

Original Article

Study on expression of IL-6/JAK/STAT3 signaling pathway in category IIIB prostatitis

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Abstract: Objective: To investigate the expression levels of interleukin (IL)-6 and downstream JAK/STAT3 signaling pathway in category IIIB prostatitis. Methods: A total of 44 patients diagnosed with category IIIB prostatitis in our hospital were selected as study group. During the same period, forty healthy people underwent physical examination in our hospital were enrolled in control group. The differences of IL-6, IL-8 and IL-2 in serum and prostatic fluid in the two groups were compared. In addition, 80 Sprague-Dawley rats were randomly divided into four groups: control group (normal saline was injected every other day), prostatitis group (animal models of category IIIB prostatitis), prostatitis + IL-6 group (IL-6 was intraperitoneally injected to rats with prostatitis every other day for one month), prostatitis + blocker group (IL-6 downstream JAK2/STAT3 signaling pathway blocker AG490 was given to rats with prostatitis every other day for one month), with 20 rats in each group. The levels of IL-6, IL-8 and IL-2 in serum of the four groups of rats were detected. And the differences of expression levels of IL-6 and downstream signaling molecules (JAK and STAT3) in prostate tissues as well as the inflammatory degree of prostate pathological tissues of rats in the four groups were compared. Results: Compared with control group, the IL-6, IL-8 and IL-2 levels in prostatic fluid of patients in study group were obviously higher (all $P < 0.01$), but the differences of those levels in serum were no significant (all $P > 0.05$). The IL-6, IL-8 and IL-2 levels in serum of rats in the four groups had no significant difference (all $P > 0.05$). However, in the prostate tissues of the four groups of rats, the numbers of IL-6 positive cells were ranked as prostatitis + IL-6 group > prostatitis group > control group > prostatitis + blocker group (all $P < 0.05$), and the levels of JAK and STAT3 in the four groups were also with the same sequence ($P < 0.05$), also, the inflammatory degrees were with the same trend. Conclusion: IL-6 and its downstream JAK/STAT3 signaling molecules present high expression in category IIIB prostatitis, and they may be involved in sterile inflammation.

Keywords: Category IIIB prostatitis, IL-6, JAK/STAT3 signaling pathway

Introduction

Category III chronic prostatitis/chronic pelvic pain syndrome is the most common chronic prostatitis, and category IIIB prostatitis is one of that without existing evidence of white blood cell in prostatic fluid [1]. Although lots of studies on category IIIB prostatitis have been carried out, its etiology and pathogenesis remain unknown. In recent years, study showed that immune reaction played an important part in the development of category IIIB prostatitis, in which IL-6 had the most concern, but specific signaling pathway of IL-6 in pathogenesis and

pathological process of chronic prostatitis/chronic pelvic pain syndrome still needed to be further explored [2]. IL-6 is an important cytokine in body's transition process from innate immunity to acquired immunity, and it played a leading role in chronic inflammation with JAK/STAT3 as its major downstream signaling pathway [3]. Accordingly, we speculated that IL-6 might be involved in the pathogenesis and progression of category IIIB prostatitis via JAK/STAT3 signaling pathway. While at present, there is no related clinical and experimental study. Therefore, preliminary discussion on it has been carried out in this study.

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Materials and methods

Subjects

This study was approved by Ethics Committee in our hospital and the informed consents were obtained from all subjects.

Forty-four patients diagnosed with category IIIB prostatitis in our hospital were selected as study group, aged 55-69 years, mean age: 61.6 ± 7.2 years. Inclusion criteria: Patients who met the diagnostic criteria of category IIIB prostatitis in *Diagnosis and Treatment of Chronic Prostatitis* set by Chinese Urological Association; patients who signed informed consents in written form. Exclusion criteria: Patients who suffered from prostate cancer; patients with severe dysfunction in heart, lung, liver, kidney or other important organs; patients who were receiving oral medication for the treatment of chronic prostatitis. In the same period, forty healthy people underwent physical examination in our hospital were enrolled in control group, aged 53-72 years, mean age: 63.5 ± 9.7 years.

Animal model and grouping

A total of 80 male Sprague-Dawley rats (weight: 250 to 300 g), which were purchased from the laboratory animal center of our hospital, were randomly divided into four groups with 20 rats in each group: control group, prostatitis group, prostatitis + IL-6 group and prostatitis + blocker group. In prostatitis group, category IIIB prostatitis animal models were established, and specific methods were described in previous experimental research [4]. Right and left ventral lobes of rat prostate were injected 3% carageenan 50 μ l, and experimental animal models were successfully established after a week. After that, intraperitoneal injection of IL-6 was given for rats in prostatitis + IL-6 group every other day for one month. In prostatitis + blocker group, JAK2/STAT3 signaling pathway blocker AG490 was given to rats with prostatitis every other day for one month. And in control group and prostatitis group, the rats were injected equal amounts of normal saline every other day for a month.

Main reagents

Enzyme Linked Immunosorbent Assay (ELISA) kits of IL-6, IL-8 and IL-2 were purchased from Beijing Biofriendship Co., Ltd. And the sources

of other reagents were as follows: AG490 from Calbiochem company, Germany; IL-6 primary antibody from Pepro Tech company, America; JAK and STAT3 primary antibodies from Cell Signal company, America; β -actin primary antibody from Santa Cruz Biotechnology company, America; all the secondary antibodies were from Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd.; cell lysis buffer (included 1 mM DTT, 5 mM EDTA and 1 \times protease inhibitor cocktail) and protein loading buffer were from Sigma-Aldrich company, America.

Detection of cytokines levels

Detection of cytokines levels in human serum and prostatic fluid: Fasting serum of the two groups of patients was collected in the morning respectively, and prostatic fluid was collected through prostatic massage. The concentrations of cytokine IL-6, IL-8 and IL-2 in serum and prostatic fluid were determined by human ELISA kits respectively, and the differences were compared.

Detection of cytokines levels in rat serum: Anesthesia was carried out for four groups of rats with 10% chloral hydrate. Then the rats were killed after serum samples were collected by carotid artery bloodletting, and their serum samples and prostate samples were kept. The concentrations of cytokine IL-6, IL-8 and IL-2 in serum of rats were detected by rat ELISA kits.

Detection of IL-6 positive cells in prostatic tissues of rats: Immunohistochemical S-P method was used to detect the protein expression level of cytokine IL-6 in prostatic tissue. Parts of rat prostatic tissues were sliced and successively incubated with rat IL-6 primary antibody, biotin-labeled goat anti-rat IgG secondary antibody and horseradish peroxidase conjugated streptavidin in room temperature for color development. Ten slices were randomly selected from each group, and three visual fields were randomly selected in each slice for high-power lens to perform data measurement and analysis, and the number of positive cells in every visual field was calculated.

Analysis of inflammation degree of prostatic tissues in rats

A part of rat prostatic samples was cut and fixed with 10% formaldehyde for 24-48 h. After HE staining of tissue slices, four visual fields were randomly selected in each slice under an

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Table 1. Comparison of cytokine levels in serum and prostatic fluid between two groups (pg/ml)

Group		Study group	Control group	t	P
Case		44	40		
IL-6	Serum	14.5±4.1	13.1±3.2	1.732	0.087
	Prostatic fluid	50.4±7.6	43.1±5.9	4.882	0.000
IL-8	Serum	296.6±35.3	285.4±29.6	1.567	0.121
	Prostatic fluid	930.2±47.5	898.9±40.7	3.227	0.002
IL-2	Serum	89.4±8.9	86.3±8.2	1.655	0.102
	Prostatic fluid	342.3±26.8	319.5±24.3	4.070	0.000

Table 2. Comparison of cytokine levels in serum of four groups of experimental rats (n=20/group, pg/ml)

Group	IL-6	IL-8	IL-2
Control group	22.4±3.6	367.9±37.4	92.4±9.8
Prostatitis group	23.2±3.9	384.5±42.7	96.5±10.2
Prostatitis + IL-6 group	24.3±5.2	377.2±56.3	95.1±11.4
Prostatitis + blocker group	21.5±4.6	342.6±33.8	89.8±8.9
F	4.382	4.956	4.663
P	0.107	0.089	0.095

optical microscope at 400 times magnification to perform data measurement and analysis, and the mean value was calculated after inflammatory cells were counted. The differences of prostate weight and inflammatory cell numbers of the four groups of rats were compared.

Expression level of JAK and STAT3 signaling pathway

Western blot method was used to detect molecular levels of JAK and STAT3 in rat prostatic tissues, and specific methods were as follows: parts of rat prostatic tissues were taken from the rats in four groups respectively, and 0.5 ml cell lysis buffer was added in the homogenized tissue, then they were boiled for five minutes to extract cell protein (20 µg protein was added for each well). Loading buffer was added in prepared samples, then they were mixed and boiled for 5 minutes for protein denaturation. After that, equal amount of mixed sample was added in each well of 7.5% polyacrylamide gel for electrophoresis. The proteins were transferred to polyvinylidene fluoride membrane, then 3% skimmed milk dissolved in phosphate buffer saline (freshly prepared) was used for blocking. Next, JAK mouse monoclonal antibody, STAT3 mouse monoclonal antibody (1:1,000) and anti-β-actin antibody (1:500) were added, then they were incubated over-

night at 4°C. After that, the proteins were washed for three times with phosphate buffer saline. Then, they were incubated after adding the horseradish peroxidase labeled secondary antibody (goat anti-rat IgG). At last, chemiluminescence and X-ray exposure were used. Image processing system (NIH image) was used for the analysis of optical density.

Statistical analysis

SPSS17.0 statistical software package was used for statistical analysis. Normality test was used for continuous data before comparison and analysis, and Levene test was used for homogeneity test of variance.

The measurement data were expressed as mean±standard deviation ($\bar{x}\pm sd$), and comparison of cases between study group and control group was performed with two independent samples t-test; one-way ANOVA and Newman-Student-Keuls test were used for the analysis of the inter-group differences among four animal groups. Enumeration data were expressed as rate, and chi-square test and chi-square partition test were used for inter-group comparison. The differences were statistically significant when $P<0.05$.

Results

Comparison of cytokine levels in serum and prostatic fluid between two human groups

Compared with control group, patients in study group had apparently higher levels of IL-6, IL-8 and IL-2 in their prostatic fluid (all $P<0.01$), while differences of those levels in serum were not significant (all $P>0.05$). See **Table 1**.

Comparison of cytokine levels in serum of experimental rats

There were no significant differences of serum IL-6, IL-8 and IL-2 levels in four groups of experimental rats (all $P>0.05$). See **Table 2**.

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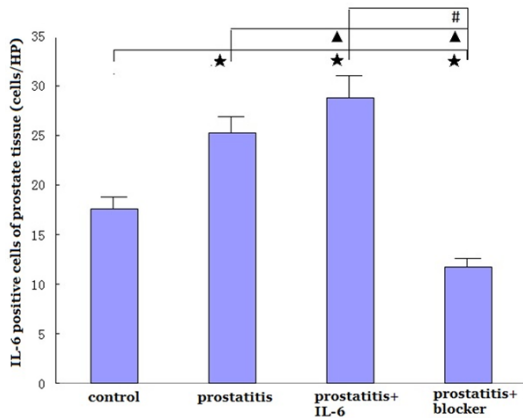


Figure 1. Comparison of numbers of IL-6 positive cells in prostate tissues of four groups of rats. Compared with control group, * $P < 0.05$; compared with prostatitis group, $\Delta P < 0.05$; compared with prostatitis + blocker group, $\# P < 0.05$.

Comparison of IL-6 levels in prostate tissues of rats

In the four groups, the amounts of IL-6 positive cells in rat prostate tissues were as follows: control group ($17.6 \pm 4.7/\text{HP}$), prostatitis group ($25.2 \pm 4.3/\text{HP}$), prostatitis + IL-6 ($28.8 \pm 5.2/\text{HP}$), prostatitis + blocker group ($11.7 \pm 4.1/\text{HP}$); namely, the order of numbers of IL-6 positive cells in these groups was: prostatitis + IL-6 > prostatitis group > control group > prostatitis + blocker group (all $P < 0.05$). See **Figure 1**.

Analysis of pathological prostatic tissue of rats

In prostatitis group, structures of rat prostate tissue were damaged with inhomogeneous lymphoid hyperplasia. Catheters were dilatated or damaged; parts of basement membrane were destroyed with visible diffuse infiltration of lymph, monocyte and other chronic inflammatory cells existing around the mesenchyme and glands. In prostatitis + IL-6 group, structures of rat prostate tissue were damaged severely with gathering inflammatory cells; plenty of glandular epithelial tissues were damaged, and lymphoid nodule or folliculus were formed also with a full view of inflammatory cells. As for prostatitis + blocker group, the inflammatory cells in rat prostatic tissues were scattered and numbered 1-10/HP. In control group, the rat prostate tissues had a relatively complete structure with neither inflammatory cell infiltration in mesenchyme or gland, nor edema in glandular cavity. See **Figure 2**.

Comparison of JAK and STAT3 levels in rat prostate tissues

The levels of JAK and STAT3 in prostate tissues of the four groups of rats were ranked as prostatitis + IL-6 group > prostatitis group > control group > prostatitis + blocker group ($P < 0.05$). See **Figure 3**.

Discussion

The immune inflammatory reaction has been proved vital to the pathogenic process of category IIIB prostatitis. As the previous studies demonstrated, after category IIIB prostatitis model rats received high level 5- α dihydrotestosterone, the cell inflammatory reaction of their prostate tissues could be significantly inhibited; therefore, many investigators believed that category IIIB prostatitis was essentially a cytotoxic immune response, but its specific pathogenesis was not yet fully defined [4-6]. The extremely complex body immune inflammatory reaction included the participation of cytokines network and downstream signal transduction, at the same time, there was a study proved that multiple cytokines networks and their interaction in the inflammatory reaction determined the outcome of overall body immune inflammation [7]. Among the cytokines, IL-6 might play a pivotal role in the pathological process of category IIIB prostatitis [8]. There were studies revealed that in patients with prostatitis, the levels of IL-6 in prostate fluid, sperm, and biopsy tissues were significantly related to their symptoms [9, 10]. Another study showed that after the treatment, the level of IL-6 in seminal plasma of patients with category IIIB prostatitis was significantly lower than that of before treatment, and it was positively correlated to clinical efficacy [11].

This study found that the levels of IL-6, IL-8 and IL-2 in prostate fluid of patients with category IIIB prostatitis were obviously higher than those of normal subjects, which was consistent with the results of previous studies [12-14]. However, when the patients were compared with the normal subjects in control group, there was no significant difference in IL-6, IL-8 and IL-2 in peripheral blood, which suggested that the immune inflammatory reaction of category IIIB prostatitis was localized in the prostate rather than the whole body [13-15]. In animal model experiment, there was no significant difference

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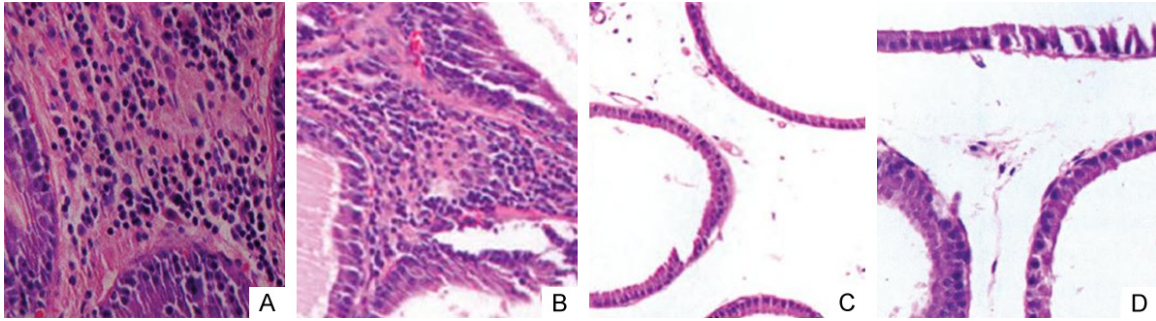


Figure 2. Inflammatory infiltration of rat prostate tissues in four groups (*400). (A) prostatitis group, (B) prostatitis + IL-6 group, (C) prostatitis + blocker group, (D) control group.

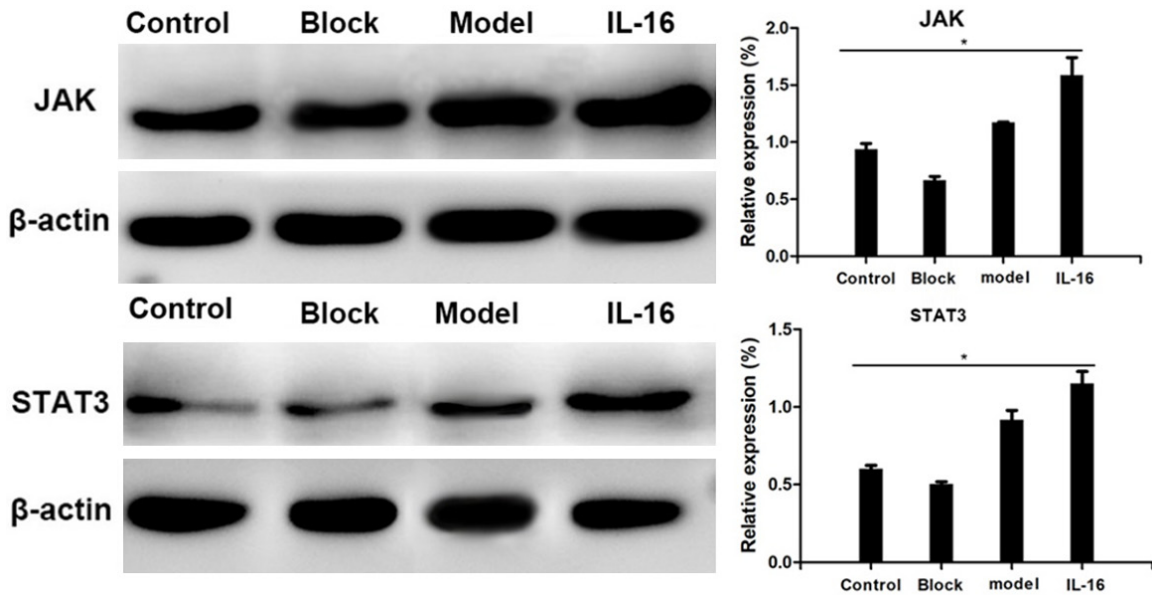


Figure 3. Comparison of JAK and STAT3 levels of rat prostate tissue in four groups *P<0.05.

in rat serum IL-6, IL-8 and IL-2 levels. Nevertheless, according to the immunohistochemistry analysis of prostate tissues, we found that the IL-6 levels of rats in prostatitis group and prostatitis + IL-6 group were higher than that in prostatitis + blocker group, which suggested that IL-6 played a role in prostate chronic immune inflammatory response and also implied that the pathological process of category IIIB prostatitis might incline to be a kind of chronic autoimmune disease.

After the combination of IL-6 and cell membrane receptor complex *in vivo*, JAK kinase was activated, and therewith STAT was also activated by tyrosine phosphorylation, and then signals were transduced to intranuclear to act the biological roles in related regulation [15]; at the same time, JAK/STAT signaling pathway was appearing a positive feedback regulation to the

cellular expression of IL-6 [16-19]. Previous studies have shown that IL-6 promotes the expression of anti-apoptotic genes such as bcl-xl, c-Jun and Fas by activating the JAK/STAT signaling pathway, which is closely related to angiogenesis, cell proliferation and apoptosis [20-22]. But it hasn't been reported by any studies that whether IL-6 can promote the pathogenesis and progression of category IIIB prostatitis by activating the JAK/STAT signaling pathway or not. Therefore, this study discussed this question by analyzing the clinical data of patients with category IIIB prostatitis in our hospital and establishing rat models of category IIIB prostatitis.

Therefore, this study suggests that IL-6 may participate in the pathological process of category IIIB prostatitis through JAK/STAT signaling pathway, but this mechanism still needs further

experiment to confirm; meanwhile, it may provide a new potential therapeutic target for the treatment of category IIIB prostatitis, and it is worthy of further researches in subsequent studies.

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Disclosure of conflict of interest

None.

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