

Original Article

Effects of fallopian tube recuperation soup on JAK2/STAT3 pathways in rats with tubal infertility

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Abstract: Objective: The aim of this study was to investigate the effects of fallopian tube recuperation soup on JAK2/STAT3 pathways in rats with salpingitis-induced infertility. Methods: Salpingitis-induced infertility models were established in 30 female rats (10 rats were retained as a control group). After successful establishment of models, rats were divided into three groups, including a fallopian tube recuperation soup group, model group, and normal group (model-free control group). Tubal specimens were collected after treatment. Changes in expression levels of JAK2 and STAT3 mRNA in oviduct tissues were detected by RT-PCR. Expression levels of JAK2, STAT3, phosphorylated JAK2 (P-JAK2), and P-STAT3 proteins in oviduct tissue were detected by Western blotting. Downstream TNF- α expression in JAK2/STAT3 signaling pathways in oviduct tissues was detected by ELISA. Results: RT-qPCR showed that expression levels of JAK2 and STAT3 mRNA in the fallopian tube recuperation soup group were significantly lower than those in the model group ($P < 0.05$). Expression levels of JAK2, STAT3, P-JAK2, and P-STAT3 proteins in oviduct tissues were significantly lower than those in the model group ($P < 0.05$). In addition, expression levels of TNF- α , detected by ELISA, in the fallopian tube recuperation soup group were significantly lower than the model group ($P < 0.05$), but not significantly different from the normal group ($P > 0.05$). Conclusion: Fallopian tube recuperation soup significantly inhibits JAK2/STAT3 pathways and regulates downstream TNF- α expression levels.

Keywords: JAK2, STAT3, TNF- α , tubal infertility, animal models

Introduction

Barrenness is generally defined as the absence of a live birth for men and women desiring a child and having been in union for more than 2 years, without the use of contraceptives. Primary infertility is failure to conceive without a previous pregnancy. Secondary infertility is failure to conceive following a previous pregnancy. According to the WHO, barrenness is defined as the absence of a live birth for 12 months [1]. Studies have shown that more than 50% of infertile couples can be attributed to female sterility, with 40% of female sterility caused by tubal factors [2]. With increasing environmental pollution and various sexually transmitted diseases in recent years, more and more women are suffering from gynecological diseases, such as pelvic inflammatory disease,

leading to occurrence of infertility. Of these gynecological diseases, salpingitis is the most common [3]. Salpingitis can cause tubal obstruction, structural abnormalities, non-specific inflammation, endometriosis, and other diseases. These diseases are the main causes of salpingitis-induced infertility, causing great impact on the reproductive health of women. Salpingitis-induced infertility has become a global reproductive health issue [4].

In terms of treatment, some scholars have proposed that hydrotubation and vaginal punctuation should be performed, under the guidance of ultrasound, to extract hydrops and promote tubal patency. Subsequently, obstruction and adhesions should be surgically separated and drugs for anti-inflammatory treatment should be administered [5]. However, another study [6]

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showed that laparoscopic surgery was more effective in the treatment of tubal hydrops. At present, laparoscopic surgery is divided into two types. The first type achieves tubal patency through plastic repair for natural pregnancy or for embryo transfer. The second type treats infertility through embryo transfer after ligation or excision of tubal adhesions and obstruction [7]. However, another study [8] found that the process of removing tubal lesions has a certain impact on prognosis. Some scholars [9] have proposed that pelvic adhesions and tubal hydrops have no impact on pregnancy results and were only related to tubal wall thickness, leading to differences in treatment of infertility at home and abroad.

According to Traditional Chinese Medicine [10], salpingitis-induced infertility is mainly caused by stasis of uterine vessels and accumulation of 'damp toxins'. In addition, Traditional Chinese Medicine has some advantages in the treatment of salpingitis-induced infertility. JAK/STAT signaling pathways are involved in immunity, proliferation, differentiation, apoptosis, and oncogenesis. The JAK/STAT signaling cascade consists of three main components: a cell surface receptor, a Janus kinase (JAK), and two Signal Transducer and Activator of Transcription (STAT) proteins [11]. Cheng et al. revealed that both EGF and LIF promote embryonic development through JAK/STAT3 signaling pathways [12]. However, the effects of fallopian tube recuperation soup on JAK2/STAT3 pathways remain unknown.

In this present study, the effects of fallopian tube recuperation soup on JAK2/STAT3 pathways in animal models of salpingitis-induced infertility were investigated.

Materials and methods

Animal sources

Thirty female specific-pathogen-free (SPF) Sprague Dawley (SD) rats were included in this study. They were healthy, sexually mature, and unfertilized by quarantine for one week; age: 8-10 weeks; weight: 250 ± 50 g. All SPF SD rats were purchased from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences. According to a random number table, rats were divided into fallopian tube recuperation soup group, model group, and normal

group (model-free group). All rats were fed in the Laboratory Animal Center of the hospital. They were free to drink water and ingest food before modeling. Day-night environmental lighting was switched every 12 hours (room temperature of animal house: 22~25C, humidity: 30-70%).

Test drugs

Fallopian tube recuperation soup was provided by the pharmacy. The composition was as follows: Angelica sinensis 15 g, rhizome of Rehmannia 15 g, Semen Persicae 15 g, Carthamus tinctorius 10 g, Radix Paeoniae Rubra 10 g, Radix Bupleuri 10 g, Ligusticum wallichii 10 g, Liquorice 6 g, Manis pentadactyla 9 g, Beautiful Sweetgum Fruit 15 g, Towel Gourd Vegetable Sponge 10 g, Chinese Honey Locust Spine 10 g, Szechwan Chinaberry Fruit 10 g, and Caulis spatholobi 15 g.

Main test reagents and strains

Escherichia coli, *Staphylococcus aureus*, and *Streptococcus* were purchased from Shanghai Kang Lang Biological Technology Co., Ltd. PBS buffer, RNA extraction reagent TRIzol, and RT-PCR kit were purchased from Invitrogen (USA). Reverse transcription kit and TaqMan miRNA kit were purchased from Applied Biosystems. RIPA reagent, rabbit anti-STAT3 antibody, rabbit anti-JAK2 antibody, phospho-STAT3, p-STAT3 antibody, phospho-JAK2, and p-JAK2 antibody were purchased from Biosystems. ELISA kit (TNF- α) was purchased from Beyotime Institute of Biotechnology.

Animal model establishment

Salpingitis-induced infertility models were established according to the "Mixed bacteria inoculation method" [13]. To anesthetize, 10% chloral hydrate (30 mg/100 g) was injected into the peritoneal cavity of rats. After the anesthetic took effect, a fine cotton thread was used to extend and fix limbs on the workbench. Hair on the abdomen was removed, the skin cleared, and iodine complex solution was used for disinfection. An incision was made, was approximately 0.8-1.0 cm long, in the middle of the lower abdomen and inspection for uterus bicornis in the abdomen was made, exposing the bilateral fallopian tubes. *E. coli*, *S. aureus*, and *Streptococcus* diluted in sterile saline, at a

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Table 1. Primer sequences

Gene	Primer	Sequence
β -actin	Upstream primer	5'-CTCACCATGGATGATGATATCGC-3'
	Downstream primer	5'-AGGAATCCTTCTGACCCATGC-3'
JAK2	Upstream primer	5'-CAGGGATCTGGCAACGAGA-3'
	Downstream primer	5'-CGCATAAATCCGCTGGTGG-3'
STAT3	Upstream primer	5'-GGAGAAACAGGATGGCCCAA3'
	Downstream primer	5'-ATCCAAGGGCCAGAAACTG-3'

ratio of 2:1:1, were used to prepare a mixture (3×10^9 cells/L). Then, 0.1 mL of the mixture was injected by syringe into bilateral fallopian tubes via the cornua uteri near the fallopian tubes and along the direction of fallopian tubes toward the ovaries. Abdominal incisions were sutured and dressings were appropriately applied. Rats were then returned to the Laboratory Animal Center for feeding and management.

Administration method

Animal in each group were subjected to washing, two times every day, continuing for 30 days. Gastric lavage dosage of 1 kg mice = Mg/60 kg \times 9 (M refers to dose of Chinese Medicine and 60 kg for adult standard weight). Rats in the fallopian tube recuperation soup group were washed with fallopian tube recuperation soup while rats in the model and normal groups were washed with an equivalent dose of distilled water (1.5 mL/100 g). Body weight and food and water intake of the animals were observed and measured every two weeks.

Tissue extraction

Rats were fasted and anesthetized 30 days after lavage. After anesthetics took effect, the rats were rapidly dissected. Fallopian tubes were searched, the surrounding ovarian tissues were peeled off, and bilateral fallopian tubes were removed and placed in a -80°C freezer.

qRT-PCR

Total RNA was extracted and reverse transcribed using TRIzol Reagent (Invitrogen; Shanghai, China). cDNA synthesis was performed, in strict accordance with instructions for use of the Prime Script RT Kit (Dalian, People's Republic of China; Takara Biotechnology). For JAK2 and STAT3 mRNA primers, GenBank was used to search the full-length

sequence of target gene mRNA. Primer and probe design software Primer Express was used to design primer sequence (Table 1). qRT-PCR was carried out on ABI Prism 7900HT Real-Time System (Applied Biosystems Inc; Shanghai, China). A control group, without reverse transcription, was included to exclude genomic DNA contamination. PCR system: Maxima SYBR Green/ROX qPCR Master Mix (2 x) (10 μ l),

PCR Forward Primer (1 μ l), PCR Reverse Primer (1 μ l), and template DNA (\leq 500 ng). Nuclease-free water was adjusted to a total volume of 20 μ l and mixed evenly. PCR conditions: pre-denaturation at 95°C for 10 minutes, at 95°C for 45 seconds, at 60°C for 30 seconds, and at 60°C for 30 seconds, with 40 cycles in total. Results are presented as Ct relative quantification of the target transcripts, using the $\Delta\Delta\text{Ct}$ method to evaluate associated alterations in expression. β -Actin served as the internal reference gene.

Expression of JAK2, P-JAK2, STAT3, and P-STAT3 proteins in rat oviduct tissues detected by western blotting

Western blotting was carried out, as described previously [14]. Briefly, oviduct tissues were lysed in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, and 0.1% SDS; pH 8.0) supplemented with a protease inhibitor cocktail (Roche). Protein concentrations were determined with BCA Protein Quantitation Kit (Genscript). Proteins were separated using 10% SDS-PAGE and blotted electrophoretically onto polyvinylidene difluoride (Immobilon) membranes with 0.45 μ m pore size. Membranes were blocked with 5% bovine serum albumin (BSA) in phosphate-buffered saline containing 0.1% Tween-20 (PBST) for 1 hour at room temperature was followed by incubation with primary antibodies against JAK2, STAT3, p-STAT3, p-JAK2, and β -actin (Santa Cruz Biotechnology, TX, USA) overnight at 4°C . Horseradish peroxidase-linked secondary antibodies (Amersham) were incubated with the membranes for 1 hour at $25 (\pm 2)^\circ\text{C}$ in PBST containing 5% BSA, followed by chemiluminescent detection.

ELISA

TNF- α levels in oviduct of rats were measured using rat-specific ELISA kits (Abcam, Cambridge, MA, USA), according to the instruction manual.

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Table 2. Expression of *JAK2* *STAT3* mRNA in oviduct tissues of animal models

Group	<i>JAK2</i> mRNA	F	P	<i>STAT3</i> mRNA	F	P
Fallopian Tube Recuperation Soup (n = 10)	1.05 ± 0.33	35.80	0.01	1.09 ± 0.11	80.06	0.01
Model group (n = 10)	3.42 ± 1.21			2.14 ± 0.35		
Normal group (n = 10)	1.01 ± 0.15			1.01 ± 0.12		

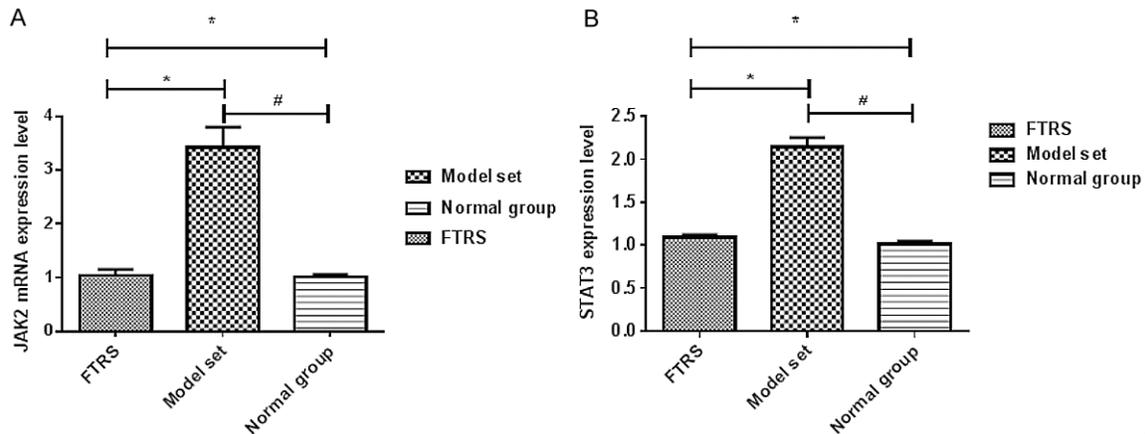


Figure 1. MicroRNA expression levels of *JAK2* and *STAT3*. A. *JAK2* mRNA expression levels in oviduct tissues of rats: RT-qPCR results showed that *JAK2* mRNA expression levels in fallopian tube recuperation soup were significantly lower than those in the model group ($t = 5.97$, $P < 0.01$). *JAK2* mRNA expression levels in the model group were significantly higher than in the normal group ($t = 6.25$, $P < 0.01$). B. *STAT3* mRNA expression levels in oviduct tissues of rats: RT-qPCR results showed that *STAT3* mRNA expression levels in animals treated with fallopian tube recuperation soup were significantly lower than the model group ($t = 9.05$, $P < 0.01$). *STAT3* mRNA expression levels in the model group were significantly higher than in the normal group ($t = 9.66$, $P < 0.01$).

Samples of 100 μ l from oviducts of rats were added to the microtiter wells and blocked with 0.5% bovine serum albumin (BSA) in phosphate buffered saline (PBS). After washing with PBS, the mAb (1:500) was added and plates were incubated at 37°C for 60 minutes. The wells were washed and then secondary antibody (goat anti-rabbit IgG-HRP, 1:1000) was added and incubated for 60 minutes at 37°C. After another washing with PBS, 100 μ l of substrate was added to the wells and incubated at room temperature for about 10 minutes. The absorbance of each well was read at 450 nm. Each sample was tested in triplicate.

Statistical analysis

SPSS 20.0 statistical package was used for statistical analysis of collected data. Results are presented as mean \pm standard deviation ($\bar{X} \pm s$). Means of several samples were compared using one-way ANOVA. Inter-group analysis of variance was performed using *F*-tests. Comparisons between groups with differences were performed with SNK-*q* tests. $P \leq 0.05$ was

considered statistically significant, $P \leq 0.01$ was considered extremely significant, and $P > 0.05$ was considered statistically insignificant.

Results

Modeling results

Histomorphological changes in fallopian tubes of 2 randomly selected rats were separately observed on days 7, 14, 21, and 28. Successful modeling was observed as peripheral tissue adhesion, edema, swelling and hydrops in fallopian tubes and exudation, adhesions, mucosal congestion, and hydrops or stenosis or even occlusion in dissected lumen by day 28.

Expression of *JAK2* and *STAT3* mRNA in oviduct tissues of animal models detected with RT-qPCR

RT-qPCR showed differences in expression of *JAK2* and *STAT3* mRNA in oviduct tissues of rats between the groups ($F = 27.96$, $P < 0.01$; $F = 64.88$, $P < 0.01$). In addition, comparisons

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Table 3. Relative expression of p-JAK2, JAK2, p-STAT3, and STAT3 proteins in oviduct tissues

Group	p-JAK2	JAK2	p-STAT3	STAT3
Fallopian Tube Recuperation Soup group (n = 10)	0.44 ± 0.12	0.40 ± 0.09	0.52 ± 0.14	0.36 ± 0.09
Model group (n = 10)	0.84 ± 0.12	0.74 ± 0.16	0.80 ± 0.11	0.73 ± 0.15
Normal group (n = 10)	0.42 ± 0.08	0.38 ± 0.07	0.49 ± 0.05	0.34 ± 0.03
F	47.84	31.81	25.64	45.94
P	0.01	0.01	0.01	0.01

showed that *JAK2* mRNA expression levels in rats in the fallopian tube recuperation soup group were significantly lower than those in the model group ($P < 0.01$), but not significantly different from that of the normal group without surgery ($P > 0.05$). *JAK2* mRNA expression levels in the model group were significantly higher than the normal group ($P < 0.01$). *STAT3* mRNA expression levels in the fallopian tube recuperation soup group were significantly lower than the model group ($P < 0.01$), but significantly higher than those in the normal group without surgery ($P > 0.05$). *STAT3* mRNA expression levels in the model group were significantly higher than the normal group ($t = 9.66$, $P < 0.01$); (Table 2 and Figure 1A, 1B).

Relative expression of p-JAK2, JAK2, p-STAT3, and STAT3 proteins in oviduct tissues of animal models

Laboratory tests of relative expression of p-JAK2, *JAK2*, p-STAT3, and *STAT3* proteins in oviduct tissues of animal models showed that expression levels of p-JAK2, *JAK2*, p-STAT3, and *STAT3* proteins in oviduct tissues in the model group were significantly higher than those in the normal group ($P < 0.01$). Expression levels of p-JAK2, *JAK2*, p-STAT3, and *STAT3* proteins in oviduct tissues in the fallopian tube recuperation soup group were not significantly different from those in the normal group ($P > 0.05$), but significantly lower than those in the model group ($P < 0.01$), (Table 3, Figure 2A-D).

Expression of TNF- α in each group

Expression of TNF- α in oviducts of rats in each group, as detected with ELISA, revealed that there were significant differences ($F = 184.64$, $P < 0.01$) in expression of TNF- α among animals in the normal, model, and fallopian tube recuperation soup groups ($F = 184.64$, $P < 0.01$). Expression of TNF- α in oviduct tissues in normal group animals was significantly lower

than the model group ($P < 0.01$) and slightly lower than the fallopian tube recuperation soup group ($P > 0.05$). There were significant differences in expression of TNF- α in oviduct tissues between the model and fallopian tube recuperation soup groups ($t = 17.08$, $P < 0.01$), (Table 4 and Figure 3).

Discussion

A common and complicated gynecological disease, infertility is a global medical social issue and an important disease affecting human reproductive health. Statistics have shown that global incidence of infertility has significantly increased in recent years, increasing year by year, followed by an increase in genetic immune diseases [15]. In Traditional Chinese Medicine theory [16], female infertility is mainly caused by obstructed Qi and blood. However, in modern medicine [17], female infertility is understood to be caused by tubal factors. One of the most important components of the female reproductive system, the oviduct is the main structure for sperm-egg binding and for transport of fertilized eggs to the uterine cavity. Studies have shown [18] that tubal lesions are mostly caused by pelvic inflammatory disease. Pelvic inflammatory disease may be caused by many factors (non-sterile intrauterine operation, lack of knowledge of sexual health, and multiple abortions). Most patients ignore the symptoms and do not seek medical advice until occurrence of severe discomfort, leading to missed optimal timing of treatment. In particular, some patients have given up follow up treatment after relief of pain, resulting in failure of a radical cure of the disease.

Manis pentadactyla, in fallopian tube recuperation soup, plays a role in promoting blood circulation to remove blood stasis. *Salvia Miltiorrhiza*, *Angelica sinensis*, and *Panax Notoginseng* play roles promoting blood circulation to remove meridian obstructions. Cooperation of the

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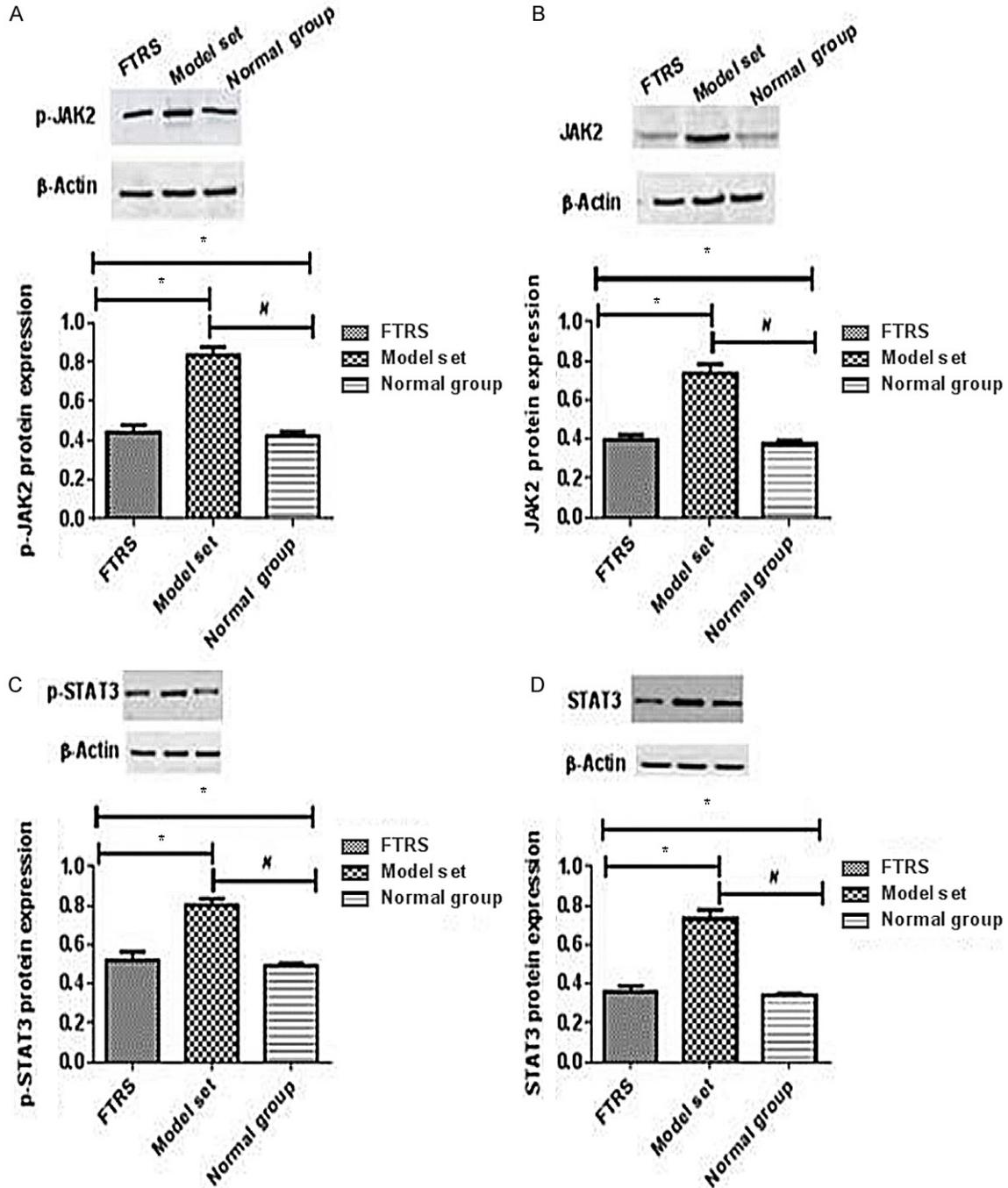


Figure 2. Protein expression levels of *JAK2* and *STAT3*. A. p-*JAK2* protein expression levels in oviduct tissues of rats. B. *JAK2* protein expression levels in oviduct tissue of rats. C. p-*STAT3* protein expression levels in oviduct tissues of rats. D. *STAT3* protein expression levels in oviduct tissues of rats.

ingredients contributes to clearing heat and promoting diuresis, stimulating blood circulation to remove blood stasis, and clearing and activating channels and collaterals [19]. This present study successfully established salpingitis-induced animal infertility models with a

mixed bacteria inoculation method, detecting relative expression of *JAK2*, *STAT3* mRNA, proteins, and $TNF-\alpha$ in rat oviduct tissues after drug treatment. It was found that $TNF-\alpha$ expression levels in rats in fallopian tube recuperation soup-treated animals were significantly lower

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Table 4. Expression of TNF- α in all groups

Group	TNF- α (ng/L)	F	P
Fallopian Tube Recuperation Soup (n = 10)	268.54 \pm 28.66	184.64	0.01
Model group (n = 10)	475.84 \pm 33.94		
Normal group (n = 10)	256.24 \pm 22.37		

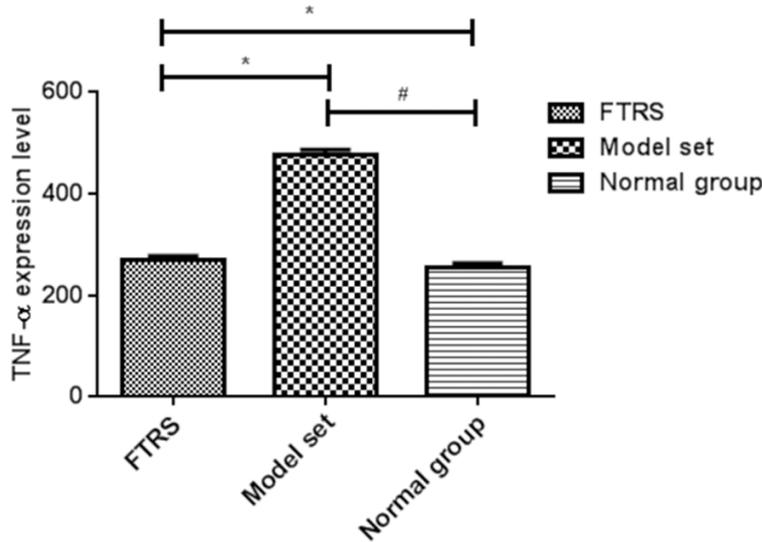


Figure 3. Expression of TNF- α in oviduct tissues of rats. Expression of TNF- α in oviduct tissues in the normal group was significantly lower than that in the model group ($t = 14.76$, $P < 0.01$). There was a significant difference in expression of TNF- α in oviduct tissue between the model group and the fallopian tube recuperation soup-treated group ($t = 17.08$, $P < 0.01$).

than those in the model group, but not significantly different from those in the normal group. As a peptide cytokine produced by monocytes-macrophages, TNF- α is an important physiological and immunological mediator with multiple biological characteristics. TNF- α is an immune protection mediator that also participates in immunopathological damage processes in the human body [20]. An earlier study [21] showed that TNF- α promoted normal ovulation and maintenance of the menstrual cycle and TNF- α overexpression was closely related to endometriosis and salpingitis-induced infertility. TNF- α overexpression indicates tubal obstruction and higher expression of TNF- α indicates more severe injury and obstruction, influencing pregnancy establishment [22]. After animal models were lavaged with fallopian tube recuperation soup, it was found that TNF- α expression was significantly reduced. TNF- α expression was not significantly different from that in the normal group, clearly demonstrating the positive effects of fallopian tube recuperation soup in

treatment of salpingitis-induced infertility.

In this study, expression levels of *JAK2*, *STAT3* mRNA and *JAK2*, p-*JAK2*, p-*STAT3*, and *STAT3* proteins in rats were detected. JAK/STAT is an important pathway of intracellular signal transduction [23]. JAKs are a class of cytosolic tyrosine protein kinases. JAK1, *JAK2*, *JAK3*, and *TYK2* are major members of the JAK family, except *JAK3* (outside of the lymphatic system and bone marrow only) which is widely expressed in the body [24]. STATs are proteins that can regulate and target genes in target cells. As the most important protein in the STAT family, *STAT3* regulates apoptosis and cell survival [25]. Many studies have shown that activation of JAK/STAT pathways is involved in regulation of inflammatory reactions, oxidative stress, and apoptosis [26]. A previous study revealed that [27] JAK/

STAT is directly involved in the regulation of inflammation, cell differentiation, and apoptosis. In addition, the TNF- α gene has a binding site at the *STAT3* locus [28]. After activation of *JAK2*, it activates downstream nuclear factor *STAT3*. Activated *STAT3* migrates to the nucleus to bind to the TNF- α gene promoter, leading to increased TNF- α expression. Detection of expression levels of *JAK2*, *STAT3* mRNA and *JAK2*, p-*JAK2*, p-*STAT3*, and *STAT3* proteins in rat oviduct tissues showed that expression levels of *JAK2* and *STAT3* mRNA in rats lavaged with fallopian tube recuperation soup were significantly lower than those in the model group, but not significantly different from the normal group. Western blotting indicated that expression levels of *JAK2*, p-*JAK2*, p-*STAT3*, and *STAT3* proteins in the fallopian tube recuperation soup group were significantly lower than the model group, but not significantly different from the normal group. The above results demonstrate the significant effects of fallopian tube recuperation soup in treatment of salpingitis-

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induced infertility and its inhibitory effects on JAK/STAT pathways.

However, there were some limitations to this study. This study was fundamental rather than clinical. It remains unclear whether fallopian tube recuperation soup could achieve the intended effects in clinical treatment. In addition, dosage of the drug should be appropriately adjusted, depending on conditions of individual patients. The timeline of this study was short. Longer periods may be required for traditional Chinese Medicine to take effect. Therefore, clinical studies should be conducted with increased sample sizes. Timely follow ups should be performed in the future to observe the effects of fallopian tube recuperation soup in treatment of salpingitis-induced infertility.

In summary, fallopian tube recuperation soup significantly inhibits *JAK2/STAT3* pathways and regulates downstream TNF- α expression.

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Disclosure of conflict of interest

None.

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