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M. F. Meek
University of Groningen

W. F. A. den Dunnen
Flevo Hospital, Almere

H. L. Bartels
University of Groningen

A. J. Pennings
University of Groningen

P. H. Robinson
University of Groningen

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PERIPHERAL NERVE REGENERATION AND FUNCTIONAL NERVE RECOVERY AFTER RECONSTRUCTION WITH A THIN-WALLED BIODEGRADABLE POLY(DL-LACTIDE-ϵ-CAPROLACTONE) NERVE GUIDE

M.F. Meek[1], W.F.A. den Dunnen[2], H.L. Bartels[3], A.J. Pennings[4], P.H. Robinson[1] and J.M. Schakenraad[5,*]

[1]Dept. Plastic and Reconstructive Surgery, [3]Lab. Animal Research, [4]Dept. Polymer Chemistry, [5]Dept. Artificial Organs, Div. Biomaterials, University of Groningen; [2]Dept. Surgery, Flevo Hospital, Almere, The Netherlands

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Abstract

The aim of this study was to evaluate functional nerve recovery after reconstruction of a 1 cm gap in the sciatic nerve of the rat, with a thin-walled biodegradable poly(DLLA-ϵ-CL) nerve guide. To evaluate both motor and sensory nerve recovery, walking track analysis and electrostimulation tests were carried out after implantation periods ranging from 3 to 26 weeks post-operatively. The first signs of functional nerve recovery could already be observed after 5 weeks. From the histological analysis, it could be concluded that most of the thin-walled nerve guides had collapsed. Despite collapsing, functional nerve recovery was relatively good after 26 weeks (motor nerve recovery 54% and sensory nerve recovery 100%), probably due to guidance of the regenerating nerve fibers along the outside of the poly(DLLA-ϵ-CL) nerve guide. This thin-walled nerve guide should, therefore, be used in combination with mechanical support.

Key Words: Nerve guide, functional nerve recovery, nerve reconstruction, electrostimulation, walking track analysis, light microscopy.

*Address for Correspondence:
Jeff M. Schakenraad
Dept. Artificial Organs, Division of Biomaterials,
University of Groningen,
Bloemsingel 10, 9712 KZ Groningen,
The Netherlands
Telephone number: +31 50 3632497
FAX number: +31 50 3633106

Introduction

The repair of transected or damaged peripheral nerves is a common clinical problem. Up to now, the most widely used technique for the reconstruction of a nerve gap is the use of autologous nerve grafts. The donor nerve is usually obtained from nerves which are functionally less important, such as the sural nerve, superficial cutaneous nerves or lateral and medial antebrachii cutaneous nerves. This technique, however, has some disadvantages: harvesting of the graft causes a sensory deficit at the cutaneous distribution site of the donor nerve and the risk of neuroma formation at the donor site. To eliminate these problems, alternative techniques, such as biodurable nerve guides constructed of silicone rubber [23, 27], acrylic polymer [37], polyethylene [8], elastomer hydrogel [20] and porous stainless steel [22], have been used to bridge the nerve gap. However, non-degradable biomaterials remain *in situ* as a foreign body, causing a chronic foreign body response with excessive scar tissue formation, resulting in compression of the regenerating nerve, ultimately limiting recovery of nerve function [23, 25, 27].

Biodegradable nerve guides provide a successful alternative. After functioning as a temporary scaffold for nerve regeneration, they gradually degrade. The use of a biodegradable nerve guide composed of an amorphous copolymer of DL-lactide and ϵ-caprolactone [poly-(DLLA-ϵ-CL)] has proven to be effective [12, 13, 14, 26]. Nerve regeneration across a 1 cm nerve gap, using a biodegradable nerve guide, was faster and qualitatively better, when compared with nerve regeneration through an autologous nerve graft [15]. Moreover, this nerve guide degrades fast and completely within 1 year [14].

Den Dunnen *et al.* [12] concluded that a nerve guide with an internal diameter of 1.23 mm and a wall thickness of 0.34 mm functioned optimally in a rat model. A nerve guide should have an internal diameter large enough to overcome problems when telescoping the nerve stumps into the lumen of the nerve guide during the implantation procedure, and should have a thin wall, that swells minimally during degradation and causes no

nerve compression. Furthermore, a too large internal diameter might stimulate fibrous tissue ingrowth into the lumen of the nerve guide, thereby possibly hampering nerve regeneration and maturation. Therefore, in the present study, we used a nerve guide with an internal diameter of 1.4 mm and a wall thickness of 0.17 mm. Recent efforts have focussed on the assessment of functional recovery following nerve injury. Since the introduction of the walking track analysis to assess motor nerve recovery in the rat by de Medinaceli *et al.* [10], this type of analysis is increasingly being used [3, 5, 6, 7, 9, 11, 18, 26, 35].

The aim of this study was to evaluate functional nerve recovery after reconstruction of a 1 cm gap in the sciatic nerve of the rat, with a thin-walled biodegradable p(DLLA-ϵ-CL) nerve guide. To evaluate both motor and sensory nerve recovery, walking track analysis and electrostimulation tests were carried out over a period, ranging from 3 to 26 weeks post-operative. Histological evaluation was carried out as a control for the functional results. Furthermore, the paws of the rats were examined for signs of automutilation.

Materials and Methods

Preparation of the nerve guides

The biodegradable nerve guide in this study was composed of a copolymer of 50% DL-lactide and 50% ϵ-caprolactone. The lactide component contained 85% L-lactide (LLA) and 15% D-lactide (DLA). The average molecular weight was 1 x 10^6 kg/kmol and the polydispersity index was 2.5.

A solution of 3 wt% of the amorphous copolymer in chloroform was prepared. This solution was dip-coated on a glass mandrel with a diameter of approximately 1.6 mm, as is described in detail by den Dunnen *et al.* [13]. This technique resulted in a nerve guide with an internal diameter of 1.4 mm and a wall thickness of 0.17 mm.

After preparation, the nerve guides were stored in 100% ethanol at 4°C. Before implantation, the nerve guides were first washed in 0.1 M sterile phosphate-buffered saline at room temperature, and then filled with 0.1 M sterile phosphate-buffered saline.

Surgical procedures

Twenty male Wistar rats, weighing approximately 200 g, were premedicated with atropine (0.25 mg/kg body weight) and anesthetized with 1% isoflurane (Forene®) and O_2/N_2O. The left sciatic nerve was exposed through a gluteal muscle-splitting incision. A 7 mm segment was then resected, leaving a gap of about 10 mm due to retraction of the nerve ends. Continuity was reestablished using a 12-mm nerve guide. During the implantation of the nerve guide, both the proximal and distal cut ends of the sciatic nerve were telescoped into the ends of the nerve guide and fixed with a single 7-0 nylon epineural suture [Auto Suture (ussc), MV 100-4 needle].

After surgery, the animals were housed in a temperature and humidity controlled room with 12-hour light cycles. All animals had access to standard rat food and water ad libitum. All procedures were carried out according to the National Guidelines for Animal Welfare.

Walking track analysis

After 3, 5, 8, 15, 21 and 26 weeks of implantation, walking track analyses were carried out to evaluate motor nerve recovery. The number of rats for each time point of walking track analysis is shown in Table 1. All rats were first allowed conditioning trials in a 8.2 x 42 cm walking track. Then photographic paper was cut to the appropriate dimensions and placed on the bottom of the track. The rat's hind feet were dipped in film developer (Ilford) slightly thickened with glycerol. The rat was permitted to walk down the track, leaving its hind feet prints on the photographic paper. Prints of both hind feet were observed on the photographic paper. From the footprints, following measurements were obtained (Fig. 1a): (1) distance from the heel to toe, the print length (PL); (2) distance from the first to the fifth toe, the toe spread (TS); and (3) distance from the second to the fourth toe, the intermediary toe spread (ITS).

All three measurements were taken from the left operated foot (OPL, OTS, OITS) as well as the contralateral non-operated foot (NPL, NTS, NITS). As a result, factors could be calculated as follows: (1) Print length factor (PLF) = (OPL-NPL)/NPL; (2) Toe spread factor (TSF) = (OTS-NTS)/NTS; (3) Intermediary toe spread factor: (ITF) = (OITS-NITS)/NITS.

Incorporating these factors into the following equation (derived by Bain *et al.* [2, 3]), the Sciatic Function Index (SFI) can be calculated as follows:

$$SFI = -38.3 \times PLF + 109.5 \times TSF + 13.3 \times ITF - 8.8 \quad (1)$$

An SFI of 0 is normal. An SFI of -100 indicates total impairment.

To obtain statistically significant data, several prints were measured for each rat. Sometimes several walks were required to obtain clear print marks. Preoperative SFI (eq. 1) of non-operated hindpaws were used as the control value for the test group (n = 20).

Electrostimulation tests

After 3, 5, 8, 15, and 26 weeks of implantation, electrostimulation tests to evaluate sensory nerve recov-

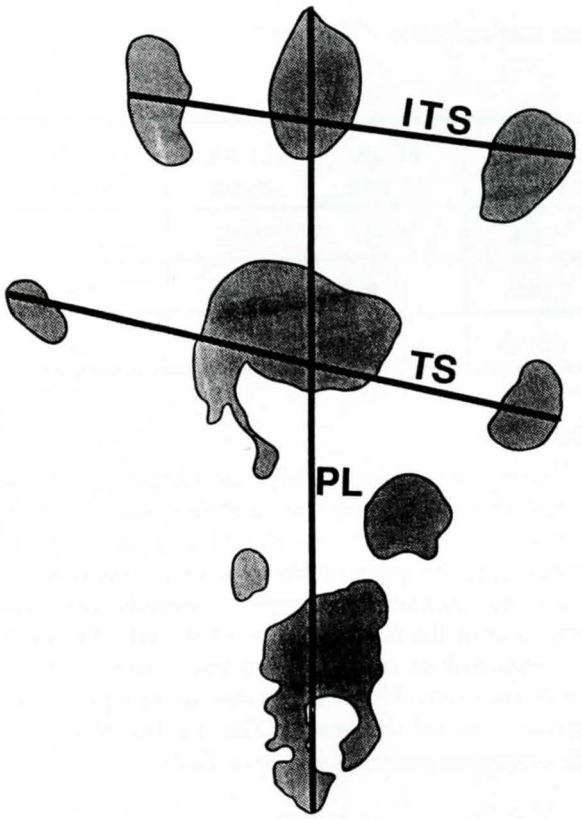

Figure 1a. Illustration showing a foot print of the rat's hind foot. To evaluate motor nerve recovery, PL: print length; TS: toe spread; IT: intermediary toe spread were measured.

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ery were carried out at three different places on the lateral side of the left (operated) foot-sole (Fig. 1b). Electrostimulation tests were carried out after 3, 5, 8 and 15 weeks of implantation (n = 3) and after 26 weeks (n = 4). An electrical stimulator with an adjustable current between 0 and 1.0 mA, was used for this purpose. A healthy rat will immediately withdraw its foot and spread its toes, when stimulated. The threshold, i.e., the lowest current causing this reflex, was evaluated and recorded. The non-operated contralateral foot-sole served as a control. The control value was calculated by dividing the outcomes of all separate measurements of the contralateral (non-operated) foot-soles at 3, 5, 8, 15 and 26 weeks by the total number of measurements. This was done to create a reliable mean value for the contralateral (non-operated) healthy paw.

Preparation for light microscopy

Histological evaluation of the nerve regeneration was carried out 3, 5, 8, 15, and 26 weeks after implantation. Evaluation was performed after 3, 5, 8 and 15 weeks of implantation (n = 3) and after 26 weeks (n = 4). After harvesting, the specimens were fixed in para

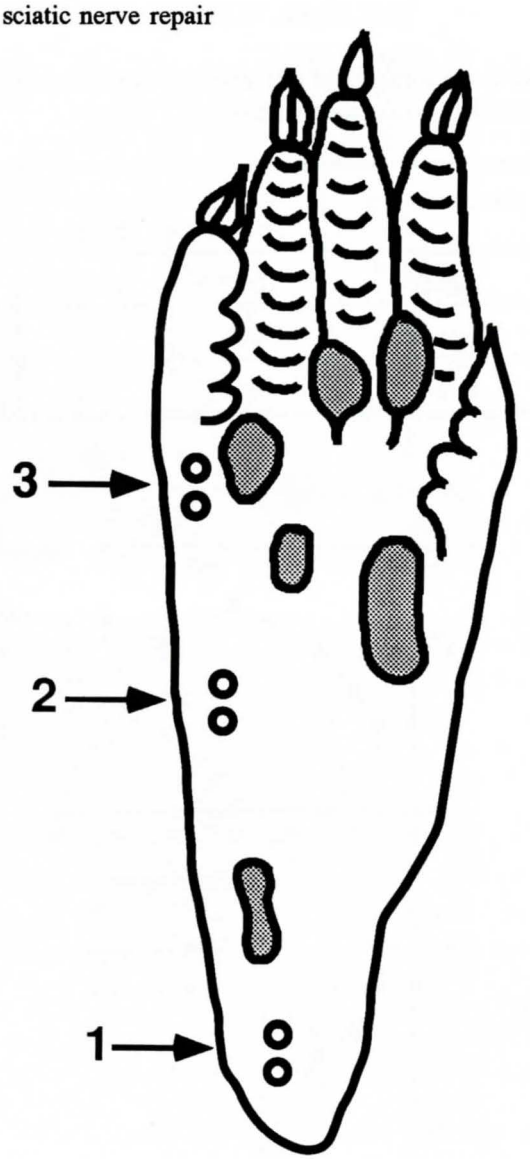

Figure 1b. Illustration showing the three different places on the plantar side of the foot, where the sensory nerve recovery was evaluated using an electrostimulator.

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formaldehyde 4% (phosphate-buffered 0.1 M, pH 7.4) for 24 hours at room temperature. Subsequently, the specimens were washed in distilled water and dehydrated in a graded ethanol series. The samples were embedded in Technovit (8100 Kulzer). Two micrometer sections were cut using a microtome (Reichert Jung 1140), with a D-knife [Spikker, Tungstencarbide edge (16/20)]. The sections were stretched in distilled water, placed on a glass slide, and allowed to dry on a 60°C hot plate for 15 minutes. The sections were routinely stained with toluidine blue and alkaline fuchsine, and evaluated for the speed and quality of the nerve regeneration.

Table 1. Percentage of rats with no, moderate and severe hindpaw automutilation. The number of rats with signs of automutilation increases with time.

automutilation	0 wk 20 rats	3 wk 20 rats	5 wk 17 rats	8 wk 14 rats	15 wk 11 rats	21 wk 8 rats	26 wk 8 rats
none	100%	100%	82%	71%	73%	63%	63%
moderate	0%	0%	12%	14%	18%	25%	25%
severe	0%	0%	6%	14%	9%	13%	13%

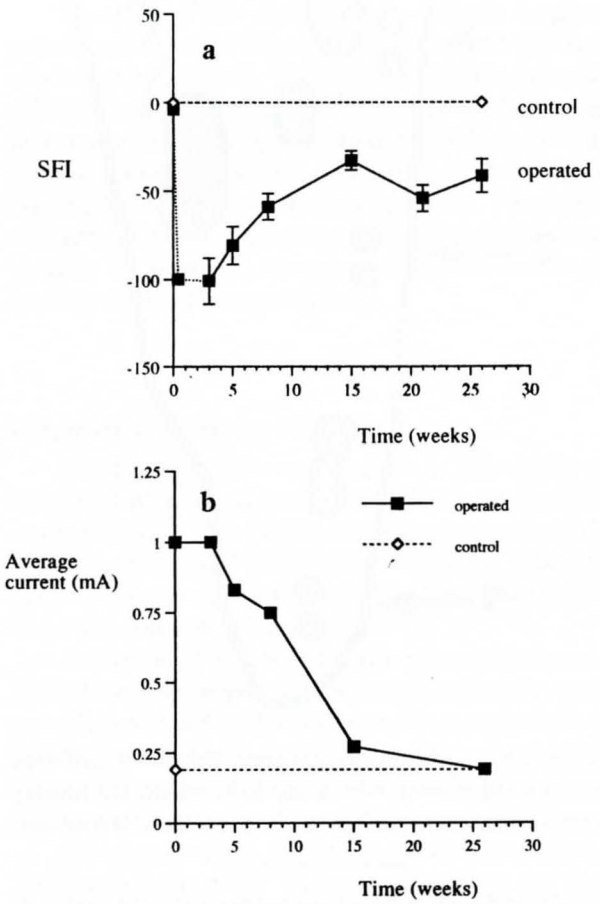

Figure 2a. Graph showing changes in the average Sciatic Function Index (SFI) and standard deviations with time. Note that the control nerve has á SFI of 0 (= normal), whereas the transected nerve has a SFI of -100 (= total impairment). After 3 weeks, the SFI starts to increase to -33 after 15 weeks. After this period, the SFI did not further increase.

Figure 2b. Graph showing the change in the average current necessary to cause a withdrawal reflex of the foot of the rat with time (the dashed line represents the control nerve; the solid line represents the operated nerve). The current necessary to cause the reflex returned to normal after 26 weeks.

Macroscopy

During this 26 week study, automutilation (only at the operated site) of the rats hind feet was observed. Therefore, after 3, 5, 8, 15, 21 and 26 weeks of implantation, the paws of the rats were examined for signs of automutilation. Superficial wounds, restricted to the nails or the cutaneous part of the rat's hindpaw, were indicated as moderate, whereas more extensive wounds (as exposed bone or the absence of a part of the paw) were scored as severe. The number of rats for each evaluation period is shown in Table 1.

Results

Walking track analysis

Reproducible walking-track patterns were obtained for all rats. The first signs of motor nerve recovery were observed after 5 weeks (Fig. 2a). After 15 weeks, a SFI of -33 was found. After this period, no further improvement of the SFI was found and after 26 weeks, the SFI was -44.

Electrostimulation tests

In the first 3 weeks after implantation of the nerve guide, the maximum current of 1.0 mA was not enough to cause a withdrawal reflex (Fig. 2b). After 5 weeks, the first signs of sensory nerve recovery could already be observed. After 8 weeks, the threshold decreased sharply to 0.27 mA at 15 weeks. After 26 weeks, the threshold returned to normal (= control value). The threshold of the contralateral control-foot was 0.19 mA.

Light microscopy evaluation

Nerve regeneration occurred in all rats. Already three weeks after implantation, the first myelinated nerve fibers could be observed in the distal nerve stump (Fig. 3b). After 26 weeks of implantation, the amount of myelinated nerve fibers in the proximal nerve stump (Fig. 3c) and the distal nerve stump (Fig. 3d) were comparable. The contralateral control nerve (Fig. 3a) contained a larger number of myelinated fibers, compared

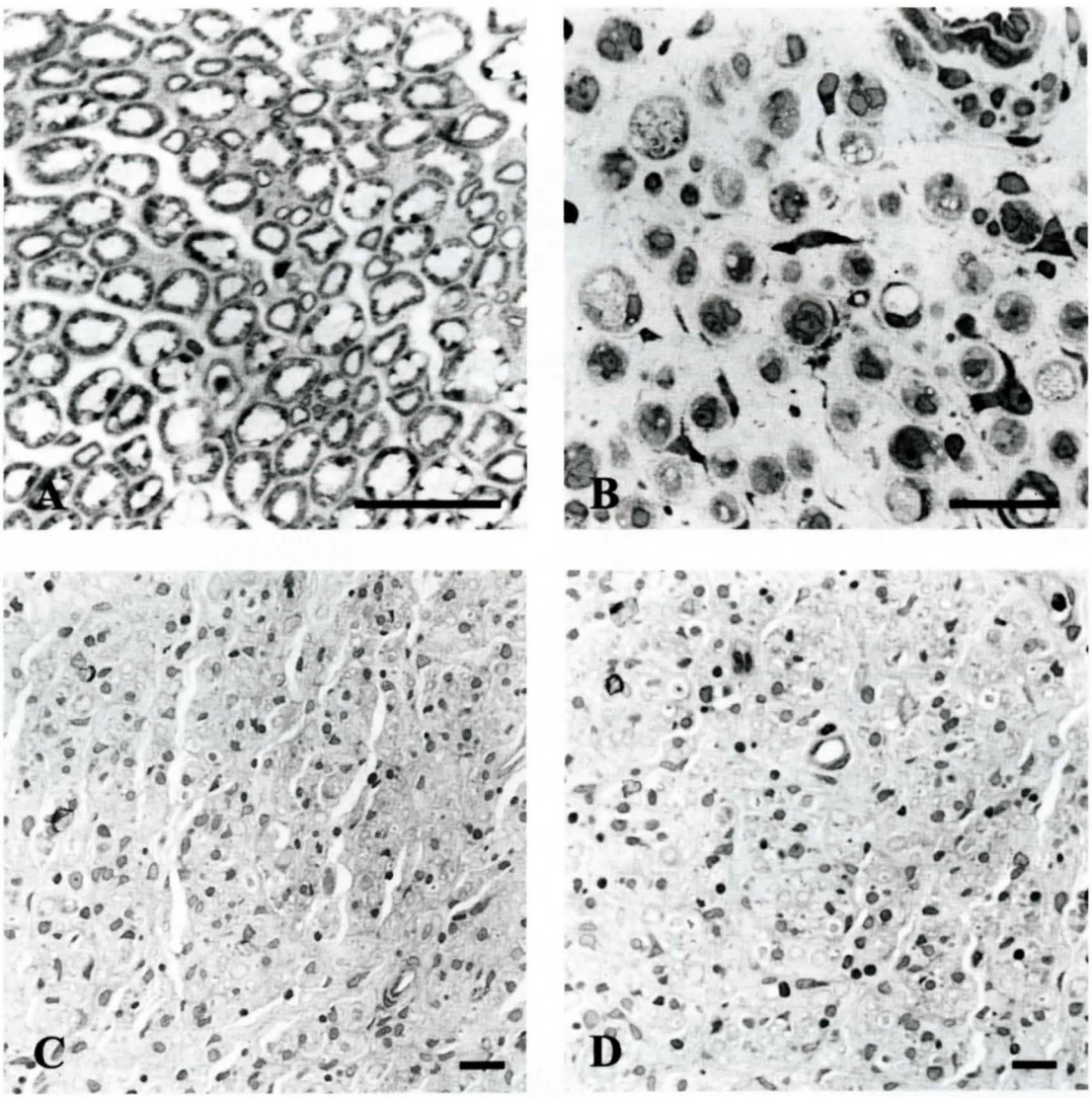

Figure 3. Light micrographs showing regenerating nerves, 3 (**Fig. 3B**) and 26 weeks (**Figs. 3C** and **3D**) after reconstruction with a thin-walled biodegradable nerve guide. The regenerated nerves were evaluated 1 mm proximal (**Fig. 3C**) and 1 mm distal (**Figs. 3B and 3D**) to the nerve guide. Note that after 26 weeks of implantation, the amount of myelinated nerve fibers in the proximal nerve stump (**Fig. 3C**) and the distal nerve stump (**Fig. 3D**) were comparable. After 26 weeks of implantation, the control nerve (**Fig. 3A**) contains a larger number of myelinated fibers. Bar = 16 μm.

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with the regenerated nerve. No neuroma formation was observed although most of the nerve guides were collapsed (Fig. 4b). The orientation of the nerve fibers was good despite the growth over the outside of the nerve guides (Fig. 4b). While functioning as a temporary scaffold, the nerve should regenerate through the nerve guide (Fig. 4a).

Macroscopic evaluation

Three weeks after nerve reconstruction, automutilation was not seen yet. However, five weeks after reconstruction, the first signs of automutilation were observed (Table 1). The number of rats with signs of automutilation increased with time, which, in turn, led to a decrease in the number of rats without any form of automutilation.

Discussion

The aim of this study was to evaluate functional nerve recovery after reconstruction of a 1 cm gap in the sciatic nerve of the rat with a thin-walled nerve guide composed of an amorphous copolymer of DL-lactide and ϵ-caprolactone (p(DLLA-ϵ-CL)). After 15 weeks, the

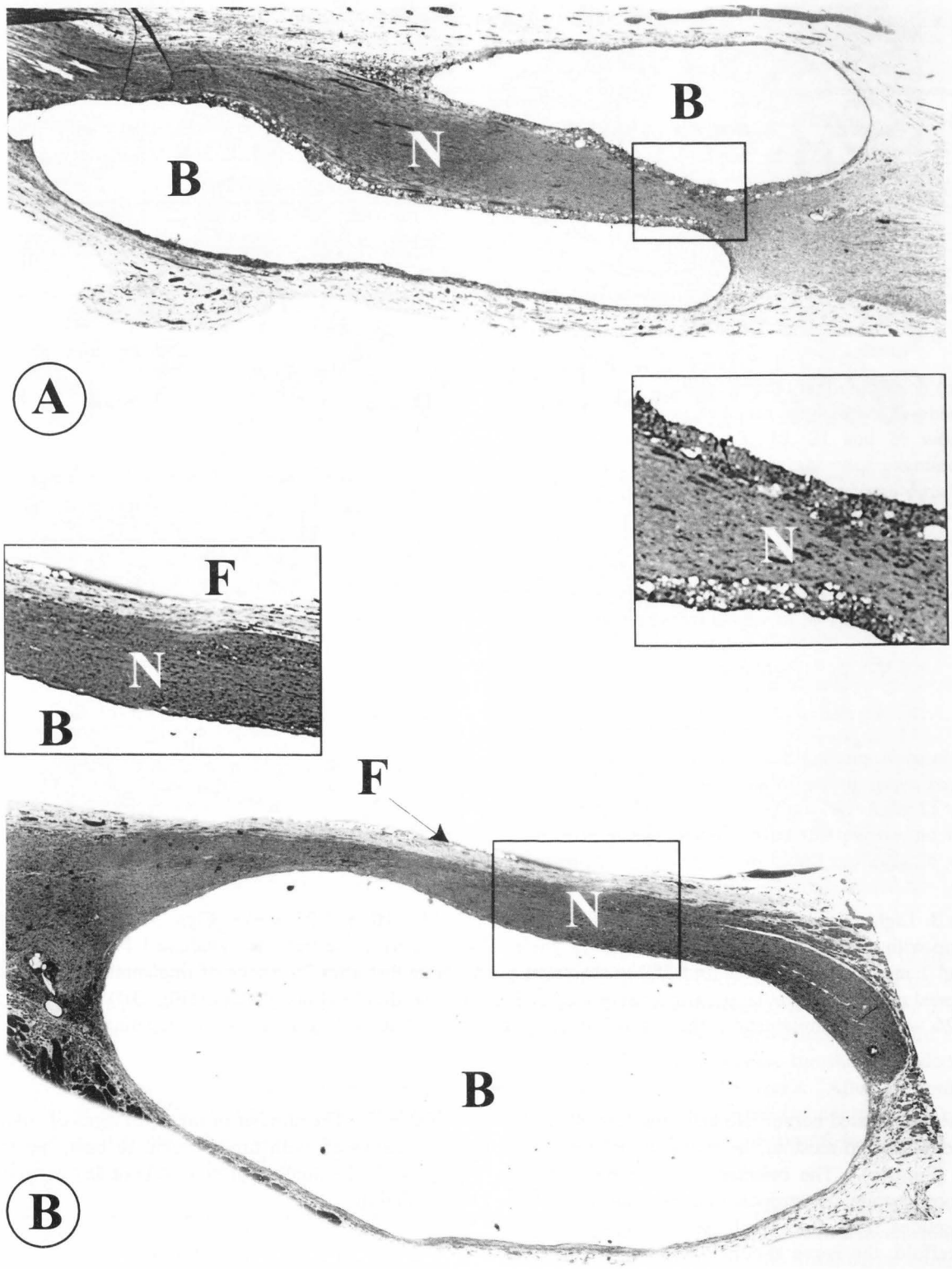

Figure 4. Light micrographs of longitudinal sections of nerve guides, showing nerve regenerating after reconstruction with a thin-walled biodegradable nerve guide. **Figure 4a** shows nerve regeneration through the nerve guide, whereas **Figure 4b** shows a collapsed nerve guide and nerve regeneration over the outside of the nerve guide. N = nerve tissue, F = fibrous tissue and B = biomaterial. Bar = 300 μm.

motor nerve function had recovered to 66% and the sensory nerve recovery returned to normal after 26 weeks. This recovery rate could have been faster if the nerve guides had not collapsed.

Walking-track analysis

In our study, a 54% motor nerve recovery was observed after 26 weeks of implantation. However, it is difficult to compare these data with results in the literature, since most studies describe either inter- and intra-observer reliability and validity of the walking track analysis, or recovery of motor nerve function after different type of nerve injuries. Hare *et al.* [19] described functional nerve recovery following epineural suturing after sciatic nerve transection. They found a SFI of -102.4 after 2 weeks and a SFI of -65.8 after 12 weeks. The SFI values never recovered to control values.

Abnormal walking-track patterns, as for example caused by automutilation of the rats hindfoot, influence the mean SFI values. A possible explanation for the fact that SFI values do not recover to control values was recently reported by Scott [32]. According to Scott [32], poor quality of nerve recovery following nerve transection may be due to: (1) the failure of regenerating axons to cross the junction between the proximal and distal nerve stumps, and (2) the mislocation of the axons within the peripheral target regions.

The failure of regenerating axons to cross a nerve gap may be due to several factors, such as the formation of scar tissue, the speed of nerve regeneration and the age of the patient. In this study, the regenerating axons failed to grow through the nerve guide towards the distal nerve stump due to collapse of the nerve guide. However, in this study, growth over the outside of the nerve guide was observed.

In rats, nerve regeneration is faster than in humans. For example, in a 1 cm gap in the rat, the nerve guide may loose its strength after 2 months. In humans however, initially there is a latent period of 3 weeks. Thereafter, nerves regenerate at a rate of approximately 1 mm per day. After bridging the nerve gap, the nerve guide should also stay intact during the first phase of maturation. Therefore, in the rat, there is less time for scar tissue formation due to the faster regeneration time.

The quality of the wound bed is also of importance in the nerve regeneration prognosis. Devitalized tissue and hematoma formation increase scarring and produce a poor nutrient bed for nerve regeneration. Any concomitant injury should be addressed and treated before nerve reconstruction is undertaken [36].

The axons that do successfully regenerate across the nerve gap are unlikely to reinnervate their original target site or even the original region. Therefore, the muscle and tendon receptors may be reinnervated by inappropriate afferent axons, and the motor unit organisation may also be altered by clumping of the muscle fibers [21]. This leads to changes in the force distribution profiles during muscle contraction, that in turn will significantly affect the mechanical input to the tendon organs [33]. As a result, between 30% and 75% of receptors may remain uninnervated, with reinnervation being more successful when the lesion is close to the target organ and the nerve comprises one, or relatively few fascicles [4, 31].

The mislocation of the afferent axons within the peripheral target regions and the uninnervated receptors have a disadvantageous effect on the utilization of proprioceptive feedback in movement control, as reflected in the walking-track patterns. This phenomenon may therefore be responsible for the fact that 15 weeks after implantation, no further improvement of the SFI was found.

Electrostimulation tests

At the end of this 26 week evaluation period, sensory nerve function was reestablished to 0.2 mA, which suggests a sensory nerve recovery approaching 100%, as measured by this technique.

However, the results of the electrostimulation test cannot be completely correlated with the return of sensation. The somatosensory cortex undergoes a complete reorganization in response to axonal injury, nerve reconstruction and axonal reinnervation of the target end-organs [38]. Although regenerating sensory nerve fibers reinnervate their specific type of end-organ, the reinnervated cutaneous fields are abnormal in terms of numbers, localizations and sizes of the somatosensory area to which they correspond. Therefore, after reinnervation, there is functional reorganization in the somatosensory cortex and multiple discontinuous patches are found [38]. Due to misdirection of regenerating nerve fibers, there is a different sensory (proprioceptive) input and therefore a different central projection. To understand the changed tactile input, the brain needs to be "reprogrammed" (cortical/central plasticity). For a complete understanding and reflection of the sensory nerve recovery, evaluation at the somatosensory cortex level should be done. The electrostimulation test is not a separate modality of sensibility, and is, therefore, used as an assessment of global sensory nerve recovery. With this measurement technique it appears that 100% sensory nerve recovery was obtained, while this would probably not be confirmed by evaluation of sensory nerve recovery at the somatosensory cortex.

Light microscopy

From this study, it can be concluded that regenerating nerve fibers can cross a 10 mm gap in the sciatic nerve of the rat within 5 weeks, although most of the

nerve guides collapsed. Some of these regenerating nerve fibers had already been myelinated within 3 weeks. In this study, a smaller number of myelinated nerve fibers was observed, compared with the study of den Dunnen *et al.* [13]. In both studies, 12 mm p(DLLA-ε-CL) nerve guides were used for the reconstruction of a 10 mm sciatic nerve defect in the rat. However, den Dunnen *et al.* [13], used a nerve guide with an internal diameter of 1.6 mm and a wall thickness of approximately 0.30 mm, while in this study, an internal diameter of 1.4 mm and a wall thickness of approximately 0.17 mm was used. Collapsing of this thin-walled nerve guide hampered the nerve regeneration and consequently, the regenerating axons had to grow along the outside of the nerve guide.

Ducker and Hayes [16] showed that tubes with thin walls were associated with less neuroma formation proximal and distal to the tube because of greater elasticity of thin-walled tubes, compared with thick-walled tubes. Den Dunnen *et al.* [13] showed that swelling of the nerve guide during degradation can have a negative effect on the speed and quality of the nerve regeneration, due to compression of the regenerating and maturing nerve fibers [12]. This is especially observed when a nerve guide with a relatively thick wall (0.68 mm) is used [12].

Therefore, in this study, a thin-walled biodegradable nerve guide was used (thin wall → less swelling and less scar tissue formation → no nerve compression). Moreover, a thin-walled biodegradable nerve guide (less biomaterial) decreases the degradation time and in turn causes less chronic foreign body reaction.

Nerve regeneration can be improved by the influence of neurite-promoting factors that positively influence axonal growth [24, 29, 34]. Besides these factors, extracellular matrix (ECM) molecules, such as collagen [28] and other proteins [1] positively influence nerve regeneration by decreasing neuronal cell death and by enhancing and directing the outgrowth and maturation of axons.

In the past, Glasby *et al.* [18] used freeze-thawed denatured autologous muscle to bridge a nerve gap. The idea behind the use of denatured muscle tissue for nerve reconstruction was that the basal lamina of the muscle tissue would direct the outgrowing nerve fibers towards the distal nerve stump.

A possible solution to improve the speed and quality of nerve regeneration through this thin-walled biodegradable nerve guide, could be the introduction of denatured muscle tissue inside the nerve guide. Besides mechanical support of the nerve guide, the longitudinally orientated basal lamina of the muscle tissue also directs the outgrowing nerve fibers towards the distal nerve stump by functioning as a scaffold for the nerve regeneration. Furthermore, the basal lamina contains collagen type IV and laminin. These ECM proteins are known to have a positive influence on the outgrowth of the damaged nerve fibers [17, 24, 30]. It is hoped that further refinements of this nerve guide will ultimately lead to a further improvement of the speed and quality of nerve regeneration and the possibility of bridging longer nerve gaps.

This study shows that good functional results can be obtained by reconstructing a 1 cm sciatic nerve defect with a thin-walled biodegradable nerve guide, although most of the nerve guides collapsed. Due to the longitudinal orientation of the nerve guide, the regenerating nerve fibers grew along the outside of the nerve guide towards the distal nerve stump. The concept behind the use of a thin-walled nerve guide (i.e., less biomaterial) is correct, but should be used in combination with mechanical support.

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