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# Exploration about changes of IL-10, NF-κB and MMP-3 in a rat model of cervical spondylosis☆

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ABSTRACT

*Objectives:* To investigate the relationship and mechanism between IL-10, NF-κB and MMP-3 in cervical degenerative disease induced by unbalanced dynamic and static forces in rats.
*Methods:* Sixty Sprague Dawley rats were randomized into test (n = 45) and control (n = 15) groups, which were randomly subdivided into three groups corresponding to one-month, three-month and six-month post-operation. Test group included 10, 15, 20 rats at corresponding postoperative stage and control group had five rats at each time point. By excising cervicodorsal muscles and ligaments of rats to establish unbalanced dynamic and static rat model in test group. The expression of IL-10, NF-κB and MMP-3 in the intervertebral disc tissue samples of both test and control group rats were detected by immunohistochemistry at one-month, three-month and six-month post-operation. The results were analyzed and compared among groups.
*Results:* Compared with the control group, the positive expression of IL-10 in test group was significantly higher at three-month ($P < 0.05$). In the same model group, IL-10 was highest at one-month. Compared with the control group, NF-kB in test group was higher at one-month, three-month and six-month. In the same model group, NF-kB was the highest at one-month, followed by the time at three-month and six-month. And, compared with the control group, MMP-3 was significantly higher in test group at one-month ($P < 0.05$).
*Conclusion:* Cervical degeneration may accompanied with the changes of IL-10, NF-κB and MMP-3.

## 1. Introduction

Cervical spondylosis is a serious and common degenerative disease, and it greatly impacts physical health and the quality of life of patients. However, the etiology and pathogenesis of cervical spondylosis have not been fully understood. It has been suggested that cervical degeneration were accompanied with the elevated of IL-10 and IL-1β which were confirmed in our preliminary experiment. At present, there are few studies on the role of IL-10 in the pathogenesis of cervical intervertebral disc degeneration and cervical spondylosis. Studer et al. (2007, 2008) found that both TNF-α and IL-1β can affect gene expression of MMP-3, TIMP-1, and ECM structural genes in vitro studies. And, it is well recognized that matrix metalloproteinases (MMPs) play vital roles in the degradation of ECM (Salminen et al., 2002; Stanton et al., 2011; Le Maitre et al., 2004). What's more, Hangai et al. (2008) considered that the progressive loss of aggrecan (Agg) and type II collagen (Col II) from the extracellular matrix (ECM) may accelerate the degenerative process in the nucleus pulposus (NP) of the intervertebral disc (IVD). At the same time, expression of MMP-1, MMP-3, and MMP-13 in chondrocytes is mediated primarily by active nuclear factor kappa B(NF-κB) (Liacini et al., 2003), So we suspected that NF-κB is an important catabolic pathway in the process of cervical degeneration. Zhongyi et al. (2015) suggests that inhibition of NF- κB pathway may protect discs from degeneration over time. And, IL-1 β promotes the activation of NF- κB, leading to upregulation of MMPs and degradation of disc matrix macromolecules Agg and collagen II. As scholars mainly concentrate on studying IL-1β, in our preliminary experiment, both IL-1β and IL-10 in test group significantly increased accompanied with the cervical degeneration. So we have reason to believe IL-10 may play the same important role in the process of IVD degeneration. At the same time, we suggest that the majority of cervical spondylosis patients may take drugs, physical therapy and other conservative treatment during

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the course which may interference the detection result to some extent. So we set up the rats' cervical spondylosis model (Zhongyi et al., 2015) by destructing the cervical equilibrium of dynamic and static forces, which will induce cervical degeneration. Dynamically detecting the positive expression of IL-10, NF-κB and MMP-3 in the intervertebral disc tissue samples of both test and control rats by immunohistochemistry. The results were analyzed and compared among groups to investigate the relationship between the changes of IL-10, NF-κB, MMP-3 and cervical degeneration,and provide laboratory basis and reference for clinical treatment of cervical spondylosis.

## 2. Material and methods

### 2.1. Animals

Sixty adult and healthy male Sprague Dawley (SD) rats (220–250 g) were randomized into test (n = 45) and control (n = 15) groups, which were subdivided into one-month, three-month and six-month post-operation. Test group included 10, 15, 20 rats at corresponding post-operative stage and control group had five rats at each time point. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Nanjing Medical University.

### 2.2. Model establishment

Rats in test group were solid-food fasted for 12 h before experiment, anesthetized with 10% chloral hydrate (0.3 ml/100 g) by intraperitoneal injection, and fixed on the operating table in the prone position with 50 ml falcon tube under the neck. The rat was unhaired on the nape of the neck, and disinfected, of which a 2–2.5 cm longitudinal incision was made at the midline to cut the skin and subcutaneous tissue. Every muscle layer was fully separated. Superficial muscles, platysma muscle, trapezius and rhomboideus, and deeper muscles, splenius cervicis muscle, longissimus capitis et atlantis, longissimus cervicis, hiocostalis cervicis and semispinalis capitis were transected successively, and 1.5 cm of those muscles were resected to avoid coalescence. At last, supraspinous ligament and interspinous ligaments from C2 to C7 were cut off before suturing skin layers successively, without removing the sutures, which came off naturally. The preparation before surgery and anesthesia for rat in pseudo-surgery (control) group was the same as rat in test group. The skin incision of rat in control group was sutured without resecting or cutting any muscle or ligament. Each rat was fed standard diet in individual cage under normal condition at 23–25 °C, with intramuscular injection of 50,000 units penicillin sodium for anti-infection after surgery.

### 2.3. Immunohistochemical analysis

Intervertebral disc samples post-fixed in 0.1 M phosphate buffer saline(PBS) containing 4% paraformaldehyde for 24 h at 4 °C and then embedded in paraffin. Paraffin embedded intervertebral disc samples were cut with a microtome, at 4 μm thick sections.

After deparaffinization, sections were rehydrated in a microwave (C21-SDHC15K, SUPOR, China)oven at 85 °C for 20 min, then xylene I, II, 100% ethanol I, II were used for 15 min each respectively to clean sections. 3% $H_2O_2$ was used for 10 min to quench endogenous peroxidase. Descending concentrations (100%, 95%, 90%, and 80%) of ethanol were used for 10 min respectively for dehydration. Sections were then rinsed two times in distilled water for 5 min each, 0.01 mol/L citrate buffer solution (pH 6.0) was added and microwaved in 'medium high' heat for 5 min and 'medium low' heat for 15 min, allowed to cool to room temperature and again rinsed 3 times in 0.1 M PBS for 5 min each. Incubation with 5% bovine serum albumin (BSA) blocking solution was done for 20 min to block non-specific binding. Sections were incubated overnight at 4 °C refrigerator(BCD-211KD3, TCL) after adding primary antibody mouse-anti-rat IL-10 (1:50, ab192271, abcam, UK), NF-kB (1:50, ab16502, abcam, UK) and MMP-3 (1:50, ab52915, abcam, UK) Sections were again rinsed 3 times in 0.1 M PBS for 5 min each, incubated with secondary antibody for 15–20 min, rinsed 3 times in 0.1 M PBS for 5 min each, incubated at 37 °C for 30 min, rinsed 4 times in 0.1 M PBS for 5 min each. Diaminobenzidine(DAB)(ZLI-9019, Zhongshanjinqiao, Beijing, China)was applied for 3–5 min until brown colored reaction product was observed. After rinsing sections in distilled water 3 more times, they were counterstained with hematoxylin (H9627, Sigma)for 10 s, rinsed 3 times in distilled water, dehydrated with ascending concentration (i.e., 80%, 90%, 95% and 100%) of ethanol for 10 min each, cleaned, mounted and then cover-slipped for immunohistochemical study.

### 2.4. Statistical analysis

Two different visual fields (×100) were randomly selected from each immunohistochemistry section, and the positive expression intensity and positive rate were observed under the microscope. Semi quantitative analysis method was used for the staining results: Scored staining intensity with percentage of positive cells. Staining intensity was scored by subtracting the background from the staining intensity showed in the majority of cells: No obvious coloring scored 0 point, canary scored 1 point, yellow scored 2 point, tan and brown scored 3 point. Percentage of positive cells was observed in two different visual fields (×100), which were randomly selected from each immunohistochemistry section: 0–5% scored 0 point, 6%–25% scored 1 point, 26%–50% scored 2 point, 51%–75% scored 3 point, > 75% scored 4 point. Staining intensity and percentage of positive cells were scored in each visual fields, both results were added together as final score: 1–4 point was weakly positive, 4–6 point was moderate positive, and 6–7 point was strong positive. The experimental data was presented as mean ± SD (x ± s).

## 3. Results

### 3.1. General observation

Forty-five SD rats in test group underwent surgery without death and were characterized with head bobbing, twisting and shaking that disappeared about one week. Seven days after surgery, five rats died and were dissected, in which cervical infection and intestinal tympaniteses were present. No more death was observed one-month, three-month and six-month post-operation.

### 3.2. Outcomes of statistical analysis

The concrete results of positive expression of IL-10, MMP-3 and NF-κB were shown in Table 1.

#### 3.2.1. IL-10

IL-10 immune positive mainly distributed in the nucleus pulposus and annulus of the intervertebral disc, a small amount of distributed in the cartilage endplate, IL-10 mainly located in cell cytoplasm with yellow and brown. The positive expression of IL-10 in control group at one-month, three-month and six-month post-operation were scored 4.83 ± 1.17, 3.80 ± 0.84, 4.00 ± 0.89. Test group: 5.42 ± 1.08, 5.17 ± 1.19$^{\#}$, 4.63 ± 0.74. Compared with the control group, the positive expression of IL-10 in test group was significantly higher at three-month ($P < 0.05$). In the same model group, IL-10 was highest at one-month (Figs. 1 and 2).

#### 3.2.2. NF-κB

NF-κB immune positive mainly distributed in the nucleus pulposus