in vivo *18*: 489-496 (2004)

Effect of Ga-As Laser on the Regeneration of Injured Sciatic Nerves in the Rat

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Abstract. *Laser irradiation is one of the therapeutic methods for the recovery of degenerated peripheral nerves. The aim of the present study was to determine if low-power laser treatment stimulates the regeneration process of damaged nerves. A standardized crush to the sciatic nerve was applied to cause extensive axonal degeneration. After this procedure, low-power infrared laser irradiation was administered transcutaneously to the injured sciatic nerve, 3 minutes daily to each of four treatment groups for 1, 3, 5 and 7 weeks, respectively. A nerve conduction study was done, and a morphological assessment was performed using both light and electron microscopy. With trauma of the nerve, both amplitude of compound motor action potential and nerve conduction velocity decreased significantly compared to the pre-trauma state. Morphologically, the numbers of myelinated axons and degenerated axons were decreased and increased, respectively, compared with the control. Typical aspects were of onion skin-type lamellation, fragmentation, edematous swelling and rarefaction in the myelin sheath. All these parameters recovered almost to the level of the pre-trauma state with laser irradiation, in direct proportion to the time spent for treatment. These results suggest that low-power infrared laser irradiation can relieve the mechanical damage of sciatic nerves and stimulate the regeneration of peripheral nerves.*

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Key Words: Laser irradiation, sciatic nerve, myelinated axon, regeneration.

Laser affects tissues differently according to the wave-length, pulse duration, pulse/energy, energy density and delivery system. Thus, finding the optimal conditions for laser irradiation is essential in the application of laser in medicine. Once the peripheral nerves are damaged, myelin sheaths and axons distal to the damaged area undergo inevitable Wallerian degeneration. This process of myelin phagocytosis incorporates Schwann cells and macrophages in its segmentation (1). Following a partial denervation of nerve, the intact and surviving motor axons in a peripheral nerve may go through distal collateral sprouting and enlargement of their motor unit size in a compensatory fashion (2). Methods of facilitating regeneration by maximizing the number of regenerated fibers are essential for a functional recovery. Surgical intervention of anastomosis (3) or titanium staples (4) (for a completely transected nerve) and laser irradiation (5) (for a partially injured nerve) are becoming increasingly popular for therapeutic purposes. However, despite advances in the medicinal and surgical management of peripheral nerve injuries, recovery is often incomplete. Therefore it would be clinically beneficial to develop new treatments to accelerate and improve the recovery process. Moreover, despite extensive studies on how axonal growth and myelin regeneration are regulated (6), few studies have adopted the electron micrograph for an in-depth analysis of sciatic nerves within the time-frame stated. Thus, the present study aimed to determine whether Ga-As (Gallium-Arsenide, wave-length: 904 nm) infrared laser irradiation could stimulate the healing process of experimentally injured rat sciatic nerves.

Materials and Methods

Animals. Since aging animals tend to show retarded regeneration of myelinated fibers after axotomy compared with young counterparts (7), we used eight-week-old male Sprague-Dawley rats

Table I. *Effect of laser irradiation on amplitude of compound motor action potential and nerve conduction velocity*[a].

Parameter	Control [b]	Crush		
		Pre-trauma [c]	Post-trauma [d*]	After treatment [e]
1 week treatment				
Amplitude [f]	11.30±1.41	18.43±1.58	8.50±2.24	12.95±3.31
NCV [g]	15.12±1.81	23.52±1.75	14.63±1.87	14.32±2.13
3 weeks treatment				
Amplitude	14.95±1.61	17.85±1.33	8.77±2.40	16.28±1.59
NCV	20.75±0.81	23.52±1.19	13.00±1.05	21.52±1.14
5 weeks treatment				
Amplitude	18.43±1.28	19.17±1.34	8.88±0.85	19.03±0.65
NCV	23.05±2.01	23.98±1.43	13.08±1.06	22.95±1.60
7 weeks treatment				
Amplitude	18.47±0.61	20.17±0.95	9.13±1.21	18.75±0.62
NCV	22.63±1.31	24.80±2.70	12.68±0.75	24.85±2.44

[a] Irradiation with Ga-As (Gallium-Arsenide, wave-length=904 nm); Number of rats used for each treatment period was 15 (total n=60); values are expressed as mean±SEM.
[b] Non-irradiated sciatic nerve of the left hindlimb, measured at the end of each treatment period.
[c] Measured before sciatic nerve injury.
[d] Measured right after sciatic nerve injury, * $p<0.05$ compared to [c].
[e] Measured at the end of each treatment period.
[f] Unit = mV.
[g] Nerve conduction velocity (m/sec).

Table II. *The change of total number of myelinated axon and degenerated axon per unit area (65,000 μm²) for each time period (mean ±SEM)* [a].

Parameter	1 wk	3 wks	5 wks	7 wks
	Control Irradiated	Control Irradiated	Control Irradiated	Control[b] Irradiated
#				
Myelinated	134.7±35.5	134.7±33.0	224.3±21.1	303.4±12.0
axon	205.7±31.0*	167.3±77.0	297.3±77.5	304.5±42.0
#				
Degenerated	63.7±16.5	77.0±19.0	60.7±5.5	20.0±2.7
axon	63.0±31.8	40.0±18.0**	31.0±8.0***	19.7±2.5

[a] Number of animals used for each treatment period was ten (total n=40); the number of myelinated axons from the normal rats (n=6) was 412.0±21.0; all the animals were from the nerve conduction study.
[b] Non-irradiated sciatic nerve of the left hindlimb, measured at the end of each treatment period.
*$p<0.001$
**$p<0.0005$
***$p<0.005$

(n=60; 220-230g) for the present study. They were housed in rooms with controlled light cycles (14 hours light and 10 hours dark) with lights on at 6 a.m. They were given water and food as desired. The Animal Care Committee at Chosun University, Korea has approved the protocols in this report.

Surgical procedures. Under intraperitoneally-injected chloral hydrate anesthesia (500 mg/kg), the area above the lower thigh was shaved and sterilized with Betadine and ethanol. An approximately 1 cm incision was made in the shaved area over the gluteus maximus muscle. The muscles were teased apart with scissor tips and the bilateral sciatic nerves were exposed. The nerves were crushed with an ordinary closed hemostat for 30 seconds, which is one of the standard methods of inducing the injury (8). The crush sites were marked by placing sutures in the epineurium immediately proximal to the crush sites. The nerves were replaced under the muscle and the incisions were sutured. The nerve damage site for each animal was kept constant by an experienced researcher.

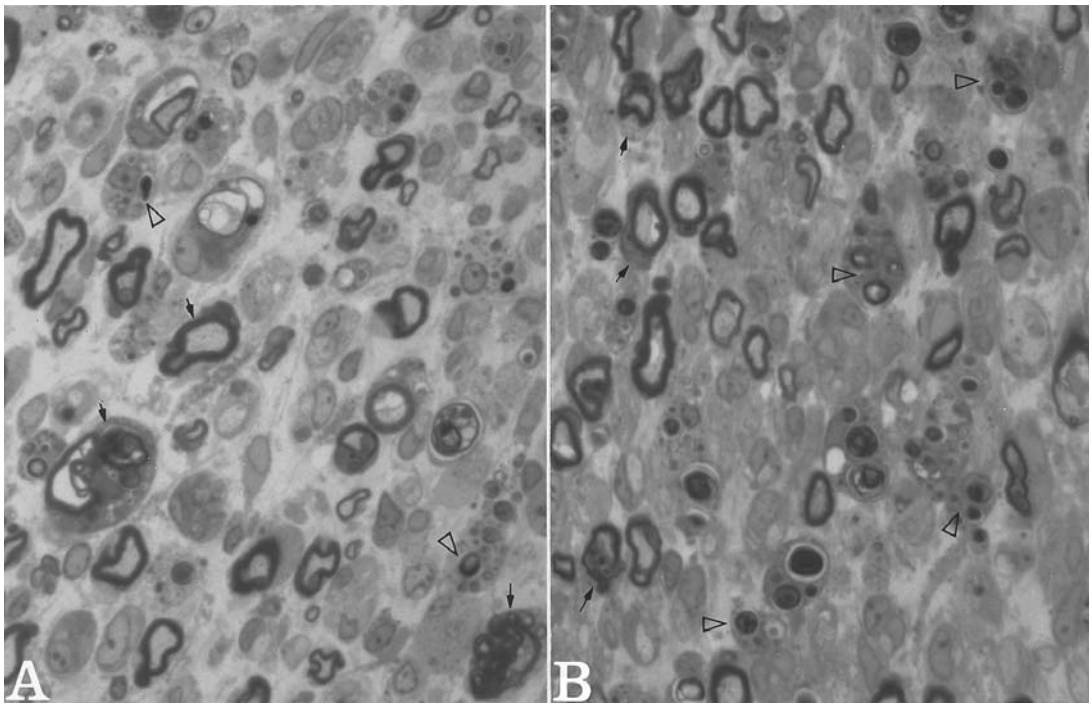

Figure 1. *1 week. A: Control group, B: Irradiated group. Degeneration of myelin sheaths(arrows) and macrophages containing fragmented myelin debris(arrow heads) are found frequently. Semi-thin section, Toluidine blue staining, x 400.*

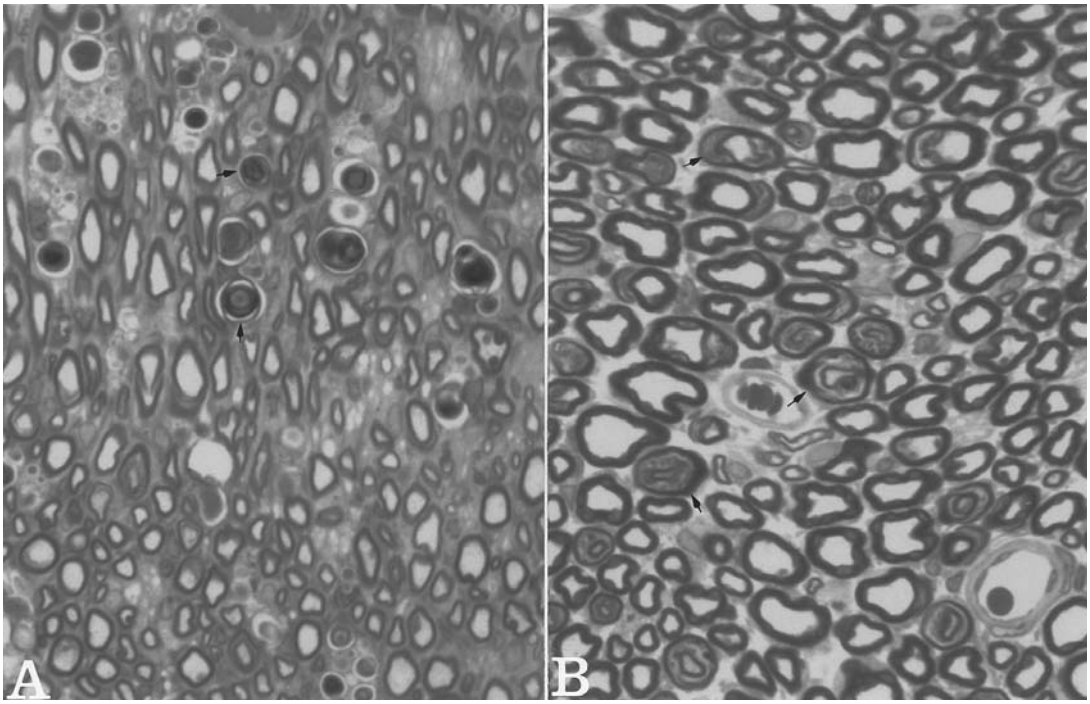

Figure 2. *5 weeks. A: Control group. Degeneration of myelin sheaths(arrows) are still noted. However, the macrophages are rarely seen. B: Irradiated group. Degeneration of myelin sheaths(arrows) are decreased profoundly. Semi-thin section, Toluidine blue staining, x 400.*

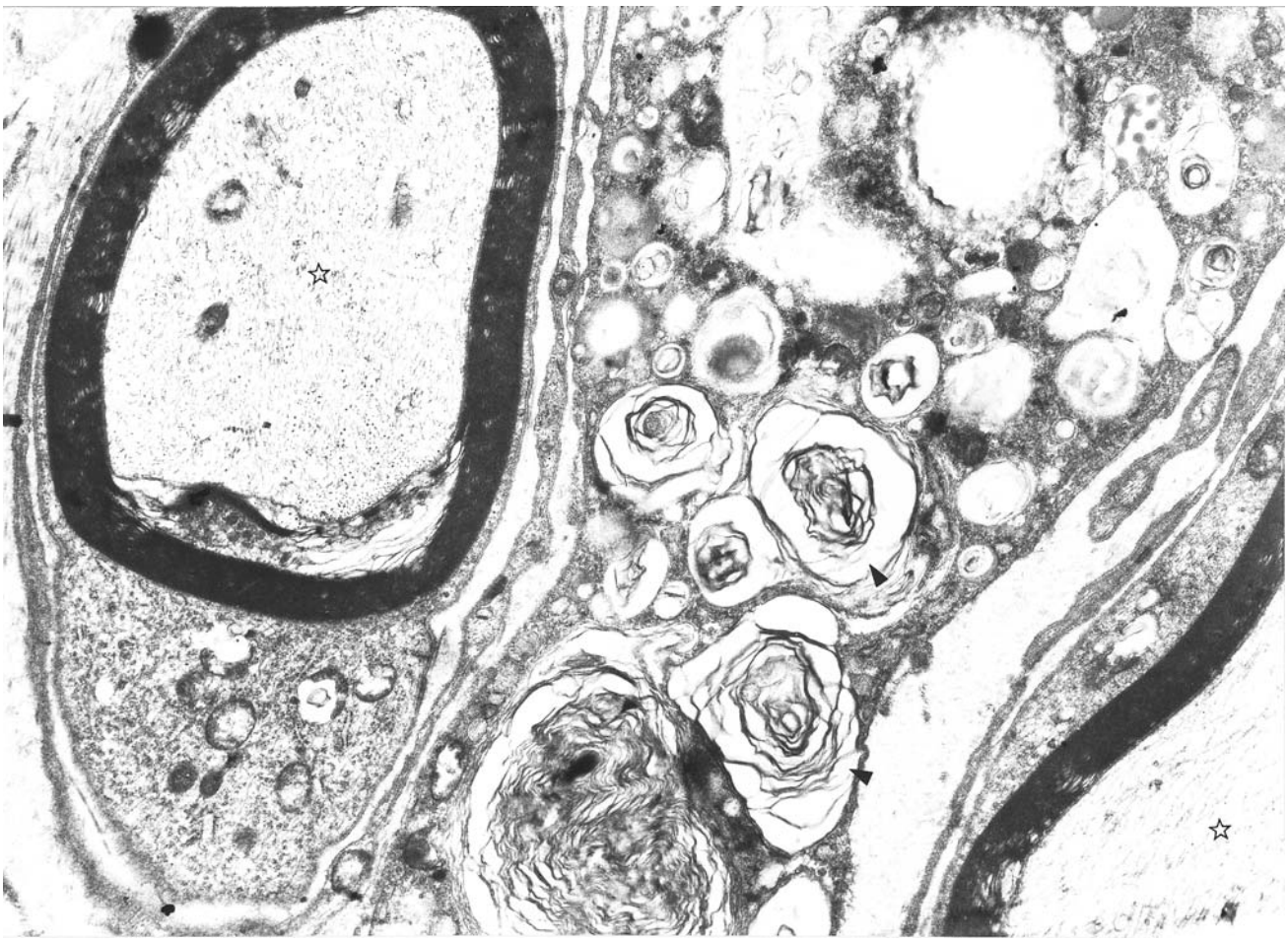

Figure 3. *1-week control group, ultrastructural findings. A macrophage containing degenerated myelin components(arrow heads) is noted. The asterisks indicate myelinated axons. Lead citrate and uranyl acetate, x 4,000.*

Laser irradiation. For the control group, the sciatic nerve of the left hindlimb was not irradiated. For the animals in the treated group, low power laser irradiation was administered transcutaneously for 3 minutes under short general anesthesia every day, using a pulse wave 904 nm, 27 mW Ga-As laser (Dens-Bio Laser, TMC, Korea). The irradiation application period was 1, 3, 5 and 7 weeks, respectively, for each of the four groups.

Electrophysiological study. A nerve conduction study was performed with an electromyographer (Multiliner, Tonnies Co, Germany) to measure the amplitude of compound motor action potential (CMAP) of the rat sciatic nerve. Each step on the monitor screen of the electromyographer was recorded as 1 millivolt, and another one step of the sweep speed was settled as 5 msec. The range of frequency was settled as 10 Hz to 10KHz. Needle electrodes were used and an active electrode was placed in the midportion of the calf muscle. While a reference electrode was placed in the sole of the paw, a ground electrode was inserted into the subcutaneous area of the thigh. Electrical stimulation was performed by a needle electrode stimulator in the subcutaneous tissue 7cm above the sciatic nerve. The frequency of nerve stimulation was two times per second and the duration was 0.1 msec. The stimulation intensity was increased to reach the maximal amplitude elicited after several stimulations of the CMAP. Peak-to-peak amplitudes were calculated by first determining the greatest magnitudes of the major initial negative and subsequent positive peaks. This amplitude is believed to represent most accurately the total number of axons with their innervated muscle fibers depolarized. Nerve conduction velocity was calculated by dividing the distance in meters an action potential traveled by the time required to cover this distance. Nerve conduction was measured in preinjury state and postinjury state. The laboratory room temperature was strictly controlled at 24 to 26˚C.

Histopathological analysis. In order to examine the histopathological aspect of the sciatic nerve, both light and electron microscopic examination were performed. For the light microscopic examination, the resected nerve trunks were cut into small pieces, each about 1 mm^3, and were immediately fixed in phosphate-buffered glutaraldehyde solution (pH 7.2) for 2 hours and later post-fixed in 1% buffered osmium tetroxide for 2 hours at 4˚C. After processing through a graded series of ethanol and propylene oxide, the tissue

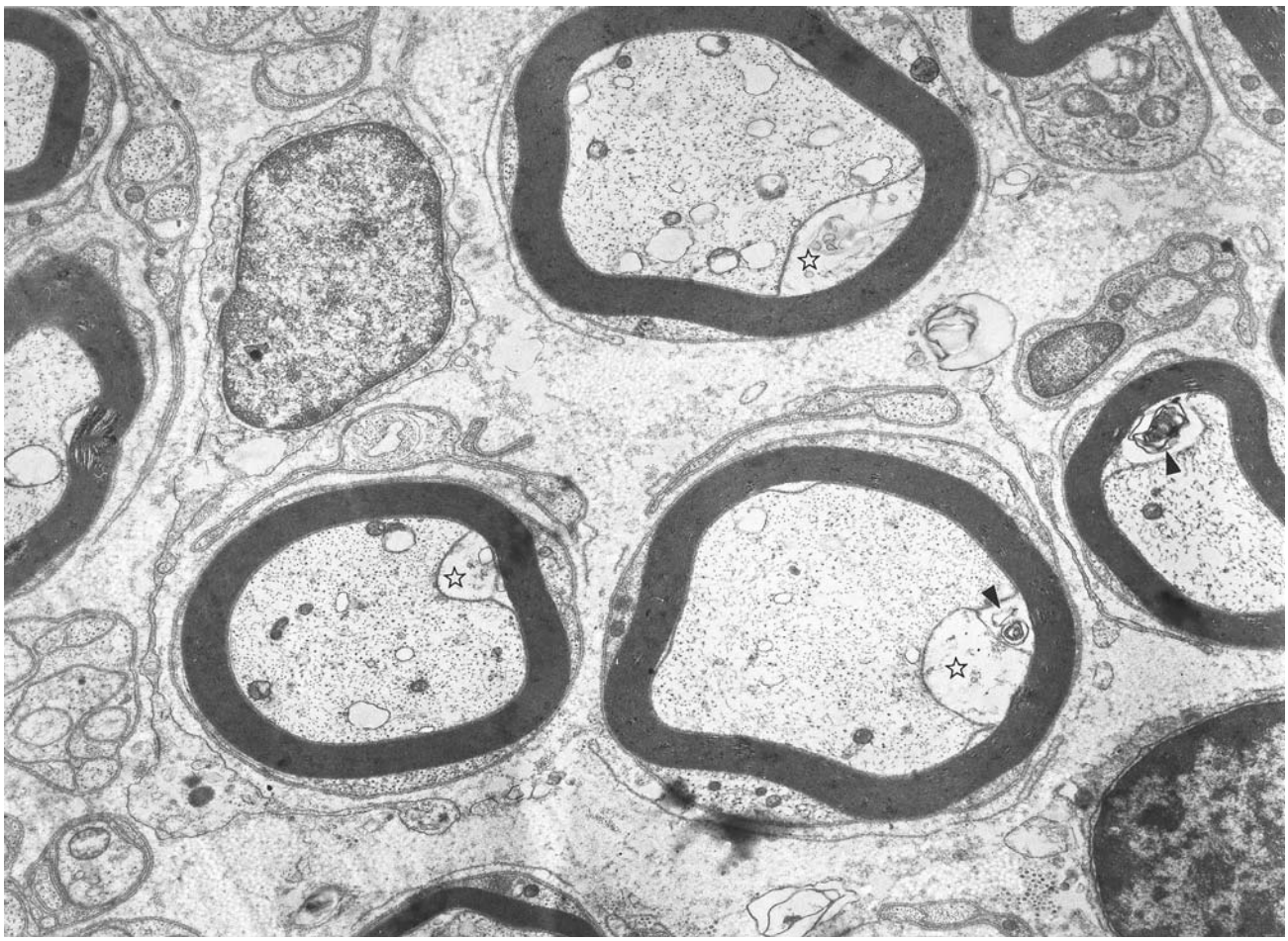

Figure 4. *7-week irradiated group, ultrastructural findings. The myelinated axons show minor changes only; axonal edema(asterisks) and myelin figure formation(arrow heads). Lead citrate and uranyl acetate, x 4,000.*

was embedded in Epon. A semi-thin (1μm in thickness) section was stained with toluidine blue for ultrastructural examination. We selected representative cross sections with a light microscope (Olympus BX-50, Japan), and attached digital image capture system MagnaFire™ SP (Optronics, LA, USA) and obtained x400 photographs. These were analyzed with an image analysis system, New IMT (VT) Image Analysis for Bio (*i*MTechnology, Daejeon, Korea). The objects for analysis were the total number of myelinated nerve fibers and the total number of degenerated myelinated nerve fibers. For the electron microscopic examination, ultra-thin (60-90 nm) sections were obtained from the Epon block described in the above paragraphs by an ultramicrotome (LKB-V, Sweden), stained with uranyl acetate and lead citrate and examined under a Hitachi H-7600 electron microscope (Hitachi, Japan).

Statistical analysis. Data are expressed as means ± SEM. One-way ANOVA and Student's *t*-test were performed using the Statistical Package for the Social Sciences (SPSS) for Windows, version 7.5 (SPSS, Korea). The Wilcoxon rank test was used for intergroup and intragroup comparisons at different times. When p was less than 0.05 it was considered significant.

Results

Electrophysiological study. The effect of laser treatment on non-irradiated nerves and on crushed nerves is summarized in Table I. As can be seen, both the amplitude of CMAP and NCV at post-trauma decreased significantly ($p<0.05$) when compared with the pre-trauma state. With the laser treatment, the injured nerves recovered significantly ($p<0.05$) to similar values as pre-trauma and for the control group. The progressive recovery was strongly correlated with the treatment period.

Histopathological analysis. At 7 weeks, the number of myelinated axons peaked and the number of degenerated axons was at a minimum (Table II). At 1 week (Figure 1), prominent degeneration of myelinated nerve fibers was observed in both the control and treatment groups. The degenerative findings of the myelin sheath surrounding the

axon consisted of onion skin-type layering, round ball-like or irregular-shaped changes, or fragmentation into small particles. At 3 weeks, degenerative changes of the myelinated nerve fibers were still frequently noted in the control group. However, they were profoundly reduced in the treatment group. At 5 weeks (Figure 2), degenerative changes of the myelinated nerve fibers were still noted frequently, not much different from the observations after 1 week or 3 weeks in the control group, but a prominent reduction of myelin degeneration was noted in the treatment group. At 7 weeks, a prominent reduction of myelin degeneration in both the control and treatment animals compared with the shorter treatment times was noted. In particular, the control group showed a nearly similar recovery pattern to the treatment group. With an electron microscope, degenerated axons were mainly observed in those nerves with large diameter axons. They showed such morphological changes as onion skin-type lamellation, fragmentation, edematous swelling and rarefaction in the myelin sheath. Moreover, most of the fragmented myelin particles were engulfed by macrophages (Figure 3). These outnumbered macrophages and the number of degenerated axons decreased significantly with the laser treatment (Figure 4).

Discussion

Laser (Light Amplification by Stimulated Emission of Radiation) is the artificial light prepared from stimulated emission and is composed of a single wave-length with high regularity. It is highly efficient energy-wise because it does not scatter, so that it can be applied in many fields including medicine. Divided according to medium, laser is largely categorized into solid laser, liquid laser and semi-conductor laser (9). Low-power laser therapy (LPLT), by definition, occurs at irradiation intensities so slow that any resulting biological effects are due to physical and/or chemical changes associated with the interaction of charged species with the electric field component of electromagnetic radiation or the absorption of specific wave-lengths of light by photoreceptors, and not simply as a result of heating. LPLT (wave-length 300-1,100 nm) has been reported to have a variety of effects on nerve function, growth and regeneration of *in vitro* and *in vivo* neural tissue (2,10-13). Pereira *et al.* (12) reported that low-power Ga-As laser stimulates fibroblast proliferation, without impairing procollagen synthesis in the inflammatory condition. The authors evaluated the effect of a LPLT using Ga-As (Gallium-Arsenide, wave-length: 904 nm) infrared laser irradiation on sciatic nerve regeneration after crush injury.

The results of the present study demonstrated that postoperative laser treatment after sciatic nerve crush resulted in improvement of electrophysiological function and morphological parameters during nerve recovery.

At 7 weeks, a prominent reduction of myelin degeneration in both the control and treatment animals compared with the shorter treatment times was noted. In particular, the control group showed a nearly similar recovery pattern to the treatment group. This correlated with the findings of the electrophysiological study. These findings suggest that natural repair of the injured myelinated nerve fibers develops vigorously around the 6th-7th week. Since the rat has a superlative capacity for nerve regeneration (14), it is necessary to demonstrate new techniques of nerve repair that would work in a short period of time. The histopathological light microscopic examination of the sciatic nerve illustrates the obvious laser-effect that was proportional to the time of treatment. On ultrastructural observation, most of the fragmented myelin particles were engulfed by macrophages. This role of macrophages is not limited to myelin phagocytosis of the degenerating process. They are also involved in the subsequent regeneration of injured peripheral nerves by secreting mitogenic factors for the proliferation of both Schwann cells (15) and fibroblasts (10). They further secrete interleukin-1 to induce the synthesis of endoneurial nerve growth factor (16,17). In the present study, the number of macrophages and degenerated axons decreased significantly with the laser treatment.

Shin *et al.* (18) reported that LPLT has an effect on the early stages of the nerve recovery process following sciatic nerve injury. They demonstrated the therapeutic effect of LPLT on neural regeneration by finding elevated immunoreactivities of growth-associated protein-43 (GAP-43), which is up-regulated during neuronal regeneration. LPLT have been applied to improve circulation, wound repair and pain control. Kao and Sheen (19) demonstrated that LPLT stimulates glutathione (GSH) production and its related enzyme activities and provided protection against oxidative damage. Interestingly enough, the large diameter axons of the nerve fibers were seen in the 7-week group of the treatment group in the present study, which is one of the indicators of regeneration quality (20).

Regardless of controversy about which wave-length is appropriate for the treatment of nerve regeneration, the low-power laser still shows a stimulatory effect on the healing of nerve wounds. It is in line with the results of Shamir *et al.* (20) that laser treatment enhanced the regenerative processes of peripheral nerves in the rat model. In addition, in our present study, the effect of laser irradiation was proportional to the treatment time. There was a parallel increase and decrease in myelinated axons and degenerated axons, respectively, compared with the time spent undergoing treatment. This whole process of recovery was proved at the level of tissue morphology. Both light and electron microscopy appeared to be equal in the normal course of nerve response to crush and regeneration. Again, recovery increased progressively with a longer treatment period.

In conclusion, low-power laser treatment certainly had an enhancing effect on the recovery of an injured peripheral nerve without ill effect and it can be assumed that this would hold true if extrapolated to the human situation.

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Received November 25, 2003
Revised January 16, 2004
Accepted March 2, 2004