Electrical stimulation enhances peripheral nerve regeneration after crush injury in rats

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Abstract. Injured peripheral nerves have the ability to regenerate; however, there is conflicting evidence with regard to whether electrical stimulation (ES) accelerates or hinders neural regeneration. To study the effect of ES on peripheral nerve regeneration following nerve crush injury, 54 Wistar rats were randomly divided into three groups (n=18/group); the control, crush and crush + ES group. Four weeks after surgery, the sciatic functional index (SFI), compound muscle action potential (CMAP) conduction velocity and amplitude in the regenerated nerve, nerve histomorphometry, and levels of myelin protein zero (P0) mRNA and protein at the crush site were assessed. The rats exposed to crush + ES had a significantly increased CMAP conduction velocity, enhanced myelin sheath thickness and increased P0 mRNA and protein levels compared with the rats exposed to crush alone. However, the CMAP amplitude and axonal diameter were similar in the crush and crush + ES rats. Findings of this study demonstrated that the application of ES (3 V, 0.1 ms, 20 Hz, 1 h) immediately after nerve injury accelerates remyelination and may provide a therapeutic clinical strategy.

Introduction

Peripheral nerve injury is common and previous studies have investigated numerous approaches to accelerate neural recovery. Low-intensity electrical stimulation (ES) has been shown to improve nerve regeneration (1-4) by increasing the expression of brain-derived neurotrophic factor (BDNF) (5,6), which is known to enhance myelin formation during the early stages of development (7). ES has also been shown to promote the expression of growth-associated genes (8,9) and signaling by neurotrophins (10). Alrashdan *et al* (11) reported that low-intensity ES for 30 min promoted nerve regeneration, while Yeh *et al* (12) observed that the timing of ES application affected the maturity of regenerating rat sciatic nerves, which indicates that early intervention after severe peripheral nerve injury may be important for recovery. Additional studies have demonstrated that ES for 1 h facilitates nerve regeneration (7,13,14). Brief pulses (duration, 0.1 ms) of suprathreshold ES (3 V, 20 Hz) have been shown to enhance remyelination and functional recovery (2,14,15), promoting the speed and accuracy of motor axonal regeneration (1,16) and sensory neuron regeneration (9). ES has also been shown to facilitate regeneration in a diabetic model (17). Thus, ES has been proposed as a therapeutic method to repair nerve lesions in a clinical setting.

However, there have also been studies which contraindicate ES therapy for peripheral nerve injury. For example, Baptista *et al* (18) demonstrated that high- and low-frequency transcutaneous electrical nerve stimulation delayed sciatic nerve regeneration following crush injury. Gigo-Benato *et al* (19) showed that ES impaired early functional recovery and exacerbated skeletal muscle atrophy after sciatic nerve crush injury in rats. Hamilton *et al* (20) revealed that ES promoted axon regeneration at the expense of decreasing the fidelity of muscle reinnervation, thus the functional recovery was unchanged. Lu *et al* (21) determined that ES was able to have a positive or negative impact on peripheral nerve regeneration and recommended that clinical trials which combine stimulation with rehabilitation should identify safe and effective parameters. Thus, whether ES therapy is beneficial after peripheral nerve trauma remains controversial and requires further investigation.

The aim of the present study was to determine whether brief ES improves functional recovery after a crush injury by promoting remyelination, which is the main process underlying the restoration of injured peripheral nerves. Myelin protein zero (P0) is a marker of axon regeneration and remyelination (22) and its mRNA and protein levels were determined using RT-PCR and western blotting, respectively, to assess recovery.

Materials and methods

Animals. Wistar rats (200 g; n=54) were obtained from the Experimental Animal Center of China Medical University (Shenyang, China; certification no. SCXK Liao 2003-0009). This study was approved by the Institutional Animal Care and

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Use Committee and the Experimental Animal Administration Committee of China Medical University; it was determined that the number of animals used in the present study and their distress was appropriately minimized.

Surgical procedure and electrical stimulation. Rats were anesthetized with 10% chloral hydrate (0.3 ml/100 g; i.p.). The right sciatic nerve was exposed and crushed for 3 min using a non-serrated clamp with a force of 54 N (23) to induce an axonotmesic lesion. The crush site was ~6 mm long, located 5 mm above the bifurcation and was sutured with 8-0 nylon as a marker. The proximal nerve trunk was electrically stimulated as described by Al-Majed *et al* (1). The rats were randomly divided into 3 groups (n=18/group); the control, crush or crush + ES group. Rats of the control group had sciatic nerve exposure with no crush procedures, while rats of the crush group had electrodes implanted with the stimulator turned off. Rats of the crush + ES group had electrodes implanted proximal to the injury site to deliver a continuous train of 20 Hz square pulses of 3 V at 0.1 ms for 1 h. The wounds were kept warm and moist with sterile saline gauze during the ES. Following completion of the ES procedure was completed, the skin was sutured with 4-0 stitches and all the rats were housed in cages with food and water *ad libitum* for 4 weeks.

Motor function evaluation using walking track analysis. An assay of motor nerve functional recovery was performed weekly for 4 weeks following surgery. The sciatic functional index (SFI) was calculated, as described by Bain *et al* (24). The hind paws of rats trained on the procedure prior to surgery were dipped in blue ink and these rats were allowed to walk down a plastic corridor (60 cm long, 10 cm wide) lined with white paper. The SFI was calculated according to the following equation: $SFI = -38.3(EPL-NPL)/NPL + 109.5(ETS-NTS)/NTS + 13.3(EIT-NIT)/NIT - 8.8$. PL was defined as the distance between the heel and the third toe, TS as the distance between the first and fifth toes and IT as the distance between the second and fourth toes. E and N represented the experimental and normal sides.

Electrophysiological assessment. Four weeks after surgery, all the rats were anesthetized and the right sciatic nerve was exposed. A bipolar stimulating electrode was placed around the sciatic nerve, proximal to the injury site and a bipolar recording electrode was placed in the gastrocnemius muscle. Compound muscle action potential (CMAP) and amplitude were recorded using an RM6240 physiological signal processing apparatus (Chengdu Instrument Factory, Chengdu, China). The distance between the two electrodes was measured and used to calculate the CMAP conduction velocity.

Nerve histomorphometry. The crush sites of sciatic nerves (n=6/group) were removed and fixed in 2.5% glutaraldehyde solution, dehydrated in graded acetone, which was then replaced with acetone, and embedded in epoxyresin for sectioning. Semi-thin cross sections (2 μm) were obtained using a microtome (UltraCut E; Leica Microsystems, Vienna, Austria) and stained with 1% toluidine blue solution for light microscopy, while ultra-thin cross sections (70 nm) were analyzed using a transmission electron microscope (TEM, JEM-1200EX; Jeol Ltd., Tokyo, Japan). Myelin sheath thickness and axonal diameter were analyzed using the MetaMorph/DP10/BX41 image analysis system.

RT-PCR. The crush sites of sciatic nerves (n=6/group) were excised and homogenized for RT-PCR. The total RNA was extracted using the TRIzol method and cDNA was synthesized using an oligo(dT)-adaptor primer. PCR was performed using a kit (Takara Biomedical Technology, Dalian, China), with the following 35 cycles: 94˚C for 30 sec, 60˚C (P0 and GAPDH) for 30 sec and 72˚C for 45 sec. GAPDH was used as an internal control. The following gene-specific primers were used: P0 forward, 5'-CTCTTCTCTTCTTTGGTGCT-3' and reverse, 5'-TTCTTATCCTTGCGAGACTC-3' (692-bp amplification fragment); and GAPDH forward, 5'-GGTGAAGGTCGGT GTGAACG-3' and reverse, 5'-CAAAGTTCTCATGGAT GACC-3' (497-bp amplification fragment). The amplification product was visualized using 1.5% agarose gel electrophoresis and analyzed with Image J software. Data were expressed as the ratio between the amplification products of P0 and GAPDH.

Western blotting. Four weeks after surgery, the crush sites of sciatic nerves (n=6/group) were excised and lysed in ice-cold RIPA buffer containing protease inhibitor (PMSF) using an ultrasonic wave disintegrator. The samples of total protein lysate (20 μg) were resolved on 10% SDS-PAGE gels by electrophoresis and transferred onto PVDF membranes. The membranes were blocked at room temperature with 20% bovine serine albumin (BSA) dissolved in Tris-buffered saline containing 1% Tween-20 (TBST) for 2 h. The membranes were then incubated with goat polyclonal antibody to P0 (1:1,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and mouse monoclonal antibody to GAPDH (1:5,000; Santa Cruz Biotechnology, Inc.) overnight at 4˚C. After rinsing with TBST three times, the membranes were incubated at room temperature for 2 h with rabbit anti-goat IgG (1:5,000; Santa Cruz Biotechnology, Inc.) and rabbit anti-mouse IgG (1:5,000; Santa Cruz Biotechnology, Inc.). Secondary antibodies were visualized using a BeyoECL Plus kit (Beyotime Institute of Biotechnology, Jiangsu, China) using ChemDoc XRS with Quantity One software (Bio-Rad, Hercules, CA, USA). Band intensities were quantified using Image-Pro Plus 6.0 software. The blots were repeated ≥3 times for each condition.

Statistical analysis. Data were analyzed using the SPSS 13.0 software, by a one-way analysis of variance (ANOVA) and post hoc multiple comparisons were assessed using Tukey's test. Results are expressed as the mean ± standard error of the mean (SEM). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Motor function evaluation. As shown in Fig. 1, the difference in the SFI between the control and experimental (crush and crush + ES) groups was statistically significant ($P<0.05$) at 1 and 2 weeks after surgery, whereas the difference between the two experimental groups was not significant ($P>0.05$). The

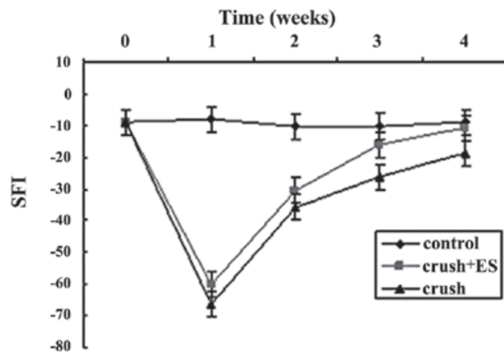

Figure 1. Weekly sciatic functional index (SFI) of rats in the 3 groups for four weeks following surgery. Bars represent standard error, n=6/group. ES, electrical stimulation.

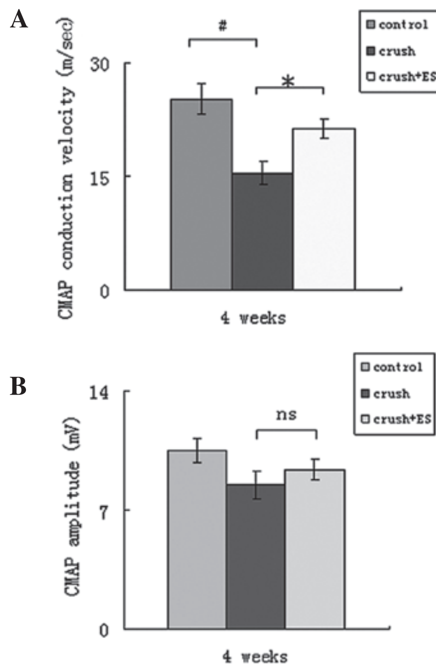

Figure 2. Electrophysiological assessment 4 weeks after surgery. (A) CMAP conduction velocity. *P<0.05 for the crush vs. the crush + ES group; #P<0.05 for the crush vs. the control group. (B) CMAP amplitude. ns, no significant difference (P>0.05) was identified between the crush and crush + ES groups. Bars represent standard error, n=6/group. ES, electrical stimulation; CMAP, compound muscle action potential.

mean SFI at 3 and 4 weeks after surgery was -25.99±3.04 and -18.55±4.10, respectively, in the crush group, whereas that of the crush + ES group was -16.15±3.95 and -10.81±4.00, respectively. There was a significant difference in the SFI between the crush and crush + ES groups (P<0.05).

Electrophysiological assessment. Four weeks after surgery, the mean CMAP conduction velocity was 25.27±2.00 m/sec in the control group, 15.34±1.50 m/sec in the crush group and 21.36±1.25 m/sec in the crush + ES group (Fig. 2A). A significant difference was observed between the control and crush groups (P<0.05) and the crush and crush + ES groups (P<0.05). However, no significant difference in CMAP conduction velocity was identified between the control and crush + ES groups (P>0.05). As shown in Fig. 2B, the mean CMAP

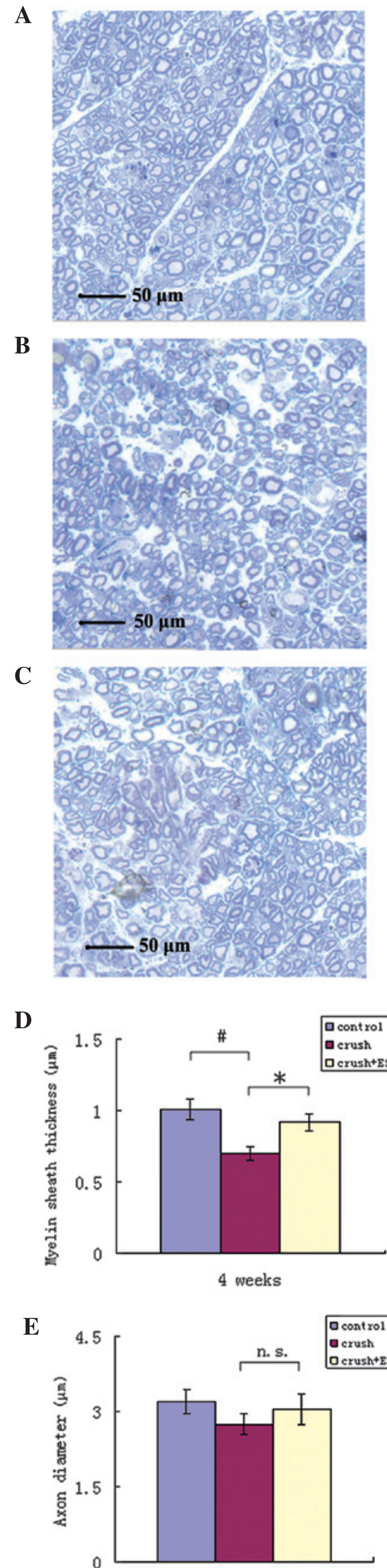

Figure 3. (A-C) Cross sections of the crush sites following toluidine blue staining, as examined under a light microscope 4 weeks after surgery in the (A) control, (B) crush and (C) crush + ES groups. Magnification, x400; scale bar, 50 μm. (D) Myelin sheath thickness. *P<0.05 for the crush vs. the crush + ES group; #P<0.05 for the crush vs. the control group. (E) Axonal diameter. ns, no significant difference was identified between the crush and crush + ES groups (P>0.05). Bars represent standard error, n=6/group. ES, electrical stimulation.

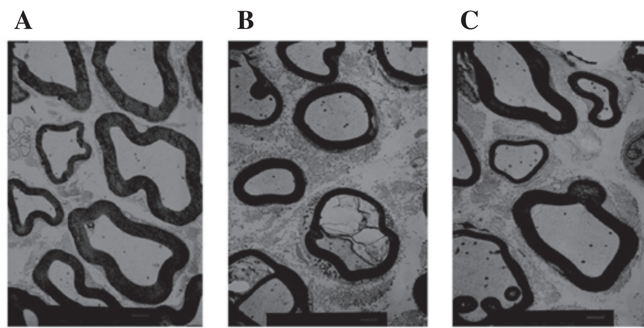

Figure 4. Images from a transmission electron microscope showing myelin sheath thickness on cross sections of the crush sites 4 weeks after surgery in the (A) control, (B) crush and (C) crush + ES groups. Magnification, x8,000. ES, electrical stimulation.

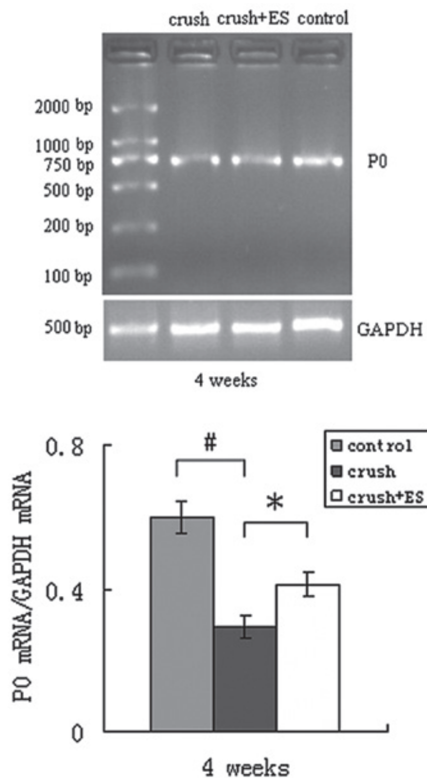

Figure 5. RT-PCR analysis of P0 mRNA/GAPDH mRNA in the crush sites of rats from the 3 groups, 4 weeks after surgery. *P<0.05 for the crush vs. the crush + ES group; #P<0.05 for the crush vs. the control group. Bars represent standard error, n=6/group. ES, electrical stimulation; P0, myelin protein zero.

amplitude was 10.5±0.7 mV in the control group, 8.5±0.8 mV in the crush group and 9.4±0.6 mV in the crush + ES group. No significant differences were found between the control and experimental groups (P>0.05).

Nerve histomorphometry. The myelin sheath thickness and axonal diameter were evaluated in the crush (Figs. 3A and 4A), crush + ES (Figs. 3B and 4B) and control groups (Figs. 3C and 4C) four weeks after surgery. The mean myelin sheath thickness was 1.01±0.07 μm in the control, 0.70±0.05 μm in the crush and 0.92±0.06 μm in the crush + ES group (Fig. 3D). A significant difference was observed between the crush and crush + ES groups (P<0.05) and the crush and control groups (P<0.05). As shown in Fig. 3E, the mean axonal diameter was 3.20±0.23 μm in the control, 2.75±0.22 μm in the crush and 3.05±0.30 μm in the crush + ES group; no significant differences were identified between the control and experimental groups (P>0.05).

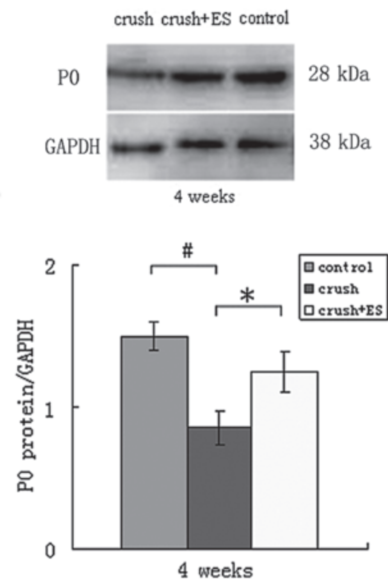

Figure 6. P0 protein/GAPDH protein levels were assessed using western blotting in the crush sites of rats in the 3 groups, 4 weeks after surgery. *P<0.05 for the crush vs. the crush + ES group; #P<0.05 for the crush vs. the control group. Bars represent standard error, n=6/group. ES, electrical stimulation; P0, myelin protein zero.

P0 mRNA and protein levels. Four weeks after surgery, the levels of P0 mRNA/GAPDH mRNA (Fig. 5) and P0 protein (Fig. 6) were significantly decreased in the crush group compared with the control group (P<0.05). The levels of P0 mRNA/GAPDH mRNA and P0 protein were increased in the crush + ES group compared with the crush group (P<0.05).

Discussion

There have been conflicting results from previous studies on the effect of ES on injured peripheral nerves (5). The present study showed that ES (20 Hz, 0.1 ms, 3 V, 1 h) enhances axonal regeneration when applied immediately after nerve injury, which supports the results of a number of previous studies (1,3,4). The SFI has often been used to assess the recovery of motor function following nerve injury. In the present study, walking track analysis was used to determine the SFI every week for 4 weeks following surgery. A significantly improved SFI was observed in the crush + ES group at 3 and 4 weeks after surgery. Further physiological and histological measures also demonstrated an improvement, with the exception of the CMAP amplitude and axonal diameter, indicating that ES is a potential early intervention therapy for peripheral nerve injury.

During the course of peripheral nerve repair, Schwann cells proliferate and form the myelin sheath to promote axonal regeneration from the proximal to the distal end. P0 is the most abundant protein within the peripheral myelin sheath (25).

P0 mediates cell-to-cell interactions via homophilic binding and stabilizes the major dense line in the peripheral nervous system, which is essential for normal myelin formation and maintenance (26). Mirsky *et al* (27) suggested that the P0 gene is upregulated during Schwann cell myelination. In the present study, P0 mRNA and protein levels were increased in the myelin sheath following ES, indicating an upregulation of the mRNA and protein levels. Combined with the enhanced CMAP conduction velocity and thickened myelin sheath following ES, these data supported our hypothesis that ES promotes Schwann cell proliferation and myelination.

One potential confounding factor in the present study was the 1 h delay for the skin to be sutured after surgery, which may have delayed axonal regeneration. Future studies should include a group that undergoes suturing immediately after the crush injury. Furthermore, it is important to note that the stimulator should be placed at an appropriate site, as described by Al-Majed *et al* (1). The stimulator may induce continuous vigorous contractions of the nearby muscles when not insulated from surrounding tissues, which may hinder regeneration and recovery.

The present study demonstrated that ES accelerates axonal regeneration after nerve injury, which may provide an early therapeutic strategy for nerve injury.

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