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Effects of repeated transection and coaptation of peripheral nerves on axonal regeneration and motoneuron survival

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Abstract *Background and purpose:* Salvage procedures for facial reanimation can involve a second neurorrhaphy operation. It remains unclear whether reuse of the original donor nerve in the salvage procedure remains likely to produce successful outcome. This study aimed to investigate the effect of repeated transection and coaptation of a nerve on axonal regrowth and motoneuron survival.
Materials and methods: The sciatic nerves of Sprague Dawley rats were transected and microsutured once (the one-time group) or repeatedly at eight-week intervals (the repeated group), and the animals remained alive for eight weeks before sacrifice. The gastrocnemius muscle was weighed, and muscle fiber diameter was measured with hematoxylin-eosin staining. Axonal count of the distal nerve stump was calculated by toluidine blue staining. Myelin thickness and axonal diameter were analyzed by transmission electronic microscopy. Finally, motoneurons were retrogradely traced to the spinal cord using Fluoro-Gold.
Results: Repeated coaptation of nerves resulted in significant decreases of the wet weight ratio of gastrocnemius and muscle fiber diameter. The axonal counts and myelin thicknesses of the distal stumps were comparable between the groups, whereas axonal diameter was significantly smaller after repeated injury. Additionally, retrograde tracing demonstrated significantly less motoneurons in the L4–L6 spinal segments of the repeatedly injured animals than that of the one-time group.
Conclusions: Compared with one-time nerve injury, repetitive transection and coaptation of nerves resulted in compromised axonal regeneration, motoneuron survival, and target muscle

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recovery. It is possible that the final functional outcome could also be compromised, and the patients should be counseled accordingly.

Introduction

Technical options for reanimation of a paralyzed face include nerve transfer and free muscle transplantation.[1-4] For recent cases of facial nerve injury, it is assumed that the facial musculature on the affected side remains viable and is able to be reinnervated.[5] Therefore, nerve repair with cross-facial nerve grafts or masseter nerve transfer is preferred.[6-8] For longstanding cases of facial palsy, it is not possible for the paralyzed mimetic muscle to regain function. Thus, microneurovascular muscle transplantation should be chosen to reinstitute major facial movement. However, both techniques carry a risk of failure, as unknown myopathy can sometimes occur. In the authors' own practice, there existed special cases in which the preoperative examination of the donor nerve function revealed no functional impairment, denervation time of the paralyzed face was less than a year, or MRI scanning suggested no tumor invasion of the trigeminal nucleus (in case of masseter nerve transfer), but the re-innervated musculature or the muscle transplant failed to generate sufficient excursion 6–12 months postoperatively. Under such circumstance, salvage procedure with muscle transfer is under consideration.[9,10] Nevertheless, it could be struggling for the surgeons and patients to decide whether to reuse the original donor nerve or to seek for another substitute to power the new muscle, as employment of a second nerve would inevitably result in additional donor site morbidity. Reuse of the primary donor nerve may be an option in this situation, but it has not been well studied thus far whether re-coaptation of the previously used nerve is as likely to gain successful outcome as the primary attempt.

One key question for reusing the primary donor nerve is whether the quality of the salvaged nerve is comparable to that of a fresh one. After transection and surgical coaptation, axons have the capacity to regenerate at a rate of approximately 1 mm per day,[11] but axonal loss can occur while the nerve is regenerating at the coaptation interface due to tributary outgrowth.[12,13] Furthermore, it has been reported that peripheral nerve injury can elicit apoptosis of motoneurons depending on injury type and the distance from the lesion site to the cell body,[14,15] undermining functional recovery. Despite these findings, there have been few investigations regarding the effect of repeated nerve injury on axonal regeneration and motoneuron survival. Some publications reported accelerated axonal growth induced by a lesion 7–14 days prior,[16,17] but recent studies have indicated loss of motoneuron and impairment of functional recovery caused by repeated crush injury.[18-20] However, to the best of our knowledge, there has been no study reporting the impact of repeated nerve transection and coaptation with longer intervals on nerve regeneration and motoneuron survival.

The present study simulated the surgical procedure by repeatedly transecting and suturing the sciatic nerve in rats at intervals of 8 weeks. We investigated axonal regeneration, muscle recovery, and motoneuron survival after one-time and repeated nerve injury and repair. The results will be helpful for the understanding of nerve regeneration after repeated injury with reasonable expectations in salvage procedures.

Materials and methods

Animals

Twenty-four male Sprague Dawley rats (200–220 g) were used for animal modeling. The animals were housed under specific pathogen-free conditions with the approval of the Institutional Animal Care and Use Committee of Shanghai Ninth People's Hospital. All procedures were performed aseptically, with animals deeply anesthetized using intraperitoneal injection of pentobarbital (40 mg/kg).

Transection and coaptation of the sciatic nerve

The animals were randomly divided into two groups: (1) one-time transection and coaptation of the sciatic nerve (the one-time group); (2) repeated transection and coaptation of the sciatic nerve (the repeated group). Briefly, the sciatic nerve of one leg was exposed and sharply transected using micro-scissors 10 mm from the piriform muscle. The nerve endings were then carefully aligned under the microscope. Standard end-to-end epineurial coaptation was performed with four interrupted 9/0 nylon sutures. The one-time group was harvested 8 weeks postinjury, whereas in the repeated group, the injury site was then sharply transected and repaired again as depicted above. The animals in the repeated group were allowed to survive for another 8 weeks and were harvested for subsequent assessment (16 weeks after the first injury, shown in Figure 1). Six animals in each group were used for histological analysis and transmission electronic microscopy, while another six in each group were used for retrograde tracing.

Histological analysis of gastrocnemius muscle

To assess denervation atrophy of the gastrocnemius muscle, bilateral gastrocnemius muscles were dissected after transcardiac perfusion with 4% paraformaldehyde under deep anesthesia, and the wet muscle weight was immediately measured. The muscle samples were then postfixed in the same fixative overnight. A $10 \times 10 \times 10$ mm^3 cubic biopsy was obtained from the mid portion of the dorsal side of the gastrocnemius, sectioned along the transversal axis after paraffin embedding, and stained with hematoxylin-eosin.

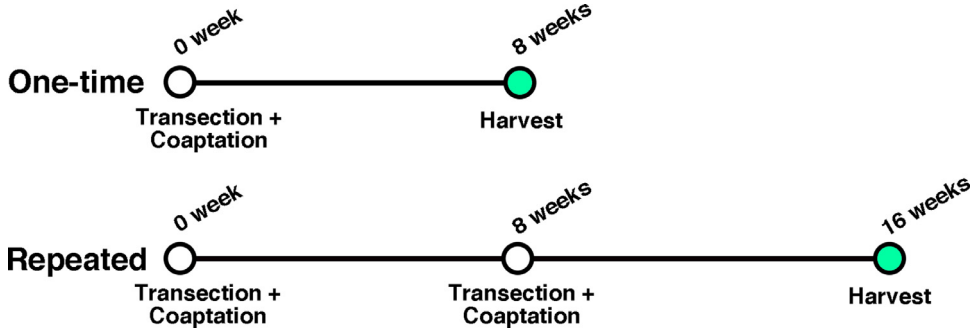

Figure 1 Schematic timeline of animal modeling demonstrating one-time or repeated transection and coaptation of the sciatic nerve with 8-week intervals. The animals were sacrificed 8 weeks after the final injury and repair.

The diameter of the muscle fibers was measured under a light microscope at high-power field (HPF) using ImageJ software (National Institutes of Health, USA). Five HPFs were randomly selected for each sample.

Transmission electronic microscopy

To analyze the myelination of the regenerated nerve, sciatic nerve samples were investigated under a transmission electronic microscope (TEM). Five-millimeter nerve specimens were harvested 2 mm distal to the injury site for transmission electronic microscopy studies, with the animals under deep anesthesia. The nerve samples were fixed with 2.5% glutaraldehyde overnight. After osmication, dehydration, and Epon embedding, 100-nm semi-thin sections were cut transversally and stained with toluidine blue. Myelinated axons in the entire cross section of the proximal and distal nerve specimen were calculated under a light microscope. Fifty-nanometer ultrathin sections were then double-stained with uranyl acetate and lead citrate before observation under a TEM (Model JEM-1200 EX, JEOL, Japan). The thickness of the myelin sheath and the diameter of the myelinated axons were measured by a blinded assessor using ImageJ (National Institutes of Health, USA).

Retrograde tracing with Fluoro-Gold

Motoneurons were labeled with Fluoro-Gold (FG; Fluorochrome Inc., Denver, CO, USA) 8 weeks after nerve injury in the one-time group and 8 weeks after the second injury in the repeated group. Briefly, after the sciatic nerve was appropriately exposed under deep anesthesia, 0.5 μL 4% FG was injected intraneurally using a micropipette that was punctured through the epineurium and advanced to the coaptation site. Forty-eight hours later, the animals were deeply anesthetized, followed by transcardiac perfusion with 4% paraformaldehyde. L4–L6 segments of the spine were dissected and post-fixed in the same solution overnight at 4 °C before dehydration with 10—30% sucrose. Fifty-micrometer frozen sections were serially cut from the spine samples longitudinally in a cryostat and mounted on glass slides. FG-labeled motoneurons were then counted using a fluoromicroscope (Leica DM2500; Leica, Germany) at an excitation wavelength of 430 nm by a blinded assessor. The number of the neurons was corrected by the method introduced by Abercrombie.[21]

Statistical analysis

All values were expressed as the mean ± standard deviation (SD). Statistical significance between the one-time group and the repeated group was verified using unpaired Student's *t*-tests. Probability (*p*) values less than 0.05 were considered statistically significant.

Results

Effect of repeated nerve transection and coaptation on muscle recovery

Peripheral nerve injury results in denervation atrophy of its innervated muscle that recovers as nerves regenerate and re-innervate.[22] In the present study, one-time transection and coaptation of the sciatic nerve elicited a significant decrease in the ipsilateral gastrocnemius muscle weight compared to the contralateral counterpart 8 weeks postinjury (1.576 ± 0.2713 g versus 2.956 ± 0.6878 g, $p = 0.0006$). Reasonably, repeated nerve transection and repair led to a significantly lower wet weight ratio in the repeated group than in the one-time group (33.31% ± 1.225% versus 56.45% ± 5.198%, $p = 0.0015$, Figure 2). Nerve injury and coaptation also caused a decline in muscle fiber diameter, and the repeated group showed significantly smaller muscle fiber diameter ratio than did the one-time group (53.62% ± 3.301% versus 81.98% ± 3.164%, $p = 0.0001$, Figure 2).

Effect of repeated nerve transection and coaptation on axon regeneration

Myelinated axons were observed on toluidine blue staining of the distal nerve stump. Repeated nerve transection and coaptation did not elicit significant variation of myelinated axon counts in the entire cross section compared to the one-time group (10,971 ± 868.0 versus 10,609 ± 846.6, $p = 0.7712$, Figure 3). Furthermore, myelin sheath thickness was comparable in both groups (417.4 ± 6.577 nm

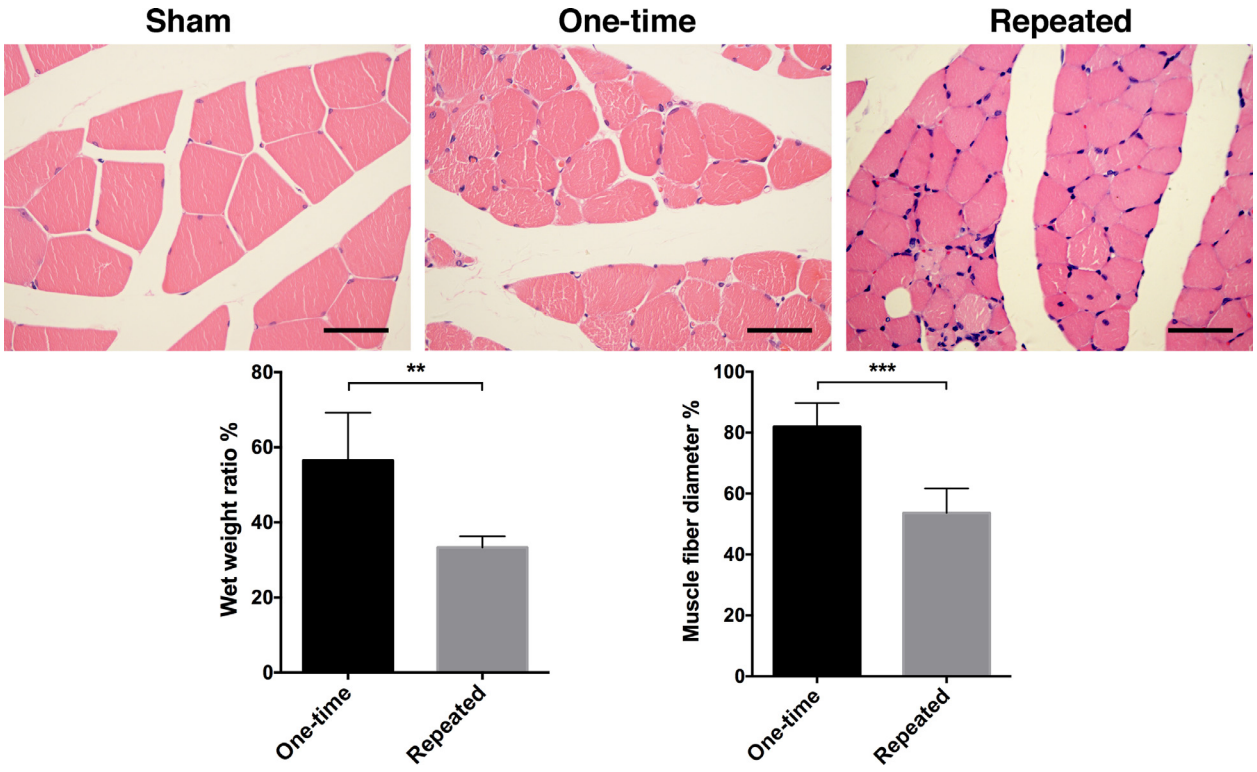

Figure 2 Repeated nerve transection and coaptation impair target muscle recovery. Representative images of gastrocnemius samples stained with hematoxylin-eosin *(above)*. Scale bars = 50 μm. The wet weight ratio *(below, left)* and the ratio of muscle fiber diameter *(below, right)* of the gastrocnemius were significantly lower in the repeated group than in the one-time group. $^{**}p < 0.01$, $^{***}p < 0.001$.

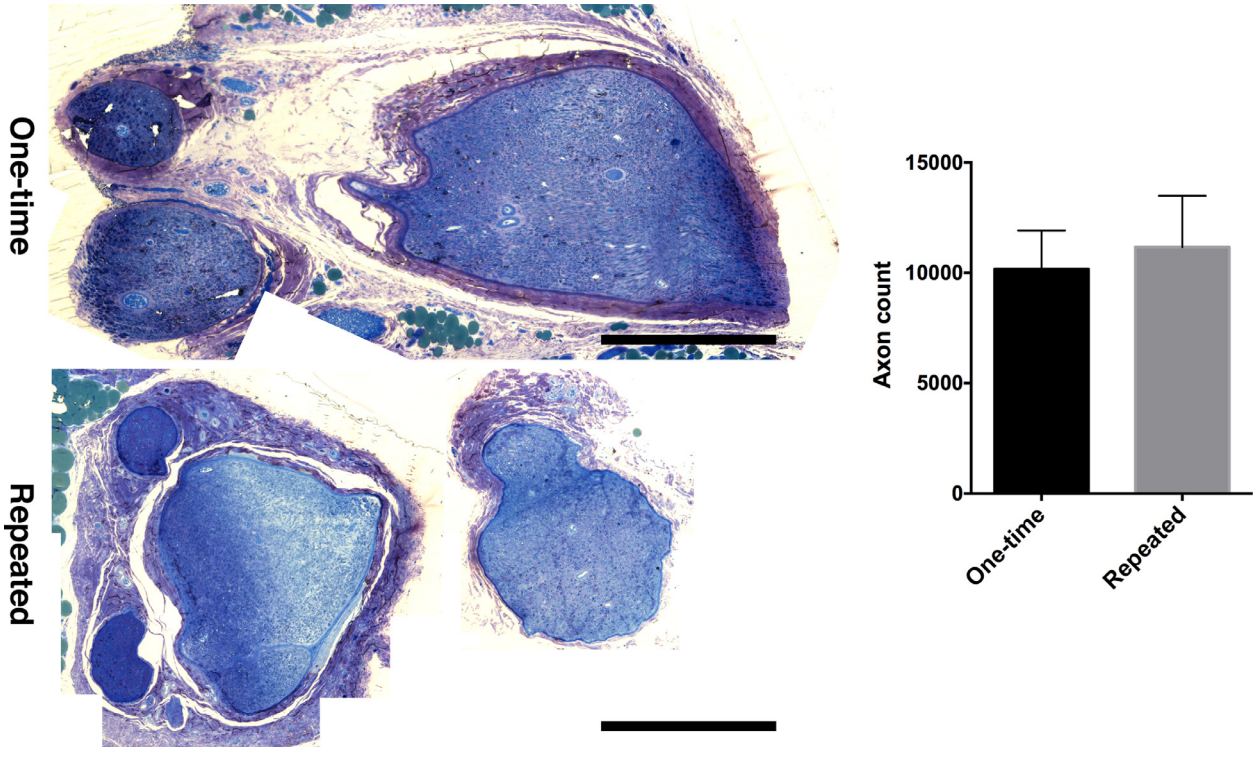

Figure 3 Repeated nerve injury has no significant impact on the number of axons in the distal nerve stump. Representative and assembled images of the entire distal nerve stump sectioned transversally and stained with toluidine blue *(left)*. Scale bars: 500 μm. The axonal count of the repeatedly transected nerve in the distal stump is comparable to that of the one-time counterpart *(right)*.

versus 440.4 ± 8.730 nm, $p = 0.0619$). However, TEM analysis revealed significantly lower axonal diameter in the repeated group than in the one-time group (1.316 ± 0.03786 μm versus 1.748 ± 0.03341 μm, $p < 0.0001$, Figure 4).

Effect of repeated nerve transection and coaptation on motoneuron survival

As shown in Figure 5, FG-labeled motoneurons were found in both groups in longitudinal sections of the spinal cord. Quantitative study revealed a lower number of FG-labeled motoneurons in animals with repeated nerve transection and coaptation (142.2 ± 12.85 versus 211.0 ± 21.92, $p = 0.0220$).

Discussion

Salvage procedure with free muscle transplantation can sometimes be necessary for facial reanimation in scenarios where the previous nerve repair fails to work.[23] It is often speculated that using a new donor nerve other than the old one is beneficial for better outcomes, but additional risk and loss of donor function should be taken into consideration. Repeated utilization of the same donor nerve is still possible, yet there is a lack of evidence regarding whether the quality of a repeatedly coapted nerve is comparable to a fresh one. Hence, further understanding of this issue could be informative and helpful in prognosticating the functional outcome in clinical practice. To date, there have been few studies addressing the impact of repeated peripheral nerve injury on nerve outgrowth and functional recovery. Most of them are not comparable with real clinical situations with regard to the type of injury or intervals between the lesions. In our study, the nerve transection model was adopted, as it simulates the actual surgical procedure. The nerves were repaired by micro-suturing as we did in the operating theater and were collected or subjected to second injury and repair after 8 weeks to allow for morphological assessment. Crucial indexes including axonal growth, motoneuron survival, and muscle reinnervation were examined because these are the key factors affecting the final functional outcome. We found a detrimental effect of repeated nerve coaptation on axonal diameter, motoneuron survival, and muscle recovery, whereas axonal number and myelin thickness were not undermined by repeated lesions.

There are numerous studies demonstrating that neonatal neurons and adult sensory neurons undergo apoptosis after peripheral nerve injury, probably due to deprivation of contact with the target organ and the absence of transported trophic factors that are vital to neuron survival.[24,25] As to motoneuron survival in adults after a peripheral lesion, the results are conflicting and could depend on the length of the remaining axons and the injury type.[26–28] Adult motoneurons appear to be less susceptible to peripheral injury-induced degeneration; however, studies have scarcely focused on the effect of repeated lesion on motoneuron survival. There is one study reporting that repeated crush injury of the hypoglossal nerve at one-week interval does not induce cell death of hypoglossal neurons in adult rats.[26] This is probably due to the less severe nerve injury (the integrity and continuity of the peripheral nerve is preserved after nerve crush injury), shorter interval, faster nerve regeneration, and shorter distance from the target organ in a hypoglossal nerve crush model. Transection and repair, on the other hand, is a direct mimic to the clinical scenarios, and our results suggested that repeated injury resulted in a significant decrease in axotomized motoneurons in the spinal cord and subsequently less nerve input to the target muscle.

There is no consensus regarding the effect of repeated nerve transection on axonal regrowth. A number of studies have demonstrated accelerated axonal elongation after a conditioning lesion that is either a crush injury or transection a few hours to 14 days preceding the test lesion.[29,30] In these studies, the intervals between the first and second injury are too short for complete nerve reinnervation of the priming injury, and such a "conditioning lesion effect" could be attributed to injury-induced alteration of metabolism in the neuron cell body that leads to augmented protein and nucleic acid synthesis.[31–33] However, it remains elusive whether axonal regrowth is influenced if the primer lesion properly regenerates after a longer interval. In such scenario, scarring and local fibrosis at the injury site could be the most important factor affecting nerve regeneration. It has been noted that scarring and fibrosis after nerve injury impede neurite growth as a mechanical block and increase branching.[34,35] Our study demonstrated that the axon count of the distal stump after two complete "injury-repair" cycles was comparable to those of the one-time group. However, distal axons in the repeated group are more likely to contain a higher portion of branched axons than are those in the one-time group. Therefore, the inhibitory effect of nerve scarring could be masked by increased nerve branching. On the other hand, repeated injury reduced the axonal diameter, in accordance with previous reports to the effect that collagen deposition at the nerve injury site leads to a reduction in axonal diameter.[36,37] Interestingly, in the present study, similar myelin thickness was shown in both groups, and repeated injury did not appear to impair myelination. It has been reported that repetitive injury induces an accumulation of supernumerary non-neuron cells such as Schwann cells (SCs) that survive for up to 24 weeks at the lesion site.[38] The SCs form onion bulb-like shapes around the myelinated axons after nerve repair. It is possible that the redundant SCs contribute to re-myelination after repetitive injury, leading to comparable myelin thickness as seen in our study.

As repeated injury hampered motoneuron survival and axonal regeneration, it is reasonable that muscle recovery following double injury was also impaired because prompt reinnervation to the denervated muscle is vital to muscle restoration. The wet weight ratio and muscular fiber diameter in the repeated group were significantly lower than those in the one-time group, possibly attributed to impaired nerve reinnervation.

The main limitation in the current study is the insufficiency of direct evidence of motor functional recovery. Although some of the pivotal determinants were analyzed, it has been reported recently that other factors such as sensory feedback also contribute remarkably to the coordination of motor function.[39] Therefore, future studies with more direct proof of the functional outcome, including muscle tetanic power or electromyogram signals, will be more

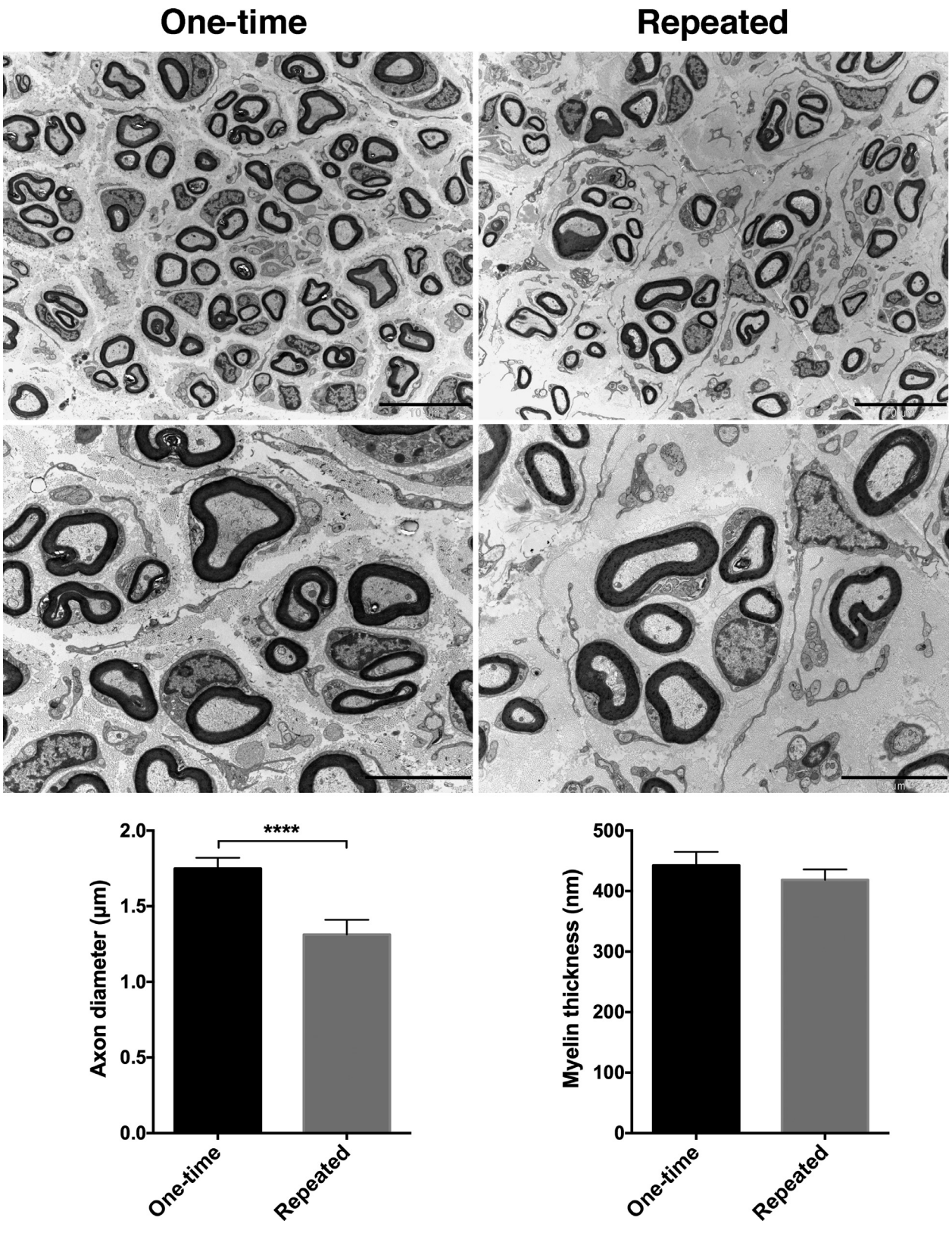

Figure 4 Effect of repeated nerve transection and coaptation on axonal regrowth analyzed under a transmission electron microscope (TEM). Representative images of ultrathin sections of the distal nerve stump under TEM *(above)*. Scale bars: 10 µm (*first row*) and 5 µm (*second row*). Repeated nerve injury significantly decreased axon diameter compared with the one-time injury *(below, left)*. $^{****}p < 0.0001$. Myelin thickness was not significantly affected by repetitive nerve transection and coaptation *(below, right)*.

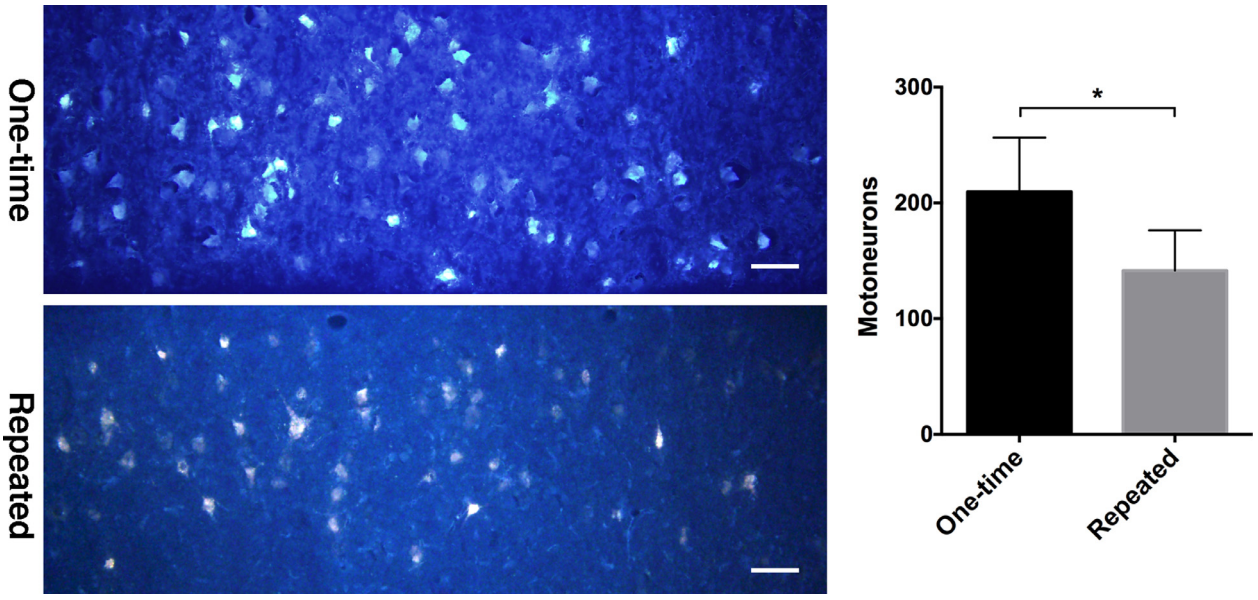

Figure 5 Repeated nerve transection and coaptation are detrimental to motoneuron survival. Representative images of L4-L6 spinal segments retrogradely labeled with Fluoro-Gold *(left)*. Scale bars: 100 μm. The number of motoneurons labeled with Fluoro-Gold was significantly lower after repeated nerve injury than after one-time injury *(right)*. * $p < 0.05$.

helpful for understanding of the final outcome. In addition, a longer time delay of 16 weeks or even longer after injury would be more constructive to functional evaluation.

In summary, our data indicate that peripheral nerve regeneration and motoneuron survival after repetitive nerve transection and coaptation could be worse compared to that after one-time injury. It is likely that the patient whose donor nerve is re-coapted may have a strong maximum contraction after salvage procedure; however, the coordination and accuracy of facial expression might be compromised due to decreased nerve input.[40] In most scenarios when salvage procedures are performed, it usually means that there are few alternatives. Therefore, it is very important to counsel the patients ahead about the possible outcomes.

Acknowledgments

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Conflict of interest

None of the authors has financial interests in any of the products, devices, or drugs mentioned in this manuscript.

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