



DOI: 10.5137/1019-5149.JTN.7800-13.1

Received: 26.02.2013 / Accepted: 26.04.2013

Published Online: 05.03.2016

*Original Investigation*

# Effect of Intrathecal Anti-Fibroblast Growth Factor-2 Antibodies on the Mechanical Allodynia and Activation of Spinal Cord Astrocytes in Rats

Jin GAO, Jiyue YOU, Ping CHEN

The First Affiliated Hospital of Chongqing Medical University, Department of Anesthesiology, Chongqing, China

## ABSTRACT

**AIM:** To investigate the effect of intrathecal anti-fibroblast growth factor-2 (FGF-2) antibodies on the mechanical allodynia and activation of spinal cord astrocytes in a rat model of neuropathic pain.

**MATERIAL and METHODS:** Forty male Sprague-Dawley rats were randomly divided into 4 groups (Each group=10 rats). Group A: Spared nerve injury (SNI) model and intrathecal phosphate-buffered saline (PBS); Group B: SNI model and intrathecal FGF-2 antibodies; Group C: Sham surgery and intrathecal PBS; Group D: Sham surgery and intrathecal basic FGF-2 antibodies. The paw withdrawal mechanical threshold (PWMT) was evaluated one day before the operation and at the 1, 4, 7, 14 and 21 days after the operation. Meanwhile, the expression of FGF-2, glial fibrillary acidic protein (GFAP) of astrocytes, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-6 (IL-6) in the L4-6 spinal cord segments were gauged at 21 days after the operation.

**RESULTS:** SNI significantly induced the mechanical allodynia and markedly increased the number of FGF-2 and GFAP positive cells and the levels of TNF- $\alpha$  and IL-6 ( $p < 0.05$ ). Intrathecal injection of FGF-2 antibodies suppressed the increase of FGF-2 and GFAP positive cells, and the levels of TNF- $\alpha$  and IL-6. These antibodies significantly attenuated SNI-induced mechanical allodynia in rats ( $p < 0.05$ ).

**CONCLUSION:** FGF-2 antibodies have the effect of analgesia on neuropathic pain in rats.

**KEYWORDS:** Fibroblast growth factor-2, Astrocyte, Neuropathic pain, Inflammatory cytokines, Spinal cord

## INTRODUCTION

Basic fibroblast growth factor-2 (FGF-2) is a pleiotropic cytokine with neurotrophic functions (6). The increase of FGF-2 causes astroglia-related mechanical hyperalgesia (5). However, the expression of FGF-2 in the spinal cord increases significantly with the spared nerve injury (SNI) and release of inflammatory cytokines, which points out that FGF-2 may closely related to the neuropathic pain. It is still controversial whether FGF-2 acts on the pain by astrocytes. This study is based on SNI model of neuropathic pain in rats. FGF-2 antibodies were injected intrathecally to observe paw withdrawal mechanical threshold (PWMT), expression of

FGF-2 and glial fibrillary acidic protein (GFAP). The results of this study elucidate the possible pathogenetic mechanisms and potential action targets of neuropathic pain in rats.

## MATERIAL and METHODS

### Animals

Forty male Sprague-Dawley rats, weighing 200~250 g at the time of surgery, were used in this study. All experimental protocols were approved by the Chongqing Medical University Institutional Laboratory Animal Care and Use Committee (ILACUC). Rats were housed 2 per cage in a 12-h



Corresponding author: Ping CHEN

E-mail: chenping\_chq@163.com

light/dark cycle with food and water ad libitum. Surgeries and experimental procedures were carried out during the light cycle. Two rats that failed to display mechanical allodynia on the first day after SNI were removed from the study.

The groups were;

Group A: SNI + phosphate-buffered saline (PBS)(n=10),

Group B: SNI + FGF-2 antibodies (n=10),

Group C: Sham surgery + PBS (n=10),

Group D: Sham surgery + FGF-2 antibodies (n=10).

In Groups B and D, 18 µg FGF-2 antibodies (drug capacity: 40 µl) were injected intrathecally at 1,6,9,13,16 and 20 days after the operation. The PWMT was evaluated one day before the operation and at 1, 4, 7, 14 and 21 days after the operation in all groups. Meanwhile, the distribution and expression of FGF-2, GFAP of astrocytes, tumor-necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in the L4~6 spinal cord segments of rats were gauged by immunofluorescence and ELISA separately at the 21 days after the operation.

#### Reagents and Instruments

FGF-2 antibodies (Santa Cruz Company, USA); GFAP antibodies (CST Company, USA); ELISA kits of rats TNF-α and IL-6 (R&D, USA); laser scanning confocal microscope (Leica Company, Germany); microliter syringe (Shanghai, China); ELISA (Tecan, Austria); Von frey filaments (North Coast, USA).

#### Spared Nerve Injury (SNI)

After 50 mg/kg intraperitoneal injection of 1% pentobarbital sodium, sciatic nerve and three branches were exposed in the left hind limb of the rats according to the Decosterd's method (2). Free nervus peroneus communis was tied with 5.0 silk in SNI groups. Besides, cut off part of the nerve at the distal ligation site remained and excessive traction was avoided. The sciatic nerve and branches were only exposed without ligation in the Sham surgery groups.

#### Intrathecal Injections (7)

Injections were performed to the rats after anesthesia with pentobarbital sodium (7). The area over the L5~L6 intervertebral space was shaved and cleaned with 95% alcohol. Injections were performed by inserting a 25-gauge, 5/8-in. needle connected to a 50-µL microliter syringe into the intravertebral space between L5 and L6 vertebrae. The insertion was perpendicular to the skin surface. When the needle entered the space, there was a sudden lateral movement of the tail, indicating a successful insertion. A constant volume was injected, and the syringe held in position for a few seconds after injection. Drug was injected within 1 minute, and the capacity was 40 µl.

#### PWMT

The PWMT of rats' left-side paws was evaluated with different strength of Von frey filaments. Rats were put in polymethyl methacrylate boxes with wire screen. Interzonal region of rats' planta pedis in left paws was stimulated according to the descending order of filaments after 15 minutes' exploratory

behavior. It was measured above 5 times continuously with every strength. The PWMT was written as the minimum with more than 50% withdrawal threshold.

#### Immunofluorescence

Rats were deeply anesthetized with 1% pentobarbital sodium (60 mg/kg), followed by 500 mL of ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Spinal cord (L4~6 segment) sample was fixed for 1.5 hours, soaked in 10% sucrose solution for more than 24 hours. Coronary frozen section was made by cryostat microtome (thickness: 14µm). These sections were immersed in PBS staining jax for 10 minutes, removed the glass slide, put them flatly in wet boxes and added some blocking reagent for an hour at room temperature. FGF-2 primary antibody (1:60) and GFAP primary antibody (1:100) were added. After that, they were incubated for the night at 4°C. And then, the primary antibody was washed with PBS at room temperature, 10 minutes×3/time. FGF-2 secondary antibodies were added in dropwise fashion. Anthocyanin 5 (1:50), GFAP secondary antibodies, Fluorescein isothiocyanate (1:50) were also added. Then, they were incubated for 1 hour in a wet box. Afterwards, the secondary antibody was washed with PBS at room temperature, 10 minutes×3/time, the slides were closed with Fluoro-Gel. Finally, the expression of astrocytes GFAP of spinal cord and FGF-2 were observed under laser scanning confocal microscope.

#### ELISA

The spine was opened after anesthesia and removed the spinal cord at L4~6 levels. The spinal tissue was grinded into the homogenate by Trace grinding device, following the instructions on the ELISA kit. When the reaction terminated, the samples' absorbance in 450 nm was measured with ELISA and drawn in standard curve. Finally, the contents of TNF-α and IL-6 in the spinal tissue were checked out according to the curve.

#### Statistical Analysis

Analysis was performed with the software SPSS 18.0. The data were presented as mean ± standard deviation ( $\bar{x} \pm s$ ). The groups were compared according to Paired Sample T test and compared them among groups according to one-way analysis of variance. When p was <0.05, the difference was statistical significant.

## RESULTS

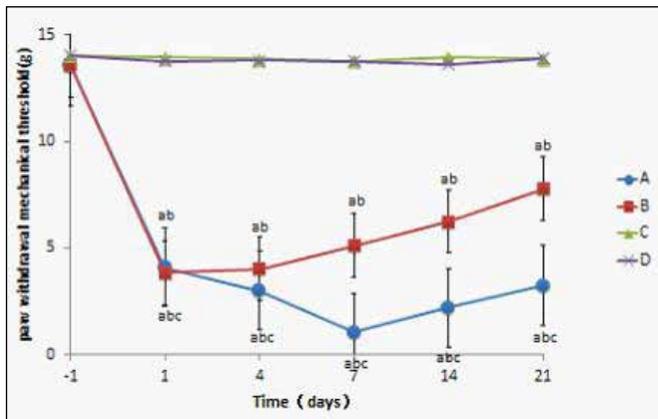
#### The comparisons of 50% PWMT before and after drug administration

The differences of pain threshold among groups were not significant ( $p > 0.05$ ) before operation. PWMT in Group C and Group D did not change significant after the operation ( $p > 0.05$ ). However, PWMT started to decrease from first day ( $p < 0.05$ ) after the operation and had a sustained downward at 4,7,14 and 21 days ( $p < 0.05$ ) in Group A. Group B also started to decrease after operation, compared with the same point of time in Group C and D before the operation. However,

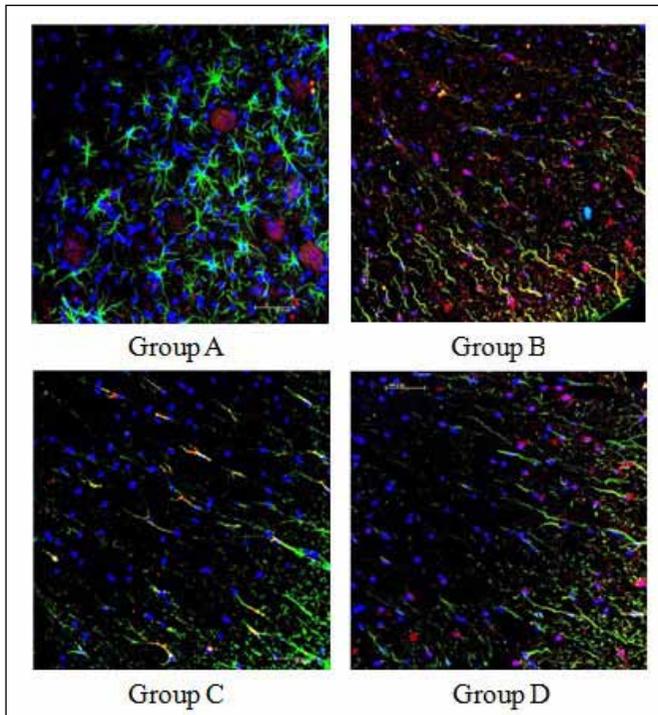
PWMT had a significant increase when compared with Group A ( $p < 0.05$ ) (Figure 1).

**The effects of FGF-2 antibodies on the expression of FGF-2 and GFAP in dorsal horn of the spinal cord**

We can learn in double immune fluorescent spinal sections that in Sham groups if GFAP of astrocytes is positive, FGF-2 also expresses positively. However, the number of positive cells and fluorescence intensity are considerably below those of SNI groups. In Group A and B, GFAP positive cells in lateral spinal dorsal horn were expressed positively, the bodies of those cells get larger. Besides, protuberances became thicker



**Figure 1:** Compared with the preoperative state, <sup>a</sup> $p < 0.05$ ; compared with group C and D, <sup>b</sup> $p < 0.05$ ; compared with group A, <sup>c</sup> $p < 0.05$ .



**Figure 2:** Expression of FGF-2 and GFAP in spinal cord dorsal horn with Laser confocal microscope.

and have more branches. Meanwhile, GFAP and FGF-2 were expressed positively in the same cells. Compared with Group C and D, it increased obviously. In Group B, the number of cells in ipsilateral spinal cord whose GFAP and FGF-2 positively decreased (Figure 2).

**The detection of FGF-2 in spinal cord by Western blot**

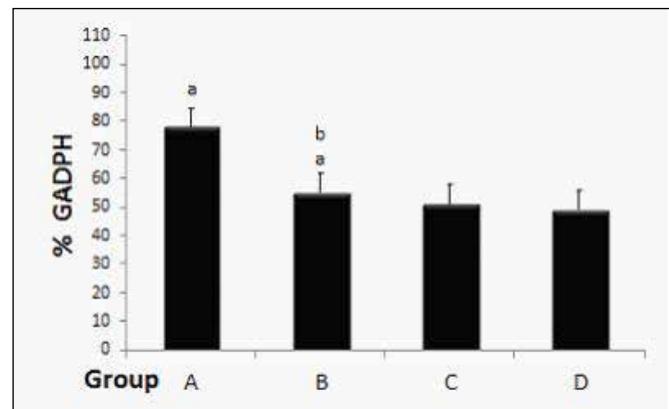
As shown in Figure 3, the FGF-2 expression in spinal cord had increased markedly in Group C and/or D when compared with Group A and/or B ( $p < 0.05$ ). However, the FGF-2 expression in group B was markedly decreased than that in group A ( $p < 0.05$ ).

**The expression of TNF- $\alpha$  and IL-6**

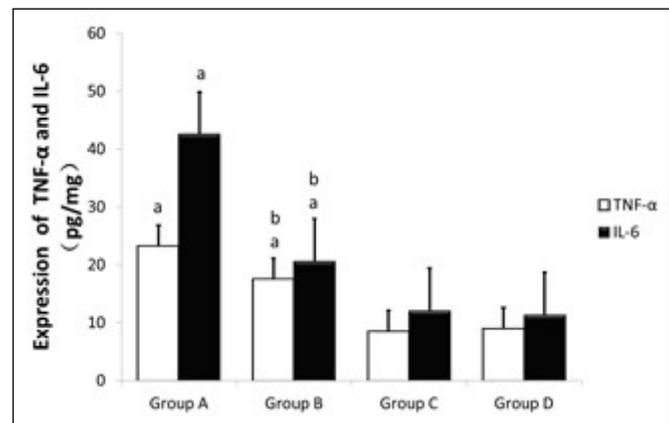
Compared with Group C and Group D, the expression of TNF- $\alpha$  and IL-6 in the spinal cord increased significantly in Group A and Group B ( $p < 0.05$ ). However, compared with Group A, the expression of spinal cord TNF- $\alpha$  and IL-6 decreased in Group B ( $p < 0.05$ ) (Figure 4).

**DISCUSSION**

Fibroblast growth factors (FGFs) are a group of structural relevant protein polypeptide which is coded by FGF gene



**Figure 3:** The expression of FGF-2 in spinal cord by western blot. <sup>a</sup> $p < 0.05$  vs. C and D group; <sup>b</sup> $p < 0.05$  vs. A group.



**Figure 4:** Expression of TNF- $\alpha$  and IL-6. Compared with group C and D, <sup>a</sup> $p < 0.05$ ; Compared with group A, <sup>b</sup> $p < 0.05$ .

family. At present the gene family has at least 23 members, FGF-2 is one of FGFs (3). At first, FGF-2 is a heparin peptide isolated from the extract of bovine pituitary and brain tissue. It is known that FGF-2 can promote the proliferation of fibroblasts (10). Recent survey shows that FGF-2 is closely related to the neuropathic pain. Madiari et al. (6) confirmed that after spinal nerve ligation, the immunoreactivity of FGF-2 will increase after one or three weeks with the effects of astrocytes whose laminae of ipsilateral cord is injured. Freed FGF-2 may further promote astrocytes' activation and make astrocytes excrete continuously. Neary et al. (9) found that nerve injury (such as sciatic nerve transection) will up-regulate FGF-2 mRNA. At the same time, the FGF-2 protein of dorsal root ganglion neuron will also increase significantly. According to our previous survey, SNI induces the injury of FGF-2 in spinal cord and the increase of the expression of inflammatory factors. The secretion of inflammatory factors fails behind that of FGF-2. However, the concrete mechanism of how FGF-2 regulates and controls neuropathic pain is still unclear.

In our study, we used Von Frey cilia and immunofluorescent double staining and we found that SNI can reduce PWMT significantly, and the number of positive cells and fluorescence intensity of FGF-2 and GFAP increased significantly. Intrathecal injection of FGF-2 antibody can increase PWMT significantly and relieve neuropathic pain that was secondary to SNI. In Group B, the expression of FGF-2 and GFAP positive cells was reduced significantly when compared with that of Group A. Therefore, FGF-2 plays an important role in neuropathic pain.

It is previously shown that astrocytes are obviously related with the appearance and continuity of chronic pain (11). GFAP is one of the commonly used markers for astrocyte characteristics. It is a sign of activated astrocytes within the spinal cord. The increase in GFAP staining is also related with the level of hyperpathia (4). When SNI induces allodynia, the number of cells expressing FGF-2 within the spinal cord is increased. Meanwhile, the number of GFAP-expressing astrocytes is also increased. Protuberances became thicker and had more branches. Besides, astrocytes were activated obviously. Intrathecal administration of FGF-2 antibody cannot suppress basal pain in normal rats. However, it can obviously suppress neuropathic pain and the expression of FGF-2 and GFAP in the dorsal horn of spinal cord. So, FGF-2 has positive feedback effects on astrocytes in the process of maintaining neuropathic pain. FGF-2 suppresses the activation of astrocytes. Therefore, FGF-2 cuts off the positive feedback and shows analgesic effects.

The function of astrocytes is related to the regulation of many neuro-hormones. At present, there are extensive concerns about various inflammatory cytokines (IL-6, TNF- $\alpha$ , IL-1 $\beta$ ), substance P and glutamic acid (1). These neuroactive substances and inflammatory cytokines, freed from neuroglial cell, have effects on algesia of spinal dorsal horn and transfer neurons. Meanwhile, positive feedback effects also further enhance the release of harmful neurotransmitters from the primary afferent nerve endings and strengthen the sensitivity and reactivity of the synaptic transmission pain

neurons by enlarging the pain degree and pain area (8). When SNI induces neuropathic pain, it also leads to the increase of the content of TNF- $\alpha$  and IL-6 in relevant section of spinal cord. Intrathecal administration of FGF-2 antibody obviously improves the allodynia and reduces the content of TNF- $\alpha$  and IL-6. Therefore, FGF-2 antibodies have analgesic effects by reducing the activation of astrocytes and cutting off secretion of inflammation factors.

## ■ CONCLUSION

FGF-2 antibodies have the effect of analgesia on neuropathic pain. The activation of astrocytes and up-regulation of the expression of TNF- $\alpha$  and IL-6 in the spinal cord may be involved in the modulation of the neuropathic pain. This provides a new therapeutic implication for neuropathic pain. However, up-regulation of TNF- $\alpha$  and IL-6 attenuates the neuropathic pain. Further studies are needed.

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