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Research on establishment of chronic stress-induced premature ovarian failure rat model and effects of Chinese medicine Munziqi treatment

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Abstract

The purposes of this study were to establish and to explore the biological basis of the chronic stress induced premature ovarian failure (POF) model, also to explore the therapeutic effects of the traditional Chinese medicine Munziq. Sexually matured female SD rats were fed with spinach and cilantro in cold and wet conditions for about 20 weeks, until a chronic stress (CS) model was established. CS rats were divided into POF stress model group and stress model group according to weekly biological characteristics and hormone level detection (LH, FSF and E2). To investigate the therapeutic effect of Munziqi, the POF disease

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stress model group was divided into the high, medium and low drug intervention groups. The results showed that chronic stresses (special food, cold, damp) can lead to POF disease; Traditional Chinese medicine Munziq not only improve the reproductive hormone level disorder, but also improve the function of the hypothalamus-pituitary-ovarian axis, its mechanism may be a change E2, LH and FSH hormone levels in serum and lower the expression of ovarian premature aging related protein PFN-1.

[Key words] chronic stress; premature ovarian failure; estrogen; PFN-1; Munziqi

1. Introduction

Premature ovarian failure (POF) is a common gynecological endocrine disorder. Premature ovarian failure (POF) refers to the phenomenon of amenorrhea before the age of 40 caused by ovarian failure (Vujovic, S. 2009; Maclaran, K., & Panay, N. 2011). Its onset is characterized by primary or secondary amenorrhea, accompanied by elevated levels of follicle stimulating hormone (FSH) and luteinizing hormone (LH), decreased levels of estrogen (E2), and varying degrees of low estrogen symptoms. Such as hot flashes, sweating, facial flushing, low sexual desire (Shelling, A. N. 2010; Otsuka, F., & Shimasaki, S. 2011). Studies have shown that the mechanism of POF is insufficient storage of primordial follicle pool, accelerated follicular atresia, altered follicle recruitment, and follicular maturation disorders, and its etiology is complicated. POF seriously affects women's quality of life and physical and mental health. Nowadays it has become a hot spot and a difficult point in reproductive health research (Hoek, A., Schoemaker, J., & Drexhage, H. A. 1997).

After encountering various adverse stimuli, the body will produce a series of responses by regulating the neuro-endocrine system, which is called stress by domestic scholars. The common biological response of stress is that after the body feels the stressor through the central system, it will initiate a series of defensive responses in biological behavior, neuroendocrine system and other aspects (Miller, G. E., Chen, E., & Zhou, E. S. 2007; Pecoraro, N. C., Reyes, F., Gomez, F., Bhargava, A., & Dallman, M. F. 2004). Among them, the most important, the most extensive and long-lasting hormone secreted by the endocrine system is brought about. These defensive responses are important because they maintaining normal biological activity. Chronic stress has become an important environmental factor for menstrual disorders, infertility, sexual dysfunction and other common diseases (Gould, E., & Tanapat, P. 1999; Tianzhu, Z., Shihai, Y., & Juan, D. 2014). However, there is limited theoretical research on the circadian rhythm disturbance and how the wet, cold and special food environment can cause

damage to the reproductive system. Therefore, it is an urgent clinical problem to explore the pathogenesis of the gonadal axis disorders induced by chronic stress and find the potential targets for its prevention and treatment.

In clinical trials, POF can be diagnosed by measuring serum FSH (FSH > 40 IU/L) and E2 (<100 pmol/L) (De Assumpcao, C. R. 2014; Coulam, C. B. et al., 1987). In addition, in POF, echocardiography such as ultrasound is also used for auxiliary diagnosis. Recent studies have shown that PFN-1 can be used as a stimulating factor was released to outside the cell, extracellular PFN-1 may by promoting phosphorylation of extracellular regulatory proteins stimulate the ovaries of smooth muscle cell proliferation and migration, thus promote the ovarian function disorder, at the same time, the plasma level of PFN-1 in the closely related with the severity of the ovarian hormone level disorder , PFN-1 and fibrosis caused by various causes and ovarian cell apoptosis is an important pathology of ovarian dysfunction occurrence and development process.

In this study, we investigated its biological basis by establishing a model of chronic stress ovarian rats and explored the possible mechanism of action of the Munziqi : the traditional Chinese medicine.

2. Materials and Methods

2.1 Animals. 90 matured female SD rats were verified by vaginal smear test provided from the Laboratory Animal Centre of Xinjiang Medical University (Certificate No. SCXK (Xin) 2011–0004), weighted (180±20) g. These rats were kept in the clean standardized laboratory condition, with an illumination from 8:00am to 8:00pm， room temperature of (25±2) °C and humidity of (55±5) % for three to five days so that they could fit in the experimental condition. In the course of study, the rats were allowed to make use of food and water ad libitum. All experiments were approved by the Ethics Committee of the Xinjiang Medical University, which were in accordance with the relevant national guidelines, including any relevant details (Muladili Yuemaier, et al., 2018).

2.2 Chemicals and reagents. Estradiol (E2) radioimmunoassay kit, follicle stimulating hormone (FSH) radioimmunoassay kit, luteinizing hormone (LH) radioimmunoassay kit (Beijing North Biotechnology Research Institute), estradiol (E2) enzyme-linked immunosorbent kit, follicle stimulating hormone (FSH) enzyme-linked immunosorbent kit, luteinizing hormone (LH) enzyme-linked immunosorbent kit, actin regulatory protein-1 (PFN1) enzyme-linked immunosorbent ELISA kit (Elatrit Biotechnology Co., Ltd.), Leica DM3000 digital microscope (Leica Microsystems, Germany). Rabbit anti-PFN1

monoclonal antibody (Abcam, UK, batch number ab136285), rabbit anti-beta-actin (beta-actin) polyclonal antibody (Bioworld Technology, USA, batch number 1102), BCA assay kit (enhanced, Biyuntian Biotechnology Research Institute, batch number P0010), PFN1 primer (Invitrogen, USA, batch number HB1503108098), reverse transcription-polymerase chain reaction (RT-PCR) kit (Beijing Tiangen Biochemical Technology Co., Ltd., batch number KR140818).

2.3 Treatments. All of 90 rats were randomly divided into a stress model group (n = 80) and a control group (n = 10). Rats in the control group were housed under normal conditions at a temperature of (25 ± 2) ° C and a humidity of (55 ± 5) % and fed normal feed throughout the study period. Rats in the stress group were housed in animal facilities at a temperature of (6 ± 1) ° C and a humidity of (80 ~ 90) %. From 8 am to 8 pm, cold natural feed (spinach and alfalfa) were fed. Both groups were modeled for approximately 20 weeks before the stress model was established(Muladili Yuemaier, et al., 2018). Then, the blood was collected from the rat iliac venous plexus and the hormone content of E2, LH and FSH in the peripheral blood was detected by radioimmunoassay. The hormone content of the rats in the stress model group was compared with the hormone content in the control group (C) group, and the combination should be combined. POF model animals were selected from the changes of estrous cycle in each group, and they were randomly divided into POF stress model group (POF group), POF drug intervention high dose group (PTH group), POF drug intervention medium dose group (PTM group), POF drug intervention in low-dose group (PTL group); Stress model animals without POF were randomly divided into stress model group (S group), stress drug intervention high-dose group (STH group), stress drug intervention medium-dose group (STM group), stress drug intervention in low dose group (STL group). (The stress model group and the stress drug intervention group were used for other experimental studies). The PTH, PTM, and PTL groups were treated with traditional Chinese medicine Munziqi administered intragastrically daily at 27 ml/kg according to the human equivalent dose calculated according to the method of Reagan-Shaw et al. The above-mentioned drug intervention groups of animals were administered with three doses of high, medium and low doses of Chinese traditional medicine. The drug intervention time was 2 weeks. During treatment, the POF group remained in a cold, humid environment as usual. Group C was fed normal feed under normal conditions. The PTH group, the PTMZ group, and the PTL group were transferred to the same normal conditions as the control group, and it was established that the stability of the model was established.

2.4 Estrous cycle and biological characterization. For the subsequent experiment, all rats were examined their estrous cycle by vaginal smear test at 10am continuously. Vaginal smears were collected and smeared on glass slides by cotton swab which was dipped in 0.9% NaCl. After air dying, the samples were stained with methylene blue in 9.5% ethanol, washed, covered with cover glass and observed under microscope. Observations of estrous cycle were followed by: proestrus, with 100% of intact live epithelial cells; estrus, with 100% of cornified epithelial cells; metestrus, with approximately 50% of cornified epithelial cells and 50% of leukocytes; diestrus, with80~100% of leukocytes The qualitative and quantitative indicators of biological characterizations were determined by weekly measurements during the study, such as daily activities, stimulus response, and quantitative indicators were measured such as animal body weight, water intake, food intake which mentioned above were the most important dialectic parameters in confirming the CS model based on Chinese medicine.

2.5 Detection of serum PFN1 and VEGF levels in rats. After the drug intervention, the peripheral blood of rats were extracted from the abdominal aorta of rats, and the serums were separated by centrifugation. The levels of PFN1 and VEGF in peripheral blood of each group were detected by enzyme linked immunosorbent assay (ELISA). According to the relevant enzyme-linked immunosorbent ELISA kit instructions.

2.6 Histological examination. After all animals were sacrificed, the ovaries of all rats were dissected and immersed in 4% neutral buffered paraformaldehyde for at least 48 hours and then embedded in paraffin. The sample was cut into 4 μm thick sections. All sections were stained with hematoxylin and eosin (H&E) and subsequently observed under a microscope and photographed.

2.7 PFN1 expression was detected by immunohistochemistry. 1 Paraffin section: cut into a thickness of about 4 μm, conventionally spread, baked; 2 dewaxing: dewaxing once with xylene and different concentrations of ethanol; 3 repair: place it at 95 °C for sodium citrate .The antigen was heat-repaired in buffer at 0.01 mol/L for 15 min; 4 inactivation: 3% H_2O_2 was added to incubate for 10 min at room temperature to inactivate endogenous peroxidase; 5 drops plus primary antibody: first add 10% goat serum at room temperature for 30 min, then add PFN1 primary antibody diluted according to a certain ratio, and incubate overnight at 4 °C; 6 drops plus secondary antibody: discard the primary antibody that was incubated overnight wash with PBS solution for 3 times, 5 min / time, add horseradish peroxidase specimen secondary antibody at 37 °C for 30 min; 7 hematoxylin counterstaining: remove, wash with PBS solution 3 times, 5 min / time, DAB color, hematoxylin counterstained, and then differentiated with 0.1% hydrochloric acid; 8 dehydrated, transparent, sealed: dehydrated with ethanol

gradient, transparent with xylene, sealed with neutral gum. Observed under the microscope, the yellow and brown particles were positive in the cytoplasm and/or nucleus. Immunohistochemical scoring: First, positive cells were scored according to staining intensity, colorless to 0, light yellow to 1 point, brown to 2 points, and brown to 3 points (staining depth should be compared with background). The percentage of positive cells is then scored, 0% to 1% = 0, 1% to 10% = 1, 11% to 50% = 2, 51% to 80% = 3, 81% to 100% = 4.

2.8 Fluorescence quantitative RT-PCR was used to determine the expression of PFN1 in rat ovarian tissue.

2.8.1 Extraction of total RNA: 0.5 g of ovarian tissue was weighed, pulverized into powder, and tissue powder was dissolved in 1 mL of Trizol to extract PFN1 RNA from rat ovarian tissue, and stored at -80 ° C until use.

2.8.2 Determination of total RNA purity and concentration: Take 5 μL of total RNA sample, add 495 μL Depc - H2O, mix well, measure the absorbance at 260 nm and 280 nm, calculate OD260 / OD280 °C; RNA concentration (μg / μL) = OD260 × 100× 40.

2.8.3 First-strand cDNA synthesis: 1 Take a centrifuge tube and add Total RNA1μg, Oligo(dT_{18}) 1μL, Depc - H2O makes up the total volume to 6μL.

2.8.4 Mix well, react at 70 °C for 5 min, then remove the ice bath immediately for 2 min; then add 5 × amplification buffer 2μL, 4 dNTPs (10 mmol / L each) 2 μL, RNA Inhibitor 0.5μL, M-MLV reverse transcriptase, 100 U.

2.8.5 Mix well, react at 60 °C for 60 min; heat at 70 °C for 5-10 min, inactivate reverse transcriptase to stop the reaction, store at 20 °C for use, and dilute 10 times for PCR amplification.

2.8.6 PCR amplification: PCR amplification was performed using GAPDH as an internal reference, gene sequences were obtained by Genbank, primers were designed using by prime premier 5 software, and primers were synthesized by Shanghai Shenggong Biotechnology Service Co., Ltd. The primer sequences and reaction conditions used in this experiment are shown in Table 1 below.

Table 1. Characteristics of PFN1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers

Gene	**Primer**	**Sequence**	**Annealing temperature**(℃)
PFN1	Forward Primer	ATGCAGGGATGATGTTCTGG	64.4
	Reverse Primer	GTGCAAAAGCCAAAGGGAG	64.3
GAPDH	Forward Primer	GACAACTTTGGCATCGTGGA	58
	Reverse Primer	ATGCAGGGATGATGTTCTGG	58

2.8.7 In a special centrifuge tube for fluorescence quantitative PCR, add: ddH2O 8.2 μL, 2 × SYBR Premix Ex Taq10.0μL, upstream primer (10pmol / μL) 0.4μL, downstream primer (10pmol/ μL) 0.4μL, c DNA 1.0μL, total volume 20.0μL.

2.8.8 The Ct value of each sample was obtained, and the normal expression group was used as a control, and GAPDH was used as an internal reference gene, and the relative expression amount of the target gene of each sample was calculated as $2^{-\Delta\Delta Ct}$. ΔΔCt =[the average Ct value of the gene to be tested - the average Ct value of the reference gene in the test group]- [the average Ct value of the target gene in the control group - the average Ct value of the reference gene in the control group]

2.9 Western blot analysis of PFN1 protein levels; The rat ovarian tissue was ground by liquid nitrogen, and the total protein of the tissue was extracted according to the kit procedure, and the protein was quantified by the BCA method. 40 μg of protein was added to the loading buffer for denaturation. SDS-PAGE gel was prepared, and the gel was electrophoresed at 100 V for 40 min. The gel was electrophoresed at 120 V for 1 h, and 200 mA was wetted for 1 h for 20 min. The protein was transferred to the PVDF membrane, and the band was observed by Ponceau red staining. TBST wash film, 5% skim milk TBST closed for 1.5 h, add one anti-temperature for 2 h, TBST wash the membrane, add secondary antibody for 1 h, TBST wash the membrane, ECL luminescence reagent luminescence, film exposure and development. The images were collected, and the results were analyzed by absorbance scanning. The ratio of

absorbance of PFN1 and VEGF protein bands to β-actin was used as the expression of PFN1 and VEGF protein.

2.10 Statistical analysis. All of the results were analyzed by SPSS 22.0 (SPSS Inc., Chicago,IL, USA) via one-way analysis of variance (ANOVA) and presented as mean± SD ($\overline{x} \pm s$), Student-Newman-Keuls test was performed for comparisons between groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1 Changes of estrus cycle in rats .After vaginal smear, staining and observation under the microscope, the estrus changes at each stage were: pre-estrus (Fig. 1a), mostly nucleated oval epithelial cells, a small number of keratinized epithelial cells and white blood cells; estrus (figure 1b): Most of them are keratinized epithelial cells, generally irregular polygons, larger, aggregated into blocks, except for a small number of nuclear epithelial cells and a small number of white blood cells; late estrus (Fig. 1c): simultaneous nucleation Epithelial cells, keratinocytes and nucleated leukocytes generally have no significant difference in number; estrus interval (Fig. 1d): most of them are nucleated white blood cells, small in size, generally round, and a small number of keratinized epithelial cells And nucleated epithelial cells, as shown in Figure 1.

3.2 Weight change. As shown in Figure 2, before the start of modeling, there was no significant difference in body weight, drinking water, food intake, urine volume and stool between rats in group C and chronic stress group ($P>0.05$). At the beginning and time, the growth rate of body weight and water consumption in chronic stress group began to slow down compared with that in group C. The difference in body weight between the two groups was statistically significant ($P<0.05$). The difference of drinking water between the two groups was statistically significant from the 8th week ($P<0.05$). The growth rate of food intake, urine volume and stool volume in the chronic stress group began to increase faster than that in the C group. There was a significant difference in the food intake between the two groups at the beginning of the week ($P<0.05$). There was a statistically significant difference between the two groups ($P<0.05$) from the 8th week. The difference in stool volume between the two groups was statistically significant ($P<0.05$). The results showed that the weight, drinking water, food intake, urine output and stool growth rate of the chronic stress group were measured by chronic stress.

3.3 Changes in biological characterization during drug intervention. As shown in Table 1-5. the changes in body weight, water intake, food intake, urine output and stool volume of the rats in each group during the 14-day period of drug intervention, the difference in body weight between the PTH group, the PTL group and the POF group after drug intervention. Statistically significant (P<0.05); in addition, it can be seen that the weight change of the POF group is lower than that of the normal group. After the administration, the weight growth rate of the rats in each dose group is better than that of the corresponding model group, suggesting that the traditional Chinese medicine Munziqi can modulate the slow rate of weight gain caused by chronic stress.

3.4 Changes in ovarian function-related hormone levels. The levels of E2, FSH and LH hormones in rat serum were determined by ELISA. As shown in Table 6, the serum E2 levels in the model control group were significantly lower than those in the normal control group (P<0.05). The levels of FSH and LH were significantly increased (P<0.05). Compared with the model control group (POF), the levels of E2, FSH and LH were significantly increased in each drug intervention group, and the levels of FSH and LH were significantly decreased (P< 0. 05), among which the high-dose drug group has the most obvious improvement effect.

*3.5 Serum levels of VEGF and PFN1.*The vascular endothelial growth factor (VEGF) content and PFN1 content in rat serum were determined by ELISA. As shown in Table 7, the serum VEGF content of the POF group was lower than that of the C group, and the serum PFN1 of the POF group was measured. The content of the rats in the C group increased. After statistical analysis, the serum VEGF levels in the POF group and the C group were statistically significant (P<0.05). After drug intervention, according to the statistical results, the POF drug intervention in each dose group the serum VEGF content could be increased, and the content of PFN1 in serum could be decreased by POF drug intervention. The difference between PTH group and POF group was statistically significant (P<0.05), although PTM and PTL groups could also increase the content, but the difference was not statistically significant (P>0.05).

3.6 Rat ovarian tissue HE staining. The ovary is the most important tissue of women. As shown in Figure 3, the ovarian follicles are seen in group C. The follicles are large and large in number, with multiple granules, abundant cytoplasm of granulosa cells, large follicular cavity and abundant follicular fluid.Cell-rich, occasionally closed follicles; POF group rats did not see obvious primary, secondary follicles, occasionally primordial follicles, cystic atresia follicles increased, vacuoles-like changes, indicating severe ovarian edema, interstitial gland hyperplasia with Fibrosis, the number of luteal tissue decreased,

with flavinization, membrane cell proliferation was obvious; After drug intervention, secondary follicles and primary follicles appeared in PTH group, the number of atresia follicles decreased, granulosa cells were abundant, and corpus luteum increased follicle development; PTM group no obvious secondary follicles, fat hyperplasia, rich luteal body, interstitial hyperplasia; no follicles in the PTL group, small follicular cavity and granule cells falling off into follicular fluid, began to appear corpus luteum.

3.7Expression of PFN1 in ovaries .As shown in Figure 4, PFN1 protein was expressed in ovarian cytoplasm of each group, and the nucleus was expressed in a small amount. The ovarian granulosa cells, interstitial and non-expression or low expression in corpus luteum were expressed. PFN1 protein was mainly expressed in ovarian cytoplasm of group C. A small amount of expression in the corpus luteum and corpus luteum; high expression in the cytoplasm, corpus luteum and interstitial of the rat in the POF group; the expression of PFN1 protein in the ovary of each group showed a different degree of decline after drug intervention. As shown in Table 8 and Figure 4, the expression of PFN1 protein in the ovary of the POF group was increased by 142.2% compared with the C group, and the difference was statistically significant (P<0.05); PTH group, PTM group, PTL The expression of PFN1 protein in the ovary of the rats was decreased by 35.12%, 18.75%, and 10.8%, respectively. The difference between the PTH group and the POF group was statistically significant (P<0.05). The PTL group and the PTM group were compared with the PTM group and the PTM group. There was no significant difference between the POF groups (P > 0.05).

3.8 Expression of PFN1 mRNA in ovary of each group. As shown in Figure 5， We initially verified the up-regulation of PFN1 gene expression by RT-PCR. The results showed that the difference was statistically significant. The abscissa in the dissolution curve represents the number of cycles, that is, the number of cycles experienced by each sample's fluorescence intensity reaching the set threshold, and the ordinate represents the fluorescence signal intensity. It can be seen that the reaction conditions of all samples are stable and reliable. From the amplification curve, the abscissa represents temperature and the ordinate represents the change in fluorescence intensity. It can be seen that all the results are single peaks, no peaks are found, and the amplification products are single; indicating that the PCR results are specific. The results are expressed by $2^{-\Delta\Delta Ct}$, the mRNA expression of PFN1 is shown in Table 9 and Figure 6; the dissolution curve and amplification curve are shown in Figure 6;

3.9 Effect of drugs on PFN1 protein in rat ovary. As shown in Figure 7-8, the expression of PFN1 protein in ovarian tissue of each group was significantly higher than that in the normal group (P<0.01). Compared with the model group,

the high-dose group significantly reduced PFN1. Protein expression ($P < 0.01$), the middle and low dose groups reduced the expression of PFN1 protein ($P < 0.05$).

4. Discussions

Stress is a non-specific reaction of the body to stressors when the homeostasis of the environment is threatened. Modern society is full of various stressors (Ye, Q., & Li, L. 2015). Women are taking more and more responsibility and pressure in society, and the body is often in a state of stress. Clinical studies have shown that women with excessively high sensory pressure have decreased levels of luteinizing hormone (LH), elevated levels of follicle stimulating hormone (FSH), decreased serum estradiol（E2） and progesterone levels, and increased ovulation cycle (Kudielka, B. M., & Wust, S. 2009). Low positive emotions can accelerate the consumption of female ovarian reserve, and chronic psychosocial stressors are independent factors related to serum FSH levels, and chronic stress is a predictor of ovarian reserve decline. Stress-induced ovarian dysfunction is characterized by dysfunction of the dominant ovarian follicles and synthetic steroid hormones. The body's response to stressors is mainly through the hypothalamic-pituitary-adrenal axis (HPA axis)-mediated adrenal glucocorticoid (GC) release and sympathetic-adrenergic system initiation response mechanisms, which regulate the hypothalamus of the female reproductive system. The pituitary-ovarian axis (HPO axis) interacts at multiple points. In recent years, with the deepening of research, the mechanism of the influence of stress on ovarian function in the hypothalamus, pituitary, and especially ovarian levels has been further revealed (Gupta, D., & Morley, J. E. 2014; Gangadharan, A., & Blair, J. 2017).

Fibrin 1 (PFN-1) also known as a cytoskeletal protein binding protein 1 is also a member of ABP (Fan, Y., & Fox, P. L. 2012; Tsujiura, & Gogoi, R. (2014).). As a member of the profibrin family, the molecular weight is about 12.15 kDa and the gene is located at 17p13.3. It is ubiquitous in eukaryotic cells and is involved in the regulation of actin skeleton remodeling, cell morphology maintenance, cell adhesion, movement, growth, division, and its ligand-dependent signal transduction providing new therapeutic ideas for clinically improving stress-induced ovarian dysfunction. The biological characterization, hormone levels and immunohistochemistry results of the rats in each group showed that the expression of PFN-1 was significantly increased by long-term chronic stress stimulation induced ovarian endothelial cell dysfunction. Further studies found that overexpression of PFN-1 promoted. Apoptosis of ovarian endothelial cells, up-regulation of intercellular adhesion molecules and vasodilators stimulate the

phosphorylation of phosphoprotein (Ding, Z.,& Roy, P. 2009; Gau, D.,& Roy, P. 2014; Turco, E. G., & Cedenho, A. P. 2013).These findings suggest that under the action of various stimulating factors, abnormally expressed PFN-1 may affect the synthesis of nitric oxide in ovarian endothelial cells by promoting ovarian cytoskeletal rearrangement, increasing stress fibers, and activating inflammation-related signaling pathways (Skare, P., & Karlsson, R. 2002; Sathish, K., & Singh, S. S. 2004). And utilization of other methods to mediate endothelial cell dysfunction. Studies have shown that PFN-1 can also be released as extracellular cytokines, and extracellular PFN-1 may promote extracellular regulation of protein kinase 1/2, ribosomal protein S6 kinase, and phosphatidylinositol 3-kinase phosphorylation stimulates proliferation and migration of ovarian smooth muscle cells, thereby promoting the progression of ovarian dysfunction, and plasma PFN-1 levels are closely related to the severity of ovarian hormone levels , PFN-1 in plasma of POF group the level of 1 was significantly increased relative to the C group, but the plasma level of PFN-1 was decreased in the drug-administered group compared with the POF group, and the high-dose administration group decreased the most (Ding, Z., & Roy, P. 2009; Bae, Y. H., & Roy, P. 2009). However, the mechanism by which extracellular PFN-1-1 activates intracellular signal transduction pathways remains unclear in terms of how PFN-1 is released extracellularly and whether PFN-1 membrane receptors are present, and studies suggest that PFN-1 may pass exosomes are released extracellularly (Bates, D. O., & Pocock, T. M. 2002; Gau, D., Veon, & Roy, P. 2017). Excessive infiltration, activated macrophages and other immune cells, abnormally produced cellular inflammatory factors and chemokines are one of the characteristics of chronic low-level inflammation. Further studies have found that elevated expression of PFN-1 can lead to ovarian blood vessels. Smooth muscle cells themselves and nuclear nucleus increase, stress fibers increase , activate signaling pathways associated with cell hypertrophy, such as protein kinases downstream of the mitogen-activated protein kinase (MAPK) signaling pathway, such as cells extracellular regulation of protein kinase 1/2, c-Jun N-terminal kinase, increased phosphorylation of p38 MAPK and increased activity of Rho kinase/Rho-associated spiral coil-forming protein kinase signaling pathways closely related to cytoskeletal regulation (Ding, Z.,& Roy, P. 2006). PFN-1A and fibrosis caused by various causes and ovarian cell apoptosis are important pathological processes in the occurrence and development of ovarian dysfunction. Existing studies suggest that PFN-1 may mediate ovarian vascular proliferation, fibrosis, and vascular migration by several pathways (Schoenwaelder, S. M., & Burridge, K. 1999): Direct activation of cellular hypertrophy-related signaling pathways, such as increased expression of PFN-1, is closely related to cell hypertrophy phosphorylation of extracellular regulated protein kinase 1/2, p38 MAPK is significantly increased in the MAPK pathway ;

through its domain binds to and interacts with target proteins, such as by phosphatidylinositol diphosphate, transcription binding of protein molecules such as factor p42POP affects the transduction of related signaling pathways in cardiomyocytes, leading to ovarian cell remodeling; by regulating cytoskeleton-mediated ovarian vascular hypertrophy, PFN-1 expression may increase it directly leads to sarcoplasmic reticulum elongation, ovarian fiber rearrangement and contraction stress increase, and further activates mechanical stress-related signaling pathways, thereby affecting ovarian physiological function. In addition, the integrity of the actin cytoskeleton plays an important role in the production of reactive oxygen species and activation of signaling pathways (Yancopoulos, G. D., & Holash, J. 2000; Cascone, I., & Bussolino, F. 2003; Broi, M. G., & Navarro, P. A. 2018). It was found that PFN-1 may cause cytoskeletal remodeling by binding to actin as a key maintenance. Activation of ROS and activation of the Rho kinase subtype A/Rho-associated helical coil-forming protein kinase pathway mediate ovarian cell hypertrophy, apoptosis, and fibrosis induced by advanced glycation end products (Weintraub, N. L., & Kader, K. N. 2006).

The results showed that the high-dose POF drug intervention significantly increased the expression of PFN-1 in rat plasma and in rat ovarian tissue after intervention with traditional Chinese medicine Munziqi. It is suggested that the occurrence of POF may affect the formation of cell membranes in each cell as the expression of PFN-1 protein decreases, which further affects cell development and thus affects follicle production. Causes disturbance of the levels of related hormones (FSH, LH, E2, P, etc.) and affects ovarian function. The Munziqi may effectively improve the expression of PFN-1, thereby effectively improving the mature development of follicles in the ovary and improving the function of ovary.

Although PFN-1 has been an important ligand for actin for many years, the understanding of PFN-1 is still bud, the concentration of PFN-1 in cells is regulated and how to precisely regulate the cytoskeleton. Dynamically changing, how does PFN-1 act as a hub of signaling pathways to regulate signal transduction in the cytoplasm and nucleus, how is PFN-1 released as a stimulator outside the cell, and how extracellular PFN-1 is the specific mechanisms of secretion, autocrine, or transport of themselves, neighboring cells and distant cells with blood flow are to be studied in depth.

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Competing interests

All authors declare that there is no conflict of interests regarding the publication of this paper.

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Table 2 Changes of body weight in drug treatment period（$\bar{x} \pm s$）

Groups（N）	D 0(g)	D 4(g)	D 8(g)	D 12(g)	D 14(g)
C（10）	366.90±28.13	365.58±34.09	367.70±32.35	368.23±32.00	370.45±33.99
POF（10）	362.40±30.34	365.50±24.15	359.80±22.42	355.40±22.67	357.80±22.78
PTH（11）	356.00±22.49	358.00±26.39	359.91±23.29	364.73±26.94$^{\#}$	363.64±30.48
PTM（10）	358.73±32.57	362.09±32.72	364.27±35.43	364.00±35.77	361.09±34.98
PTL（11）	360.91±29.68	361.90±26.65	365.09±27.56	364.82±26.31$^{\#}$	366.09±27.75
F	0.858	1.066	1.245	1.227	0.995
P	0.555	0.395	0.284	0.294	0.447

Note：Compared with group C, **P*<0.05; compared with POF group, $^{\#}$*P*<0.05

Table 3 Changes of water intake in drug treatment period（$\overline{x} \pm s$）

Groups（N）	D 0(mL)	D 4(mL)	D 8(mL)	D 12(mL)	D 14(mL)
C（10）	38.51±3.75	40.00±3.82	36.00±3.20	46.00±4.05	42.00±3.90
POF（10）	34.81±2.04*	33.00±2.25*	26.00±1.64*	35.00±2.28*	31.00±1.97*
PTH（11）	29.35±2.48#	29.09±2.16#	28.64±1.90#	34.55±2.60	35.73±2.74
PTM（10）	30.10±3.10#	28.18±2.53#	27.80±2.77	36.14±3.69	34.16±3.49#
PTL（11）	29.00±2.33	31.81±2.31	28.47±2.02#	35.66±2.46	34.03±2.35#
F	20.410	22.358	18.980	12.607	12.533
P	0.000	0.000	0.000	0.000	0.000

Note：Compared with group C, *P<0.05; compared with POF group, $^{\#}P$<0.05

Table 4 Changes of food intake in drug treatment period（$\overline{x} \pm s$）

Groups（N）	D 0(g)	D 4(g)	D 8(g)	D 12(g)	D 14(g)
C（10）	21.19±2.03	18.50±1.65	20.40±1.96	22.20±1.95	21.50±2.00
POF（10）	35.39±2.41*	35.00±2.21*	31.10±2.08*	33.80±2.20*	32.00±2.04*
PTH（11）	26.63±1.98#	23.91±1.59#	19.55±1.59#	20.36±1.53#	23.55±1.98#
PTM（10）	24.72±2.24#	22.05±2.16#	21.41±2.13#	21.33±2.08#	24.23±2.33#
PTL（11）	29.36±2.13#	25.30±2.82#	25.86±2.02#	24.78±1.71#	25.66±2.01#

F	51.298	61.938	55.635	63.425	46.597
P	0.000	0.000	0.000	0.000	0.000

Note：Compared with group C, *P<0.05; compared with POF group, $^{\#}P$<0.05

Table 5 Changes of urine volume in drug treatment period（$\overline{x} \pm s$）

Groups（N）	D 0(mL)	D 4(mL)	D 8(mL)	D 12(mL)	D 14(mL)
C（10）	10.45±2.43	10.20±1.57	11.08±1.06	11.29±1.28	10.92±0.76
POF（10）	14.08±0.79*	13.28±2.31*	14.15±0.53*	14.01±1.07*	14.95±1.54*
PTH（11）	12.50±1.56#	12.06±1.47	11.45±1.34#	12.01±0.85#	13.07±1.08#
PTM（10）	12.28±1.31#	12.46±0.86	12.04±0.67#	12.28±1.05#	12.93±1.05#
PTL（11）	13.08±1.34	13.06±0.95	12.98±1.25#	12.42±2.18#	12.85±0.58#
F	3.828	3.871	7.808	4.686	9.181
P	0.001	0.001	0.000	0.001	0.000

Note：Compared with group C, *P<0.05; compared with POF group, $^{\#}P$<0.05

Table 6 Changes of stool quantity in drug treatment period（$\overline{x} \pm s$）

Groups（N）	D 0(g)	D 4(g)	D 8(g)	D 12(g)	D 14(g)
C（10）	9.83±0.85	9.90±0.78	10.02±0.81	9.93±0.89	10.14±0.92
POF（10）	13.20±1.25*	12.99±1.38*	13.15±1.03*	14.18±1.45*	13.69±1.19*
PTH（11）	11.95±1.03#	11.93±1.62#	12.05±0.83#	11.98±0.96#	12.03±1.52#
PTM（10）	12.01±1.34#	12.02±1.09	11.98±1.32#	12.36±1.23#	12.99±0.98
PTL（11）	11.20±0.98#	11.25±1.17#	11.84±1.07#	12.50±1.05#	12.87±0.83
F	8.679	6.748	7.091	9.645	7.918
P	0.000	0.001	0.001	0.000	0.000

Note：Compared with group C, *P<0.05; compared with POF group, $^{\#}P$<0.05

Table 7 Changes of the hormone level change rate of rats in each group ($\overline{x} \pm s$)

Groups	E2(pg/mL)	FSH(mlU/mL)	LH(mlU/mL)
C	49.17±5.87	2.34±0.67	3.72±1.13
POF	21.09±7.80*	8.57±0.89*	8.84±1.51*
PTH	38.81±6.11#	4.14±0.35#	4.75±0.45#
PTM	33.97±5.56#	4.19±0.59#	5.45±0.87#
PTL	31.97±4.63#	5.14±0.76#	5.72±1.17#

Note：Compared with group C, *P<0.05; compared with POF group, $^{\#}P$<0.05

Table 8 Changes of serum VEGF and PFN1 of rats in each group ($\bar{x} \pm s$)

Group	N	VEGF（Pg/mL）	PFN1/ (ng/mL)
C	7	264.48±24.94	1.75±0.22
POF	8	165.57±18.58*	3.91±0.25
PTH	10	191.21±29.25$^{\#}$	2.76±0.29
PTM	9	187.57±22.34	3.74±0.23
PTL	9	171.94±19.74	3.83±0.13
F		8.698	166.796
P		0.000	0.015

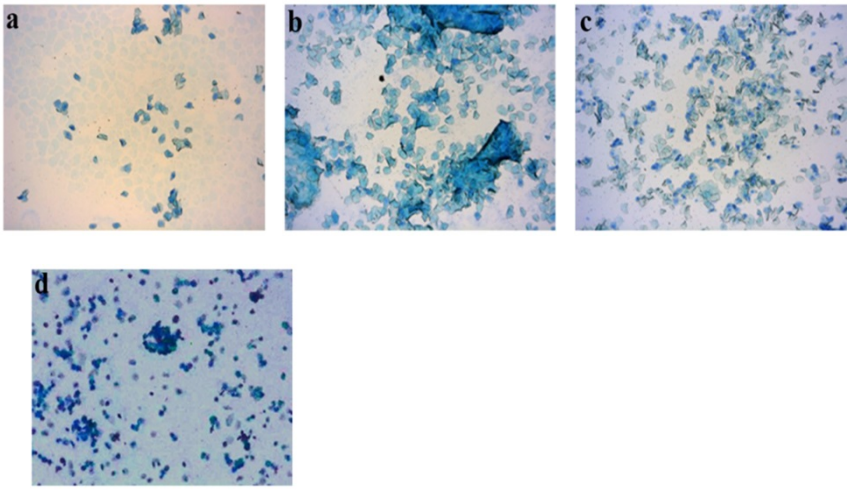
Note：Compared with group C, *P<0.05; compared with POF group, $^{\#}P$<0.05

Tab.9 Expression of PFN1 in ovarian tissue of rats in each group($\bar{x} \pm s$)

Groups	N	Expression of PFN1	F value	P value
C	10	3.86±0.56	76.266	0.000
POF	10	9.31±0.61*		

PTH	10	6.89±0.32#
PTM	9	7.84±0.24#
PTL	11	8.40±0.13

Note: Comparison with group C, *P<0.05; Comparison with group POF, $^{\#}P$<0.05;



Note： a: pre-estrus; b: estrus; c: late estrus; d: estrus

Figure 1: Changes of estrus cycle of rats （×100）

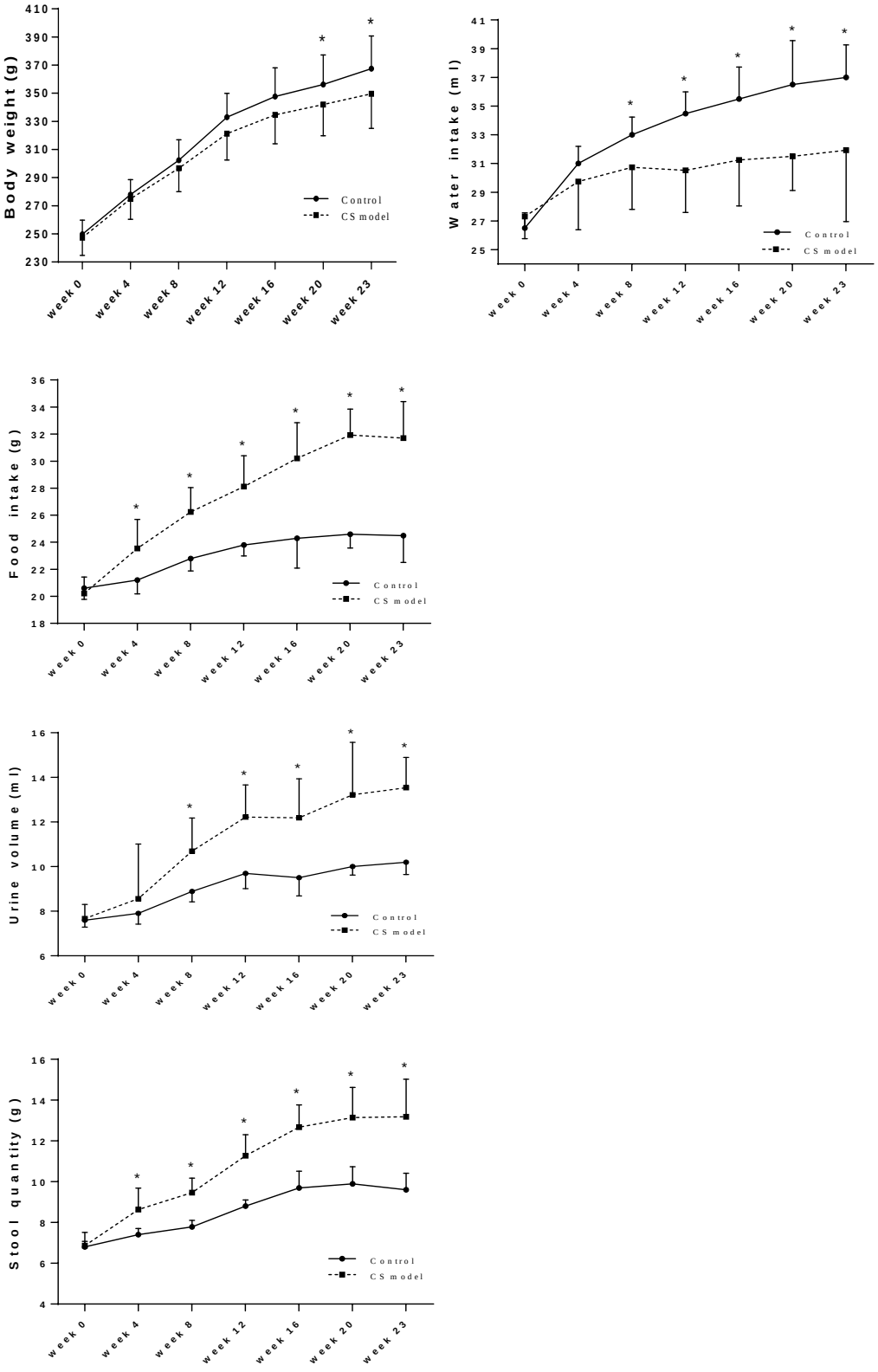


Figure 2: Changes of biological characterizations, body weight (a), food intake (b), water intake (c), urine volume (d), and Stool quantity (e) during modeling period. Asterisks show significant differences when APS model group was compared with control group in different time period (*$P < 0.05$; mean ± SD).

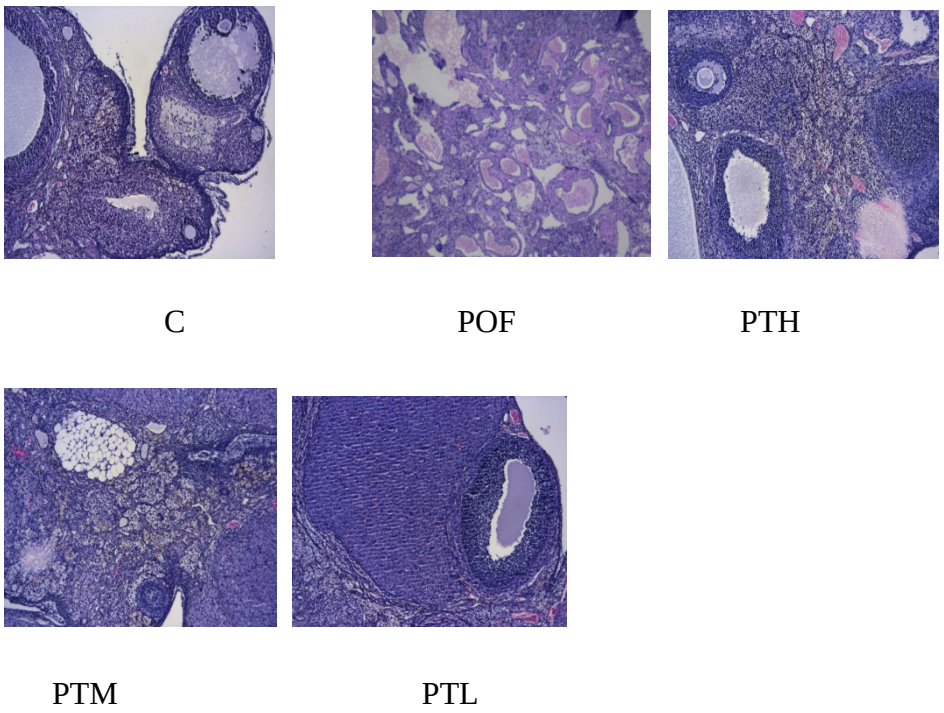


Figure 3: H&E staining of ovaries of each group rats (×100)

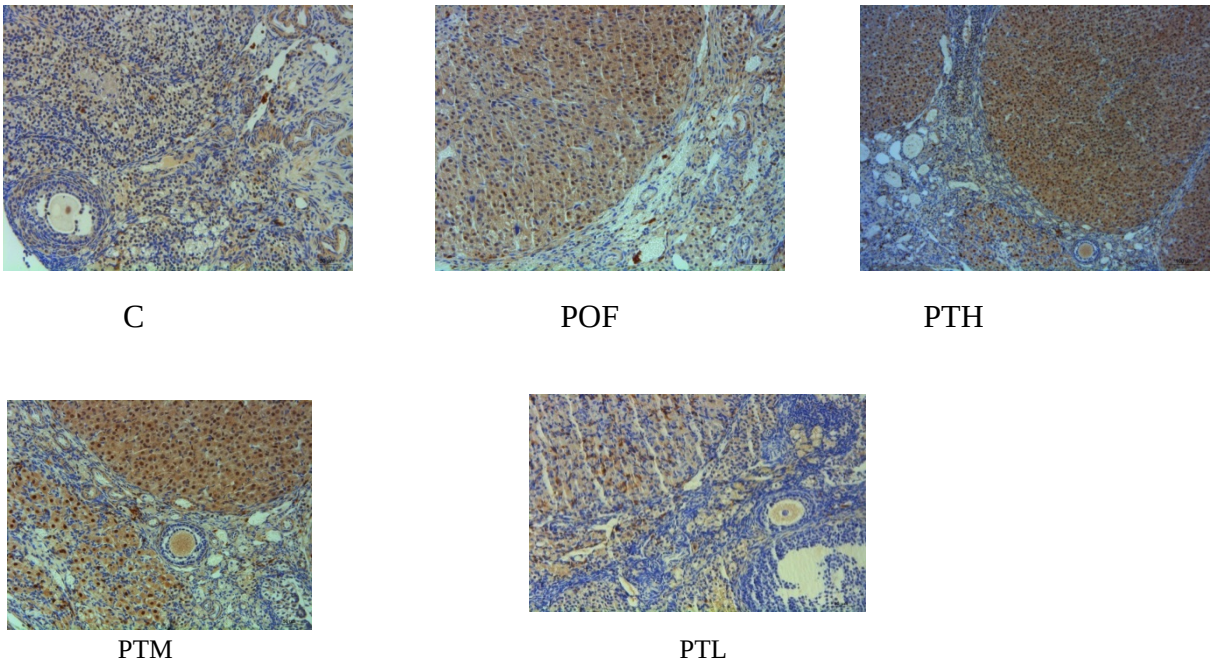


Figure 4: Expression of PFN1 in ovarian tissue of rats in each group（200×）

Note： normal group（group C）；POF disease group(POF)；high doses of BMq treatment group(PTH)；middle doses of BMq treatment group(PTM)；low doses of BMq treatment group(PTL)；

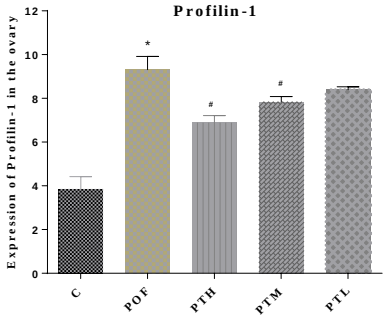
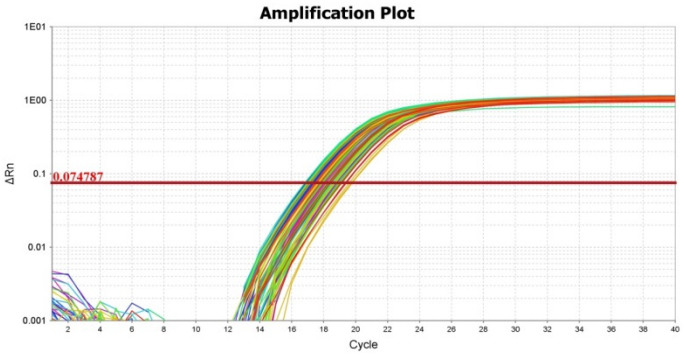
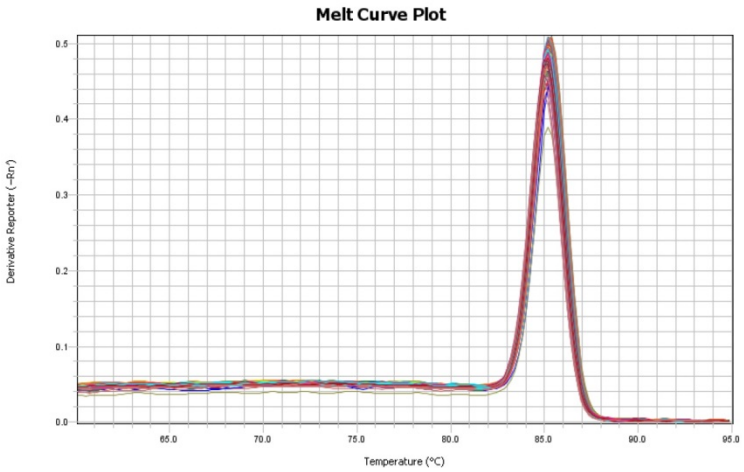


Figure 5: Expression of PFN1 in ovarian tissue of rats in each group



a1



a2

Figure 6: Melt curve andamplification curve of samples

Note：Figure 6 Real-time PCR melt curve and amplification curve. a1 and a2 represent the amplification curve and dissolution curve of PFN1 mRNA respectively.

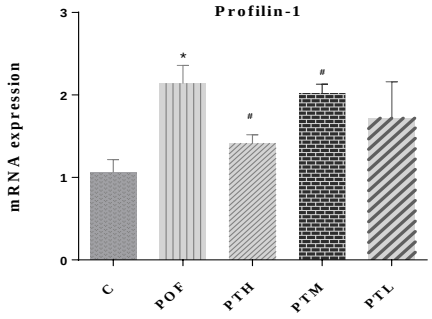


Figure 7: mRNA expression levels in ovary of each group of rats

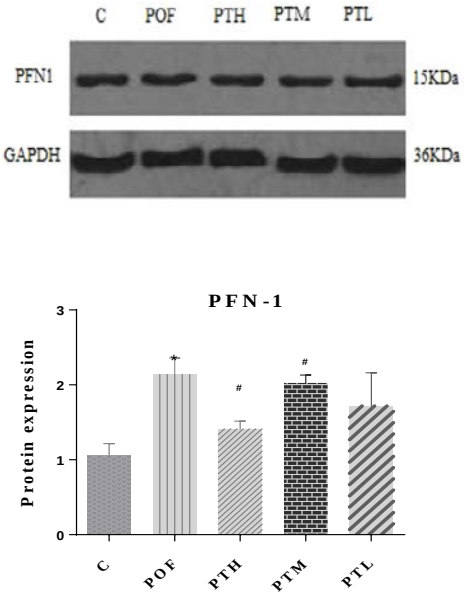


Figure 8: Expression of PFN1 protein in ovary of each group of rats