

Article

Functional and Morphological Outcomes of Duration-Dependent Electrical Stimulation in Silicone Conduit-Mediated Peripheral Nerve Repair in Rats

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Abstract

Peripheral nerve regeneration is most rapid during the early post-injury period but gradually slows over time, often limiting functional recovery. Electrical stimulation (ES) delivered via percutaneous needle electrodes has been shown to modulate the local neural microenvironment and promote axonal regeneration; however, the optimal temporal window and duration of stimulation remain unclear. This study aimed to evaluate the time-dependent effects of needle-based ES on peripheral nerve regeneration in a rat model of sciatic nerve transection, using a well-established silicone nerve conduit as a stable and reproducible non-biodegradable repair model. Female Sprague–Dawley rats underwent sciatic nerve transection and repair. Postoperatively (PO), animals were randomly assigned to control (C) needle insertion or needle-based ES groups, receiving stimulation for either 3 weeks (C-3W-PO and ES-3W-PO, respectively) or 7 weeks (C-7W-PO and ES-7W-PO, respectively). Functional recovery was evaluated using cold plate latency and rotarod performance tests. Electrophysiological assessments included measurements of nerve conduction velocity (NCV), compound muscle action potential amplitude, and muscle action potential (MAP) area. Histomorphometric analysis of regenerated nerve tissue quantified total nerve cross-sectional area, endoneurial space, axon number, and axon density. Retrograde labeling with fluoro-gold (FG) was used to quantify reinnervated motor neurons. Immunohistochemical analyses of calcitonin gene-related peptide (CGRP) and macrophage-associated markers were conducted to assess sensory neuropeptide expression and immune cell infiltration within the regenerated nerve. ES significantly improved both sensory and motor recovery in a duration-dependent manner. Behavioral data showed increased cold pain thresholds and improved motor coordination in ES groups, with the most pronounced functional gains observed in the ES-7W-PO group. Electrophysiological measures revealed higher NCV, amplitude, and MAP area in ES-treated animals, with the most pronounced improvements at 7 weeks. Morphologically, ES enhanced nerve regeneration, as evidenced by increased total and endoneurial areas, axonal counts, and axon density. FG-labeled neuron counts were significantly elevated in ES groups, indicating enhanced motor reinnervation. At



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3 weeks, ES induced higher CGRP expression and macrophage density, suggesting transient activation of sensory-associated and pro-regenerative immune responses during the early post-injury phase. These findings demonstrate that ES accelerates peripheral nerve repair in rats and that sustained stimulation across the early regenerative window yields superior structural and functional outcomes.

Keywords: inflammation; electrical stimulation; peripheral nerve injury; regeneration; silicone rubber conduit; time duration

1. Introduction

Peripheral nerve injury presents a significant clinical challenge due to its profound impact on both motor and sensory function [1,2]. Although the peripheral nervous system has an intrinsic capacity for regeneration, the repair process is slow and often incomplete, particularly in cases involving large nerve gaps. Electrical stimulation (ES) has emerged as a promising strategy to enhance peripheral nerve regeneration by promoting neuronal activity, guiding axonal growth, and accelerating functional recovery [3,4]. However, the optimal timing for applying ES to achieve maximal therapeutic benefit remains unclear.

In preclinical models, such as rats with a 1 cm sciatic nerve gap, the timing of ES plays a critical role in its efficacy. Peripheral nerve regeneration is a staged process involving initial inflammation, axonal sprouting, and remyelination. Each of these phases may respond differently to electrical cues [5,6], making it essential to identify a therapeutic window that maximizes repair while minimizing adverse effects such as fibrosis or axonal misdirection [7].

Accumulating evidence suggests that the biological effects of ES on peripheral nerve regeneration are highly dependent on the timing and duration of stimulation [8]. Several experimental and clinical studies have demonstrated that brief or early-phase ES, applied immediately or shortly after nerve injury or repair, can enhance axonal sprouting, accelerate target reinnervation, and improve functional recovery by promoting neurotrophic factor expression and activity-dependent signaling. In contrast, prolonged or chronic ES applied during later stages of regeneration has been reported to produce variable or diminishing benefits, potentially due to saturation of activity-dependent pathways, altered inflammatory responses, or maladaptive remodeling. Despite these observations, the optimal temporal window and duration of ES remain incompletely defined, and direct comparisons between short-term and extended stimulation paradigms within the same experimental framework are limited. This unresolved issue highlights a critical knowledge gap regarding how ES duration influences regenerative efficacy across different stages of the nerve repair process.

Previous studies have shown that the regenerative outcome is influenced by various stimulation parameters, including frequency, intensity, duration, and the specific phase of nerve healing during which stimulation is applied [9,10]. While some evidence supports the benefits of early stimulation during the inflammatory phase, others highlight the potential of delayed stimulation during remyelination to enhance recovery [11,12]. Nonetheless, the precise temporal window that yields optimal regenerative outcomes remains to be determined.

In the present study, a silicone nerve conduit was selected as a well-established and biologically inert guidance model. Silicone conduits provide mechanical stability and consistent lumen geometry without introducing confounding bioactive or degradable material effects. This design choice enabled systematic evaluation of the temporal effects of

ES on nerve regeneration under controlled and reproducible conditions, independent of material-driven biological cues [13].

To address this knowledge gap, the present study examines the effects of invasive, needle-based ES on peripheral nerve regeneration in a rat model of sciatic nerve transection, where the nerve is repaired using a silicone nerve conduit. By comparing regenerative outcomes following short-term (3 weeks) versus extended (7 weeks) stimulation protocols initiated one week postoperatively (PO), this study aims to elucidate the temporal dynamics of ES-mediated nerve repair and to identify the optimal therapeutic time window for enhancing functional and structural recovery after peripheral nerve injury.

2. Materials and Methods

2.1. Ethical Statement

This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Academies Press, Washington, DC, USA). All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of China Medical University, Taichung, Taiwan (Approval Number: CMUIACUC-2024-241, Date: 2 April 2024).

2.2. Experimental Design and Surgical Procedure

Female Sprague–Dawley rats were anesthetized, and the right sciatic nerve was transected to create proximal and distal nerve stumps. Both nerve ends were secured into a silicone rubber conduit. The conduit used was a Helix Medical Silicone Tube (Helix Medical, Inc., Carpinteria, CA, USA) with a length of 12 mm, an inner diameter of 1.47 mm, and an outer diameter of 1.96 mm. The silicone conduit used in this study was a commercially available, standardized nerve guidance conduit with fixed inner diameter and wall thickness, and was therefore not considered an experimental variable in the present study. The sciatic nerve was completely transected using microsurgical scissors. Both proximal and distal nerve stumps were inserted approximately 1 mm into a 12 mm silicone conduit and secured with 9-0 nylon epineurial sutures, creating an inter-stump gap of approximately 10 mm within the conduit (Figure 1). This transection model was selected to provide a stringent regenerative environment, in which axonal regrowth depends on guidance through the conduit rather than preserved endoneurial architecture. The suture was then tied with a single knot outside the conduit to secure the nerve in place. After surgery, the rats' body weights were recorded, and they were returned to their cages. Body temperature was maintained with a heat lamp until the rat recovered. Postoperative care included administration of an analgesic and Pamoxicillin® (amoxicillin trihydrate, 1.5 g/60 mL) dissolved in drinking water and provided ad libitum to minimize pain and prevent infection. At experimental endpoints, animals were deeply anesthetized and euthanized by transcardial perfusion with saline followed by paraformaldehyde, in accordance with institutional IACUC and AVMA guidelines.

Animals were randomly assigned to four experimental groups ($n = 10$ per group): C-3W-PO and C-7W-PO, which received control (C) needle insertion without ES for 3 and 7 weeks, respectively; and ES-3W-PO and ES-7W-PO, which received needle-based ES for the corresponding durations. All interventions were initiated 7 days after implantation of the sciatic nerve conduit. Accordingly, stimulation was terminated at postoperative week 4 in the 3-week groups and at postoperative week 8 in the 7-week groups.

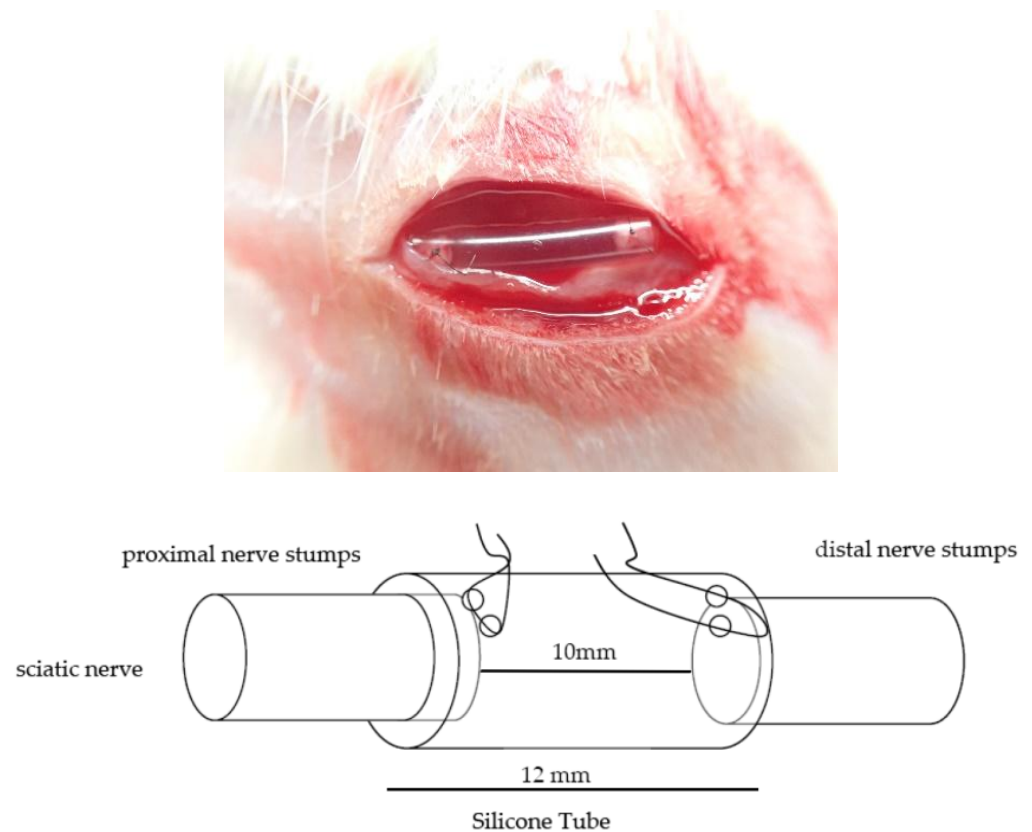


Figure 1. The picture and diagrams to scale of the implanted material in the animal.

2.3. Needle-Based ES Protocol

One week after surgery, sterile disposable stainless-steel needle electrodes (28G, 12 mm length, 0.35 mm diameter; Chian Huei, Taiwan) were percutaneously inserted into the posterolateral hip region and the lateral aspect of the knee region on the operated limb, corresponding to the proximal and distal segments of the sciatic nerve pathway. Needles were inserted to a depth of approximately 0.5–1.0 cm.

For ES, a Trio 300 stimulator (Ito Co., Tokyo, Japan) was used in constant-current output mode. The positive and negative leads were connected to the proximal and distal needle electrodes, respectively (Figure 2). Continuous square-wave stimulation was delivered at a frequency of 2 Hz and an intensity of 1 mA, adjusted to elicit mild visible muscle contraction without causing discomfort. Each stimulation session lasted 15 min and was administered three times per week. ES was administered three times per week on fixed, alternate days (Monday, Wednesday, and Friday) throughout the designated treatment period.

The ES parameters (2 Hz, 1 mA) were selected based on prior studies demonstrating that low-frequency, low-intensity stimulation enhances peripheral nerve regeneration by modulating Schwann cell activity, promoting neurotrophic factor release, and facilitating axonal elongation while avoiding overstimulation-induced tissue damage. Although a wide range of ES regimens has been reported in the literature, this protocol represents a well-established and physiologically relevant approach for conduit-based sciatic nerve repair. Future studies may further optimize stimulation parameters to maximize specific regenerative outcomes.

Following completion of the assigned stimulation duration, functional, electrophysiological, histomorphometric, and immunohistochemical assessments were conducted as described below (Figure 3).

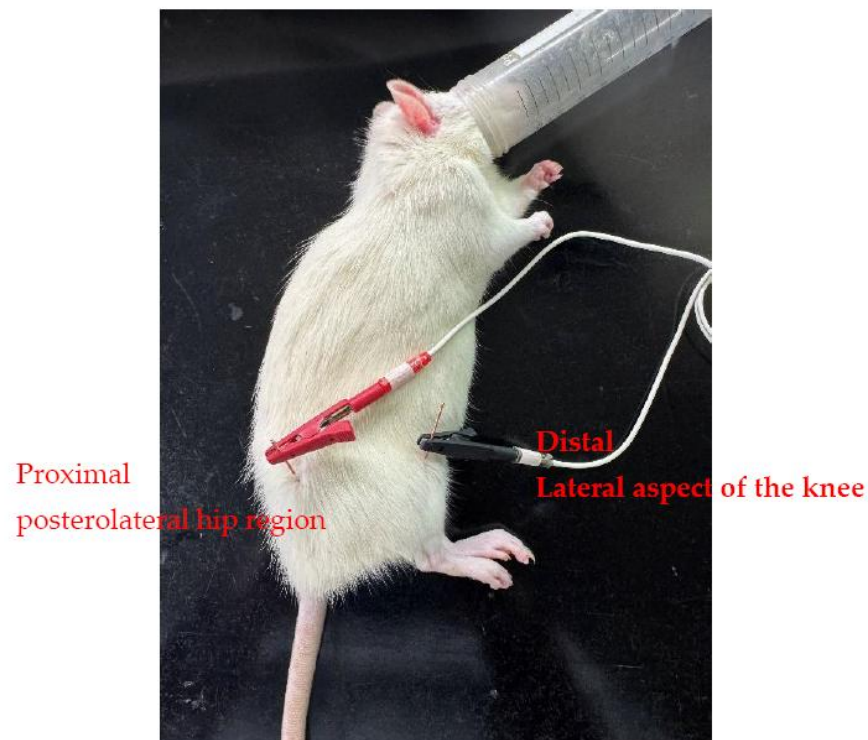


Figure 2. The diagrams to scale of electrode positions.

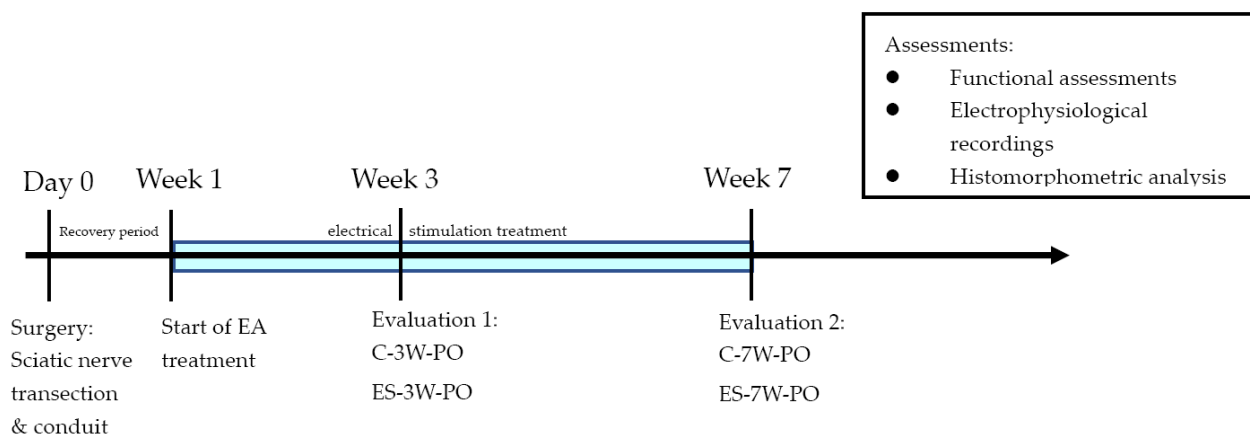


Figure 3. A schematic illustration summarizing the animal model and experimental protocol.

2.4. Thermal Hyperalgesia

Thermal nociceptive sensitivity was evaluated using a Hargreaves analgesia meter (IITC Life Sciences, SERIES8, Model 390G, Woodland Hills, CA, USA) to measure heat-induced pain responses. A focused radiant heat source (40 °C) located beneath a glass floor was directed at the plantar surface of the rat's hind paw. The withdrawal latency, i.e., the time taken for the rat to withdraw its paw, was measured using a stopwatch. A 30 s cut-off time was set to prevent tissue damage. Withdrawal latency values were compared among experimental groups.

2.5. Cold Allodynia

Cold sensitivity was assessed using a hot/cold plate system (Panlab, Harvard Apparatus, Holliston, MA, USA). The cold plate was pre-cooled to 4 °C, and the rat was placed inside an acrylic chamber on the plate. The number of hind paw withdrawals or lifts within

a 3 min test period was recorded, along with the latency to the first paw lift, representing the cold response threshold.

2.6. Motor Coordination Test

Motor coordination was evaluated using an accelerating rotarod device (Rotamex Rotarod, Columbus Instruments, Columbus, OH, USA). Rats were placed on a rotating rod that started at 4 rpm, with the speed increasing by 2.5 rpm every 10 s. The time each rat remained on the rod and the maximum speed tolerated before falling were recorded as indices of motor coordination and balance.

2.7. Electrophysiological Assessment

Following behavioral tests, compound muscle action potentials (CMAPs) were recorded using a Biopac Systems, Inc. (Goleta, CA, USA) evoked potential system. The recording electrodes (positive and negative) were inserted into the tendon and belly of the tibialis anterior muscle at the distal end of the nerve conduit. The stimulating electrode was placed at the proximal end of the nerve conduit. Upon ES, the regenerated nerve transmitted signals to the muscle, and the resulting CMAP waveforms were recorded. The following parameters were analyzed, including MAP area, nerve conduction velocity (NCV), amplitude, and latency.

2.8. Muscle Weight Measurement

After euthanasia, the gastrocnemius muscles were excised from both the operated (right) and contralateral (left) hind limbs. After removing blood and connective tissue, the wet weights of the muscles were measured. The muscle weight ratio was calculated by dividing the weight of the right (experimental) gastrocnemius by that of the left (control) muscle.

2.9. Fluoro-Gold Injection and Retrograde Tracing Procedure

A Hamilton micro-syringe (Hamilton Company, Reno, NV, USA) was used to inject fluoro-gold solution (FG, Fluorochrome, Denver, CO, USA) into the common peroneal and tibial nerves. Five days later, animals were perfused transcardially with saline, followed by paraformaldehyde. The ipsilateral L4 and L5 dorsal root ganglia (DRG) were harvested. Frozen sections of the spinal cord and DRG were examined under a fluorescence microscope (Olympus CKX41, Center Valley, PA, USA) to assess retrograde labeling.

2.10. Evaluation of Regenerated Nerve Tissue

After euthanasia, the nerve conduit, along with the regenerated nerve tissue, was harvested and initially fixed in 2.5% glutaraldehyde (GA) for 24 h. The middle third of the regenerated nerve within the conduit was then isolated and post-fixed in 2.5% GA for 1–2 days. Subsequently, the tissue was treated with 1% osmium tetroxide for 1 h, dehydrated through a graded ethanol series (70–100%), and embedded in resin. The resin blocks containing the nerve tissue were hardened in a 60–70 °C oven for approximately 48 h. Semi-thin cross-sections (1 µm) were cut and stained with Toluidine Blue, which clearly highlights myelin sheaths and Schwann cells. Digital photomicrographs were taken using a microscope equipped with a digital camera at magnifications of 100× and 400×. Quantitative analysis of immunoreactive cells was performed using Optical Imaging software on the 100× images.

2.11. Immunohistochemistry (IHC)

The L4–L6 spinal segments and dorsal root ganglia were collected, fixed, dehydrated, embedded, and sectioned. Immunohistochemical staining for calcitonin gene-related pep-

tion (CGRP) was performed using the Novolink™ Max Polymer Detection System [14,15]. First, endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide, and non-specific antibody binding was reduced using Protein Block (RE7102; Novocastra). Sections were incubated with a primary anti-CGRP polyclonal antibody (1:1000; Calbiochem, Darmstadt, Germany), followed by Post Primary Block (RE7111; Novocastra, Newcastle upon Tyne, UK), and then a secondary antibody using the Novolink Polymer (RE7112, Newcastle upon Tyne, UK). Diaminobenzidine was used for color development, and hematoxylin was used for counterstaining as a background control. CGRP expression was quantified as the CGRP-positive area ratio (%) using standardized image analysis parameters. All analyses were performed by two independent, blinded observers, and the averaged values were used for statistical evaluation.

For macrophage immunofluorescence staining, tissue sections were pre-treated with 10% bovine serum albumin containing 0.4% Triton X-100 for 1 h [16,17]. The sections were incubated overnight at 4 °C with primary antibodies: anti-CD68 (1:200; Serotec, Hercules, CA, USA) and anti-Iba1 (1:100; Bioss, Woburn, MA, USA). After three washes with phosphate-buffered saline, sections were incubated with appropriate secondary antibodies conjugated to Alexa Fluor 488 or 594 (1:500; Abcam, Cambridge, UK) for 1 h at room temperature in the dark. Finally, coverslips were mounted using an aqueous mounting medium (ScyTek, Logan, UT, USA), and images were acquired using a laser scanning confocal microscope (Leica SP2/SP8X, Leica Microsystems, Wetzlar, Germany).

2.12. Statistical Analysis

The total area of regenerated nerve tissue, endoneurial area, and number of regenerated axons were calculated using color image analysis software (Image-Pro Lite, Media Cybernetics, Rockville, MD, USA). All data were analyzed using SPSS statistical software version 10.0. One-way analysis of variance (One-way ANOVA) was performed to evaluate statistical differences among groups. A significance level (α) of 0.05 was set, and a p -value less than 0.05 was considered statistically significant.

3. Results

3.1. Thermal and Cold Hyperalgesia Test and Motor Coordination Test

In the thermal hyperalgesia test (Figure 4A), paw withdrawal latency decreased from 3 weeks to 7 weeks across all groups. The C-3W-PO group exhibited the longest latency, whereas the ES-7W-PO group had the shortest latency, suggesting a trend toward reduced thermal pain tolerance after prolonged ES. However, no statistically significant differences were observed among the groups.

In the cold hyperalgesia test (Figure 4B), paw withdrawal latency increased notably after 7 weeks of treatment. The ES-7W-PO group exhibited the longest latency, significantly higher than both the C-3W-PO and C-7W-PO groups ($p < 0.05$ and $p < 0.01$, respectively). This suggests that extended ES effectively alleviates cold hypersensitivity over time.

In the mechanical test (Figure 4C), the number of foot retractions over a 3 min period showed no statistically significant differences among the four groups. Although the ES-3W-PO group exhibited a slightly higher number of foot retractions compared to the other groups, the variability was large, and no consistent trend was observed. These findings suggest that ES did not significantly alter mechanical sensitivity in this test.

In the rotarod test assessing motor coordination (Figure 4D), the ES-3W-PO group exhibited a significantly longer latency to fall compared with the C-3W-PO group ($p < 0.05$), indicating improved motor performance following short-term ES. No significant differences were observed among the remaining groups. Overall, rotarod performance remained stable

across all experimental conditions, indicating that neither needle insertion nor ES had an adverse effect on motor coordination.

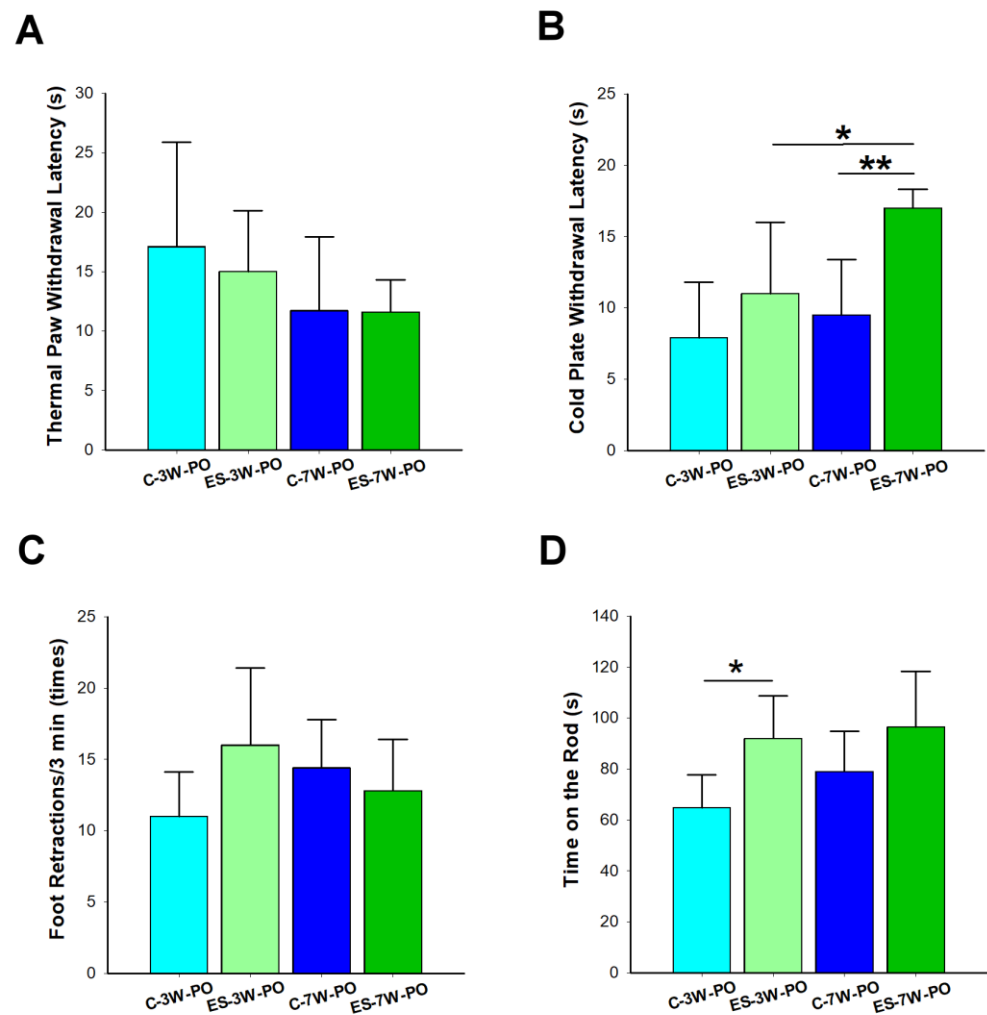


Figure 4. Effects of ES on sensory sensitivity and motor coordination in silicone conduit-bridged rats. **(A)** Thermal hyperalgesia test: Paw withdrawal latency gradually decreased from 3 to 7 weeks post-operation in all groups, with the C-3W-PO group showing the highest latency and the ES-7W-PO group the lowest. No significant differences were observed among the groups. **(B)** Cold hyperalgesia test: Paw withdrawal latency significantly increased in the ES-7W-PO group compared to the C-3W-PO (* $p < 0.05$) and C-7W-PO (** $p < 0.01$) groups, indicating reduced cold sensitivity following prolonged ES. **(C)** Mechanical sensitivity test: No statistically significant differences were observed in the number of foot retractions among the groups. While the ES-3W-PO group showed slightly higher retraction counts, the data were highly variable. **(D)** Rotarod test: The ES-3W-PO group exhibited significantly improved motor coordination, remaining longer on the rotating rod than the C-3W-PO group (* $p < 0.05$). No significant differences were noted among the other groups. Data are presented as mean $\pm$ SD ($n = 10$ per group).

3.2. Gastrocnemius Muscle Weight Changes

No significant differences were observed between the groups (Figure 5). Although a trend toward increased muscle weight in the ES-7W-PO group is apparent, the high variability and lack of statistical significance preclude any definitive conclusions about this effect.

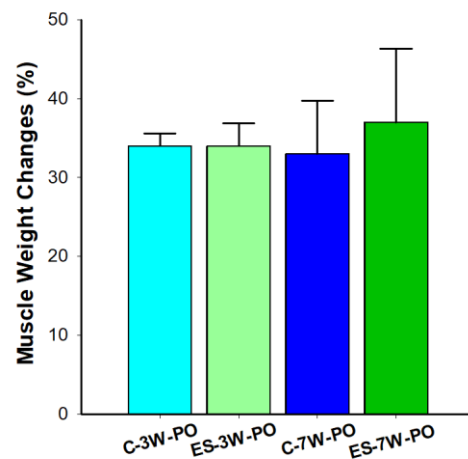


Figure 5. Gastrocnemius muscle weight following ES in silicone conduit-bridged rats. The bar graph presents the mean gastrocnemius muscle weight ($\pm$ SD) in each group at 3 and 7 weeks post-operation ($n = 10$ per group). No statistically significant differences were observed among the groups, indicating that ES did not produce a measurable effect on muscle mass restoration during the study period.

3.3. Electrophysiological Testing

The NCV results show statistically significant improvements in both the ES-3W-PO and ES-7W-PO groups compared to their respective C-3W-PO and C-7W-PO controls ($p < 0.01$) (Figure 6A). This suggests that the ES had a positive effect on nerve conduction regardless of the duration.

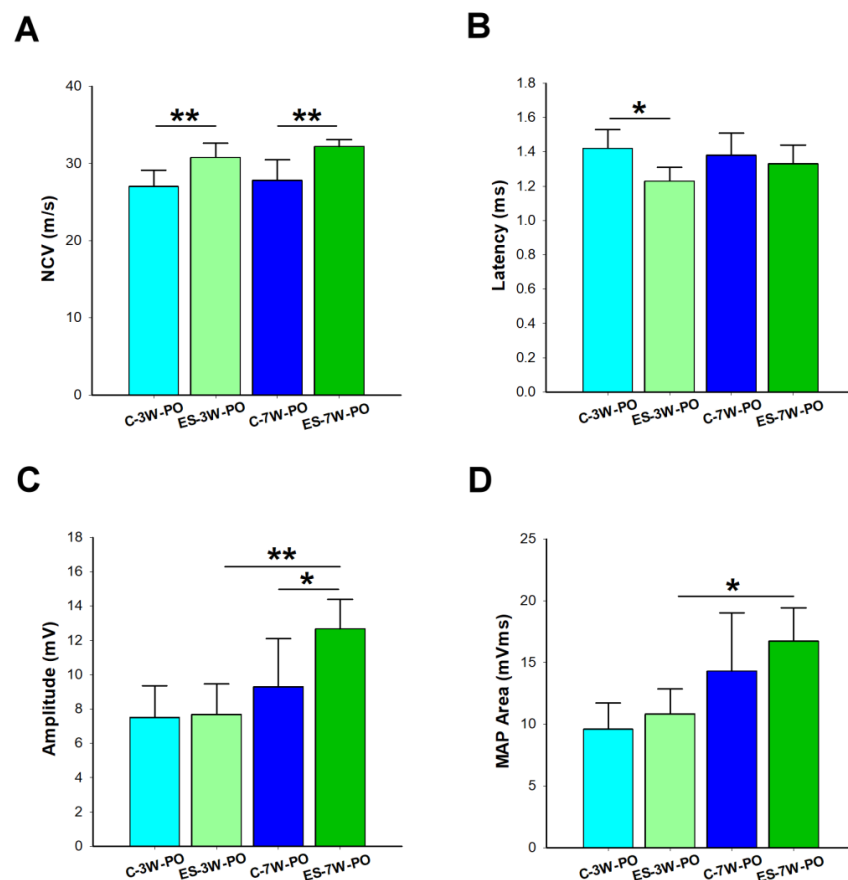


Figure 6. Electrophysiological assessment of nerve regeneration following ES in silicone conduit-bridged rats. (A) Nerve conduction velocity (NCV), (B) latency, (C) amplitude, and (D) muscle action potential (MAP) area were measured at 3 and 7 weeks post-operation. EA treatment significantly increased NCV in both ES-3W-PO and ES-7W-PO groups compared to their respective controls (** $p < 0.01$). Latency was

significantly reduced in the ES-3W-PO group compared to the C-3W-PO group (* $p < 0.05$), with no significant difference observed at 7 weeks. Amplitude was significantly higher in the ES-7W-PO group compared to both C-7W-PO (* $p < 0.05$) and ES-3W-PO (** $p < 0.01$), indicating enhanced nerve response strength. Similarly, the ES-7W-PO group exhibited a significantly larger MAP area than ES-3W-PO (* $p < 0.05$), suggesting improved motor nerve activation with prolonged treatment. Values are mean $\pm$ SD ($n = 10$).

The latency results indicate a significant reduction in latency in the ES-3W-PO group compared to the C-3W-PO group ($p < 0.05$) (Figure 6B), suggesting improved nerve function following ES at 3 weeks. No significant differences in latency were observed between the C-7W-PO and ES-7W-PO groups.

The amplitude results show a significant increase in the ES-7W-PO group compared to both the ES-3W-PO ($p < 0.01$) and C-7W-PO ($p < 0.05$) groups (Figure 6C). This suggests that extended ES enhances nerve response strength. No significant changes were observed at 3 weeks.

The MAP area results reveal a significant increase in the ES-7W-PO group compared to the ES-3W-PO group ($p < 0.05$) (Figure 6D). This indicates enhanced regenerated nerve activity with extended ES. No significant differences were observed at the 3-week time point.

3.4. Analysis of Fluoro-Gold Retrograde Tracing

Figure 7A shows FG-labeled cross-sections of neuronal tissues from different experimental groups, used to evaluate the extent of neuronal regeneration via retrograde tracing. The bright fluorescent spots represent labeled neurons that have successfully re-established connections. The ES-3W-PO group displays more labeled neurons compared to the C-3W-PO group, indicating enhanced early-stage regeneration. The ES group continues to show superior regeneration at 7 weeks. Regardless of treatment, more neurons are labeled at 7 weeks than at 3 weeks, suggesting progressive regeneration over time.

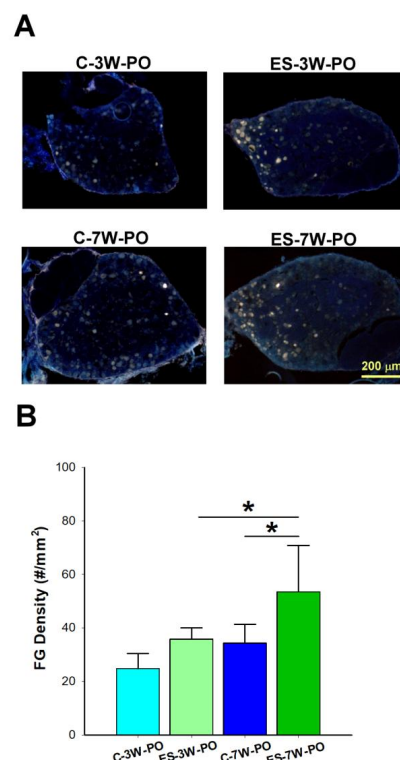


Figure 7. FG retrograde tracing of motor neuron regeneration in rats treated with ES. (A) Representative images of FG-labeled motor neurons in the spinal cord and (B) quantification of FG-labeled neuron density at 3 and 7 weeks post-operation. The ES-7W-PO group showed a significantly higher

density of FG-labeled neurons compared to both ES-3W-PO and C-7W-PO groups (* $p < 0.05$), indicating enhanced axonal regeneration and target reinnervation following extended ES. No significant differences were observed among groups at 3 weeks. Values are mean $\pm$ SD ($n = 10$). Scale bar = 200 μm .

FG retrograde tracing results demonstrate that extended ES enhances motor neuron regeneration. Quantification of FG-labeled neurons revealed a significant increase in FG density in the ES-7W-PO group compared to both the ES-3W-PO and C-7W-PO groups (both $p < 0.05$) (Figure 7B), indicating improved axonal regeneration and reinnervation. No significant differences were observed between groups at the 3-week time point, suggesting that the regenerative benefits of ES become more pronounced with prolonged treatment.

3.5. Evaluation of Regenerating Nerve Tissue Sections

Representative micrographs of regenerated nerve cross-sections were analyzed. These images reflect the structural integrity and maturity of regenerated peripheral nerves (Figure 8A). The C-3W-PO group exhibited sparse and loosely organized nerve fibers, with irregular and unevenly thick myelin sheaths, indicating an early phase of regeneration with incomplete structural development. In contrast, the ES-3W-PO group showed improved tissue compactness, more clearly defined myelin sheaths, and better fiber alignment, suggesting that ES accelerates early regenerative processes. By 7 weeks, both treatments showed marked improvement. The C-7W-PO group presented an increased number of myelinated fibers with more uniform organization compared to the C-3W-PO group, indicating delayed yet progressive nerve repair. Notably, the ES-7W-PO group displayed the most robust regeneration, characterized by densely packed, large-diameter axons with uniformly thick and clear myelin sheaths, indicating advanced maturity and structural restoration. These findings demonstrate that ES facilitates enhanced structural regeneration of peripheral nerves compared with non-stimulated controls, with the regenerative benefits becoming more evident over time.

Quantitative analysis of the total cross-sectional area of regenerated nerve tissue (Figure 8B) demonstrated that the ES-3W-PO group exhibited a significantly larger total nerve area compared with the C-3W-PO group ($p < 0.05$), indicating that early application of ES enhanced nerve regeneration. In addition, the C-7W-PO group showed a significantly greater total nerve area than the C-3W-PO group, suggesting progressive spontaneous nerve tissue growth over time following conduit-mediated repair. No significant difference was observed between the ES-3W-PO and ES-7W-PO groups, indicating that the regenerative benefit conferred by ES was established during the early post-injury period and sustained with extended duration.

The endoneurial area analysis of regenerating nerve tissue (Figure 8C) shows a significant increase in the ES-3W-PO and C-7W-PO groups compared to the C-3W-PO group (both $p < 0.01$), indicating that both ES and longer recovery time independently promote endoneurial regeneration. The ES-7W-PO group also demonstrated a higher endoneurial area, though not significantly different from the ES-3W-PO or C-7W-PO groups, suggesting a sustained effect of ES on nerve tissue repair. These results highlight the early and lasting impact of ES on enhancing the structural integrity of regenerating nerves.

The axon number analysis (Figure 8D) shows a significant increase in axon count in the ES-3W-PO and C-7W-PO groups compared to the C-3W-PO group (both $p < 0.01$), indicating that both ES and extended recovery promote axonal regeneration. Furthermore, the ES-7W-PO group exhibited significantly higher axon numbers than both the ES-3W-PO ($p < 0.01$) and C-7W-PO ($p < 0.05$) groups, suggesting that prolonged ES has a cumulative and superior effect on axonal growth. These findings highlight the significant role of ES in promoting axonal regeneration, particularly with prolonged treatment duration.

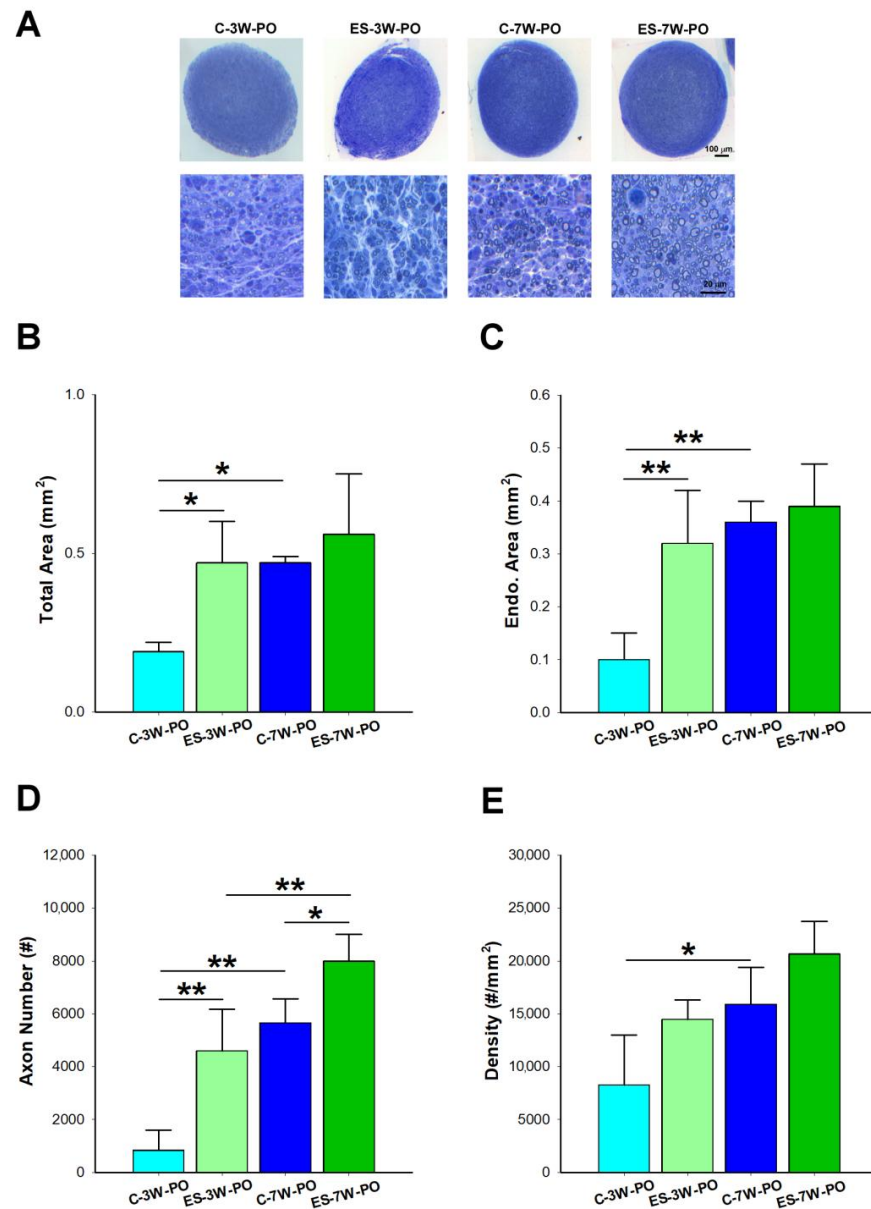


Figure 8. Morphometric analysis of regenerating nerve tissue in rats following ES at different post-operative durations. **(A)** Representative transverse semi-thin sections of regenerated sciatic nerves stained at low (**top row**) and high (**bottom row**) magnification. The C-3W-PO group exhibits loosely organized nerve fibers with sparse myelination. In contrast, the ES-3W-PO group exhibits improved structural organization, characterized by denser endoneurial regions. Both the C-7W-PO and ES-7W-PO groups exhibit enhanced regeneration, with the ES-7W-PO group demonstrating the most compact and well-myelinated nerve fibers, indicative of a more advanced recovery. **(B)** Quantification of total nerve area reveals significant enlargement in the ES-3W-PO group compared to C-3W-PO (* $p < 0.05$), and in the C-7W-PO group compared to C-3W-PO (* $p < 0.05$), indicating early and time-dependent enhancement of nerve tissue regeneration. **(C)** Endoneurial area is significantly greater in both ES-3W-PO and C-7W-PO groups compared to C-3W-PO (** $p < 0.01$), suggesting that ES and longer recovery independently promote structural nerve repair. **(D)** Axon number is significantly increased in the ES-3W-PO and C-7W-PO groups compared to C-3W-PO (** $p < 0.01$). The ES-7W-PO group shows the highest axon count, significantly greater than both ES-3W-PO (** $p < 0.01$) and C-7W-PO (* $p < 0.05$), indicating cumulative benefits of prolonged ES. **(E)** Axon density is significantly higher in the C-7W-PO group than in the C-3W-PO (* $p < 0.05$). Although the ES-7W-PO group showed the highest mean density, differences were not statistically significant. Values are presented as mean $\pm$ SD ($n = 10$ per group).

Axon density analysis (Figure 8E) revealed a significantly higher axon density in the C-7W-PO group compared with the C-3W-PO group ($p < 0.05$), indicating a time-dependent increase in axonal regeneration in the absence of ES. Among all experimental groups, the ES-7W-PO group exhibited the highest axon density; however, this increase did not reach statistical significance when compared with the other groups.

3.6. CGRP Expression

Immunohistochemical staining was performed to assess the expression of CGRP, a neuropeptide closely associated with nerve regeneration and sensory recovery. Representative spinal cord sections from each group (C-3W-PO, ES-3W-PO, C-7W-PO, and ES-7W-PO) are shown in Figure 9A. CGRP-positive regions are indicated by the brown-stained areas within the dorsal horn of the spinal cord. Visually, the ES-treated groups (especially ES-3W-PO and ES-7W-PO) show darker and more extensive staining, indicating elevated CGRP expression.

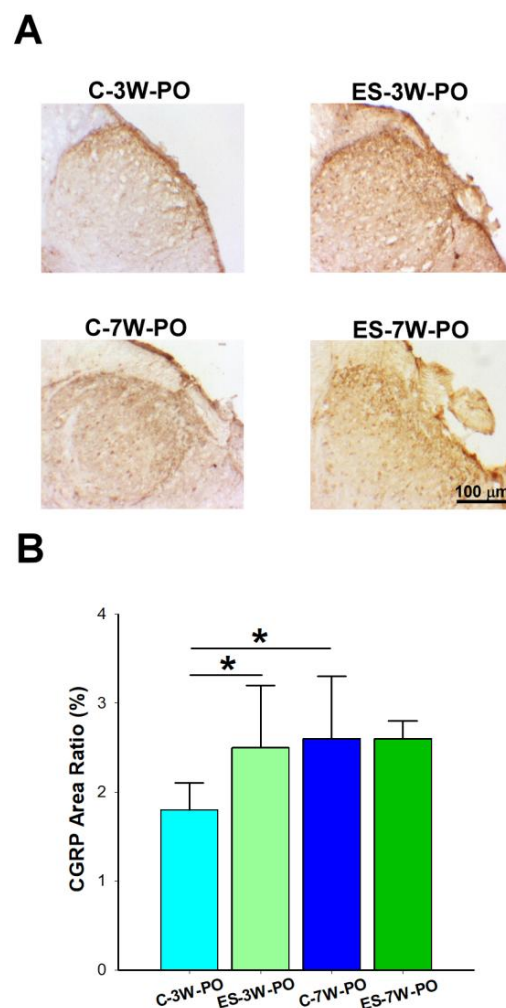


Figure 9. CGRP expression in regenerating sciatic nerve tissue following ES at different post-operative durations. (A) Representative IHC images showing CGRP-positive nerve fibers. (B) Quantification of CGRP expression based on CGRP area ratio (%) reveals a significant increase in the ES-3W-PO group compared to the C-3W-PO group ($* p < 0.05$), indicating that short-term ES enhances CGRP expression, which is associated with nerve regeneration and sensory recovery. The C-7W-PO group also exhibited significantly higher CGRP levels than the C-3W-PO group ($* p < 0.05$), suggesting time-dependent upregulation. Values are presented as mean $\pm$ SD ($n = 10$ per group). Scale bar = 100 μ m.

Quantitative analysis of CGRP expression, presented as the CGRP area ratio (%) (Figure 9B), demonstrated a significantly higher CGRP expression in the ES-3W-PO group compared with the C-3W-PO group ($p < 0.05$), indicating that ES upregulated CGRP expression during the early post-injury period. In addition, the C-7W-PO group exhibited significantly greater CGRP expression than the C-3W-PO group ($p < 0.05$), reflecting a time-dependent increase in sensory neuropeptide expression in the absence of ES.

3.7. Macrophage Density

The macrophage density image shows immunohistochemical staining of nerve tissue cross-sections from four experimental groups (Figure 10A). Brown-stained circular or oval cells represent macrophages, indicating immune cell infiltration at the site of nerve regeneration. In the ES-3W-PO group, a noticeably higher number of macrophages is observed, with densely packed, darkly stained cells dispersed throughout the tissue, suggesting heightened immune activity. In contrast, the C-3W-PO and C-7W-PO groups show fewer and more sparsely distributed macrophages, indicating lower levels of immune response. The ES-7W-PO group shows moderate macrophage presence, with more cells than in C-7W-PO but fewer than in ES-3W-PO.

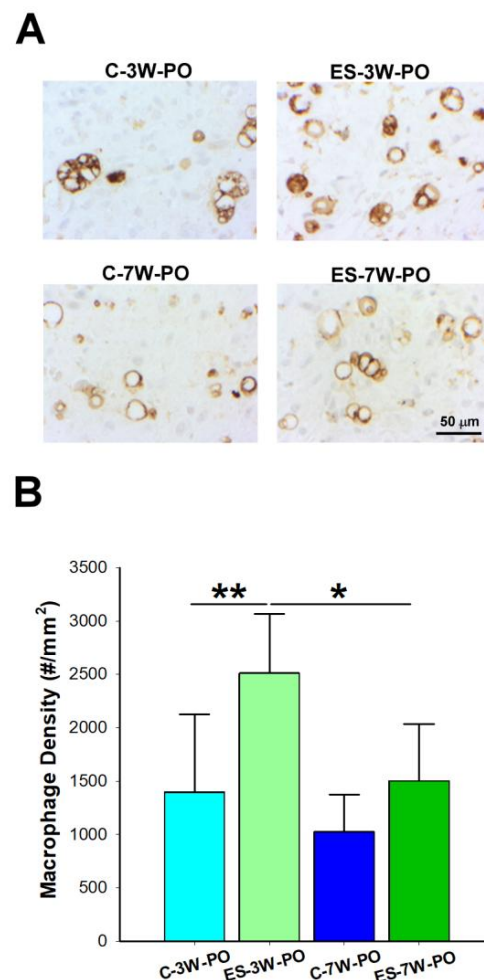


Figure 10. Effects of ES on macrophage infiltration in silicone conduit-bridged rats. (A) Representative immunohistochemical images showing macrophages in regenerated nerve tissue at 3 and 7 weeks post-operation. (B) Quantification of macrophage density reveals a significantly higher infiltration in the ES-3W-PO group compared to the C-3W-PO group (** $p < 0.01$) and the ES-7W-PO group (* $p < 0.05$), indicating enhanced immune cell recruitment at the early stage of ES. Data are presented as mean $\pm$ SD ($n = 10$ per group). Scale bar = 50 μ m.

The quantitative analysis of macrophage density results (Figure 10B) demonstrates a significant increase in macrophage presence in the ES-3W-PO group compared to both the C-3W-PO ($p < 0.01$) and ES-7W-PO ($p < 0.05$) groups, indicating that ES at the early-stage post-operation enhances immune cell recruitment, which is crucial for debris clearance and initiating nerve repair. Although the ES-7W-PO group showed a moderate increase in macrophage density relative to C-7W-PO, this difference was not statistically significant. These findings suggest that ES stimulates an early inflammatory response that may contribute to improved nerve regeneration.

4. Discussion

Peripheral nerve regeneration occurs most rapidly during the early weeks following injury but slows significantly as recovery progresses. Within the first few weeks post-injury, key regenerative events, such as rapid axonal sprouting, cellular proliferation, and growth cone formation, occur [18,19]. This early phase is vital, as it enables injured axons to begin bridging the lesion site with support from Schwann cell recruitment and the development of a permissive extracellular matrix [20]. ES has been shown to enhance these early regenerative processes by accelerating axonal growth and improving the alignment and orientation of regenerating fibers, leading to faster functional recovery [21]. Needle-based ES was employed to ensure precise and reproducible control of stimulation parameters, allowing focused evaluation of the temporal effects of ES during the early regenerative phase. Although emerging technologies such as piezoelectric conduits offer minimally invasive alternatives, they introduce additional material-related variables that were beyond the scope of the present study [22].

We acknowledge that silicone conduits are no longer favored for clinical application due to their propensity to elicit foreign body reactions and the requirement for secondary removal surgery. In the present study, silicone was deliberately selected as an experimental control model to provide mechanical stability and reproducible geometry, thereby allowing isolation of the temporal effects of ES independent of material degradation or bioactivity.

After the initial few weeks, the rate of regeneration declines as the repair process transitions into more complex phases such as remyelination and the re-establishment of functional synapses [23]. These later stages, typically extending to 8 weeks or beyond, are marked by gradual improvements in fiber maturation and functional integration. Although ES can continue to influence repair by promoting myelination and maintaining axonal integrity, its impact tends to be less pronounced in this phase [24]. This shift in regenerative dynamics highlights the importance of stimulation timing; however, whether delayed ES may exert distinct or complementary effects during later stages of repair remains to be determined [25].

Importantly, macrophage density should not be interpreted as a direct or standalone indicator of nerve regeneration. Rather, macrophage recruitment reflects the inflammatory and remodeling microenvironment that supports debris clearance, Schwann cell activation, and subsequent axonal regrowth during peripheral nerve repair. Our findings indicate that ES enhances peripheral nerve regeneration predominantly during the early phase of recovery within the observation window of this study. Although gait analysis is commonly used to assess locomotor recovery following peripheral nerve injury, it was not included in the present study. In severe transection models bridged with nerve conduits, gait parameters are often influenced by compensatory mechanisms rather than direct neural regeneration. Therefore, we prioritized electrophysiological, histomorphometric, and retrograde tracing outcomes to objectively assess nerve repair. Future studies incorporating gait analysis may provide additional insight into functional motor adaptation following ES [26].

Gastrocnemius muscle weight was included as a gross indicator of denervation-related atrophy; however, it does not capture qualitative changes in muscle architecture such as fiber-type composition, fiber cross-sectional area, or fibrosis. Therefore, the absence of significant differences in muscle weight should not be interpreted as evidence of preserved muscle structure or complete reinnervation. Future studies incorporating muscle histology and fiber-type analysis are necessary to establish a direct structural link between nerve regeneration and functional recovery. Gastrocnemius muscle weight primarily reflects gross muscle atrophy and reinnervation status, which generally lag behind early neural and histological changes during peripheral nerve regeneration. Therefore, although ES influenced axonal regeneration and neurohistological outcomes, the difference between 3-week and 7-week ES may not have been sufficient to generate a measurable divergence in muscle mass within the experimental timeframe. Moreover, muscle weight is influenced by multiple factors, including the duration of denervation, reinnervation efficiency, and animal activity, which may reduce its sensitivity to differences in ES timing [27].

ES promoted earlier improvements in functional recovery, as evidenced by significantly increased NCV and reduced latency at 3 weeks. These electrophysiological improvements were supported by morphological enhancements, including larger total and endoneurial nerve areas, increased axon counts, and a greater density of FG-labeled neurons. The ES groups also exhibited elevated CGRP expression at 3 weeks, suggesting earlier activation of sensory and motor axon regeneration pathways. Additionally, ES significantly increased macrophage density during the early stage, which likely contributed to a more pro-regenerative inflammatory microenvironment. Cold and heat hyperalgesia are mediated by distinct sensory fiber populations and molecular pathways, which may differ in their regenerative capacity and responsiveness to ES. While prolonged ES improved cold sensitivity, thermal nociception showed a non-significant trend toward reduced latency, suggesting modality-specific sensory modulation rather than uniform normalization. In addition, heat withdrawal responses may be influenced by inflammatory components or central sensitization, which could be differentially affected by ES. These findings highlight the complexity of sensory recovery and warrant further investigation. Macrophages were identified using pan-macrophage markers (CD68 and Iba1), and phenotype-specific M1/M2 polarization was not examined. Accordingly, macrophage density was interpreted as an indicator of the regenerative microenvironment rather than a direct marker of regeneration itself. Future studies incorporating polarization markers may provide further insight into the immunomodulatory mechanisms of ES. Accordingly, macrophage density in the present study was evaluated in conjunction with functional, electrophysiological, and histomorphometric outcomes, rather than as an isolated measure of regenerative efficacy [28].

A limitation of this study is the absence of a surgery-only control group without subsequent percutaneous intervention. Although a needle-only control was included to account for nonspecific procedural effects, needle insertion itself may induce biological responses that could modulate nerve regeneration. Therefore, the observed effects should be interpreted as resulting from percutaneous ES rather than electrical current alone. Future studies incorporating surgery-only, needle-only, and ES groups will be necessary to fully disentangle these effects.

As regeneration continued to 7 weeks, many key indicators, such as CGRP expression and macrophage density, either increased or plateaued in both ES and control groups, implying that time itself plays a substantial role in supporting recovery. However, the additive benefits of ES were most notable during the early phase, and the differences between groups became less pronounced by 7 weeks. This pattern suggests that ES may preferentially influence early regenerative events, although the limited number of time

points precludes definitive conclusions regarding acceleration versus prolongation of regeneration. A limitation of this study is the fixed early initiation of ES at 7 days post-injury. While this design allowed focused evaluation of ES duration during the early regenerative phase, it does not address whether delayed stimulation may also confer benefits during later stages, such as axon maturation or remyelination. Thus, the proposed “optimal time window” should be interpreted within the early post-injury context studied. Future investigations incorporating delayed ES onset are necessary to fully assess its therapeutic potential and clinical applicability.

ES consistently enhanced electrophysiological outcomes across both time points. It significantly improved NCV at 3 and 7 weeks, reduced latency at 3 weeks, and markedly increased amplitude and MAP area by 7 weeks, indicating improved motor function and nerve integrity. Latency primarily reflects conduction velocity and myelination status, which tend to plateau at later stages of peripheral nerve regeneration. Accordingly, by 7 weeks post-operation, spontaneous recovery in the control group may have reached a level comparable to that of the ES-treated group, thereby reducing detectable differences in latency. Compared to the control groups, ES groups showed faster and more robust electrophysiological recovery. Furthermore, FG-labeled neuron density, i.e., a marker of axonal regeneration and target reinnervation, was significantly elevated in ES-treated animals, particularly at 8 weeks, suggesting a sustained but time-dependent effect of ES on axonal regeneration. Retrograde tracing analysis was focused on the L4–L5 segments, which represent the primary origins of the rat sciatic nerve. Although L3 and L6 roots may occasionally contribute, they were not included to maintain analytical consistency. Future studies may expand the analysis to additional segments.

These results align with previous reports, which have shown that early, short-term ES can accelerate both functional and structural nerve repair [3,4,9,19,21]. Consistent with findings from rodent models of peripheral nerve injury [29], our ES-treated groups exhibited increased NCV, reduced latency, higher amplitudes, and greater MAP area, particularly at early time points [30]. The elevated CGRP expression and macrophage density observed at 3 weeks are comparable to results from diabetic and corneal nerve injury models, where immediate ES enhanced macrophage recruitment and CGRP-mediated neuroregeneration [31]. Although routine Hematoxylin and Eosin (H&E) staining can provide a general overview of tissue inflammation, this study focused on immunohistochemical detection of macrophages using CD68 and Iba1 to achieve greater specificity. Silicone conduits are considered biologically inert, and no evidence of persistent foreign body-related inflammation was observed. Future studies may include H&E staining as a complementary approach to further characterize inflammatory responses. Furthermore, our histological findings, including enlarged total and endoneurial areas, higher axon counts, and greater axon density, corroborate the structural benefits reported in other ES studies at 4–6 weeks post-injury [9,10,19]. Axon counts were compared among injured experimental groups, and uninjured sciatic nerve controls were not included. Future studies incorporating normal nerve baseline data may further contextualize the extent of regeneration. The increased FG-labeled neuron counts in ES groups are also consistent with earlier evidence of augmented retrograde labeling following brief stimulation [29].

Although some studies have suggested that ES may yield prolonged regenerative benefits [3,9,32], our data indicate that ES-related differences were most apparent during the early post-injury period and diminished by later stages within the timeframe examined [9,32]. Accordingly, the present findings support the hypothesis that ES preferentially enhances early-stage regeneration, rather than conclusively demonstrating an absence of later effects. We hypothesize that ES promotes peripheral nerve regeneration by enhancing neuroimmune interactions, particularly through modulation of macrophage activity and

neurotrophic factor signaling, thereby creating a permissive microenvironment for axonal growth and alignment.

Based on the comparison of outcomes at 3 and 7 weeks post-injury, ES appeared to exert more prominent effects during the earlier phase of regeneration, with group differences diminishing at later stages. However, given the limited number of time points assessed, this observation should be interpreted cautiously. Whether ES accelerates the regenerative process, alters its temporal profile, or influences later stages such as remyelination and functional stabilization requires further investigation using a more detailed time-course analysis.

Recent advances in hydrogel-based nerve guidance conduits have introduced material-driven strategies to enhance peripheral nerve repair, including 4D-printed shape-morphing hydrogels that enable sutureless self-folding conduits and wet-adhesive conductive hydrogels that integrate electrical conductivity with strong tissue adhesion [33]. These platforms provide dynamic mechanical and electroactive cues that actively participate in the regenerative process. In contrast, the silicone conduit used in the present study offers an inert and mechanically stable framework, allowing isolation of the biological effects of externally applied ES. Functionalized hydrogel conduits, therefore, represent promising alternatives that could be combined with tailored ES paradigms in future studies to achieve synergistic regenerative outcomes.

5. Conclusions

In summary, ES enhances both functional and structural aspects of peripheral nerve regeneration, with its effects being most evident during the early stages of recovery within the studied timeframe. The time-dependent nature of ES efficacy underscores the importance of early intervention to maximize therapeutic benefit. Our results support existing evidence that brief ES can accelerate axonal repair and reinnervation and demonstrate that ES facilitates this process by promoting faster nerve conduction, improved electrophysiological responses, and enhanced axonal and tissue regeneration. Notably, several improvements—such as increased nerve conduction velocity, greater endoneurial space, higher axon counts, and elevated CGRP expression—were observed during the early post-injury period. While continued recovery occurred over time, the additional benefits associated with ES became less distinct at later stages, suggesting a predominant influence on early regenerative events rather than definitive prolongation of regeneration. The ES-7W-PO group exhibited robust regenerative outcomes across multiple measures; however, whether prolonged ES confers sustained or delayed benefits beyond the observation period requires further investigation. Collectively, these findings highlight the therapeutic potential of ES as a time-sensitive intervention for enhancing peripheral nerve repair, while emphasizing the need for future studies incorporating extended time-course analyses and delayed stimulation paradigms.

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Institutional Review Board Statement: This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Academies Press, USA). All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of China Medical University, Taiwan (Approval Number: CMUIACUC-2024-241).

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are included in the article; further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

ANOVA	Analysis of Variance
C	Control
CGRP	Calcitonin Gene-Related Peptide
ES	Electrical Stimulation
FG	Fluoro-Gold
IHC	Immunohistochemistry
MAP	Muscle Action Potential
NCV	Nerve Conduction Velocity
SD	Sprague–Dawley (rats)
PO	Post-Operation

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