Correlation of pain with Substance P and neurokinin-1 receptor in the L5-S2 spinal cord in rats with chronic nonbacterial prostatitis

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Abstract

Background

To investigate the correlation of the pain with the expression of SP and NK-1R in the posterior horn of the L5-S2 spinal cord in a rat model of chronic nonbacterial prostatitis (CNP) at different time points of modeling.

Methods

Forty adult male SD rats were randomly divided into four equal groups: control group, 45 day (d) group, 60 d group, and 90 d group, and proteins were obtained from the prostatic tissue of another 30 rats. The CNP model was established by intraperitoneal injection of 0.5 mL diazepam vaccine and intradermal injection of a mixed solution of 1 mL prostatein extract and complete adjuvant at a 1:1 ratio. The control group rats were injected with the same volume of normal saline. At 45, 60, and 90 days after modeling, we measured the paw withdrawal threshold (PWT) of the rats, detected the level of TNF- α , IL-1 β , IL-2, and IL-10 in the prostate tissues by ELISA, observed the histomorphological changes in the prostate by transmission electron and light microscopy, and detected the expression of SP and NK1-R in the L5-S2 spinal cord by immunohistochemistry.

Results

The levels of TNF- α , IL-1 β , IL-2, and IL-10 in the prostate tissue were markedly higher in the CNP models than those in the control group (all $P < 0.05$); this difference was Most significant at 90 days (all $P < 0.05$). Immunohistochemistry showed that the expressions of SP and NK-1R were remarkably higher in the CNP model groups than in the control (all $P < 0.05$), with the highest expression found at 90 days. Light microscopy revealed no inflammatory cell infiltration in the prostate tissue of the control rats, and obvious edema and increased lymphocyte count were observed as the duration of modeling increased. Transmission electron microscopy showed inflammatory changes in the prostate tissue of the model rats; peritubular interstitial edema was most obvious at 90 days, with widened gaps between the peritubular cells and epithelial base and increased numbers of fibroblasts and collagen fibrils.

Conclusion

There was a correlation between the pain and the expressions of SP and the NK-1R in the L5-S2 spinal cord of the rats.

Background

Chronic prostatitis (CP) is a common disease of the urological and reproductive systems of adult men[1]and accounts for about one quarter of outpatients[2]. Chronic nonbacterial prostatitis (CNP) is the most common type and accounts for 90% of all CPs[3,4]. About 50% of men are affected by CNP[5]. At present, the pathogenesis, pathophysiology, and the underlying mechanisms of CNP are not very clear[6].The clinical symptoms of CNP are complicated, and pain is the most obvious manifestation; however, the diagnosis and treatment are still medical challenges. Some scholars believe that the pain is not necessarily caused by the prostatic lesions and may instead be related to the abnormal nerve conduction pathways and regulatory mechanisms that govern the prostate, which are likely due to secondary lesions of the L5-S2 spinal cord

segment[7]. In this study, an autoimmune method was used to establish a CNP rat model. The expression of Substance P (SP) and its neurokinin-1 receptor (NK-1R) in the spinal dorsal horn of the L5-S2 spinal cord at different time periods were evaluated to explore the possible mechanisms of pain.

Methods

Experimental animals

In all, 70 healthy adult male SD rats weighing 250–300 g were provided by Qinglongshan Animal Breeding Farm in Jiangning District, Nanjing City (certificate number: SCXK (Su) 2018-0001). The experimental operation process complies with the experimental animal ethics requirements of Shanghai University of Traditional Chinese Medicine (ethics approval number: SZY201807016).

Model building and grouping

Thirty male SD rats weighing 280–300 g were sacrificed by grabbing the head of the rats with one hand and the tail of the rats with the other, and pulling with both hands at the same time until their cervical spines were broken, and the lower abdomen skin was disinfected. The prostate tissue was stripped and fully washed with cold physiological saline and a physiological saline solution (containing 0.5% TritonX-100, American Sigma). The prostate tissue was then homogenized (with the lower part of the glass homogenizer in ice) and centrifuged at $3000 \times g$ for 30 min at 4°C; The centrifuged supernatant was extracted, and the biuret method was used to determine the protein concentration (Biuret kit, Nanjing Jiancheng Technology Co., Ltd.). The prostate protein solution was diluted to 40 g/L with 0.1 mol/L PBS (pH 7.4) for subsequent use.

Forty male SD rats weighing 250–280 g were randomly divided into four groups: one control group and three model groups (45 days [d], 60 d, and 90 d), with 10 rats in each group. On days 0 and 30, animals in each group were anesthetized with ether. The model groups were injected intraperitoneally with 0.5 mL of diazepam vaccine (Wuhan Institute of Biological Products), while intradermal injections of 1 ml Freund's Complete Adjuvant (American Sigma) at a ratio of 1:1 and a suspension of prostate protein extract. The control group was injected with the same volume of saline.

Detection of paw withdrawal threshold (PWT)

The PWT was measured by Chaplan et al's method[8]. All rats were placed in a translucent glass cage, and Von Frey filaments were used to stimulate the midfoot of the rats' hind limbs. The slight bending of the cilia was used as the complete force standard to bend the fibers into an S shape for 6–8 s, use fiber filaments to stimulate 2, 5, 6, 8, 10, 15, 26 g for 2 s in sequence, at intervals of 15 s, for 5 consecutive times. The minimum number of grams that caused 3/5 leg lifts was defined as PWT. The PWT was measured at 45, 60, and 90 days after the start of modeling.

Light microscopy, transmission electron microscopy, and SP and NK-1R immunohistochemistry

On days 45, 60, and 90 after modeling, the rats died of torn cervical spine, and the prostate tissue of each rat from all the groups was collected. Part of the prostate tissue was fixed with 4% neutral formaldehyde solution at room temperature for 24 h, followed by dehydration in a graded series of ethanol, and xylene treatment. After being embedded in paraffin, the tissues were sectioned into 4-μm-thick slices, stained with hematoxylin-eosin

(HE), and observed under a light microscope (Japan Olympus Optical Ltd). The remaining part of the prostate tissue was cut into small pieces measuring 1 mm×1 mm×1 mm, submerged in 4.9% glutaraldehyde and 1% acetic acid (pH 7.4) solution for double fixing, dehydrated with ethanol and acetone, embedded in Epon 812 epoxy resin, and sectioned. Ultrathin sections were stained with uranium acetate and lead citrate, and the microstructure was observed by transmission electron microscopy (Japan HITACHI company).

The spinal cord was removed and fixed in 4% paraformaldehyde phosphate buffer and kept in the refrigerator 4°C overnight. The L5-S2 spinal cord segment was isolated, and continuous cross-sections were obtained using a vibrating microtome, and floating immunohistochemistry on freely floating fixed tissue sections (American Sigma) was performed to detect the optical density (OD) values of SP and NK-1R immunopositive reactions.

Determination of tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), interleukin-2 (IL-2), and interleukin-10 (IL-10) in prostate tissues

Briefly, 1 mL of 0.5% TritonX-100 normal saline per 100 mg of prostate tissue was added to prepare the tissue homogenate, and centrifuged at 3000 $\times g$ for 15 min. The concentrations of TNF- α , IL-1 β , IL-2, and IL-10 in the prostate tissues was measured in an appropriate amount of supernatant by using ELISA method (American Sigma).

Statistical analysis

Descriptive and differential statistical analysis of the data were conducted. For normal distribution, the mean and standard deviation were used to describe the centralized and discrete trends, respectively. The variances were the same. A completely random design analysis of variance was used, and the SNK-q method was used to compare pairs. The test standard was $\alpha=0.05$. The SAS9.1 software (North Carolina State University, United States) was used for all statistical analysis.

Results

PWT changes

Compared with the control group, the PWT of rats in the 45 d, 60 d, and 90 d groups decreased. The PWT in the 90 d group was significantly lower than that in the 45 d and 60 d (all $P<0.05$) groups (Table 1). The pain sensitivity of rats appeared to be enhanced after modeling, and the pain was more sensitive with increasing modeling time.

Table 1 Paw withdrawal threshold (PWT) of the rats in the control and different modeling groups ($\bar{x} \pm s$)

Group	n	PWT(g)
Control	10	73.34±6.86
Model		
45 d	10	48.25±6.65 ^a
60 d	10	42.61±4.93 ^{ab}
90 d	10	25.82±5.73 ^{abc}

a: $P < 0.05$ vs. the control group; b: $P < 0.05$ vs. the 45 d group; c: $P < 0.05$ vs. the 60 d group.

Pathological examination of prostate tissue under light microscopy

The prostate tissue of the control group appeared normal (Fig. 1A). Lymphocyte infiltration around the capillaries and irregular glandular cavity were visible in the prostate tissue in the CNP rat model at 45 days after modeling (Fig. 1B). Interstitial edema, large numbers of lymphocyte infiltration around the capillaries, and invasion of the local glandular epithelium into the prostate cavity were more obvious in the CNP model, with part of the basement membrane widened or destroyed and intraluminal secretions increased or decreased, at 60 days after modeling (Fig. 1C). A large number of lymphocytes and mast cells were found in the CNP model, with a few neutrophils and plasma cells where the epithelium appeared segmental necrosis and shedding. The glandular cavity was dilated, no secretions were seen in part of the prostate cavity, and interstitial collagen fibers showed proliferation at 90 days (Fig. 1D).

A: No inflammatory cells infiltration was seen in the prostate tissue of the control rats. B: Lymphocytes were visible in the prostate tissue from the rat model of chronic nonbacterial prostatitis (CNP) at 45 days after modeling. C: Edema was more obvious in the CNP rat model, with large numbers of lymphocytes at 60 days after modeling. D: A large number of lymphocytes and mast cells were found in the CNP rat model, with a few neutrophils and plasma cells at 90 days. The black arrows indicate lymphocytes. The glandular cavity was expanded and collagen fibers were proliferated. Bar=50 μ m.

TEM pathological results of prostate tissue

The structure of the prostate was normal in the control group, with a small number of mitochondria, rich rough endoplasmic reticulum, developed Golgi complex, and a large amount of secretions in the glandular cavity (Fig. 2A). At 45 days after modeling, epithelial cells proliferated and the interstitial space widened, endoplasmic reticulum was abundant, and mast cells were visible in the interstitial space (Fig. 2B). Infiltration of macrophages and lymphocytes was seen around capillaries at 60 days after modeling, with widened epithelial space of the prostate and exacerbated peritubular and interstitial edema (Fig. 2C). Interstitial edema was more pronounced, with the space between the peritubular cells and the base of the epithelium was widened, and the number of fibroblasts and surrounding collagen fibrils increased at 90 days after modeling (Fig. 2D).

Comparison of TNF- α , IL-1 β , IL-2, and IL-10 concentrations in prostate tissue of rats in each group

Compared with the control group, the concentrations of TNF- α , IL-1 β , IL-2, and IL-10 in the prostate tissue of each model group was significantly increased. However, this increase was most significant in the 90 d group (all $P<0.05$) (Table 2). Inflammation was seen in the prostate tissue of rats after modeling, and the inflammation became more severe as the modeling time increased.

Table 2 Levels of TNF- α , IL-1 β , IL-2, and IL-10 in the prostate tissue of rats in the control and different modeling groups ($\bar{x}\pm s$)

Group	n	TNF- α (pg/ml)	IL-1 β (pg/ml)	IL-2(pg/ml)	IL-10(pg/ml)
Control	10	41.34 $\pm$ 2.21	15.57 $\pm$ 1.02	68.86 $\pm$ 7.21	90.09 $\pm$ 16.85
Model					
45 d	10	72.01 $\pm$ 2.23 ^a	38.75 $\pm$ 3.74 ^a	121.17 $\pm$ 9.49 ^a	118.05 $\pm$ 12.53 ^a
60 d	10	78.32 $\pm$ 3.23 ^{ab}	43.90 $\pm$ 2.44 ^{ab}	125.43 $\pm$ 10.12 ^{ab}	147.10 $\pm$ 8.91 ^{ab}
90 d	10	95.97 $\pm$ 7.06 ^{abc}	54.61 $\pm$ 1.92 ^{abc}	157.39 $\pm$ 9.52 ^{abc}	281.97 $\pm$ 9.31 ^{abc}

a: $P<0.05$ vs. the control group; b: $P<0.05$ vs. the 45 d group; c: $P<0.05$ vs. the 60 d group.

Immunohistochemical results of SP and NK-1R in the L5-S2 spinal cord of rats in each group

Light microscopy showed that both SP and NK-1R immunoreactants were densely packed in the dorsal horn of the spinal cord, which was distributed in a brown-yellow cord-like form, while other parts were either not colored or pale yellow (Figs. 3 and 4). The OD value of each model group was significantly higher than that of the control group, while the OD value of the 90 d group was higher than that of the remaining three groups (Table 3 and 4) (all $P<0.05$), which suggested that SP synthesis in the L5-S2 spinal dorsal horn in CNP rats increased, causing the receptor to be up-regulated. This was more significant with increasing modeling time.

Table 3 Optical density of Substance P (SP) in the posterior horn of the L5-S2 spinal cord in different groups of rats ($\bar{x}\pm s$)

Group	n	L5	L6	S1	S2
Control	10	0.10 $\pm$ 0.02	0.10 $\pm$ 0.02	0.09 $\pm$ 0.01	0.09 $\pm$ 0.01
Model					
45 d	10	0.17 $\pm$ 0.03 ^a	0.17 $\pm$ 0.03 ^a	0.14 $\pm$ 0.01 ^a	0.15 $\pm$ 0.02 ^a
60 d	10	0.19 $\pm$ 0.02 ^{ab}	0.21 $\pm$ 0.06 ^{ab}	0.18 $\pm$ 0.04 ^{ab}	0.18 $\pm$ 0.03 ^{ab}
90 d	10	0.28 $\pm$ 0.03 ^{abc}	0.27 $\pm$ 0.03 ^{abc}	0.26 $\pm$ 0.03 ^{abc}	0.26 $\pm$ 0.03 ^{abc}

a: $P<0.05$ vs. the control group; b: $P<0.05$ vs. the 45 d group; c: $P<0.05$ vs. the 60 d group.

Table 4 Optical density of neurokinin-1 receptor (NK-1R) in the posterior horn of the L5-S2 spinal cord in different groups of rats($\bar{x}\pm s$)

Group	n	L5	L6	S1	S2
Control	10	0.08±0.01	0.08±0.01	0.07±0.01	0.07±0.01
Model					
45 d	10	0.15±0.01 ^a	0.16±0.01 ^a	0.14±0.01 ^a	0.14±0.01 ^a
60 d	10	0.19±0.01 ^{ab}	0.19±0.01 ^{ab}	0.18±0.04 ^{ab}	0.18±0.01 ^{ab}
90 d	10	0.26±0.01 ^{abc}	0.26±0.01 ^{abc}	0.25±0.01 ^{abc}	0.24±0.01 ^{abc}

a: **P**<0.05 vs. the control group; b: **P**<0.05 vs. the 45 d group; c: **P**<0.05 vs. the 60 d group.

Discussion

The disease course of CNP is long and presents with complex symptoms[9], which seriously affect patients' quality of life and physical and mental health[10-15]. Further, it is also associated with shame[16]. According to global statistics, the incidence of CNP ranges from 2.2% to 9.7%, with an average prevalence of 8.2%[17]. The pathogenesis and pathophysiology of CNP are not very clear, and the treatment is challenging[18]. Persistent and intractable pain that is difficult to control with drugs is a characteristic feature of CNP. It is also the most important symptom that affects the quality of life of patients and the main reason for outpatient visits[19], while CNP is as severe as active Crohn's disease, myocardial infarction, and unstable angina[20].

CNP pain sensitivity is increasing, which is mostly chronic, persistent, spontaneous, recurrent and intractable in the pelvis. Some scholars believe that inflammation is only one of the causes of prostate pain[21], whereas others suggested that while CNP pain may be due to a single cause, there were many factors that contributed to the gradual development of a chronic neuropathic state as the disease progressed[22]. This pain may be related to secondary lesions of the L5-S2 spinal segment that dominated the prostate[23]. After Chen et al. chemically stimulated the bladder, prostate, and involved pain sites (root of tail) in rats, the expression of c-fos-positive cells in the lumbosacral spinal cord was abundant, while the distribution of c-fos-positive cells was similar in these three areas[23]. Zhang et al.'s study showed that highly expressed SP was involved in the generation and maintenance of neuropathic pain[24]. Xu et al. found that plasma SP levels in patients with CNP were significantly higher than those in the control group, suggesting that the expression of SP was closely related to the occurrence and development of CNP. Through further analysis, they found that plasma SP levels gradually increased with the progression of the disease which suggested a certain relationship between the SP levels and degree of the patient's condition[25].

SP is an important pain transmitter. When noxious stimuli appear, SP is transmitted to the central nervous system. At the same time, the reverse release of SP was also found in the damaged local tissues, which can cause neurogenic inflammatory reactions that are closely related to the occurrence of inflammatory pain, hyperalgesia, and neurogenic pain and are involved in the transmission of peripheral pain stimuli to the center[26]. SP mainly exists on the C fibers of the spinal cord and is released in the spinal dorsal horn, where SP receptors are rich, and activates NK-1R to transmit pain information. Some scholars believe that the combination of SP and NK-1R mediates the production of pain[27]. When the nerve is stimulated, SP is released at the central and peripheral ends and combines with NK-1R to exert a physiological role. After being released from the central

segment, SP can directly or indirectly participate in pain transmission by promoting the release of glutamic acid and aspartic acid[28]. Through *in vivo* experiments, some researchers have found the lowest limit of L- and T-type calcium channel blockers affecting the release of SP from rats injected with formalin, by detecting the internal changes of neurokinin receptors[29].

In this study, different degrees of chronic inflammation and proliferative pathological changes were observed in the prostate of rats in different groups. While the levels of TNF- α , IL-1 β , IL-2, and IL-10 increased, the PWT in rats decreased, which suggested an increased sensitivity to pain, thereby indicating that the CNP model established by the autoimmune method was successful. The expressions of SP and NK-1R in the dorsal horn of L5-S2 spinal cord in the 45 d, 60 d, and 90 d groups were higher than those in the control group, the difference was statistically significant ($P < 0.05$); this result was consistent with the findings of Xu[25] and Liu[30]. SP can also stimulate the secretion of cytokines such as TNF- α and IL-1 β , which further aggravate chronic inflammation of the prostate and cause increased pain. The PWT of the 45 d, 60 d, and 90 d groups was lower than the PWT of the control group. The PWT of the 90 d group was lower than those of the 45 d and 60 d group ($P < 0.05$), further proving that SP can aggravate the degree of pain in CNP models.

CNP is a slow and chronic disease with very complex and pathological processes. In this rat model of CNP, we extended the disease duration to 45, 60, and 90 days to investigate the expression of SP and NK-1R in the posterior horn of the L5-S2 spinal cord at the different time points of modeling. The results were significantly different for the studied time points, suggesting that the expressions of SP and NK-1R are a dynamic process, which is more in line with clinical manifestations.

Conclusion

In summary, the expression of SP and NK-1R in the spinal dorsal horn of L5-S2 of CNP rats is up-regulated, and the changes are different at different time periods, which is closely related to the refractory pain of chronic prostatitis. The conclusion of this study suggests that clinical research on new drugs that adjust nerves or adjust inflammatory cytokines to relieve pain from chronic prostatitis, the specific mechanism of which needs to be further studied.

Abbreviations

SP: substance P; NK-1R: neurokinin-1 receptor; CNP: chronic nonbacterial prostatitis; PWT: paw withdrawal threshold; CP: chronic prostatitis

Declarations

Acknowledgement

Not applicable.

Authors' contributions

Concept and design: Xinhua Zheng and Liya Hao. Collection and assembly of data: Junfeng Zhang, Xianguang Bai, Li Liu, Jinxu Qi and Zhonglin Wang. Data analysis and interpretation: Xinhua Zheng, Liya Hao, Junfeng

Zhang,Xianguang Bai,Li Liu and Jinxu Qi. Model making:Wanjie Zhang and Yubin Zuo.Manuscript writing: All authors. Final approval of manuscript: All authors.

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

Ethics approval and consent to participate

All protocols in this study have been approved by the Experimental Animal Ethics Committee of Shanghai University of Traditional Chinese Medicine (ethics approval number: SZY201807016). The research also complies with the "Guidelines for the Care and Use of Laboratory Animals" published by the National Institutes of Health (NIH No. 85-23, revised in 1996).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Results of HE staining of the prostate tissue from different groups of rats (scale bar: 50 μ m). A: No inflammatory cells infiltration was seen in the prostate tissue of the control rats. B: Lymphocytes were visible in the prostate tissue from the rat model of chronic nonbacterial prostatitis (CNP) at 45 days after modeling. C: Edema was more obvious in the CNP rat model, with large numbers of lymphocytes at 60 days after modeling. D: A large number of lymphocytes and mast cells were found in the CNP rat model, with a few neutrophils and plasma cells at 90 days. The black arrows indicate lymphocytes. The glandular cavity was expanded and collagen fibers were proliferated. Bar=50 μ m.

Figure 2

Prostate tissues from different groups of rats as observed under transmission electron microscopy A: The structure of the prostate was normal in the control group. B: Mast cells were observed at 45 days after modeling. The yellow arrow indicates mast cells. C: Widened intercellular space of the epithelium along with lymphocytes and macrophages were seen at 60 days after modeling. D: Fibrosis and edema were found in the Prostate interstitial at 90 days after modeling.

Figure 3

Expression of Substance P (SP) in the posterior horn of the L5-S2 spinal cord in different groups of rats (IHC: 40×) A: L6 in the control group; B: L6 in the 60 d modeling group; C: S2 in the control group; D: S2 in the 90 d modeling group.

Figure 4

Expression of neurokinin-1 receptor (NK-1R) in the posterior horn of the L5-S2 spinal cord in different groups of rats (IHC: 40×) A: L5 in the control group; B: L5 in the 90 d modeling group; C: S2 in the control group; D: S2 in the 60 d modeling group.