

The Experimental Study of the Influence of Yanlingdan Prescription on the Apoptosis Protein Fas/FasL Expression of Ovarian Granular Cells in Mice with Premature Ovarian Failure

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Abstract. Objective: The purpose of this research is to observe the influence of Yanlingdan prescription on the apoptosis protein Fas/FasL expression of ovarian granular cells in mice with autoimmune premature ovarian failure and investigate the mechanism of this prescription in the treatment of premature ovarian failure. Method: Taking the Zona pellucida3 of mice as the antigen, the female mice were injected subcutaneously with this drug to make mice immune so as to become the mice with premature ovarian failure. Blank control group, model group, Yanlingdan group and western medicine group are set. After two weeks of dosing mice, we use immunohistochemical method to detect the expression level of apoptosis related protein Fas/FasL of ovarian granular cells. Results: The expression level of apoptosis related protein Fas/FasL in model group is obviously higher than the blank control group ($P < 0.01$). Compared with model group, the FAS and FASL protein expression of mice' follicle in Yanlingdan group and western medicine group weakens obviously ($P < 0.01$). When the expression of Fas and FasL in Yanlingdan group compares with the normal control group, the difference has no statistical significance ($P > 0.05$). Conclusion: Yanlingdan prescription can effectively suppress the expression of FAS/FASL protein of follicular granular cells and have inhibitory effects on the apoptosis of ovarian granular cells in mice with premature ovarian failure.

Introduction

Premature Ovarian Failure (POF) refers to the ovarian function failure which is resulted by follicular atresia before the age of 40. The clinical manifestations of patients are the high gonadotropin level, low oestrogen level, amenorrhoea, infertility and various perimenopausal period symptoms. With the change of the living environment, dietary habit and work stress of modern society, the morbidity of premature ovarian failure has increased gradually[1]. Modern research shows that[2] the excessive apoptosis of ovarian granular cells is one of the main reasons to lead to premature ovarian failure. Ovarian granular cells can not only secrete estrogen and progesterone hormone but also play an important role in the process of development and maturity of follicle, therefore the adjustment of the apoptosis of follicular granular cells may provide new target spot for the treatment of premature ovarian failure. Through the animal experiment, this research explores the influence of Yanlingdan prescription on the molecular regulation mechanism of the apoptosis pathway which is mediated by Fas in ovarian granular cells and investigates the mechanism of action of this prescription.

Materials and Methods

Animal. The animal center of Qiqihar Medical University provides 7-8 weeks old 60 SPF BABL/C female mice and 10 male mice. The weight of mice is 18~22g. The certification number of the animal is SCXK(black)2015-0004. Females and males were reared in separate rearing cages at a rate of 4 to 1 in order to induce normal estrous cycles in mice. Exfoliated cells of the vagina were made into smears every day in order to observe the estrus cycle of mice under the microscope. Mice with regular estrous cycle are selected to conduct the experiment (The calculation is based on two estrous cycles).

Experimental Medicine. The first one is traditional Chinese medicine preparations. Yanlingdan prescription: rehmannia 12g, Chinese yam 12g, sweet potato 12g, Chinese dodder 12g, Eucommia ulmoides Oliver 12g, fleece-flower root 6g, black ants 6g, oyster 6g, the fruit of Chinese wolfberry 6g, the root of red-rooted salvia 6g, 6 Cyperus rotundus, root of membranous milk vetch 6g, Codonopsis pilosula 6g, liquorice 3g. These medicines are provided by the bureau of traditional Chinese medicine in Qiqihar. The dosage form is granules. And it is made into the solvent which is equivalent to 1.08g/L crude drugs. It should be reserved in the refrigerator of 4°C. The second one is western medicine. Estradiol Valerate Tablets which are provided by Guangzhou Branch of Bayer Medicine and Health Care Co.,Ltd. Each tablet is 1mg. It is diluted by tri-distilled water. The concentration of it is 0.006mg/ml.

Kit. The zona pellucida polypeptide solution of mice: The 330th~342th amino acid sequence (NSSSSQFQIHGPR) of the zona pellucida3(ZP3) of mice. The purity of it is greater than 95%. It is produced by Chinese Peptide Co.,Ltd. Freund's complete adjuvant and Freund's incomplete adjuvant which are provided by Beijing Bioss Biotechnology Co.,Ltd. FAS and FASL primary antibodies which are provided by Wuhan Boster Biological Technology Co.,Ltd.

Experimental Instruments. BX53 optical microscope (Japanese Olympus Company). RM2235 freezing microtome (German Lecai company).

Methods

POF Model Equipment. Tri-distilled water is added to 2mg ZP3 zona pellucida polypeptide powder to make into the solution. The selected 1 ml solution is diluted until 6 ml solution. Then solution and Freund's complete adjuvant are made into the immunoreagent at a ratio of 1:1. Solution and Freund's incomplete adjuvant are made into the immunopotentiator at a ratio of 1:1. Model is made according to the literature method[3]. Double rear soles and abdominal cavity of mice are subcutaneously injected 0.15ml immunoreagent. After 14 days, double rear soles and abdominal cavity of mice are subcutaneously injected 0.15ml immunopotentiator to strengthen immunity.

Groups and Feeding Drug. 40 mice with regular estrous cycle are divided into blank control group, model group, Yanlingdan group and western medicine group. In addition to the blank control group, double rear soles and abdominal cavity of mice in model and other two groups are subcutaneously injected 0.15ml immunoreagent. The mice in the blank control group are injected 0.15ml normal saline. After 14 days, double rear soles and abdominal cavity of mice in model and other two groups are subcutaneously injected 0.15ml fresh immunopotentiator. The mice in the blank control group are interjected 0.15ml normal saline again. Yanlingdan prescription group was given 2 times clinical equivalent dosage (1.08ml/20g) Yanlingdan prescription liquor by gavage. The western group is given 0.4ml/20 gestradiol valerate by gavage. The blank group and model group are given normal saline by gavage. Each group is given twice a day for 15 days.

Observation Index. The first one is estrous cycle observation. The estrous cycle of autoimmune POF model mice is observed[4]. Vaginal cells smears are inspected at 9:00 every morning in order to observe and compare the changes of the estrous cycle of mice in each group. The judgement basis of the estrous cycle: There are a large number of nucleated epithelial cells and a small amount of keratinized epithelial cells in the prophase of estrus. There are a large number of keratinized epithelial cells in estrus. In the late stage of estrus, there are mainly keratinized epithelial cells accompanied by a few white blood cells. There are a large number of leukocytes during diestrus.

The second one is ovarian Fas and FasL proteins expression detection. After the mice were killed, aseptic ovarian tissues were reserved. They are fixed by paraformaldehyde and embedded and cut into slices by paraffin. The expression of Fas and FasL proteins in the ovary was detected by immunohistochemistry according to the kit instruction. The third one is a statistical method. SPSS18.0 statistical software is used. Measurement data is expressed by using mean \pm standard deviation ($\bar{x} \pm s$). Analysis of variance was used in multiple groups. SNK method is used in the comparison between two groups. The difference has statistical significance ($P < 0.05$).

Results

Comparison in General Condition. The mice in the blank group have glossy fur, good eating, regular bowel movements, quick movement and quick reaction. The fur of mice in model group fur is lackluster and sparse, erect. They eat very little. Their stool is thin. They are tired, apathetic. They have less independent activities and like to stay with each other and curl up.

Comparison in Estrous Cycle. The estrous cycle of normal mice is 4~5 days. The diestrus is 1.5~2.5 days. After the model is made, the estrous cycle of mice in the model group is prolonged. The period appears irregular disorder and the cycle was prolonged to 7~10 days. Most of them stay at diestrus. The disorder order of the estrous cycle of mice in Yanlingdan treatment group is improved. There is a regular estrous cycle alternation and the time is 6 ~ 9 days.

The Influence of Yanlingdan Prescription on the Expression of Fas and FasL Proteins of Follicular Granular Cells in Mice with Autoimmune Premature Ovarian Failure. The expression of FAS protein of follicular granular cells of mice in blank control group is little and the expression of FASL protein is weakly positive. Compared with the blank group, the expression of FAS and FASL proteins of follicular granular cells of mice in the model group is obviously increased ($P < 0.01$). Compared with the model group, the expression of FAS and FASL proteins of follicular granular cells of mice in Yanlingdan group and western medicine group is obviously decreased ($P < 0.05$). It shows that Yanlingdan group and western medicine group both can restrain the excessive apoptosis of follicular granular cells through decreasing the expression of FAS and FASL proteins of follicular granular cells of mice.

Table 1. The influence of the Wubie oral liquid on the expression of ovarian FAS/FASL protein of mice with autoimmune premature ovarian failure.

Groups	The number of ovaries	FAS	FASL
Blank control group	10	0.200 \pm 0.008	1.532 \pm 0.441
Model group	10	0.468 \pm 0.021 ▲	2.344 \pm 0.345 ▲
Yanlingdan group	10	0.208 \pm 0.141 ■ ▼	1.610 \pm 0.341 ■ ▼
Western medicine group	10	0.242 \pm 0.126 ■ ■	1.527 \pm 0.362 ■

Note: The model group compares with the blank control group ▲ $P < 0.01$; Yanlingdan group and western medicine group compare with the model group ■ $P < 0.01$; ■ ■ $P < 0.05$; Yanlingdan group compares with the western medicine group ▼ $P > 0.05$.

Conclusion

Ovarian development is a process of continuous change, in which granular cells and oocytes begin to interact with each other. Granular cells supply 85% of required nutrients for oocyte development [5, 6]. It has been proved[7-10] that granular cell apoptosis occurs during the process of follicular

atresia and the granular cell apoptosis is an important and direct reason which leads to follicular atresia. The system belongs to the tumor necrosis factor receptor family and the FASL is the only natural ligand of it. At present, the signal path of cell apoptosis, which is mediated by Fas/FasL system, is one of the important ways of cell apoptosis. The results show that FAS and FASL proteins are weakly expressed in ovarian granular cells of the normal control group, but the model group was the opposite. The expression of the two of model group is enhanced. After the treatment, the expression intensity of gene proteins of Yanlingdan group and western medicine group is decreased, but the intensity is between the normal control group and the model group. There are significant differences between them. From it we can see that Yanlingdan can adjust the apoptosis gene proteins of ovarian granular cells. Its mechanism of action may be to disturb and restrain cell apoptosis which is induced by signal transfer system and prevent the open of dead passage through increasing the expression of apoptosis-suppressing gene FAS protein in ovarian granular cells so as to restrain the apoptosis of ovarian granular cells, promote the secretion of oestrogen and progesterin and improve the endocrine function of ovary.

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