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Study on Sympathetic Nerve Pain Conduction during Discogenic Low Back

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Abstract

Objective: To explore the functions of the sympathetic nerves during discogenic low back pain.

Method: Adult Wistar rats were divided into three groups: in one group, the sympathetic nerve fiber was preserved; in the second group, the sympathetic nerve fiber was severed; and in the third group, the sympathetic nerve fiber was preserved on one side and severed on the other side. Sections of the spinal ganglion samples from all rats were subject to horseradish peroxidase (HRP) retrograde tracing, retrograde fluorescence double labeling, and immunohistochemical methods for PAP and SP detection.

Result: The number of HRP-positive cells was significantly higher in the sections from samples where the sympathetic nerve was preserved. HRP-positive and SP-positive doubly labeled cells were found in spinal ganglions on both sides. In spinal ganglions on the right side of L2, fluorescence doubly-labeled cells were found, some of which contained calcitonin gene-related peptide.

Conclusion: Discogenic low back pain is a type of referred pain caused by lesions of the lumbar intervertebral disc that is conducted by sympathetic nerves on both sides and mainly implicates (lumbar) areas innervated in segments by the L1 and L2 lumbar nerve posterior ramus. Conduction of pain by sympathetic nerve fibers in the lumbar paravertebral sympathetic trunk is the major mechanism underlying discogenic low back pain.

Keywords: Paravertebral sympathetic trunk, discogenic low back pain, spinal ganglion, referred pain, neuropathic pain.

1. Introduction

Modern imaging technologies such as CT and MRI and emerging treatment methods have made it easier to diagnose and treat lumbar intervertebral disc prolapse in patients who suffer from lumbocrural pain (Marchi L. et al., 2012) (Manchikanti L. et al., 2010). However, there are increasing reports about patients with low back pain typically associated with disc prolapse, who do not show any abnormalities on CT, MRI and other examinations. Alternatively, lesions of intervertebral discs are believed to be one of the main reasons for low back pain (Mooney V., 1987); mechanical stimulation of or injection of salt water or contrast agent into the intervertebral discs can all cause low back pain (Kuslich SD. et al., 1991; Moneta GB. et al., 1994). Lesions of the lumbar intervertebral discs are often observed on the caudal side of the L4-5 and L5-S1 discs, and low back pain often involves pain in the areas innervated by the posterior ramus of the lumbar nerves in the upper part. However, it is not clear how the posterior ramus of the upper lumbar nerves can trigger low back pain, as this cannot be clearly explained based on mechanical oppression. Therefore, it is important to understand nerve distribution in the caudal part of the lumbar intervertebral disc and posterior

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longitudinal ligament, in order to elucidate the mechanism underlying discogenic low back pain.The posterior longitudinal ligament is thought to be innervated by branches of the sinu-vertebral nerve. There is some debate among scholars about the origin of the sinu-vertebral nerve. While some believe that is composed of branches extending from the anterior lumbar nerve branch and the sympathetic nerve (Schliessbach J. et al., 2010)(Rennie C. et al., 2013)(Bogduk N. et al., 1988)(Bogduk N. , 1983), others believe that the two roots that form the sinu-vertebral nerve do not originate separately from the spinal nerve and the sympathetic nerve, but rather both originate from either the spinal nerve or the sympathetic nerve (Groen GJ. , 1990). Moreover, the distribution and branching of the sinu-vertebral nerve after it enters the vertebral canal are also not very clear. The most common belief is that after entering the vertebral canal, the sinu-vertebral nerve approaches the posterior longitudinal ligament and divides into an ascending branch, a descending branch and a transverse branch; these branches communicate not only with branches on the opposite side but also with branches of the neighboring anterior and posterior segments (Bogduk N. , 1983)(Edgar MA & Ghadially JA, 1976). In 1990, Kojme (1990) proved that the ascending and the descending branches send out transverse branches, and that the ascending, descending and transverse branches together form the posterior longitudinal ligament nerve network. In addition, some scholars reported that another type of nerve fiber that was different from the sinu-vertebral nerve is distributed behind the lumbar intervertebral discs; these never fibers extend from the anterior spinal nerve branch or the communicating branch and are directly distributed in the area at the back of and outside the annulus fibrosus. Nakamuka (1996) believes that the shallow annulus fibrosus layer and the posterior longitudinal ligament nerve network are composed of nerve fibers sent out by spinal ganglions on both sides and in multiple segments and that a portion of the nerve fibers inside the lumbar paravertebral sympathetic trunk are sympathetic.

Some scholars proposed that a large amount of clustered non-myelin free nerve endings, which are typically associated with nocireceptors, are present in the lumbosacral region. Moreover, Stilweel reported that posterior longitudinal ligaments have distributions of both unmyelinated fibers and myelinated fibers with only one type of ending—free nerve endings. However, Kojima (1990) recently reported the presence of round or oval nerve endings in addition to free nerve endings in the deep web at the intervertebral disc region of the posterior longitudinal ligament, but only an extremely small amount of these nerve endings was found. With regard to the intervertebral discs, most scholars have proved that nerve fibers enter the superficial layer of the annulus fibrosus, especially its posterior superficial layer, where there are comparatively many free endings; however, there is no nerve distribution in the deep part of the annulus fibrosus and the nucleus pulposus (Kojima Y. et al., 1990) (Yoshizawa H. et al., 1980). Physiological studies report that pain actuations are mainly conducted by non-myelinated or thin-myelin Aδ and C fibers that are comparatively slim; therefore, slim nerves with free endings are probably distributed in the posterior part of the intervertebral disc and the posterior longitudinal ligament. In 1993, Minaki (1993) provided evidence for the distribution of C fibers in the area that conducts pain. In agreement with this, in recent years, nerve fibers in the lumbar intervertebral discs have been reported to contain SP and CGRP, which are molecules required for pain actuation (Ashton IK. et al., 1994) (McCarthy PW. et al., 1991). All these results seem to indicate that stimulation of the posterior lumbar intervertebral discs and the posterior longitudinal ligaments is closely related to the occurrence of lumbocrural pain.

The traditional view is that discogenic low back pain is mainly conducted by the spinal nerve branch of the sinu-vertebral nerves (Yoshizawa H. et al., 1980). However, in recent years, there has been some speculation that the afferent branch of the sympathetic nerves performs the most important functions in the conduction of low back pain (Suseki K. et al., 1997)(Brena S.F. et al., 1980)(Ohtori S. et al., 2009)(Groen G.J. et al., 1990). The human sympathetic trunk originates only from the spinal cord, at the T1-L2 segments (Williams PL. et al., 1989). Foerster have proved that the L2 nerves are distributed in the lumbar area in segments. Moreover, Takahash (1996) reported referred inguinal pain in rats injected with capsaicin in the anterior L5-6 intervertebral disc. This referred pain in the inguinal region caused by lesions in the anterior intervertebral disc was further proved using electrophysiological experiments (Takahashi Y. et al., 1998).

Studies have shown that the L5-6 lumbar facet joint and the posterior part of the L5-6 intervertebral discs are also innervated by nerve fibers in the sympathetic trunk on both sides and in multiple segments (Nakamura S. et al., 1996) (Suseki K. et al., 1987) (Raoul S. et al., 1987) (Higuchi K & Sato T., 2002) (Aoki Y. et al., 2004). Lesions of the lumbar intervertebral discs mostly occur in the posterior part of the lumbar vertebral discs, while low back pain is mostly seen in the low back region and the buttocks; therefore, it is important to study the neuroanatomical relationship between the posterior part of the lumbar intervertebral discs and the low back region and the buttocks in order to study discogenic low back pain. This study uses retrograde horseradish peroxidase (HRP) and fluorescence double labeling in combination with immunohistochemical methods to study the internal relationship between nerve distributions in both regions and to further explore the mechanism of discogenic low back pain.

2. Materials and Methods

2.1. HRP retrograde tracing method

Healthy adult Wistar rats (purchased from the Animal Experiment Center of the Second Affiliated Hospital of Harbin Medical University) weighing 250-300 g, regardless of gender, were used for the experiments. Three groups of seven rats each were used for the HRP tracing experiment.

In group 1 (control group in which the sympathetic trunk was preserved) (Figure 1), the rats were anesthetized by injection of 0.6% sodium pentobarbital into the abdominal cavity, after which the lumbar intervertebral discs were resected from the back to expose the right caudal sidewalls of the L5-L6 intervertebral discs. From 1.0 mm to the right of the posterior midline, 1 μL of 30% horseradish peroxidase (HRP) (R.Z > 3.0, provided by Shanghai Lizhu Dongfeng Biotechnology Co. Ltd.) was injected into the right posterior sidewall of the L5-L6 intervertebral discs using a microsyringe, under direct observation. After 48 h, the rats were anesthetized again and were intravenously administered 500 ml of stationary liquid (2% paraformaldehyde buffered with 0.1 mol/L phosphate solution, pH7.4, 4°C) for an hour. Immediately after the injection, spinal ganglions on both sides of L1 and L2 were resected and fixed in the abovementioned stationary liquid for 3 h; the spinal ganglions were then immersed overnight in a 0.1 mol/L phosphate buffer (pH7.4, 4°C) containing 30% sucrose. Each sample was then used to make 7-10 sections that were 40 μm thick, using a crytostat. All sections were then treated with tetramethylbenzidine (TMB) according to the Mesulam (1978) method, for HRP tracing. In 24 h, the stained cells could be observed with the naked eyes, and the number of cells was counted.

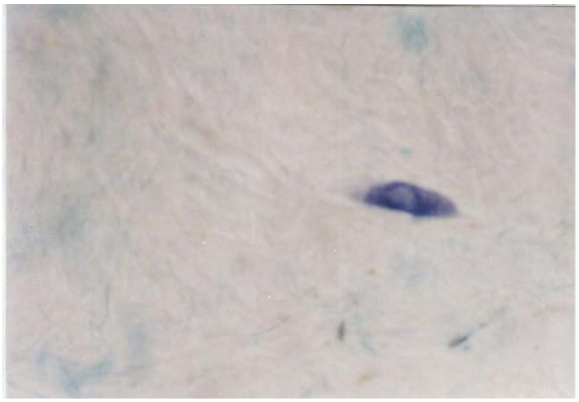

Figure 1: **HRP-Positive Cells in The L1-2 Spinal Ganglia of Rats (TMB staining)**

In group 2 (experimental group in which the sympathetic trunk was severed), the rats were anesthetized as described for group 1; then, the sympathetic trunks alongside both sides of L1-L6 were severed via the abdominal cavity. The remaining steps are the same as those described for group 1.

In group 3 (control group in which only the sympathetic trunk along the right side was severed), the rats were anesthetized as described before, and the sympathetic trunk along the right side of L1-L6 was severed via the abdominal cavity, with the sympathetic trunk on the left side preserved. The remaining steps are the same as those for the other groups.

There was another group of seven Wistar rats that underwent the same procedures described for group 1. The steps that follow are different. After TMB color development, the sections were fixed via immersion in cobalt chloride containing DAB, and subject to the PAP method. This is the procedure: The sections were treated with 0.2% Triton X-100 for 30 min and then 1:50 normal goat serum for another 30 min. They were then incubated for 72 h with rabbit anti-SP serum, after which the sections were treated with goat anti-rabbit immunoglobulin G for 1 h and then the peroxidase-anti-peroxidase complex for 1 h. They were then subject to color development reactions using dimethylbenzidine and hydrogen peroxide. The sections were flushed with phosphoric acid saline

buffer (pH7.4) between treatments. After the reactions, the samples were dehydrated, transparentized, section-sealed, and observed under a light microscope.

In order to confirm the immunological specificity of the experiments, two separate experiments were conducted: (1) Replacement experiment: normal rabbit serum was replaced with rabbit anti-SP serum. (2) Absorption experiment: SP antigen was used to absorb rabbit anti-SP serum, which was left overnight in a refrigerator; it was centrifuged the next day, with the supernatant was used as the anti-SP serum. Remaining procession of the two control groups are the same with those of the experiment groups.

2.2. Retrograde fluorescence double-labeling method

Seven Wistar rats were anesthetized by abdominal injection with 10 g/L pentobarbital sodium (40 mg/kg). Then, an incision was made along the center to expose the dorsal muscles on the right. The posterior ramus of the L2 nerves were then exposed from the dorsal muscle on the right corresponding to the L2 segment (positioned based on the last segment of the thoracic vertebra). Then, 1 μL of 20 g/L fast blue (FB) was injected with a microsyringe into the right posterior ramus under a microscope. After 40 h, the rats were anesthetized again, and their lumbar vertebral discs were resected to expose the right posterior sidewall of the L5-6 vertebral discs. At a point 1.0 mm to the right of the central line, 2 μL of 10 g/L nuclear yellow (NY) was injected using a microsyringe into the right posterior sidewall of the L5-6 vertebral discs under direct observation. After 8 h, the rats were again anesthetized and then injected with the stationary liquid mentioned before. Immediately after the injection, spinal ganglions on both sides of L1-L6 were resected and immersed in 0.1 mol/L phosphate buffer (pH7.4, 4°C) containing 300 g/L sucrose. Four hours later, four frozen serial sections were made, each 20 μm thick. The sections were mounted on gelatin slides for observation and recording under a fluorescence microscope with UV filter (356 nm).

We then carried out immunohistochemial experiments on the sections. Calcitonin gene-related peptide (CGRP, 1:2000) was used as the primary antibody; goat anti-rabbit immunoglobulin G was used as the secondary antibody; and rabbit anti-PAP compound (1:200) was used as the tertiary antibody. Color development reactions were induced using 0.1 M Tris2-HCL buffer solution containing 0.05% DAB and 0.01% H2O2. In order to confirm immunological specificity of the experiment, the two-control replacement and absorption experiments mentioned before were conducted.

2.3. Statistical analysis

SPSS statistical analysis software (11.0) was used to carry out all statistical analyses. All data were expressed as mean ± standard deviation (X ± S). Analysis of variance and F examination were adopted for comparison of the sympathetic trunk preserved group and the sympathetic trunk severed group. t' examination was used for comparison between the control groups. $P < 0.05$ indicates statistical significance.

3 Result

3.1. HRP tracing

HRP-positive cells were found in spinal ganglions on both sides of L1 and L2 in rats of all groups: a large amount of blue or deep blue granules (products of the HRP-TMB reaction) were found in the cytoplast (Figure 1). The HRP-positive cells were mostly medium to small in size, with diameters ranging from 20 to 40 μm. In the group in which the sympathetic trunk was preserved(Figure 2), the difference in the amount of HRP-positive cell between both sides was not significant ($P > 0.05$), but the difference was obviously greater than that observed in the group in which the sympathetic trunk was severed; the difference between the two groups was significant ($P < 0.01$; Tables 1 and 2). In rats of the control group, the amount of HRP-positive cells in spinal ganglions on the severed side (right side) was significantly lower than that on the preserved side (left side) ($P < 0.01$; Table 3).

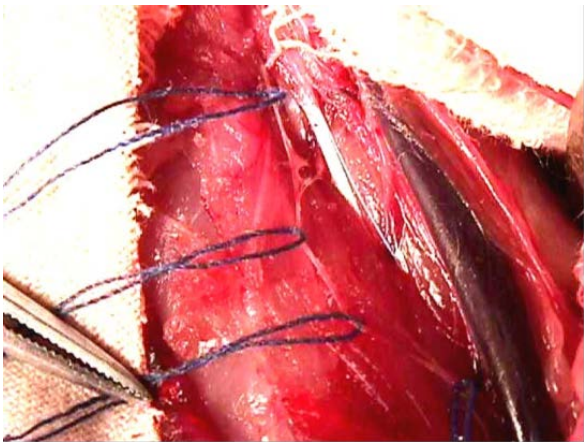

Figure 2: **Lumbar Paravertebral Sympathetic Trunk (l1-l6) On the Right Side**

Table 1: **Comparison of The Number Of hrp-Positive Cells in The L1-2 Spinal Ganglia of Rats Between the Control Group (Preserved Sympathetic Trunk) And Experimental Group (Severed Sympathetic Trunk)**

Spinal ganglion	Control group		Experimental group	
	No. of sections	No. of HRP-positive cells	No. of sections	No. of HRP-positive cells
Left L1*	58	22.948 ± 7.681	55	13.164 ± 5.284
Left L2**	55	27.382 ± 6.988	51	13.275 ± 4.928
Right L1△	57	22.491 ± 7.089	58	13.328 ± 4.861
Right L2△△	56	29.304 ± 9.688	53	12.943 ± 5.051

*F = 289.02, P = 0.0001(<0.01); **F = 663.58, P = 0.0001 (<0.01); △F = 293.21, P = 0.0001 (<0.01); △△F = 442.40, P = 0.0001(<0.01)

Table 2: **Comparison of The Number Of HRP-Positive Cells in The Left and Right L1-2 Spinal Ganglia in Rats of The Control Group (Preserved Sympathetic Trunk)**

Spinal ganglion	Left		Right	
	No. of sections	No. of HRP-positive cells	No. of sections	No. of HRP-positive cells
L1*	58	22.948 ± 7.681	57	22.491 ± 7.089
L2**	55	27.382 ± 6.988	56	29.304 ± 9.688

*T′ = 0.3314, P = 0.7409 (>0.05); non-parametric test: P = 0.5626(>0.05)

**T′ = -1.1795, P = 0.2410(>0.05); non-parametric test: P = 0.2996(>0.05)

Table 3: **Comparison of The Number Of HRP-Positive Cells in The Left and Right L1-2 Spinal Ganglia in Rats of The Control Group (Sympathetic Trunk Preserved on The Left Side and Severed on The Right Side)**

Spinal ganglion	Left		Right	
	No. of sections	No. of HRP-positive cells	No. of sections	No. of HRP-positive cells
L1*	58	24.793 ± 6.717	59	15.763 ± 4.443
L2**	58	30.621 ± 8.391	57	14.509 ± 4.115

*T′ = -8.5623, P = 0.0001(<0.01); non-parametric test: P = 0.0001(<0.01)

**T′ = -13.1068, P = 0.0001(<0.01); non-parametric test: P = 0.0001(<0.01)

Some of the doubly-labeled cells were positive for PAP, as evidenced by the presence of dark brown granules. No positive reactions were observed in the control experiments. (Figure 3)

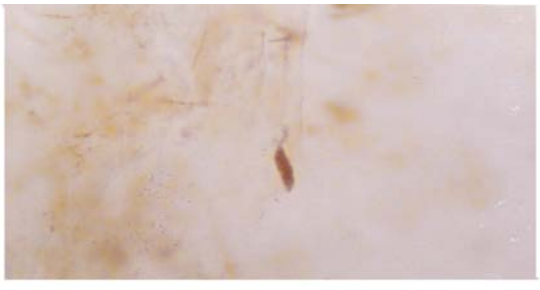

Figure 3: **HRP-SP Double-Labeled Cells in The L1-2 Spinal Ganglia of Rats (TMB And PAP Staining)**

3.2. Fluorescence double-labeling

In spinal ganglions of the L2 segment on the right, some FB singly-labeled cells or NY singly-labeled cells as well as FB/NY doubly-labeled cells could be seen (Figure 4). The cytoplasm of FB singly-labeled cells was stained blue, with no fluorescence visible in the nucleus, because of which the cells appear hollow. The cytoplasm of NY singly-labeled cells did not exhibit fluorescence, while the nucleus was stained yellow. The cytoplasm of FB/NY doubly-labeled cells was stained blue, and the nucleus was stained yellow. On the right side of L2, the FB/NY doubly-labeled cells comprised 3.41% of the total labeled cells. Except for spinal ganglions on the right side of the L2 segment, doubly-labeled cells were not observed in spinal ganglions of any other segment.

Some of the doubly-labeled cells were positive for PAP, as evidenced by the presence of dark brown granules. No positive reactions were observed in the control experiments.

Figure 4: **FB/NY Doubly-Labeled Cells on The Right Side of The L2 Spinal Ganglia of Rats**

4 Discussion

Our study provides evidence for the existence of an anatomical relationship between nerve fibers that conduct pain in the lumbar paravertebral sympathetic trunk and those that conduct pain in the posterior ramus of the lumbar nerves. Two types of fluorescence dyes were separately injected into the posterior ramus of the second lumbar nerves on the right side and into the sidewall at the right back of the L5-6 intervertebral discs; doubly-labeled cells were found in the right L1 and L2 spinal ganglions and some of these doubly-labeled cells showed positive immunohistochemcial reactions. Therefore, our results indicate that while some of the nerve cells in the L1 and L2 spinal ganglions send out their branches to form lumbar nerves, they may also send out branches that become a part of the sympathetic nerve fibers in the lumbar paravertebral sympathetic trunks that may conduct pain. These results are supported by several other studies in the field. In 1991, Kuslich and other scholars (1991) discovered during the course of their clinical practice that in patients given local anesthesia for an operation, mechanical stimulation of the inflammatory nerve roots may cause sciatica while stimulation of the exterior layer of the annulus fibrosus and posterior longitudinal ligament may cause low back pain. This not only indicates that lesions of lumbar intervertebral discs may cause low back pain, but also suggests that there may be a close neuroanatomical relationship between areas where low back pain occur and the exterior layer of the annulus fibrosus as well as the posterior longitudinal ligaments. Moreover, Nakamuka (1996) believes that low back pain involves the areas innervated in segments by the posterior ramus of the L1 and L2 lumbar nerves, since low back pain is often seen in the low back region and buttocks, where these nerves are usually distributed.

Existing research has shown that (Raoul S. et al., 003; Manchikanti L. et al., 2010) the posterior part of the lumbar intervertebral discs and posterior longitudinal ligaments are dually innervated by L-3 segmental sinu-vertebral nerves and sympathetic nerves. Sympathetic nerves originate from the segmental spinal ganglions above L2 and are capable of transmitting pain signals (Ohtori S. et al., 2007; Gillette RG. et al., 1987); stimulation of the lumbar paravertebral sympathetic trunks may induce lumbar pain while blocking lumbar paravertebral sympathetic nerves may relieve the low back pain. Based on these results, we think that lesions of the lower lumbar intervertebral discs may induce pain in areas where upper lumbar nerve branches are distributed.

Based on the current results and those reported previously,(Suseki K. et al., 1997)(Takahashi Y. et al., 2009)(Morinaga T. et al., 1996)(Takahashi Y. et al., 2000)(Chen J. et al., 2008)(Nakamura S. et al., 1996)(Konnai Y. et al., 2000)(Murata Y. et al., 2000), we have come to the conjecture that discogenic low back pain is a type of referred pain and that its occurrence can be explained by the convergence—projection theory of referred pain. Specifically, lesions of the lumbar intervertebral discs may be implicated in pain actuations in the annulus fibrosus and posterior longitudinal ligaments (Yukawa Y. et al., 1997)(Peng B. et al., 2007)(Peng B. et al., 2006)(Carragee EJ & Hannibal M., 2004); this type of pain stimulation is then conducted, via the sympathetic nerve fibers in the lumbar paravertebral sympathetic trunk, to the L1 and L2 spinal ganglions; some of these sympathetic nerves conduct the pain along the posterior ramus of the L1 and L2 lumbar nerves to the low back region, where they release pain signals at their endings, which further induce the generation of pain actuation; these actuations are then conducted to the nerve center along local sensory endings to generate referred low back pain. The conclusion indicates that stimulation or oppression of a patient's lumbar nerve roots may only cause skelalgia, but also low back pain (the sympathetic nerves are not inflicted); at the same time, the conclusion also explains why stimulation of the intervertebral discs may cause low back when corresponding segmental nerve roots are completely blocked.

In support of this conclusion, there are clinical reports about some patients with lumbar intervertebral prolapses who complain of inguinal and lower abdomen pain. Recently, some scholars have proved that this type of pain is actually referred pain associated with lumbar intervertebral disc lesions, and that the conducting nerves are the sympathetic nerves in the lumbar paravertebral sympathetic trunk. (Takahashi Y. et al., 2009; Morinaga T. et al., 1996; Takahashi Y. et al., 2000).

Discogenic low back pain has features such as inaccurate positioning and absence of clear points of tenderness, which are also characteristics of referred pain. Therefore, we believe that if the role of referred pain is taken into consideration and further studied in the context of discogenic low back pain, it could lead to new breakthroughs in the diagnosis and treatment of lumbocrural pain.

Conflict of interest statement

The authors declare no conflict of interests.

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