



Experimental evaluation of nerve graft orientation in a rat model: Functional and histological insights

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The incidence of peripheral nerve injuries is high within the field of orthopedics. The prevalence of peripheral nerve injuries in developed countries is approximated at 13 to 23 per 100,000 individuals, with a 1.64% increase observed in those sustaining extremity trauma.^[1,2] Currently, epineural repair constitutes the established procedure for nerve reconstruction.^[3] Nevertheless, the existence of a defect at the injury site may hinder complete repair. For these cases, the use of autologous nerve grafts represents the accepted optimal approach to preserving nerve continuity.^[4] The use of autografts presents benefits such as the creation of a basal lamina-lined conduit to facilitate axon regeneration and the reduction of immunological reactions. In clinical practice, the sural nerve is the most frequently utilized autograft, owing to its harvestable

ABSTRACT

Objectives: This study aims to investigate the effect of nerve graft orientation on nerve regeneration in a rat sciatic nerve defect model and to compare functional and histological outcomes of normal and reverse orientation grafts, focusing on sensory and motor recovery.

Materials and methods: A total of 30 Wistar Albino rats were divided into three equal groups: a control group, a normal graft orientation group (Group A), and a reverse graft orientation group (Group B). A 10-mm sciatic nerve defect was created in the surgical groups, and the graft was applied with epineural coaptation. Functional recovery was evaluated using extensor postural thrust (EPT), pinprick, and hot plate tests, while histological analysis involved axon counts, myelin sheath thickness measurements, and the axon count change ratio.

Results: No significant difference was observed between Group A and Group B in motor function recovery as evaluated by the EPT test ($p>0.05$). However, Group A showed improved sensory recovery compared to Group B in the pinprick test ($p=0.028$). Histologically, both groups demonstrated similar ratios of axon count and myelin sheath thickness between proximal and distal segments ($p>0.05$).

Conclusion: Normal autograft orientation demonstrated superior sensory recovery, while no significant differences were observed in motor function or histological results. These findings highlight the importance of graft orientation for sensory regeneration and underscore the need for future studies to explore the long-term effects of graft orientation and its implications for human nerve repair, particularly in larger defects and clinical scenarios.

Keywords: Nerve graft orientation, peripheral nerve regeneration, rat model, sciatic nerve defect, sensory recovery.

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length (up to 30-40 cm) and minimal associated donor-site morbidity.^[5] Alternative donor nerves for autografting include the medial antebrachial cutaneous, lateral antebrachial cutaneous, dorsal

antebrachial, and lateral femoral cutaneous nerves.^[6] Further research is needed to definitively establish the optimal orientation of nerve grafts.

While previous studies have explored various aspects of nerve grafting, including graft material selection, the impact of graft orientation has received limited attention.^[7] The impact of graft orientation on nerve regeneration warrants further investigation to optimize clinical outcomes, particularly for substantial nerve injuries. This investigation sought to address a gap in the existing literature by assessing the functional and histological outcomes of utilizing either standard or reversed nerve graft orientations within a rat sciatic nerve defect model.

In this experimental study, we aimed to investigate the effect of nerve graft orientation on nerve regeneration in a rat sciatic nerve defect model and to compare functional and histological outcomes of normal and reverse orientation grafts, focusing on sensory and motor recovery.

MATERIALS AND METHODS

Experimental design and nerve grafting model

In this experimental study, a total of 30 Wistar Albino rats (350 to 400 g) were used. The study was approved by the Selçuk University Experimental Medicine Application and Research Center Experimental Animals (date: 31.12.2021, no: 2021-66). All experimental protocols adhered to international ethical guidelines and the Guide for the Care and Use of Laboratory Animals.

The rats were randomly divided into three equal groups including 10 rats in each group: two surgical groups (Groups A and B) and one control group. The control group underwent no surgical procedures. In Groups A and B, a 10-mm defect was created in the right sciatic nerve, and the excised nerve was grafted with epineural repair in either normal (Group A) or reverse orientation (Group B). The subjects were monitored for 12 weeks postoperatively, during which functional and histological assessments were performed. All groups were standardized except for graft orientation to isolate its effect on nerve regeneration. Functional assessments included extensor postural thrust (EPT), pinprick, and hot plate tests, alongside gastrocnemius muscle mass measurements as described by Koka and Hadlock.^[8] Histological analysis compared axon count and myelin sheath thickness changes between groups.

Surgical procedure

Under 10 mg/kg xylazine anesthesia, the sciatic nerve was exposed, and a 10-mm segment was excised. The skin incision line was marked (Figure 1a). Following surgical prep, the sciatic nerve was exposed and explored (Figure 1b). Sciatic nerve trifurcation was identified; incisions 1 and 2 cm proximal were planned. A 10-mm nerve segment was to be excised. The sciatic nerve was incised with a single motion at two different points using a No.11 scalpel blade (Figure 1c). Epineural end-to-end coaptation was performed using microsurgical techniques with four sutures on each side (Figure 1d). Group A received grafts in the normal orientation, while Group B received grafts in the reverse orientation.

Evaluation methods

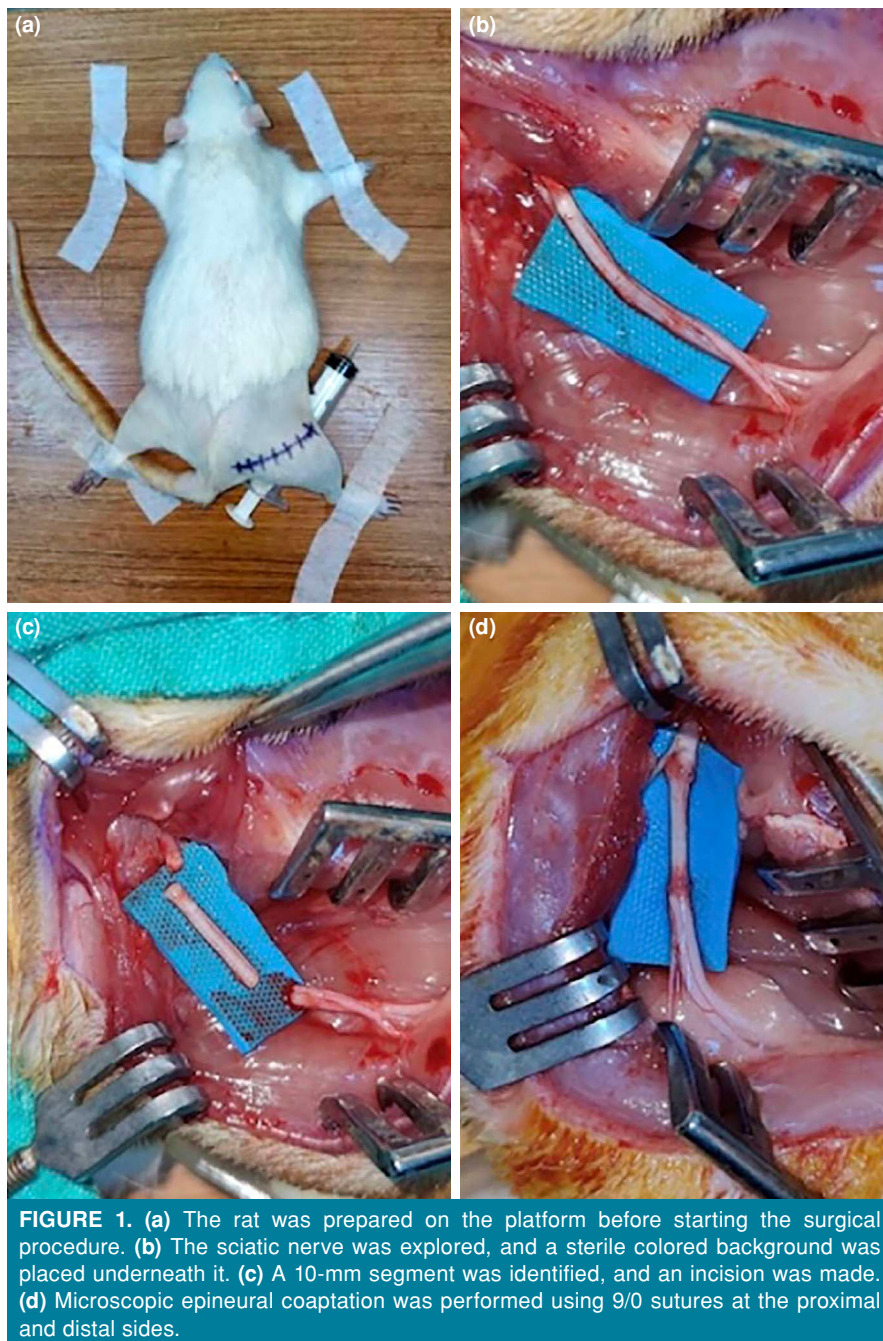
Functional assessments: Functional recovery was evaluated using EPT, pinprick, and hot plate tests. For EPT, the force exerted by each hind limb on a precision scale (Figure 3) was measured, and functional deficits were calculated as percentages. The pinprick test assessed sensory response, scoring withdrawal reflexes from 0 to 3 based on stimulus location, while focusing on the sciatic nerve's lateral innervation (Figure 4). The hot plate test recorded reflexive withdrawal times to a 56°C stimulus, with a 12-sec cut-off to prevent injury (Figure 5).

Gastrocnemius weight ratio: Gastrocnemius muscles were excised and weighed bilaterally to calculate weight ratios, indicating muscle atrophy or recovery based on nerve regeneration (Figure 6).^[9]

Histological examination: Following euthanasia, sciatic nerves were excised, fixed, and sectioned for staining with hematoxylin-eosin, Toluidine blue, and Luxol fast blue. Axonal and myelin sheath morphology was analyzed under light microscopy (Figures 7 and 8). Myelin was quantified using Luxol fast blue, revealing structural changes and demyelination. Measurements were taken from proximal, mid-graft, and distal sections to evaluate regeneration. All study data was evaluated by a blinded researcher to avoid bias among the groups.

Statistical analysis

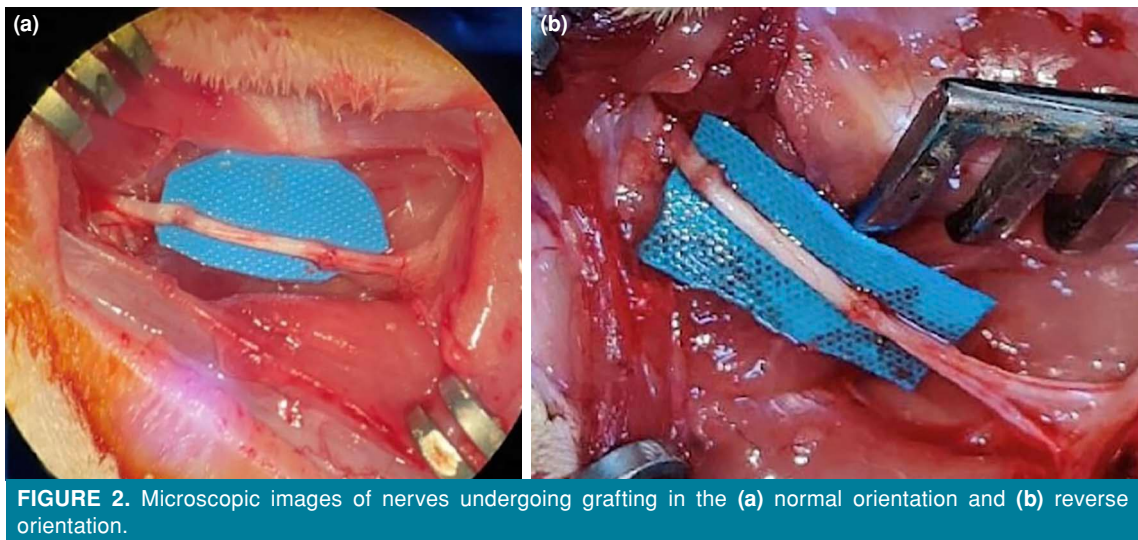
Statistical analysis was performed using the IBM SPSS version 26.0 software (IBM Corp., Armonk, NY, USA). Normality distribution and homogeneity of variance of the data were assessed using the Kolmogorov-Smirnov and Levene tests, respectively. Following these tests, one-way analysis



of variance (ANOVA) was applied. In cases where the analysis yielded statistically significant results, Fisher's least significant difference (LSD) post-hoc analysis was used for pairwise comparisons. In cases where the normality assumption was not met, the non-parametric Kruskal-Wallis test was applied, and subsequently, the Mann-Whitney U test was used for pair wise comparisons. A p value of <0.05 was considered statistically significant.

RESULTS

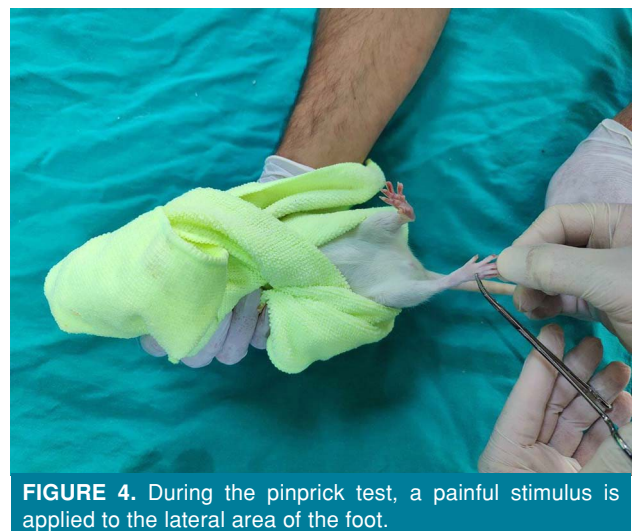
Evaluation of the results revealed no statistically significant difference in EPT between groups undergoing grafting in normal versus reverse orientations ($p=0.679$). Similarly, no statistically significant difference in gastrocnemius weight ratios was observed between groups receiving grafts in normal versus reversed orientations ($p=0.716$).



Since both the EPT and gastrocnemius weight ratio evaluate motor capabilities, the motor function results were similar for both groups.

An assessment of sensory functions was conducted via pinprick and hot plate testing. A comparative analysis of pinprick test scores revealed Group A's superiority over Group B. There

was no statistically significant difference in pinprick test scores between the control group and Group A ($p > 0.05$). However, a comparison of hot plate test scores revealed a statistically significant reduction in withdrawal latency for Group A relative to Group B ($p = 0.007$ and $p = 0.001$). The experiment was stopped in Group B after four rats did not react within 12 sec and failed to remove their feet from the hot plate, thus preventing potential tissue damage. While Group A exhibited superior performance in duration, statistical analysis revealed no significant difference between the groups ($p > 0.05$). In terms of sensory functions, the group with grafting in the normal orientation demonstrated superior functional scores compared with the group with grafting in the reverse orientation.



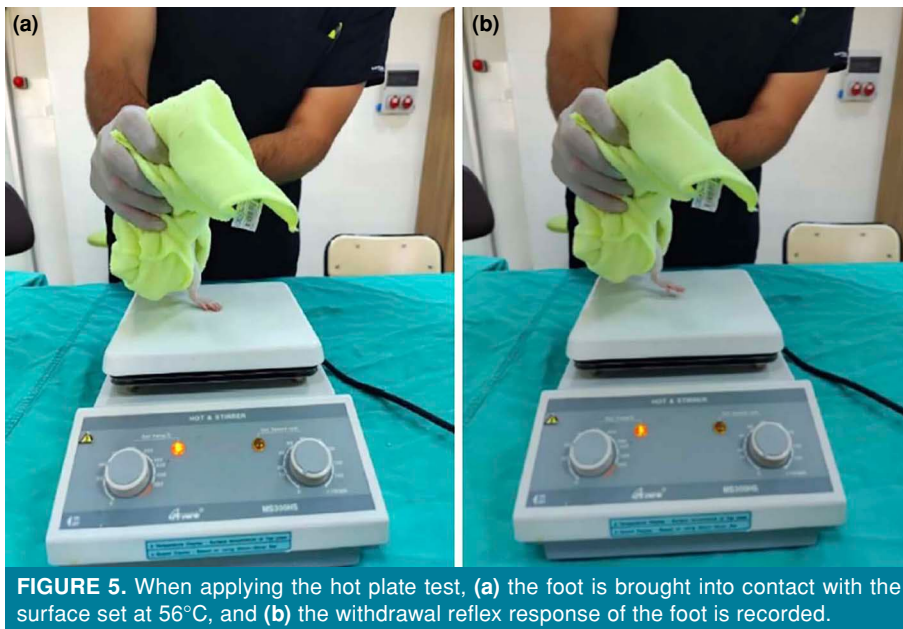


FIGURE 5. When applying the hot plate test, (a) the foot is brought into contact with the surface set at 56°C, and (b) the withdrawal reflex response of the foot is recorded.

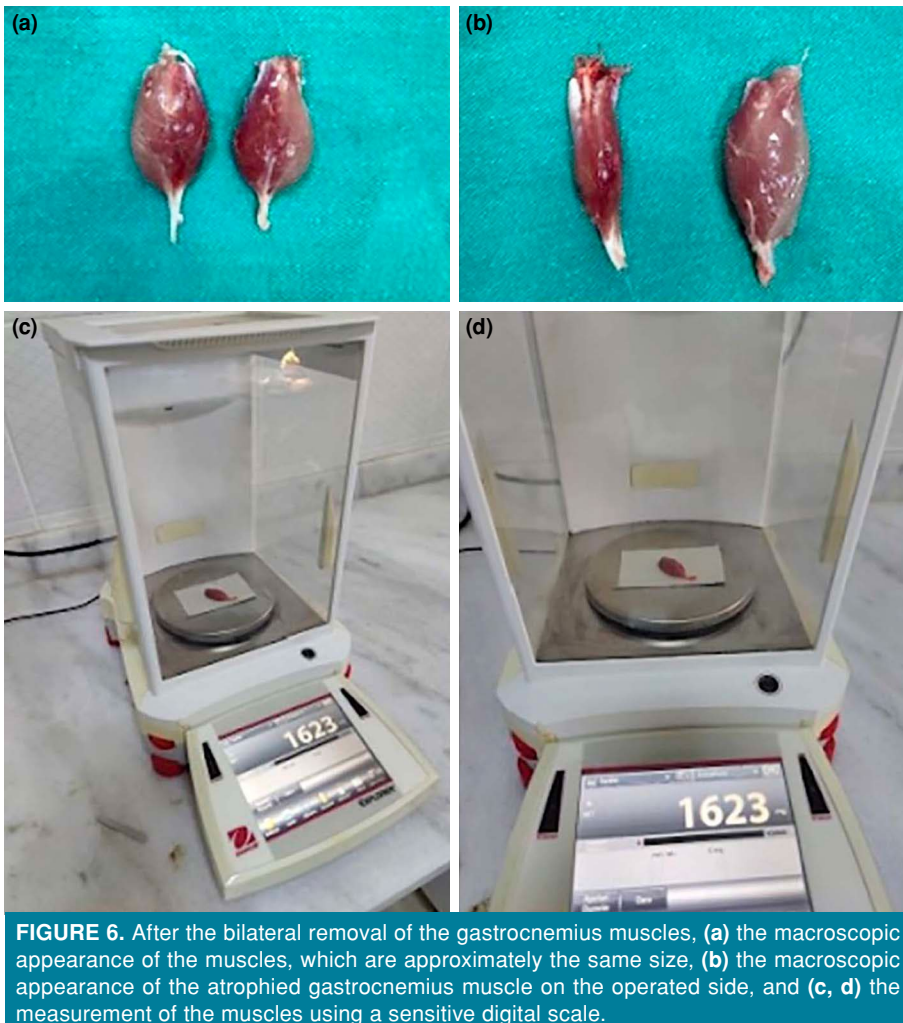


FIGURE 6. After the bilateral removal of the gastrocnemius muscles, (a) the macroscopic appearance of the muscles, which are approximately the same size, (b) the macroscopic appearance of the atrophied gastrocnemius muscle on the operated side, and (c, d) the measurement of the muscles using a sensitive digital scale.

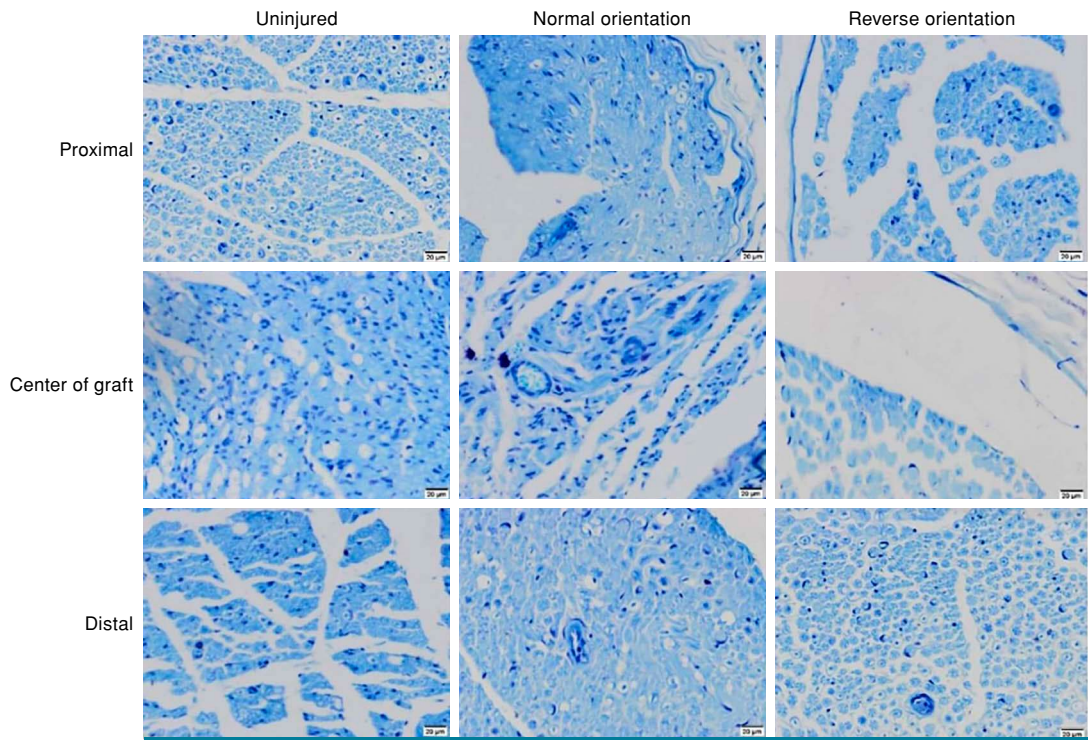


FIGURE 7. Microscopic images of cross-sections taken from the proximal side, middle part of the graft and distal side for all three groups in Toluidine Blue sections.

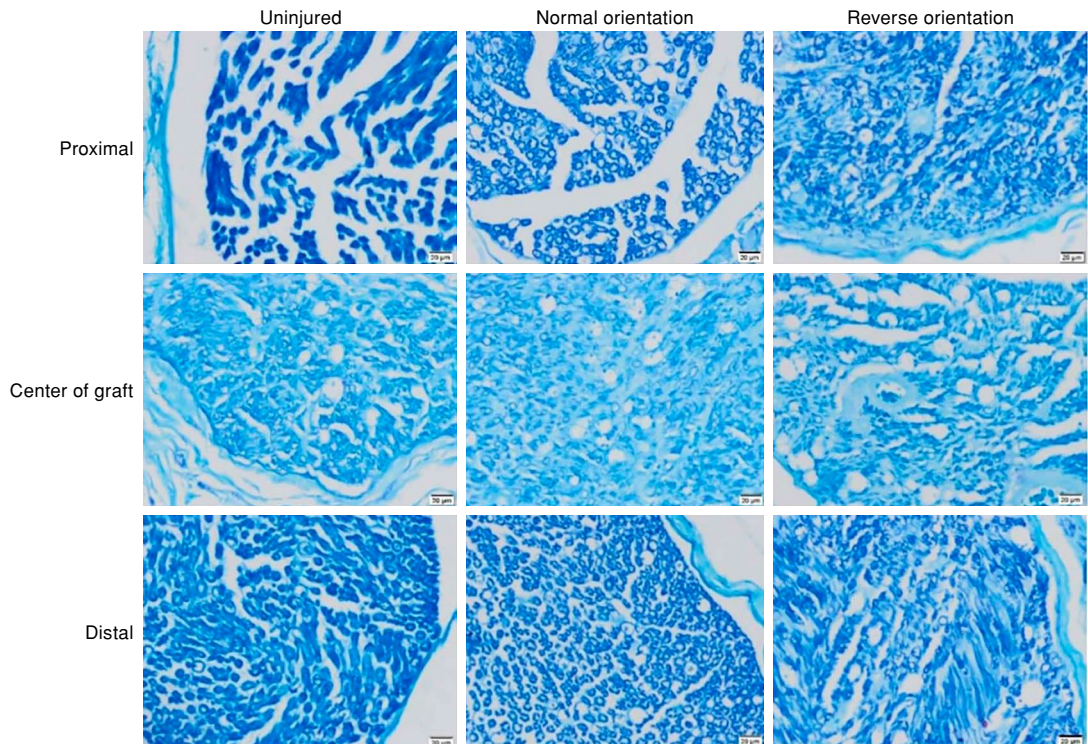


FIGURE 8. Microscopic images of cross-sections taken from the proximal side, middle part of the graft and distal side for all three groups in Luxol fast blue sections.

Histological analysis was conducted on sections stained with Toluidine blue and Luxol fast blue. Proximal, middle, and distal graft sections underwent assessment. Toluidine blue was used to evaluate axon counts. The axon count ratio was determined by computing the number of distal axons divided by the number of proximal axons. There was no statistically significant difference in axon count change index between the two groups ($p>0.05$). Luxol fast blue was used to assess myelin sheath thickness. The ratio of distal to proximal myelin sheath thickness was calculated and designated as the myelin sheath thickness ratio. Analysis indicated no significant difference in myelin sheath thickness ratio between the groups ($p>0.05$).

Based on histological data, no significant difference was observed in distal axon counts and myelination between grafts with normal and reversed orientations ($p>0.05$). Histomorphometric analysis revealed no correlation between graft orientation and outcome.

The control group exhibited statistically significantly superior performance in terms of thrust force ratio compared to Groups A and B ($p<0.05$). However, no significant difference was observed between Group A and Group B ($p=0.679$).

No significant difference in pinprick scores was found between the control group and Group A ($p>0.05$). Conversely, a statistically significant difference was noted between the control group and Group B ($p=0.028$). Moreover, Group A exhibited significantly superior pinprick test results than Group B ($p=0.017$).

Comparing the groups according to the response time of withdrawing feet in the hot plate test, significant differences were observed. A statistically significant difference in latency to withdraw from the hot plate was observed between the control group and Groups A ($p=0.007$) and B ($p=0.001$). However, no significant difference was detected between Group A and Group B ($p>0.05$).

Considering the gastrocnemius weight ratios, the control group had significantly higher ratios compared with Groups A and B ($p<0.005$). However, no significant difference in gastrocnemius weight ratio was found between Groups A and B ($p=0.716$).

The control group exhibited a significantly different axon count compared to Group A and Group B ($p<0.05$). Nonetheless, no statistically significant difference in axon count

alterations was observed between Group A and Group B ($p>0.05$).

A statistically significant difference in myelin sheath thickness ratios was observed between the control group and both Group A and Group B ($p=0.024$). However, there was no significant difference between Group A and Group B regarding myelin sheath thickness ratio ($p>0.05$).

DISCUSSION

In this study, we investigated the effect of nerve graft orientation on functional and histological recovery in a rat model of sciatic nerve injury. Our study results showed that graft orientation did not significantly affect motor function recovery, as both Group A (normal orientation) and Group B (reverse orientation) demonstrated similar outcomes in the EPT test. However, Group A exhibited superior sensory recovery, particularly in the pinprick test ($p=0.028$), indicating that normal orientation may facilitate improved sensory regeneration. Histological outcomes, including axon counts and myelin sheath thickness, were comparable between the two groups, suggesting that graft orientation does not significantly influence these structural parameters.

Following nerve injury, axonal sprouts from the proximal stump require proper guidance to reach the distal target organ. Misalignment during nerve repair may prevent axons from appropriately connecting to their targets, resulting in complications such as neuroma formation.^[10] Distally, the degenerative processes of Wallerian degeneration play a critical role in creating an environment conducive to regeneration, with Schwann cells, macrophages, and phagocytes clearing debris and forming Büngner bands which guide regenerating axons.^[11-13] Based on these mechanisms, our findings suggest that the alignment of grafts in the normal orientation better facilitates sensory axonal regeneration, potentially due to improved structural and biological cues at the repair site.

Numerous factors impact nerve regeneration, including the injury type, patient attributes, and surgical methodology. Compressive injuries of low energy, characterized by preserved nerve continuity, usually exhibit superior outcomes compared to those of high energy with substantial nerve damage.^[14] Regarding surgical factors, early repair reduces fibrosis and tension at the repair site, while precise fascicular alignment

minimizes axonal misrouting and poor functional outcomes.^[15,16] Surgical repair was achieved through the placement of four epineural sutures at each repair site, thereby optimizing alignment and mitigating neuroma formation. Significantly, no neuromas were found upon macroscopic or microscopic examination of the repaired nerves.

A key contribution of this study is its role in standardizing experimental approaches to nerve grafting.^[17] While previous studies have investigated graft orientation, variability in repair techniques and graft selection has limited their generalizability.^[18-27] Our study employed a sciatic nerve graft in a controlled setting to avoid confounding factors, such as diameter mismatches or immune responses, allowing for a more direct evaluation of the effects of graft orientation.

In clinical practice, peripheral nerve injuries often require donor nerves, such as the sural nerve to bridge large defects. These clinical scenarios introduce additional complexities, including diameter mismatches, fascicular misalignment, and structural thinning of donor nerve segments.^[14] While our experimental model used a controlled setup with a sciatic nerve graft, this may not fully replicate the challenges faced in clinical applications. To illustrate, larger defects exceeding 1 cm introduce greater risks of misalignment, tension at the repair site, and functional loss. Future studies should incorporate models using donor nerves, such as the sural nerve, in both normal and reversed orientations to evaluate these additional variables.

As the defect size increases, the orientation and continuity of fascicles become more challenging to maintain. Distal segments of peripheral nerves, which are commonly used in longer grafts, exhibit structural thinning and reduced fascicular density, limiting their regenerative capacity. These factors were not addressed in this study due to the use of a relatively short graft length (10 mm). Larger defects, exceeding 1 cm, may amplify the challenges associated with fascicular thinning and misalignment. Additionally, tension and mismatch at the repair site may further impede axonal regeneration and functional recovery. Advanced techniques such as three-dimensional fascicular mapping and the use of biomaterials that mimic the structural properties of nerves, may provide solutions to these challenges in future studies.

Electrophysiological evaluations, including electromyography (EMG) and nerve conduction

studies, are well-established methods for assessing functional recovery in animal models.^[28,29] While our study relied on behavioral and histological assessments, incorporating these techniques could provide additional insights into the integrity and functionality of regenerating nerves. Their inclusion in future research would strengthen the translational relevance of the findings and improve the robustness of the conclusions.

Nonetheless, this study has several limitations. First, the follow-up period was relatively short (12 weeks), although it was sufficient to observe the primary regenerative processes in a rat model where axonal regeneration occurs at a rate of 2 to 3.5 mm/day.^[28,29] Second, the use of a graft length equal to the defect size may not fully reflect clinical practices, where grafts are typically 10 to 20% longer to reduce tension at the repair site.^[30] Third, the study also did not include assessments of the opposite healthy leg, which could have provided insights into the systemic effects of recovery on the healthy limb and body after surgical intervention. Furthermore, the absence of detailed three-dimensional fascicular analysis of the sciatic nerve segment limits the ability to fully understand the structural alignment and regenerative mechanisms at play. Future studies should aim to address these limitations by incorporating longer grafts, donor nerve models, bilateral assessments, and advanced imaging and electrophysiological techniques to enhance the clinical relevance of the findings.

In conclusion, this study demonstrates that nerve graft orientation does not significantly impact motor function recovery, but may have a notable influence on sensory outcomes. Both functional and histological findings suggest that normal graft orientation provides an advantage in sensory regeneration, potentially due to improved fascicular alignment and biological guidance. Future studies should investigate the long-term effects of graft orientation on nerve regeneration to better understand its role in clinical scenarios, particularly for sensory recovery. Incorporating advanced assessment techniques, such as nerve conduction studies and three-dimensional fascicular mapping, could provide deeper insights into the structural and functional outcomes of nerve repair. Additionally, evaluating the influence of graft length and the use of donor nerves in larger defects would be helpful to bridge the gap between experimental findings and clinical applications. These findings lay the groundwork

for refining peripheral nerve repair strategies, contributing to improved outcomes for patients with nerve injuries.

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions: Was involved in protocol development, gaining ethical approval, all surgical procedures and data analysis: M.E.; Was involved microscopic surgical procedures: E.S.E.; Researched literature and conceived the study: S.S., E.E., E.O., S.Ç., A.G. All authors reviewed and edited the manuscript and approved the final version of the manuscript.

Conflict of Interest: The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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