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DIVERSE TYPES OF EPINEURAL CONDUITS FOR BRIDGING SHORT NERVE DEFECTS. AN EXPERIMENTAL STUDY IN THE RABBIT

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In this study the process of peripheral nerve regeneration through an epineural flap conduit was examined using four groups of 126 New Zealand rabbits. There were three study groups (A, B, and C) and 1 control group (D). A 10-mm long sciatic nerve defect was bridged either with 3 variations of an epineural flap (Groups A, B, and C) or with a nerve autograft (Group D). Animals from all groups were examined 21, 42, and 91 days postoperatively to evaluate nerve regeneration employing light microscopy and immunocytochemistry. Nerve regeneration was studied in transverse sections at 3, 6, and 9 mm from the proximal stump. The gastrocnemius muscle contractility was also examined prior to euthanasia at 91 days postsurgery in all groups using electromyography. Immunohistochemical, histochemical and functional evaluation showed the presence of nerve regeneration resembling the control group D, especially in group A, where an advancement epineural flap was used. In this experimental model an epineural flap can be used to bridge a nerve defect successfully. © 2007 Wiley-Liss, Inc. Microsurgery 27:000–000, 2007.

Peripheral nerve injuries are common and often leave considerable and/or permanent disability.

Reports of successful peripheral nerve repair appear in literature since the 19th century, but surgical treatment of nerve injuries remained unsuccessful until nerve grafting and microsurgical techniques had been employed. Not all nerve injuries are amenable to direct end-to-end repair. Significant loss of nerve tissue necessitates use of a nerve graft, while nerve regeneration through a large peripheral nerve gap will not be successful unless a conduit is used.

Autogenous nerve grafting is the most commonly used procedure to repair a neural gap providing good end result, but it is hampered by donor site morbidity and limited availability.[1–5] The use of interfascicular or group fascicular cable grafts is the gold standard for the management of peripheral nerve defects against which all new techniques have to be compared.

Nerve regeneration can occur through neural and nonneural tissues used as conduits. In several experimental models the search for an optimal nerve conduit material led to the use of autogenous and, more recently, artificial materials in association with regeneration promoting (neurotrophic) factors.[6–15]

Clinical implementation of conduits has focused on the use of autogenous tissue (veins, arteries, pseudosheaths, nerve grafts) and occasionally of artificial conduits (silicone and polyglactine mesh).[2,3,16] Conduit materials do not seem to improve the outcome significantly compared with conventional nerve grafting. The major obstacle in the use of conduits is the limitation in the defect size that can be successfully bridged and in humans it is ~2.5 cm. In larger defects typical nerve grafting is necessary.

The epineurium may serve as an autologous conduit and may facilitate bridging of nerve defects. The use of the epineural cuff technique was described by Snyder et al.[17–18] and used in animals and humans. Since then this technique was validated in animal models.[19–21] The use of the epineurium as a tubular free nerve conduit has been also examined in the rat sciatic nerve defect model.[22]

In the present study three variations of an epineural flap were used to bridge a short nerve defect in the rabbit sciatic nerve defect model.

MATERIALS-METHODS

This study was approved by the Veterinary Directorate, in compliance with the EEC Directive 609/86, and the National Research Council's guide for the care and use of laboratory animals was followed. In this study 126 white New Zealand rabbits weighing between 3 and 3.5 kg were used. The animals were allocated to four groups. In each of the three study groups (Groups A, B, and C) 36 animals were included while the remaining 18 animals served as control (Group D). In all groups a 10-mm sciatic nerve

[1]Hand Surgery and Microsurgery Department, KAT Hospital, Athens, Greece
[2]Experimental Research Unit, Elpen Pharma Co., Athens, Greece
[3]Orthopaedic Department, University of Ioannina Medical School, Greece
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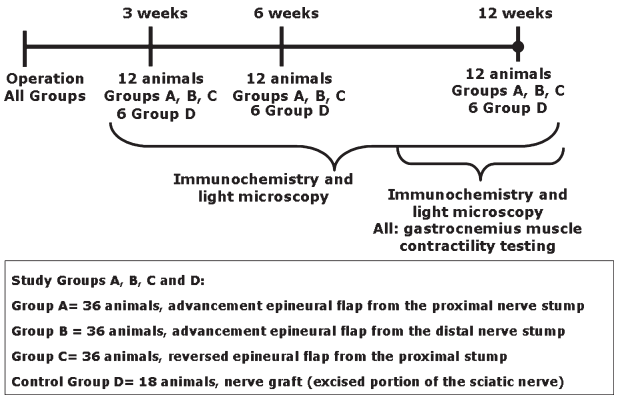

Figure 1. Schematic representation of the study protocol, time course and evaluation methods.

defect was created and bridged either with three variations of an epineural flap (Groups A, B, and C) or a nerve graft (Group D) (Fig. 1). In all groups the sciatic nerve was exposed under general anesthesia using fentanyl & fluanison and midazolam. Anesthesia was maintained using 2% halothane delivered with a mask. The sciatic nerve was exposed under the surgical microscope using 16× magnification. A 10-mm nerve defect was created proximal of sciatic nerve bifurcation using a scalpel. An advancement epineural flap harvested from the proximal nerve stump (Fig. 2) and from the distal nerve stump was used in groups A and B, respectively. In these cases the epineural flap was completely detached from the nerve and was used to bridge the defect. In group C a specially designed reversed epineurial flap harvested from the proximal stump was used (Fig. 3). The epineurium retained its attachment to the distal edge of the proximal nerve stump. In the control group D the defect was bridged using the excised portion of the sciatic nerve sutured at its original site.

The surgical technique of epineural flap harvesting was as follows. Following exposure of the sciatic nerve and creation of the defect a 10-mm flap was designed on the epineurium of the proximal nerve stump in group A and a similar flap was designed on the distal stump in group B. Using the surgical microscope, a 2-mm bridge of the dorsal epineurium containing the main nerve artery was preserved while the rest of the epineurium was excised, taking care to avoid injury to the underlying nerve fascicles. Additionally, a 2-mm bridge of the epineurium located at the rim of the proximal (group A) or distal (group B) nerve stumps was preserved to facilitate flap suturing. Surgical dissection started in a dorsal longitudinal direction and continued circumferentially to remove the epineurium. The excised epineurium was then used to bridge the nerve defect. To prevent collapse of the conduit and to facilitate suturing, a 2-mm diameter silicon tube was temporarily inserted within the conduit and between the two nerve stumps, and it was removed before the final suturing of the epineural tube. The proximal and distal edge of the epineurium was secured to the proximal and distal nerve stumps using four 10-0 Ethilon (Johnson & Johnson, Norwood, MA) stitches. The longitudinal flap edges were also approximated using five to seven stitches, respectively. The space within the conduit was filled with a blood clot (Figs. 4a and 4b) to prevent immediate postoperative collapse of the conduit wall.

In group C the epineurium of the proximal nerve stump was not completely excised but its distal attachment was preserved (Fig. 5). The epineurial flap was reversed pivoting on its distal attachment and sutured on the distal nerve stump with epineurial sutures. In this case the length of the flap was 12 mm to accommodate the reversing process.

In group D the 10-mm defect was repaired using the previously resected nerve segment, which served as an autologous graft, using four epineurial stitches at each suture line.

After the operation the muscles and the skin were sutured and the animals were left to recover from anesthesia.

POSTOPERATIVE EVALUATION

The process of nerve regeneration was studied at various time intervals using immunochemistry, light microscopy and measurement of the gastrocnemius muscle contractility. After 3, 6, and 12 weeks 12 animals from groups A, B, and C and six animals from the control group D were sacrificed. The nerve defect area was exposed and the grafted area was excised. Six specimens were used for light microscopy examination and six specimens for immunochemistry. At 12 weeks all animals underwent examination of the gastrocnemius muscle contractility in both limbs prior to euthanasia.

Nerve regeneration was studied in 1 μm transverse sections at a distance of 3, 6, and 9 mm from the proximal stump (three specimens for each group designated S3, S6, S9) and in longitudinal sections stump (three specimens for each group). The epineurium conduit was resected and immersed in 2.5% glutaraldehyde. After fixation in 1% osmium tetroxide and dehydration in ethanol the specimens were embedded in Agar 100. Finally, the specimens were stained with toluidine blue and examined by light microscopy.

Quantitative morphometry was performed measuring the number of myelinated axons per mm^2 and the mean axon diameter in every section, taking into account the mean value of 10 measurements.

Similar with light microscopy six specimens from each group were examined using immunochemistry. The harvested conduit was rinsed in ice-cold PBS and embedded in Tissue Tek O.C.T. Compound 4583 (10.24% w/w poly-

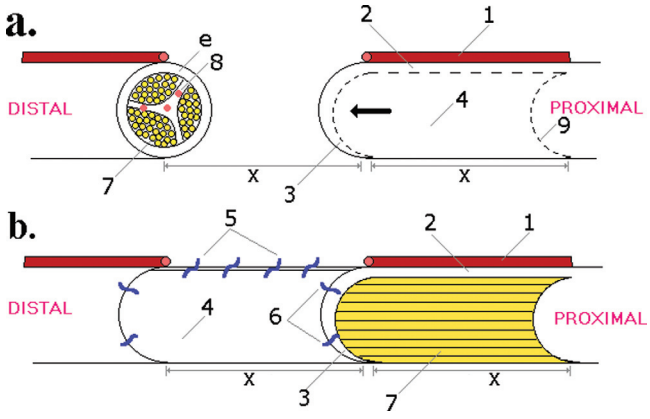

Figure 2. A 10-mm sciatic nerve defect was repaired with an epineural advancement flap harvested from the proximal nerve stump (group A). Proximal = proximal nerve stump, Distal = distal nerve stump, e = epineurium, 1 = main longitudinal nerve artery, 2 = perivascular preservation zone, 3 = 2-mm epineurium preservation zone, 4 = epineural advancement flap, 5, 6 = epineural stay sutures, 7 = nerve fascicles, 8 = intranervous vascular plexus, 9 = outline of epineurium detachment, x = 10-mm long nerve defect.

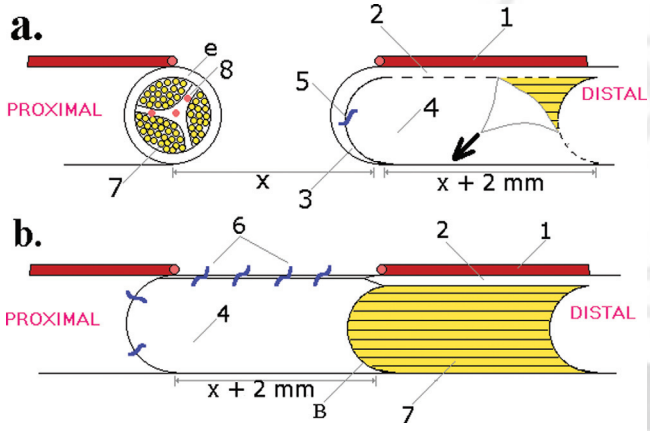

Figure 3. A 10-mm long sciatic nerve defect was repaired with a reversed epineural advancement flap harvested from the proximal nerve stump (group B). Proximal = proximal nerve stump, Distal = distal nerve stump, B = base of the reversed epineural flap, *e* = epineurium, 1 = main longitudinal nerve artery, 2 = perivascular preservation zone, 3 = 2 mm epineurium preservation zone, 4 = epineural advancement flap, 5, 6 = epineural stay sutures, 7 = nerve fascicles, 8 = intranervous vascular plexus, 9 = outline of epineurium detachment, x = 10-mm long nerve defect.

vinyl alcohol, 4.26% w/w polyethylene glycol, 85.50% w/w inactive substances, Miles, USA). Three, 3 μm thick transverse and three 10-mm long longitudinal sections were cut on a cryostat. After fixation in 2.5% paraformaldehyde, the sections were exposed to primary antibodies (DAKO) to identify the components of the newly formed nerve, including 68 KD neurofilament protein, fibrinogen, fibrin, and fibronectin.

The immunocytochemistry and light microscopy findings at three and six weeks were only qualitatively analyzed.

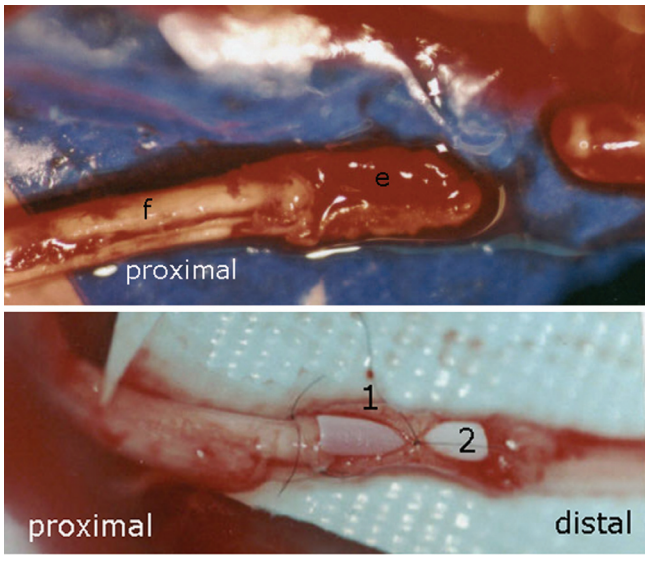

Figure 4. Intraoperative photograph of the epineural flap technique. In the upper part the epineurium (e) is harvested from the proximal nerve stump and is prepared to create the epineural conduit. Nerve fascicles (*f*) from the proximal stump are evident. In the lower part, the proximal and distal nerve stumps are evident. A silicone tube (2) is temporarily used to prevent epineural flap (1) collapse. The flap is sutured with 10–0 nylon stitches to form a tubular conduit. The silicon tube is removed prior to conclusion of the repair and the conduit is filled with a blood clot to prevent collapse.

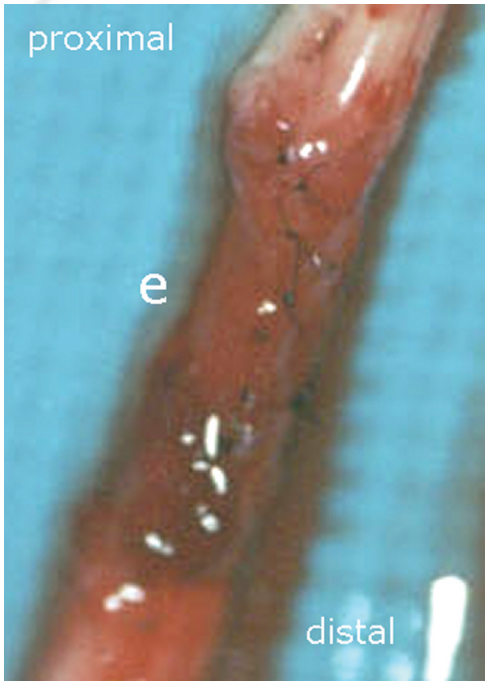

Figure 5. Final appearance of the sciatic nerve defect bridged with the epineural flap (e). A blood clot occupies the conduit to prevent collapse.

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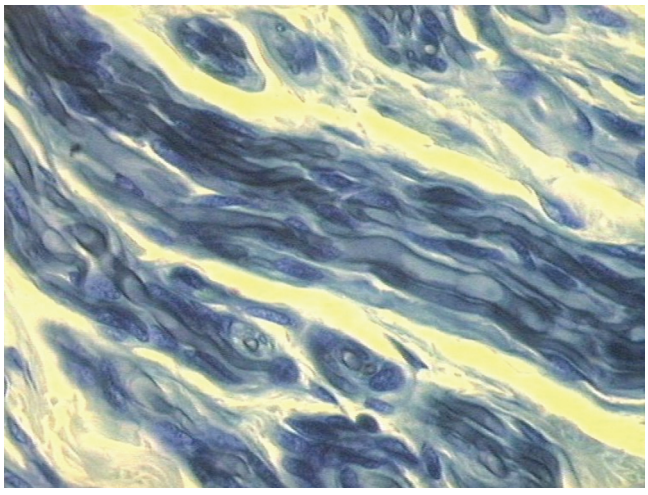

Figure 6. Longitudinal section of the myelin sheaths at 6 weeks. Fixation with osmic acid and stained with toluidine blue (magnification ×400).

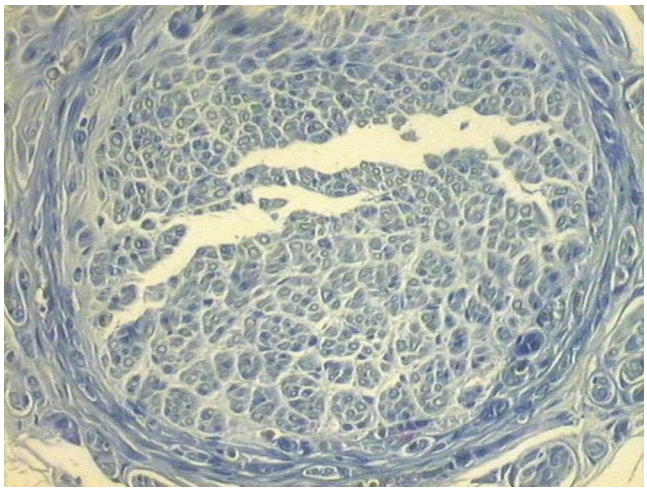

Figure 7. Appearance of the myelin sheaths 13 week after the operation. There is a tendency to mini-fasciculation in cross sections. Fixation with osmic acid and stained with toluidine blue (magnification ×25).

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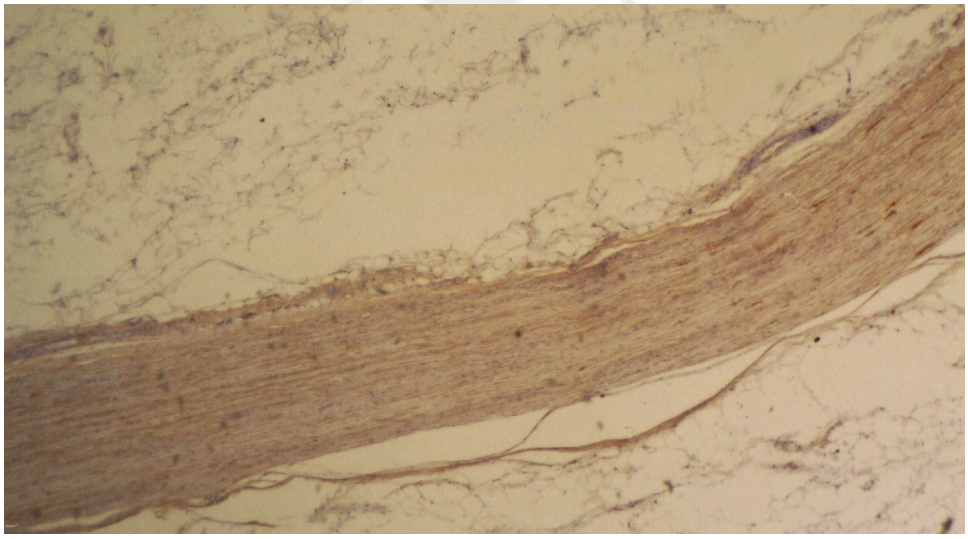

Figure 8. The entire conduit is occupied by stained neurofilament protein (DAKO) at 13 weeks (magnification ×25).

Twelve weeks after surgery the animal was anesthetized and both sciatic nerves and gastrocnemius muscles were exposed. The body temperature was kept constant using a heating lamp. On both sides the nerves were stimulated with electrodes at the proximal and distal ends of the nerve gap and the onset of the compound muscle action potential was recorded with needle electrodes placed in the gastrocnemius muscle, 10 mm below the tibial tubercle. Supramaximal electrical stimuli were delivered proximal to the nerve repair site or the respective intact nerve location by a Grass-SD-9 stimulator at a frequency of 100 Hz for 0.6 ms and the gastrocnemius electrode was recording transmitted evoked potentials. Latency was defined as the conduction time from the onset of the stimulus to the beginning of the initial deflection of motor response (called also compound motor action potential, CMAP) and the amplitude was measured from peak to peak. The amplitude of the motor response (or CMAP) is roughly proportional to the number of muscle fibers that respond to the nerve impulse. The ratio of the compound muscle action potential between the operated and the normal limb (P-ratio) was recorded.[11,17] After completion of the electrophysiologic testing an overdose of pentobarbital was administered and the nerve gap site was excised and processed for light microscopy and immunochemistry as described above. The quantitative histomorphometric and electromyographic data were statistically compared using ANOVA and the significance level was set at $P = 0.05$.

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Table 1. Quantitative Histomorphometry Results

	Normal	Group A advancement	Normal	Group B reversed distally	Normal	Group C reversed proximally	Normal	Group D control group
Mean axonal diameter (μm) (mean ± SD)	6.53 ± 0.92	3.36 ± 0.27[a,b]	6.9 ± 0.53	3.37 ± 0.38[a,b]	6.95 ± 0.62	3.13 ± 0.4[a,b]	6.72 ± 0.74	4.53 ± 0.97[b]
Number of myelinated axons/mm^2 (mean ± SD)	11.5 ± 1.05	6.33 ± 1.86[a,b]	11.66 ± 1.36	6.16 ± 1.47[a,b]	11 ± 0.89	4.16 ± 1.47[a,b]	11.58 ± 1.16	7.66 ± 1.07[b]

[a] *Statistically significant difference compared with the control group D.*
[b] *Statistically significant difference compared with the normal, contralateral sciatic nerve.*

RESULTS

Nerve regeneration through the epineural tube was successfully accomplished. There was no neuroma formation nor evidence of inflammatory reaction. The results of histomorphometry are presented in Table 1. T1

The mean axonal diameter and the number of myelinated axons/mm^2 were significantly lower in all groups compared with the normal contralateral sciatic nerve. In groups A, B, and C both parameters were significantly lower compared with the control group D (nerve autograft). The number of myelinated axons in group C was significantly lower compared with groups A and B (4.16 ± 1.47 compared with 6.33 ± 1.86 and 6.16 ± 1.47, respectively, $P < 0.05$), although this was not the case when the mean axonal diameter was taken into account.

On light microscopy examination of the regenerated nerve several observations were evident. Three weeks after the operation, presence of myelin sheaths was evident throughout the nerve section with extensive areas of connective tissue between the axons. In the longitudinal sections new myelinated axons could be seen throughout the conduit which appeared thicker at the proximal third of the conduit. At six weeks, the myelin sheaths (Figure 6) F6 looked thicker than before and there was a clear tendency to mini-fasciculation in cross sections (Figure 7). After 13 F7 weeks the axons constituted a new structure closely resembling the normal nerve.

Using immunocytochemistry the epineural conduit space was filled with fibrin and fibronectin matrix as part of the healing process, while S100 stain for Schwann cells was positive. Bunger bands (lines with Schwann cells) appeared on the third week along the conduit. Schwann cell proliferation preceded axonal growth (sprouting). The proximal 2/3 of the newly formed structure showed positive staining for neurofilament proteins. The proximal third was profoundly stained while the middle third was less intensively stained, representing progressive axon advancement. At six weeks, fibrin and fibronectin were still present but their matrix was attenuated. After 6 and 13 weeks entire conduit was occupied with stained neurofilament protein (Figure 8). F8

Regarding gastrocnemius muscle contractility the amplitude of the motor response in mV was expressed as the ratio between the operated and the normal side. The amplitude of the gastrocnemius muscle contraction ranged between 5.3 ± 1.2 mV and 21.8 ± 3.9 mV. The mean amplitude of the motor response (compound motor action potential, CMAP) of the gastrocnemius muscle in the operated compared with the contralateral normal limb 13 weeks after surgery (expressed as a percentage, P-ratio) was (60.33 ± 4.25)% in group A (periosteum advance- AQ2 ment), (42.1 ± 7.33)% in group B (distally reversed periosteum), (58.7 ± 5.66)% in group C (proximally reversed

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periosteum) and (64.67 ± 6.41)% in group D (control group, nerve graft). There was no significant difference between groups A, C, and D but only between these groups and group B ($P > 0.05$).

DISCUSSION

In this study a short nerve defect was bridged using various types of epineural flaps. The results of this type of conduit were comparable with those provided by typical nerve grafting.

During the last decade the principles of nerve injury and repair have been refined based on the improved knowledge of nerve biology.[1–3,22,23] The ability of an injured nerve to regenerate depends on the degree of continuity remaining in the nerve trunk following injury. Treatment of peripheral nerves injuries when a nerve gap is present is a great challenge.[1–3]

The anatomy and the physiological role of the epineurium are well described. The epineurium is a loose connective tissue layer, which surrounds the fascicles carrying the blood supply, protecting them against stretching and external trauma. The epineurium may be classified as external and internal which defines fascicular groups.[1] Harvesting of the epineurium does not hinder nerve function.

In our study when the nerve defect was bridged with a free epineural flap the resultant nerve regeneration closely approximated that of the control group, achieving 93% for muscle contractility and 81% for the light microscopy parameters. In the same group the regenerated nerve reached 60.33% of the normal nerve values concerning the contractility force and 55% concerning the two parameters of the light microscopy evaluation. Morphologically, the nerve regeneration proceeded in an orderly fashion with the progressive appearance and maturation of axons. When the epineural flap was harvested from the distal nerve stump the results were inferior compared with the proximally harvested flap. This may be due to its reduced thickness or may be attributed a more serious effect on the nerve regeneration process. In the latter flap type the contractility of the gastrocnemius muscle on the injured side reached only 42.1% of the normal contralateral muscle. The use of a distally attached epineural flap (group C) did not improve the results. Based on these results harvesting of the epineurium of the proximal nerve stump is preferable.

Epineurium flaps harvested from the proximal neural stump may serve as biological conduit for bridging short nerve defects. The advantage of this technique is the evasion from donor site morbidity, which accompanies nerve graft harvesting. There are no additional costs and the injury to the nerve from the harvesting procedure seems to be recoverable. Disadvantages of the technique are the increased operative time and the necessity for microsurgical skills. The technique may be accompanied with the infusion of a neurotrophic factor (NGF) in the epineural conduit to improve nerve regeneration.

Selection of the appropriate animal model is crucial in experimental nerve injury research. Our study was initially performed on rats but in almost all animals the iatrogenic nerve injury was significant because of the delicate structure of the rat sciatic nerve. In this model deduction of useful information deemed precarious. In another study an epineurial tube has been employed in rats to bridge short nerve defects.[24] In this study the epineurial tube was not filled with any material to prevent wall collapse but the epineurium tube led to improved functional and morphological results compared with vein autografts.[24] The rabbit experimental model was chosen because it has a well-developed epineurium, which can be harvested without significantly injuring the nerve.

Our technique is different than the epineural sleeve technique.[20,21] In our experiment a 10-mm defect was created and the epineurium was completely detached in groups A and B from the proximal and distal nerve stump respectively and tabularized to bridge the nerve defect. In group C the epineurium from the proximal stump was mobilized preserving only 2 mm and it was reversed to facilitate gap bridging. In the epineural sleeve technique a 2-mm defect in the rat sciatic nerve is created, the epineurium is rolled back distally and then pulled over the proximal nerve stump to bridge the small nerve defect and sutured with 10–0 sutures. The gap between the nerve ends with the epineural sleeve technique is minimal.

Autogenous nerve grafting provides the best functional results regarding muscle response and neural regeneration but the proximally harvested epineural conduit provides comparable results. Addition of a neurotrophic agent may improve the results and take advantage of the lesser donor site morbidity.

CONCLUSIONS

- A rabbit model was developed to evaluate the process of peripheral nerve regeneration through an epineural flap conduit.
- Three variations of an epineural flap, proximally or distally based, were compared with a control group. Nerve regeneration was examined using light microscopy, immunocytochemistry and electromyography.
- Nerve regeneration occurs in an orderly fashion across the epineural flap, especially when advanced from the proximal nerve stump.
- An epineural flap may alternatively used to bridge short nerve defects.

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