

Thesis of Doctor Degree

**Effects of Chiropractic Manipulation on the Expressions of
NADPH-Diaphorase and Nitric Oxide Synthase in the Spinal
Motor Neurons following Knee Joint Immobilization in the
Guinea Pig**

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Hanseu University
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Abstract

The aim of the present study was to investigate effects on manipulative therapy in neurochemical changes of NADPH-d and NOS of the spinal motor neurons after right knee joint immobilization (RKJI) and in ultrastructural changes of the femoral nerves innervating the muscles acting on RKJI. A total of 15 guinea pigs were used and divided into 5 groups; control, 8I-2M group, 8I-2N group, 4I-2M group and 4I-2N group. Immunohistochemistry was performed to detect NADPH-d and NOS. Ultrastructures of the femoral nerves were examined. NADPH-d and NOS were not expressed in the ventral horn of control and experimental groups, but were expressed or not in the dorsal horn according to the duration of release after RKJI and the presence or absence of manipulative therapy. In case of NADPH-d, nonspecific activity was seen in the 8I-2M group and control, low level activity was seen in the 8I-2N and 4I-2M groups and high level activity was seen in the 4I-2N group. In case of NOS, nonspecific activity was seen in the 8I-2M group and control, high level activity was seen in the 8I-2N group and low level activity were seen in the 4I-2M and 4I-2N groups. Ultrastructures of the femoral nerves in experimental groups had partial demyelination and condensed clumps in axon. Effects on manipulative therapy after RKJI were confirmed from expression of NADPH-d and NOS in the dorsal horn of the lumbosacral spinal cord. Manipulative therapy was more effective against a long-term immobilization than a short-term immobilization.

Key Words : Knee Joint, Manipulative Therapy, NADPH-diaphorase, Nitric Oxide Synthase, Immobilization

I. Introduction

Knee joint immobilization is common medical practice used to manage musculoskeletal injuries, however Immobilization of the knee joint for a period generally causes degenerative alterations in many articular tissues (He & Dishman, 2009). Together with the articular tissues, neural tissue in the knee joint will also be altered after knee joint immobilization (Fuglsang-Frederiksen & Scheel, 1978; Michinaka et al., 1997; Kanemura et al., 2002). Progressive degeneration of articular tissues by immobilization of the knee joint, especially long-term immobilization appears to result in the Knee joint problems. Knee joint problems are one of the most common ailments of the musculoskeletal system and can produce pain (Xiaohua et al., 2008). Chiropractic manipulative therapy can be helpful in alleviating some of the pain in knee joint problems (Xiaohua et al., 2008), and are also effective on extremity conditions (Hoskins et al., 2001; Nelson et al., 2005).

Recent interest in nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) and nitric oxide synthase (NOS) has been focused on its expression in motor neurons of the central nervous system (CNS) after peripheral nerve injury (Gonzalez et al., 1987; Lei et al., 1992; Wu & Li, 1993; Hama & Sagen, 1994; Wu et al., 1994; Hee et al., 1997). NADPH-diaphorase had been detected in a subpopulation of neurons in the CNS and myentric plexus (Bredt et al., 1990; Hee et al., 1997). NOS is an enzyme for generation of nitric oxide (NO) which is implicated in a multiplicity of biological functions (Vincent & Hope, 1992; Pannu & Singh, 2006). At the CNS, it is known that, low concentrations of NO promote neuronal survival and growth (Dimmeler & Zeiher, 1999; Xiong et al., 1999), while high concentrations exert toxic effects and neuron death (Bao & Liu, 2002; Shibata & Kobayashi, 2008). Several studies have demonstrated NO involvement in neurodegenerative disorder causing neurons death such

as Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis (Good et al., 1998; Tabner et al., 2002; Ischiropoulos & Beckman, 2003). The induction and up-regulation of NADPH-d and NOS have been suggested to be related to neuronal degeneration and to be resulted from RKJI (Li et al., 1993; Wu & Li, 1993; Hee et al., 1997; Dawson et al., 2002).

Many of studies on the knee joint, muscles acting on the knee joint, the nerve supply to the knee joint and neuroanatomical and neurophysiological changes as the result of the knee joint immobilization in the knee joint have been carried out in different species, including the rats, cats, monkeys, rabbits and humans (Fuglsang-Frederiksen & Scheel, 1978; Kennedy et al., 1982; Dorn et al., 1991; Hildebrand et al., 1991; Lee et al., 1991; Dougherty et al., 1992; Gardner, 1995; Michinaka et al., 1997; Kanemura et al., 2002; He et al., 2009). However, studies on the effects of chiropractic manipulative therapy in the knee joint problems are rarely. The aim of the present study was to investigate effects on manipulative therapy in neurochemical changes of NADPH-d and NOS of the spinal motor neurons after RKJI, in ultrastructural change of nerves innervating the muscles acting on the right knee joint immobilization (RKJI).

II. Methods

1. Animals

This study was approved by HanSeo University Graduate school of Health Promotion, S. Korea. A total of 15 guinea pigs of both sexes weighing 350-450g were used in the present study Table 1. All experiments were carried out in accordance with the guidelines regarding the care and use of animals for experimental procedures established by National Institutes of Health. Every step was taken to minimized animals suffering and reduced the number of animals used.

Table 1. Numbers of Used Animals in Each Group

Group	Control	8I-2M	8I-2N	4I-2M	4I-2N
Number	3	3	3	3	3

8I-2M, 2 weeks release and manipulative therapy after 8 weeks RKJI; 8I-2N, 2 weeks release only after 8 weeks RKJI; 4I-2M, 2 weeks release and manipulative therapy after 4 weeks RKJI; 8I-2N, 2 weeks release only after 4 weeks RKJI.

2. Right Knee Joint Immobilization and Manipulative Therapy

The fur around the right knee joint was shaved before immobilization. Two padded plastic splints were placed on the medial and lateral sides of the knee joint and were fixed with tape around the limb. The right knee joint from the lower 1/3 of thigh to the upper 1/3 of the leg was fixed in an extended position (150°) with two splints. After RKJI, the foot skin color and movements of the knee and hip joints were observed. The knee joint of animals were immobilized for 4 weeks (2 groups) and 8

weeks (2 groups) and then released. Released one group had manipulative therapy for 2 weeks and the other group left freely. Manipulative therapy was performed by repetitive operation including contraction and extension of the right knee joint. Released day 1 had 5 times manipulative therapy (5°), released day 2 had 10 times (10°), day 3 had 15 times (15°), days 4-14 had 20 times manipulative therapy (20°-90°).

3. General Histological Methods

Animals were anesthetized with ketamine (0.2ml/100g, Yuhan, KOR) and perfused via the left ventricle with 0.9% saline (4°C) followed by 4% paraformaldehyde (4°C) in 0.1 mol phosphate buffer (PBS, pH 7.4) for 30 min. The lumbosacral spinal cords from L5 to S1 were removed and postfixed in the same fixative for 2-3h, then were stored overnight in 20% sucrose in 0.1M PBS at 4°C. The spinal cords were cut transversely to a thickness of 30 μ m in a cryostat. Sections were mounted on gelatin coated slides and dried at room temperature for NADPH-d histochemistry and nitric oxide synthase (NOS) immunohistochemistry.

4. NADPH-d Histochemistry

Sections of the lumbosacral spinal cord for NADPH-d histochemistry were incubated at room temperature for 60mins in a solution containing 0.1M Tris-HCl buffer (pH 7.6), 1mg/ml β -NADPH (Sigma, USA; Lot 82H7010, 1.2mM), 0.5mg/ml nitroblue tetrazolium 100 (Sigma, USA), then washed in 0.1M PBS (pH 7.4) and distilled water, and then were examined with a microscope.

5. Nitric Oxide Synthase Immunohistochemistry

Sections of the lumbosacral spinal cord for NOS immunohistochemistry were washed with PBS (pH 7.4) containing 0.2% Triton X-100 (Sigma, USA) and placed in 0.3% H₂O₂ for 20 min to block endogenous peroxidase activity. After 3 rinses in PBS, sections were placed in 10% normal goat serum in 0.1M PBS (pH7.4) for 1 hr. Sections were then incubated overnight at room temperature in rabbit polyclonal anti-bNOS (Chemicon, USA) at dilution 1:70. To detect the reaction product from antigen-antibody bindings, a biotinylated goat antirabbit secondary antibody and a standard ABC kit (Vector Laboratories, USA) were used. Diaminobenzidine tetrahydrochloride (DAB) was used as the peroxidase substrate. The sections were washed with PBS for 3 times, and were examined with a microscope.

6. Electron Microscope of Femoral Nerve

Quadriceps were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in Millonig's phosphate buffer (pH 7.4) for 2hr. The tissues was post-fixed with 1.3% osmium tetroxide in the same buffer for 2hr, dehydrated with a series of the graded ethyl alcohol and acetone, and embedded in epoxy resin. Blocks were trimmed and cut transversely to thickness of 60-90nm by an ultramicrotome. Thin sections of 60-90nm were double stained with uranyl acetate and lead citrate for observation of an electron microscope. All of the thin sections were examined with a JEOL 100S transmission electron microscope.

7. Measurement of Area of Femoral Muscular Fiber

Sections for measurement of area in femoral muscular fibers were stained with 5% toluidine blue, washed with distilled water and then were examined with a microscope. Perfectly cross sectionalized 50 muscular fibers having round form were randomly

selected and measured in area with Motic Image Plus 2.0 (Motic Ltd., Taiwan).

8. Statistical Analysis

Areas of 50 femoral muscular fibers in quadriceps were measured. All measured data was assessed by analysis of the Excel statistical package (Microsoft). Student's t-test was used for the comparison of two groups ($p < 0.05$). Values were represented as mean \pm SD.

III. Results

1. Motor Behavior

After RKJI, the foot skin color and swelling of the immobilized limbs were checked daily to ensure adequate blood supply and nerve integrity of the limbs. Since the knee joints were immobilized in extended position, activity of the immobilized knee was totally lost. Movements at the hip joint were retained. Although quantifiable data on spontaneous movements were not made, movement was clearly reduced or appeared totally lost in guinea pigs with RKJI. In released animals after 8 weeks and 4 weeks of immobilization, spontaneous movements were gradually regained during the first week and returned to normal in the second week.

2. NADPH-d Histochemistry

NADPH-d was not expressed in the ventral horn of the lumbosacral spinal cord of non-immobilization control and all experimental groups (data not shown). In the dorsal horn of the lumbosacral spinal cord, NADPH-d was expressed or not expressed according to the duration of release after RKJI and the presence or absence of manipulative therapy Fig. 1. Nonspecific activity on NADPH-d labeled neurons was seen in the non-immobilization control Fig. 1A and 8I-2M group having 2 weeks release and manipulative therapy after 8 weeks RKJI Fig. 1B, low level activity was seen in the 8I-2N group having 2 weeks release only after 8 weeks RKJI Fig. 1C and 4I-2M group having 2 weeks release and manipulative therapy after 4 weeks RKJI Fig. 1D, and high level activity was seen in 4I-2N group having 2 weeks release only after 4 weeks RKJI Fig. 1E.

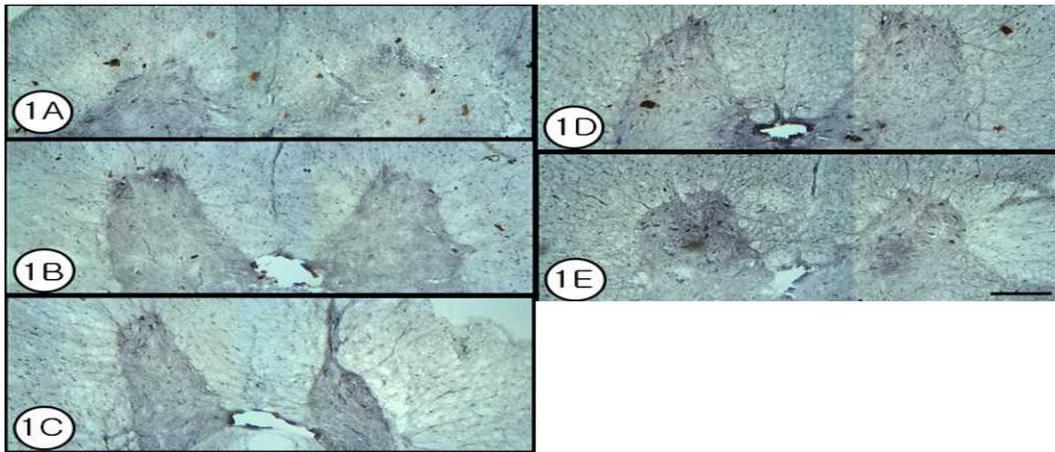


Fig. 1. Light Micrographs Showing Histochemistry for NADPH-d in the Dorsal Horn of the Lumbar Spinal Cord. Nonspecific activity on NADPH-d labeled neurons is seen in the non-immobilization control (A) and 8I-2M group (B), low level activity is seen in the 8I-2N (C) and 4I-2M groups (D), and high level activity is seen in 4I-2N group (E). 8I-2M, 2 weeks release and manipulative therapy after 8 weeks RKJI; 8I-2N, 2 weeks release only after 8 weeks RKJI; 4I-2M, 2 weeks release and manipulative therapy after 4 weeks RKJI; 4I-2N, 2 weeks release only after 4 weeks RKJI. Scale bar=100 μ m.

3. Nitric Oxide Synthase Immunoreactivity

NOS was not expressed in the ventral horn of the lumbar spinal cord of the non-immobilization control and all experimental groups as those of NADPH-d histochemistry (data not shown). In the dorsal horn of the lumbar spinal cord, NOS was expressed or not expressed according to the duration of release after RKJI and the presence or absence of manipulative therapy Fig. 2, but was distinguished from those of NADPH-d. Nonspecific activity on NOS labeled neurons was seen in the non-immobilization control Fig. 2A and 8I-2M group Fig. 2B, High level activity was seen in 8I-2N group Fig. 2C and low level activity was seen in 4I-2M group Fig. 2D and 4I-2N group Fig. 2E.

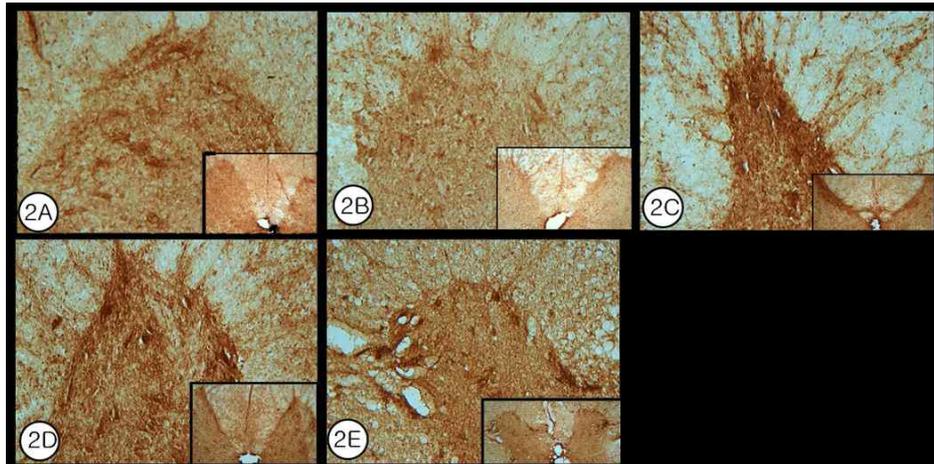


Fig. 2. High Magnified Micrographs Showing Immunohistochemistry for NOS in the Dorsal Horn of the Lumbosacral Spinal Cord. Nonspecific activity on NOS labeled neurons is seen in the non-immobilization control (A) and 8I-2M group (B), high level activity is seen in the 8I-2N group (C) and low level activity is seen in 4I-2M (D) and 4I-2N groups (E). Inset show low magnified image of the transverse section of the dorsal horn. 8I-2M, 2 weeks release and manipulative therapy after 8 weeks RKJI; 8I-2N, 2 weeks release only after 8 weeks RKJI; 4I-2M, 2 weeks release and manipulative therapy after 4 weeks RKJI; 4I-2N, 2 weeks release only after 4 weeks RKJI. Scale bar=100 μ m.

4. Ultrastructures of Femoral Nerves

Axon and myelin sheath of the femoral nerves in the non-immobilization control and all experimental groups were observed Fig. 3. Non-immobilization control had sound axon and myelin sheath Fig. 3A, but the other experimental groups including 8I-2M Fig. 3B, 8I-2N Fig. 3C, 4I-2M Fig. 3D and 4I-2N Fig. 3E had modified axon and myelin sheath, having many of vacuoles and condensed clumps in axon and partial demyelination. The extent of modification in axon and myelin sheath are similar to all experimental groups.

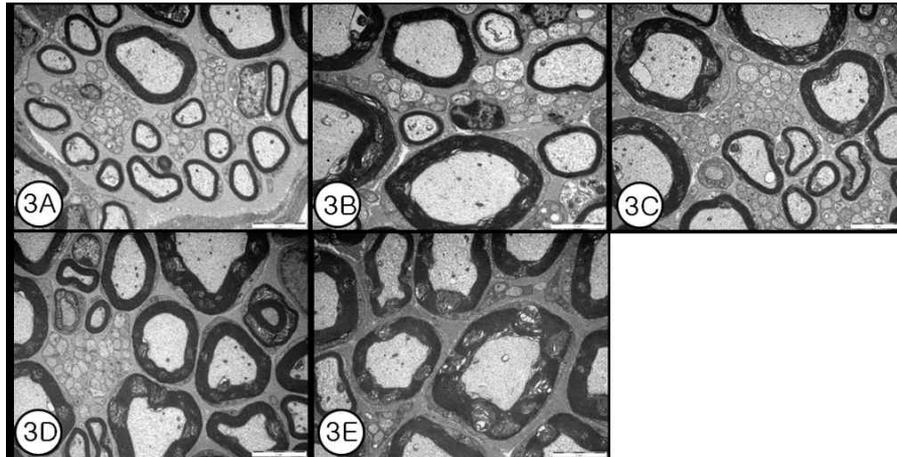


Fig. 3 Electron Micrographs Showing the Femoral Nerves. Sound axon and myelin sheath are seen in the non-immobilization control (A). Many of vacuoles and condensed clumps in axon and partial demyelination are seen in all experimental groups, 8I-2M (B), 8I-2N (C), 4I-2M (D) and 4I-2N (E). The extent of modification and morphologic characteristics in axon and myelin sheath are similar to all experimental groups. 8I-2M, 2 weeks release and manipulative therapy after 8 weeks RKJI; 8I-2N, 2 weeks release only after 8 weeks RKJI; 4I-2M, 2 weeks release and manipulative therapy after 4 weeks RKJI; 4I-2N, 2 weeks release only after 4 weeks RKJI. Scale bars=2 μ m.

5. Area of Femoral Muscular Fibers

Femoral muscular fibers having round from in each group were randomly selected and measured in area. Measured value of 8I-2M group was significantly large and distinguished from those of the non-immobilization control and the other experimental groups ($p < 0.05$). Measured value of 8I-2N, 4I-2M and 4I-2N groups were similar to with each other and significantly smaller than the non-immobilization control Fig. 4. Although measured value of 4I-2M group was not distinguished from those of 4I-2N group, measured value of 8I-2M group was not only distinguished from those of 8I-2N group but also those of the non-immobilization control.

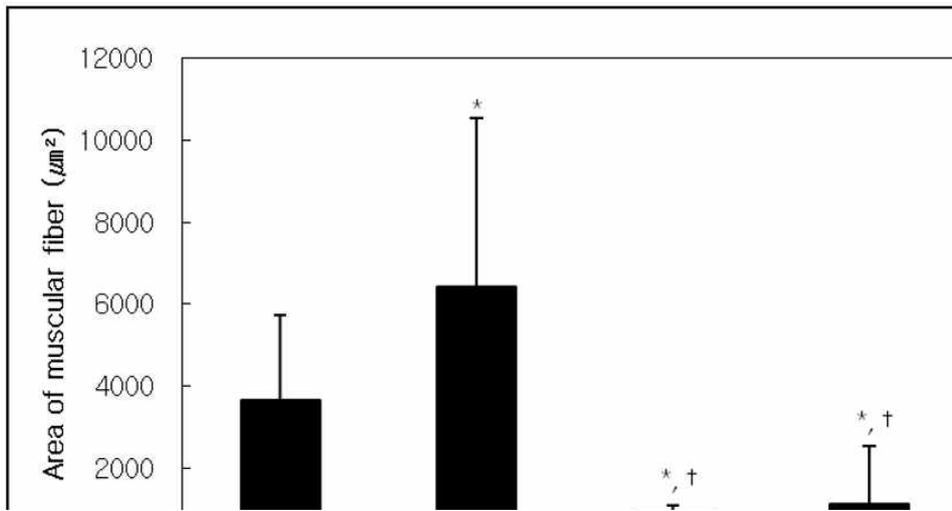


Fig. 4 Measurements of Area of the Muscular Fibers in the Quadriceps. Large measured values in area of the muscular fibers of 8I-2M group are not only distinguished from non-immobilization control but also the other experimental groups. Measured values in area of the muscular fibers of 8I-2N, 4I-2M and 4I-2N groups are similar to with each other. Data are mean±SD (n=50). * p <0.05 compared to the non-immobilization control. † p <0.05 compared to the 8I-2M group. 8I-2M, 2 weeks release and manipulative therapy after 8 weeks RKJI; 8I-2N, 2 weeks release only after 8 weeks RKJI; 4I-2M, 2 weeks release and manipulative therapy after 4 weeks RKJI; 4I-2N, 2 weeks release only after 4 weeks RKJI.

IV. Discussion

Changes of NADPH-d and NOS by manipulative therapy and release after RKJI were examined in the ventral horn and the dorsal horn of the lumbosacral spinal cord.

Previous study showed that NADPH-d and NOS were expressed in the ventral horn of lumbosacral segments after RKJI (Hee et al., 1997). According to this study, under RKJI condition, expression of NADPH-d and NOS were progressive enhanced in the ventral horn and under release condition, NADPH-d and NOS reactivity were prominently reduced in the ventral horn neurons. Also enhanced NADPH-d expression in the ventral horn motoneurons following peripheral nerve lesions has been reported (Gonzalez et al., 1987; Li et al., 1993; Wu et al., 1994). Present study showed that NADPH-d and NOS were not expressed in the ventral horn of the lumbosacral spinal cord regardless of the duration of release after RKJI and the presence of manipulative therapy, however, NADPH-d and NOS were expressed or not expressed in the dorsal horn of the lumbosacral spinal cord according to the duration of release after RKJI and the presence or absence of manipulative therapy. Also NADPH-d expression in the dorsal horn of the aged lumbosacral spinal cord under the non-stressed condition has been reported (Tan et al., 2006). Thus, it seems that such differences of the expressed regions are due to the released condition after RKJI, namely, NADPH-d and NOS are expressed in the ventral horn of the lumbosacral spinal cord under the stressed condition such as RKJI, while were expressed in the dorsal horn under the freely released condition.

NOS, an enzyme for generation of nitric oxide (NO) has 3 known isoforms of NOS, that is, the neuronal (nNOS), the endothelial (eNOS) and the inducible (iNOS) form, are found in nervous tissue (Bredt & Snyder, 1992; Vincent & Hope, 1992; Martin et al., 2005; Pannu & Singh, 2006; Sung et al., 2010). The different NOS isoforms changed

reciprocally during motor neurons apoptosis; nNOS protein was upregulated, whereas iNOS protein was downregulated. But, the residual iNOS contributes more than the upregulated nNOS to the injury-induced transient increase in NADPH-d activity and NO production (Martin et al., 2005). The iNOS has been found to be a major factor to initiation of the CNS inflammatory or degenerative conditions through the production of excessive NO (Pannu & Singh, 2006). It has been well known that NO generated by iNOS has both neuroprotective and neurotoxic effects in the central nervous system (Bredt & Snyder, 1992; Vincent & Hope, 1992; Pannu & Singh, 2006). NO from different cellular sources functions as a neurotransmitter and also promotes neuronal survival and growth under low concentrations (Dimmeler & Zeiher, 1999; Xiong et al., 1999). However, in high concentrations, it may exert toxic effects leading neurons to death (Bao & Liu, 2002; Shibata & Kabayashi, 2008). NADPH-d had been detected in a subpopulation of neurons in the CNS and myenteric plexus (Bredt et al., 1990). Also NADPH-d expression in the aged lumbosacral spinal cord has been reported (Tan et al., 2006). Changes in the neurochemical properties of NADPH-d after spinal cord injury may be mediated by pathological changes in the target organs and/or spinal cord (Tan et al., 2006). These results demonstrate that expression of NOS in the dorsal horn of the present study under the non-stressed condition without RKJI may serve for neuroprotective effects, that is, serve as part of a defense mechanism of neuron and of their subsequent recovery with the restoration of muscle function. Expression of NADPH-d in the dorsal horn also may induce by NOS having neuroprotective effects. However, function of NADPH-d and NO in the dorsal horn of the lumbosacral spinal cord still remains speculative.

NADPH-d activity has been used as a marker for NOS in neurons (Toung et al., 1997). It has been argued that NADPH-d activity in the fixed brain is used as a marker for nNOS, and NADPH-d and nNOS are actually the same enzyme (Dawson et al., 1991). In contrast with the former studies, there is evidence that NADPH-d activity is

not always a specific histochemical marker for the NO and these activities are not always co-localized (Kemp et al., 1988; Bredt et al., 1991; Matsumoto et al., 1993; Tracey et al., 1993; Doone et al., 1999). Suggested that NOS represents only a fraction of the total cellular NADPH-d activity and that these activities are not always co-localized. In the present study, there were a difference in expression of NADPH-d and NO in the dorsal horn of the lumbosacral spinal cord according to the duration of release after RKJI and the presence or absence of manipulative therapy. In case of NADPH-d in the dorsal horn, nonspecific activity was seen in the 8I-2M group and non-immobilization control, low level activity was seen in the 8I-2N and 4I-2M groups and high level activity was seen in the 4I-2N group. In case of NOS in the dorsal horn, nonspecific activity was seen in the 8I-2M group and non-immobilization control, high level activity was seen in the 8I-2N group and low level activity were seen in the 4I-2M and 4I-2N groups. Thus, NADPH-d histochemistry and NOS immunohistochemistry do not absolutely label the same neuron population.

The present study demonstrated that manipulative therapy effective against the Knee joint problems having progressive degeneration by the knee joint immobilization. According to our data, Groups with manipulative therapy, 8I-2M and 4I-2M had nonspecific or low level activity of NADPH-d and NOS in the dorsal horn of the lumbosacral spinal cord than groups without manipulative therapy, 8I-2N and 4I-2N. Especially the 8I-2M group as the non-immobilization control had only nonspecific activity of NADPH-d and NOS in the dorsal horn of the lumbosacral spinal cord. From the above results, manipulative therapy was effective in RKJI and more effective against a long-term RKJI than a short-term RKJI. Thus, expression of NADPH-d and NOS in the dorsal horn of the lumbosacral spinal cord could be regarded as one of estimated instruments for effects of manipulative therapy. Similar results were obtained in measured value of area in the femoral muscular fibers. Although values of area in the femoral muscular fibers of 4I-2M group were not distinguished from those of 4I-2N

group, significantly large values of area in the femoral muscular fibers of 8I-2M group were not only distinguished from those of 8I-2N group but also those of non-immobilization control. These results demonstrate that measured value of area in the femoral muscular fibers showed effects of manipulative therapy in RKJI and more effective against a long-term RKJI than a short-term RKJI. However, in observation of ultrastructures of the femoral nerves, all experimental groups had partial demyelination and condensed clumps in axon, and the extent of modification and morphological characteristics in axon and myelin sheath are similar to all experimental groups. So that, ultrastructures of the femoral nerves had not difference according to the presence or absence of manipulative therapy.

V. Conclusions

The present study demonstrates that manipulative therapy is effective against Knee joint problems having a progressive degeneration by the knee joint immobilization. Especially manipulative therapy is more effective against a long-term immobilization than a short-term immobilization. Expression of NADPH-d and NOS in the dorsal horn of the lumbosacral spinal cord could be regarded as one of estimated instruments for effects of manipulative therapy.

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국문 초록

본 연구는 오른쪽 슬관절 고정 (RKJI) 후 재활도수치료가 오른쪽 슬관절 고정 (RKJI) 후 척추운동신경세포의 NADPH-d와 NOS의 신경화학적 변화와 RKJI에 작용하는 대퇴 신경이 초미세구조 변화에 어떤 영향을 미치는 지에 대해 조사하였다. 기니피그의 15마리를 대조군, 8I-2M 그룹, 8I-2N 그룹, 4I-2M 그룹, 4I-2N 그룹으로 나누었다. 면역조직화학법으로 NADPH-d와 NOS를 검출과 대퇴 신경의 초미세 구조를 검사하였다.

NADPH-d와 NOS는 대조군과 실험군의 앞쪽뿔에 나타나지 않았으나 등쪽뿔에서는 RKJI 후 작용 시간에 따라사와 재활도수치료 유무에 따라 나타나거나 나타나지 않았다. NADPH-d의 경우에는, 대조군과 8I-2M그룹에 특별한 변화가 없었으며, 8I-2N과 4I-2M 그룹에는 낮은 움직임이 보였고, 4I-2N 그룹에서는 높은 움직임이 보였다. NOS의 경우에는 대조군과 8I-2M에서 특별한 변화가 없었고, 8I-2N그룹에서는 높은 움직임이 보였으며, 4I-2M과 4I-2N 그룹에서는 낮은 움직임이 관찰되었다. 실험군의 대퇴 신경의 초미세 구조는 부분적인 탈수초와 응결된 축색 돌기 덩어리를 가지고 있었다.

RKJI의 후 재활도수치료의 효과는 요천추 척수의 등쪽뿔에서 NADPH-d와 NOS의 발현을 확인할 수 있었다. 재활도수치료는 단기간 고정보다 장기간 고정이 더 효과적으로 사료된다.

Key Words : 슬관절, 재활도수치료, NADPH-diaphorase, Nitric Oxide Synthase, 고정