Successful regeneration of the peripheral nerve with proximal injury has remained a challenging situation. Regenerating axons have a limited time to reach the end organs. In these cases, distal end-to-end (ETE) nerve transpositions and end-to-side (ETS) repairs have been used to overcome the problem. However, in these repair techniques, the distal end is used for reconstructions. The side-to-side (STS) repair technique leaves both injured nerve ends free and thus offers a tool for further nerve reconstructions. Only a few studies have been made with the STS nerve repair technique. In clinical reports, sensory regeneration and motor regeneration were noticed.

The objective of the present experimental study is to compare comprehensively nerve regeneration between the STS, ETS, and ETE repair techniques.

METHODS

Animals

Eighty female young adult Wistar rats (Harlan Laboratories Netherlands B.V., Melderslo, The Netherlands)

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weighing 300 to 340 g were used. The local laboratory animal care committee approved the experiment, which followed the principles of laboratory animal care.

**Operative Procedure**

The animals were randomly divided into 10 groups (Table 1). They were anesthetized with an intraperitoneal injection of 5 μg/kg medetomidine hydrochloride (Domitor; Orion Oy, Espoo, Finland) and 750 μg/kg ketamine hydrochloride (Ketalar; Pfizer Oy, Helsinki, Finland). The same investigator (H.R.) carried out all operations with microsurgical instruments and a surgical microscope (Zeiss, Jena, Germany). The bifurcation of the common peroneal nerve (CPN) and tibial nerve (TN) was exposed (Fig. 1). The CPN was transected 5 mm distally to the bifurcation, and the neurorrhaphy was performed with four 10-0 sutures (Nylon; S&T AG, Neuhausen, Switzerland). In the ETS group, a 2-mm long epineural window was performed to both the CPN and TN 15 mm distally to the bifurcation, and the neurorrhaphy was performed with four 10-0 sutures. In the ETE group, the CPN transection was repaired with four 10-0 sutures. In the STS group, a 2-mm long epineural window was performed to both nerve ends of the CPN and, in the STS repair group, the proximal end of the CPN and both nerve ends of the TN similarly to the previous group, and the neurorrhaphy with the distal end of the CPN was performed with four 10-0 sutures. In the sham-repaired group, the sciatic nerve trunk was revealed and left intact. The wounds were closed in separate layers with 5-0 nylon sutures (Deknatel Bondek Plus; Teleflex Medical, Durham, N.C.). The analgesic treatment was ensured by a subcutaneous injection of 5 mg/kg carprofen (Rimadyl; Vericode Ltd., N.C.).

**Walk Track Analysis**

The walk track analysis was performed before the operation and 2, 4, and 6 weeks postoperatively on all animals and, further, 8, 12, 16, 20, and 26 weeks postoperatively on animals with a longer follow-up period. The print length (PL; distance between the heel and third toe) and the toe spread (TS; distance between the first and fifth toe) were measured from the footprints. The results were calculated as a mean value of 3 measurements. The peroneal function index (PFI = 174.9 [(EPL − NPL)/NPL] + 80.3 [(ETS − NTS)/NTS] − 13.4) was calculated. “N” refers to the normal, unoperated side, and “E” refers to the experimental side. The investigator had passed the self-education test to minimize interobserver differences.

**Sample Preparation**

The animals were killed at 6 or 26 weeks (Table 1) with an intraperitoneal injection of 60 mg/kg sodium pentobarbital (Mebunat; Orion Oy). In 7 of 8 animals, the tissues were fixed with intracardiac perfusion of phosphate-buffered formalin. The operated nerves and tissue samples of the long peroneal muscle were removed and fixed in phosphate-buffered formalin. The sites of nerve samples are shown in Figure 1. Nerve and muscle samples were embedded in paraffin. From the paraffin blocks, 4-μm-thick sections were cut both for morphometry with neurofilament immunohistochemistry–stained sections and for histology with hematoxylin and eosin–stained sections.

One animal of 8 per group was perfused intracardially with Millonig phosphate buffer and glutaraldehyde. Nerve and muscle samples were removed and postfixed with osmium tetroxide and embedded in epon. One-micrometer-thick sections were cut and stained with toluidine blue for qualitative histologic study.

**Neurofilament Protein Immunocytochemistry**

Four-micrometer-thick sections were cut from the paraffin blocks. The staining was performed with a biotin-free Poly-HRP-Anti-Mouse kit (BrightVision; Immunologic BV, Duiven, The Netherlands) according to the protocol of the manufacturer. Mouse monoclonal neurofilament (200 and 68 kDa) Ab1 (Clone 2F11) antibody (Thermo Fisher Scientific, Fremont, Calif.) was applied and incubated. Normal antibody diluent (Immunologic BV) was used to dilute and stabilize horseradish peroxidase conjugates. The sections were then incubated with peroxidase-compatible chromogen (Bright-DAB; Immunologic BV) and finally counterstained and cover slipped.

**Morphometry**

Morphometry was performed with neurofilament-stained sections. The whole-nerve cross-sections of immunohistochemically stained samples were photographed...
with the AxioVert 200M microscope and AxioCam HRc microscope camera (Carl Zeiss, Göttingen, Germany). The images were stitched as a mosaic image by using AxioVision software (Carl Zeiss, Jena, Germany). The digitalized images of the subperineural areas of the nerve cross-sections were processed with imaging software (Graphics Suite X6/Photo-Paint; Corel Corp., Ottawa, Ontario, Canada). Morphometric measurements were done with BioImageXD.\textsuperscript{18} The nerve area ($\mu m^2$), nerve fiber count, and areas of nerve fibers ($\mu m^2$) were measured. The following outcomes were calculated: total fiber area (sum of fiber areas [$\mu m^2$]), fiber density (fiber count/nerve area [number/$mm^2$]), the mean fiber area (total fiber area/fiber count [$\mu m^2$]), and the percentage of the fiber area (total fiber area/nerve area $\times 100$).

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.). The results are expressed as means and SD. $P$ values smaller than 0.05 were considered statistically significant.

The comparisons between the groups of the walk track analysis were done with analysis of covariance for repeated measurements after adjustment for baseline PFI values. The heterogeneous autoregressive covariance structure was used to consider the correlation between observations in these longitudinal data.

In the morphometric analysis, the groups were compared with two-way analysis of variance (ANOVA). Unrepaired groups with only a long follow-up period were compared with other groups with one-way ANOVA. Comparison between the 2 different sites of the CPN of the STS group was performed with the paired $t$ test.

In the comparisons of the fiber area, there was a dependency between the observations because of the thousands of values measured from each animal. It was taken into account with the linear mixed model with the random intercept for animal. The data were normally distributed after log$_{10}$-transformation.

The effect of multiple comparisons in the analyses mentioned above was considered by using Tukey–Kramer and Dunnett adjustments.

The correlations between the PFI and morphometric outcomes were calculated with Pearson correlation coefficients.

The sample size of 8 animals per group was calculated from the expected difference in the walk track analysis. The sample size 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 intact controls and the intervention groups. This expected difference is based on the following assumptions:

A mean (SD) PFI value of $-10$ (3) among the intact control animals from pilot studies by the investigators.

A mean (SD) PFI value of $-25$ (13) among animals undergone ETE repair reached values of $-28.41$ (4.16) at 30 days, $-22.92$ (3.62) at 60 days, $-13.94$ (2.68) at 150 days,\textsuperscript{19} and $-14.5$ (3.9) at 12 weeks.\textsuperscript{20} ETS repair reached the PFI value of $-77.0$ (11.5) at 6 weeks and $-37.3$ (13.5) at 12 weeks\textsuperscript{20} and $-48.5$ (SEM, 2.2) at 28 weeks.\textsuperscript{21}

RESULTS

Two animals did not wake up from anesthesia and were excluded from the study. The sample size of 7 gives 0.86 power to the test. No cases of autotomy or flexion contracture were detected.

Walk Track Analysis

The STS and ETS groups did not differ significantly at any time point. From 6 weeks onward, PFI was better in the ETE group compared with the STS and ETS groups. PFI of the STS and ETS was significantly better when compared with the unrepaired group from 12 weeks onward. The PFI values at 12 weeks were as follows: STS, $-40.3$ (12.2); ETS, $-42.6$ (17.3); ETE, $-19.1$ (5.7); sham repaired, $-12.6$ (1.4); and unrepaired, $-75.8$ (12.0; Fig. 2).

Morphometry

CPN

All intervention groups showed significantly higher values of the fiber count, total fiber area, fiber density, and percentage of the fiber area when compared with the unrepaired group both at 6 and 26 weeks (Fig. 3). At 26 weeks, there were no significant differences between the STS and ETS groups in any outcome. At 6 and 26 weeks, the fiber count, total fiber area, fiber density, and percentage of the fiber area of the ETE reached significantly higher values than the ETS (all $P < 0.02$) and STS (all $P < 0.001$) with the exception of a nonsignificant difference with the total fiber area ($P = 0.06$) at 6 weeks between the ETE and ETS (Fig. 3).

The mean nerve area of the STS, ETS, and ETE did not differ at 6 weeks, but at 26 weeks, the STS was significantly smaller than the ETE. There were no differences between the ETS and STS groups (Fig. 3).

In the STS group, the morphometric analysis of the CPN was performed on both sides of the neurorrhaphy (Fig. 1). The fiber count, fiber density, and percentage of the fiber area were significantly higher on the distal side compared with the stump (Fig. 3).

The mean nerve fiber areas did not differ between the STS, ETS, and ETE groups (Fig. 4). In all 3 groups, the values of the mean fiber area and percentage of the fiber area were significantly higher at 26 weeks compared with 6 weeks (group by time interaction effect, $P = 0.01$).

All morphometric parameters of the distal CPN at 26 weeks (Table 2) correlated with PFI: nerve area (Pearson correlation, 0.73; $P < 0.001$), fiber count (0.82; $P = 0.000$), mean fiber area (0.68; $P < 0.001$), total fiber area (0.77; $P < 0.001$), fiber density (0.77; $P < 0.001$), and percentage of the fiber area (0.80; $P < 0.001$).

TN

The mean fiber area of the ETS was smaller at 6 weeks when compared with the STS, ETE, and sham repaired (all $P < 0.002$) and at 26 weeks when compared with the ETE group ($P = 0.03$; Fig. 4).
The values of the mean nerve area, fiber count, fiber density, and percentage of the fiber area (Fig. 5) did not differ significantly between the STS, ETS, and ETE groups both at 6 and 26 weeks. The mean fiber count values of the TN at 26 weeks were as follows: STS, 5,064 (542); ETS, 5,026 (384); ETE, 5,272 (411); intact, 5,158 (232); sham repaired, 5,138 (284); and unrepaired, 5,301 (295).

**Qualitative Light Microscopy**

In the TN proximal to the neurorrhaphy, axon density seemed normal in all groups. In the STS and ETS groups, some axon sprouts were observed outside the perineurium at 6 and 26 weeks. The mean fiber count values of the TN at 26 weeks were as follows: STS, 5,064 (542); ETS, 5,026 (384); ETE, 5,272 (411); intact, 5,158 (232); sham repaired, 5,138 (284); and unrepaired, 5,301 (295).

Distal to the neurorrhaphy in the CPN, the epineurium seemed normal, and no misdirected axons were seen outside the perineurium in the STS and ETS groups. Axon density looked similar in both groups (Fig. 6). Mild changes of fibrosis could be seen in the STS group at 26 weeks. In the ETE group, axon density seemed to be high and myelin sheaths thicker compared with the STS and ETS groups.

Distal to the neurorrhaphy in the TN in the STS and ETS groups, small amounts of axon sprouts were seen in the peripheral areas inside the perineurium; otherwise, the view looked normal at 6 and 26 weeks.

In the long peroneal muscle, there were focal signs of atrophy in both the STS and ETS groups (Fig. 7) at 6 and 26 weeks. In the ETE group, only some atrophic muscle fibers could be observed. In the unrepaired group, there were changes in advanced muscle denervation.

**DISCUSSION**

The objective of the present study was to compare the STS, ETS, and ETE repair techniques comprehensively. Previously, Yüksel et al. studied STS neurorrhaphy experimentally. However, in their study, CPN was transected only 3 weeks after the primary operation. Furthermore, Ladak et al. used nerve grafts between the donor and the recipient nerve in their distal neurorrhaphy. To our knowledge, this study is the first experimental examination with a similar model in STS and ETS repairs. In the present study, the donor-side window was identical in the STS and ETS repairs, and on the recipient side, the nerve end was used similarly in the ETS and ETE repairs.

All 3 repair groups showed functional recovery. From 12 weeks onward, the functional results of the 3 intervention groups were significantly better compared with the unrepaired control group. The functional results did not differ between the STS and ETS groups. In the present study, the results of the walk track analysis at 26 weeks had a signifi-
Fig. 3. Results of the morphometric analyses of the common peroneal nerve. The fiber count (B), total fiber area (C), fiber density (D), and percentage of the fiber area (E): values of all intervention groups at 26 weeks were significantly higher when compared with the unrepaired group ($P < 0.001$). There were no significant differences between the STS and ETS groups in any outcome at 26 weeks. Both at 6 and 26 weeks, the ETE group showed significantly higher values than the STS and ETS groups in the fiber count, total fiber area, fiber density, and percentage of the fiber area values, with the exception of the nonsignificant difference in the total fiber between the ETE and ETS at 6 weeks. On the distal side of STS neurorrhaphy, the fiber count, fiber density, and percentage of the fiber area were significantly higher compared with the nerve stump. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and +++ $P < 0.001$, comparison of experimental groups to the unrepaired group at 26 weeks. Error bar, ± 1 SD.
cant correlation with the morphometric parameters. It is clear that although there is robust axonal regeneration in the distal nerve stump, but appropriate connections to the muscles are not reached, functional regeneration remains poor, and there is no correlation between the outcomes.24–26

The present results of the walk track analysis are in accordance with those of previous studies. In our study, the PFI of the STS at 26 weeks (−36.6) was in line with the study by Yüksel et al at 28 weeks (−30.4). The PFI of the present ETS group (−41.2) at 26 weeks is also comparable with the results of the previous ETS studies: Eren et al,21 −48.5 after 28 weeks; Liu et al,20 −37.3, and Ozmen et al,27 −54.8 after 12 weeks.

In the morphometric analysis, we analyzed the whole cross-sections of nerves, which ensures unbiased analysis of different nerve regions. Immunohistochemical staining was conducted with neurofilament antibody, which allows the calculation of even small and unmyelinated axon sprouts. The protocol allowed to add axons to the mask and to remove nonaxonal particles from the mask. The morphometric parameters, histological findings, and functional results were superior in the ETE repair compared with the STS and ETS repairs. It can be explained by a better axonal flow from the transsectional donor nerve end compared to the epineural window. On the recipient side, the transectional nerve end (ETS) showed no advantage compared with the window (STS), as in the long-term, there were no significant differences between the STS and ETS groups in the morphometric parameters, histologic degenerative signs of the muscle, and functional results.

Table 2. Results of Morphometric Analyses of Common Peroneal Nerve

| Nerve Area, μm² | Fiber Count | Mean Fiber Area, μm² | Total Fiber Area, μm² | Fiber Density, n/mm² | Percentage of Fiber Area, % |
|----------------|-------------|----------------------|-----------------------|---------------------|-----------------------------|
| **6 wk**       |             |                      |                       |                     |                             |
| Side-to-side repair | 119,401 (43,997) | 1,380 (439)         | 2.2 (0.60)            | 3,196 (1,617)      | 12,529 (3,579)             | 2.9 (1.8)                  |
| End-to-side repair | 108,847 (32,490) | 2,220 (323)         | 2.4 (0.48)            | 5,465 (1,385)      | 22,061 (6,950)             | 5.4 (1.8)                  |
| End-to-end repair | 99,392 (20,071)  | 3,258 (507)         | 2.7 (0.45)            | 8,752 (2,524)      | 35,087 (2,843)             | 8.7 (1.2)                  |
| Sham-repaired controls | 123,264 (23,092) | 2,325 (120)         | 11.3 (1.7)            | 26,321 (3,873)     | 19,418 (3,544)             | 21.6 (3.0)                 |
| **26 wk**       |             |                      |                       |                     |                             |
| Side-to-side repair | 52,354 (13,412)  | 881 (344)           | 3.5 (0.55)            | 3,218 (1,593)      | 16,653 (4,050)             | 6.0 (2.1)                  |
| Stump | 100,778 (47,083)  | 480 (294)           | 4.1 (1.0)             | 2,150 (1,272)      | 5,641 (2,287)              | 2.4 (1.1)                  |
| End-to-side repair | 56,357 (9,154)   | 1,096 (260)         | 4.2 (0.65)            | 4,640 (1,382)      | 19,554 (3,698)             | 8.2 (1.9)                  |
| End-to-end repair | 87,720 (10,430)  | 3,195 (233)         | 3.8 (0.48)            | 12,231 (1,976)     | 36,779 (4,400)             | 14.0 (2.0)                 |
| Sham-repaired controls | 98,282 (18,260) | 2,135 (154)        | 10.5 (1.4)            | 22,429 (3,586)     | 22,304 (4,036)             | 23.0 (1.8)                 |
| Intact controls | 88,153 (22,767)  | 2,298 (150)         | 10.3 (1.3)            | 23,796 (3,963)     | 27,115 (4,778)             | 27.6 (3.5)                 |
| Unrepaired controls | 35,969 (27,287) | 65 (40)            | 1.7 (0.45)            | 120 (93)           | 1,966 (1,017)              | 0.32 (0.15)                |

Data are expressed in terms of mean (SD).
Considering these 2 results, it seems that the donor side of the neurorrhaphy is decisive to the end result when all 3 repair techniques are compared. After successful end-organ connection, axons will maturate and the myelin layer will thicken. Our morphometric and histological findings showed that during regeneration from 6 to 26 weeks, the axons grew in size in the STS, ETS, and ETE groups (Fig. 4) and the myelin layer seemed thickened (Fig. 6).

Fig. 5. Results of the morphometric analysis of the donor tibial nerve. The biopsy sites are seen in Figure 1. Fiber count (A) and percentage of the fiber area (B) values of different groups did not differ significantly from each other at 6 and 26 weeks. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Error bar, ± 1 SD.

Fig. 6. Nerve cross-sections of side-to-side repair (left) and sham repair (right) at 6 (above) and 26 weeks (below) postoperatively. Clusters of regenerative axon sprouts (arrows) can be seen 5 mm distal to the site of operation at 6 weeks (above left). At 26 weeks, axons are myelinated and larger in size (below left). Well-preserved nerve fibers in the sham-repaired group at 6 (above right) and at 26 weeks (below right): toluidine blue staining.
To clarify the mechanism of regeneration in the STS repair, nerve samples were taken from both sides of the neurorrhaphy of the CPN. The values of the fiber count, fiber density, and percentage of the fiber areas were significantly higher on the distal side compared with the stump. Thus, the results of regenerating axons in the STS group cannot be explained with contamination.

According to the previous reports, donor muscle denervation has been reported to be negligible from 3 to 12 months after ETS repair. However, signs of acute donor muscle denervation and decrease in the number of myelinated nerve fibers distal to ETS neurorrhaphy compared with the proximal values have been reported. In our study, a histologically small amount of axon sprouts was noticed in the TNs of both STS and ETS groups as slight signs of donor nerve injury both at 6 and 26 weeks. Although deliberate injury to the donor nerve was avoided at operations, it is obvious that axonal injury cannot be completely excluded when epineural windows are created.

Despite encouraging results, we are aware that nerve regeneration is faster with rats compared with humans. Despite the known “blow through effect,” we considered it important to get long-term results as well to ensure the stability of the regeneration results. Form the methodological point of view, quantitative morphometry was performed with neurofilament staining, which cannot distinguish the myelin sheath. Further studies are warranted to analyze the development of myelin thickness and differentiation of sensory and motor axon sprouting.

The present results are in accordance with previous promising clinical results. According to the literature with ETS repair and our findings with STS neurorrhaphy, the noted number of axons may be limited to ensure sufficient regeneration. The purpose of STS and ETS repairs is to serve axon sprouts into the severed nerve and end organ rapidly enough after nerve injury. These so-called “baby-sitting” procedures aim to maintain the growth-supporting atmosphere in the distal nerve stump and to reduce muscle atrophy. When compared with the ETS technique, the advantage of the STS technique is that it leaves both nerve stumps available for further reconstructions. Further studies are needed to optimize the size of epi- or perineural windows to enhance regeneration and to combine the STS technique to proximal ETE repair.

CONCLUSIONS

Nerve regeneration was compared between STS, ETS, and ETE techniques. The present results with the walk track analysis and the morphometric and histological findings showed that nerve regeneration occurs in all 3 groups. STS repair showed similar regeneration when compared with ETS repair.
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