Brief electrical stimulation accelerates axon regeneration and promotes recovery following nerve transection and repair in mice

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Brief Electrical Stimulation Accelerates Axon Regeneration and Promotes Recovery Following Nerve Transection and Repair in Mice

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Background: Clinical outcomes following nerve injury repair can be inadequate. Pulsed-current electrical stimulation (ES) is a therapeutic method that facilitates functional recovery by accelerating axon regeneration. However, current clinical ES protocols involve the application of ES for 60 minutes during surgery, which can increase operative complexity and time. Shorter ES protocols could be a strategy to facilitate broader clinical adoption. The purpose of the present study was to determine if a 10-minute ES protocol could improve outcomes.

Methods: C57BL/6J mice were randomized to 3 groups: no ES, 10 minutes of ES, and 60 minutes of ES. In all groups, the sciatic nerve was transected and repaired, and, in the latter 2 groups, ES was applied after repair. Postoperatively, changes to gene expression from dorsal root ganglia were measured after 24 hours. The number of motoneurons regenerating axons was determined by retrograde labeling at 7 days. Histomorphological analyses of the nerve were performed at 14 days. Function was evaluated serially with use of behavioral tests up to 56 days postoperatively, and relative muscle weight was evaluated.

Results: Compared with the no-ES group, both ES groups demonstrated increased regeneration-associated gene expression within dorsal root ganglia. The 10-minute and 60-minute ES groups demonstrated accelerated axon regeneration compared with the no-ES group based on increased numbers of labeled motoneurons regenerating axons (mean difference, 202.0 [95% confidence interval (CI), 17.5 to 386.5] and 219.4 [95% CI, 34.9 to 403.9], respectively) and myelinated axon counts (mean difference, 559.3 [95% CI, 241.1 to 877.5] and 339.4 [95% CI, 21.2 to 657.6], respectively). The 10-minute and 60-minute ES groups had improved behavioral recovery, including on grid-walking analysis, compared with the no-ES group (mean difference, 11.9% [95% CI, 3.8% to 20.0%] and 10.9% [95% CI, 2.9% to 19.0%], respectively). There was no difference between the ES groups in measured outcomes.

Conclusions: A 10-minute ES protocol accelerated axon regeneration and facilitated functional recovery.

Clinical Relevance: The brief (10-minute) ES protocol provided similar benefits to the 60-minute protocol in an acute sciatic nerve transection/repair model and merits further studies.

Peripheral nerve injuries are common, affecting 2.6% of patients with upper-extremity trauma and 1.2% of those with lower-extremity trauma. Despite repair of nerve injuries, clinical outcomes can be inadequate. Slow axonal growth can be a cause of inadequate recovery, and, for severe axonotmetic (Sunderland III) or neurotmetic (Sunderland IV and V) nerve injuries, axon outgrowth across a repair site occurs at different rates in a staggered fashion, further delaying regeneration and recovery. Thus, strategies for accelerating axonal regeneration would improve surgical management.

Therapeutic electrical stimulation (ES) protocols, with ES being applied intraoperatively during nerve surgery, are a promising strategy. Animal data have demonstrated that the application of pulsed-current ES for 60 minutes accelerates the number of motor and sensory axons regenerating and reaching their appropriate end-organ targets. ES achieves these effects by augmenting early expression of regeneration-associated genes (RAGs) within neurons, including brain-derived neurotrophic factors (BDNFs). From these promising data, clinical trials involving the use of a 60-minute ES protocol have demonstrated
improved outcomes after nerve surgery from as early as 2010, yet only 3 additional trials have been performed since 2010. A factor that may limit translation of ES is the current utilization of a 60-minute stimulation period. Care providers will find a lengthy ES protocol during surgery to be an obstacle as it can increase operative time, which negatively affects costs and complication risks, or necessitate more complex recovery procedures to implement ES protocols. Shorter ES protocols, therefore, could increase translation.

In the present study, we assessed whether a shorter ES protocol could promote improved nerve regeneration and recovery in the context of a repaired traumatic nerve injury. We hypothesized that a shorter ES protocol could improve nerve regeneration and functional recovery and tested our hypothesis with use of a mouse sciatic nerve transection and repair model.

Materials and Methods

Experimental Design

All experiments were approved by the Washington University in St. Louis Institutional Animal Care and Use Committee and performed in compliance with the National Institutes of Health guidelines. Ninety-nine male C57BL/6J mice (age, 7 weeks; weight, 20 to 25 g) (Jackson Laboratories) were used to evaluate the effects of ES on nerve regeneration (Fig. 1), 20 mice were used to determine an evaluation end point for axon regeneration, and 5 mice were used as controls for gene analysis. For the primary experiments, mice were randomized to 3 groups: (1) no ES, (2) 10 minutes of ES, and (3) 60 minutes of ES. Ten minutes was chosen as the lower limit for ES based on previous studies. Mice were monitored daily in a temperature-controlled central animal facility with free access to chow and water.

Previous studies assessing the effects of electrical stimulation on nerve regeneration after repair showed that the primary effect was to accelerate axon regeneration, which led to functional recovery. Therefore, we designed the study with specific end points representing early and still-ongoing axon regeneration in order to determine whether the application of ES accelerated axon regeneration compared with that after repair alone. Gene analysis was performed on dorsal root ganglia at 24 hours, as neuron bodies are affected by ES within the first 48 hours. As this particular nerve injury and repair model was unlikely to show differences between groups regarding histological findings at late end points, axon regeneration was measured at early end points as it could detect differences in rates of regeneration. Functional recovery alone was measured to a longer end point as this longer end point would still capture whether accelerated axon regeneration led to differences in recovery.

Surgical Procedures

Mice were anesthetized with a cocktail of ketamine (100 mg/kg) (Fort Dodge Animal Health) and dexmedetomidine (0.5 mg/kg) (Pfizer Animal Health). The right sciatic nerve was exposed under sterile conditions, transected 2 mm proximal to the sciatic trifurcation, and repaired immediately with 11-0 nylon epineural

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Fig. 1
Schematic illustrating the experimental design and the allocation of animals for the various outcome measures, with end points indicated for each metric.

Fig. 2
Photograph made during the experimental procedure. The sciatic nerve was transected and repaired 2 mm proximal to the sciatic trifurcation. ES was applied after repair for 10 or 60 minutes at a location 2 mm proximal to the repair site, with a hook electrode being used to secure the nerve and a return electrode placed proximal to the injury in the fascia.
sutures (Sharpoint). For applicable groups, ES was performed as described previously. A stainless-steel wire electrode (Component Supply Company) was hooked around the sciatic nerve 2 mm proximal to the repair site. A return electrode was placed into subcutaneous tissue. The electrodes were connected to the ES device (Checkpoint Stimulator/Locator; Checkpoint Surgical). ES was delivered for 10 or 60 minutes (16 Hz, 0.5 mA) (Fig. 2). After ES, the electrodes were removed and the fascia and skin were closed with 6-0 nylon sutures (Ethicon). Postoperative pain was managed with buprenorphine SR (sustained-release) (0.05 mg/kg) (ZooPharm). At terminal end points, mice were killed with administration of sodium pentobarbital (>200 mg/kg).

**Gene Analysis (qRT-PCR) of Dorsal Root Ganglia**

Gene expression from dorsal root ganglia was assessed 24 hours postoperatively; this end point was chosen on the basis of previous studies. Total RNA was prepared from combined L3 and L4 dorsal root ganglia explants from each animal. RNA was extracted, measured, and reverse-transcribed to cDNA as described previously. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed with use of a StepOnePlus thermocycler (Applied Biosystems) using Taqman Master Mix (Applied Biosystems) reagents with the following primer pairs: Bdnf (Mm04230607_s1), Atf3 (Mm00476033_m1), Il6 (Mm00446190_m1), Csf1 (Mm00432684_m1), Ccl2 (Mm00441242_m1), and Actb (Mm02619580_g1) (Thermo Fisher Scientific). Genetic expression levels were normalized to Actb. Relative gene expression changes were calculated from gene expression levels obtained from dorsal root ganglia of uninjured mice.

**Retrograde Labeling of Motoneurons**

Retrograde labeling of motoneurons regenerating their axons was performed on anesthetized mice in which the sciatic nerve was re-explored and transected 3 mm distal to the original repair site. The proximal cut end was placed in a well of petroleum jelly (Vaseline; Unilever) filled with 4% Fluoro-Gold (Sigma-Aldrich). After 45 minutes, the well and Fluoro-Gold were removed, and the wounds were closed. One week later, spinal cords were harvested and fixed in 4% paraformaldehyde in phosphate-buffered saline solution (PBS) overnight, followed by immersion in 30% sucrose in PBS for 24 hours. Tissues were frozen in OCT Compound (VWR) and cut into 30-μm longitudinal sections on glass slides. The total number of Fluoro-Gold-labeled cell bodies on every section was counted with use of an Olympus IX81 microscope (Olympus).

**Histomorphological Analysis**

Histomorphological analyses of the sciatic nerve 3 mm distal to the repair site were performed 14 days postoperatively, as described previously. Briefly, harvested sciatic nerves were fixed in 3% glutaraldehyde, post-fixed in 1% osmium tetroxide, dehydrated,
embedded in epoxy resin, and sectioned on an ultramicrotome to yield 1-μm cross-sections. Slides were counterstained with 1% toluidine blue and analyzed at 1,000× with a Laborlux S microscope (Leitz). Slides were assessed with use of custom histomorphometry software (Clemex Vision Professional; Clemex Technologies). Histological measures were calculated with use of >75% of the cross-sectional area of the nerve.

**Grid-Walking Analysis**

A grid-walking test was performed twice per week from preoperatively (Day 0) to 56 days postoperatively. Mice were acclimated to an elevated wire mesh with a grid size measuring 2.5 × 2.5 cm for 5 minutes before recording of the total number of steps of the ipsilateral hindlimb for 3 minutes. The number of total steps and slipped steps missing the mesh and going...
through the grid were counted, and the proportion of foot faults was reported.

**Mechanical and Cold Sensitivity Analyses**
Mechanical and cold sensitivity analyses were performed at a 56-day end point. Mice were acclimated to an elevated meshed metal grid for 1 hour before measuring paw-withdrawal thresholds with use of von Frey filaments (0.02 to 2.56 g) (TouchTest; North Coast Medical), where diminished sensation was indicated by an ipsilateral:contralateral ratio of <1.26,32. Cold sensitivity was assessed by means of acetone-evoked evaporative cooling27. A drop of acetone was released onto the plantar surface of the hindlimb, and the time of response was measured.

**Muscle Wet Weight**
The tibialis anterior and gastrocnemius muscles were dissected and weighed 56 days postoperatively. The ipsilateral:contralateral ratio was calculated.

**Statistical Analysis**
The primary outcome during experimental planning was myelinated axon counts26. A power analysis (alpha = 0.05, power = 0.8, effect size = 0.86) was performed considering the effect of ES on regenerated myelinated axon counts27 with use of G*power 3.1.9.7 (Heinrich Heine University). Five mice per group were required to detect significant differences. Therefore, the group size for each experiment was at least 5 mice.

For comparisons of retrograde labeling, histomorphometry, sensitivity tests, and muscle wet weight, 1-way analysis of variance (ANOVA) followed by Tukey-Kramer post hoc testing was used. In the grid-walking analysis, following 2-way repeated-measures ANOVA, Tukey-Kramer post hoc testing was used for multiple comparisons between groups at each time point. All statistical analyses were performed with use of JMP software (version 13; SAS Institute). The level of significance was set at p < 0.05. The data in the figures are expressed as the mean and the standard deviation (SD), whereas the data in the Results section are expressed as the mean difference and the 95% confidence interval (CI).

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**Results**

**Gene Analysis of Dorsal Root Ganglia**
Dorsal root ganglia harvested at 24 hours from all 3 groups revealed that nerve injury elevated the expression of regeneration-associated genes, *Bdnf* and *Atf3*, as well as inflammatory genes, *Il6*, *Csf1*, and *Ccl2* (Fig. 3). However, both the

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**TABLE I Histomorphometric Data of Distal Repaired Sciatic Nerves at 14 Days Postoperatively**

<table>
<thead>
<tr>
<th>Outcome Metric</th>
<th>Uninjured</th>
<th>No ES</th>
<th>10-Minute ES</th>
<th>60-Minute ES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myelinated axon count (no.)</td>
<td>4,521 ± 468</td>
<td>496 ± 218</td>
<td>1,055 ± 378§</td>
<td>835 ± 239†</td>
</tr>
<tr>
<td>Myelinated axon density (axons/mm²)</td>
<td>29,822 ± 2,685</td>
<td>3,133 ± 1,377</td>
<td>6,344 ± 3,024†</td>
<td>5,531 ± 2,029</td>
</tr>
<tr>
<td>Percent nerve area (%)</td>
<td>72.14 ± 2.04</td>
<td>3.22 ± 1.62</td>
<td>7.13 ± 3.65†</td>
<td>5.15 ± 1.89</td>
</tr>
<tr>
<td>Fiber width (µm)</td>
<td>4.78 ± 0.24</td>
<td>3.21 ± 0.41</td>
<td>3.32 ± 0.14</td>
<td>3.02 ± 0.22</td>
</tr>
<tr>
<td>G-ratio (Ratio)</td>
<td>0.60 ± 0.02</td>
<td>0.51 ± 0.06</td>
<td>0.49 ± 0.03</td>
<td>0.54 ± 0.03</td>
</tr>
</tbody>
</table>

*All data are expressed as the mean and the standard deviation (n = 10 for each group). †p < 0.05, †p < 0.01, §p < 0.001 compared with the no-ES group (1-way ANOVA followed by a post hoc Tukey-Kramer test).
10-minute-ES and 60-minute-ES groups revealed significantly elevated expression of Bdnf (mean difference, 1.04 [95% CI, 0.44 to 1.64] and 1.54 [95% CI, 0.94 to 2.13], respectively), Atf3 (mean difference, 22.9 [95% CI, 3.6 to 42.1] and 32.3 [95% CI, 13.0 to 51.6], respectively), and Il6 (mean difference, 13.4 [95% CI, 4.5 to 22.2] and 11.1 [95% CI, 2.3 to 19.9], respectively), in comparison with the no-ES group. Expression levels of Csf1 in the 60-minute-ES group (mean difference, 1.78 [95% CI, 0.83 to 2.72]) and Ccl2 in the 10-minute-ES group (mean difference, 2.38 [95% CI, 0.47 to 4.28]) were significantly higher than that in the no-ES group. Gene expression levels were not significantly different between the 10-minute-ES and 60-minute-ES groups.

**Motor Nerve Regeneration**

Retrograde labeling of motoneurons regenerating their axons from the repair site without ES revealed staggered axon regeneration. There were significantly greater numbers of labeled motoneurons 14 days compared with 7 days postoperatively (mean difference, 355.4 [95% CI, 217.7 to 493.1]) (Figs. 4-A, 4-B, and 4-C), but there were no differences after 14 days. Based on these results, we assessed the number of motoneurons regenerating axons in the ES groups at 7 days postoperatively. The numbers of labeled motoneurons in the 10-minute-ES and 60-minute-ES groups were significantly greater than those in the no-ES group (mean difference, 202.0 [95% CI, 17.5 to 386.5] and 219.4 [95% CI, 34.9 to 403.9], respectively). No significant difference was observed between the 10-minute-ES and 60-minute-ES groups (Figs. 4-D and 4-E).

**Histomorphological Analysis**

Histomorphological analyses of the sciatic nerve 3 mm distal to the repair site at 14 days revealed ongoing axon regeneration, as normal sciatic nerve contains ~4,500 myelinated axons. Myelinated axon regeneration in the ES groups was increased compared with the no-ES group (Fig. 5, Table I). The 10-minute-ES and 60-minute-ES groups had significantly greater myelinated axon counts than the no-ES group (mean difference, 559.3 [95% CI, 241.1 to 877.5] and 339.4 [95% CI, 21.2 to 657.6], respectively). Myelinated axon density and percent nerve area in the 10-minute-ES group were significantly higher than those in the no-ES group (mean difference, 3,211.3 axons/mm² [95% CI, 718.7 to 5,703.9 axons/mm²] and 3.91% [95% CI, 1.08% to 6.74%], respectively). No significant differences were observed among the 3 groups in terms of fiber width and g-ratio.

**Figs. 6-A through 6-D** Illustrations showing that ES improved motor recovery. Foot faults represent steps that miss the grid rungs and enter the grid pattern. Preoperative values are represented at Day 0. **Fig. 6-A** Schematic of the grid-walking test, which involves motor coordination as well as proprioceptive and other sensory feedback. **Figs. 6-B, 6-C, and 6-D** Quantification of foot faults (proportion of foot fault steps to total steps for the affected limb). All data are expressed as the mean and the standard deviation (n = 10 per group). **Fig. 6-B** †P < 0.05 between Day 7 and all time points from Day 28 onward in the no-ES group. P < 0.05 between Day 7 and all time points from Day 17 onward in the 10-minute-ES group. ‡P < 0.05 between Day 7 and all time points from Day 17 onward in the 60-minute-ES group. #P < 0.05 between the no-ES group and both ES groups from Day 17 onward. The arrows indicate that significant differences continued until 56 days postoperatively. **Figs. 6-C and 6-D** Bar graphs showing the quantification of foot faults in the no-ES and ES groups at Days 17 and 56, respectively. *P < 0.05. **P < 0.01. **P < 0.01.

**Figs. 7-A and 7-B** Bar graphs illustrating that ES improved mechanical sensitivity but not cold sensitivity. All data are expressed as the mean and the standard deviation (n = 5 per group). **Fig. 7-A** Mechanical sensitivity of the affected foot based on von Frey monofilament tests at 56 days postoperatively. In uninjured nerve (dashed line), there is no ipsilateral/contralateral difference in the paw-withdrawal threshold ratio (ratio = 1). **Fig. 7-B** Cold allodynia responses to the application of cold acetone to the affected foot, measured at 56 days postoperatively. Mean data for the uninjured (contralateral) nerve (n = 15) are represented by the dashed line.
The relative gastrocnemius muscle weight was significantly greater for the 10-minute-ES and 60-minute-ES groups as compared with the no-ES group (mean difference, 0.08 [95% CI, 0.01 to 0.16] and 0.10, [95% CI, 0.03 to 0.18]). Similarly, the relative tibialis anterior muscle weight was significantly greater for the 10-minute-ES and 60-minute-ES groups as compared with the no-ES group (mean difference, 0.16 [95% CI, 0.07 to 0.24] and 0.14 [95% CI, 0.05 to 0.22] respectively) (Fig. 8).

**Discussion**

The present study demonstrated that both the 10-minute and 60-minute ES protocols accelerated axon regeneration, thereby promoting improved functional recovery, compared with that in the no-ES group. Furthermore, there were no obvious differences between the ES groups in any measured outcome.

To our knowledge, only 4 clinical trials have investigated the use of ES as an intervention in nerve surgery since 2010, all of which utilized a 60-minute ES protocol\(^\text{14-17}\) , which was reviewed in detail by Ransom et al\(^\text{17}\). Those results suggested a slow adoption of ES in the clinic, which could be improved by simplifying the application of ES to the treatment of nerve injuries. The present study is the first to demonstrate that ES protocols of <60 minutes can be effective for promoting regeneration and recovery\(^\text{22-24,26}\). A 10-minute ES protocol involving the use of direct current has been previously shown to promote improved axon regeneration in vitro and in vivo\(^\text{22-24}\). The present study was different from those studies in that we considered a 10-minute pulsed-current ES protocol using a clinically available nerve stimulator and compared the results with the more common 60-minute ES protocol.

The findings observed in association with the 10-minute ES protocol are supported by previous literature\(^\text{6-10}\). Pulsed-current ES protocols applied for 60 minutes increase regeneration-associated gene expression earlier than that after injury alone\(^\text{11}\) and accelerate the rate at which axons cross a nerve repair site\(^\text{6-7}\), similar to our observations. These outcomes have been associated with improved functional recovery\(^\text{6-7}\). Therefore, our findings suggest that ES facilitating earlier axon regeneration results in axons reaching end-organ targets earlier and thereby improves functional recovery.

The mechanism promoting the regenerative effects for the 10-minute ES protocol is unclear. The present study suggests that regeneration was improved via a similar mechanism as that associated with 60-minute ES, with ES-generated action potentials leading to increased expression of growth-associated genes and, in turn, accelerated axon regeneration\(^\text{11,13,36}\). While this direct connection cannot yet be proven, studies applying optogenetics have offered insights. In optogenetic studies, the number of pulses generating action potentials, rather than duration, was the predominant cue for promoting axon growth from neurons\(^\text{37}\). Optical stimulation of neurons generating far fewer pulses than 1 hour at 20 Hz still resulted in increased axon growth in vitro compared with no pulses\(^\text{37}\). Therefore, a...
10-minute ES protocol may stimulate sufficient evoked-action potentials to elicit neurons to enhance axon growth.

Our results also suggest that ES could involve non-neuronal cells that may play a role in nerve regeneration, as inflammatory genes became elevated relative to the no-ES group. ES therapies are associated with an increase in macrophage accumulation and increased macrophage accumulation and the responses of the macrophages can promote nerve regeneration. However, the current study is limited in terms of our ability to draw conclusions as the histomorphometry techniques that were utilized to describe nerve regeneration could not quantify inflammatory cells. In addition, the assessors of the histomorphometry were not blinded to treatment group, representing a potential source of detection bias.

The present study had several additional limitations. Only male sex was considered, and future studies will need to consider any sex effects due to ES. Additionally, the animal injury and repair model represents a “best-case scenario” in terms of regeneration as nerve transaction followed by immediate repair and then application of ES is ideal. Future studies would benefit from considering clinical scenarios, such as blunt trauma, which may result in a larger zone of injury or delayed repair to determine if this ES protocol could still provide therapeutic benefit.

In conclusion, a brief 10-minute ES protocol promoted nerve regeneration and functional recovery, similar to a 60-minute ES protocol. Brief (10-minute) ES protocols merit further studies.

References


