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LONG-TERM EVALUATION OF RAT PERIPHERAL NERVE REPAIR WITH END-TO-SIDE NEURORRHAPHY

ABSTRACT

This study was designed to assess long-term reinnervation of end-to-side neurorrhaphy in the rat. The cut right peroneal nerve was repaired and sutured to the side of the intact tibial nerve. Both the extent of reinnervation and the integrity of the intact donor nerve were evaluated in 48 Sprague-Dawley rats randomly treated with fresh or delayed nerve repair with or without perineurotomy. Evaluations included nerve conduction velocity (NCV) of both the peroneal and tibial nerves, dry muscle weight, and histologic examination (neurofilament stain and morphometric assessment) at 8 and 12 months postoperatively. Although animals treated with perineurotomy tended to have better NCV and dry muscle weight recovery than those without, the difference was not statistically significant. No difference was observed between fresh and predegenerated nerve repair. The mean total (all four subgroups) NCV recovery rates were 87 percent and 94 percent for the peroneal nerve, and 93 percent and 95 percent for the tibial nerve, compared to the contralateral intact nerves, at 8 and 12 months, respectively. Tibialis anterior muscle mass measurements revealed a recovery in dry muscle weight of about 85 percent and 89 percent at 8 and 12 months, respectively, compared to the intact contralateral tibialis anterior muscles. Histologic studies with neurofilament staining revealed numerous axons at the distal end of the peroneal nerve in all groups, indicative of myelinated axonal regeneration. Morphometric analysis demonstrated that the presence of a window in the perioneurium improved the histologic picture. The mean number of myelinated fibers at 12 months postoperatively was significantly higher in animals with a perineurotomy window (compared to without) in both fresh and predegenerated nerve repair subgroups, respectively ($p < 0.05$). These results indicated that end-to-side neurorrhaphy permits axonal regeneration from the intact donor nerve and is associated with satisfactory recovery. The effect of the procedure on the donor nerve was negligible.

Recovery following nerve injury, using traditional end-to-end neurorrhaphy, is dependent on an adequate donor source. Recent studies have suggested that suturing the distal portion of the injured nerve to the side of an intact nerve also can produce nerve regeneration. End-to-side nerve coaptation is able to attract axonal sprouting from the intact donor nerve. In most previous reports, however, only early events of neural reinnervation were assessed, while the long-term results of reinnervation remain to be established.[1–9] The present study was designed to evaluate the long-term effects of end-to-side nerve coaptation on both the extent of reinnervation and the integrity of the intact donor nerve in a rat model. Information of this kind would have practical implications for the potential clinical application of this procedure.

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MATERIALS AND METHODS

TREATMENT GROUPS. Forty-eight Sprague-Dawley female rats (200 to 250 g) were randomly divided into four groups, in which an end-to-side nerve coaptation was performed immediately (fresh) or following nerve degeneration (predegenerated), with or without a perineurotomy window in both cases (fresh no window, FNW; fresh window, FW; predegenerated no window, PNW; predegenerated window, PW). Observations were made at 8 and 12 months after end-to-side nerve coaptation.

SURGICAL TECHNIQUES. The surgical techniques have been previously described.[10] Briefly, animals were anesthetized with sodium pentobarbital 3 to 5 mg/100 g body weight intraperitoneally. The right hind limb was shaved and washed with povidone-iodine (Betadine), and treated in a sterile manner thereafter. The sciatic nerve, including the common peroneal and tibial nerve, was exposed, and hemostasis was maintained by electric cauterization. In animals treated with predegeneration, the common peroneal nerve was sharply transected 5 mm below its bifurcation from the sciatic nerve. The proximal peroneal nerve stump was then ligated, reflected superiorly, and implanted into the adjacent soft tissue to prevent autoreinnervation with the distal stump. After 2 weeks of degeneration, the predegenerated distal part of the peroneal nerve was sutured to the intact tibial nerve, with or without a neurotomy in the perineurium, in an end-to-side fashion, using 10-0 microsurgical suture (Ethilon) under X16 magnification. End-to-side nerve coaptation was performed in a similar fashion without predegeneration of the nerve (fresh) in a second group of animals. The wound was closed with skin clips and the animals were maintained on a 12-hr light/dark cycle with food and water *ad libitum*.

NERVE CONDUCTION VELOCITY (V) EVALUATION. Nerve stimulation and recordings were carried out with the use of an electromyograph (Tönnies DA II, Frieburg, Germany) at three locations: 1) proximal to the site of end-to-side nerve coaptation; 2) distal to the site of end-to-side nerve coaptation on the peroneal nerve; and 3) distal to the site of end-to-side nerve coaptation on the tibial nerve. The distance between proximal and distal sites was 10 mm. For the peroneal nerve, the recording pin was placed into the tibialis anterior muscle and, for the tibial nerve, the recording pin was placed into the gastrocnemius muscle. Electric stimulation by square-wave pulses of 0.2 ms widths for twitch contractions were given at 2 mv. Contralateral side values were also recorded in the same manner and used as intra-animal controls. Motor nerve conduction velocity was calculated with the formula: MNCV(m/s) = 10 mm/proximal latency − distal latency (ms), and compared to the contralateral side to determine the rate of recovery.[2,11–13]

DRY MUSCLE WEIGHTS (DMW). After electrophysiologic examination, the entire tibialis anterior muscles from both hind limbs were carefully harvested and placed in 10 percent paraformaldehyde. After 4 days of fixation, the dry muscle weights were determined to the nearest 10^{-3} g using an electronic balance (FX-300, Japan). The weight of the muscle reinnervated by end-to-side nerve coaptation was expressed as a percentage of contralateral tibialis anterior muscle weight (i.e., [operated muscle weight/weight of normal muscle] × 100).[12,13]

HISTOMORPHOMETRY. Nerve specimens were cut 5 mm distal to the site of end-to-side neurorrhaphy for both peroneal and tibial nerves, fixed in cacodylate buffered 4 percent glutaraldehyde, and routinely prepared for toluidine blue staining. Control samples from the contralateral intact peroneal and tibial nerves were harvested and prepared in a similar manner. Post fixation was done in 1 percent osmium tetroxide. The specimens were then imbedded in Epon 812, cut into 1-μm sections, stained with toluidine blue, and mounted on a microscopic slide. Photomicrographs from different fields of cross sections were taken at a magnification of X600 and 10 × 14 cm photomicrographs were used to count myelinated fibers. For each specimen, five photo micrographs were counted to obtain the mean number of fibers per section. After axon counts, the photomicroraphs were scanned for quantitative image analysis. Morphometric analysis of myelinated fibers was performed using an image analyzer (Analyze, Biomedical Imaging Resource, Mayo Foundation, supplied by CN Software). For each specimen, 20 myelinated fibers were randomly selected for assessment of the following parameters: (1): mean total fiber area = myelin area + axon area; (2): mean myelin area = total area − axon area; (3): G index = axon area/myelin area. Two specimens from each experimental group were routinely prepared for transmission electron microscopy (TEM, JEM-100 X. II. Electron Microscope. Japan) to examine ultrastructure.

The entire area at the site of end-to-side nerve coaptation was carefully harvested and prepared for longitudinal sections. The nerve section was placed in 10 percent paraformaldehyde for a minimum 2 days fixation. The nerve was then imbedded in paraffin, and cut into 2 to 3 μm longitudinal sections from the attachment site, so that the section included the proximal tibial nerve, the end of the peroneal nerve that was attached to the tibial nerve, and the distal end of tibial nerve. Three sections were stained with a modified silver impregnation (Bio-Optica, Milan, Italy) to delineate the axons in the distal stump of the peroneal nerve. Another three sections were treated with an immunoperoxidase technique (ABC),

using an antibody against the 70 kDa neurofilament protein (NF, DAKO), to confirm the presence of axonal sprouts from the intact tibial nerve to the peroneal nerve segment.

Statistical Analysis. Motor nerve conduction velocity and muscle weight measurements are expressed as a percentage of the contralateral side and recorded as the mean ± SD. It has been suggested that percentage values minimize variations between animals and are more useful than absolute values for comparison among groups.[11,12] Analysis of variance (ANOVA) was used to evaluate differences between group means. A p value of 0.05 was accepted for statistical significance.

RESULTS

All animals survived and no ulcers or autotomies were observed. Biopsy showed that the peroneal nerve was well-attached to the tibial nerve at the site of end-to-side neurorrhaphy in all groups. No dehiscence or neuromas were observed.

Nerve Conduction Velocity. NCV recovery rates at 8 and 12 months are shown in Table 1. The total recovery of NCV of the peroneal nerve was 87 percent at 8 months. Animals treated with perineurotomy tended to perform better than those without, although the difference was not statistically significant. No difference was observed between fresh nerve repair and predegenerated repair. At 12 months, the total recovery of NCV for the peroneal nerve was 94 percent. NCV recovery rates of the tibial nerve were 93 percent and 95 percent at 8 and 12 months, respectively, suggesting that the procedure had a negligible effect on the function of the tibial nerve long-term. No difference was observed between tibial nerves treated with or without a perineurotomy.

Dry Muscle Weights. The dry muscle weights of the tibialis anterior muscles were expressed as a percentage of values at the contralateral unoperated side (Table 2). At 8 months, the total recovery rate was about 85 percent and, at 12 months, the total recovery rate increased to about 89 percent. The perineurotomy groups demonstrated somewhat better recovery than those not treated with perineurotomy, but the difference was not statistically significant. The differences between fresh and predegenerated groups at 8 and 12 months were also not statistically significant.

Silver Impregnation and Neurofilament Staining. Silver and neurofilament staining demonstrated that there were numerous regenerating axons originating from the site of the end-to-side nerve coaptation to the distal stump of the peroneal nerve. The presence of axons in the distal stump of the peroneal nerve was demonstrated by silver staining, which is specific for nerve fibers. In all groups, numerous axons were stained brown-black; these axons originated from the site of attachment and extended toward the distal stump of the peroneal nerve (Fig. 1). Also, extensive nerve fibers that were neurofilament protein positive could be observed in longitudinal sections of all groups. These nerve fibers originated from the site of end-to-side neurorrhaphy and extended to the distal stump of the peroneal nerve, regardless of whether a perineurotomy window was present. Both stains showed a "transitional region" where the axons were not organized in an orderly fashion and were interlaced with connective tissue at the site of attachment. When the fibers entered into the distal peroneal nerve stump, the nerve fiber organization appeared to return to normal (Fig. 2).

Histomorphometry and TEM Assessment. The number of myelinated axons per photomicrograph are summarized in Figure 3. At 8 and 12 months, the

Table 1. Nerve Conduction Velocity at 8 and 12 months in End-to-side Nerve Coaptation

Groups	*Peroneal*	F	P	NW	W	*Tibial*	F	P	NW	W
8 months										
FNW	83.7 (10.4)					91.0 (10.2)				
FW	85.0 (11.8)					93.8 (8.7)				
PNW	85.8 (10.1)					92.5 (10.7)				
PW	93.3 (9.4)					93.3 (9.4)				
TRR	87.0 (10.4)	84.4*	89.6	84.6#	89.2	92.7 (9.7)	92.4*	92.9	91.8#	93.6
12 months										
FNW	93.1 (7.2)					95.0 (8.7)				
FW	94.5 (5.6)					96.3 (5.2)				
PNW	93.0 (7.6)					96.7 (7.5)				
PW	94.8 (7.8)					93.3 (9.4)				
TRR	93.9 (7.1)	93.8*	93.9	93.1	94.7	95.3 (7.7)	95.7*	95.0	95.9#	94.8

Mean ± SD expressed as a percentage of contralateral unoperated peroneal nerve.

F (fresh repair), P (predegenerated repair), NW (no perineurotomy window), W (perineurotomy window), FNW (fresh repair with no perineurotomy window), FW (fresh repair with perineurotomy window), PNW (predegenerated repair with no perineurotomy window), PW (predegenerated repair with perineurotomy window), and TRR (total recovery rate).

*$p > 0.05$ F vs. p; #$p > 0.05$ NW vs. W

Table 2. Dry Weights of Tibialis Anterior Muscle

Groups	*8 months*					*12 months*				
	Tibialis	F+	P	NW*	W	*Tibialis*	F+	P	NW*	W
FNW	77.2 (10.6)					88.1 (6.4)				
FW	85.8 (10.5)					91.5 (7.0)				
PNW	88.7 (12.0)					84.9 (4.7)				
PW	87.8 (7.9)					90.7 (8.3)				
TRR	84.9 (10.1)	81.5	88.3	83.0	86.8	88.8 (6.6)	89.8	87.8	86.5	91.1

Means ± SD expressed as a percentage of the weight of contralateral normal muscles.

F (fresh repair), P (predegenerated repair), NW (no perineurotomy window), W (perineurotomy window), FNW (fresh repair with no perineurotomy window), FW (fresh repair with perineurotomy window), PNW (predegenerated repair with no perineurotomy window), PW (predegenerated repair with perineurotomy window), and TRR (total recovery rate).

+: $p > 0.05$ F vs. P (8 and 12 months).

*: $p > 0.02$ NW vs. W (8 months); $p > 0.05$ (12 months).

numbers of myelinated nerve fibers had not increased and tended to remain stable. With the exception of the perineurotomy group at 12 months, no significant differences were observed between the groups with or without a window in the perineurium, and between the fresh and delayed repair groups. The mean total area (MTA) of myelinated fiber in the FW and PW groups at both 8 and 12 months, was superior to both no-window (FNW and PNW) groups. Overall, the mean total area of myelinated fibers in the perineurotomy group was better than in the group without perineurotomy, although the difference was not statistically significant ($p > 0.10$, Fig. 4). The mean myelin area (MMA) of nerve fibers is summarized in Figure 5. Both MTA and MMA showed better results at 12 months, compared to 8 months, indi-

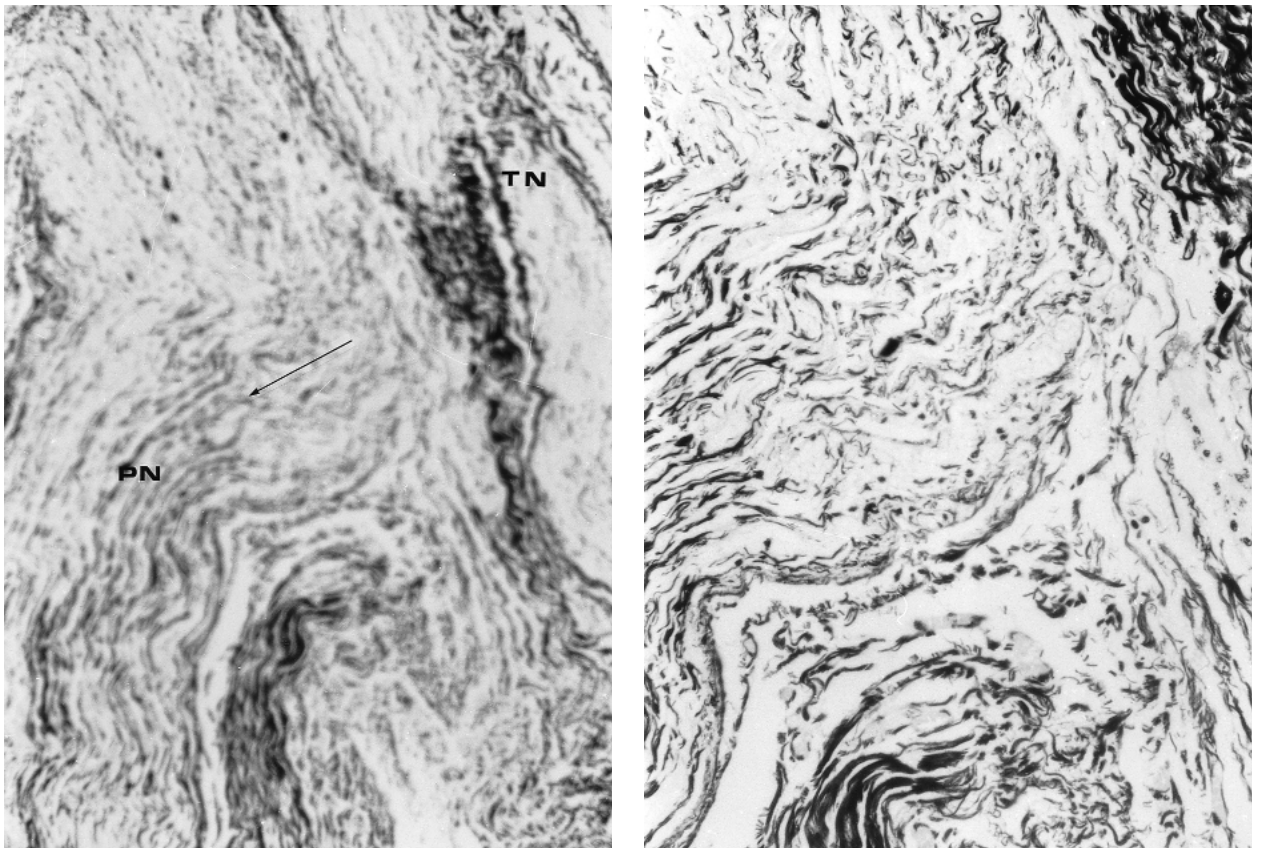
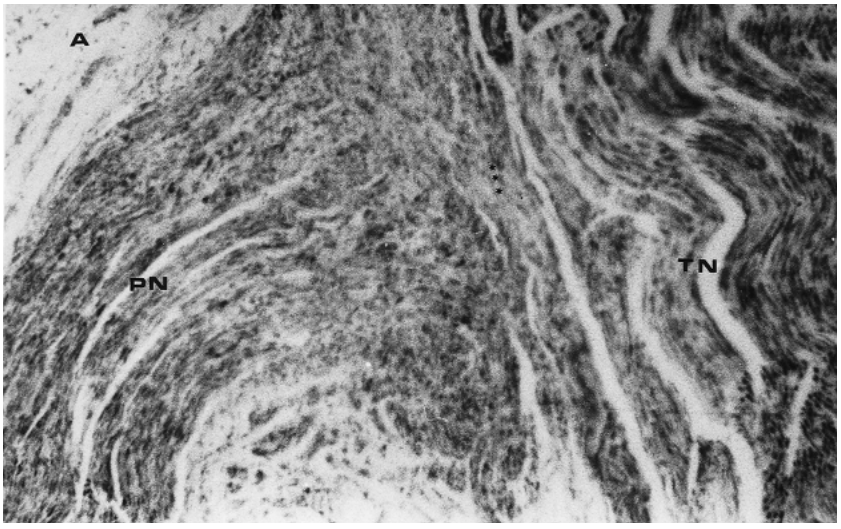
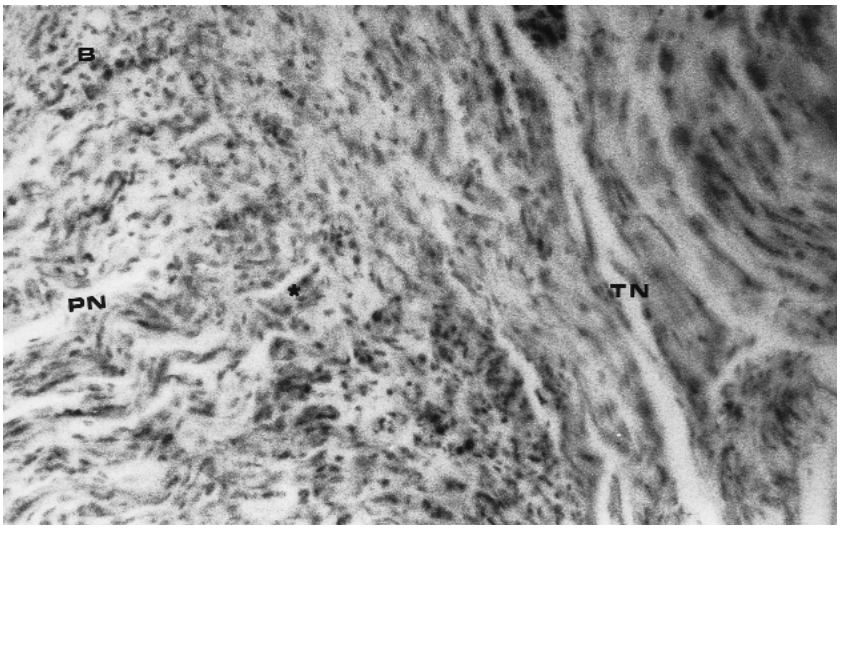


Figure 1. A–B, Color photomicrographs of silver impregnantion; longitudinal section through the site of end-to-side nerve coaptation of fresh window group; 12 months postoperative. A, There are numerous sprouting nerve fibers originating from the tibial nerve (TN) to peroneal nerve (PN), as shown by the arrow. B, High magnification showing numerous axons emanating from the tibial nerve to the peroneal nerve by collateral sprouting. (original magnification: A, X100; B, X200)



A



B

Figure 2. A, B, Photomicrographs of immunocytochemical staining with neurofilament protein; longitudinal section through the site of end-to-side nerve coaptation of pre-degenerated perineurotomy group; 8 months postoperative. A, There are extensive sprouting nerve fibers that neurofilament protein stained positively. The asterisks show the site of anastomosis. B, High magnification showing the "transitional region" (asterisk). (PN = peroneal nerve; TN = tibial nerve; original magnification: A, X100; B, X200)

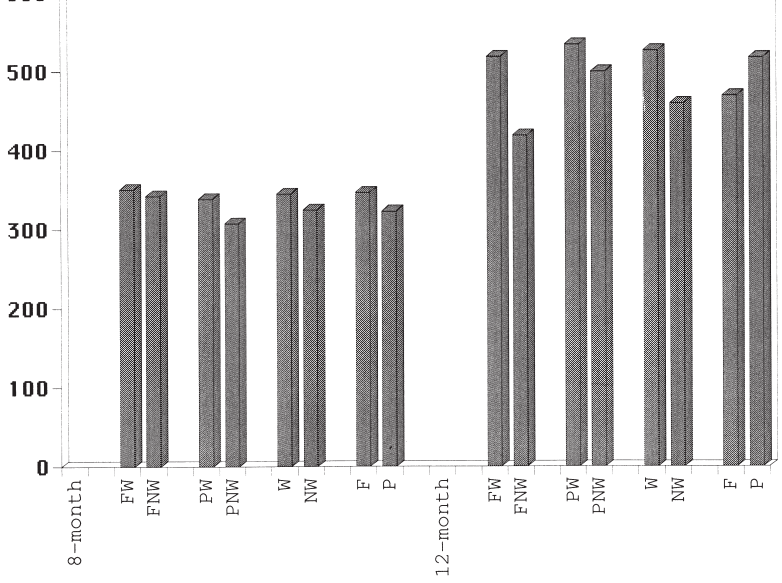


Figure 3. Mean number of myelinated fibers in different groups, 8 and 12 months postoperatively, per photo [FW (fresh repair with perineurotomy window), FNW (fresh repair with no perineurotomy window), PW (predegenerated repair with perineurotomy window), PNW (predegenerated repair with no perineurotomy window), W (perineurotomy window), NW (no perineurotomy window), F (fresh repair), and P (predegenerated repair)]. ($p > 0.05$ W vs. NW and F vs. P (8 months); $p < 0.05$ W vs. NW and PW vs. PNW; $p > 0.05$ F vs. P (12 months)

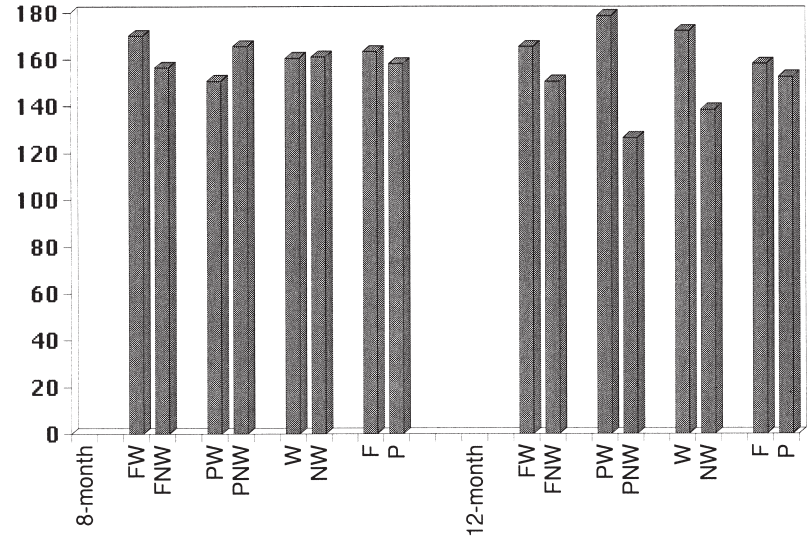


Figure 4. Mean total area (pixel) of myelinated fibers at 8 and 12 months postoperatively. [FW (fresh repair with perineurotomy window), FNW (fresh repair with no perineurotomy window), PW (predegenerated repair with peirneurotomy window), PNW (predegenerated repair with no perineurotomy window), W (perineurotomy window), NW (no perineurotomy window), F (fresh repair), and P (predegenerated repair)]

cating that the regenerating nerve fibers in the distal stump of the peroneal nerve had a higher degree of maturation at 12 months than at 8 months. Similarly, the perineurotomy groups were superior to the groups without perineurotomy. The G index (the ratio between axon area and myelin area) reflects the degree of maturation of the regenerating nerve fibers. At 8 months, the G index reached 1, and no further change was observed by 12 months postoperatively (Fig. 6). TEM evaluation indicated that most regenerating nerve fibers in the distal stump of the peroneal nerve and myelination were fairly mature. No Wallerian degeneration was observed in the donor tibial nerve (Fig. 7).

DISCUSSION

To date, the long-term results in both the extent of reinnervation and the influence on the donor nerve following end-to-side nerve coaptation have not been well-documented.[1–9] The present results demonstrated that NCV recovered to 87 percent and 94 percent of the contralateral intact controls by 8 and 12 months, respectively. Dry muscle weight recovered to 89 percent of controls by 1 year postoperatively. At this time, no significant difference was observed between fresh and delayed repair. On the other hand, the perineurotomy groups showed better recovery than those groups without perineurotomy.

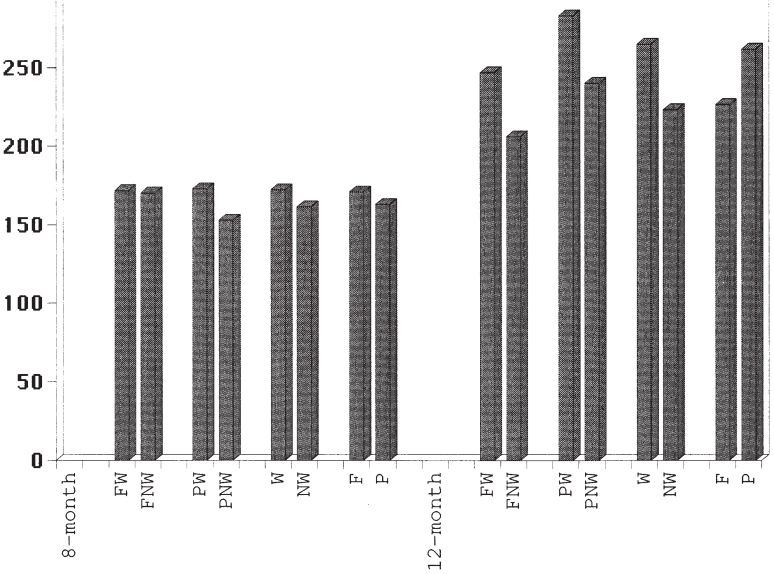


Figure 5. Mean myelin area (pixel) of myelinated fibers at 8 and 12 months postoperatively. [FW (fresh repair with perineurotomy window), FNW (fresh repair with no perineurotomy window), PW (predegenerated repair with perineurotomy window), PNW (predegenerated repair with no perineurotomy window), W (perineurotomy window), NW (no perineurotomy window), F (fresh repair), and P (predegenerated repair)]

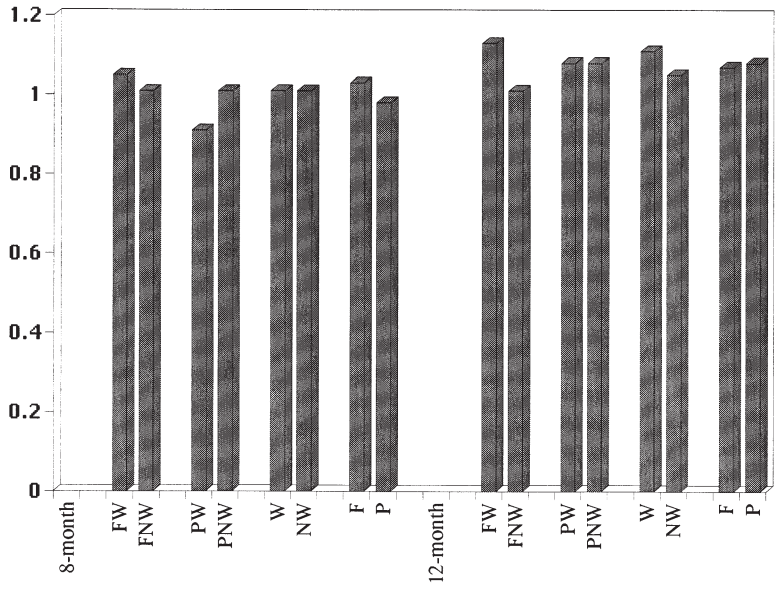
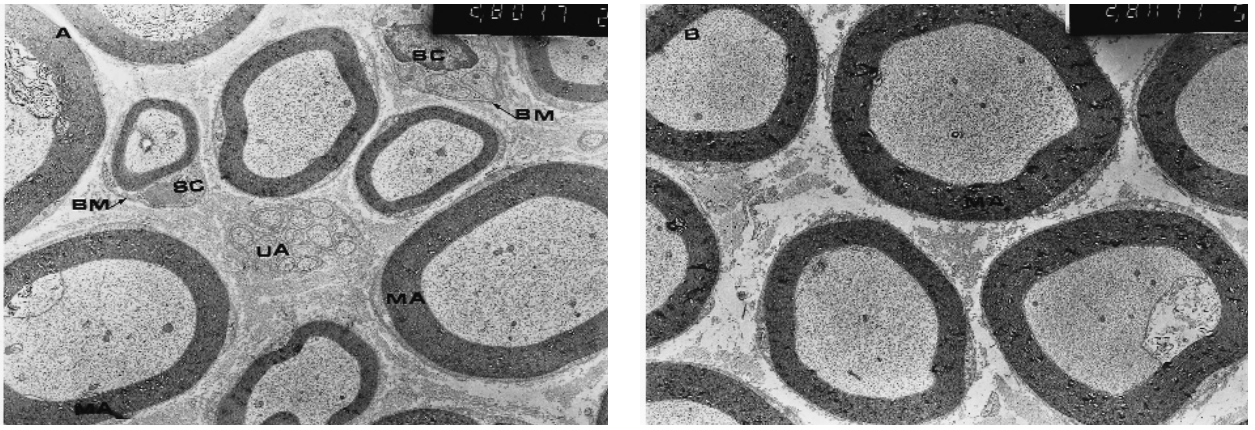


Figure 6. G index is shown at 8 and 12 months postoperatively. [FW (fresh repair with perineurotomy window), FNW (fresh repair with no perineurotomy window), PW (predegenerated repair with perineurotomy window), PNW (predegenerated repair with no perineurotomy window), W (perineurotomy window), NW (no perineurotomy window), F (fresh repair), and P (predegenerated repair)]

The donor nerve recovered to 95 percent by 1 year postoperatively, suggesting that the effect of end-to-side nerve coaptation on the donor tibial nerve was minimal at long-term follow-up. This is in contrast to the early negative effects on donor-nerve function, which has been previously reported to be 88 percent of controls at 2 months postoperatively.[10]

The thickness of the myelin sheath is considered to be a valuable parameter in determining the degree of maturation of the regenerated axon. The mean myelin area in animals treated with perineurotomy was better than in those without the perineurotomy window. Corresponding to myelin area, the G index also showed that the ratio between the axon area and the myelin area was nearly 1:1, indicating that myelination had reached near maturation, when compared to early results in which the G index was only between 0.39 to 0.81. This suggests that the G index is time-dependent and increases as maturation occurs over time.

The possibility of parasitic neurotization from other sites, such as the proximal transected nerve segment and neighboring muscles innervated by the posterior tibial nerve, cannot be ruled out at long-term follow-up after end-to-side nerve coaptation.[3,14–16] In the present study, this type of parasitic neurotization was apparently prevented by the ligation of the proximal stump of the peroneal nerve, its reflection superiorly, and its implantation into the adjacent soft tissue. In addition, the muscles innervated by the posterior tibial nerve were separated by intermuscular septa. The possibility of parasitic neurotization from the proximal stump of the peroneal nerve has been previously ruled out by the use of a



A B

Figure 7. Electron micrographs 5 mm distal to end-to-side neurorrhaphy, 12 months postoperative. A, Fresh perineurotomy group, showing myelinated axons (MA) and unmeylinated axons (UA). The myelinated axons vary in size with a fairly mature profile. A basement membrane (BM) surrounds the Schwann cell (SC) plasma membrane. B, Showing no degeneration in donor tibial nerve. (original magnification X2800)

fluorescent double-labeling technique.[17] We hypothesize that this is attributed to the sprouts needing to penetrate barriers surrounding the intact and degenerating axons in order to enter the pathway to the denervated end-plate. Among these physical barriers to nodal sprout growth is the fine basement membrane on the surface of the Schwann cells.[14]

In most previous studies, the effects of end-to-side nerve coaptation have been assessed at 2 weeks, and at 1 to 6 months postoperatively.[2–10] The findings at such early stages may not give an accurate prediction of long-term results, as the rate of peripheral nerve regeneration is very slow, compared to other tissues. It is known that the number of axons distal to the site of repair in the rat sciatic nerve increases markedly up to 3 months, plateaus at 6 to 9 months, and returns to about normal levels by 1 year.[18] The regenerative ability in the rat is greater than that reported in primates.[19] When compared to early postoperative results at 2 and 3 months,[10] we find that the number of myelinated fibers was not increased at 8 and 12 months. In fact, it was slightly reduced, indicating that the number of myelinated fibers tends to return to normal. This may reflect a pruning mechanism,[20] whereby there is a larger number of regenerating axons penetrating the site of coaptation during the early stages of nerve regeneration. This is thought to reflect the fact that functional recovery requires many more regenerated axons to replace the portion lost below the site of injury, and is attributed to the fact that misdirected axons cannot mature when they fail to enter their original endoneurial tube and reach their programmed end-organ. Rather, these misdirected axons degenerate and are pruned away. Only those axons which make the correct connections with peripheral targets appear to receive the necessary trophic substances that promote maturation. This may explain, in part, why axon counts often do not accurately reflect final function.[21,22] Axonal sprouting generates more axons than usual, and is more likely to occur when axons are not regenerating well or fail to meet their programmed target organs. In this regard, recovery appears to be more dependent on the regenerating axon finding a functional Schwann cell column, rather than on the actual numbers of the axons.

To extrapolate the present findings to clinical application, it should be noted that the regenerative capacity of the rat is much more vigorous than in humans. In end-to-side nerve coaptation without perineurotomy, the regenerating axons must pierce three to four different conjunctival layers, including the basal lamina, endoneurium, peri- and epineurium.[23,24] It has been shown that the epi- and perineurium are thinner in rats than in humans. Although the axons of collateral sprouting can penetrate these conjunctival tissues in the rat, it is not clear whether this is the case clinically; some authors have indicated that regenerating axons may not be able to penetrate the conjunctival layers, especially the basal lamina.[25] Neurotrophic factors, such as NGF, FGF, and insulin-like factor,[26–30] the implantation of cultured Schwann cells at the repair site,[31–34] or vascularized grafting[35] may be necessary to promote nerve regeneration, by providing not only the necessary neurotrophic factors, but also an adhesive surface for the migration of the growth cone and the insuring of an adequate blood supply to the nerve trunk.

In conclusion, our long-term results demonstrated that end-to-side nerve coaptation could induce axonal sprouts from the donor nerve. No measurable effects on the nerve were observed at long-term follow-up. Overall, the presence of a window in the perineurium was associated with better histologic results, although no difference was observed at long-term follow-up between fresh and delayed repair. This procedure opens new, promising prospects for clinical nerve repair, especially in cases where the proximal nerve segment is unavailable, such as in facial palsy and global brachial plexus avulsion injuries.

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