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EXPERIMENTAL STUDY

Effects of different Chinese herbal prescriptions on cytokines in autoimmune prostatitis rats

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Abstract

OBJECTIVE: To observe and compare the effects of Chinese herbal prescriptions for promoting blood circulation, clearing heat, removing toxicity, and dispersing stagnated liver-*Qi* on cytokines in model rats with experimental autoimmune prostatitis (EAP) to provide an experimental basis for the use of Chinese herbal prescriptions in the treatment of chronic prostatitis.

METHODS: One-hundred and ten male Wistar rats were randomly divided into 11 groups: blank group; model group; Huoxuehuayu (promoting blood circulation to remove blood stasis) high, middle, and low dose groups; Qingrejiedu (clearing heat and removing toxicity) high, middle, and low dose groups; and Shuganliqi (dispersing stagnated liver-*Qi*) high, middle, and low dose groups. Except the blank group, rats in all groups were injected subcutaneously in multiple points on days 0 and 30 with prostatic protein extractive solution (60 mg/mL), and intraperitoneally injected with diphtheria-pertussis and tetanus vaccine (DPT vaccine) to establish the EAP model. Model rats were administrated high, middle, and low doses of Chinese herbal prescriptions and were sacrificed after 4 weeks. Pathological changes in the prostate gland were observed with HE staining and changes in serum interleukin-6 (IL-6), interleukin-8 (IL-8), and prostaglandin E_2 (PGE_2) levels were detected with enzyme-linked immunosorbent assay.

RESULTS: Compared with the blank group, serum PGE_2, IL-6, and IL-8 levels in the model group were significantly higher ($P < 0.05$). Compared with the model group, serum PGE_2, IL-6, and IL-8 levels in the Qingrejiedu low dose and middle dose groups were significantly lower ($P < 0.05$), with the lower dose having a more obvious effect. Serum PGE_2, IL-6, and IL-8 levels in the Huoxuehuayu high dose group ($P < 0.05$), IL-6 and IL-8 levels in the Huoxuehuayu middle dose group ($P < 0.05$), and the IL-8 level in the Huoxuehuayu low dose group were sig-

nificantly lower than those in the model group ($P < 0.05$). There were significant differences in PGE_2 and IL-6 levels among the different dose groups of Shuganliqi drugs ($P < 0.05$). Compared with the model group, serum PGE_2, IL-6, and IL-8 levels in the Shuganliqi high dose group ($P < 0.05$) and IL-8 level in the Shuganliqi low dose group were significantly lower ($P < 0.05$), while the Shuganliqi middle dose group did not change significantly ($P > 0.05$).

CONCLUSION: Therefore, in TCM treatment of autoimmune prostatitis, different treatment methods should select different doses. For prescriptions that clear heat and remove toxicity, low doses should be used. For prescriptions that promote blood circulation to remove blood stasis and for prescriptions that disperse stagnated liver-*Qi*, high doses should be used.

Key words: Autoimmune disease; Prostatitis; Models, animal; Dinoprostone; Interleukins

INTRODUCTION

Chronic prostatitis is one of the most common prostate diseases seen in clinic. Traditional Chinese Medicine (TCM) has therapeutic effects on the disease, but as there is little experimental research and the therapeutic mechanisms are unclear, TCM popularization and application is hindered. In recent years, immune response in the role of pathogenic mechanisms of prostatitis has been increasingly studied, particularly with experimental autoimmune prostatitis (EAP). Research indicates that the prostate gland has a local immune function for protecting the genital system against bacteria and other pathogenic microorganisms.[1] Abnormalities in the local immune function of the prostate tissue can induce corresponding changes in its physiological function, and even a series of pathological changes when it is severe, indicating immune factors play an important role in its genesis, development and transformation. In the present study, an experimental autoimmune prostatitis rat model was established and treated with different Chinese herbal prescriptions. Changes in serum prostaglandin E_2 (PGE_2), interleukin-6 (IL-6), and interleukin-8 (IL-8) before and after treatment were detected to observe the therapeutic effects of different Chinese herbal prescriptions and different doses on autoimmune prostatitis. These results give evidence for the drug mechanism of action and provide a theoretical basis for the selection of different TCM therapeutic methods and doses.

MATERIALS AND METHODS

Experimental animals

Two-hundred male Wistar rats, Specific pathogen free grade, were supplied by the Experimental Animal Center (SCKX [Xin] 2011-0001), and raised in the Department of Experimental Animal Science Research, the Medical Research Center, The First Affiliated Hospital of Xinjiang Medical University. The body weight in 90 rats ranged from 300-350 g, and in 110 rats ranged from 180-200 g. After the 110 male Wistar rats, aged 3 months, were adaptively raised for 1 week, they were divided into 11 groups according to a random number table. The groups were: blank group; model group; Huoxuehuayu high-, middle-, and low-dose groups; Qingrejiedu high-, middle-, and low-dose groups; and Shuganliqi high-, middle-, and low-dose groups, with 10 rats in each group, except the blank group, which had nine rats (one rat died in raising). Intraperitoneal injection of the mixture (0.4 mL/100 g) of ketamine, diazepam, and atropine was used for anesthesia before the rat was sacrificed. Administration and experimental studies all met the Requirements for Administration of Experimental Animals (No. IACUC-20111125007) stipulated by the Ethics Committee of Administration and Use of Experimental Animals, the First Affiliated Hospital, Xinjiang Medical University.

Drugs and reagents

The prescription for promoting blood circulation to remove blood stasis (Huoxuehuayu) was composed of: Fuling (*Poria*) 10 g, Taoren (*Semen Persicae*) 10 g, Mudanpi (*Cortex Moutan Radicis*) 10 g, Chishao (*Radix Paeoniae Rubra*) 10 g, Guizhi (*Ramulus Cinnamomi*) 9 g, Zhiqiao (*Fructus Aurantii Submaturus*) 5 g, Danggui (*Radix Angelicae Sinensis*) 9 g, Chuanxiong (*Rhizoma Chuanxiong*) 9 g, Yanhusuo (*Rhizoma Corydalis Yanhusuo*) 9 g, Xiangfu (*Rhizoma Cyperi*) 9 g, and Honghua (*Flos Carthami*) 9 g.

The prescription for dispersing stagnated liver-*Qi* (*Shuganliqi*) was composed of: Wuyao (*Radix Linderae Aggregatae*) 6 g, Muxiang (*Radix Aucklandiae*) 9 g, Juhe (*Semen Citri Reticulatae*) 10 g, *Qi*ngpi (*Fructus Citri Reticulatae Immaturus*) 10 g, Xiaohuixiang (*Fructus Foeniculi*) 3 g, Zhiqiao (*Fructus Aurantii Submaturus*) 10 g, Chuanlianzi (*Fructus Toosendan*) 10 g, Yujin (*Radix Curcumae Wenyujin*) 10 g, Baishao (*Radix Paeoniae Alba*) 12 g, and Chaihu (*Radix Bupleuri Chinensis*) 9 g.

The prescription for clearing heat and removing toxicity (Qingrejiedu) was composed of: Jinyinhua (*Flos Lonicerae*) 15 g, Yejuhua (*Flos Dendranthematis Indici*) 10 g, Pugongying (*Herba Taraxaci Mongolici*) 10 g, Zihuadiding (*Herba Violae Philippicae*) 10 g, Dongkuiguo (*Fructus Malvae Verticillatae*) 10 g, Yiyiren (*Semen Coicis*) 10 g, and Jiangcan (*Bombyx Batryticatus*) 6 g.

All herbs were purchased from Tongrentang Haozhou Co. (Haozhou, China). Complete Freund's adjuvant (FCA) (batch No. 101M8714) was from Sigma Co.,

(St. Louis, MO, USA). Diphtheria-pertussis and tetanus vaccine (DPT vaccine) (batch No. 20110620-4) was from Wuhan Institute of Biological Products (Wuhan, China). Rat PGE_2 (batch No. DRE20017), IL-8 (batch No. DRE20033), and IL-6 (batch No. DRE20064) kits for enzyme-linked immunosorbent assay (ELISA) were purchased from Shanghai Xiangsheng Biological Products Co., (Shanghai, China). TritonX-100 (batch No. T7200) was purchased from Beijing Solar bio-Science and Technology Co., Ltd. (Beijing, China).

Instruments

The 3-18 k high speed refrigerated centrifuge (Sigma, Germany), the RV10 rotary evaporator. (IKA Co., Germany), and the enzyme-linked immunoassay detector was from BIO-RAD Co., (Hercules, CA, USA).

Establishment of the EAP rat model

Except for the rats in the blank group, rats in other groups were modeled respectively on days 0 and 30 (60 mg/mL), and intraperitoneally injected with diphtheria-pertussis and tetanus vaccine (DPT vaccine)[3,4]

Administration method

The administration dose of the rat was calculated according to the equivalent body surface area method in The Experimental Methodology of Pharmacology.[5,6] The amount of crude drugs and administration doses needed for the Huoxuehuayu, Qingrejiedu, and Shuganliqi high-, middle-, and low-dose groups were calculated with equal concentration methods. The drug solutions were diluted to appropriate concentrations with a rotary vaporizer on a water bath, respectively corresponding to three-, two-, and one-fold the equivalent dose, calculated according to the body surface area between the human and the animal. The administration was given by intragastric perfusion (equal concentration method) from the second day (on day 31) after the second injection of modeling, once each day, for 28 days.

Preparation of rat prostate protein extractive solution

Ninety male Wistar rats, weighing 300-350 g, were intraperitoneally anesthetized under aseptic conditions and a median incision on the lower abdomen was made. The prostate tissue was removed without the adhesive tissue, weighed, and then equal volume of saline containing 0.5% Triton X-100 (Sigma, Germany) (preliminary high temperature sterilization) was added. The tissue was homogenized with a glass homogenizer in an ice-water bath, and centrifuged at 10 000 r/min (3000 ×*g*) for 30 min at 4 ℃. The supernatant was removed for detection of protein with the biuret method,[2] and the protein concentration of the extracted solution was diluted to 60 mg/mL with PBS buffer.

Hematoxylin-eosin (HE) staining for observation of pathological changes in the prostate

Rats were anesthetized intraperitoneally on the second day after the final administration, and the abdominal cavity was explored and blood was taken from the abdominal aorta. Then, the prostate was removed and rinsed with 0.9% saline, immersed in 10% neutral formaldehyde for fixation, and embedded in paraffin. After 4-μm sections were made and stained with hematoxylin-eosin, changes in histopathologic changes of the mesenchyme and parenchyma were observed under a light microscope.

Detection of serum cytokines with ELISA

Serum IL-6, IL-8, and PGE_2 contents in the rats were detected according to manufacturer's directions.

Statistical analysis

SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA) was used. For measurement data, one-way analysis of variance was used for comparison between the groups, and $P < 0.05$ was regarded as a statistically significant difference. For paired comparison between groups, least significant difference was used when variance was homogenous, and the Games-Howell method was used when the variance was heterogeneous.

RESULTS

Pathological changes among the groups

In the blank group, the prostate tissues of the rats were intact, and fibers between acinuses and the smooth muscle were normally distributed, with no infiltration of inflammatory cells in the lumen of the gland and mesenchyme. There was also no hyperemia or edema. In the model group, the lumen of the gland in the rats' prostate tissues were dilated and enlarged, with secretion visible in the lumen, and dilated blood vessels with hyperemia. The epithelial cells of the acinus had obvious papillary hyperplasia, the mesenchyme had obvious fibrosis, and there were a great number of lymphocytes and plasma cells. Compared with the model group, after administration of the drugs for promoting blood circulation to remove blood stasis, clearing heat and removing toxicity, and dispersing stagnated liver-*Qi*, the proliferation of epithelial cells was improved, the secretion of the cells was reduced, the inflammation disappeared to varying degrees, and the lumen of the gland was enlarged. Epithelial cells mildly proliferated with a regular arrangement, and there was less secretion in the lumen. These changes were particularly obvious in the Qingrejiedu low-dose group, the Huoxuehuayu high-dose group, and the Shuganliqi high-dose group, and there were more reddish-staining secretions in the lumen, similar to those in the blank group (Figure 1).

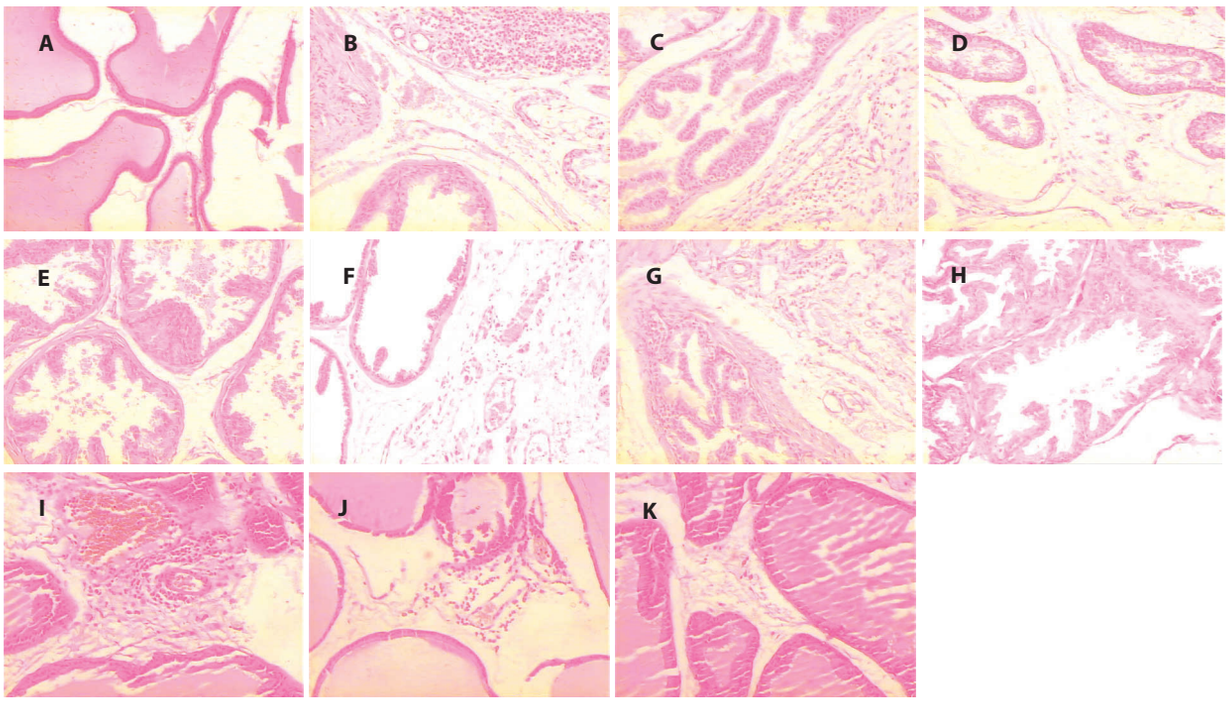

Figure 1 Pathological changes in the groups (HE staining, ×100)
A: blank group; B model group; C: Qingrejiedu low dose group; D: Qingrejiedu middle dose; E: Qingrejiedu high dose; F: Huoxuehuayu low dose; G: Huoxuehuayu middle dose; H: Huoxuehuayu high dose; I: Shuganliqi low-dose group; J: Shuganliqi middle-dose group; K: Shuganliqi middle-dose group. HE: hematoxylin-eosin.

Effects of prescriptions on serum PGE_2 levels in the EAP rats

The serum PGE_2 level in the model group was significantly higher ($P < 0.05$) compared with the blank group. After treatment with different drugs, serum PGE_2 levels decreased with significant statistical differences among the different doses of the three drugs ($P < 0.05$). Serum PGE_2 levels in the Qingrejiedu low- and middle-dose groups, the Huoxuehuayu high-dose group, and the Shuganliqi high-dose group were lower than those in the model group ($P < 0.05$). Serum PGE_2 levels in the Qingrejiedu groups increased along with increased dosage, while serum PGE_2 levels in the Huoxuehuayu groups and the Shuganliqi groups decreased along with decreased dosage. There were significant differences in decreases in serum PGE_2 levels among the three low-dose groups ($P < 0.05$), with the best effect in the Qingrejiedu group. There was no significant difference in decrease of PGE_2 levels among the three middle-dose groups ($P > 0.05$). There was a significant difference among the three high-dose groups in decrease of serum PGE_2 levels, with the best effect in the Huoxuehuayu group (Table 1).

Effects of prescriptions on serum IL-6 level in EAP rats

Compared with the blank group, the IL-6 level was significantly higher ($P < 0.05$) in the model group. After treatment with different drugs, the serum IL-6 levels all significantly decreased among the different doses of the three drugs ($P < 0.05$). Serum IL-6 levels in the Qingrejiedu low and middle-dose groups; the Huoxuehuayu middle and high dose groups; and the Shuganliqi high-dose group were lower than those in the model group (all $P < 0.05$). The IL-6 levels in the Qingrejiedu groups increased along with increases in dosage. IL-6 levels in the Huoxuehuayu groups gradually decreased along with increased dosage. There were significant differences among the three low dose groups ($P < 0.05$), with the best effect in the Qingrejiedu group, and with no differences among the three middle- and high-dose groups ($P > 0.05$) (Table 2).

Effects of prescriptions on serum IL-8 level in EAP rats

Compared with the blank group, serum IL-8 was significantly higher in the model group ($P < 0.05$). After

Table 1 Changes in serum prostaglandin E_2 levels in EAP rats after treatment with different doses of drugs (ng/L)

Group	Low dose (n=10)	Middle dose (n=10)	High dose (n=10)	Blank (n=9)	Model (n=10)
Qingrejiedu	382±48[a]	405±42[a]	434±52[b]	340±30[a]	468±54
Huoxuehuayu	440±46	433±54	362±55[ab]	340±30[a]	468±54
Shuganliqi	428±55	428±56	403±32[a]	340±30[a]	468±54

Notes: Qingrejiedu (clearing heat and removing toxicity, 4 weeks); Huoxuehuayu (promoting blood circulation to remove blood stasis, 4weeks); Shuganliqi (dispersing stagnated liver-*Qi*, 4 weeks). EAP: experimental autoimmune prostatitis. Compared with the model group, [a]$P < 0.05$; Compared with the low dose group, [b]$P < 0.05$.

treatment with different drugs, serum IL-8 levels were significantly lower in the Qingrejiedu low- and middle-dose groups, the Huoxuehuayu high-, middle-, and low-dose groups, and the Shuganliqi low- and high-dose groups as compared with the model group ($P < 0.05$). There were no significant differences among the three different doses of the three prescriptions in effect on serum IL-8 ($P > 0.05$)(Table 3).

DISCUSSION

Chronic prostatitis is a common disease of the prostate, possibly caused by infection, urine reflux, abnormal immunity, stress, and other factors.[7] Developments in molecular biology and immunology have changed research focus from the definition of cellular components to regulation of mediators of inflammatory response, with emphasis on the immune regulatory mechanisms of inflammatory response.[8] The cytokines related with the inflammatory response include interferon, the interleukin family, and tumor necrosis factor. The inflammatory factors involved in the present study were PGE_2, IL-6, and IL-8. Injury of local tissue or inflammation can produce and release algogenic chemical substances and PGE_2. PGE_2 promotes leukotaxis, strengthens capillary permeability, accelerates release of lysosomes and pyrogenic action, and is involved in the entire inflammatory response.[9] IL-6 is a cytokine that produced as an autocrine or paracrine. IL-6 can strength the production of IL-2 and T cells,[10] and can induce neutrophilic granulocyte inflow into the prostate tissue and activate T, B, and NK cells, promote degranulation of neutrophilic granulocytes, and release lysosomes. These effects aggravate infiltration of inflammatory cells, and promote inflammation.[11] IL-8, a pro-inflammatory factor, is produced by mononuclear leukocytes, lymphocytes, granulocytes, and endothelial cells, and is produced by endothelial cells, macrophages, and other cells after inflammatory stimulation.[12] IL-8 promotes chemotaxis causing the neutrophilic granulocytes and mononuclear leukocytes to leave the blood and cause inflammation.

In the present study, in the prescription for clearing heat and removing toxicity, Jinyinhua (*Flos Lonicerae*), Zihuadiding (*Herba Violae Philippicae*), Yejuhua (*Flos Dendranthematis Indici*) mainly clear heat and remove toxicity, cool blood, and reduce swelling. Meanwhile, adjuvant drugs Dongkuiguo (*Fructus Malvae Verticillatae*) and Yiyiren (*Semen Coicis*) clear heat, eliminate damp, and activate blood circulation to reduce swelling. The whole prescription clears damp heat to remove external pathogens and improve stagnation, which restores the normal physiologic state of the prostate. In the present study, it was found that after treatment with the prescription for clearing heat and removing toxicity, serum PGE_2, IL-6, and IL-8 levels were significantly lower than those in the model group. This result indicates that the prescription has regulatory action on algogenic and inflammatory factors. Previous research on Jinyinhua (*Flos Lonicerae Japonicae*), in the treatment of a fever rat model, has found that different doses of Jinyinhua can eliminate inflammation.[13] Zihuadiding (*Herba Violae Philippicae*) can clear heat and remove toxicity, cool blood, and reducing swelling.[14] Zihuadiding (*Herba Violae Philippicae*) can also strengthen the immunity of organisms and cells.[15] In TCM theory, drugs that are bitter-cold in taste and nature can spoil appetite, which influences therapeutic effect. Therefore, in this experiment, the high prescription dosage for clearing heat and removing toxicity could not significantly decrease PGE_2, IL-6, and IL-8 levels in the model mouse, while the low dose could.

In the prescription for promoting blood circulation to remove blood stasis, Taoren (*Semen Persicae*), Honghua (*Flos Carthami*), and Chishao (*Radix Paeoniae Rubra*) mainly promote blood circulation to remove blood sta-

Table 2 Changes in serum interleukin-6 levels in EAP rats after treatment with different prescriptions (ng/L)

Group	Low dose (n=10)	Middle dose (n=10)	High dose (n=10)	Blank (n=9)	Model (n=10)
Qingrejiedu	100±11[a]	108±13[a]	116±14[b]	75±8[a]	128±18
Huoxuehuayu	123±19	115±7[a]	109±10[ab]	75±8[a]	128±18
Shuganliqi	122±21	121±13	121±12[a]	75±8[a]	128±18

Notes: Qingrejiedu (clearing heat and removing toxicity, 4 weeks); Huoxuehuayu (promoting blood circulation to remove blood stasis, 4weeks); Shuganliqi (dispersing stagnated liver-*Qi*, 4 weeks). EAP: experimental autoimmune prostatitis. Compared with the model group, [a]$P < 0.05$; Compared with the low dose group, [b]$P < 0.05$.

Table 3 Changes in serum interleukin-8 levels in EAP rats after treatment with different prescriptions (ng/L)

Group	Low dose (n=10)	Middle dose (n=10)	High dose (n=10)	Blank (n=9)	Model (n=10)
Qingrejiedu	638±170[a]	669±125[a]	669±137	654±254[a]	811±61
Huoxuehuayu	663±144[a]	655±72[a]	649±128[a]	654±254[a]	811±61
Shuganliqi	669±122[a]	675±150	660±40[a]	654±254[a]	811±61

Notes: Qingrejiedu (clearing heat and removing toxicity, 4 weeks); Huoxuehuayu (promoting blood circulation to remove blood stasis, 4weeks); Shuganliqi (dispersing stagnated liver-*Qi*, 4 weeks). EAP: experimental autoimmune prostatitis. Compared with the model group, [a]$P < 0.05$.

sis, resolve masses, and disperse stagnation. Zhiqiao (*Fructus Aurantii Submaturus*), Xiangfu (*Rhizoma Cyperi*), and Yanhusuo (*Rhizoma Corydalis Yanhusuo*) disperse the stagnated liver-*Qi* and regulate *Qi*, remove stagnancy, resolve mass, promote blood circulation, and improve local micro-circulation. Mudanpi (*Cortex Moutan Radicis*) and Chishao (*Radix Paeoniae Rubra*) cool blood, clear heat, reduce swelling, remove stagnated heat, and relieve degree of fibrosis. Guizhi (*Ramulus Cinnamomi*) warms *Yang*, removes blood stasis, and regulates *Yin* and *Yang*. The entire prescription promotes blood circulation and removes blood stasis, disperses the stagnated liver-*Qi*, removes blood stasis, and clears heat. The results of the study indicate that after administration of the prescription for promoting blood circulation and removing blood stasis, serum PGE_2, IL-6, and IL-8 levels in the rat were lower than those in the model group. The levels were lower when the dosage was increased. The mechanism for treatment of prostatitis is possibly that the drugs for promoting blood circulation and removing blood stasis can improve microcirculation, increase local blood perfusion, and promote drug entrance into the gland lumen. These effects relieve chronic inflammatory obstructions of the prostate, unblock the prostate lumen,[16] regulate the secretory function of the prostate, increase immunity, stimulate proliferation of the reticuloendothelial system, promote phagocytosis of white cells, and alleviate dysfunction of the pelvic floor muscles.[17]

In the prescription for dispersing stagnated liver-*Qi*, Wuyao (*Radix Linderae Aggregatae*) and Xiaohuixiang (*Fructus Foeniculi*) disperse the stagnated liver-*Qi*, dispel cold, and relieve pain. Juhe (*Semen Citri Reticulatae*), Qingpi (*Fructus Citri Reticulatae Immaturus*), and Chuanlianzi (*Fructus Toosendan*) relieve stagnated liver-*Qi*, disperse stagnation, relieve pain, and promote blood circulation. Baishao (*Radix Paeoniae Alba*) nourishes liver blood, relieves spasms and pain, and avoids consumption of liver-*Yin* owing to the over pungent and dispersing action of the drugs. The entire prescription disperses the stagnated liver-*Qi*, and relieves stagnation and pain. In the study, it was found that after treatment with the drugs for dispersing the stagnated liver-*Qi*, serum PGE_2, IL-6, and IL-8 levels were lower as compared with the model group, with the higher dose having a larger effect. The treatment is mainly based on the TCM theory that "the Liver Meridian goes along the *Yin* organs, and is in charge of dispersing." Pain and distending pain in the perineum or the low abdomen are the main manifestations of chronic prostatitis. Pharmacologic study indicates that Chaihu (*Radix Bupleuri Chinensis*) in the prescription can: relieve spasms; act as a tranquilizer; act as an anti-inflammatory, analgesic, anti-viral, and anti-histamine; induce the production of interferon; and promote immune function.[18,19] Yujin (*Radix Curcumae Wenyujin*) promotes blood circulation and removes blood stasis, clears heart-fire to relieve depression, promotes *Qi* circulation to relieve pain, and has immunosuppressive action in regulating immune function.[20] In addition, Yujin (*Radix Curcumae Wenyujin*) can promote diuresis, relieve stranguria, and help to improve the stimulating symptoms of prostate in urination.

The above results indicate that the drugs for clearing heat and removing toxicity (low dose), the drugs for promoting blood circulation and removing blood stasis (high dose), and the drugs for dispersing stagnated liver-*Qi* (high dose) can alleviate infiltration of inflammatory cells and proliferation of fibrous tissue in prostatitis rats. These drugs can also restore the secretory function of the prostate epithelial cells, and strengthen the anti-inflammatory action and the ability of the prostate to restore injured tissue. These effects have a protective effect on the prostate, which provides supporting evidence for the use of Chinese drugs in treating autoimmune prostatitis.

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