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The inhibitory effect of neurotropin on inflammation in rats with lumbar disc herniation based on the c-JNK/CXCL1 signaling pathway

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ABSTRACT

This study aimed to investigate the inhibitory effect and mechanism of neurotropin on inflammation in rats with lumbar disc herniation. For this purpose, forty-eight rats were randomly divided into sham group, autologous nucleus pulposus transplantation model group (NP group), neurotropin treatment group (NP+NT group), and solvent [normal saline (NS)] control group (NP+NS group). After 7 days of intervention, the mechanical paw withdrawal threshold (PWT) and thermal paw withdrawal latency (PWL) of the rats were measured, and the expression levels of Iba-1, c-JNK and CXCL1 in spinal cord tissues were measured by Western blot. The levels of tissue-associated inflammatory and anti-inflammatory factors in the spinal cord were detected by ELISA. Results showed that Neurotropin significantly alleviated mechanical and thermal hyperalgesia induced by NP transplantation and reduced levels of Iba-1, c-JNK, and CXCL1 proteins in the spinal cord tissue. In addition, neurotoxins also lowered concentrations of the inflammatory factors IL-1β, IL-6 and TNF-α. It was concluded that Neurotropin has an inhibitory effect on lumbar disc herniation-induced spinal cord inflammation through inhibition of the c-JNK/CXCL signaling pathway.

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Introduction

Lumbar disc herniation (LDH), a common disease in clinical practice, is featured by the long treatment and high recurrent rate (1), which is found to be caused by the degeneration of lumbar disc herniation, with the manifestation of protrusion of the nucleus pulposus due to the rupture of the fibrous ring that further stimulates or suppress the nerve root, vessels and spinal cord, eventually resulting in the back pain, sometimes accompanied by the radiating pains in lower limbs.

Neurotropin (NTP), as a kind of non-protein physiologically active substance isolated from the skin dermis of rabbits with inflammation due to the vaccination of vaccinia virus, is found to be active in analgesia, rectifying the disturbance of vegetative nerve function and peripheral circulation, antagonizing the allergic reaction, regulating the immune function and repairing the cell injury (2). Clinically, NTP has been noted to be effective in the treatment of back or leg pains caused by LDH, the strain of lumbar muscles and hyperostosis (3). However, how NTP ameliorates the pains caused by LDH remains unclear, so we, in this study, prepared the LDH models on rats via autograft of nucleus pulposus (NP) to investigate the inhibitory effect of NTP on the inflammation and the underlying changes in c-JNK/CXCL1 signal pathway in LDH rats.

Materials and methods

Animals

Adult, male, Sprague-Dawley (SD) and specific pathogen-free (SPF) rats (weight between 200 and 250 g) were purchased from Guangdong Medical Laboratory Animal Center (Animal production license: SCXK (Guangdong)2019-0035) and fed at a 12/12 day/night cycle and 25°C, with free access to food and water. All animal-related experiments were approved by the Animal Ethical Committee of Guangdong Medical University.

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Reagents

Major reagent: Neurotropin was purchased from Nippon Zoki Pharmaceutical Co., Ltd OnoGreeneryFactory. ELISA kit was purchased from Boster Biotech Co., Ltd. The rabbit anti-rat anti-Iba-1 and CXCL1 monoclonal antibodies were purchased from Abcam (US).

Model establishment, grouping and treatment

LDH models were prepared by using the autologous transplantation of nucleus pulposus. In brief, rats were anesthetized by injection of pentobarbital sodium, followed by the semi-laminectomy for L4 – L5 segment by exposing the nerve root of L5. Rats were fixed in the supine position, and from the caudal vertebra, NP (about 5 mg) was placed on the exposed L5 nerve root slightly. Finally, muscle and skin were sutured layer by layer. Rats in the Sham group received the same operation but with no transplantation of NP on the nerve root. Forty-eight rats were divided randomly into 4 groups: Sham group, NP group, NP + NT group and NP + NS group. Rats in the NP + NT group would take NP at 1 h after the model establishment by intraperitoneal injection at a dose of 1 mL/kg for consecutive 7 days, and those in the NP + NS group would take NS in the same amount.

Detection of paw withdrawal threshold and thermal withdrawal latency

The classical up-down method and heat plate (7370, UgoBasile) were adopted to detect the paw withdrawal threshold (PWT) (4) and paw withdrawal latency (PWL) (5) as the previously mentioned methods, and any paw withdrawal or licking feet was taken as positive reactions.

Detection of pro-inflammatory cytokines in the spine

L4 – L5 spine was taken from rat and homogenized in PBS, followed by centrifugation to obtain the supernatant. The supernatant was subjected to the ELISA and the optical density of samples was determined at 450 nm to calculate the concentration of IL-1β, IL-6 and TNF-α in tissue.

Western blotting

Rats were anesthetized by intraperitoneal injection of pentobarbital sodium to open the vertebra to obtain the L4 – L5 segment which was placed in the liquid nitrogen rapidly. Then, cornu dorsale medullae spinalis were separated and placed in the pre-cooled lysis buffer for homogenizing to obtain the total protein. Then, the protein concentration was determined by using the BCA method, and, accordingly, proteins were taken for the SDS-PAGE and then transferred onto the PVDF membrane, where the unoccupied sites were blocked in 5% non-fat milk for 1 h. Proteins on the membrane were incubated with the primary antibodies at 4°C overnight at a dilution of 1:1000. The membrane was then rinsed in PBST to remove the unbound antibodies and then incubated with the secondary antibodies at a dilution of 1:1000 at room temperature for 6 h. Thereafter, the resulting immunoblots were further subjected to enhanced chemiluminescence to develop the protein bands on the membrane. With β-actin as endogenous control, the expression of Iba-1, p-c-JNK and CXCL1 was analyzed.

Statistical analysis

In this study, SPSS 13.0 software was utilized to perform the statistical analysis. Data were expressed in form of mean ± standard deviation (SD), while the comparison of data among groups was performed with the one-way ANOVA, followed by the LSD-t test for pairwise comparison. The difference at $P < 0.05$ had statistical significance.

Results and discussion

Comparison of the general condition of rats among groups

Rats in the Sham group performed normally, with normal intake and activities; those in the NP group acted sluggishly, with a significant reduction in activities, food intake and weight, while rats in the NP + NT group had quite opposite changes in indexes above.

Comparison of PWT and PWL of rats among groups

As compared to the Sham group, rats in the NP group had sharp decreases in PWT and PWL after transplantation of NP ($P < 0.01$), lasting for 15

postoperative days; in comparison with the NP + NS group, rats in the NP + NT group, after seven days of intraperitoneal injection of NT, showed significant increases in PWT and PWL ($P < 0.05$; Figure 1). Thus, NT could increase the PWT of NP rats, with relief of allergy to mechanical pains and hypersensitivity to heat pains.

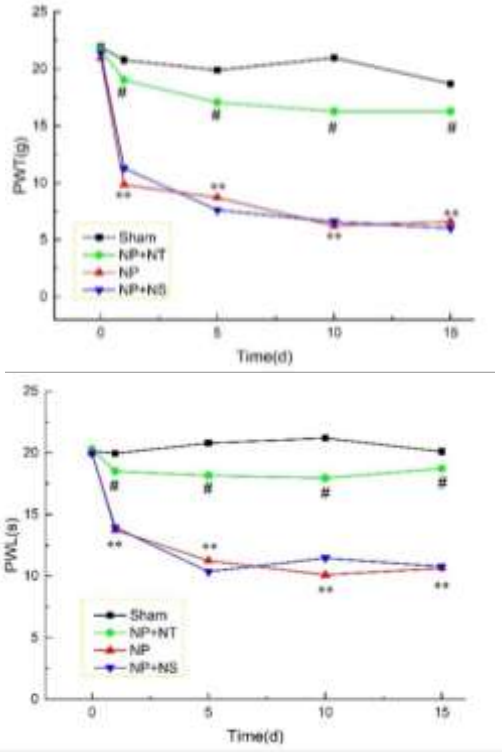

Figure 1. NT prolongs the PWT and PWL of NP rats. In comparison with the Sham group, rats in NP group had sharp decreases in PWT and PWL, while NP could reverse. ** $P < 0.01$ *vs.* the Sham group, # $P < 0.05$ *vs.* the NP + NS group.

Comparison of IL-1β, IL-6 and TNF-α levels in the spine of rats among groups

As compared to the Sham group, rats in the NP group had a significant increase in the levels of IL-1β, IL-6 and TNF-α ($P < 0.05$), while those in the NP + NT group experienced sharp decreases as compared to the NP + NS group ($P < 0.05$; Figure 2). As such, NT is able to reduce the levels of IL-1β, IL-6 and TNF-α in the spine of rats, thus inhibiting the nerve inflammation caused by NP transplantation.

Comparison of levels of Iba-1, p-c-JNK and CXCL1 in spine tissues of rats

Compared to the sham group, Iba-1, p-c-JNK and CXCL1 in rats of NP group were elevated significantly; in comparison with the NP + NS group, NT treatment decreased the levels of Iba-1, p-c-JNK and CXCL1 (Figure 3).

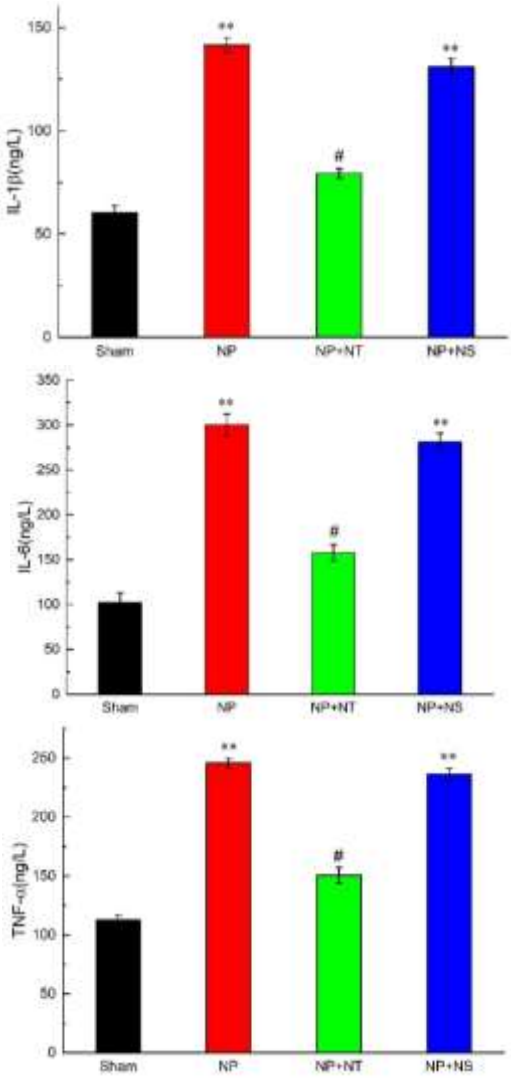

Figure 2. NT inhibits the nerve inflammation caused by NP transplantation in rats. In comparison with the Sham group, rats in the NP group had evident increases in levels of IL-1β, IL-6 and TNF-α, while NT treatment reduced the levels. N = 4, ** $P < 0.01$ *vs.* the Sham group; # $P < 0.05$ *vs.* the NP + NS group.

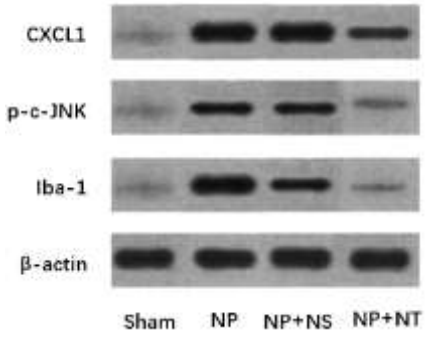

Figure 3. Expression of Iba-1, p-c-JNK and CXCL1 in cornu dorsale medullae spinalis of rats

LDH, as a common condition in clinical orthopedics, is mainly caused by the protrusion of the nucleus pulposus that suppresses the spinal nerve and cauda equina that may result in the dysneuria, with the symptoms of back pains, leg pains or incontinence of urine and feces, and any delay of treatment may involve the dysfunction in key organs, like heart and brain (6). At present, clinical medication for LDH includes hormones, non-steroidal anti-inflammatory drugs and opiates. According to the published literature (7), NT could inhibit or delay LDH and alleviate the pains or numbness caused by LDH. Thus, we wish to probe the role of NT with LDH rats to provide theoretical evidence for clinical application.

NT has been found to be able to facilitate the growth of axons and proliferation of Schwann cells and inhibit the increases in membrane potentials of nerve cells caused by hypoxia; moreover, it also has the analgesic effect by activating the descending pain-inhibition system and inhibiting the release of bradykinin; furthermore, it could modify the nerve symptoms by adjusting the function of the vegetative nervous system and regulate the immune system (8). Research (9) has shown that NT in combination with mecobalamin can mitigate the pains of LDH, with the recovery of impaired nerves and relieve numbness and paresthesia.

Interleukin (IL) has been reported to affect the activity of matrix metalloproteinases (MMPs) and suppress the synthesis of proteoglycan in MMPs to affect the intervertebral disc. IL-6, as a kind of important inflammatory cytokine, can recruit the inflammatory factors and reduce the TIMP-1 activity and synthesis. Any reduction of TIMP-1 could trigger the changes in proteins inside the intervertebral disc, resulting in the loss of elasticity of the fibrous ring and curbing the constraint of the fibrous ring on the nucleus pulpous, eventually developing LDH (10). IL-1β, as reported, could induce the generation of IL-1α, IL-6, TNF-α and PGE2, presenting a much stronger algesic effect that IL-1α (11). TNF-α, by promoting the release of inflammatory factors, can accelerate the proliferation of osteoclast and apoptosis of chondrocytes, thus leading to degenerative changes in the intervertebral disc (12). LDH rats, as reported (13), present increases in the IL-1α, IL-6 and TNF-α, while the corresponding treatment could reverse such changes, which coincides with our findings that NT could reduce the levels of IL-1α, IL-6 and TNF-α, suggesting that NT could suppress the nerve inflammation caused by NP transplantation.

JNK, as a pivotal member of MAPK, is a kind of signal transduction molecule in cells that play a key role in post-traumatic stress reaction that can induce apoptosis and also associate with proliferation (14). In cells affected by the stimuli of bleeding or pains, c-JNK phosphorylation increases, which promotes the transcription of c-JNK, thereby regulating the post-traumatic stress reaction and inflammation (15). Moreover, p-c-JNK has a higher expression in the degenerative intervertebral disc as compared to the normal intervertebral disc, suggesting that p-c-JNK may relate to the degenerative changes in the intervertebral disc (16). Iba-1, as a marker of glial cells, has been found to be able to inhibit the activation of Iba-1 to relieve the generation of chronic pains (17). Niu H *et al*. (18) found that the gavage of Zhuijianpan Pill could reduce the expression of Iba-1 and CXCL-1 in cornu dorsale medullae spinalis. Similar results were also obtained in this study – NT could reduce the levels of Iba-1, p-c-JNK and CXCL1 as compared to the NP group, suggesting that NT has an analgesic effect by regulating c-JNK/CXCL1 signal pathway.

In conclusion, NT could inhibit the spinal inflammation caused by intervertebral disc degeneration, which may be achieved by regulating c-JNK/CXCL1 signal pathway. Our findings may provide novel theoretical evidence for clinical treatment of LDH.

Acknowledgments

None.

Conflict interest

None.

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