Thesis of Doctor Degree

Study in the Chiropractic Manipulation Attenuating the Thermal Hyperalgesia by Diminishing the Inflammatory Factors in the Sciatic Nerve Injury Rats

Takashi Inouchi

The Graduate School of

Hanseo University

Department of Chiropractic

2015. 8.

Study in the Chiropractic Manipulation Attenuating the Thermal Hyperalgesia by Diminishing the Inflammatory Factors in the Sciatic Nerve Injury Rats

Supervised by Professor HanSuk Jung

Dissertation submitted to the Doctor degree by

Takashi Inouchi

2015. 8.

The Graduate School of

Hanseo University

Department of Chiropractic

Takashi Inouchi

Dissertation submitted to the Doctor degree by

Takashi Inouchi is approved.

Chairman/Advisor JooHyun Ham *signature*

Advisory Committee HanSuk Jung *signature*

Advisory Committee SeungHae Han *signature*

Advisory Committee DongHeui Kim *signature*

Advisory Committee YongSeok Lee *signature*

2015. 8.

The Graduate School of

Hanseo University

Contents

Figure Contents

Abstract

The present study evaluated the effect of instrument-assisted spinal manipulation on neuropathic pain after the sciatic nerve crush injury in rats. After creating a sciatic nerve injury in Sprague-Dawley rats, rats were randomly assigned into 5 groups (n=7, respectively): Sham group, Sciatic nerve injury group, Sciatic nerve injury and single impulse thrust treated group, Sciatic nerve injury and five impulse thrusts treated group, and Sciatic nerve injury and ten impulse thrusts treated group. After one day, we applied the instrument-assisted spinal manipulation at the level of L6-S1 using an Impulse Adjusting Instrument® once a day for 7 consecutive days in the respective groups. Thermal hyperalgesia was assessed using a plantar test apparatus, and the expressions of tumor necrosis factor alpha (TNF-α) and cyclooxygenase-2 (COX-2) in the sciatic nerve were determined by western blot. Finally, the expressions of c-Fos and nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d), a neural marker of pain, in the spinal cord (L4-L5) and the ventral lateral periaqueductal grey (vlPAG) were measured using immunohistochemistry. Sciatic nerve crush injury significantly decreased the thermal withdrawal threshold, and increased the expressions of TNF-α and COX-2 in injured sciatic nerves (p<0.05). On the other hand, instrument-assisted spinal manipulation significantly increased the latency time, and suppressed the expressions of TNF-α and COX-2 (p<0.05). Moreover, instrument-assisted spinal manipulation significantly inhibited the expressions of c-Fos and NADPH-d in the dorsal horn of spinal cord and vlPAG (p<0.05). When comparing the treatment groups, the five impulse group showed a trend for benefit over the single thrust or ten thrust group. From these results, it can be inferred that instrument-assisted spinal manipulation may ameliorate the neuropathic pain through suppressing the expressions of inflammation-related factors after sciatic nerve crushed injury. However, further studies

are needed to identify the exact molecular mechanisms of spinal manipulation on the neuropathic pain.

Key Words : Sciatic Nerve Injury, Tumor Necrosis Factor Alpha, Cyclooxygenase-2, c-Fos, Nicotinamide Adenine Dinucleotide Phosphate-diaphorase

I. Introduction

Neuropathic pain is associated with injury of the peripheral or central nervous system and is clinically classified into stimulus-independent pain, regarding the spontaneously ongoing pains, and stimulus-dependent pain called evoked pains depending on stimulus, meaning the hyper-excitability in the nervous system (Coderre & Katz, 1997; Jensen et al., 2001). c-Fos expression is used as an appropriate marker of neuronal activation after noxious stimulation (Hunt et al., 1987). Originally, c-Fos is an immediate early gene, and its expression is triggered by stimuli-induced changes in the metabolic activity of neurons under various conditions (Dragunow et al., 1989). The expression of c-Fos is also used as the useful parameters for the study of the neuronal response to a painful experience (Coggeshall, 2005; Takeda et al., 2009). Nitric oxide (NO) is reported to be associated with nociceptive signaling in chronic neuropathic pain models (Lam et al., 1996; Miclescu & Gordh, 2009). Nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) is the enzyme responsible for NO synthesis (NOS), and thus is regarded to be equivalent to NOS (Hope et al., 1991). Moreover, many studies reported that pro-inflammatory factors such as TNF-alpha (TNF-α) and cyclooxygenase-2 (COX-2) are involved in the development of neuropathic pain (George et al., 2004; Moini et al., 2014).

Neuropathic pain is considered to be difficult to treat because of its complex etiology and mechanism including neurotransmitter systems, receptors and cell types (Jensen et al., 2001; Jain, 2008). Because of the poor efficacy and side effects of pharmacological management in neuropathic pain, non-pharmacological approaches have been on the rise. Actually, regular exercise, transcutaneous electrical nerve stimulation, transcranial direct current stimulation, acupuncture and ultrasound have been proved to be effective in alleviating chronic and/or pathological pain (Nizard et al., 2012; Almeida et al., 2015).

Spinal manipulation therapy has also been reported to be effective in alleviating acute low back pain and thus to improve neck pain, sciatica and chronic low back pain (Hurwitz et al., 1996; Koes et al., 1996). Practically, spinal manipulation is commonly used to alleviating pain in the United States (Nahin et al., 2009). Spinal manipulation is suggested to activate the diffuse descending pain inhibitory neurons located in the periaqueductal gray matter (PAG) through stretching the ligaments, disks, joint capsules or muscles, and this may explain the reason pain can be alleviated by nociceptive stimulation at another site (Terrett & Vernon, 1984; Willer et al., 1984; Vicenzino et al., 1998). However, the mechanisms of spinal manipulation how to reduce pain and disability are not clear. Thus, the present study investigated the mechanisms by which spinal manipulation can relieve the neuropathic pain induced by sciatic nerve injury in rats using the adjusting instrument with which spinal manipulation is considered to have the equal effect compared to manual manipulation (Huggins et al., 2012). In this study, behavioral test, immunohistochemistry and western blot were performed.

II. Materials and Methods

1. Animals and Experimental Groups

Female Sprague-Dawley rats weighing 280±10g (12 weeks of age) were used. The experimental procedures were performed in accordance with the animal care guideline of National Institutes of Health (NIH) and the Korea Academy of Medical Sciences. The animals were house at controlled temperature (23±2°C) and maintained under light-dark cycles, each consisting of 12h of light and 12h of darkness (lights on from 07:00 to 19:00h), with food and water made available ad libitum. The rats were randomly divided into five groups (n=7 in each group): the sham operation group (Sham), the sciatic crushed nerve injury group (SNI), the sciatic crushed nerve injury and single impulse thrust-treated group (SNI+Impulse 1), the sciatic crushed nerve injury and five impulse thrusts-treated group (SNI+Impulse 5), and the sciatic crushed nerve injury and ten impulse thrusts-treated group (SNI+Impulse 10).

2. Surgical Procedure for Induction of Sciatic Nerve Injury

To induce crushed injury on the sciatic nerve in rats, the previously described surgical procedure was performed (Byun et al., 2005) (Fig. 1). In brief, the right sciatic nerve was exposed by incision on the gluteal muscle under anesthesia using Zoletil 50® (50mg/kg; Virbac Laboratories, Carros, France). The sciatic nerve was carefully exposed and then crushed for 30sec using a surgical clip (pressure: 125g; Fine Science Tool Inc., San Francisco, CA, USA). The crushed location was between the sciatic notch and the point of trifurcation. And then, the surgical wound was sutured and recovered. The rats in the sham operation group, the sciatic nerve exposed, however the nerve was not

crushed.

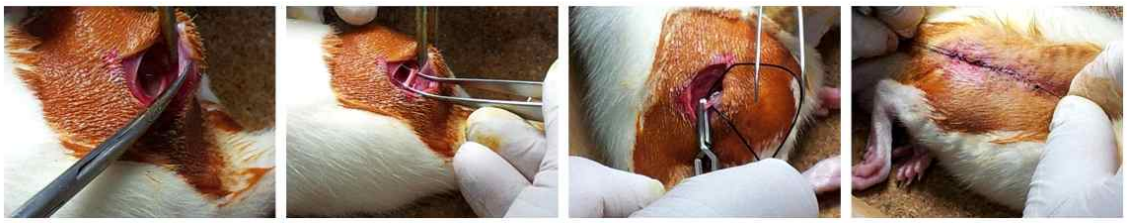

Fig. 1. Induction of Sciatic Crushed Nerve Injury.

3. Chiropractic Manipulation Using the Adjusting Instrument

In this study, chiropractic adjusting instrument (Impulse Adjusting Instrument®, Neuromechanical Innocations, LLC, Phoenix, AX, USA, Fig. 2a) was used for manipulation. Manipulation was applied to the between the level of L6-S1 at an angle of approximately 90°, with the animal held in prone position by an assistant Fig. 2b. The spinal manipulation interventions were conducted once a day for 7 consecutive days, consisting single impulse thrust (6Hz), 5 impulse thrusts (6Hz), and 10 impulse thrusts (6Hz), according to the respective groups. Each impulse thrust of chiropractic adjusting instrument includes the force 2N for 2msec. The force 2N corresponds to the about 70 percentages of animal body weight. The rats in the sham operation group received no manipulation.

Fig. 2. Instrument-Assisted Spinal Manipulation.; (a) Impulse Adjusting Instrument® (Neuromechanical Innovations, LLC, Phoenix, AX, USA) (b) Illustration of the adjustments applied to the level of L6-S1.

4. Plantar Test for Measurement of Thermal Hyperalgesia

Thermal hyperalgesia was measured using a Plantar test algesimeter (Ugo-Basile, Comerio, Italy). Briefly, rats were placed into a plastic box and acclimated to the testing space for at least 5min before starting behavioral test. After acclimation, radiant heat was applied to the ipsilateral hindpaw plantar surface until the rat withdrew its paw. A photoelectric cell automatically tuned the heat source off when the reflected light beam was interrupted (for example, when the animal lifted its paw) and the time (seconds) was recorded as the paw withdrawal latency (PWL). Intensity was set to low power (40mW/cm^2) with a heating rate of 1°C/sec, to induce activation of unmyelinated fibers (Le Bars et al., 2001).

5. Tissue Preparation

The animals were sacrificed immediately after performing the behavioral test. The animals were anesthetized using Zoletil 50® (10mg/kg, i.p.; Virbac Laboratories), transcardially perfused with 50mM phosphate-buffered saline (PBS), and fixed with a freshly prepared solution consisting of 4% parafomaldehyde in 100 Mm phosphate buffer

(PB, pH 7.4). For immunohistochemistry, the brain and spinal cord were dissected and postfixed in the same fixative overnight and transferred into a 30% sucrose solution for cryoprotection. Serial coronal section of 40μm thickness was made with a freezing microtome (Leica, Nussloch, Germany). The ventrolateral periaqueductal gray matter (vlPAG) was selected from the midbrain region spanning from Bregma -7.64 to -8.00 mm. Then, the dorsal horn of the spinal cord was selected from the L4-L5 regions. In each region, ten sections were collected on average from each rat.

For western blot, the sciatic nerves were dissected and then were immediately frozen at -70°C.

6. Immunohistochemistry for c-Fos and NADPH-d

To analyze the degree of the expressions of c-Fos and nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) in the pain related regions (vlPAG and the dorsal horn of the L4-L5 spinal cord), we performed immunohistochemistry as previously described (Kim et al., 2012).

In immunohistochemistry for c-Fos, free-floating tissue sections were incubated overnight with rabbit anti-c-Fos antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at a dilution of 1:1,000, and the sections were then incubated for 1 hour with biotinylated anti-rabbit secondary antibody (Vector Laboratories Inc., Burlingame, CA, USA). The sections were subsequently incubated with avidin-biotin-peroxidase complex (Vector Laboratories Inc.) for 1 hour at room temperature. Immunoreactivity was visualized by incubating the sections in a solution consisting of 0.05% 3,3-diaminobenzidine and 0.01% H_2O_2 in 50 mM Tris-buffer (pH7.6) for approximately 3 minutes. The sections were then washed three times with PBS and mounted onto gelatine-coated slides. The slides were air-dried overnight at room temperature, and coverslips were mounted by using Permount mounting medium (Thermo Fisher Scientific

Inc., Waltham, MA, USA).

For measurement of NOS activity in the vlPAG and the dorsal horn of the L4-L5 spinal cord, immunohistochemistry for NADPH-d was performed according to the previous study (Kim et al., 2012). Briefly, free-floating sections were incubated at 37°C for 60 minutes in 100mM PBS (pH 7.4) containing 0.3% Triton X-100, 0.1mg/mL nitroblue tetrazolium, and 0.1mg/mL β-NADPH. The sections were then washed three times with PBS and mounted onto gelatin-coated slides. The slides were air dried overnight at room temperature, and coverslips were mounted by using Permount mounting medium (Thermo Fisher Scientific Inc.).

The number of c-Fos- and NADPH-d-positive cells in the vlPAG, and spinal cord (L4-L5) regions were counted hemilaterally through a light microscope (Olympus Co., Tokyo, Japan). The area of the vlPAG, and spinal cord (L4-L5) regions from each slice was measured by using an Image-Pro Plus computer-assisted image analysis system (Media Cybernetics Inc., Silver Spring, MD, USA) attached to a light microscope (Olympus Co.).

7. Western Blot Analysis

The sciatic nerves were homogenized on ice, and lysed in a lysis buffer containing 50mM HEPES (pH7.5), 150mM NaCl, 10% glycerol, 1% Triton X-100, 1mM PMSF, 1mM EGTA, 1.5mM MgCl2・6H2O, 1mM sodium orthovanadate, and 100mM sodium flouride. Protein content was measured using a Bio-Rad colorimetric protein assay kit (Hercules, CA, USA). Protein (20μg) was separated on SDS-polyacrylamide gels and transferred onto a nitrocellulose membrane. Mouse beta-actin antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat tumor necrosis factor alpha (TNF-α) (1:1000; Santa Cruz Biotechnology), and goat cyclooxygenase-2 (COX-2) (1:1000; Santa Cruz Biotechnology) were used as the primary antibodies. Horseradish

peroxidase-conjugated anti-mouse antibody for beta-actin (1:5000; Vector Laboratories), and horseradish peroxidase-conjugated anti-goat antibody (1:5000; Santa Cruz Biotechnolog) for TNF-α and COX-2 were used as the secondary antibodies.

Experiments were performed in normal laboratory conditions and at room temperature, except for the transferred membranes. Transferred membranes were performed at 4ºC with the cold pack and pre-chilled buffer. Band detection was performed using the enhanced chemiluminescence (ECL) detection kit (Santa Cruz Biotechnology). To compare the relative expression of proteins, the detected bands were calculated densitometrically using Molecular AnalystTM, version 1.4.1 (Bio-Rad).

8. Statistical Analysis

The results were expressed as the mean±standard error of the mean (S.E.M.). The data were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's post-hoc test. Differences among groups were considered statistically significant at $p<0.05$.

III. Results

1. Effect of Instrument-Assisted Spinal Manipulation on Thermal Hyperalgesia

Thermal hyperalgesia was measured using a plantar test and represented as the paw withdrawal latency (PWL) Fig. 3. The PWL of the sciatic crushed nerve injury group significantly decreased compared to the sham operation group. The PWLs were 23.70±1.45 sec, 15.07±0.67 sec, 17.60±1.17 sec, 20.40±1.71 sec, and 17.28±2.20 sec in Sham, SNI, SNI+Impulse 1, SNI+Impulse 5, and SNI+Impulse 10, respectively. In the present results, there was significant difference in the PWLs of groups. The induction of sciatic nerve injury significantly decreased the PWL, compared to rats in Sham. On the other hand, application of instrument-assisted spinal manipulation accelerated the increment of PWL in the sciatic crushed nerve injury rats ($p<0.05$). Especially, the five impulse thrusts increased the PWL near the similar level to Sham. These results means that instrument-assisted spinal manipulation can alleviate the pain induced by sciatic crushed nerve injury, and especially the five impulse thrust may be the most effective in relieving the neuropathic pain.

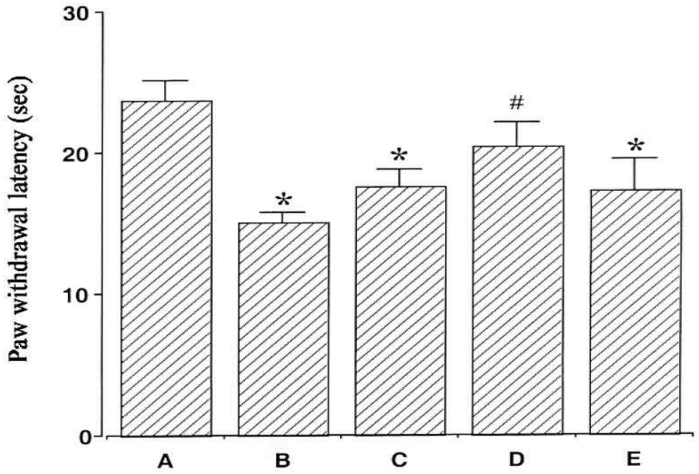

Fig. 3. Effect of Instrument-Assisted Spinal Manipulation on Thermal Hyperalgesia. (A) the sham operation group (Sham), (B) the sciatic crushed nerve injury group (SNI), (C) the sciatic crushed nerve injury and single impulse thrust-treated group (SNI+Impulse 1), (D) the sciatic crushed nerve injury and five impulse thrusts-treated group (SNI+Impulse 5), (E) the sciatic crushed nerve injury and ten impulse thrusts-treated group (SNI+Impulse 10). All data are represented as mean±standard error of the mean (S.E.M.). *represents $p<0.05$ compared to Sham. #represents $p<0.05$ compared to SNI.

2. Effect of Instrument-Assisted Spinal Manipulation on Expression of TNF-α in Sciatic Crushed Nerve Injury

To ascertain the effect of instrument-assisted spinal manipulation on the inflammatory factors related to the pain, the expression of TNF-α in the proximal region of the crushed sciatic nerve was analyzed by western blot Fig. 4. The relative expressions of TNF-α were 3.21±0.21, 2.31±0.32, 1.99±0.21, and 2.10±0.31 in SNI, SNI+Impulse 1, SNI+Impulse 5, and SNI+Impulse 10, when the expression Sham was 1.00. Induction of crushed nerve injury remarkably increased the expression of TNF-α in the proximal region of injury site, compared to Sham ($p<0.05$). On the other hand, the expression of TNF-α was significantly suppressed by application of instrument-assisted spinal manipulation ($p<0.05$). Especially, five impulse thrusts showed most potent suppressive

effect on TNF-α expression. From these results, it can be inferred that the instrument-assisted spinal manipulation can suppress the expression of inflammatory factor in the proximal region of the crushed sciatic nerve.

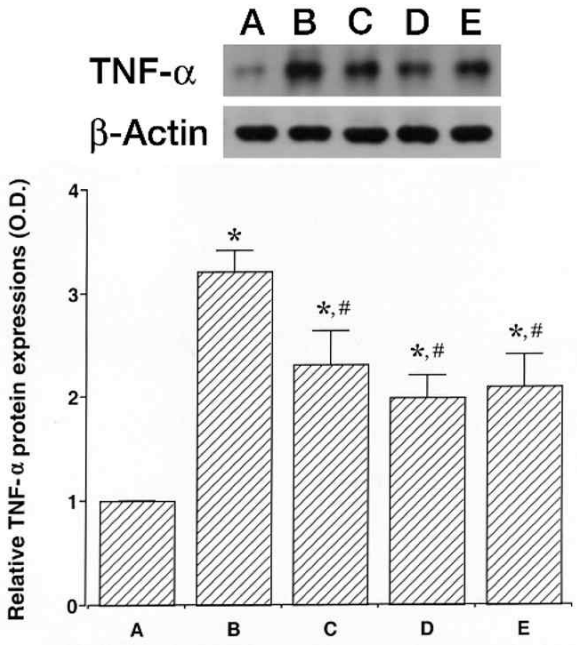

Fig. 4. Effect of Instrument-Assisted Spinal Manipulation on Expression of TNF-α in Sciatic Crushed Nerve Injury. β-Actin was used as an internal control (43kDa). (A) the sham operation group (Sham), (B) the sciatic crushed nerve injury group (SNI), (C) the sciatic crushed nerve injury and single impulse thrust-treated group (SNI+Impulse 1), (D) the sciatic crushed nerve injury and five impulse thrusts-treated group (SNI+Impulse 5), (E) the sciatic crushed nerve injury and ten impulse thrusts-treated group (SNI+Impulse 10). Upper: The results of band detection using the enhanced chemiluminescence (ECL) detection kit. Lower: The relative expression of TNF-α protein. All data are represented as mean±standard error of the mean (S.E.M.). *represents $p<0.05$ compared to Sham. #represents $p<0.05$ compared to SNI.

3. Effect of Instrument-Assisted Spinal Manipulation on Expression of COX-2 in Sciatic Crushed Nerve Injury

Similarly, the expression of COX-2, the other inflammatory factor, in the proximal

region of the crushed sciatic nerve was significantly enhanced by the sciatic nerve injury in comparison with Sham (p<0.05). However, application of instrument-assisted spinal manipulation significantly reduced the expression of COX-2, and the five impulse thrusts was the most effective in suppressing the expression of COX-2 (p<0.05). Specifically, the relative expressions of COX-2 were 2.08±0.09, 1.43±0.05, 1.24±0.02, and 1.80±0.11 in SNI, SNI+Impulse 1, SNI+Impulse 5, and SNI+Impulse 10, when the expression Sham was 1.00 Fig. 5. The present results show that spinal manipulation effectively suppressed the expression of COX-2 related to the inflammation.

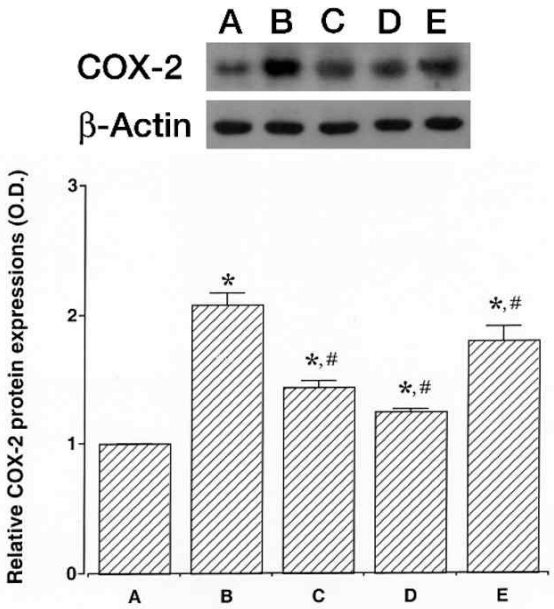

Fig. 5. Effect of Instrument-Assisted Spinal Manipulation on Expression of COX-2 in Sciatic Crushed Nerve Injury. β-Actin was used as an internal control (43kDa). Upper: The results of band detection using the enhanced chemiluminescence (ECL) detection kit. Lower: The relative expression of COX-2 protein. (A) the sham operation group (Sham), (B) the sciatic crushed nerve injury group (SNI), (C) the sciatic crushed nerve injury and single impulse thrust-treated group (SNI+Impulse 1), (D) the sciatic crushed nerve injury and five impulse thrusts-treated group (SNI+Impulse 5), (E) the sciatic crushed nerve injury and ten impulse thrusts-treated group (SNI+Impulse 10). All data are represented as mean±standard error of the mean (S.E.M.). *represents p<0.05 compared to Sham. #represents p<0.05 compared to SNI.

4. Effect of Instrument-Assisted Spinal Manipulation on the c-Fos Expressions in the L4-L5 Spinal Cord Regions and vlPAG

Photomicrographs of c-Fos-positive cells in the vlPAG and dorsal horn of the spinal cord were presented in Fig. 6. In the L4-L5 spinal cord regions, the number of c-Fos-positive cells was 13.40±1.35/section in Sham, 309.39 ± 17.10/section in SNI, 199.53±33.43/section in SNI+Impulse 1, 57.92±6.77/section in SNI+Impulse 5, and 203.17±23.84/section in SNI+Impulse 10. In the vlPAG, the number of c-Fos-positive cells was 51.22±4.56/section in Sham, 256.00±13.06/section in SNI, 219.75±8.17/section in SNI+Impulse 1, 157.22±14.32/section in SNI+Impulse 5, and 228.58±8.04/section in SNI+Impulse 10.

These results showed that c-Fos expressions in the vlPAG and the dorsal horn of spinal cord were enhanced by induction of sciatic crushed nerve injury. On the other hand, application of spinal manipulation significantly decreased the sciatic nerve injury-induced c-Fos expressions in the vlPAG and the dorsal horn of spinal cord related to the pain ($p<0.05$). The suppressive effect of instrument-assisted spinal manipulation appeared most potent in the five impulse thrusts.

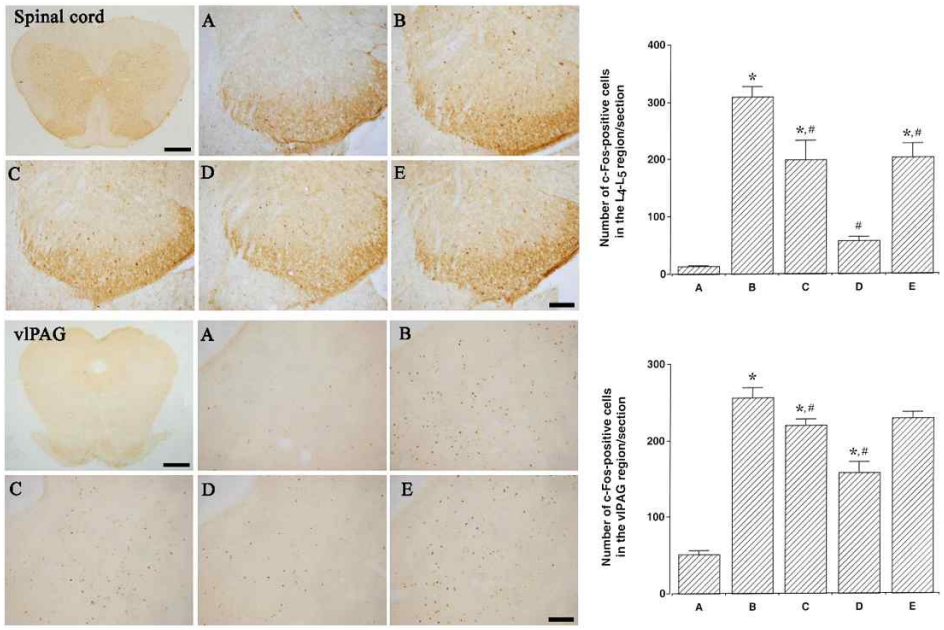

Fig. 6. Effect of Instrument-Assisted Spinal Manipulation on the c-Fos Expressions in the L4-L5 Spinal Cord Regions and vlPAG.: Representative photomicrographs of c-Fos-positive cells. The sections were stained for c-Fos immunoreactivity (brown). The scale bar represents 100μm. Left: Number of c-Fos-positive cells in each group. (A) the sham operation group (Sham), (B) the sciatic crushed nerve injury group (SNI), (C) the sciatic crushed nerve injury and single impulse thrust-treated group (SNI+Impulse 1), (D) the sciatic crushed nerve injury and five impulse thrusts-treated group (SNI+Impulse 5), (E) the sciatic crushed nerve injury and ten impulse thrusts-treated group (SNI+Impulse 10). All data are represented as mean±standard error of the mean (S.E.M.). *represents $p<0.05$ compared to Sham. #represents $p<0.05$ compared to SNI.

5. Effect of Instrument-Assisted Spinal Manipulation on the NOS Expressions in L4-L5 Spinal Cord Regions and the vlPAG

Photomicrographs of NADPH-d-positive cells in the vlPAG and dorsal horn of the spinal cord were presented in Fig. 7. In the L4-L5 spinal cord regions, the number of NADPH-d-positive cells was 7.93±1.07/section in Sham, 28.00±1.97/section in SNI, 22.28±1.26/section in SNI+Impulse 1, 15.10±1.89/section in SNI+Impulse 5, and 19.43±2.20/section in SNI+Impulse 10. In the vlPAG, the number of NADPH-d-positive

cells was 28.86±3.15/section in Sham, 82.82±4.60/section in SNI, 67.79±3.97/section in SNI+Impulse 1, 48.55±4.38/section in SNI+Impulse 5, and 60.11±4.27/section in SNI+Impulse 10.

Like the expression of c-Fos, sciatic crushed nerve injury significantly enhanced the expressions of NADPH-d in the vlPAG and L4-L5 spinal cord regions, compared with Sham, but application of instrument-assisted spinal manipulation significantly reduced the expressions of NADPH-d in these regions ($p<0.05$). Finally, the five impulse thrusts was the most effective in suppressing the expression of NADPH-d among other impulse thrusts.

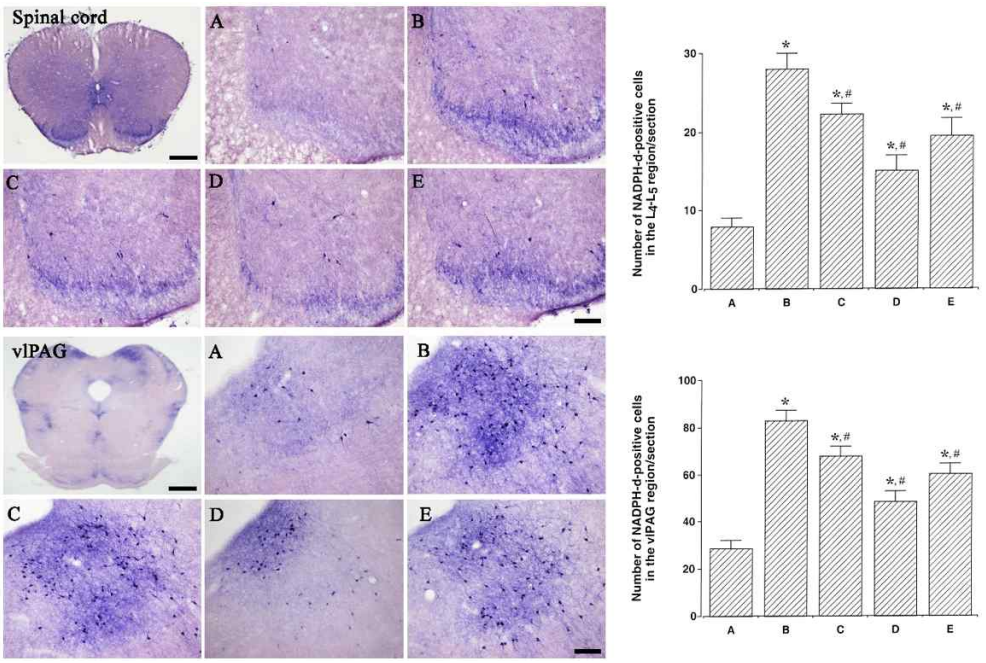

Fig. 7. Effect of Instrument-Assisted Spinal Manipulation on the NADPH-d Expressions in the L4-L5 Spinal Cord Regions and vlPAG. Right: Representative photomicrographs of NADPH-d-positive cells. The sections were stained for NADPH-d immunoreactivity (blue). The scale bar represents 100 μm. Left: Number of NADPH-d-positive cells in each group. (A) the sham operation group(Sham), (B) the sciatic crushed nerve injury group (SNI), (C) the sciatic crushed nerve injury and single impulse thrust-treated group (SNI+Impulse 1), (D) the sciatic crushed nerve injury and five impulse thrusts-treated group (SNI+Impulse 5), (E) the sciatic crushed nerve injury and ten impulse thrusts-treated group (SNI+Impulse 10). All data are represented as mean±standard error of the mean (S.E.M.). *represents $p<0.05$ compared to Sham. #represents $p<0.05$ compared to SNI.

IV. Discussion

Maves et al. (1993) suggested that sciatic nerve ligation of rats is the most commonly used model for neuropathy study because it resembles human neuropathy caused by trauma of peripheral nerves, and is reliable and easily reproducible. Peripheral nerve injury causes a neuropathic pain characterized by allodynia and hyperalgesia. Actually, sciatic nerve injury is reported to induce a cold allodynia and thermal hyperalgesia in rats (Kanyadhara et al., 2014). The behavioral test in the present study also revealed that sciatic crushed nerve injury significantly increased the thermal sensitivity Fig. 3. The sciatic crushed nerve injury also enhanced the expressions of TNF-α and COX-2 involved in the inflammatory process Fig. 4 & Fig. 5.

Many studies report that inflammatory processes play a crucial role in the development of neuropathic pain after nerve damage (George et al., 2004; Moini Zanjani et al., 2014). Zelenka et al. (2005) reported that intraneural inection of proinflammatory cytokines such as TNF-α and interleukin-1 beta (IL-1β) induced neuropathic pain. The early and transient upregulation of TNF protein may precede and parallel the development of pain-related behavior after sciatic nerve injury, supporting that TNF is essential in the neuropathic pain generation, and thus anti-TNF interventions may be effective in preventing the development of neuropathic pain (George et al., 2004). Schäfers & Sommer (2007) also reported that blockade of these cytokines or application of anti-inflammatory cytokines reduces pain. Such peripheral mechanism of proinflammatory cytokine action in neuropathic pain may be associated with the induction of COX-2 leading to prostaglandin synthesis in damaged nerves (Sommer & Kress, 2004). COX-2 is the inducible prostaglandin-synthesizing enzyme and constitutively expressed in the central nervous system of rats (Beiche et al., 1998). COX-2 is also induced and/or increased by inflammation or administration of cytokines

such as IL-1β, contributing to inflammatory pain hypersensitivity (Samad et al., 2001). Although the mechanisms by which COX2 is involved in neuropathic pain or analgesic action are poorly understood, prostaglandin E_2 (PGE_2) is considered to be a major factor because it increases neuronal activity and hyperalgesia (Minamietal., 1994; Ahmadietal., 2001). Actually, injured sciatic nerves produce PGE_2 through a mechanisms related to COX-2 activity, and such upregulation of COX-2 and PGE_2 in injured nerves is long-lasting and plays an important role in the development of neuropathic pain. Thus many studies reported the effectiveness of COX-2 inhibitors to alleviate the neuropathic pain (Muja & DeVries, 2004; Ma et al., 2010; Ma et al., 2012). Similarly, our study showed that instrument-assisted spinal manipulation significantly suppressed the up-regulations of TNF-α and COX-2 in injured sciatic nerves, which contributed to the alleviation of thermal hyperalgesia Fig 3-5.

c-Fos, an immediate-early gene, is used as the marker of neuronal activation following noxious stimulation, and thus many studies on neuronal response to a painful experience reported the expression of c-Fos in the dorsal horn of spinal cord and brain areas, such as the anterior cingulate cortex, hypothalamic paraventricular nucleus, and periaqueductal grey lateral ventral nucleus (PAG) (Hunt et al., 1987;, Nishimori et al., 2002; Coggeshall, 2005; Takeda et al., 2009). Spinal neurons expressing c-Fos after peripheral noxious stimulation project to structures in the midbrain, especially the PAG, which play an important role in controlling the pain (McMahon & Wall, 1985; Harris, 1999; Heinricher et al., 2009). The PAG interconnected with the hypothalamus and limbic forebrain areas such as the amygdala directly receives spinomesencephalic input, and projects to the rostral ventromedial medulla (RVM). The RVM, in turn, sends the output to dorsal horn laminae playing an important role in nociceptive function (Heinricher et al., 2009). Thus, many studies reported that noxious stimulation including nerve injury enhanced the expression of c-Fos in the dorsal horn of spinal cord and PAG (Nishimori et al., 2002; Gholami et al., 2006). Originally, c-Fos is involved in the

signal transduction cascade linking extracellular events to long-term intracellular adaptations (Harris, 1998). Fos also contribute to long-term modulation of spinal nociceptive pathways through involvement in the alteration in the spinal nociceptive circuits causing hyperalgesia or allodynia (Zimmermann & Herdegen, 1994). Terayama et al. (2014) reported that injury to tibial nerve enhanced c-Fos expression in the spinal dorsal horn, and convergent nociceptive input through neighboring intact nerve may partially contribute to the augmentation of c-Fos in the spinal dorsal horn and the neuropathic pain induced by peripheral nerve injury. In the present study, crushed sciatic nerve injury induced the augmentation of c-Fos expression in the spinal dorsal horns and ventrolateral PAG (vlPAG), but instrument-assisted spinal manipulation significantly suppressed the sciatic nerve injury-induced c-Fos expressions in these major nociceptive centers, the vlPAG and the spinal dorsal horn Fig. 6.

Some researchers suggested an association between NO and nociceptive signaling in neuropathic pain models by upregulating NOS expression in neurons of spinal dorsal horn (Lam et al., 1996; Miclescu & Gordh, 2009). Lam et al. (1996) reported that formalin injection increased the number of neuronal NOS (nNOS)-positive neurons at the L4-L5 dorsal horn, suggesting that NO may be involved in the mechanism of hyperalgeisa. Mor et al. (2011) also reported that rats with pain and disability induced by chronic constriction injury of the sciatic nerve injury showed the increased iNOS in the vlPAG. Thus, NOS inhibitors were reported to be effective in suppressing the neuropathic pain (Chapman et al., 1995; Miclescu & Gordh, 2009). NO immunoreactivity is associated with the NADPH-d enzyme, serving as a histochemical marker for neurons that produce NO (Hope et al., 1991). Noxious visceral stimulation significantly increased the NADPH-d-positive neurons in PAG (Rodella et al., 1998). Damasceno et al. (2013) also reported that paradoxical sleep deprivation promoting hyperalgesia increased the number of NADPH-d-positive cells in the dorsolateral PAG, and this might be involved in the increased pain sensitivity. Our results also showed

that sciatic nerve injury significantly enhanced the expressions of NADPH-d in the vlPAG and L4-L5 spinal cord regions, but application of spinal manipulation significantly reduced the expressions of NADPH-d in these regions Fig. 7.

V. Conclusion and Limitation

The present results showed that sciatic crushed nerve injury induced the thermal hyperalgesia by enhancing the TNF-α and COX-2 in the injured nerves and c-Fos and NO in the major nociceptive centers, such as the spinal dorsal horns and vlPAG. However, instrument-assisted spinal manipulation remarkably alleviated the neuropathic pain derived from sciatic nerve injury through suppressing the inflammatory factors in the injured nerves and neuronal activity in the spinal dorsal horns and vlPAG. Many studies reported effectiveness of spinal manipulation on relieving neck pain, sciatica and chronic low back pain (Childs et al., 2004; Chu et al., 2014). Some studies suggested that spinal manipulation activated the diffuse descending pain inhibitory neurons located in the PAG through stretching the ligaments, disks, joint capsules or muscles (Terrett & Vernon, 1984; Willer et al., 1984; Vicenzino et al., 1998). Coronado et al. (2012) suggested that spinal manipulation might have a favorable effect by increasing pressure pain threshold at the remote sites of stimulus application, and this reduction in pain sensitivity following spinal manipulation may be related to the modulation of afferent input or central nervous system processing of pain. However, previous studies did not suggest the mechanism of spinal manipulation in the molecular and biological aspects. On the other hand, our study suggested the therapeutic potential of the instrument-assisted spinal manipulation in alleviating the neuropathic pain by presenting molecular and biological evidences. But, this study did not investigate the effects of spinal manipulation on the other factors related to the neuropathic pain including IL1β and in other brain regions, such as the anterior cingulate cortex and hypothalamic paraventricular nucleus. Therefore, further studies are required to elucidate more exact mechanisms by which spinal manipulation alleviate the neuropathic pain induced by the nerve injures.

References

Ahmadi S, Lippross S, Neuhuber WL, Zeilhofer HU: PGE2 selectively blocks inhibitory glycinergic neurotransmission onto rat superficial dorsal horn neurons. Nat Neurosci 5(1): 34-40, 2002.

Almeida C, DeMaman A, Kusuda R, Cadetti F, Ravanelli MI, Queiroz AL, Sousa TA, Zanon S, Silveira LR, Lucas G: Exercise therapy normalizes BDNF upregulation and glial hyperactivity in a mouse model of neuropathic pain. Pain 156(3): 504-513, 2015.

Beiche F, Klein T, Nüsing R, Neuhuber W, Goppelt-Struebe M: Localization of cyclooxygenase-2 and prostaglandin E2 receptor EP3 in the rat lumbar spinal cord. J Neuroimmunol 89(1-2): 26-34, 1998.

Byun YH, Lee MH, Kim SS, Kim H, Chang HK, Lee TH, Jang MH, Shin MC, Shin MS, Kim CJ: Treadmill running promotes functional recovery and decreases brain-derived neurotrophic factor mRNA expression following sciatic crushed nerve injury in rats. J Sports Med Phys Fitness 45(2): 222-228, 2005.

Childs JD, Fritz JM, Flynn TW, Irrgang JJ, Johnson KK, Majkowski GR, Delitto A: A clinical prediction rule to identify patients with low back pain most likely to benefit from spinal manipulation: a validation study. Ann Intern Med 141(12): 920-928, 2004.

Chu J, Allen DD, Pawlowsky S, Smoot B: Peripheral response to cervical or thoracic spinal manual therapy: an evidence-based review with meta analysis. J Man Manip Ther 22(4): 220-229, 2014.

Coderre TJ, Katz J: Peripheral and central hyperexcitability: differential signs and symptoms in persistent pain. Behav Brain Sci 20(3): 404-419, 1997.

Coggeshall RE: Fos, nociception and the dorsal horn. Prog Neurobiol 77(5): 299-352, 2005.

Coronado RA, Gay CW, Bialosky JE, Carnaby GD, Bishop MD, George SZ: Changes in pain sensitivity following spinal manipulation: a systematic review and meta-analysis. J Electromyogr Kinesiol 22(5): 752-767, 2012.

Damasceno F, Skinner GO, Araújo PC, Ferraz MM, Tenório F, de Almeida OM: Nitric oxide modulates the hyperalgesic response to mechanical noxious stimuli in sleep-deprived rats. BMC Neurosci 14(1): 92, 2013.

Dragunow M and Faull R: The use of c-fos as a metabolic marker in neuronal pathway tracing. J Neurosci Methods 29(3): 261-265, 1989.

George A, Buehl A, Sommer C: Wallerian degeneration after crush injury of rat sciatic nerve increases endo- and epineurial tumor necrosis factor-alpha protein. Neurosci Lett 372(3): 215-219, 2004.

Gholami S, Lambertz D, Hoheisel U, Mense S: Effects on c-Fos expression in the PAG and thalamus by selective input via tetrodotoxin-resistant afferent fibres from muscle and skin. Neurosci Res 56(3): 270-278, 2006.

Harris JA: Using c-fos as a neural marker of pain. Brain Res Bull 45(1): 1-8, 1998.

Heinricher MM, Tavares I, Leith JL, Lumb BM: Descending control of nociception: Specificity, recruitment and plasticity. Brain Res Rev 60(1): 214-225, 2009.

Hope BT, Michael GJ, Knigge KM, Vincent SR: Neuronal NADPH diaphorase is a nitric oxide synthase. Proc Natl Acad Sci USA 88(7): 2811-2814, 1991.

Huggins T, Boras AL, Gleberzon BJ, Popescu M, Bahry LA: Clinical effectiveness of the activator adjusting instrument in the management of musculoskeletal disorders: a systematic review of the literature. J Can Chiropr Assoc 56(1): 49-57, 2012.

Hunt SP, Pini A, Evan G: Induction of c-fos-like protein in spinal cord neurons following sensory stimulation. Nature 328(6131): 632-634, 1987.

Hurwitz EL, Aker PD, Adams AH, Meeker WC, Shekelle PG: Manipulation and mobilization of the cervical spine. A systematic review of the literature. Spine 21(15): 1746-1760, 1996.

Jain KK: Current challenges and future prospects in management of neuropathic pain. Expert Rev Neurother 8(11): 1743-1756, 2008.

Jensen TS, Gottrup H, Sindrup SH, Bach FW: The clinical picture of neuropathic pain. Eur J Pharmacol 429(1-3): 1-11, 2001.

Kanyadhara S, Dodoala S, Sampathi S, Punuru P, Chinta G: Ethanolic extract of Aloe vera ameliorates sciatic nerve ligation induced neuropathic pain. Anc Sci Life 33(4): 208-215, 2014.

Kim SE, Shin MS, Kim CJ, Park JH, Chung KJ, Jung H, Kim KH, Lee JH, Ko IG: Effects of tamsulosin on urinary bladder function and neuronal activity in the voiding centers of rats with cyclophosphamide-induced overactive bladder. Int Neurourol J 16(1): 13-22, 2012.

Koes BW, Assendelft WJ, van der Heijden GJ, Bouter LM: Spinal manipulation for low back pain. An updated systematic review of randomized clinical trials. Spine 21(24): 2860-2873, 1996.

Lam HH, Hanley DF, Trapp BD, Saito S, Raja S, Dawson TM, Yamaguchi H: Induction of spinal cord neuronal nitric oxide synthase (NOS) after formalin injection in the rat hind paw. Neurosci Lett 210(3): 201-204, 1996.

Le Bars D, Gozariu M, Cadden SW: Animal models of nociception. Pharmacol Rev 53(4): 597-652, 2001.

Ma W, Chabot JG, Vercauteren F, Quirion R: Injured nerve-derived COX2/PGE2 contributes to the maintenance of neuropathic pain in aged rats. Neurobiol Aging 31(7): 1227-1237, 2010.

Ma W, St-Jacques B, Duarte PC: Targeting pain mediators induced by injured nerve-derived COX2 and PGE2 to treat neuropathic pain. Expert Opin Ther Targets 16(6): 527-540. 2012.

Maves TJ, Pechman PS, Gebhart GF, Meller ST: Possible chemical contribution from chromic gut sutures produces disorders of pain sensation like those seen in man.

Pain 54(1): 57-69, 1993.

McMahon SB, Wall PD: Electrophysiological mapping of brainstem projections of spinal cord lamina I cells in the rat. Brain Res 333(1): 19-26, 1985.

Miclescu A, Gordh T: Nitric oxide and pain; Something old, something new. Acta Anaesthesiol Scand 53(9): 1107-1120, 2009.

Minami T, Nishihara I, Uda R, Ito S, Hyodo M, Hayaishi O: Characterization of EP-receptor subtypes involved in allodynia and hyperalgesia induced by intrathecal administration of prostaglandin E2 to mice. Br J Pharmacol 112(3): 735-740, 1994.

Moini Zanjani T, Ameli H, Labibi F, Sedaghat K, Sabetkasaei M: The Attenuation of Pain Behavior and Serum COX-2 Concentration by Curcumin in a Rat Model of Neuropathic Pain. Korean J Pain 27(3): 246-252, 2014.

Mor D, Bembrick AL, Austin PJ, Keay KA: Evidence for cellular injury in the midbrain of rats following chronic constriction injury of the sciatic nerve. J Chem Neuroanat 41(3): 158-169, 2011.

Muja N, DeVries GH: Prostaglandin E (2) and 6-keto-prostaglandin F (1alpha) production is elevated following traumatic injury to sciatic nerve. Glia 46(2): 116-129, 2004.

Nahin RL, Barnes PM, Stussman BJ, Bloom B: Costs of complementary and alternative medicine (CAM) and frequency of visits to CAM practitioners: United States, 2007. Natl Health Stat Report (18): 1-14, 2009.

Nishimori T, Ikeda T, Terayama R, Ishida Y, Nakamura T, Otahara N: Effect of ionotropic glutamate receptor antagonists on Fos-like immunoreactivity in the dorsal horn following transection of the rat sciatic nerve. Brain Res 934(1): 81-86, 2002.

Nizard J, Lefaucheur JP, Helbert M, de Chauvigny E, Nguyen JP: Non-invasive stimulation therapies for the treatment of refractory pain. Discov Med 14(74):

21-31, 2012.

Rodella L, Rezzani R, Agostini C, Bianchi R: Induction of NADPH-diaphorase activity in the rat periaqueductal gray matter after nociceptive visceral stimulation. Brain Res 793(1-2): 333-336, 1998.

Samad TA, Moore KA, Sapirstein A, Billet S, Allchorne A, Poole S, Bonventre JV, Woolf CJ: Interleukin-1beta-mediated induction of Cox-2 in the CNS contributes to inflammatory pain hypersensitivity. Nature 410(6827): 471-475, 2001.

Schäfers M, Sommer C: Anticytokine therapy in neuropathic pain management. Expert Rev Neurother 7(11): 1613-1627, 2007.

Sommer C, Kress M: Recent findings on how proinflammatory cytokines cause pain: peripheral mechanisms in inflammatory and neuropathic hyperalgesia. Neurosci Lett 361(1-3): 184-187, 2004.

Takeda R, Watanabe Y, Ikeda T, Abe H, Ebihara K, Matsuo H, Nonaka H, Hashiguchi H, Nishimori T, Ishida Y: Analgesic effect of milnacipran is associated with c-Fos expression in the anterior cingulate cortex in the rat neuropathic pain model. Neurosci Res 64(4): 380-384, 2009.

Terayama R, Kishimoto N, Yamamoto Y, Maruhama K, Tsuchiya H, Mizutani M, Iida S, Sugimoto T: Convergent Nociceptive Input to Spinal Dorsal Horn Neurons After Peripheral Nerve Injury. Neurochem Res Nov 28. [Epub ahead of print] 2014.

Terrett AC, Vernon H: Manipulation and pain tolerance. A controlled study of the effect of spinal manipulation on paraspinal cutaneous pain tolerance levels. Am J Phys Med 63(5): 217-225, 1984.

Vicenzino B, Collins D, Benson H, Wright A: An investigation of the interrelationship between manipulative therapy-induced hypoalgesia and sympathoexcitation. J Manipulative Physiol Ther 21(7): 448-453, 1998.

Willer JC, Roby A, Le Bars D: Psychophysical and electrophysiological approaches to

the pain-relieving effects of heterotopic nociceptive stimuli. Brain 107 (Pt 4): 1095-1112, 1984.

Zelenka M, Schäfers M, Sommer C: Intraneural injection of interleukin-1beta and tumor necrosis factor-alpha into rat sciatic nerve at physiological doses induces signs of neuropathic pain. Pain 116(3): 257-263, 2005.

Zimmermann M, Herdegen T: Control of gene transcription by Jun and Fos proteins in the nervous system; Beneficial or harmful molecular mechanisms of neuronal response to noxious stimulation?. APS 3(1): 33-48, 1994.

국문 초록

본 연구는 좌골신경 압박손상 후 신경성 통증을 가진 흰쥐를 대상으로 척추 교정기구 효과에 대해 실험하였다. Sprague-Dawley 흰쥐에 좌골신경 손상을 가한 후, 무작위로 대조군, 좌골신경 손상 그룹, 좌골신경 손상을 가진 1번 임펄스 교정 그룹, 좌골신경 손상을 가진 5번 임펄스 교정 그룹, 좌골신경을 가진 10번 임펄스 교정 그룹으로 나누었다 (n=7).

하루에 한번씩 7일 연속으로 각각의 그룹에게 요추 제6번과 천추 제1번 부위에 척추 교정기구인 Impulse Adjusting Instrument® (임펄스)를 사용하였다. 족저 실험 장치를 이용하여 열통각과민을 평가하였고. 특수 단백질 검출 검사로 인해 tumor necrosis factor-alpha (TNF-α)와 cyclooxygenase-2 (COX-2)는 좌골신경에 발현한다는 것을 알아냈다. 마지막으로 요추 제 4-5번 척수에 c-Fos와 NADPH-diaphorase (NADPH-d)으로 신경 통증 표시 하였으며, 면역 조직화학법을 이용하여 중심회색질 (vlPAG)를 측정하였다.

좌골신경 압박손상 시 좌골신경에서 TNF-α와 COX-2는 발현을 증가시키고 열의 도피반응 시간을 감소시켰다 (p<0.05). 반면에, 임펄스 교정 치료 횟수가 늘어날수록 TNF-α와 COX-2의 발현이 감소하였다 (p<0.05). 더욱이 임펄스 교정 치료는 c-Fos와 NADPH-d, vlPAG의 발현이 척수의 등쪽뿔에서 억제되는 것을 알 수 있다 (p<0.05).

각 치료 그룹들을 비교해 보았을 때, 1번 또는 10번 임펄스 교정 그룹보다 5번 임펄스 교정 그룹에서 높은 효과가 있었음을 알 수 있다. 그 결과로, 좌골 신경 압박 손상 시 염증 관련 인자가 발현 억제하는 것을 통하여 척추 교정 기구는 신경성 통증에 완화할 수 있다고 볼 수 있다. 그러나 추가 연구에는 신경성 통증이 척추 교정의 정확한 분자 메카니즘을 파악하는 것이 필요하다고 사료된다.

Key Words : 좌골 신경 손상, Tumor Necrosis Factor-alpha, Cyclooxygenase-2, c-Fos, NADPH-diaphorase