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Coaptation region selection and functional monitoring in direct muscle neurotization: An experimental study with sprague-dawley rats

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Abstract

This study aimed to identify suitable muscle regions for direct muscle neurotization and the effects of muscle neurotization on functional status. This is an experimental in vivo study. Forty 40 Sprague-Dawley rats were divided into 5 groups, and each group included 8 rats. Muscle neurotization was not performed in group 1. In group 2, end-to-end anastomosis was performed between the free endings of the peroneal nerve and the tibial nerve. In groups 3, 4, and 5, the peroneal nerve was implanted inside a 2 mm pouch opened at the proximal, median, and distal ends of the medial gastrocnemius muscle, respectively, and dissected. The results were assessed using the modified Sihler staining method and electromyography. In the rats in group 1, which did not undergo any muscle neurotization procedure, new nerve branches did not form, and peripheral nerves branched out and became thinner. In group 2, the peroneal nerve and tibial nerve endings were anastomosed using the end-to-end technique, and a few new nerve branches formed. The placement of the free ending of the peroneal nerve in the rats in group 3, 4, and 5 in the pouches opened in the proximal, median, and distal parts of the medial gastrocnemius muscle and its end-to-side dissection were associated with the formation of new nerve branches. Among groups 3, 4, and 5, where direct muscle neurotization was performed, the highest rate of new nerve branch formation was in group 3. The muscle neurotization procedure applied to the proximal, median, and distal regions of the gastrocnemius muscle in this study facilitated the formation of new nerve branches, whereas the best outcome was achieved in the proximal region of the muscle and by the end-to-side dissection of the nerve ending when end-to-end nerve ending anastomosis was excluded.

Keywords: Coaptation, muscle neurotization, nerve injury, nerve repair, peripheral nerve

Introduction

Neurotization is defined as the implantation of nerve fascicles into a target tissue (e.g., skin, muscle, cornea) in cases where primary nerve repair is not possible [1,2]. This process may result in the recovery of sensation and tropism [3]. Wallerian degeneration, which is seen as a result of the cutting of axons, is a cause of various neurodegenerative disorders [4], and neurorrhaphy is not possible due to the injury or absence of the distal ending of the nerve [5]. In direct muscle neurotization, distal nerve fascicles are implanted into the muscle to achieve axonal sprouting (reinnervation) in the neuromuscular interface [6]. Primary nerve repair is the gold standard in nerve reconstruction [7]. In cases of gaps between nerve endings, surgical interventions involving direct muscle neurotization, nerve transfer, or nerve grafts can facilitate the optimal closure of these gaps [8]. Thus, axons help the functionalization of sensation by extending toward a recipient nerve, and this process enables the repair of posttraumatic denervation.

Although they cause sensory loss, albeit to a small extent, at the donor site, end-to-end nerve repairs are frequently preferred, and their advantage is that they result in collateral sprouting in sensory nerves without an epineural window [7,9]. Sensory loss

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is associated with the development of various chronic ulcers, trauma development, infection, tissue ischemia, and when it is not prevented, amputation requirement [10,11]. In this context, in proximal sciatic nerve injuries, the limited access to peroneal nerves can be compensated by implanting the saphenous branch of the femoral nerve into the distal of the tibial nerve or into the sural nerve. As a result of this, sensory reconstruction can be achieved in cases of spinal cord injuries or lower extremity peripheral nerve injuries [12].

In 1908, Hacker successfully achieved reinnervation by implanting the motor branch of the accessory nerve originating from segments from the junction of the spinal cord and medulla oblongata to the C6 level into the same muscle of a 12-year-old pediatric patient (trapezius muscle) [13]. On the other hand, peripheral nerve damage can occur as a result of the contraction of muscle fibers in the new terminal plates where skeletal muscle fibers and peripheral nerve interact [14]. This issue limits the usage area of neurotization in human studies. We conducted this experimental in vivo study to select the most suitable muscle region for the implantation of nerve fascicles in direct muscle neurotization and determine the functional outcomes of muscle neurotization applied to this region.

Material and Methods

Ethical Issues

Before starting the study, the necessary ethical permissions were obtained from the Animal Experiments Local Ethics Committee at Adıyaman University (Date: 08/02/2024, Protocol No: 2024/005). In all steps of the study, the conditions recommended by the European Commission were followed.

Design and Animal Material

This is an experimental in vivo study. The sample of the study included 40 adult male and more than 2 weeks Sprague-Dawley rats weighing 350-400 g on average. Anesthesia was induced in the rats by the intramuscular injection of 5 mg/kg xylazine and 50 mg/kg ketamine [15]. Each rat was kept in a separate cage without bedding material (60x50x60 cm height). Light/dark cycles of 12/12 h were implemented at a constant temperature of 19±2°C and a humidity rate between 50% and 70%. Water and a standard pellet diet were provided ad libitum. Histopathological examinations were performed in the laboratories of the Department of Histology and Embryology at the Faculty of Medicine at Adiyaman University.

Surgical Procedure

The hair in the surgical incision site was shaved, and the area was disinfected using betadine. A longitudinal incision was made to separate the region between the vertebral head of the biceps femoris and the superior gluteal muscles along the posterior distal thigh of the hindlimbs. A 1.5-2 cm segment of the sciatic nerve was isolated at the level where it branched out toward the peroneal and tibial nerves. The rats were divided into 5 groups, each group included 8 rats, and the procedures performed in the groups are described below.

Group 1: Proximal tibial nerve resection was performed on a 1 cm segment of the gastrocnemius muscle, the nerve endings were left loose without muscle neurotization, and the incision was closed.

Group 2: End-to-end 11-0 prolene sutures were applied to the free ending of the peroneal nerve and the branch of the tibial nerve entering the medial gastrocnemius, and anastomosis was performed. The incision was then closed.

Group 3: The free ending of the cut peroneal nerve was implanted into (end-to-side dissected) a 2 mm pouch opened in the proximal end of the medial gastrocnemius muscle. Three 11-0 prolene sutures were applied, and the incision was closed.

Group 4: The free ending of the cut peroneal nerve was end-to-side dissected and implanted into a 2 mm pouch opened in the median end of the medial gastrocnemius muscle. Three 11-0 prolene sutures were applied, and the incision was closed.

Group 5: The free ending of the cut peroneal nerve was implanted into (end-to-side dissected) a 2 mm pouch opened in the distal end of the medial gastrocnemius muscle. Three 11-0 prolene sutures were applied, and the incision was closed.

To alleviate surgical pain, in the postoperative stage, carprofen (5.0 mg/kg; Rimadyl, Pfizer, New York, NY) was administered by 1 subcutaneous injection every 12 h. At the 12th postoperative week, the rats were sacrificed.

Histomorphometric Evaluation

In both hindlimbs, the gastrocnemius medialis muscle was dissected along with its nerve pedicle and fixated in 10% formaldehyde. The specimens were transported to the Department of Histology and Embryology at the Faculty of Medicine at Adıyaman University.

The Sihler staining technique was performed in all specimens. In this technique, while all specimens are made transparent, the nerves with an intramuscular course preserve their three-dimensional spatial position and appear in a darker color. This way, the entire neural network, including terminal endings, can be observed [16].

Modified Sihler Staining Method

The specimens were fixated in 10% formaldehyde. The fixated specimen was washed under running water for 1 h. It was kept in 3% Potassium Hydroxide Solution in Ethanol c(KOH) (for each 100 ml 3% c(KOH), 3 drops of 3% H2O2 are added) for at least 3 weeks. The c(KOH) solution was replaced every day. The procedure was completed when the specimen became transparent. The macerated specimen was kept in Sihler solution I (1:1:6 glacial acetic acid: glycerin: 1% aqueous chloral hydrate) for 2 weeks for decalcification. The decalcified specimen was kept in Sihler solution II (1:1:6 Erlich's hematoxylin: glycerin: 1% aqueous chloral hydrate) for 5 weeks for staining. A period of two weeks is recommended for staining [16-18]. In this study, after staining, the specimens were taken back into Sihler solution

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I to rid them of excess stain. This process took 2 days. Some sources, on the other hand, have stated that 1-2 h is sufficient for this process [16-18]. As a consequence, when the muscle was stained in a light purple color, and the nerves were stained in a dark color, the process ended. The stained specimens were washed again under running water for 1 h. They were then kept in 0.05% lithium carbonate solution for 1 h. Clarification was performed by keeping the specimens in glycerin containing a few crystals of thymol at increasing glycerin concentrations of 40%, 60%, 80%, and 100%, 1 day for each concentration. The successfully stained specimens showed dark blue nerves and translucent non-nerve tissues. The specimens that were preserved in glycerin were trimmed and made ready for examination and photography under a stereomicroscope at x100 magnification.

Electromyography

Electromyography (EMG) is used to measure the bioelectric potential created by nerves and muscles. Bioelectric potentials have values ranging from 10μV to 10mV, and they are displayed in waves and graphs by amplification. When a skeletal muscle contracts, motor unit action potentials occur in the form of sharp declines and peaks on the isoelectric line, and these denote the "amplitude". As the degree of the muscle's contraction and tension increases, the amplitude also rises. The time it takes for amplitude formation to occur after an electrical stimulus is called the "latent period". A prolonged latent period indicates nerve and muscle injury [19].

Electrophysiological Evaluation

For the electrophysiological examinations in this study, an EMG Natus Keypoint (Keypoint net v3. 3, Denmark) device was utilized. Recordings were made using 13-mm-long twisted-pair needle electrodes at a diameter of 0.4 mm. The electrodes were placed into the medial gastrocnemius muscle. A 13-mm-long subdermal single needle electrode was used as the ground electrode, and this electrode was placed under the skin along the tibial bone. The peroneal nerve was stimulated by placing 13-mm-long twisted-pair needle electrodes at a diameter of 0.4 mm placed under the skin in the region where it emerged out of the tibial bone. The stimulation process was carried out with stimuli in the form of square waves of 60 μs so that the peroneal nerve would be maximally stimulated using the minimum intensity that would not disperse around the region (1.5-8mA). Stimulation was performed at least 3 times (5-10 sec pause among stimulations) for each of the right and left sides of each rat. The motor responses with negative onset and the shortest latency, the response with the highest amplitude known as 'base to peak', and the time it took the first response to occur after the stimulus (latency) were recorded [20,21].

Statistical Analysis

The collected data were analyzed using the Statistical Package for the Social Sciences (SPSS) 25.0 IBM (Armonk, NY) program. The results were evaluated in a 95% confidence interval and at a significance level of $p<0.05$. Descriptive statistics (mean, standard deviation, minimum, and maximum values) were calculated for the electroneurophysiological parameters. One-way analysis of variance (ANOVA) was used to identify differences in parameter values. The post hoc Scheffe test was used to identify the source of any significant differences detected in the ANOVA.

Results

Figure 1 shows the morphological view of the gastrocnemius muscle, sciatic nerve, tibial nerve, peroneal nerve, and sural nerve of a rat. It is clearly seen that the placement of the nerves inside and outside of the gastrocnemius muscle was demonstrated and the morphological relationships between the muscles and nerves are observed (Figure 1).

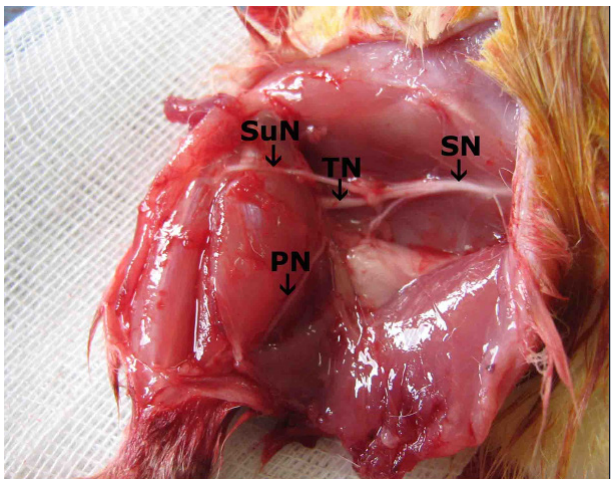

Figure 1. Morphology of the gastrocnemius muscle and three different nerves; SN: sciatic nerve, TN: tibial nerve, PN: peroneal nerve, SuN: sural nerve

Figure 2 displays the morphology of the peripheral nerves of the rats in Group 1. The nerves in the gastrocnemius muscle, which divided into 3 branches, turned into peripheral nerves, became thinner, and formed further branches. The nerve endings that did not undergo muscle neurotization did not form new nerve branches. These branches were divided into three subbranches during their course within the muscle, which showed that peripheral nerves branched out and became thinner before they ended. No new nerve branch formation was observed in Group 1 because the nerve ending was left loose, and no muscle neurotization was performed (Figure 2).

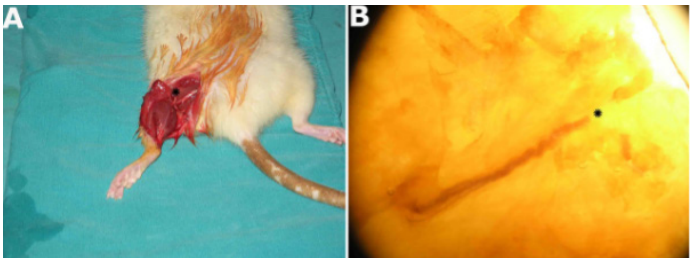

Figure 2. Morphology of the peripheral nerves of the rats in group 1; *Free ending of the peroneal nerve

Figure 3 shows the very thin nerves branching out of the peroneal and tibial nerves of the rats in Group 2. New nerve branches developing due to the end-to-end anastomosis of the

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free ending of the peroneal nerve and the tibial nerve branch entering the medial gastrocnemius muscle were identified in Group 2 (Figure 3).

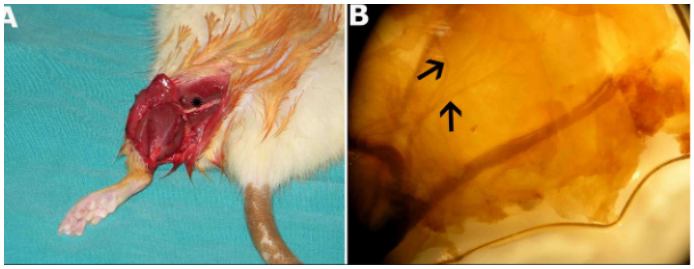

Figure 3. Very thin nerves branching out of the peroneal and tibial nerves of the rats in group 2; * End-to-end anastomosis of the free ending of the peroneal nerve and the tibial nerve branch entering the medial gastrocnemius muscle; → New nerve branches forming out of the remnants of denervated nerves

Figure 4 shows the new nerve branches coming out of the peroneal nerves of the rats in Group 3. Noticeably increased new nerve branch formation out of the remnants of denervated nerves was observed in Group 3, in which the free ending of the peroneal nerve was dissected for implantation into the proximal end of the medial gastrocnemius muscle (Figure 4).

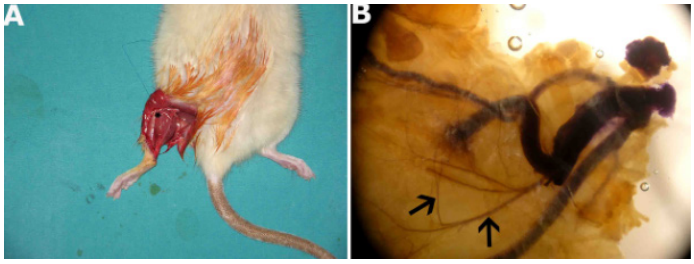

Figure 4. New nerves branching out of the peroneal nerves of the rats in group 3; * Dissected part of the free ending of the peroneal nerve in the proximal end of the medial gastrocnemius muscle; → New nerve branches forming out of denervated nerve remnants (noticeably intense new nerve branch formation)

Figure 5 shows the new nerve branches coming out of the peroneal nerves of the rats in Group 4. Noticeably increased new nerve branch formation out of the remnants of denervated nerves was observed in Group 4, in which the free ending of the peroneal nerve was dissected for implantation into the median end of the medial gastrocnemius muscle (Figure 5).

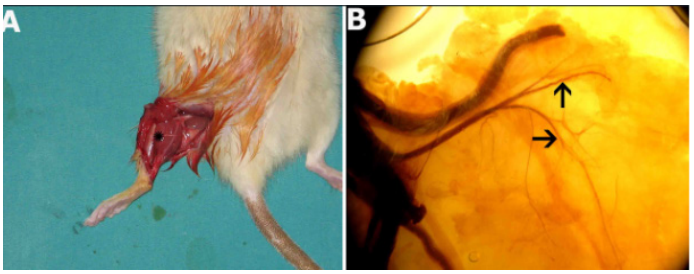

Figure 5. New nerves branching out of the peroneal nerves of the rats in group 4; * Dissected part of the free ending of the peroneal nerve in the median end of the medial gastrocnemius muscle; → New nerve branches forming out of denervated nerve remnants (noticeably intense new nerve branch formation)

Figure 6 shows the new nerve branches coming out of the peroneal nerves of the rats in Group 5. The new nerve branches forming out of the remnants of the denervated nerves as a result of the dissection of the free ending of the peroneal nerve for implantation into the distal end of the medial gastrocnemius muscle were fewer than those in Groups 3 and 4 (Figure 6).

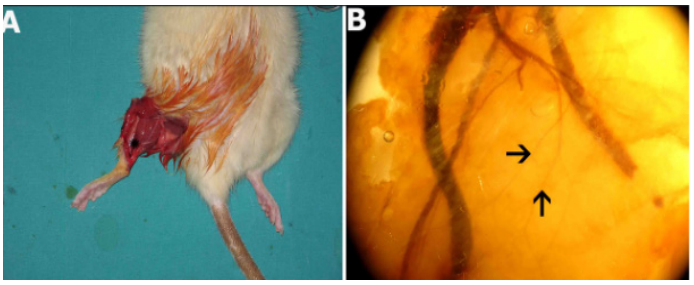

Figure 6. New nerves branching out of the peroneal nerves of the rats in group 5; * Dissected part of the free ending of the peroneal nerve in the distal end of the medial gastrocnemius muscle; → New nerve branches forming out of denervated nerve remnants

Table 1 presents the results of the electroneurophysiological evaluations of the groups that were performed using EMG. No statistically significant difference was found among the groups in terms of single impulse and tetanic impulse values. In the comparisons of the groups in which muscle neurotization was performed, the single twitch and tetanic force values of Group 3 were significantly higher than those in Groups 4 and 5, and the values of Group 2 were higher than 3 (p=0.000).

Table 1. Results of electroneurophysiological evaluations performed by electromyography

Groups	Single impulse (V)	Single twitch (Gr)	Tetanic impulse (Hz)	Tetanic force (Gr)
	x̄±Sd (Min, max)	x̄±Sd (Min, max)	x̄±Sd (Min, max)	x̄±Sd (Min, max)
Group 1	1.13±0.1 (1.05, 1.23)	44.8±25.01 (5, 78.05)	29.8±1.2 (27, 32)	114.23±46.23 (160.47, 68)
Group 2	1.15±0.09 (1.08, 1.22)	88.95±47.5 (19.27, 163.33)	30±1 (29, 31)	179.37±76.62 (59.92, 269.97)
Group 3	1.1±0.07 (1.03, 1.19)	84.56±57.22 (31.83, 219,21)	29.6±1.11 (28, 31)	171.85±84.3 (54.15, 317.58)
Group 4	1.16±0.08 (1.1, 1.23)	50.04±24.04 (38, 86.08)	30.3±0.96 (29, 31)	124.44±52.84 (67.47, 192.7)
Group 5	1.14±0.06 (1.07, 1.24)	49.82±30.66 (6.91, 87.99)	30.2±0.99 (28, 30)	123.7±97.47 (18.75, 322.2)
Test and sig	F=0.211, p=0.187	F=0.416, **p=0.000****	F=0.517, p=0.091	F=0.908, **p=0.000****
Post Hoc	-	Group 2>Group 3>Groups 4 and 5>Group 1		Group 2>Group 3>Groups 4 and 5>Group 1

Single impulse: The product of the force acting on the peroneal nerve and the duration of the action; Single impulse: The fibers of the medial gastrocnemius muscle rapidly contract and relax when they are stimulated by a single electrical impulse at sufficient voltage, One action potential results in one contraction followed by relaxation; Tetanic impulse: Linear and continuous contraction in the medial gastrocnemius muscle; Tetanic force: The force created by the contraction of the medial gastrocnemius muscle in tetanic contractions; F: one-way ANOVA, **p<0.01

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Discussion

The results of this study demonstrated that nerve endings did not create new nerve branches when muscle neurotization was not performed in rats (Group 1). It is seen that the primary repair of nerve endings by end-to-end anastomosis (Group 2), as in some other studies in the literature [7,9] was a more effective treatment method than the methods in the other groups.

Direct muscle neurotization is a method that involves the opening of distal nerve fascicles and their direct implantation into the target muscle to recover sensory and functional capacity, and it provides axonal sprouting in the neuromuscular interface. Its indications usually include surgical or traumatic neuromuscular injuries, as well as avulsion injuries affecting the distal nerve. In this sense, it is performed as a rescue operation and an auxiliary treatment method rather than a primary method [22]. This situation makes it a necessity to conduct relevant studies mostly on animals. Nevertheless, many clinical problems continue to develop in the repair of peripheral nerve injuries and during the recovery period. These problems include those based on findings that nerve graft surgery, which is a method preferred in cases where primary nerve repair is not possible, is associated with multiple fibroses accompanying multiple nerve coaptations [23,24].

Ninety years after the end-to-side nerve repair procedure performed by Ballance et al., on a patient with facial palsy [25], Viterbo et al., formed a bridge between two transected nerves using the intact tibial nerve and the proximal of the peroneal nerve, and conduction to the anterior tibial muscle via this bridge was observed [26]. In another study, the proximal of the tibial nerves of rats was resected, coaptation was performed by opening a perineural window on the side walls of the peroneal nerve, and similar results to those in primary nerve repair were obtained [27]. In a systematic review, 49 preclinical studies that included 1425 animals in total were analyzed, and techniques that were frequently used for nerve coaptation were reported as tissue adhesives, micro sutures, mechanical methods, and lasers [28]. The suture material that is used for nerve coaptation affects neurorrhaphy. However, the aforementioned systematic review did not reveal a certain finding as the examined studies employed various types of sutures. Some previous studies have emphasized that end-to-end nerve repair is associated with better outcomes in terms of neurorrhaphy compared to end-to-side repair [29-31].

The process of axonal elongation and the functionalization of sensation occurs very slowly, and the rate of this process is approximately 1 mm/d' on average [32]. If there is a peripheral nerve injury in the proximal, and the region from the site of injury to the distal muscle innervation area is large, nerve regeneration may take years. In this study, the abundance of the new nerve branches forming as a result of the dissection of the free ending of the peroneal nerve for end-to-side implantation into pouches opened in the proximal (Group 3), median (Group 4), and distal (Group 5) of the medial gastrocnemius muscle was lower compared to their abundance in Group 2. Moreover, the neurotization values closest to the neurophysiological results of Group 2 were in Group 3. Groups 4 and 5 showed muscle injury and denervated muscle characteristics. Such a histological appearance is among the known properties of denervated muscle [33,34], and denervated ends are associated with fibrosis development and muscle atrophy [23,24,32]. These interrelated structures lead to morbidities characterized by sensory and functional disorders. Furthermore, considering that the proximal of this muscle can be vascularized more during circulation, the observation of the best neurotization outcomes in Group 3 was inevitable. The massed action potential (M-wave) develops by the stimulation of motor nerves in muscles. The changes in the amplitude of the M-wave as a result of stimulation occur on the local level, they are usually more intense in the proximal of the muscle, and they are distributed along the electrode channels. This set of processes is compatible with the mechanical properties of muscle fibers [35,36]. Accordingly, the finding of significantly higher single twitch and tetanic force values in Group 3 in comparison to Groups 4 and 5 was in agreement with the information in the literature.

In the treatment of muscle atrophy, coaptation can be applied by using end-to-side neurorrhaphy between a healthy donor nerve and a damaged nerve. This way, a paralyzed muscle can be allowed to regain sensation and function. An increase in muscle mass can be achieved by collateral motor neuron sprouting from the epineural window of the healthy donor nerve to the damaged nerve [15,37,38]. Several previous studies have reported that direct muscle neurotization increases sensitivity to acetylcholine and provides nerve conduction again [39]. In this way, the muscle becomes sensitive to ectopic stimulation. Sprouted axon fibers are directed towards the muscle tissue where nerve conduction has been interrupted [20,21,40]. In this study, end-to-end anastomosis was performed between the free ending of the peroneal nerve and the tibial nerve branch entering the medial gastrocnemius of the rats in Group 2. It was observed that this procedure resulted in the formation of new nerve branches, and these results supported the results of previous studies in the relevant literature. This study has several limitation principles. The sample of this study consisted of rats and the results cannot be generalized to human subjects. In addition, lack of motor function tests didn't perform. These issues were accepted as limitation principles.

Conclusion

In this study, the nerve map of the gastrocnemius muscle was created using the Sihler staining method in a rat model. Additionally, it was shown that the implantation and dissection of the peroneal nerve into pouches opened in varying parts of the medial gastrocnemius muscle (proximal, median, and distal) resulted in different outcomes. In this context, the proximal part of the gastrocnemius muscle seemed more advantageous. Based on these results, we believe that in cases where the end-to-end anastomosis of nerve endings is not possible, performing neurotization to the proximal of the muscle with the free ending of the nerve will be more beneficial.

Conflict of Interests
The authors declare that there is no conflict of interest in the study.

Financial Disclosure
This study was funded by the Scientific Research Projects Directorate Adiyaman University to meet the cost of Sprague-Dawley Rats and laboratory expenses.

Ethical Approval
Before starting the study, the necessary ethical permissions were obtained from the Animal Experiments Local Ethics Committee Adıyaman University (Date: 08/02/2024, Protocol No: 2024/005). In all steps of the study, the conditions recommended by the European Commission were followed.

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