Effects of Low Power Laser on Pain Response and Axonal Regeneration in Rat Models with Sciatic Nerve Crush Injury

This study purposed to examine the effect of low power laser on pain response and axonal regeneration. In order to prepare peripheral nerve injury models, we crushed the sciatic nerve of Sprague–Dawley rats and treated them with low power laser for 21 days. The rats were divided into 4 groups: normal group (n=10); control group (n=10) without any treatment after the induction of sciatic nerve crush injury; experimental group I (n=10) treated with low power laser (0.21mJ/mm²) after the induction of sciatic nerve crush injury; and experimental group II (n=10) treated with low power laser (5.25mJ/mm²) after the induction of sciatic nerve crush injury. We measured spontaneous pain behavior (paw withdrawal latency test) and mechanical allodynia (von Frey filament test) for evaluating pain behavioral response, and measured the sciatic function index for evaluating the functional recovery of peripheral nerve before the induction of sciatic nerve crush injury and on day 1, 7, 14, and 21 after the induction. After the experiment was completed, changes in the H & E stain and toluidine blue stain were examined histopathologically, and changes in MAG (myelin associated glycoprotein) and c-fos were examined immunohistologically. According to the results of this study, when low power laser was applied to rat models with sciatic nerve crush injury for 21 days and the results were examined through pain behavior evaluation and neurobehavioral, histopathological and immunohistochemical analyses, low power laser was found to affect pain response and axonal regeneration in both experimental group I and experimental group II. Moreover, the effect on pain response and axonal regeneration was more positive in experimental group I to which output 0.21mJ/mm² was applied than in experimental group II to which 5.25mJ/mm² was applied.

Key words: c-fos; MAG; Paw Withdrawal Latency Test; Sciatic Function Index; Von Frey Filament Test

INTRODUCTION

Peripheral nerves can be easily damaged by crush, compression, stretching, and avulsion(1). When nervous tissues have been damaged by compression or otherwise, primarily, morphological changes occur in the nervous tissue and secondarily, physiologic changes occur in the nervous tissues. These changes cause disorders in both sensory nerves and motor nerves leading to functional problems clinically(2).

Damage to peripheral nerves brings about partial or total loss of conducted motor, sensory and autonomic functions depending on the sizes of denervated segments and the degrees of damage. This is because of the apoptosis of neurogens that lost axons, the degeneration of nerve fibers distant from damaged regions and the interruption of the continuity of axons(3). Damage to the peripheral nerve systems brings about major personal or social losses when seen from the view point of life with secondary problems such as material losses of functions, permanent physical function, and psychosocial interference(4). This study purposed to evaluate the effect of low power laser on pain response and axonal regeneration in a rat model with sciatic nerve crush injury.
sensory and motor functional disorders and neuropathic pain(4). When nerves have been damaged due to ischemic damage and inflammatory responses, even adjacent proximal regions and neuronal bodies are denaturalized and damaged neurons are regenerated centering on some surviving cells to undergo the process of recovery. However, even when recovered, not only the functions cannot be completely restored but also serious disorders are caused(5). To review changes resulting from damage to peripheral nerves, the axons, i.e., neurites separated from cell bodies due to the damage are gradually degenerated as they lose functions and sometimes, inflammatory responses progress because of macrophages gathering due to the intracellular substances released while axons are degenerated(6). Damaged regions may be reinvested by the regeneration of damaged axons or the collaterals branches formed by intact axons in the vicinity(7). After crushed damage to peripheral nerves, a series of changes are triggered in the cell bodies of the damaged nerves, sensory neurons and motor neurons to promote nerve survival and axon regeneration in the region of the lesion so that functions are ultimately recovered(8). These changes are triggered through the activation of gene programs and the process of signal transduction in local cells(9). As changes in nerves, cell adhesion molecules are expressed by Schwann cells and glycoproteins and fibronecrtin increase(10), to induce the regeneration of axons(11).

Neuropathic pain is a sort of pain accompanying the aforementioned diseases that appears when peripheral nerves have been partially damaged due to diverse causes such as infectious diseases and trauma. Since this neuropathic pain not only appears continuously even when the disease or trauma that caused the damage to nerves has been completely healed but also is not relieved by treatment, it is recognized as a serious clinical problem(12). Damage to peripheral nerves causes neuropathic pain that includes spontaneous pain which is felt even without any external stimuli, hyperalgesia which is felt stronger than normal from harmful stimuli and allodynia which is felt from harmless stimuli(13). It is not yet clear which neurotransmitter is involved in pain transmission or generation processes in neuropathic pain(14). Although no clear mechanism of neuropathic pain generation processes has been established(15), changes in peripheral nerves’ ion channels, changes in pain related receptors in sensory neurons in the spinal cord and changes in the interneurons in the spinal cord that adjust pain or changes in descending pain control pathways have been suggested as the mechanisms(16). It has been reported that the activity of microglia plays important roles in the induction and maintenance of neuropathic pain resulting from nerve damage and induces hyperalgesia and allodynia(17). Not only nerve system pathways but also Schwann cells and satellite cells in spinal ganglia, the constituents of the peripheral immune system, microglia in the spinal cord and astrocytes are involved in neuropathic pain. Microglia are a sort of neuroglia and they serve the roll of macrophages in the central nervous system(18).

Although there are many reports on the effects of low power lasers on peripheral and central nerves and skin wounds, most of those indicated that low power lasers were directly associated with irradiated tissues or affected the regions below the irradiated skin(19). In neurons, low power lasers bring about the formation of axons and dendrites, the proliferation of Schwann cells and astrocytes and biochemical changes in neurons to prevent the decomposition of axons and the formation of scars thereby preventing the degenerative changes of axons and promoting the generation of axons(20). Although it has been reported that low power lasers promote nerve regeneration processes and enhance the function to reduce nerves inflammatory responses, studies that proved pain and regeneration related effects of laser treatment are quite insufficient yet.

The proliferation of Schwann cells in damaged sciatic nerves has been known as an indicator to identify axonal regeneration(21). Schwann cells synthesize adhesive molecules on the surfaces of plasma membranes to become the frame of regenerated axons and produce diverse neurotrophic factors(22). Axons regenerated after damage occur in the nodes of Ranvier and are given to the proximal neuromere through the distal neuromere of damaged regions so that nerves are regenerated up to target organs(23). Regenerated axons grow in Schwann cells and along the inside surfaces of the basal plates of Schwann cell columns. This morphological characteristics mean that both Schwann cells and the basal plates of Schwann cell columns are the roots of axon regeneration and the basal plates of Schwann cells serve important roles in nerve regeneration(24). Myelin associated glycoproteins continuously exist in the peripheral nervous system thanks to Schwann cells and in the central nervous system thanks to oligodendrocytes and these are perfect structures that are essential for long-term maintenance during the initial stage of myelination(25). As one of methods to test the degrees of pain and analgesic effects, a morphological
method to observe c-fos which is a product of immediate early genes by immunohistochemical staining has been developed and is widely used currently(26). It is known that c-fos does not appear in spinal ganglion neurogens that are primary neurogens but mainly appears in pain transmitting neurogens in the spinal cord and is expressed in other pain related regions in the brain(27).

METHODS

Subjects

Fifty healthy and mature Sprague–Dawley male rats aged 8 weeks and weighed 230±10g raised under the same conditions were used and of them, 10 rats that died or were unsuitable for the purpose of the experiment were excluded. During the experimental period, water and feed were supplied without any restriction, temperatures in the laboratory were maintained at 23±2°C, humidity was maintained at 55±10%, a constant intensity of illumination was maintained and light cycles and dark cycles were adjusted to 12 hours to conform with the life cycle of rats thereby maintaining a constant life habit of the rats. The experimental animals were randomly assigned to a normal group(n=10), a control group(n=10), experimental group(n=10) and experimental group II (n=10). After causing damage to the sciatic nerve by compression, the control group was not treated and experimental group I and experimental group II were applied with low power laser treatment at different intensities five times a week for 15 minutes/time for 21 days.

Measurement

Induction of damage to the sciatic nerve

To induce general anesthesia, 1mg/kg of Zoletil® (Virbac, France) in 0.5ml/kg of saline solution was used intra–abdominally in each of the animals in each experimental group. Damage to the peripheral nerve was induced by temporarily compressing the sciatic nerve. The skin was incised around 2–2.5cm on the region of the incisura on the hipbone and muscles in the buttocks and the hamstring were detached to expose the sciatic nerve. The distal part at around 5–6mm from the region where the exposed nerve came out from the incisura on the hipbone was carefully lifted and compression damage was induced on the left side of it for 30 seconds using hemostatic forceps. To cause damage to the nerve, force was applied to the hemostatic forceps in three steps(28).

Low power laser treatment

For laser treatment, the right sciatic nerves of experimental group I and experimental group II were cut using a Hixson, TN 37343 (Chattanooga group, USA) and lasers were perpendicularly irradiated on the sutured region five times a week for 15 minutes a time for 21 days at a dose of 21mJ/mm² in experimental group I and at a dose of 5.25mJ/mm² in experimental group II (29).

Voluntary pain behavior evaluation(paw with draw–al latency test)

To observe the effect of laser on pain in terms of ethology, paw withdrawal latency time of the hind limbs at the damaged side was measured using an IITC Model 39 hot plate (Life Science Instruments, USA) in the normal group, the control group, experimental group I and experimental group II before the damage and one day, seven days, 14 days and 21 days after the damage. First, the rat was induced to adapt to a hot plate not heated for 30 minutes. Then, the rat was placed on a hot plate heated to a constant temperature(51.2°C) and the rat's paw withdrawal latency time of the damaged side was measured. At this time, the rat's behavior of taking off the paw from the hot plate to move was excluded from the measurement and the measurement was performed within 30 seconds to prevent damage to tissues.

Mechanical alldynia assessment(von Frey filamenet test)

To assess hyperalgesia caused by mechanical stimuli(touch test™ sensory evaluator), the rat was put into an acrylic box(20×25×13cm) having an iron wire net floor and the box was installed 35cm away from the ground. Then a sloped mirror was installed below the box so that the animal's behavioral reactions could be effectively observed. As mechanical stimuli, von Frey filaments (North Coast Medical Inc, USA) were made to stand vertically and used to stimulate the third metatarsal region of the right paw where nerve damage had been induced in order to find the minimum bending force that would induce the reaction to actively withdraw the paw at least three times when stimulated five times with von Frey filaments having 1.0, 2.0, 4.0, 6.0, 8.0, 10.0, 15.0 and 26.0g of bending force respectively and determine it as a paw withdrawal mechanical threshold(PWT, g)(30).
Sciatic function index (SF1)

After smearing the soles of the rat, the rat was made to walk on a pathway made to be 10cm × 20cm × 80cm with white paper spread on the floor to obtain footprints. First, each rat was made to walk two times so that the rat could become familiar with the direction of the pathway and then made to walk five times to record footprints. At this time, in order to make the rat maintain an accurate and constant gait pattern, unspecified influential factors were removed by controlling factors such as noises in surroundings before inducing the rat to move(SF1).

Hematoxyline & eosin staining

To induce general anesthesia of each of the experimental animals, 11mg/kg of Zoletil™ (Virbac, France) in 5ml/kg of saline solution was used intra-abdominally in each of the animals. Sciatic nerve tissues were excised and post fixed by setting the tissues in 10% paraformaldehyde solution for 24 hours at 4°C. After being post fixed, the tissues were automatically treated through dehydration, decolorization and infiltration processes for 14 hours using an automatic tissue processor(4640B, Sakura, Japan). Thereafter, the tissues were made into paraffin blocks while using an automatic embedding device(Tissue-Tex, Japan). The produced paraffin blocks were sliced into 4μm thick slices at intervals of 180μm using a rotary microtome(Rotary Microtome 2040, Japan), put into and taken out from a floating water tank and then attached to gelatin coated slides. Thereafter, after normal H & E staining, dehydration and decoloration processes were implemented, the slices were sealed using Canada balsam(Sigma, USA).

Toluidine blue staining

The sciatic nerves were pre-fixed for two hours in a 2% glutaraldehyde solution, fixed in an 1% OsO₄ solution, washed using cacodylate buffer(pH 7.4, 0.1 M, 4°C), dehydrated using 70% ethanol, interfused with propylene oxide, embedded into Epon and thermally polymerized for 72 hours at 60°C. The Epon blocks were made into 2~3μm thick semi-thin sections using an ultramicrotome(LKB−V, Sweden), stained with 1% toluidine blue and observed.

Western blot analysis(MAG)

The extracted sciatic nerves were immediately put into storage at −70°C and the tissues kept frozen were washed three times with PBS and homogenized at 4°C. Thereafter, the tissues were dissolved in a 4°C dissolution buffer(50mM HEPES(pH 7.5), 150mM NaCl, 10% glycerol, 1% Triton X−100, 1mM PMSF, 1 mM EGTA, 1.5mM MgCl₂, 6H2O, sodium orthovanadate, 100mM sodium fluoride) by making them react for 30minutes in the buffer. Thereafter, the solution was put into centrifugal separation for 20minutes at 4°C, 15,000rpm and the supernatant liquid was extracted. The protein concentration of the dissolved tissues was quantified using the Bradford(Bio−Rad protein assay) method in the supernatant liquid and the extracted protein was loaded on SDS polyacrylamide gel in the same quantity and then electrophoresis was performed with the gel. Thereafter, the gel was transferred to nitrocellulose membranes (Whatman GmbH, Dassel, Germany). To prevent non−specific combination with antibodies, the membranes were made to react with 5% nonfat dried milk in TBS for one hour at 4°C, washed with TBS and made to react in rabbit polyclonal anti−MAG1:1,000, Santacruz Biotechnology, USA) for 24 hours at 4°C. To check if specific reactions had occurred, the membranes were made to react with secondary antibodies, anti−rabbit Ig G(1:1,000, Santacruz Biotechnology ,USA) for one hour and exposed to radiation films using an enhanced chemical luminescence kit(RPN 2106, Amersham life science, Inc., USA) to check the band. To ensure that the same quantities of proteins were loaded, the membranes were compared through monoclonal anti−β actin(1:50, 1:5,000, Sigma, USA), Goat anti−mouse Ig G(BD bioscience, Flanklin lakes, NJ, USA) antibodies.

Immunohistochemical staining(c−fos)

When the experiment had been completed, the skin of the experimental animal covering the spine was excised using scissors and the middle region of the sacral vertebra was incised to expose the spinal canal. A 21 gauge needle was inserted through the spinal canal, a syringe containing a cooled saline solution was attached to the needle to inject the saline solution. Then, the entire region of the spinal cord was made to be exposed through the opening on the cervical vertebrae and only the lumbar spinal cord region was extracted. The extracted spinal cord was fixed for 2~24 hours in a 3% paraformaldehyde, 3% glutaraldehyde, 0.1% picric acid fixing solution and sliced into 5μm thick slices using a rotary microtome(Sakura 2040, Japan). The sliced tissue slices were washed many times with phosphate buffered saline(PBS) and treated for one hour with 1% sodium borohydride in order to remove remaining fixing solution components. As a pre−treatment process for immunohistochemical staining, the tissue slices were treated with a 0.3% hydrogen peroxide solution for 20 minutes. After washing the tissue slices many
times again with PBS, the tissue slices were cultured in normal Blocking Serum for 20 minutes using a Novostain Super ABC kit (Novocastra Lab., Benton Lane, UK), treated with anti c-fos antibodies Sigma, F7799, USA) diluted to 1:1,000 overnight, washed with PBS and then cultured with diluted biotinylated secondary antibodies for 30 minutes. The tissue slices were washed again with PBS, cultured with the novostain super ABC reagent for 30 minutes and washed with PBS. For color formation, the tissue slices were applied to a DAB (Sertec Ltd, BUF021B, UK) for 10 minutes and counterstained with Mayer’s Hematoxyline(Sigma, MHS-32, USA), washed for 5 minutes with flowing water. Then, the slide samples were dried and sealed for observation after a normal dehydration process. An optical microscope (Olympus BX 50, Japan) was used for morphological observation and images were taken with the CCD camera(Toshiba, Japan) installed on the microscope. To check the number of cells that showed reactions in the slices that had undergone immunohistochemical staining processes, c-fos was analyzed through image analysis.

Data Analysis

In this study, statistical analysis was conducted using the SPSS 14.0 version. Mean values and standard deviations of individual experimental results were shown. One-way ANOVA was conducted to test the statistical significance of the relationship between the values of individual experimental groups over time. Paired t-tests were conducted to test the significance of the relationship between the mean values of individual experimental groups. The significance of the relationships between other variables of individual experimental groups was tested through one-way ANOVA and Tukey’s multiple range tests were conducted as ex post facto tests. The statistical significance intensity in the tests was set to p<0.05.

RESULTS

Voluntary Pain Behavior Assessment (paw withdrawal latency test)

In the voluntary pain behavior assessment, the control group where SNI was induced showed significantly (p<0.001) decreased paw withdrawal latency time compared to the normal group at all measuring time points and thus was identified as sensitively reacting to pain. Experimental group I and experimental group II irradiated with low power laser showed significant (p<0.001) increases compared to the control group with 6.86±13 in experimental group I and 4.30±16 in experimental group II compared to 3.58±97 in the control group at day 7; 8.26±54 in experimental group I and 6.13±25 in experimental group II compared to 3.78±19 in the control group at day 14; and 10.08±43 in experimental group I and 8.39±62 in experimental group II compared to 4.20±45 in the control group at day 21. However, the values were not as large as those of the normal group. In comparison between experimental group I and experimental group II, although experimental group I showed a pattern of increasing in values compared to experimental group II, no significance could be observed (Table 1).

Mechanical Allodynia Assessment (von Frey filament test)

Paw withdrawal latency time (sec) was measured to assess mechanical allodynia. Based on the results, it was identified that the control group where SNI was induced showed significantly (p<0.001) decreased paw withdrawal latency time compared to the normal

<table>
<thead>
<tr>
<th>Group</th>
<th>1 day</th>
<th>7 days†††</th>
<th>14 days*</th>
<th>21 days§§§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>13.64±.64</td>
<td>14.05±.67</td>
<td>13.77±.79</td>
<td>13.68±.67</td>
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<tr>
<td>Control</td>
<td>3.34±.45***</td>
<td>3.58±.97***</td>
<td>3.78±.19***</td>
<td>4.20±.45***</td>
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<tr>
<td>Experimental I</td>
<td>3.50±.57</td>
<td>6.86±13***</td>
<td>8.26±54**</td>
<td>10.08±43***</td>
</tr>
<tr>
<td>Experimental II</td>
<td>3.74±.24</td>
<td>4.30±.16***</td>
<td>6.13±.25***</td>
<td>8.39±.62***</td>
</tr>
</tbody>
</table>

Each value represents the Mean±SD
Tested by One-way ANOVA: * p<0.05, ** p<0.01, *** p<0.001
Normal compare with control: * p<0.05, ** p<0.01, *** p<0.001
Post-hoc test was by Tukey’s multiple range test, control compared with experimental I and experimental II
††† p<0.001, ** p<0.01, †† p<0.05
group at all measuring time points thereby showing decreased reactions to sensory stimuli, Experimental group I and experimental group II irradiated with low power laser showed significant increases compared to the control group with 5.71±1.38(p<0.01) in experimental group I and 4.28±1.38(p<0.05) in experimental group II compared to 2.40±1.13 in the control group at day 14 and 8.71±2.87(p<0.01) in experimental group I and 7.14±1.95(p<0.01) in experimental group II compared to 2.49±1.06 in the control group at day 21. However, the values were not as large as those of the normal group. In comparison between experimental group I and experimental group II, experimental group I showed a pattern of significant increases(p<0.01) in values compared to experimental group II (p<0.05) at day 14 and also experimental group I showed a pattern of significant increases(p<0.01) in values compared to experimental group II (p<0.01) at day 21 (Table 2).

Table 2. The withdrawal threshold to mechanical allodynia in induced by sciatic nerve injury in rats (sec)

<table>
<thead>
<tr>
<th>Group</th>
<th>1 day</th>
<th>7 days</th>
<th>14 days##</th>
<th>21 days###</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>22.70±5.31</td>
<td>23.80±4.64</td>
<td>22.86±5.37</td>
<td>22.86±5.37</td>
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<tr>
<td>Control</td>
<td>1.70±3.20***</td>
<td>2.14±0.69***</td>
<td>2.40±1.13***</td>
<td>2.49±1.06***</td>
</tr>
<tr>
<td>Experimental I</td>
<td>1.76±3.10</td>
<td>2.30±1.05</td>
<td>5.71±1.38''</td>
<td>8.71±2.87''</td>
</tr>
<tr>
<td>Experimental II</td>
<td>1.70±3.20</td>
<td>2.40±3.12</td>
<td>4.28±1.38'</td>
<td>7.14±1.95''</td>
</tr>
</tbody>
</table>

Mean±SD

Sciatic Function Index(SFI)

Based on the results of SFI tests, it was observed that the control group where SNI was induced showed significantly(p<0.01) decreased sciatic function indexes compared to the normal group and thus showed declined motor functions. Of experimental group I and experimental group II irradiated with low power laser, only experimental group I showed significant increases with ~50.15±10.53 compared to the control group with ~65.87±12.18 at day 14 and both experimental group I and experimental group II showed significant increased compared to the control group with ~29.26±11.38(p<0.01) in experimental group I and ~38.01±12.91(p<0.01) in experimental group II compared to ~62.55±11.03 the control group at day 21. However, the values were not as large as those of the normal group. In comparison between experimental group I and experimental group II, experimental group I showed a pattern of significant increases(p<0.05) compared to experimental group II at day 14 and also experimental group I showed a pattern of significant increases(p<0.01) compared to experimental group II (p<0.01) at day 21 (Table 3).

Table 3. The changes of sciatic function index (score)

<table>
<thead>
<tr>
<th>Group</th>
<th>1 day</th>
<th>7 days</th>
<th>14 days##</th>
<th>21 days###</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>-8.48±2.50</td>
<td>-8.99±3.55</td>
<td>-8.31±1.53</td>
<td>-8.80±0.00</td>
</tr>
<tr>
<td>Control</td>
<td>-68.60±12.96***</td>
<td>-66.43±10.17***</td>
<td>-65.87±12.18***</td>
<td>-62.55±11.03***</td>
</tr>
<tr>
<td>Experimental I</td>
<td>-66.25±13.45</td>
<td>-61.74±12.31</td>
<td>-50.15±10.53’</td>
<td>-29.26±11.38’’</td>
</tr>
<tr>
<td>Experimental II</td>
<td>-68.47±13.03</td>
<td>-62.68±7.00</td>
<td>-55.64±11.33</td>
<td>-38.01±12.91’’</td>
</tr>
</tbody>
</table>

Mean±SD

Histopathologic Examination

Findings from hematoxylin & eosin staining
Sciatic nerves were extracted from individual groups at 21 days after the therapeutic treatment was completed to make tissue slices and normal H & E staining was conducted. In the staining, the degrees of degeneration of axons in the sciatic nerves
were observed in all induced groups. Although severe sciatic nerve axon degeneration and vacuolization could be observed in the control group, the findings of axon degeneration decreased in experimental group I and experimental group II and vacuolization could be hardly observed. In particular, almost normal forms of sciatic nerves could be observed in experimental group I and slight axon degeneration could be observed in experimental group II (Fig. 1).

Fig. 1. The histological finding of sciatic nerve in each groups(H & E stain, ×100, 21 days)
A : Normal(Normal rat)
B : Control (sciatic nerve injury(SNI) rat)
C : Experimental I (SNI+low power laser(0.21mJ/mm²) on the injured side)
D : Experimental II (SNI+low power laser(5.25mJ/mm²)
on the injured side)

Findings from toluidine blue staining
Based on the results of toluidine blue staining, irregular cell shapes, the phenomenon of vacuolization that appears due to partial necrosis, degeneration where the sizes decrease and axon disappearance were observed in all of the control group, experimental group I and experimental group II and cells that were not clearly stained could be observed. These histomorphological changes were observed the most clearly in the control group and it could be observed that morphological changes in axons were relatively improved in experimental group I and experimental group II compared to the control group. In particular, decreases in cell degeneration and axon increases in number were observed in experimental group I compared to experimental group II (Fig. 2).

Fig. 2. The histological finding of sciatic nerve in each groups(toluidine blue stain, ×200, 21 days)

Immunological Test
Western blot analysis(MAG)
To observe the effects of low power laser on the expression of MAG protein expression after SNI induction, western blot analysis was conducted on spinal cord tissue samples separated at 21 days after damage. Larger amounts of MAG expressed could be observed in experimental group I and experimental group II irradiated with low power laser compared to control group. In comparison between experimental group I and experimental group II, more MAG proteins expressed were observed in experimental group I than in experimental group II (Fig. 3).

Fig. 3. Myelin associated protein expression of spinal cord in each groups
Immunohistochemical findings from changes in c-fos expression

To observe the effects of low power laser on the expression of c-fos in the tissues of the normal group, control group, experimental group I, experimental group II, immunohistochemical tests were conducted on the spinal cord tissue samples separated at day 14 and day 21 after the damage.

Based on the results of the immunohistochemical tests, it could be observed that c-fos expression decreased in experimental group I and experimental group II irradiated with low power laser compared to control group both at day 14 and at day 21. In addition, when experimental group I and experimental group II were compared with each other, it could be observed that c-fos expression decreased more in experimental group I than in experimental group II (Fig. 4).

Fig. 4. The immunohistochemical finding of c-fos reaction in spinal cord (immunohistochemical stain, × 200, 21 days)

DISCUSSION

As methods to test the degrees of pain and analgesic effects, test methods to measure behavior to avoid harmful stimuli such as tail-flick reflex, hot plate tests, von Frey filament tests, pinprick tests and acetone spray tests and methods to measure nerve conduction speeds electrophysically are frequently used(32). In the experiment, paw withdrawal latency time was measured to assess voluntary pain behavior and based on the results, it was identified that the time significantly increased in experimental group I and experimental group II irradiated with low power laser compared to the control group and the time increased a little in experimental group I compared to experimental group II at days 7, 14 and 21 but no significance was observed. In addition, paw withdrawal latency time was also measured to assess mechanical allodynia and based on the results, significant increases were identified in experimental group I and experimental group II irradiated with low power laser compared to the control group at days 14 and 21 after the induction and the time significantly increased in experimental group I compared to experimental group II at days 14 and 21 after the induction. This means that low power laser treatment healed hyperalgesia conditions resulted from nerve damage and thus hyperalgesia was relieved. Peripheral nerve recovery related functional assessments are divided into those of motor nerve recovery and sensory nerve recovery. In relation to motor nerve recovery, sciatic function index(SFI), a functional assessment of nerve damage have been known to be the most reliable test method in rodents and thus functional assessments in relation to regeneration after nerve damage has become possible(33). In this experiment, sciatic function indexes were measured to assess functional recovery. Sciatic function indexes show the ratio of lacking functions when normal gaits are regarded as 0% and thus an SFI value of 0 indicates normal conditions and a value of ~100 indicates complete damage. Therefore, irradiating low power laser to rats with sciatic nerves damaged by compression and calculating SFI values by period can produce objective indicators to assess the recovery of motor functions. Based on the results of the experiment, the value significantly increased(p<0.05) in experimental group I only compared to the control group at day 14 after the damage and significantly increased in experimental group(p<0.001) and experimental group II (p<0.01) compared to the control group at day 21 after the damage. In comparison between experimental group I and experimental group II, experimental group I showed a pattern of significant increases (p<0.001) in values compared to experimental group II (p<0.05) at day 14 and also experimental group I showed a pattern of significant increases(p<0.001) in values compared to experimental group II (p<0.01) at day 21. These results mean that low power laser recovered motor nerve functions after sciatic nerve damage. In histological findings from sciatic nerves, Schwann cell proliferations were observed in experimental group I and experimental group II applied with low power laser at day 21 after the damage and thus clear regeneration could be seen. Furthermore,
findings of decreasing Wallerian degeneration were shown and inflammation cell infiltration remarkably decreased and the degree of destruction of surrounding tissues was very low or disappeared and thus it could be observed that the tissues were coming close to normal tissues. MAG expression decreased by sciatic nerve damage by compression and this decrease in MAG expression means that axon regeneration was suppressed by autoimmune responses. However, low power lasers showed effects to increase MAG expression that had been induced by sciatic nerve damage by compression. This result indicates that low power lasers suppress autoimmune responses and enhance axon regeneration.

c-fos which is a cell early expression gene that makes cells express early when tissue damage or inflammation has been induced is generally known as a transcription factor that adjusts early gene expression that induces long-term functional changes in the nervous system by being bound to the DNA-binding protein or DNA in the catalytic regions of target genes (34). Once expressed, c-fos enters into cell nuclei and form protein compounds to be bound to the DNA adjusting unit called AP-1 site to interact with other genes (35). In this study too, immunohistochemistry was conducted in relation to c-fos as a cytogenetic indicator of sciatic nerve damage. When the peripheral nerve has been damaged, damage to the synapses of neurons that go through the spinal ganglion is caused in the anterior horn neurons in the spinal cord and cell losses are caused due to direct damage to α-motor neurons in the posterior horn neurons in the spinal cord, It has been reported that when the sciatic nerve was ligated, lamina I~III neuron degeneration occurred in the posterior horn of the spinal cord of rats from two weeks later (36). In this study, c-fos expression increased in the spinal cord because of compression damage to the sciatic nerve, This increase in c-fos expression means that nerve activity increased because of continuous pain. However, low power laser showed effects to suppress the c-fos expression induced by compression damage to the sciatic nerve, This result indicates that low power lasers suppress pain and as a result, nerve decreases so that c-fos expression is adjusted. On reviewing existing studies on nerve regeneration by low power lasers, it can be seen that the intensity of low power laser shows differences between researchers and thus the intensity should be standardized. In this study, experimental group I was irradiated at a dose of 0.21 mJ/mm² and experimental group II was irradiated at a dose of 5.25 mJ/mm². It was identified that low power lasers positively affected pain reactions and axon regeneration in both experimental group I and experimental group II and that low power lasers affected pain reactions and axon regeneration more positively in experimental group I applied with an output of 0.21 mJ/mm² than in experimental group II applied with an output of 5.25 mJ/mm². Therefore, both 0.21 mJ/mm² and 5.25 mJ/mm² had effects to reduce pain and regenerate axons while 0.21 mJ/mm² was more effective than 5.25 mJ/mm². However the mechanism that makes 0.21 mJ/mm² more effective than 5.25 mJ/mm² requires more studies.

In the results of this study, the proliferation of Schwann cells that serve important roles in reducing inflammations and regenerating axons during low power laser treatment after peripheral nerve damage could be identified and based on the study mentioned above, it is assumed that low power lasers relieve pain and promote nerve regeneration when peripheral nerves have been damaged. Although later changes could not be seen due to the limited experimental period, it could be seen that low power laser treatment neurologically and histologically affected pain reactions and the promotion of axon regeneration. It is considered that the periods and methods of application of low power lasers should be changed diversely to examine related morphological and functional changes so that clinical studies can be multilaterally conducted on patients using the results.

CONCLUSION

In this study, to examine the effects of a low power laser intervention method for 21 days on neuropathic pain control and sciatic nerve axon regeneration in rat models with sciatic nerve damage induced by compression, assessments of behavioral responses to pain, nervous ethological assessments, histopathological examinations and immuno-histological tests of MAG and c-fos were conducted and the following results were obtained.

1. In the results of paw withdrawal latency test for assessing spontaneous pain behavior, experimental group I and experimental group II showed a significant increase in spontaneous pain behavior compared to the control group on day 7, 14 and 21 (p<0.001), but in comparison between experimental group I and experimental group II, spontaneous pain behavior was higher in experimental group I than in experimental group II but the difference was not significant.
2. In the results of von Frey filament test for assessing mechanical allodynia, experimental group I and experimental group II showed a significant increase compared to the control group on day 14 (p<.001 and p<.05, respectively), and on day 21 (p<.001 and p<.01, respectively). In comparison between experimental group I and experimental group II, experimental group I (p<.001) showed a significantly higher increase than experimental group II (p<.05) on day 14, and experimental group II (p<.001) showed a significantly higher increase than experimental group II (p<.01) on day 21 as well.

3. In the results of sciatic functional assessment for assessing neuro behavioral response, only experimental group I showed a significant increase compared to the control group on day 14 (p<.05), and both experimental group I (p<.001) and experimental group II (p<.01) showed a significant increase compared to the control group on day 21. In comparison between experimental group I and experimental group II, experimental group I (p<.05) showed a significantly higher increase than experimental group II on day 14, and experimental group II (p<.001) showed a significantly higher increase than experimental group II (p<.01) on day 21.

4. According to histological findings from examining the progression of axonal degeneration in the sciatic nerve through H & E staining, severe axonal degeneration and vacuolization of sciatic nerve were observed in the control group, but axonal degeneration decreased and vacuolization was hardly observed in experimental group I and experimental group II. Particularly in experimental group I the form of sciatic nerve was almost normal, and in experimental group II mild axonal degeneration was observed.

5. In the results of toluidine blue staining, histomorphological changes showing cellular degeneration and axonal loss were most clear in the control group, and the morphological change of axon was relatively milder in experimental group I and experimental group II compared to that in the control group. In particular, experimental group I showed reduced cellular degeneration and an increased number of axons compared to experimental group II.

6. MAG expression was higher in experimental group I and experimental group II treated with low power laser than in the control group, and in comparison between experimental group I and experimental group II it was higher in experimental group I than in experimental group II.

7. In the results of immunohistochemical test, c-fos expression was lower in experimental group I and experimental group II than in the control group on both day 14 and 21. In comparison between experimental group I and experimental group II, it decreased more in experimental group I than in experimental group II.

Based on the results of assessments of behavioral responses to pain, nervous ethological assessments, histopathological examinations and immunohistochemical tests mentioned above, it was identified that when low power lasers had been applied to rat models with sciatic nerves damaged by compression for 21 days, the low power lasers affected pain reactions and axon regeneration in both experimental group I and experimental group II while being more effective in experimental group I applied with an output of 0.21mJ/mm² than in experimental group II applied with an output of 5.25mJ/mm².

REFERENCES