TG via subcutaneous injection. Therefore, oxidative stress might be one potential mechanism of POI caused by TG.

Acknowledgements

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Disclosure of conflict of interest

None.

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