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Thirty minutes of low intensity electrical stimulation promotes nerve regeneration after sciatic nerve crush injury in a rat model

Mohammad S. Alrashdan[1], Jong-Chul Park[1], Mi-Ae Sung[2], Sang Bae Yoo[3], Jeong Won Jahng[3], Tae Hyung Lee[4], Sung-June Kim[4], Jong-Ho Lee[1,2]

[1]Department of Oral and Maxillofacial Surgery, [2]Department of Craniofacial Structure and Functional Biology, [3]Dental Research Institute, School of Dentistry, Seoul National University, Seoul, Korea; [4]Department of Electrical Engineering, School of Electrical and Computer Science, Seoul National University, Seoul, Korea

Abstract

We investigated whether electrical stimulation (ES) applied directly for 30 minutes after crushing injury to the sciatic nerves of rats could improve nerve regeneration. Two groups of animals were used in this study (n = 20 each): the ES group received 30 minutes of low intensity ES (20 Hz pulse rate, 2 uA amplitude) immediately after a standard crush injury, while the control group received no stimulation after injury. Both groups were followed up for three weeks. The sciatic function index (SFI) was calculated weekly. Mean conduction velocity (MCV) and peak voltage (PV) were calculated, and the sensory neurons in L4 and L5 dorsal root ganglia (DRG) were traced with Fluorogold in retrograde fashion and quantified at the end of the follow up period. Histomorphometric studies were also carried out in both groups. The ES group showed improved functional and sensory recovery compared to the control group three weeks after injury. SFI, MCV and the number of retrogradely labeled sensory neurons were significantly higher in the ES group. Additionally, axon counts, myelin thicknesses and G-ratio values were also higher in the ES group. Quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) showed an elevated expression of brain derived neurotrophic factor (BDNF) in DRG sensory neurons of the ES group five days post-injury. Here, we present the first evidence that the application of ES for 30 minutes immediately following crush injury is effective to promote nerve regeneration in a rat sciatic nerve model.

Keywords: electrical stimulation; nerve regeneration; rat sciatic nerve, crush injury.

Introduction

The use of electrical stimulation (ES) to promote peripheral nerve regeneration after injury has gained growing attention following reported positive results in both motor and sensory neurons (Roman *et al*., 1987; Pomeranz and Campbell, 1993; Al-Majed *et al*., 2000 a,b; Gordon *et al*., 2003; Mendoca *et al*., 2003; Brushart *et al*., 2005; English *et al*., 2006; Geremia *et al*., 2007; Ahlborn *et al*., 2007). Since Nix and Hopf (1982) found that ES improved nerve regeneration and motor function recovery in the soleus muscles of rabbits after axonotmesis, extensive studies have been carried out to further investigate the effects of ES on the process of peripheral nerve regeneration and to elucidate the molecular mechanisms by which electrical fields may improve it. Gordon and colleagues were leaders in this field, performing several studies suggesting that ES enhances the expression of growth associated genes and factors, specifically *brain derived neurotrophic factor* (*BDNF*) and its *tyrosine receptor kinase B* (*trkB*), in addition to pronounced up-regulation of cytoskeletal proteins tubulin and actin and growth associated protein (GAP-43), all of which in turn support the regeneration process. (Al-Majed *et al*., 2000b, 2004; Gordon *et al*., 2003; Geremia *et al*., 2007).

Several studies on the effects of ES on peripheral nerve regeneration have utilized the crush injury model (Pomeranz and Campbell, 1993; Mendoca *et al*., 2003), which was used widely as an axonotmetic model because it allows evaluation of both motor and sensory nerve function (De Medinaceli *et al*., 1982, 1984; Bain *et al*., 1989; Dellon and Mackinnon, 1989; Shen and Zhu, 1995, Oliveira *et al*., 2001; Varejao *et al*., 2004; Luis *et al*., 2007; Mazzer *et al*., 2008). Functional and morphometric studies are usually used for nerve regeneration assessment and methods including: the sciatic function index (SFI), and electrophysiological tests are often employed (Varejao *et al*., 2004; Asenio-Pinilla *et al*., 2009; Vivo *et al*., 2008). SFI analyzes rats'

hind paw prints during gait as an indicator of sciatic nerve motor function and is well-correlated to morphometric results in rat models (Oliveira *et al*., 2001).

In the same field, retrograde labeling of injured nerve axons by the use of fluorescent tracers, especially Fluorogold, have been recently used for quantification of regenerated sensory neurons (Geremia *et al*., 2007).

Among studies regarding ES effects on peripheral nerve regeneration, some investigators have used implantable electric stimulators able to deliver a constant electrical current for a given period of time (Nix and Hopf, 1982; Roman *et al*., 1987; Pomeranz and Campbell, 1993; Mendoca *et al*., 2003). More recently, single–time application of ES has been adopted (Brushart *et al*., 2005; Ahlborn *et al*., 2007; Vivo *et al*., 2008; Asenio-Pinilla *et al*., 2009), which, for obvious reasons, has more clinical relevance. Geremia *et al*. (2007) reported that single application of ES for 1 h directly after nerve transection and repair was optimal to promote sensory neuronal regeneration.

Different authors have employed different electrical current intensities, ranging from 1 μA (Pomeranz *et al*., 1984; Mendoca *et al*., 2003) or 1.5 μA (Shen and Zhu, 1995) up to 10 μA (Roman *et al*., 1987; Pomeranz and Campbell, 1993). Most previous experiments using a single ES application utilized a 1 h protocol (Al-Majed *et al*., 2000a; Brushart *et al*., 2005; English *et al*., 2006; Ahlborn *et al*.,2007; Vivo *et al*., 2008; Asenio-Pinilla *et al*., 2009). In an attempt to modify this protocol, we investigated whether using ES for less than 1 h (here, 30 minutes) delivered according to our protocol would have any effect on nerve regeneration in rats after a crush injury.

Materials and methods

Experimental design

A total of 40 six-week old male Sprague-Dawley rats (250-300 g) were used in this experiment, distributed equally into two groups, the control group and the ES group (n = 20 each). Rats were kept in cages (3 animals/cage) with free access to rat chow and water prior to and after surgery.

A double-hook shaped electrode was designed and fabricated in the Department of Electrical Engineering, School of Electrical and Computer Sciences, Seoul National University. The electrode was made of tungsten (0.3 mm OD) and had two stimulation sites, positive and negative, that were designed to be positioned around the sciatic nerve to deliver ES. The electrode connectors were connected to a biphasic current stimulator chip that was designed using a 0.8- m high voltage complimentary metal-oxide semiconductor fabrication process at Austria Micro-Systems Corporation. The circuit configuration was adapted to generate biphasic pulses and an electrostatic discharge protector was built at all input and output pads of the chip to protect the circuit from external shock or electrostatic discharge.

Prior to surgery, animals were trained to walk across a transparent confined walking track (100cm long, 15cm wide) with a dark shelter at the end and white paper placed on the floor, and preoperative hind paw prints were obtained using the method described by Jolicouer *et al*. (1979) by applying liquid blue ink to the rats' hind paws and allowing the rats to walk across the track leaving their foot prints on the paper. Pre- and post-operative footprint track data were later used to calculate the SFI.

All animal surgical and experimental procedures were carried out in accordance with the care guidelines of the laboratory of animal resources of Seoul National University, Seoul, Korea.

Surgical procedure

Animals were anesthetized with an intraperitoneal injection of a 4:1 mixture of ketamine HCl (100mg/kg, ketamine hydrochloride, Ketara®, Yuhan, Korea) and xylazine hydrochloride (5mg/kg, Rumpun®, Bayer, Korea). The surgical field was prepared by hair trimming and applying 10% povidone iodine to the lateral aspect of the left thigh. The left sciatic nerve was exposed by a posterolateral longitudinal straight incision from the greater trochanter to the lateral condyle of the left femur and then by blunt dissection between the gluteus maximus and quadriceps muscles.

When the sciatic nerve was exposed and detached from the surrounding tissues, a standard surgical hemostat was used to create a crush injury at a distance of about 10mm proximal to the trifurcation. A defect of 3mm length was produced.

The injury site was labeled under 16X magnification of surgical microscope (Carl Zeiss, Germany) by introducing a single 9-0 nylon (Ethicon®,UK) epineural stitch at the distal limit of the injury for later identification.

Immediately after the creation of the injury in the ES group, ES was commenced by applying the electrodes 5 mm proximal to the injury site. Switching the power key in the electric stimulator produced a biphasic current pulse (100 μs pulse width, 20 Hz pulse rate, 2 μA amplitude) that was applied to the nerve for 30 minutes. The surgical wound was kept moist throughout the stimulation period by covering it with wet sterile gauze to avoid drying of the

underlying tissues. Immediately after ES was completed, the wound was closed in a single layer using 4-0 nylon sutures (Ethicon®,UK) and further antisepsis with povidone iodine solution was applied. In the control group, the wound was closed immediately following the injury by the same manner.

Animals were followed up for three weeks for assessment of nerve regeneration.

Quantification of BDNF and trkB expression by real-time RT-PCR

Five animals from each group were used to study the expression of endogenous *BDNF* and its receptor *trkB*, by means of quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR-. Five days after surgery, the animals were anesthetized, and L4, L5 and L6 DRG were harvested, directly moved to a liquid nitrogen container (-196°C), and stored at -80°C until further processing. The RT-PCR technique was, briefly, as follows: RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The purified RNA was DNase treated using an RQ1 RNase-Free DNase kit (Promega, Madison, WI, USA). mRNA encoding *BDNF* and *trkB* receptor was reverse transcribed to cDNA using a first-strand synthesis kit (Invitrogen). The amount of cDNA was also quantified using real-time PCR. The following primers were used to amplify specific cDNA regions of the transcripts of interest: *BDNF* (5´-ACCATAAGGACGCGGACTTG-3´) *tyrosine receptor kinase B full length* (*trkBFL*) (5´-GGCCAGATGCAGTGCTGAT-3´ and 5´-CATGCCTGCTGCGATTTG-3´), and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) (5´-AGCAAGAGAGAGGCCCTCAGT-3´ and 5´-TTGTGAGGGAGATGCTCAGTGT-3´). GAPDH quantification was used as an internal control for normalization. Percentage differences of mRNA levels over control values were calculated using the ΔCt method according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA). PCR reactions were independently repeated at least twice.

Footprint recording and SFI calculation

Preoperative footprints were recorded as previously described. After surgery, the procedure was repeated weekly for both groups until the end of the follow up period.

For each footprint, the following parameters were measured; print length (PL, the longitudinal distance between the tip of the longest toe and the heel), toe spread (TS, the distance between the first and fifth toes) and intermediate toe spread (IT, the distance between the second and fourth toes), for both the normal (N) and the experimental (E) paws.

Based on these parameters, SFI was calculated according the formula suggested by De Medinaceli *et al*. (1982) and later modified by Bain *et al*. (1989):

$$SFI = -38.3 \times EPL\text{-}NPL/NPL + 109.5 \times ETS\text{-}NTS/NTS + 13.3 \times EIT\text{-}NIT/NIT - 8.8.$$

SFI was designed to be an indicator of nerve function, with values around -100 indicating total loss of function and values around 0 indicating normal function.

SFI values were compared between the two groups at weekly intervals starting from the first week to the third week.

Electrophysiological tests

At the end of the third postoperative week, five rats from each group were anesthetized using the same mixture as for previous operations. A stimulating hooked platinum bipolar electrode was placed around the sciatic nerve at its emergence from the greater trochanter, and another recording electrode was placed around the nerve at a point about 15mm distal to its emergence, thereby insuring that the injury site was located between the two electrodes. Electrical current application started with a duration of 1ms and an intensity of 10 μA produced by an electric stimulator (EMG100C, Biopac Systems, Inc., USA). The intensity was gradually increased until the supramaximal stimulation that ensured maximal amplitude was reached (1-2 mA). Signals were then amplified 1000 times with a differential amplifier (Plexon, Dallas, TX, USA). Thereafter, the recorded signals were digitally converted with an MP 150 (Biopac Systems Inc,CA, USA) and the latency period was measured. The mean conduction velocity (MCV) was calculated as the distance between the electrodes divided by the latency period (m/s). Peak voltage (PV) was defined as the maximum voltage from the baseline (mV). A heating lamp was used to keep the rat's body temperature at approximately 37°C during the tests.
MCV and PV were measured and compared between both groups.

Retrograde labeling and quantification of sensory neurons

Retrograde labeling of back-labeled sensory neurons was performed as described by Geremia *et al*. (2007). 3 weeks postoperatively, the sciatic nerves of three animals in each group were labeled with 4% Fluorogold (Fluorochrome, LLC, Denver, CO, USA), while a fourth rat served as a negative control

using distilled water (DW). Fluorogold was shown to be effectively endocytosed and retrogradely transported through nerve axons and provides long-lasting labeling of nerve cells (Schmued and Fallon, 1986; Yoshida *et al*., 1988; Brushart *et al*., 2005; Geremia *et al*., 2007).

Sciatic nerves were sharply cut at a distance 10 mm distal to the injury site, and soaked in 4% FG for 30 m in a Vaseline well and then were thoroughly irrigated with physiologic saline, reflected back and the wound was closed. For the negative control, the sciatic nerve was processed as above but was instead soaked in DW. The wound was covered with wet sterile gauze during the procedure. Five days later the animals were deeply anesthetized and access to the left ventricle was gained to perform transcardiac perfusion. First, a pre-perfusion solution (50 ml/100g body weight) of 0.9% NaCl with 1% heparin was infused using a peristaltic pump and then an ice cold fixation solution of 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, (100ml/100g body weight) was used for tissue fixation. L4 and L5 DRG were then harvested and post-fixed in 4% paraformaldehyde overnight and were transferred to PBS solution (pH 7.4) until further processing. One day before sectioning, DRG were transferred to 30% sucrose solution.

DRGs were serially sectioned into fresh frozen 20μm thick sections using a cryocut microtome (Leica CM3050S Cryostat, Leica Microsystems, Wetzlar, Germany) at -18°C. Sections were collected and mounted on glass slides that were covered with Permount® coverslips (Fisher, CA , USA) for later examination.

A laser scanning confocal microscope (Olympus, FV-300, Tokyo, Japan) was used to capture images of DRG sections at 20x magnification. The three sections with the greatest area (usually those obtained from the middle of the DRG) were selected from each DRG, and the back-labeled neurons in these sections were counted. The total number of counted neurons in each DRG was used for comparison between the two groups.

Histomorphometric studies

Six rats from each group were anesthetized; the sciatic nerves were resected and properly identified. The nerves were immediately immersed into a fixation solution containing 2.5% gluteraldehyde in PBS at 4°C for 24 h.

Each nerve was divided into a proximal, an intermediate and a distal portion. Only the distal portion (5mm distal to the injury) was used for light and electron microscopy examination. The proximal and intermediate portions were used for assessment of nerve regeneration at and proximal to the injury site (details not shown).

The distal segment (5mm long) was then identified and post-fixed with 2% osmium tetroxide for 2 h, thereafter it was washed with a PBS (pH 7.4) solution and dehydrated in graded concentrations of ethanol, for 5 minutes each, and then in 10 minutes in absolute alcohol. The segment was then routinely processed and embedded in epoxy resin.

Serial transverse semi-thin sections of 2μm thickness were cut with an ultramicrotome (Leica, Ultracut, UCT, Austria) and stained with1% Toluidine blue for light microscopy examination. Images were captured using a specialized SPOT RT™-KE color mosaic system, (Diagnostic Instruments Inc., Sterling Heights, MI, USA) connected to a light microscope (Olympus, BX41-TF, Tokyo, Japan) and analyzed by OPTIMAS Ver. 6.5 (Image Processing Solutions Inc., North Reading, Mass, USA) software.

For each section, the total fascicular area was measured. The internal epineural edge of each fascicle was outlined with the computer mouse at a 40× magnification and the fascicle area was automatically shown. The procedure was repeated until all fascicles areas were obtained. The sum represented the total area of the given section. Thereafter, three representative fields were randomly selected at 200× images of the same section for axon counting, provided that the area of the fields was at least 50% of the total fascicular area of the given section. Mean axon density was calculated by dividing the total number of axons within the three fields by their area sum (N/mm^2). Finally, the total axon count (N) was estimated by multiplying the mean axon density by the total fascicular area.

For calculation of myelin thickness and G-ratio, ultra thin sections were cut and then double stained with uranyl acetate and lead citrate. The sections were analyzed using a transmission electron microscope (TEM; JSM 1200 IIEX, JEOL, Japan), and after image capture, myelin thickness was measured using the same software mentioned. The G-ratio was defined as the ratio of axon diameter to the myelinated fiber diameter.

Data analysis was carried out using StatView software (Version 5.0.1, SAS, Institute, Cary, NC, USA).

All data were presented as mean values with standard errors of mean (SEM).One-way ANOVA followed by Fisher's PLSD test was used to compare SFI values between the two groups at each time interval, as well as to MCV and PV three weeks post-injury.

One-way ANOVA followed by Fisher's PLSD test was also used to examine the statistical significance

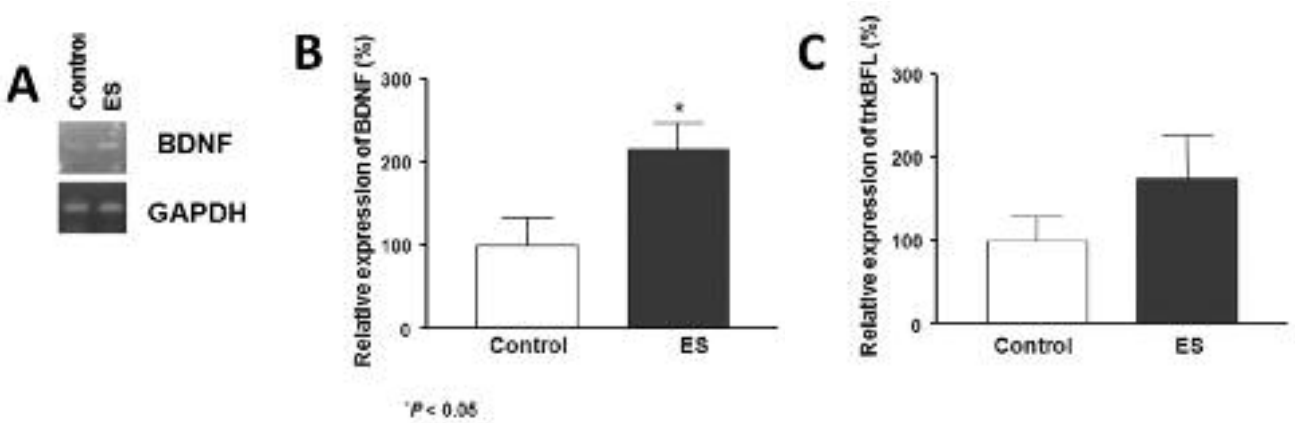

Fig. 1. — ES effects on *BDNF* and *trkB* expression in L4,L5 and L6 DRG neurons. (A) RT-PCR analysis of *BDNF* and *ttrkBFL* was performed on total mRNA five days following the injury. Results are representative of two independent experiments. Higher expression of *BDNF* is seen in ES group. *BDNF* (B) and *trkBFL* (C) mRNA expression was quantified using RT-PCR. Values are shown as means ± SEM normalized to GAPDH from at least two independent trials ($n \geq 2$).

of differences in expression of *BDNF* and *trkB* between the two groups, five days postoperatively.

The Mann-Whitney U test for nonparametric analysis was used to compare the number of FG back-labeled neurons, axon counts, axon density and myelin thickness means between the two groups at the end of third week postoperatively.

Values of $p < 0.05$ were considered statistically significant.

Results

BDNF and trkB levels

Quantitative RT-PCR showed higher levels of both *BDNF* and *trkB* mRNA by in the ES group 5 days after injury. The difference in *BDNF* expression was statistically significant ($p < 0.05$. Fig. 1).

Functional assessment, SFI

An average of 3 imprints for each rat was selected for Sciatic Function Index (SFI) calculation at each time interval. Accordingly, a total of 360 footprints (180 per group) were considered for evaluation by the same observer during the study.

Preoperative SFI means were around 0 for both groups, indicating normal function. One week following the crush injury, means for control and ES groups decreased sharply (-85.4 ± 1.39 and -89.0 ± 1.81 respectively) as a result of the axonotmesis and consequent loss of function. Recorded footprints were noticeably longer and the toe spread was smaller than in preoperative footprints. During the next two weeks, both groups showed progressive improvement of SFI, which was more pronounced between the second and third weeks. At the end of the second week, there was no significant difference between the two groups, as both showed only a slight functional recovery. At the end of the third week, however, the ES group showed a marked recovery reaching -38.5 ± 3.1, compared to -53.17 ± 2.4 for the control group. By the end of the third week the statistical difference between the two groups was clearly significant ($p < 0.001$, Fig.2). Comparison of footprints between the two groups 3 weeks postoperatively, showed that in the ES group, experimental and normal imprints were much alike, while in the control group, the experimental footprint was still longer with less toe-spreading potential than its normal counterpart (Fig. 3A & B).

Electrophysiological assessment

Supramaximal stimulation for both groups was reached at an intensity of 100μA. Following proper amplification of the stimulation signals and digital recording, MCV and PV were calculated. 3 weeks after the crush injury MCV in the control group, was 14.16 ± 1.84 m/s, while in the ES group it was about twice this value (28.8 ± 4.86 m/s) (Fig. 4A). The difference was statistically significant ($p < 0.05$), which shows a better functional recovery in the ES group. As for PV, values for control group ranged from 0.69-2.41 mV, while those for ES group were between 1.48-2.84 mV. The mean PV for ES group

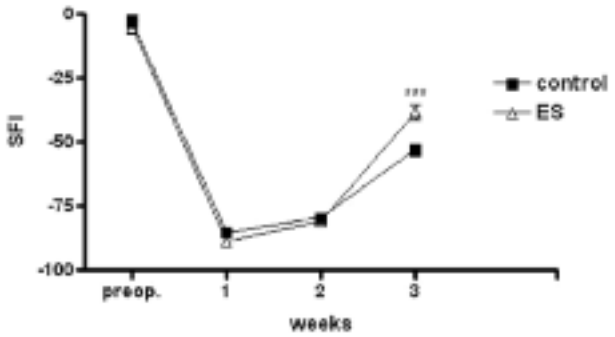

Fig. 2. — Graph of weekly SFI of both groups. Starting from normal preoperative values (around 0), SFI of both groups sharply declined 1 week after injury, indicating severe loss of function. 2 weeks after injury, both showed a slight functional improvement, without any significant difference. Three weeks postoperatively, the ES group recovered dramatically, while the control group showed a moderate functional recovery. ***($p \leq 0.001$).

was higher than that of the control group (ES: 2.0 ± 0.23, control:1.16 ± 0.2 mV) but the difference between the two was not significant ($p > 0.05$, Fig. 4B).

Retrograde labeling

In an attempt to increase the accuracy of the counting procedure, we took a number of measures. The person who was in charge of the counting was unaware of the groups and only clearly fluorescent cells were considered for counting (Fig. 5A & B).

Mean counts of back-labeled neurons were higher in the ES group than in the control group (326 ± 20 compared to 229 ± 32/ DRG; $p < 0.05$, Fig. 6), showing that the number of sensory neurons that regenerated their axons through the crushing site was significantly augmented by the use of ES.

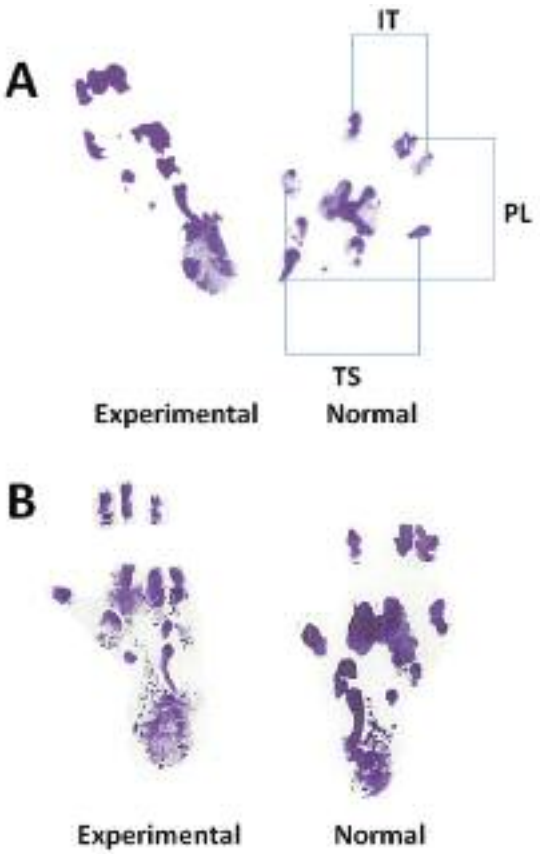

Fig. 3. — Representative footprints of control group (A) and ES group (B), 3 weeks postoperatively. Differences in experimental footprint shape and toe spread show lesser degree of recovery in the control group. Parameters used to calculate SFI are presented on the normal footprint of the control group, PL (print length), TS (toe spread) and IT (intermediate toe spread).

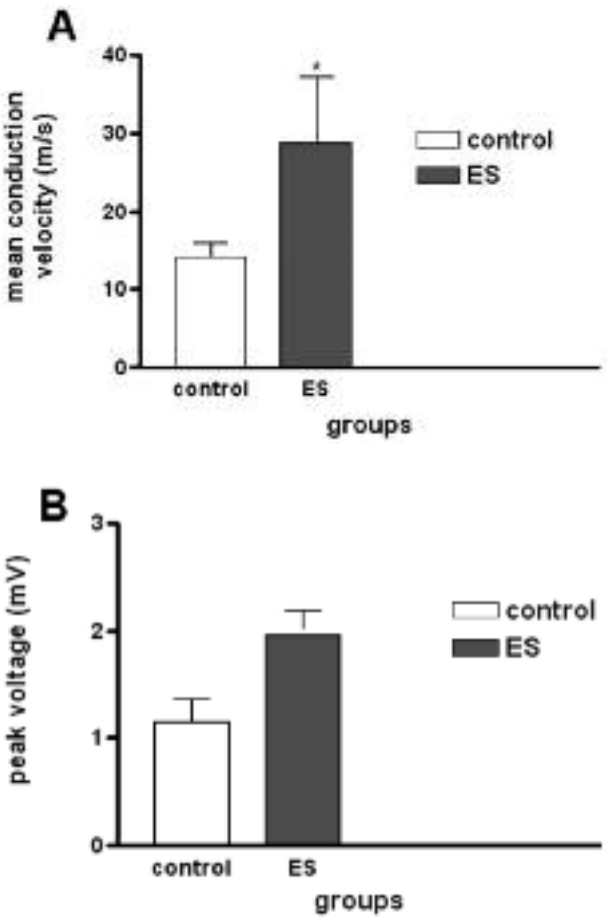

Fig. 4. — Electrophysiological results for both groups, 3 weeks after the injury.

A. Mean conduction velocity (MCV). *($p \leq 0.05$).

B. Peak voltage (PV).

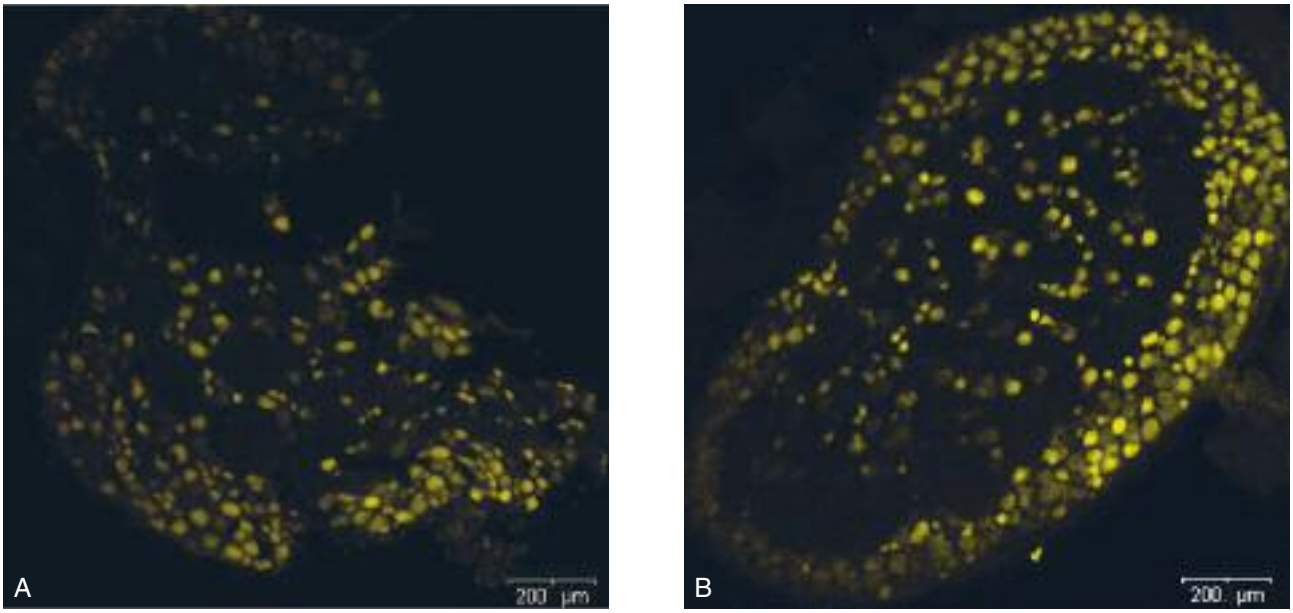

Fig. 5. — Fluorescence photomicrographs of 20 µm sections cut from L4 DRG from control (A) and ES (B) groups, showing fluorescent (yellow color) cell bodies of sensory neurons that were retrogradely labeled with FG, three weeks following the crush injury.(scale bar 200 µm).

Histomorphometric evaluation

The total fascicular area of the ES group was greater than that of the control group (0.84 ± .09 and 0.7 ± 0.7mm^2, respectively, ($p > 0.05$).

In the control group, the total axon counts were between 3,112-7,171 axons, whereas the densities were 5,665-9,420 N/mm^2. In the ES group, the total axons ranged between 6,010 and 8,855 axons, and the densities were between 7,041-11,625 N/mm^2 (Fig. 7A). There was a significant difference between total axon counts of the two groups (control mean = 5,273 ± 552, ES mean = 7,208 ± 381, $p < 0.05$), however, the difference was not significant when axon densities were compared (Fig. 7B) (control mean = 7,541 ± 497, ES mean = 8,990 ± 749, $p > 0.05$).

For the measurement of myelin thickness, axons surrounded by uniform-thickness myelin layers were chosen. The mean myelin thickness in the control group was 0.35 ± 0.01 µm (range: 0.25-0.49 µm), while in ES group it was 0.41 ± 0.02 µm (range: 0.26-0.56 µm) (Fig. 7C), the difference was statistically significant, ($p < 0.01$).

The average G-ratio was 0.69 ± 0.01 (range: 0.58-0.75) in the control group and 0.74 ± 0.02 (range: 0.56-.81) in the ES group (Fig. 7D). The difference between the two groups was significant ($p < 0.05$). The broad range seen for both groups is probably an indicator of the myelin regeneration process in both groups that is more pronounced in the ES group. The

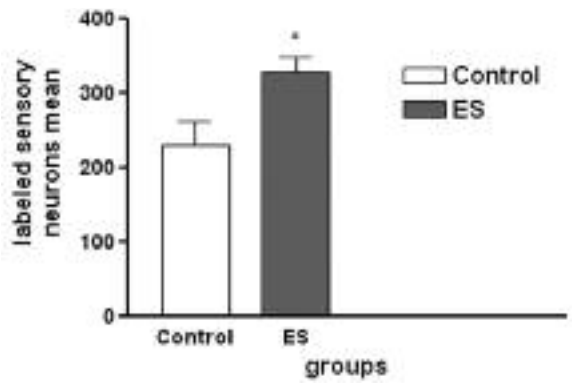

Fig. 6. — Mean counts of FG back-labeled DRG neurons, three weeks postoperatively. *($p \leq 0.05$).

histological picture of both groups was quite similar with mixed myelinated and unmyelinated fibers of variable diameters undergoing an obvious regeneration processes within a Wallerian degeneration background (Fig. 8A & B).

Discussion

The SFI is widely used for motor function assessment after sciatic nerve injury (Varejao *et al*., 2001, 2004; Luis *et al*., 2007). Moreover, SFI can help in overall sciatic nerve function evaluation, as a rat's hind paw motor function relies partly on plantar

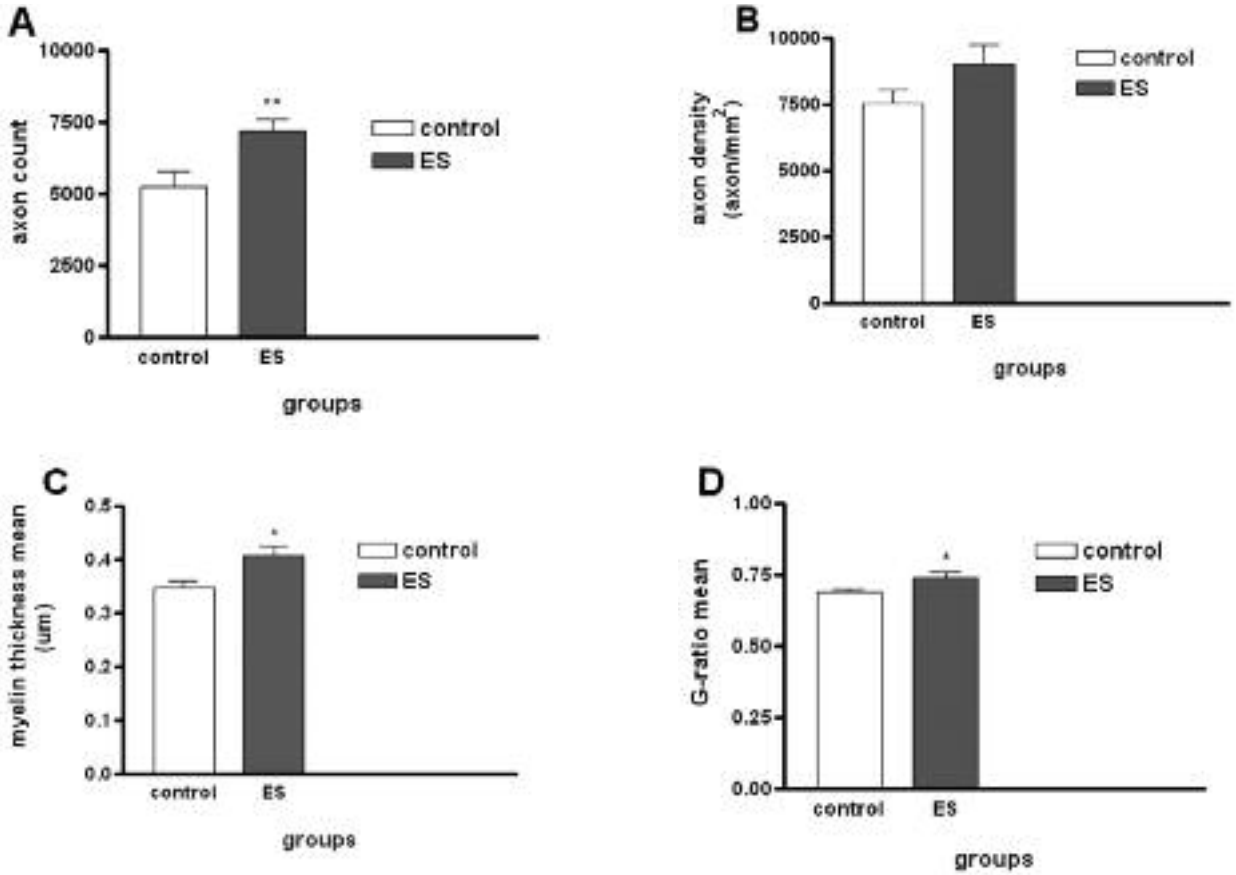

Fig.7. — Histomorphometric results in both groups. Significantly higher axon counts (A) myelin thickness means (C) and G-ratio means (D) were seen in the ES group, indicating a better histomorphometric recovery. All values are presented as mean ± SEM, *($p \leq 0.05$), **($p \leq 0.01$).

A. Total axon counts (*N*).

B. Axon densities (N/mm^2).

C. Myelin thickness means (μm).

D. G-ratio means.

sensitivity (Mendoca *et al*., 2003). In a recent study, SFI evaluation was shown to be a fully reproducible and reliable method from the third post-injury week (Monte-Raso *et al*., 2008). We studied SFI in the two groups, at weekly intervals from crushing injury until three weeks later. Both groups showed progressive recovery of motor function, especially between the second and third weeks. However, the dramatic improvement of SFI in the ES group was much more pronounced than that seen in the control group ($p < 0.001$), indicating a better functional recovery.

The SFI results were further confirmed by an MCV electrophysiological test, which depends largely on fiber diameter and myelination, and measures the functional recovery mainly in the fastest fibers (Hursh, 1939; Dorfman, 1990; Vleggeert-Lankamp, 2007).

Measured MCV in the ES group was more than twice that of the control group, which indicated that the regeneration process after injury, including remyelination of fibers was more efficient in the ES group, as shown in the histomorphometric analysis.

Another valuable assessment method focused on sensory neuron regeneration. A review of the literature regarding quantification of rat sciatic nerve sensory neurons showed a wide variability in results (Swett *et al*., 1991; Devor and Govrin-Lippmann, 1985a,b). Swett's (1991) work represents one of the most widely accepted reports in this field. They reported that 99% of all rat sciatic DRG perikarya are located in the L4 and L5 DRG, and the estimate of the total DRG neurons contributing to sciatic nerve is thought to be around 10,500 in adult rats. The technique used in the current study was a simple one that counted back-labeled neurons only in

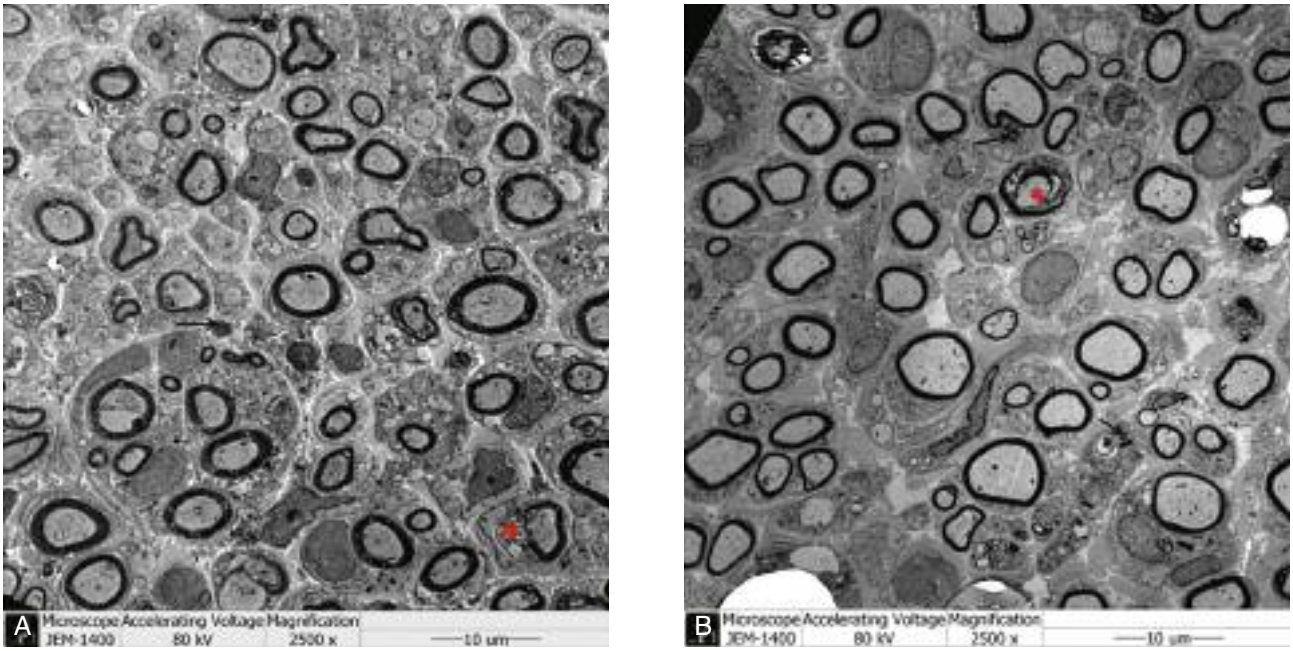

Fig. 8. — Representative photomicrographs of histologic features of both groups in thin sections cut distal to the crush injury site, three weeks postoperatively. The control group (A) and ES group (B) showed nearly similar features, characterized by the presence of small and large diameter myelinated and unmyelinated fibers. Some degenerated fibers are being phagocytosed by macrophage-like cells (asterisks) and dispersed myelin remnants are seen in the background (arrows), indicating an active Wallerian degeneration process. (Scale bar 10 µm)

representative sections of DRGs to facilitate comparison, and the results showed significantly higher counts of retrogradely-labeled neurons in the ES group. Nevertheless, a 3-D based stereological quantification method would be ideal for such studies.

A considerable body of evidence exists now about the key role of *BDNF* and its receptor *trkB* in mediating ES positive effects on nerve regeneration in both sensory and motor neurons.

Al-Majed *et al*. (2000b) demonstrated by *in situ* hybridization that ES increases *BDNF* and *trkB* mRNA expression, in addition to other regeneration-associated genes, in motor neurons. Similar results have been seen in sensory neurons; where 1 h of ES resulted in upregulation of *BDNF* immunoreactivity (Geremia *et al*. 2007). Moreover, when endogenous *BDNF* was blocked by a functional blocking antibody administered during the first three days post-injury, the augmenting effects of ES on axon regeneration were abolished (Tyreman *et al*., 2008; Gordon *et al*., 2009).

In the current report, quantification of *BDNF* and *trkB* levels in DRG sensory neurons by means of real time RT-PCR showed higher detectable levels by the fifth day postoperatively in the ES group, reflecting a higher axonal plasticity induced by ES.

Our histomorphometric studies showed that at the third week post injury, both groups were undergoing a Wallerian degeneration process as seen in the photomicrographs. Myelinated and unmyelinated fibers showing variable degrees of dystrophy with the presence of macrophage-like cells and signs of microfasciculation were seen in both groups, indicating an active regeneration process. This was consistent with data from a previous study based on the same model (Mendoca *et al*., 2003).

ES resulted in significantly higher axon counts than the control group. However, our data regarding axon counts and densities differed from previous reports (Mendoca *et al*., 2003; Varejao *et al*., 2004), which showed higher axon density means, mainly due to differences in crushing instruments or follow up periods.

The average myelin thickness and G-ratio were also higher in the ES group. These findings correlate well with previous functional assessment results, especially for SFI and MCV. An overall consideration of all these data presents evidence that our protocol of 30 minutes of low intensity ES was effective in promoting motor functional as well as sensory recovery.

Recently, researchers have been focusing on improvements and modifications of original techniques

in order to put their results into clinical use. An important aspect of ES to promote nerve regeneration is the duration of stimulation and long-term versus short-term stimulation protocols. Although some investigators managed to use implantable electrical stimulators without any side effects (Al-Majed *et al*., 2000a; Mendoca *et al*., 2003; Pomeranz and Campbell, 1993), their protocols were far from being applicable in a clinical environment.

Gordon and colleagues progressively reduced the duration of stimulation and proved that 1 h of ES is effective for promoting motor as well as sensory nerve regeneration (Al-Majed *et al*., 2000a; Geremia *et al*., 2007). They mentioned that shorter stimulation periods were ineffective, but no details were provided (Gordon *et al*., 2003).

During their experiments, Al-Majed *et al*. (2000a) first chose a stimulation period of two weeks, as this was the expected period for motor nerve regeneration in rat femoral nerve model after transection and repair (Brushart, 1988, 1993). They found that this period was effective to accelerate axonal regeneration so that all motor neurons regenerated their axons within 3 weeks. In the same study, they demonstrated that short term stimulation for 1 h was as effective as the long term stimulation protocol (Al-Majed *et al*., 2000a). When different stimulation duration protocols were studied in sensory neuron regeneration, however, the results were different. Geremia *et al*. (2007) found that 1 h of ES was optimal for promoting sensory neuronal regeneration, and longer stimulation periods were not as effective. A possible explanation for this is the different sensitivity of *trkB* receptors to released neurotrophic factors. Following the up-regulation of neurotrophic factors and trk receptors as a result of ES, the receptors of motor neurons remain sensitive to neurotrophic factors while those receptors on sensory neurons are rapidly down regulated if ES continues for more than 1 h (Gordon *et al*., 2009). This may answer the question why longer than 1 hr of ES can be of no effect or even inhibitory to peripheral nerve regeneration, but it gives no clue to whether or not shorter periods of ES are effective in promoting nerve regeneration. At a molecular level, we have shown that 30 minutes ES was sufficient to significantly elevate endogenous *BDNF* levels in sensory neurons. This was consistent with previous findings, where ES for 1 h modulated the cell body response to injury as shown by the upregulation of *BDNF* expression in sensory neurons (Geremia *et al*., 2007).

One of the major criticisms to the crushing injury model is the possibility that some nerve fibers are not directly damaged during injury and may only undergo a Sunderland type I lesion. Thus the subsequent functional or sensory recovery is not a true reflection of an axotomized neurons regeneration process (Varejao *et al*., 2004). The use of a nerve transection and repair model would have obliterated this pitfall. However, we were trying to establish the possibility that ES for less than 1 h combined with the inherent capacity for nerve regeneration after axonotmesis (Sunderland, 1951, 1990) would have any positive effects on recovery.

Another shortcoming of the current report is that it did not include an assessment of nerve fiber diameter and its relationship to the G-ratio. While this important evaluation parameter was a key part of some previous histomorphometric studies that evaluated the crush injury model (Mazzer *et al*., 2008; Varejao *et al*., 2004; Luis *et al*., 2007), we were more concerned with the functional and sensory recovery (assessed through the SFI, electrophysiological tests and retrograde tracing of sensory neurons), and correlations with relevant morphometric features were highlighted where appropriate.

Conclusion

In this study we investigated the effects of low intensity electrical stimulation for 30 minutes on the regeneration process in rat sciatic nerves after crush injury, and provided some evidence that functional and sensory recovery were improved by the third post-injury week. Elevated levels of *BDNF* expression were also evident by RT-PCR five days postoperatively in sensory neurons. The future use of a nerve transection and repair model, together with more extensive histomorphometric assessment will alleviate some imperfections of this study and give us the chance to further explore the effects of less than 1 h of ES on peripheral nerve regeneration, in order to make ES a viable therapeutic modality for clinical application in the near future.

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J.-H. Lee, DDS, Ph.D.
Department of Oral and Maxillofacial Surgery,
School of Dentistry, Seoul National University
275-1 Yeongun-dong, Jongno-gu,
Seoul, 110-768, Rep .of Korea
E-mail: leejongh@snu.ac.kr