In proximal nerve injuries, the main clinical problem is nerve regeneration: how to reach the end organs in sufficient time before muscle atrophy occurs. Distal end-to-end nerve transpositions and end-to-side (ETS) repair have been used in these situations, but with these techniques, the distal end of the nerve is, at least partially, reserved for neurorrhaphy and, thus, cannot be used for further reconstructions. The side-to-side (STS) nerve repair technique, which leaves the distal nerve end free, was introduced by Yüksel et al\(^1\) in 1999. They reported the effects of different size epineural windows and degree of axonal injury of STS repair on nerve regeneration and donor nerve morbidity. 

**Background:** Side-to-side (STS) neurorrhaphy can be performed distally to ensure timely end-organ innervation. It leaves the distal end of the injured nerve intact for further reconstruction. Despite encouraging clinical results, only few experimental studies have been published to enhance the regeneration results of the procedure. We examined the influence of different size epineural windows and degree of axonal injury of STS repair on nerve regeneration and donor nerve morbidity.

**Methods:** Three clinically relevant repair techniques of the transected common peroneal nerve (CPN) were compared. Group A: 10-mm long epineural STS windows; group B: 2-mm long windows and partial axotomy to the donor tibial nerve; and group C: 2-mm long windows with axotomies to both nerves. Regeneration was followed by the walk track analysis, nerve morphometry, histology, and wet muscle mass calculations.

**Results:** The results of the walk track analysis were significantly better in groups B and C compared with group A. The nerve fiber count, total fiber area, fiber density, and percentage of the fiber area values of CPN of the group C were significantly higher when compared with group A. The wet mass ratio of the CPN- innervated anterior tibial muscle was significantly higher in group C compared with group A. The wet mass ratio of the tibial nerve- innervated gastrocnemial muscle was higher in group A compared with the other groups.

**Conclusions:** All three variations of the STS repair technique showed nerve regeneration. Deliberate donor nerve axotomy enhanced nerve regeneration. A larger epineural window did not compensate the effect of axonal trauma on nerve regeneration. (Plast Reconstr Surg Glob Open 2016;4:e1180; doi: 10.1097/GOX.0000000000001180; Published online 22 December 2016.)
Materials and Methods

Animals

Twenty-four female young adult Sprague Dawley rats (Central Animal Laboratory, University of Turku, Turku, Finland) weighing 242 to 293 g were used in the present study. The National Animal Experiment Board approved all interventions, the analgesic treatment, and animal care. The animals were fed laboratory chow and allowed to drink tap water freely.

Operative Procedure

The animals were randomly divided into 3 groups. Anesthesia was carried out with an intraperitoneal injection of 5 μg/kg medetomidine hydrochloride (Domitor; Orion Oyj, Espoo, Finland) and 750 μg/kg ketamine hydrochloride (Ketalar; Pfizer Oy, Helsinki, Finland). The fluid balance was maintained perioperatively with a 5-mL subcutaneous injection of 9 mg/mL sodium chloride (Fresenius Kabi AB, Uppsala, Sweden). The left common peroneal nerve (CPN) was ligated with 2 sequential 8-0 polyamide sutures (Nylon; S&T AG, Neuhausen Switzerland) 5 mm distally to the bifurcation of the left CPN and tibial nerve (TN). The CPN was transected between the ligations. In group A, 10-mm-long epineural windows were performed microsurgically to the distal CPN and to the contralateral side.6 The investigator was blinded to know the intervention groups when analyzing the footprints. He had passed the self-education test of the walk track analysis.7

Sample Preparation

At 12 weeks, the animals were terminal anesthetized with an intraperitoneal injection of 60 mg/kg sodium pentobarbital (Mebunat; Orion Oyj).

Seven of 8 animals per group were perfused intracardially with 4% phosphate-buffered formalin. The operated nerves, the tibial anterior and gastrocnemial muscles of the operated limb, and the corresponding tissues from the contralateral side were carefully excised with microsurgical instruments and operating loupes. The muscles were weighed with a balance (PG403-S DeltaRange; Mettler-Toledo GmbH, Greifensee, Switzerland). Tissue samples for further studies were immersion fixed in phosphate-buffered formalin overnight. Nerve biopsy sites are seen in Figure 1. From the paraffin blocks, 4-μm-thick sections were cut for neurofilament immunohistochemistry or hematoxylin and eosin staining.

Table 1. Results of Morphometric Analyses of Common Peroneal and Tibial Nerve

| Nerve Area (μm²) | Fiber Count | Mean Fiber Area (μm²) | Total Fiber Area (μm²) | Fiber Density (n/mm²) | Percentage of Fiber Area (%) |
|------------------|-------------|----------------------|------------------------|----------------------|-----------------------------|
| **Common peroneal nerve** | | | | | |
| Group A | 87,251 (19,648) | 1,930 (364) | 3.2 (0.51) | 6,131 (1,762) | 22,991 (7,101) | 7.1 (1.6) |
| Group B | 89,815 (15,809) | 2,683 (515) | 3.4 (0.38) | 9,363 (3,043) | 29,813 (9,081) | 10.2 (2.2) |
| Group C | 85,718 (15,007) | 2,866 (310) | 3.6 (0.71) | 10,257 (2,287) | 33,984 (4,743) | 12.3 (3.7) |
| **Tibial nerve** | | | | | |
| Group A | 315,264 (59,736) | 5,772 (513) | 11.3 (2.66) | 65,276 (17,784) | 18,853 (5,380) | 20.4 (2.7) |
| Group B | 295,929 (62,227) | 6,188 (759) | 9.77 (4.26) | 60,154 (25,590) | 21,588 (4,094) | 20.0 (6.8) |
| Group C | 297,575 (32,041) | 5,937 (677) | 9.99 (1.40) | 59,100 (9,415) | 20,232 (3,366) | 19.9 (2.2) |

Data are expressed in terms of mean (SD).
One of 8 animals per group was perfused intracardially with 4.4 mL 0.1 M Millonig phosphate buffer and 0.6 mL 25% glutaraldehyde. The samples were postfixed with osmium tetroxide, dehydrated, and embedded in epon. One-micrometer sections were stained with toluidine blue. The qualitative analysis was performed with toluidine blue–stained sections.

**Neurofilament Protein Immunocytochemistry**

Morphometry was performed with immunohistochemically stained samples of 7 animals of 8 per group. The stainings were performed with a biotin-free Poly-HRP-Anti-Mouse kit (BrightVision; Immunologic BV, Duiven, The Netherlands) according to the instructions of the manufacturer. Mouse monoclonal neurofilament (200 and 68kDa) Ab1 (Clone 2F11) antibody (Thermo Fisher Scientific, Fremont, Calif.) was applied and incubated. Normal antibody diluent (Immunologic BV) was used to dilute and stabilize HRP conjugates. The sections were then incubated with peroxidase-compatible chromogen (Bright-DAB; Immunologic BV).

**Morphometry**

The whole-nerve cross-sections of immunohistochemically stained samples were photographed with an AxioCam HRc camera (Carl Zeiss, Göttingen, Germany) connected to an AxioVert 200M microscope (Carl Zeiss). The images were stitched as a mosaic image by using AxioVision software (Carl Zeiss). The digitalized images of subperineural, transsectional areas of the nerves were processed by using imaging software (Graphics Suite X6/Photo-Paint; Corel Corp., Ottawa, Ontario, Canada). From the final grayscaled images, the nerve area (mm²), nerve fiber count, and areas of the nerve fibers (μm²) were measured with BioImageXD. The following outcomes were calculated: total nerve fiber area (sum of nerve fiber areas [μm²]), nerve fiber density (nerve fiber count/nerve area [number/mm²]), mean nerve fiber area (total nerve fiber area/fiber count [μm²]), and percentage of the nerve fiber area (total nerve fiber area/nerve area × 100; Table 1).

**Statistical Analysis**

The statistical analyses were done with SPSS (version 21; IBM Corp., Armonk, N.Y.) and SAS System for Windows (version 9.4: SAS Institute Inc., Cary, N.C.). On the basis of the power analysis, the sample size of 7 animals per group gives 90% power and a type I error rate of no more than 5% to detect a difference of 15 or more in the mean PFI values between the groups. This presumption is based on our previous study in which the STS repair group reached a PFI value of −40.3 ± 12.2 SD in a 12-week follow-up. In the present study, a sample size of 8 animals was selected. An experienced statistician supervised the statistical analysis. Differences with \( P \) values less than 0.05 were considered statistically significant. Type 3 tests of fixed effects were used to reveal a significant difference between the intervention groups.

The comparisons between the groups of the results of the walk track analysis were analyzed with the analysis of covariance for repeated measurements, which was adjusted for baseline PFI values. Heterogeneous autoregressive covariance structure was used to consider the correlation between repeated measurements in these longitudinal data. The Tukey-Kramer adjustment was used to control the effect of multiple comparisons.
The nerve fiber count, nerve fiber density, total nerve fiber area, percentage of the nerve fiber area, and nerve area outcomes are expressed as mean values (SD). The groups were compared using the two-way analysis of variance analysis with Tukey–Kramer adjustment for multiple comparisons. Comparison of the distal site of neurorrhaphy of CPN to the distal stump of CPN (Fig. 1) was performed with the paired t test.

In comparisons of the nerve fiber area, the data were well correlated because of the thousands of values from each animal. This was taken into account using the linear mixed model with random intercept for animal. The fiber area values were normally distributed after log10-transformation. The effect of multiple comparisons was taken into account with Tukey–Kramer and Dunnett adjustments.

The wet mass ratios were compared using the Kruskal–Wallis test and the Mann–Whitney U test with Bonferroni adjustment for multiple comparisons.

RESULTS

Walk Track Analysis

The change in the PFI values was different between the groups (group by time interaction effect, P < 0.001). At 12 weeks, groups B and C showed significantly higher values than group A (PFI: A, −48.8 [10.7]; B, −35.7 [9.1]; C, −37.0 [6.3]; Fig. 2). The values of groups B and C did not differ significantly. The intervention groups reached better results than the unrepaired group (PFI, −75.8 [12.0]). The result of the unrepaired group is derived from our previous study.5

Morphometry

CPN

The nerve fiber count values of groups B and C were significantly higher compared with group A (both P < 0.007). The total nerve fiber area, nerve fiber density, and percentage of the nerve fiber area of group C were significantly higher when compared with group A (Fig. 3). The mean nerve fiber area values did not differ between the 3 groups.

In groups B and C, nerve biopsies of CPN were taken from both sides of neurorrhaphy (Fig. 1). The nerve fiber count, nerve fiber density, and percentage of the nerve fiber area were significantly higher distal to the neurorrhaphy compared with the distal stump in both groups. The nerve area of the distal stump was larger compared with the distal site in group B. The values of the distal stump did not differ between groups B and C (Fig. 3).

TN

The nerve area, nerve fiber count, total nerve fiber area, nerve fiber density, and percentage of the nerve fiber areas did not differ significantly between the groups. The mean values of the nerve fiber area of group A were higher compared with the values of groups B and C (both P < 0.0001), but there was no difference between groups B and C.

Light Microscopy

The distal stump of CPN (proximal to the side of neurorrhaphy; Fig. 1): there were no differences between the groups. Several axons and plenty of fibrosis were seen in the specimens of all groups.

Distal CPN: in all groups, a number of axons could be seen, part of them well myelinated. A small amount of fibrous tissue was present, and in more distal sections, the amount of fibrosis was further decreased. In groups B and C, the axonal regeneration seemed better compared with group A (Fig. 4).

Distal TN: there were very few signs of nerve injury in group A. Occasionally, in groups B and C, the axons seemed to be smaller in the lateral zones compared with the central zones.

Anterior tibial muscle: the size of the muscle fiber was slightly decreased, and some angular-shaped cells could be seen in all groups. The changes were focal in groups B and C but wider in group A. The histology of the specimens of the contralateral unoperated side looked normal (Fig. 5).
Gastrocnemial muscle: the general appearance was normal in group A. There were focal areas of atrophy in groups B and C, but as a whole, these findings seemed mild (Fig. 5).

**Muscle Mass Calculations**

In group C (57.2% [3.5]), the anterior wet mass ratio of the tibial muscle was significantly higher compared with group A (46.1% [6.3]). There was no difference between
groups A and B (51.8% [4.3]) and B and C, respectively (Fig. 6).

In group A (79.0% [5.4]), the wet mass ratio of the gastrocnemial muscle was significantly higher compared with the other groups. No difference was seen between groups B (67.6% [9.3]) and C (70.2% [4.6]; Fig. 6).

**DISCUSSION**

Regeneration through neurorrhaphy of peripheral nerve repair is a topic of continuous interest among scientists and surgeons. The mechanism of regeneration after STS and ETS repairs is not, so far, clearly understood. There is controversy to what extent nerve regeneration occurs without axonal injury of the donor and recipient nerves. Previously, it was reported that nerve regeneration occurs through neurorrhaphy of ETS repair even without a window through collateral sprouting, and intact axons were presumed to produce branches from nodes of Ranvier. On the other hand, it has been reported that axonal injury in the donor nerve is a prerequisite for axonal sprouting.

Various techniques have been introduced with ETS repair in attempt to enhance nerve regeneration. Yan et al reported that the number of regenerating nerve fibers increased when the size of the epineural window was enlarged. In the present study, STS repair with 10-mm epineural windows (group A) was compared with a 2-mm window with deliberate axonal injury in the donor.
nerve (group B) and to 2-mm window with axonal injury in both the donor and recipient nerves (group C). All groups showed regeneration when compared with the un-repaired nerve. The results are in accordance with previous studies with STS repair. The mean nerve fiber counts of the groups with donor-side axotomy were significantly higher when compared with the bare window group. Thus, a larger epineural window did not compensate the effect of donor nerve axotomy on axonal flow to the recipient nerve. Furthermore, the nerve fiber count distal to the repair was not significantly increased when the axotomy was added also to the recipient nerve. The results of the walk track analysis are in relation to the number of regenerating nerve fibers as PFI was significantly higher in the groups with donor-side axotomy compared with the mere epineural window group.

In the present study, the wet muscle mass ratio of the CPN–innervated anterior tibial muscle was significantly higher in group C (57 %) with axotomies on both nerves compared with group A (46 %). The results are in accordance (or better) with those of the previous ETS repair studies: Ozmen et al16 (2004): 47% at 12 weeks and Tarasis et al18 (1998): 26% at 24 weeks. Yan et al14 (2002) found a correlation between the size of the epineurial window and the muscle mass. Also in the present study, the signs of denervation were wider in muscles with mere epineural windows compared with the muscle specimens of the axonal injury groups.

The present results indicate that in STS repair, the donor-side degree of axonal trauma was decisive to the recruitment of the number of nerve fibers to the regenerating nerve. In ETS neurorrhaphy, collaterally regenerated axons are said to be able to form functional connections with new end organs after relinquishing their original connections.19 If this conclusion is correct, every axon forming a functional connection via the recipient nerve is ultimately from the donor nerve. This is in accordance with the present results as the nerve fiber areas were significantly smaller in donor TNs with axotomy (Fig. 5). Furthermore, the muscle mass ratio of the donor nerve–innervated gastrocnemius was significantly lower (B: 68% and C: 70 % vs A: 79%), and focal signs of denervation could be seen microscopically in muscle specimens in groups with axotomy. Essential in optimizing the size and depth of the donor-side window is to find out if the achievable benefits of recipient nerve regeneration exceed the possible donor-site morbidity.

The present results increase the understanding of STS repair and elucidate the possibilities and drawbacks of deliberate axotomy in neurorrhaphy. In this study, the axotomy was intentionally relatively large, a half of the nerve, and it is not acceptable in humans. Besides comparable repair results with ETS neurorrhaphy, STS neurorrhaphy can be combined with other reconstructive techniques as it leaves the end of the distal nerve stump available. Although STS repair alone may not have enough regenerative potential for sufficient functional recovery, it may have potential to reduce muscle atrophy and maintain a growth-supportive atmosphere for further reconstructions. Thus, we aim to study STS neurorrhaphy as part of the treatment of proximal nerve injuries.

The present results together with those of our previous study5 provide a scientific basis for the regeneration capacity of the STS repair technique. In the present study, regeneration was followed by morphometry and wet muscle mass calculation at 12 weeks. Although the walk track analysis was repeated 2, 4, 6, 8, and 12 weeks postoperatively, another shorter follow-up period for morphometry would have ensured the results. In further studies, more detailed information about axonal regeneration will be gained if unmyelinated axons are assessed with electron microscopy, and sensory and motor axons are distinguished with retrograde labeling studies.

**CONCLUSIONS**

The present study on STS nerve repair was conducted to examine the requirements for the increase of axonal supply to the recipient nerve and to improve the func-
tional results. We conclude that STS repairs both with and without deliberate axonal injury showed regeneration of the nerve and functional improvement. A larger epineurial window could not compensate the result of better regeneration, which was achieved with partial donor nerve axotomy.

Henriki Rönkkö, MD
Hatanpää City Hospital
P.O. Box 437
FIN-33101 Tampere, Finland
E-mail: ronkko.j.henrikki@student.uta.fi

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