SUBTHRESHOLD CONTINUOUS ELECTRICAL STIMULATION FACILITATES FUNCTIONAL RECOVERY OF FACIAL NERVE AFTER CRUSH INJURY IN RABBIT

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ABSTRACT: We sought to determine whether electrical stimulation (ES) with subthreshold, continuous, low-frequency impulses is a viable clinical method for improving functional recovery after facial nerve crush injury. In 10 rabbits, bilateral crush injuries were made on the facial nerve by compression for 30 s with mosquito forceps, causing complete facial paralysis. Subthreshold continuous direct current ES with 20-Hz square-wave pulses was applied to the proximal stump on one side for 4 weeks. Vibrissae movement returned significantly earlier on the ES side, with a less variable recovery time. Electrophysiologically, the stimulated side had a significantly shorter latency, longer duration, and faster conduction velocity. Light and transmission electron microscopy revealed that the electrical stimulation also markedly decreased Wallerian degeneration. The average numbers of fluorescent, double-labeled nerve cells were significantly different between the ES and non-ES sides. This study shows that subthreshold, continuous, low-frequency ES immediately after a crush injury of the facial nerve results in earlier recovery of facial function and shorter overall recovery time.

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Acute facial paralysis, such as Bell's palsy, is an emotionally and psychologically traumatic injury. The annual incidence of acute facial paralysis is about 23–25 cases per 100,000 population.[1] Most cases of facial neuropathy recover with various treatment modalities, but permanent sequelae remain in approximately 16% of cases.[2] Synkinesis, a problem of facial movement control in which abnormal movements accompany intended voluntary facial movements, is a distressing consequence of acute facial palsy. Although there is no direct evidence for delayed recovery as an independent causative factor in synkinesis, it does correlate with longer recovery times.[3,4] There is a continuing need for new methods to decrease the incidence of facial sequelae during recovery and to enhance early recovery after facial nerve injury.

Electrical stimulation (ES) is being studied as a potential intervention for nerve regeneration. Neuromuscular ES has been used as a rehabilitation technique to enhance the function of paralyzed muscles in patients with facial nerve paralysis.[5] ES affects various properties of denervated muscles, including the distribution of acetylcholine receptors,[6] contractile properties of the muscle,[7] and endplate morphology.[8] Direct ES of a damaged nerve has also been assessed for improving functional recovery. Many reports have indicated that, in the central nervous system, electrical fields are involved in controlling aspects of development and regeneration.[9] Furthermore, both therapeutic and functional ES have been developed for patients with spinal cord injuries.[10]

Recently, ES of damaged peripheral nerves was reported to promote nerve regeneration. Brief supramaximal ES of the femoral nerve at proximal injury sites accelerated axonal regeneration and preferential motor reinnervation in rats.[11] ES has also been shown to promote regeneration of sensory axons and to improve selective reinnervation by regenerating sensory fibers in rats.[12] However, to our knowledge, no study has shown definitive benefits of ES in the final outcome of acute facial palsy.[13] Previous studies have demonstrated only that ES applied as suprathreshold impulses carried out briefly or daily for a few minutes can effectively stimulate damaged nerves, or they have compared the recovery of facial nerve function with and without ES. Lal et al.[14] showed that brief ES accelerated the recovery of the semi-eyeblink reflex in rats with a facial nerve crushing injury; however, overall recovery of the facial nerve was not significantly improved by ES.

In contrast to suprathreshold impulses, ES designed with subthreshold intensity and delivered in a continuous 20-Hz train by an implantable stimulator may benefit the nervous system, as even extremely weak electrical currents can affect the nervous system and induce axonal regeneration following trauma.[15] We used a continuous, low-frequency impulse in this study because it is a physiologically relevant frequency of motor neuron discharge.[16]

The objective of this study, which was performed in rabbits, was to determine whether subthreshold, continuous, low-frequency-impulse electrical stimulation (SCLES) has the potential to become a viable clinical method for improving functional recovery after a crushing injury of the facial nerve.

Abbreviations: ANOVA, analysis of variance; BDNF, brain-derived neurotrophic factor; ES, electrical stimulation; FR, Fluoro-Ruby; SCLES, subthreshold , continuous, low-frequency-impulse electrical stimulation; TB, True Blue; TEM, transmission electron microscopy

Key words: electrical stimulation, facial nerve, recovery, threshold, Wallerian degeneration

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METHODS

Animals. Ten male New Zealand white rabbits, 3 months old and weighing 2.5–3.0 kg (mean weight 2.73 kg), were provided with a standard diet and water. They were kept under a 12/12-h light/dark cycle at 22°C in 45% humidity-controlled rooms. Experiments were approved by the local ethics committee (Health Science Laboratory Animal Services) under the Korean guidelines for animal experimentation.

Surgical Procedure. After premedication by intramuscular administration of Zoletil (tiletamine chloridrate and zolazepan chloridrate, 1.5 mg/kg), the rabbits were anesthetized by endotracheal administration of enflurane (2 MAC/kg) and intravascular iodic thiopentone (250 mg/kg). Surgical procedures were performed using an operating microscope under sterile conditions. Curvilinear incisions were made on both cheeks, from the anterior margin of the external meatus to the homolateral mandibular corner of each side. After exposure of 20 mm of the buccal facial nerves, they were atraumatically divided into ventral and dorsal branches.

To ensure that the crushing injuries were identical on both sides of the face, the nerve trunks were clipped using the same pressure ($\backsim$40 N) for 30 s, with the beaks of a mosquito forceps.[17] A stainless-steel needle electrode was implanted in the lateral side of the nerve trunk, 5 mm proximal to the injury site. The electrode was tunneled from the anterior margin of the external meatus to the back of the head, where it exited from the subcutaneous area and was connected to a nerve stimulator. After closure of the surgical incisions, 150,000 U of benzathine penicillin and 150,000 U of procaine penicillin were administered intramuscularly, which was repeated every 24 h for three additional doses.

Two randomly selected rabbits per week were euthanized at weeks 1, 2, 3, and 4 after the crushing injury, and the stump of the facial nerve and the innervated muscle were inspected.

Electrical Stimulation. The electrical stimulator (version 1; Kwangwoo Medix, Inc.) could generate a pulse of 10-ms duration every 50 ms and could regulate the stimulus voltage between 20 mV and 20 V and the frequency between 20 Hz and 5 kHz. The connector pins of the Teflon-coated needle electrode were inserted alongside the facial nerve of the rabbit and sutured just below the exit from the skin; the electrode was attached to the positive and negative leads of the electrical stimulator. The side of the face that was to receive ES was selected randomly.

Table 1. Six-point scale of rabbit vibrissae movement.

Grade	Evaluation
1	Normal active movement
2	Noticeable movement of nasal ridge, intact movement of whiskers
3	Detectable movement of nasal ridge, intact movement of whiskers
4	Noticeable movement of whiskers, no movement of nasal ridge
5	Slightly detectable movement of whiskers, no movement of nasal ridge
6	No movement

Immediately after nerve injury, the average current needed to obtain minimal muscle contraction was determined (average preoperative threshold 2.00 ± 0.43 mA, $n = 16$), and ES was delivered in continuous, 20Hz, rectangular, monophasic spikes with a subthreshold pulse (average 1.4 mA, 10 ms). ES was continued for a maximum of 4 weeks.

Functional Assessment. The recovery of facial nerve function on each side was assessed daily. The recovery of vibrissae movement was assessed using videography and was scored on a 6-point scale (Table 1). A video recording of the nasal ridge and whiskers was made for 1 min, from positions in front of and above the head.

Electrophysiological Tests. Electroneurography was performed with monopolar Teflon-coated needle electrodes placed around the injured facial nerve of the rabbit. An active recording electrode was placed lateral to the nasal ala, with a reference electrode a few centimeters distal, and the ground was placed in a convenient location. The facial nerve was excited just proximal to the injury site. To produce electroneurographic activity, we used a variable square-wave current (from 1.6 mA to 11 mA, 10 ms). All stimuli were delivered at one per 50 ms. The resulting compound muscle action potentials were used for comparison of facial nerve function between pre- and post-crushed status on the same side, and between the sides with and without ES. The threshold, amplitude, and latency of the compound action potentials were estimated at days 1–5 post-injury, and then every week for 4 weeks.

Histological Study. The stumps of the facial nerve, including the injury sites, were harvested from both sides at weeks 1, 2, 3, and 4 after the crushing injury. The specimens, which included the stump of the facial nerve and the innervated muscles, were fixed overnight in buffered 2% glutaraldehyde, 2% paraformaldehyde, and 0.5% $CaCl_2$. Semithin cross-sections of the muscle were placed in 2% osmium tetroxide in phosphate buffer for

2 h, dehydrated through a graded ethanol series, cleaned with toluene, and embedded in plastic epoxy. Sections of 1-μm thickness were stained with toluidine blue.

The harvested nerves of three crushed samples with SCLES and three without ES were examined under a light microscope (Olympus BX-40) and scanned for distinct histological features to confirm that no region of the nerve cross-section had been excluded. The total numbers of myelinated and unmyelinated axons were counted using ImageJ software.

For transmission electron microscopy (TEM), three ultrathin sections (1-nm thickness) from each specimen were prepared on single-slotted copper grids and stained with Reynold's lead citrate and uranyl acetate. Specimens were examined under a Zeiss electron microscope. Micrographs were taken at magnifications of 3000× and 5000×, along with serial photographs of the entire cross-section of all three samples, to look for alteration or disorganization of the myofibrils and mitochondrial matrices, and for electron-dense granules.

Fluorescent Retrograde Axonal Tracing of Facial Nerve. For the labeling procedures, two rabbits were selected. Innervated muscle was exposed, and 45 μl of tracer solution was distributed by injection over five regions within a muscle using a 25-gauge needle. Prior to utilizing the needle for injection, the whole system was filled with normal saline to minimize injection error arising from air compression.

Sequential application of double retrograde labeling using fluorescent retrograde tracers was used to allow an optimal evaluation of pre- and postoperative distribution of myelinated fibers. True Blue (TB; Sigma-Aldrich, USA) as a first tracer was injected into innervated muscle 3 days before nerve injury to label original nerve fiber, and Fluoro-Ruby (FR; Fluorochrome, USA) as a second tracer injected into innervated muscle 3 days after nerve injury to label preserved or regenerated nerve fiber[18,19] at both sides, to compare double-labeled myelinated fibers.

Seven days after second tracer injection, the distal stumps from crushed facial nerve were harvested from both sides. Specimens were fixed overnight in buffered 2% glutaraldehyde, 2% paraformaldehyde, and 0.5% $CaCl_2$, then cryprotected in 15% sucrose in phosphate buffer overnight (both 4°C). The facial nerve was frozen in isopentane cooled with liquid nitrogen. Sections of the facial nerve were cut at 30 μm in the horizontal plane using a cryostat and mounted serially on slides.

Fluorescent-labeled cells in the nerve fiber were identified under a fluorescent microscope (LSM 510; Zeiss) and were evaluated for the presence and extent of fluorescence using a flouroscein isothiocyanate and rhodamine fluorescent filter. The fluorescence intensity of double-labeled nerve fiber of ES side per square millimeter was automatically compared with nerve fiber of the non-ES side using Z-stack imaging analysis (version 3.0; Zeiss).

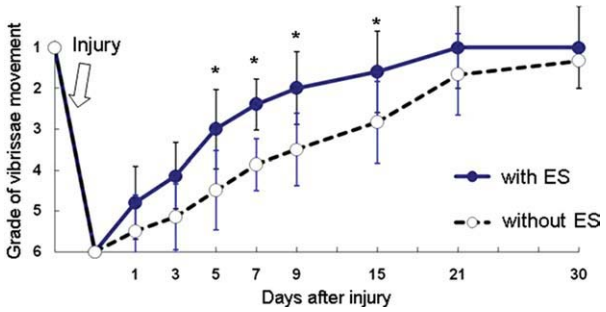

FIGURE 1. Functional recovery of vibrissae movement after a crushing injury. Vibrissae movement was completely paralyzed with the crushing injury and recovered over time. The recovery rate was faster with ES than without ES ($n = 8$ for each group; $^*P < 0.05$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Statistical Analysis. An independent-sample t-test was used to compare facial nerve function and electroneuronography at different time-points. The Kruskal–Wallis analysis of variance (ANOVA) test was used to compare time to complete recovery. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Functional Recovery of Vibrissae Movement after Crushing Injury. On the side without ES, vibrissae movement recovered completely within 31.93 ± 6.48 days after the crushing injury, even when facial function was initially grade 6 (complete facial paralysis). The recovery of vibrissae movement improved continuously following facial nerve injury.

In contrast, the time for complete recovery on the SCLES side was 20.84 ± 4.02 days, significantly shorter than that on the non-ES side [Kruskal–Wallis ANOVA: $H(3) = 10.78$, $P < 0.05$]. The recovery rate of vibrissae movement was highest within 3 days on both sides; the largest difference between the two sides occurred between days 5 and 15 and was statistically significant (independent-sample t-test: $P < 0.05$; Fig. 1). The video presentation shows the difference in functional recovery of vibrissae movement between the two sides at 10 days after injury of facial nerve (see Video 1 in supplementary material).

Electroneurographic Recovery of Compound Action Potential after Crushing Injury. The threshold of the compound action potential was 2.0 ± 0.43 mA before the crushing injury and increased to 6.0 ± 0.7 mA at 1 day after the injury. The threshold on

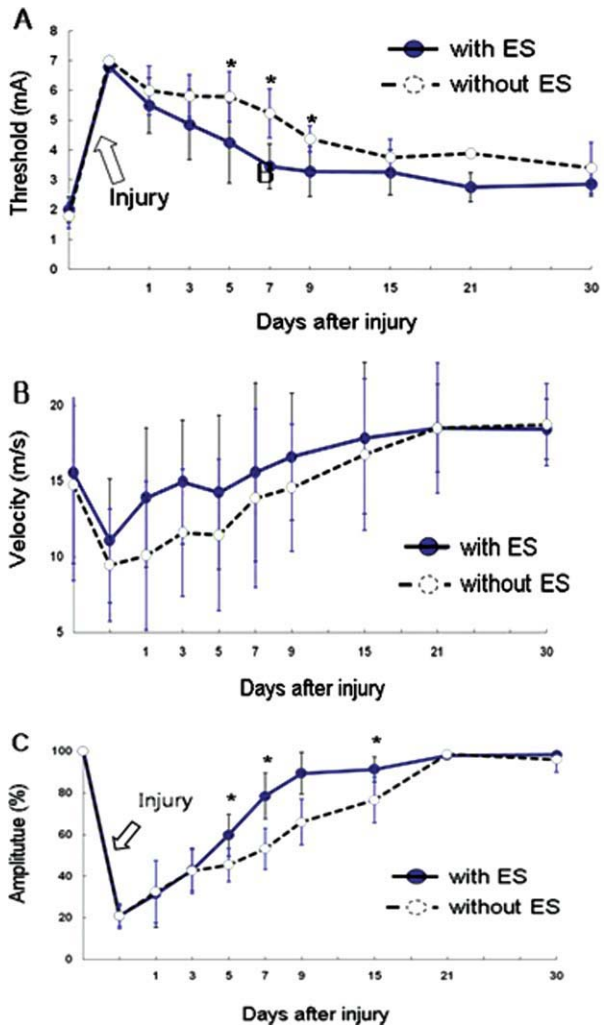

FIGURE 2. Electroneurographic recovery of the compound action potential after a crushing injury. Changes in **(A)** threshold, **(B)** nerve conduction velocity, and **(C)** amplitude after a crush injury of the facial nerve in rabbits ($n = 8$ for each group; $^*P < 0.05$ by independent-sample *t*-test). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the SCLES side then rapidly decreased to 3.45 ± 0.75 mA by 7 days after injury, whereas the threshold recovery time on the non-ES side was significantly longer, with the threshold reaching 3.4 ± 0.8 mA at 30 days after injury (independent-sample *t*-test: $P < 0.05$ for 5, 7, and 9 days after injury; Fig. 2A).

The conduction velocity of the facial nerve was slowest on day 1 after the crushing injury. The largest differences between the two sides were seen on days 2, 3, and 5, and these differences were statistically significant. The velocity recovered completely on both sides within 20 days after injury (Fig. 2B).

The maximal amplitude of the compound action potential was lowest on day 3 after injury, and statistically significant differences in maximum amplitude were seen between the two sides on days 7 and 10 (independent-sample *t*-test: $P < 0.05$ for both). The amplitude on both sides recovered completely within 20 days after injury (Fig. 2C).

Histological Recovery of Facial Nerve and Innervated Muscle. Light microscopy revealed numerous unmyelinated Schwann cells, outgrowing fibroblasts, and amorphous granules in the sample from the unstimulated side at 2 weeks after crushing (Fig. 3A). At the same time, a large number of healthy, myelinated Schwann cells and well-preserved endoneurium were visible in the sample from the stimulated side (Fig. 3B). In five randomized fields of the samples viewed at 200× magnification, there were 324 ± 48 myelinated Schwann cells from the non-ES side and 894 ± 156 myelinated Schwann cells from the ES side.

To assess the effects of ES on the microstructure of the injured facial nerve, the distal stump, injured site, and proximal stump of harvested nerves were examined by TEM. On the side without ES, the nerves showed widespread Wallerian degeneration at the crush-injured site and distal

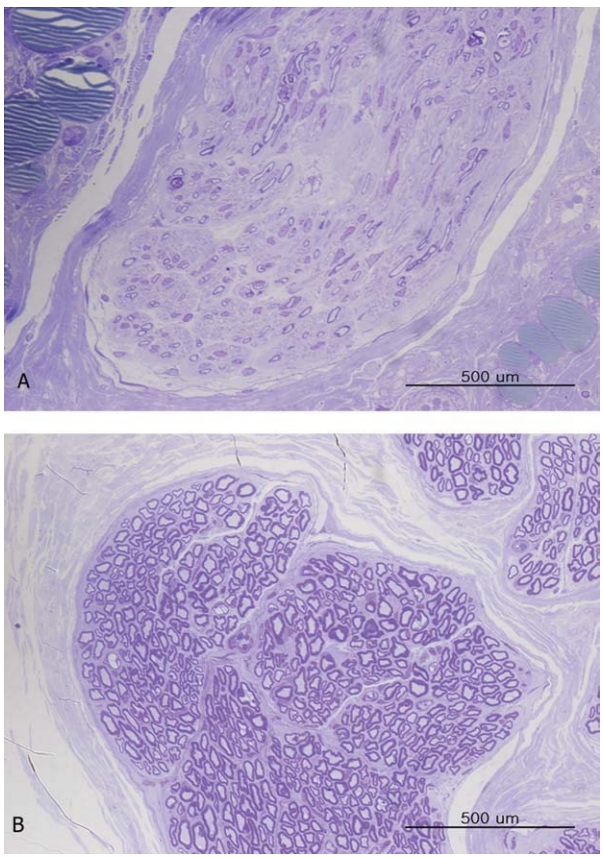

FIGURE 3. Light microscopic findings of the injured facial nerve 2 weeks after a crushing injury (original magnification ×200; toluidine blue stain). **(A)** Unmyelinated Schwann cells and amorphous granules are visible in a sample from the side with no electrical stimulation. **(B)** The myelin sheath is well preserved in a sample from the side with electrical stimulation. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

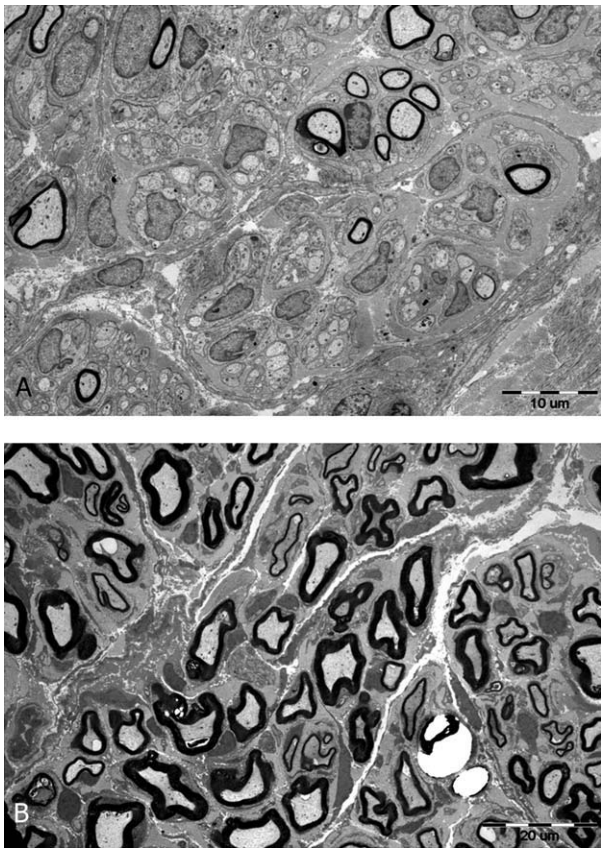

FIGURE 4. TEM images of the facial nerve 2 weeks after a crush injury. Samples from the side without **(A)** and the side with **(B)** electrical stimulation at the neural segment located 5 mm distal from the crush injury site (original magnification ×3000).

stump, as indicated by an increased number of unmyelinated Schwann cells as well as intratubal macrophages with multiple vacuoles that contained myelin debris. Fibroblasts and agranular mononuclear leukocytes were also visible. Some degeneration could be found even at the proximal stump of the harvested nerves (Fig. 4A).

By comparison, the nerves from the ES side exhibited partial or markedly decreased Wallerian degeneration at the injured site and distal stump of the facial nerve, and there were Schwann cells with preserved shape covered by firm myelin sheaths. No Wallerian degeneration was observed at the proximal stump of the nerve, in contrast to that seen on the non-ES side (Fig. 4B).

We also examined the effect of ES on facial muscle morphology. The ultrastructure of the nasalis muscles of the rabbits at 3 weeks after injury showed dramatic differences with ES. The muscle fibers of the nonES side showed alterations in the myofibrils, including focal or widespread disorganization and/or disruption. Mitochondrial matrices were replaced by spaces with amorphous granular material and small, electron-dense granules (Fig. 5A). In contrast, muscle cells from the ES side showed normal Z-band myofibrils, which appeared as distinct structural units with many mitochondria (Fig. 5B).

Double Labeling Nerve Fiber of Distal Stumps from Crushed Facial Nerve. Schwann cells labeled with TB were characterized by their green fluorescence and those labeled with FR were characterized by their red fluorescence.

The double-labeled photographs using TB as a first tracer and FR as a second tracer were used to compare the ES side and non-ES sides. The ES side showed strongly positive TB-labeled and FR-labeled cells. Small differences in the staining intensity in double-labeled nerve fibers were found in the histogram of cross-sectional intensity. The non-ES side had weakly positive TB-labeled and FR-labeled cells compared with the ES side (Fig 6A–F). A wider gap of intensity in double-labeled nerve fibers was found in the histogram of cross-sectional intensity (Fig. 7A, B). The average intensity levels of fluorescent, double-labeled nerve cells were significantly different between the ES and non-ES sides (LSM510 program; Zeiss), as shown in their histograms.

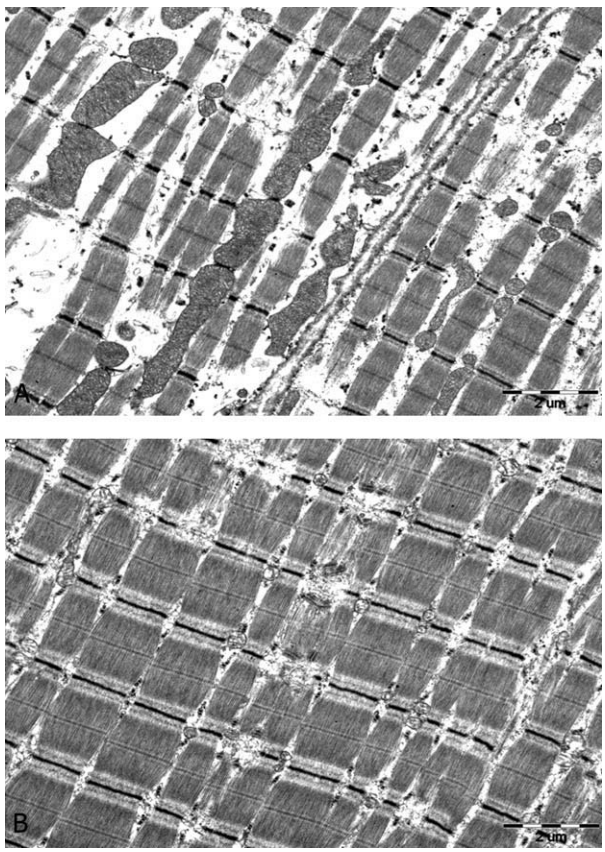

FIGURE 5. TEM images of muscle fibers innervated by the facial nerve 2 weeks after a crush injury. Samples from the side without **(A)** and the side with **(B)** electrical stimulation.

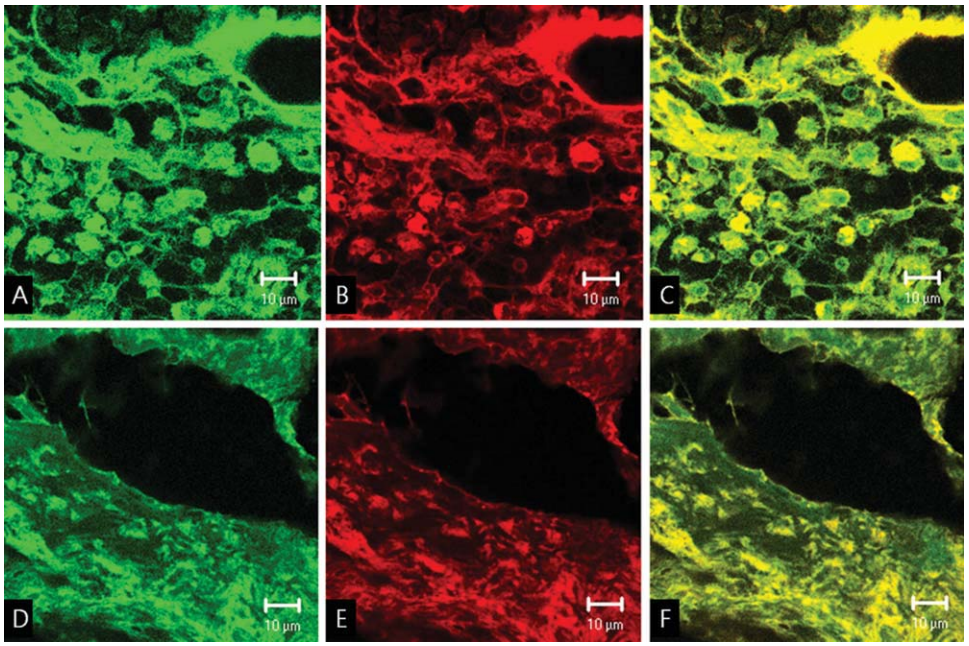

FIGURE 6. Double-labeled nerve fiber of the distal stumps from crushed facial nerve. True Blue as a first tracer **(A, D)**, Fluoro-Ruby as a second tracer **(B, E)**, and double- labeled Schwann cells **(C, F)** shown for the comparison of the ES side **(A–C)** and non-ES side **(D–F)**. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

DISCUSSION

Electrical stimulation is a potential candidate for therapeutic intervention after peripheral nerve injury. Direct ES applied to injured peripheral nerves is known to enhance neuronal regeneration of peripheral motor and sensory nerves. Brief administration of ES at the time of nerve repair promoted fast and accurate motor axonal regeneration of the rat femoral nerve,[11] and ES also restored the specificity of sensory axon regeneration in rats.[12] Lal et al.[14] demonstrated that brief ES (30 min/day) reduced the recovery time for the semi-eyeblink reflex, a marker of early recovery after crushing injury, in rats, although the time for complete recovery of vibrissae movement was not significantly reduced with ES. In this study, we showed that continuous ES (24 h/day, 7 days/week) applied to injured facial nerves accelerated their functional recovery. More specifically, electrophysiological studies demonstrated that the recovery rates of nerve conduction velocity, amplitude, and threshold were faster with ES than without ES, especially during the first 5 days after injury. A histological study also suggested that ES may prevent or reduce Wallerian degeneration following neural damage. Acute facial paralysis, such as Bell's palsy, recovers spontaneously in most cases, but unexpected sequelae remain in some patients. Synkinesis, as a consequence of misdirected neural regeneration, can occur due to severe deterioration of the damaged nerve or prolonged recovery time following facial nerve injury. Shortening the recovery time by applying ES may decrease the incidence of facial sequelae following injury and help with overall recovery of facial function. The difference in histological findings between the ES and non-ES sides is more prominent than that in the electrophysiological findings, as shown in Figures 3 and 4. Histological studies are not always coincident with electrophysiological studies because partially damaged or regenerated nerve fibers can still conduct electrical impulses. As a result, electrophysiological tests can overestimate recovery more than histological findings.

In contrast to prior studies, we chose to use a subthreshold impulse, determined after measuring the minimal amplitude required to evoke a muscle twitch. Although the neuromuscular system as a whole needs suprathreshold ES for activation, the subthreshold impulse focused on the nerve facilitated its regeneration despite the low electrical current. SCLES has the potential to accelerate functional recovery of injured facial nerves, as shown here, and it may be useful clinically without causing discomfort or muscular contraction. Moreover, marked microscopic differences in muscle structure were observed between tissue treated with SCLES versus without ES. This suggests that even subthreshold impulses can affect properties of denervated muscles, possibly enough to prevent synkinesis.

Several mechanisms have been proposed to explain the biological effects of direct ES. Acetylcholine receptors, voltage-dependent calcium channels, and calcium release from intracellular stores have been shown to control electrical effects

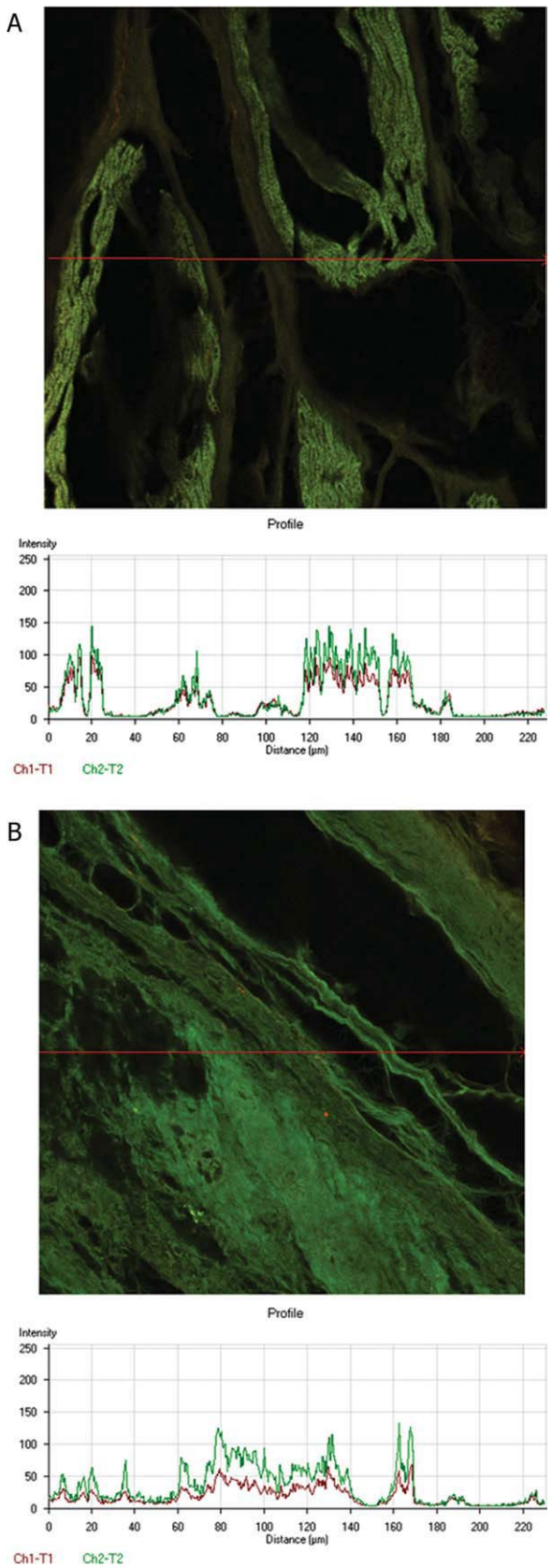

FIGURE 7. Histogram of intensity of fluorescent, double-labeling tracer. The photography showed the gap difference of ES side **(A)** and non-ES side **(B)**. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

in embryonic spinal neurites in *Xenopus laevis*.[6,7] Other possible mechanisms include reduction in the number of astrocytes at the injury site[20] and changes in posttraumatic blood flow.[2,1] Al-Majed et al. reported that ES enhanced the expression of regeneration-associated genes, such as brain-derived neurotrophic factor (BDNF) and GAP-43, to accelerate regeneration in the peripheral nervous system.[2,2] We are currently investigating the mechanism of facial nerve regeneration by ES, and our preliminary data have shown upregulation of BNDF with ES in injured facial nerves.

To determine whether ES accelerated axon outgrowth or the rate of axon regeneration after nerve injury, we evaluated the effect of subthreshold, low-frequency ES on the number of myelinated Schwann cells with well-preserved axons, as determined by electron microscopy, at the injury site and distal nerve stump. We found that this ES helped to preserve the myelination of Schwann cells. Thus, rather than accelerating regeneration or reinnervation of motor nerves, ES appeared to protect myelinated axons from deterioration after injury. It appears to reduce or prevent Wallerian degeneration based on our morphological results. The double labeling of nerve fibers of the distal stumps from crushed facial nerve showed the number of double-labeled axons was higher on the ES side than the non-ES side. These data also support our hypothesis that subthreshold ES of the facial nerve helps preserve myelination of Schwann cells.

Because we stimulated the proximal portion of the injured facial nerve in this study, our model cannot be directly applied to a facial nerve lesion in the temporal bone, such as that in Bell's palsy. However, ES also prevented Wallerian degeneration in the area proximal to the injured site. Accordingly, ES applied to the extratemporal portion of the facial nerve may still be useful for disorders such as Bell's palsy. Another limitation of our study is that a crushing injury leads to incomplete facial palsy, which recovers to some degree with no treatment. We are currently studying the effect of subthreshold, continuous ES in a mouse model of Bell's palsy using viral inoculation.

In conclusion, we found that subthreshold, continuous, low-frequency ES immediately after a crushing injury of the facial nerve resulted in quicker recovery of facial function. These results suggest that continuous subthreshold ES may be a useful treatment modality for acute facial nerve palsy.

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