Early sensory protection in reverse end-to-side neurorrhaphy to improve the functional recovery of chronically denervated muscle in rat: a pilot study

Laboratory investigation

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Object. Early innervation by sensory nerves has been proposed to prevent atrophy of chronically denervated muscle, but conventional end-to-end (ETE) neurorrhaphy has been demonstrated to have adverse effects on muscle contractile function. The aim of the present study was to investigate the potential for modified sensory nerve protection in reverse end-to-side (ETS) neurorrhaphy as a way of improving the functional recovery of denervated muscle.

Methods. Four groups of rats underwent surgical denervation of the tibial nerve projecting to the right hindlimbs (Group 1, unprotected controls; Group 2, positive control [immediate repair without delayed denervation]; Group 3, ETS-protected; and Group 4, ETE-protected). The proximal and distal stumps of the tibial nerve were ligated in all animals except for those in the immediate-repair group. Other animals underwent denervation without sural nerve protection, or with ETE or ETS neurorrhaphy. The ETE- and ETS-protected and unprotected groups underwent an additional surgery in which the trimmed proximal and distal tibial nerve stumps were sutured together. After 3 months of recovery, the tibial function index was determined, and electrophysiological, histological, and morphometric parameters were assessed.

Results. Significant muscle atrophy was observed in the unprotected group, while a well-preserved ultrastructure was observed for the gastrocnemius muscle in the ETE- and ETS-protected groups. Enhanced recovery in the ETS-protected group was indicated by the tibial function index, motor nerve conduction velocity, muscle contractile force tests, and the histological results. In contrast, early sensory nerve protection in ETE neurorrhaphy impaired the recovery of the regenerated axons and diminished the contractile force of the denervated muscle.

Conclusions. Early sensory protection in reverse ETS neurorrhaphy is an effective method for improving the functional recovery of chronically denervated muscle following peripheral nerve injury in rats.

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Abbreviations used in this paper: AChR = acetylcholine receptor; ETE = end-to-end; ETS = end-to-side; MNCV = motor nerve conduction velocity; PBS = phosphate-buffered saline; TFI = tibial function index.
before reinnervation is achieved by the native axon. As an alternative, a procedure known as reverse end-to-side (ETS) neurorrhaphy connects the end of a foreign nerve to the distal nerve stump of the injured nerve, thus exchanging the recipient and donor nerves used in the standard ETS technique. Using this method, denervated muscles are protected until axons of the native nerve reach the target, bypassing the need for a second operation.\(^{26}\)

In the present study, it is hypothesized that the early sensory protection conferred by the reverse ETS technique can promote the functional recovery of chronically denervated muscle. To test this, a rat model of chronic denervation was generated in which the denervated targets were protected by foreign sensory nerves using ETE or reverse ETS neurorrhaphy. The functional recovery of the denervated muscle was evaluated by various tests.

**Methods**

**Animals**

Adult female Sprague-Dawley rats weighing 200–250 g were housed under controlled, pathogen-free conditions with free access to pellet food and water. Every effort was made to minimize animal suffering and reduce the number of animals used, according to the Chinese guidelines for the care and use of laboratory animals. All animal surgeries were conducted under the Institutional Animal Care Guidelines, and ethics approval was obtained from the Administration Committee of Peking University People’s Hospital.

**Surgical Procedures**

Rats were randomly divided into 4 groups of 6 animals each. Surgeries were performed on the right hindlimb of each animal. Preoperatively, animals were anesthetized by an intraperitoneal injection of sodium pentobarbital (30 mg/kg). The right sciatic nerve was exposed through a longitudinal incision posterior and parallel to the femur. The nerve was dissected distal to its trifurcation to free the tibial, peroneal, and sural nerves.

Rats in Groups 1 and 2 were subjected to the following procedures. The tibial nerve was transected 6.5 mm proximal from the entry point of the nerve into the gastrocnemius muscle. The proximal stump of the nerve was doubly ligated and sewn into a blind-ending silicone cap to prevent regenerating fibers from reaching the distal stump. The capped proximal stump was then sutured onto the superficial surface of the biceps femoris muscle (Fig. 1A). Group 1 served as unprotected controls; Group 2 served as a positive control (immediate repair without delayed denervation). In these animals, the epineural sheath of the proximal stump was attached to the distal stump of the tibial nerve with a 10-0 nylon suture (Fig. 1B).

For Group 3 (ETS-protected), an epineural window was created on the distal side of the tibial nerve at a site 3.5 mm proximal from the entry point of the tibial nerve into the gastrocnemius muscle. The sural nerves were divided, and the proximal ends were sutured by reverse ETS to the epineural window. The tibial nerves were then transected 6.5 mm proximal to the nerve’s entry point into the gastrocnemius muscle. Both proximal and distal nerve stumps were covered with a silicone cap to prevent spontaneous reinnervation by the tibial nerve (Fig. 1C). For Group 4 (ETE-protected), the tibial nerve was transected 3.5 mm proximal to the entry point of the nerve into the gastrocnemius muscle. The proximal stump of the sural nerve was sutured to the distal stump of the tibial nerve in an ETE neurorrhaphy (Fig. 1D). The surgical wound was irrigated thoroughly and closed in layers, and the animals were allowed to recover.

Three months after the initial surgery, animals in Groups 1, 3, and 4 underwent a second surgical procedure. Following anesthetization, the scar of distal tibial stump and the neuroma of proximal tibial stump were trimmed. The two stumps were sutured together in an ETE neurorrhaphy (Fig. 1). The animals were allowed to recover until the testing phase.

**Walking Track Analysis**

Walking track analyses were performed 12 weeks after the surgery (the second surgery for Groups 1, 3, and 4). Animals were allowed a conditioning trial on a confined walking track (10 × 60 cm) darkened at one end. The bottom of the track was covered with white paper. The animals’ hindlimbs were dipped in black ink before they were placed at the entrance of the track to initiate walking. Footprints on the track were selected for measurement at the time of testing, based on the clarity and completeness of the print from a period of brisk walking. Paired footprint parameters for print length (PL; distance from heel to toe), toe spread (TS; distance from first to fifth toes), and intermediary toe spread (IT; distance from second to fourth toes) were recorded for the normal control foot of each animal’s left hind leg (that is, NPL, NTS, and NIT) and the corresponding right experimental foot (that is, EPL, ETS, and EIT).

The tibial function index (TFI) was calculated according to the Bain-Mackinnon-Hunter formula:

\[
TFI = -37.2 \times (EPL - NPL)/NPL + 104.4 \times (ETS - NTS)/NIT + 45.6 \times (EIT - NIT)/NIT - 8.8.
\]

The investigators who assessed all the outcomes were blinded to the experimental group assignment of each animal.

**Electrophysiological Tests and Determination of Muscle Contractile Force**

The electrophysiological recordings and muscle contractile force tests were conducted after the walking track analysis. The tibial nerve on the surgery side (that is, the right side) was exposed, and the stimulating electrodes were applied to the nerve trunk at positions proximal and distal to the repair site. The recording electrode was placed in the gastrocnemius muscle, while the ground electrode was placed in the subcutaneous tissue between the stimulating and recording electrodes. Six continuous rectangular pulses (0.1-msec duration, 5 mV, 10 Hz) were generated using the Medelec Synergy system (Oxford Instruments Inc.). The motor nerve conduction velocity (MNCV) was determined by dividing the distance between the two stimulation sites by the difference in conduction time.
Recovery of muscle strength was determined by measuring twitch and tetanic tensions in the gastrocnemius muscle. The muscle was freed from surrounding tissue, leaving intact only the proximal point of attachment. The knee and foot were immobilized with clamps, and the distal tendon of the gastrocnemius muscle was connected to force transducers (MLT500/D, ADInstruments) using a nylon ligature. Hooked stimulating electrodes were placed on the tibial nerve trunk proximal to the ETE coaptation site. A single maximal stimulus was delivered to the tibial nerve, and the twitch tension of the entire (medial and lateral) muscle was recorded at the optimal muscle length. Tetanic tension tests were subsequently performed with a 50-Hz stimulation. The monitoring data were recorded and analyzed using Scope software version 3.6.12.

Morphological Evaluation of the Gastrocnemius Muscle

Following the electrophysiological recordings, the gastrocnemius muscle was dissected and tissue was obtained from the midbelly region and fixed in 4% paraformaldehyde solution in phosphate-buffered saline (PBS). The tissue sample was divided into two parts. The first sample was washed in water, dehydrated in a graded ethanol series, cleared in xylene, embedded in paraffin, and cut into 5-mm transverse sections. After H & E staining, the sections were photographed with a DFC 300FX color digital camera (Leica) to allow measurement of the cross-sectional area of muscle fibers. Images were taken of 4 random fields (Leica Q550 IW; Leica Imaging Systems Ltd.) in 4 sections from every specimen, and analyzed with Leica QWin software (Leica Imaging Systems Ltd.). The second sample was flash frozen in isopentane, cooled with solid CO₂, and stored in a sealed plastic tube at −80°C. A series of 5 longitudinal 50-μm sections for each sample were cut on a freezing microtome (Frigocut) at −20°C and collected in PBS. Sections were rinsed twice in PBS, followed by 0.4% Triton X-100 in PBS, and then incubated in 1% bovine serum albumin and 0.05% NaN₃ in PBS for 1 hour. The postsynaptic acetylcholine receptor (AChR) was identified by staining with rhodamine-conjugated α-bungarotoxin. Labeling of this site was achieved by 4–6 hours at room temperature. Sections were washed several times over 1–2 hours in PBS and mounted with Vectashield (Vector Laboratories). The samples were imaged using a DMR fluorescence microscope. Images were acquired using a DMR Fluorescent Microscope (Leica), with an

![Fig. 1. Schematic representation of surgical procedures producing chronic denervation of the gastrocnemius muscle (GM) in the right hindlimb of rats: Group 1, denervation without nerve protection (A); Group 2, immediate tibial nerve (TN) repair (B); Group 3, delayed tibial nerve repair with sensory nerve protection from the sural nerve (SN) by ETE neurorrhaphy (ETE-protected) (C); and Group 4, delayed TN repair with sensory nerve protection from the sural nerve by reverse ETS neurorrhaphy (ETS-protected) (D). PN = peroneal nerve.](image-url)
excitation wavelength of 488 nm. Postsynaptic receptors apposing motor endplates were digitally measured using SigmaScan Pro 5 (Systat Software Inc.).

**Histological Analysis of Nerve Regeneration**

As gastrocnemius muscles were dissected, segments of the tibial nerve 2 mm proximal and 2 mm distal to the ETS coaptation site near its entry point into the muscle were obtained. The segments were fixed for 24 hours in 1% osmium tetroxide, dehydrated with ethanol and embedded in paraffin. The paraffin blocks were cut into 2 mm transverse sections using an ultramicrotome. Five sections from each nerve segment were randomly selected for analysis. Images were acquired on a fluorescence microscope with a DFC 300FX color digital camera. The total number and distribution of the myelinated axons, fiber and axon diameters, and myelin thickness were determined from the images. Morphometric measurements were made using IPP6.0 software. The shortest lengths of the outer and inner margins of the myelin sheath were used to determine the fiber and axon diameters, which were then used to calculate myelin thickness.

**Statistical Analysis**

Data were analyzed with SPSS 16.0 software (SPSS Inc.). Mean differences were determined by 1-way ANOVA and further analyzed with the post hoc Scheffe and Student-Newman-Keuls tests. A p value < 0.05 was considered significant for all statistical comparisons. All values are presented as the mean ± SD.

**Results**

**General Observations**

None of the animals had systemic or regional inflammation or other postsurgical complications. Although the locomotor function of the operated limb was gradually restored, recovery was greater for rats that had undergone nerve protection (both ETE and ETS).

**Tibial Function Index**

The walking track analysis showed that the TFI in the immediate-repair group gradually increased following surgery repair. The TFI values of the ETS-protected, ETE-protected, and unprotected rats were poor due to denervation and gradually increased following the second surgical repair. The TFI values at Week 16 were similar in these 3 groups, which were significantly lower than the TFI in immediate-repair group. After 20 weeks, the TFI
Reverse end-to-side protection for chronic denervation

Motor Nerve Conduction Velocity and Muscle Contractile Force

The MNCVs of the immediate-repair, unprotected, ETS-protected, and ETE-protected groups were 33.88 ± 11.99, 24.93 ± 8.69, 30.25 ± 12.65, and 16.90 ± 7.92 m/sec, respectively (Fig. 3). There was no significant difference in MNCV between the immediate-repair and ETS-protected groups, but these two groups had a significantly higher MNCV than the unprotected and ETE-protected groups (p < 0.05), with the ETE-protected group having the lowest value.

The measurements of contractile force, which combines twitch and tetanic tensions, are shown in Table 1.

<table>
<thead>
<tr>
<th>Group</th>
<th>Twitch (N)</th>
<th>Tetanic (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETS-protected</td>
<td>0.98 ± 0.38†</td>
<td>3.79 ± 1.25†</td>
</tr>
<tr>
<td>ETE-protected</td>
<td>0.75 ± 0.15*</td>
<td>2.75 ± 0.27*</td>
</tr>
<tr>
<td>unprotected control</td>
<td>0.64 ± 0.20</td>
<td>1.67 ± 0.16</td>
</tr>
<tr>
<td>immediate-repair</td>
<td>1.31 ± 0.27‡</td>
<td>4.52 ± 0.57‡</td>
</tr>
</tbody>
</table>

* p < 0.05 versus immediate-repair group.
† p < 0.05 versus ETE-protected group.
‡ p < 0.05 versus unprotected control.

The twitch tensions in animals that underwent immediate nerve repair reached 1.31 ± 0.27 N, which was two times higher than values measured in the unprotected group (0.64 ± 0.20 N; p < 0.05). The twitch tension in the ETS-protected group (0.98 ± 0.38 N) was greater than that in ETE-protected animals (0.75 ± 0.10 N). There was no significant difference in twitch tension between the unprotected and ETE-protected groups. Tetanic tension reached its highest value in the immediate-repair group (p < 0.05), followed by the ETS-protected group, while there was no significant difference between the ETE-protected and unprotected groups.

Evaluation of the Gastrocnemius Muscle Ultrastructure

Upon gross examination of the gastrocnemius muscle, the muscle fibers and surrounding connective tissue in the immediate-repair group were indistinguishable from those of normal tissue (Fig. 4A). The muscle ultrastructure was also preserved in ETE- and ETS-protected animals, although a greater variation in fiber cross-sectional area and a slight increase in the amount of connective tissue were observed (Fig. 4C and D). However, the difference in fiber cross-sectional area between the two protected groups was not statistically significant (Table 2). In contrast, the muscle in the unprotected group had large regions of atrophied fibers and connective tissue hyperplasia (Fig. 4B). The average cross-sectional area of muscle fibers was significantly smaller in the unprotected group than it was in nerve-protected groups (Table 2).

In the evaluation of motor endplates, a strikingly abnormal morphology at postsynaptic AChR sites was observed in all groups. The majority of the AChR plaques in the unprotected group appeared to be less delineated, small, flat, and slender. The average area of AChR sites was smaller in the unprotected group (157.17 ± 101.47 μm²) than it was in nerve-protected groups. In contrast, AChR plaques in protected groups were well defined, similar to those in the immediate-repair control group, displaying thick fringes and a few small, round cupulae with distinct contours. The difference in the areas of AChR sites for the two protected groups was not statistically significant (Table 2).

 Histological Analysis of Nerve Regeneration

The number of myelinated axons at the proximal region of the tibial nerve was similar for all the groups.
The tibial stump distal to the suture site in each group revealed a regeneration of clustered myelinated fibers. The immediate-repair group had a greater number of regenerated myelinated axons (1923 ± 623) than the protected groups, which displayed robust myelination and axonal integrity. The number of regenerated myelinated axons in the ETS- and ETE-protected groups (995 ± 430 and 985 ± 264, respectively) was lower than that in the unprotected group (1760 ± 544; Fig. 5). Light microscopic examination provided evidence of myelin and axon degeneration, which had uneven distribution and low density in ETE-protected animals. A low level of myelin degeneration was observed in the ETS-protected group, while the myelin distribution in the unprotected group was not very uniform.

Animals in the immediate-repair group had greater axon diameter, axon area, and myelin thickness than those in the delayed-repair groups (Table 3). The mean axon diameters in the ETS-protected and unprotected groups were similar and were larger than those in the ETE-protected group (p < 0.05). The mean axon area in the ETE-protected group was smaller than it was in ETS-protected and unprotected groups (p < 0.05); there was no significant difference between the latter two groups. Myelin thickness in the ETE-protected group was significantly lower than in the ETS-protected and unprotected groups (p < 0.05) and was similar between these latter two groups.

**Discussion**

Peripheral nerve injury usually results in poor functional recovery when reinnervation is delayed, either due to the timing of the surgical intervention or the long distance that must be traveled by the regenerating nerve fiber. A suboptimal outcome can be attributed to degeneration of the distal nerve stump and atrophy of the chronically denervated muscle. Therefore, reinnervation within a limited time after injury is essential for maximum functional recovery. One solution, the so-called babysitter technique, is to provide the denervated segments with trophic support from a foreign donor nerve until the native axons regenerate to reach the target. However, adverse effects on muscle force recovery have been reported after the use of the conventional babysitter technique, which involves an ETE neurorrhaphy. In the present study, this technique was modified and performed as a reverse ETS neurorrhaphy. The extent of functional recovery in gastrocnemius muscle chronically denervated after tibial nerve injury was compared for groups of rats subjected to conventional ETE or ETS neurorrhaphy.

Analysis of the data revealed that muscle fiber architecture was preserved by early sensory innervation from either ETE or ETS neurorrhaphy, with ETS neurorrhaphy promoting greater functional recovery after reinnervation by native axons. However, contradicting the prevailing view, early sensory nerve protection in ETE neurorrhaphy impaired myelinated axon regeneration and the functional recovery of the denervated muscle.

The absence of trophic and substrate support from the distal nerve stumps and the atrophy of the denervated target muscle together undermine the reinnervation of a muscle from regenerated axons of native motoneurons. Following acute nerve injury, Schwann cells in the distal nerve stumps undergo proliferation and phenotypic changes in growth factor production to create a local environment to be favorable for axonal regeneration. However, these changes are transient and gradually decrease with prolonged denervation, while chronically denervated muscles become less receptive to regenerated motor axons.

**TABLE 3: Assessment of the regenerated tibial nerve parameters**

<table>
<thead>
<tr>
<th>Group</th>
<th>Axon Diameter (μm)</th>
<th>Axon Area (μm²)</th>
<th>Myelin Thickness (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETS-protected</td>
<td>2.75 ± 1.15†</td>
<td>6.01 ± 5.94†</td>
<td>0.72 ± 0.26†</td>
</tr>
<tr>
<td>ETE-protected</td>
<td>1.90 ± 0.72‡</td>
<td>2.29 ± 2.16‡</td>
<td>0.63 ± 0.15‡</td>
</tr>
<tr>
<td>unprotected</td>
<td>2.63 ± 0.79</td>
<td>4.63 ± 4.17</td>
<td>0.77 ± 0.23</td>
</tr>
<tr>
<td>immediate-repair</td>
<td>3.08 ± 1.09‡</td>
<td>7.77 ± 6.54‡</td>
<td>1.13 ± 0.23‡</td>
</tr>
</tbody>
</table>

* p < 0.05 versus immediate-repair group.
† p < 0.05 versus ETE-protected group.
‡ p < 0.05 versus unprotected control.
that reach the target muscle due to a significant loss of viable muscle cells resulting from a combination of fiber necrosis, connective tissue hyperplasia, and depletion of satellite cells.24,25 The loss of input from neurotransmitters, neurotrophic factors, and other signals also contributes to muscle fiber atrophy and diminished receptivity to regenerated axons.25 The deterioration of the motor endplate also undermines functional recovery following prolonged denervation.38 In the present study, unprotected muscles had large numbers of atrophied fibers and connective tissue hyperplasia, which is in agreement with previous findings.3,13,22 In contrast, with sensory nerve protection the ultrastructure and cross-sectional area of the target muscle, as well as the areas postsynaptic to the motor endplate, were preserved. Although the exact mechanism by which early innervation by foreign nerves preserves the capacity for muscle reinnervation is not fully understood, it may maintain endoneurial pathways and prolong the growth-supportive phenotype of Schwann cells to allow timely reinnervation.39 Foreign axons may support denervated muscle fibers directly, by supplying trophic factors or, indirectly, by promoting repair of the endoneural sheath, thus enhancing the regeneration of the native nerve.22,25 Timely reinnervation induces Schwann cell proliferation20 and the expression of neurotrophic factors such as NGF, BDNF, GDNF, and pleiotrophin, their receptors, and also neuregulin and the cognate receptor.10,24 All of these factors, which are associated with neuronal survival and axonal growth in the peripheral nervous system, may promote the reestablishment of a functional connection at the neuromuscular junction.16,15

The conventional babysitter technique makes temporary use of a foreign donor nerve to neurorhize the distal nerve stump in ETE neurorrhaphy. When regenerated axons from the native proximal nerve stump reach the target muscle, the donor nerve is cut and cross-sutured with the native nerve stump.3,13,19 In this method, distal nerve stumps undergo Wallerian degeneration twice prior to target reinnervation by native axons, adversely affecting axonal regeneration and functional recovery. It was previously reported that two sequential episodes of skeletal muscle denervation and reinnervation resulted in greater force and power deficits than a single occurrence of peripheral nerve injury and repair in rats.23 The present results confirm and extend the previous findings that sensory protection in ETE neurorrhaphy impairs the functional recovery of the denervated muscle because it is subjected to two cycles of denervation and reinnervation. As an alternative to this approach, a modified procedure was used to connect the donor nerve to the distal nerve stump in a reverse ETS neurorrhaphy. In this method, denervated muscles are protected until axons of the native nerve reach their target, eliminating the need for a second operation.26 The results of this study showed that ETS protection was the superior method, as indicated by the TBI, MNCV, muscle contractile force, and histological analysis. Thus, reverse ETS neurorrhaphy is effective in promoting the functional recovery of chronically denervated muscle.

In general, animals subjected to sensory nerve protection regenerated fewer myelinated axons. The ETE-protected groups had smaller mean axon diameters and areas of myelinated fiber, in addition to a thinner myelin sheath. These findings indicate that the presence of sensory nerve axons did not significantly improve motor axon regeneration and myelination but instead undermined both. A few studies have shown that the early presence of sensory axons may induce a change in Schwann cell properties in a mixed nerve stump, which impairs motor axon regeneration and myelination. Schwann cells express distinct sensory or motor characteristics that support regeneration in a phenotype-specific manner.12 The motor phenotype can be modified toward the sensory phenotype through forcible innervation by sensory axons;12 however, sensory nerves may induce a phenotype in Schwann cells that makes them less capable of regenerating motor axons.21 This distinction between sensory and motor Schwann cells may explain the less robust reinnervation that was observed after long-term denervation in the absence of sensory axons. Thus, neurotization by a mixed nerve may be more effective in promoting reinnervation than neurotization by a sensory nerve. This possibility is the subject of future studies.

Conclusions

Early innervation with foreign sensory axons helped to preserve the ultrastructure of chronically denervated muscle fiber architecture. Early sensory protection provided by reverse ETS neurorrhaphy promoted the functional recovery of an injured tibial nerve in rats. This technique could be a useful approach to the restoration of muscle function following peripheral nerve injury. The potential clinical applicability warrants further investigation.

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Disclosure

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Author contributions to the study and manuscript preparation include the following. Conception and design: Jiang, Li, Zhang. Acquisition of data: Li, Yin. Analysis and interpretation of data: Jiang, Li, Han, Kou. Drafting the article: Li, Zhang. Critically revising the article: all authors. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Jiang. Statistical analysis: Yin, Han, Kou. Administrative/technical/material support: Yin, Han, Kou. Study supervision: Jiang, Zhang, Yin.
References


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