CHAPTER 7

Functional assessment of sciatic nerve reconstruction: biodegradable poly(DLLA-ε-CL) nerve guides versus autologous nerve grafts

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Abstract

The aim of this study was to compare functional nerve recovery after reconstruction with a biodegradable p(DLLA-ε-CL) nerve guide filled with modified denatured muscle tissue (MDMT), or an autologous nerve graft. We evaluated nerve recovery using walking track analysis (measurement of the sciatic function index (SFI)) and electrostimulation tests. Functional nerve recovery after reconstruction with a biodegradable p(DLLA-ε-CL) nerve guide filled with MDMT was faster when compared with nerve reconstruction using an autologous nerve graft. We conclude that in case of a short nerve gap in the rat, reconstruction can best be carried out using a p(DLLA-ε-CL) biodegradable nerve guide filled with MDMT.

Introduction

The most widely used technique for the reconstruction of a peripheral 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. This technique, however, has some disadvantages: harvesting of the graft causes sensory deficit at the donor site and the risk of neuroma formation. One alternative to eliminate these problems is the use of biodegradable nerve guides.1 After functioning as a temporary scaffold for nerve regeneration, they gradually degrade. Using a biodegradable nerve guide composed of an amorphous copolymer of DL-lactide and ε-caprolactone [p(DLLA-ε-CL)] has proven to be effective in rats in case of a 1 cm nerve gap.2 This nerve guide degrades completely within 1 year.3 Nerve regeneration across a 1 cm nerve gap, using a biodegradable nerve guide, proved faster and qualitatively better, as compared to nerve regeneration through an autologous nerve graft.4

The addition of nerve growth stimulation factors can be of special importance when bridging longer nerve gaps. Several researchers have studied the influence of growth factors,5 extracellular matrix molecules (ECM),6-8 and freeze-thawed muscle tissue on peripheral nerve regeneration.9 Most factors positively influence peripheral nerve regeneration. Recently, we evaluated different preparation techniques of denatured muscle tissue,10 aiming at an open structure of the ECM and an intact basement membrane. The addition of modified denatured muscle tissue (MDMT) inside a nerve guide creates a three-dimensional structure, providing the possibility of reconstruction after a longer nerve gap. We filled the biodegradable p(DLLA-ε-CL) nerve guide with MDMT,11 and demonstrated an increase in the speed of nerve recovery compared with an empty nerve guide. Full restoration of sensory and motor nerve functions after peripheral nerve repair often fails.12 An important aspect when comparing different techniques is to evaluate functional nerve recovery with quantitative methods. One way to evaluate the recovery of sensory function of the sciatic nerve is the withdrawal test, originally described by Young and
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Medawar. Withdrawal responses can be evoked by stimulating the foot-sole with an electric current (the so-called electrostimulation test), as was described by De Koning et al. and thereafter used by others. Rat walking track analysis for the assessment of sciatic nerve function has been well documented following nerve injury and repair. Since the introduction of this method to assess motor nerve recovery in the rat by De Medinaceli et al., this form of analysis is increasingly being used. Furthermore, due to the modification of the formula by Bain et al., walking track analysis provides detailed and reliable data on motor nerve function. The aim of this study was to compare functional nerve recovery in the rat after reconstruction with a thin-walled biodegradable p(DLLA-ε-CL) nerve guide filled with MDMT (15 mm nerve gap) with an autologous nerve graft (12 mm nerve gap). Walking track analysis and electrostimulation tests were carried out after implantation periods ranging from 2 to 15 weeks after reconstruction of the nerve defect. In addition, the rats were examined for signs of automutilation.

Materials and Methods

Preparation of Nerve Guides

The biodegradable nerve guides in this study were 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 nerve guide was made by a dip-coating technique, which is described in detail by Den Dunnen et al. 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 (RT), and then filled with MDMT.

Preparation of Denatured Muscle Tissue

Muscle tissue specimens were harvested from the back of male Wistar rats, stored at -20 °C for 24 hr, and placed in demineralised water at 4 °C for 24 hr. These freeze-thawed (denatured) muscle tissue specimens were then treated using the following technique: the sample was first placed in 16% acetic acid, at RT for 30 min, placed under vacuum (10 mBar) (Brand RS-4, Wertheim, Germany) for 1 hr, and finally placed in 0.1 M phosphate buffer at RT for 1 hr to clean the muscle tissue, as was described in more detail by Meek et al.

This modified denatured muscle tissue was then cut to the appropriate dimensions and put into the nerve guide, with the basal lamina oriented longitudinally leaving approximately 1.5 mm at both nerve guide ends.

Surgical Procedures

Male Wistar rats (n = 30), weighing approximately 250 g, were premedicated with atropine (0.25 mg/kg body weight) and anesthetised with 1% isoflurane (Forene®, Abbott Laboratories Ltd., Queenborough, Kent, UK) and O₂/N₂O. The left sciatic nerve was exposed through a superficial gluteal muscle-splitting incision. A 12 mm segment was then resected, leaving a gap of about 15
mm due to retraction of the nerve ends. In group A (n = 12); continuity was re-established by interposing an 18 mm nerve guide, filled with MDMT (Fig. 1, group A). Both the proximal and distal ends of the sciatic nerve were telescoped into the ends of the nerve guide and fixed with a single 9-0 nylon epineural suture [Auto Suture Company, Norwalk, CT, USA (ussc), MV 100-4 needle]. In group B (n = 18); the 12 mm nerve segment, which was resected from the left sciatic nerve, was reversed and re-implanted as an autologous nerve graft (Fig. 1, group B). For fixation, the proximal and distal ends of the sciatic nerve were dissected free and two 9-0 nylon epineural sutures [Auto Suture (ussc), MV 100-4 needle] were used. It should be noted that a “mixed” monofascicular (“ideal”) nerve graft, containing both sensory and motor nerve fibers was used for the reconstruction of the resected monofascicular nerve, both with similar diameters. In group C (n = 6); the rats were not operated and served as controls. Surgical procedures were performed under an operation microscope (Zeiss OPMI-6, Weesp, The Netherlands) and sterile techniques were used throughout the procedure. After surgery, the animals were housed in a temperature- and humidity-controlled room with 12 hr light/dark cycles and they had access to standard rat food and water ad libitum.

**Functional Assessment**

**Walking Track Analysis**

Walking track analysis was performed at 2, 3, 4, 5, 7, 8, 10, 12, and 15 weeks after surgery. The rats were first allowed conditioning trials in a 8.2 x 42-cm walking track. Photographic paper was placed on the bottom of the track. The rat’s hind feet were dipped in film developer, slightly thickened with glycerol. The rat was allowed to walk down the track (Fig. 2A), leaving its hind feet prints on the photographic paper. From the footprints, several measurements were taken and the so-called sciatic function index (SFI) was calculated. This procedure is described in detail by Meek et al.17 The SFI was calculated using the formula developed by Bain et al.22,24

**Electrostimulation Tests**

Two, 3, 4, 5, 7, 8, 10, 12, and 15 weeks after surgery, electrostimulation was carried out on the lateral side of the left (operated) foot-sole, as described in detail by Meek et al.17 (Fig. 2B). A healthy rat immediately withdraws its foot and spread its toes after stimulation. The threshold, i.e. the lowest current causing this reflex at the operated side, was recorded.
Automutilation
The paws of the rats were examined weekly for signs of automutilation. Superficial wounds restricted to the cutaneous part of the rat’s hindpaw were indicated as moderate, whereas more extensive wounds showing exposed bone or the absence of a part of the paw were scored as severe.

Statistical Analysis
All data for group A and B were submitted to linear regression analysis.

Results

Functional Evaluation
Preoperative SFI’s in rats of groups A and B did not differ from the control group (group C). In group B, three rats were excluded from evaluation: two rats showed extreme plantar flexion contractures of the operated hindpaw and clear print marks could not be obtained in these rats; in the other rat, automutilation of the complete lateral side of the footsole occurred.

Walking Track Analysis
The SFI’s obtained from the walking track patterns using photographic paper are shown in Figure 3A.
In group A, good quality walking tracks could be recorded from all rats. The first signs of recovery in the walking track patterns were already observed after three weeks. During the evaluation period, the SFI significantly increased with time to approximately -43 after 12 weeks. After 12 weeks, the walking pattern had greatly improved, but had not attained normal values. In group B, from the start, the SFI did not show any significant recovery and after 7 weeks, the SFI still was -96. No further increase of the SFI was observed thereafter and from week 10, most footprints could not be measured anymore because of dragging of the operated foot resulting in smearing of the prints. In group C, reproducible walking track patterns could be measured in all rats. The mean SFI for perfect functioning hindpaws did not significantly change during the study and varied from -6 to -12, with a mean value of -8.6.

Electrostimulation Tests
The current (mA) necessary to cause a withdrawal reflex of the foot is outlined in Figure 3B. In group B one rat was excluded from evaluation because of automutilation of the complete lateral side of the footsole.
In group A, 3 weeks after implantation, the first signs of sensory nerve recovery could be observed. Thereafter, the threshold decreased gradually to approximately 0.4 mA after 12 weeks. In group B, there were no significant signs of sensory nerve recovery in the first 7 weeks after reconstruction (the
sensory nerve recovery could be observed. After 15 weeks, the threshold had decreased to approximately 0.4 mA. In group C, the threshold of this control group was 0.1 mA throughout the whole study.

**Automutilation**

In group A, severe automutilation was not observed in any of the rats. In two rats, however, superficial wounds restricted the cutaneous part of the fourth and fifth toe (moderate automutilation) appearing 5 weeks after operation (Fig. 4B). The toes of these rats also showed shorter nails. In group B, first rats with automutilation were observed as soon as two weeks after operation. One rat showed automutilation of the complete lateral side of the footsole (Fig. 4C) and was therefore excluded from further testing. One other rat showed automutilation at the skin of the fourth toe, but walking track analysis could still be obtained from this rat. After 4 weeks, two other rats showed exposed bone of the third and fourth toe. The rat with automutilation of the fourth toe in week 2, now also showed moderate automutilation of the third toe. After 7 weeks of reconstruction, we observed that the rat with moderate automutilation in week 4 also showed complete loss of the fourth toe (severe automutilation). Furthermore, another rat showed severe automutilation of the third, fourth, and fifth toes. After 10 weeks, four rats with severe automutilation were excluded from further testing. In group C, no automutilation was observed (Fig. 4A).

**Statistical Analysis**

Linear regression analysis was per
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Fig. 4. Photograph showing the rats’ hindpaws. A: Intact hindpaw. B: Moderate automutilation in group A, 7 weeks after implantation. Only a cutaneous part of the rat’s toes of the operated hindpaw is removed. C: Severe automutilation 2 weeks after reconstruction in group B. The complete lateral side of the rat’s foot is removed.

formed, and showed that motor and sensory nerve recovery in group A during the 12 weeks of study was highly significant: $P < 0.001$. In group B, no significant improvement of the SFI could be obtained during the study. Sensory nerve recovery was significant in group B: $P = 0.005$.

Discussion

Although autologous nerve grafting is the most widely used technique for peripheral nerve reconstruction, the results are often disappointing and full recovery of nerve function is seldom achieved. Therefore, several alternative techniques have been developed which are more or less successful. Biodegradable nerve guides potentially provide a successful alternative. The objective of using biodegradable p(DLLA-$\varepsilon$-CL) nerve guides is that the nerve guide degrades after guiding the regenerating nerve fibers towards the distal nerve stump. This generally prevents the formation of a neuroma-in-continuity and ingrowth of fibrous tissue. In addition, the amount of scar tissue formed after reconstruction is decreased by using a single suture to fix the proximal and distal nerve stumps into the lumen of the nerve guide. The use of this type of biodegradable nerve guide has proven to be effective in animal studies. From previous studies it could be concluded that tube dimensions and swelling of degrading biomaterials are important factors influencing the final outcome of the nerve regeneration. Den Dunnen et al. used biodegradable nerve guides with an internal diameter of 1.5 mm and a wall thickness of 0.3 mm, which so far proved optimal in a rat model. A nerve guide with a wall thickness of 0.17 mm tended to collapse, unless some mechanical support inside the lumen of this thin-walled nerve guide was used such as MDMT. We used this type of guide in group A. Immediately after surgery, all rats in group A and B dragged their operated
paws. Incomplete nerve recovery as indicated by an abnormal walking track pattern, or a delayed or absent withdrawal reflex may be due to the failure of regenerating axons to cross the junctions between the proximal and distal nerve stumps. Axonal misdirection within the peripheral target regions (which leads to so-called cross-innervation) may be another cause for incomplete return of function. After 3 weeks of reconstruction, the walking track pattern in group A showed improvement, whereas the walking pattern in group B showed no improvement at all (Fig. 3). The speed of recovery and the quality of the walking pattern thereafter was superior in group A.

Hare et al. described functional nerve recovery following fascicular sciatic nerve repair using epineural sutures. Their improvements in SFI measurements were hampered by the formation of contractures. In our study this phenomenon was also observed in group B but not in group A. Dellon and Mackinnon found that 1 year after nerve repair, chronic foot deformities resulted in gait patterns that hamper walking track assessment. Prolonged muscle denervation may result in diminished motor nerve recovery, which in turn also may lead to abnormalities in the walking track analysis. Kobayashi et al. showed that a delay in nerve repair for longer than 2 weeks resulted in a significant reduction in rat muscle mass. Restrepo et al. showed results similar to those in the present study. They used electromyography recordings to assess relative effectiveness of nerve repair. After reconstruction of 25 mm defects of the sciatic nerve, they found that at all time points (1-11 weeks), motor reinnervation was more advanced on the nerve repaired with an empty perineurial tube compared to that after a conventional nerve graft. Lin et al. compared functional nerve recovery in a group of rats with a nerve graft (10 mm of sciatic nerve was transected) with an ungrafted group in which the nerve gap was left unrepaird. They found no return of the SFI after 16 weeks of reconstruction in the grafted group. It can therefore be concluded that the results after grafting nerves is inferior to the results after the use of nerve guides in case of short peripheral nerve defects in the rat. The local application of small electrical stimuli to the rat’s footsole is a noninvasive, rapid, easy, and very precise method to evaluate the return of sensory nerve function after nerve injury. Our results suggest that in group A and B, sensory nerve fibers regenerated faster as compared to motor nerve fibers. The results in the present study may imply that the regenerating axons in group B needed more time to cross the junction between the proximal and distal nerve stump. In a previous, unpublished study we hypothesized that the return of sensory function, as measured by the electrostimulation tests, does not necessarily imply the outgrowth of fibers from the sciatic nerve. The return of sensory function could also be explained by collateral sprouts from intact fibers in the skin surrounding the denervated zone, or a combination of both. An increased delay in nerve recovery might result in an increased formation of intraneural fibrosis. This in turn may narrow the endoneurial tubes in the distal nerve stump through which the regenerating axons have to grow, thereby
limiting recovery of nerve function. This has also been demonstrated by den Dunnen et al.\textsuperscript{4}

The results in the present study indicate that the biodegradable nerve guide filled with MDMT leads to improved functional nerve recovery. One possible explanation could be that regenerating nerve fibers grow out faster. The purpose of the use of both the nerve graft and the MDMT, is to offer a scaffold for axonal growth between the proximal and distal nerve stump. In nerve grafts, regeneration of axons is guided by endoneural tubes (e.g., Schwann cell basement membranes).\textsuperscript{40} MDMT also contains basement membranes oriented in a longitudinal direction. These basement membranes contain laminin, which has neurite-promoting activity\textsuperscript{41} and stimulates Schwann cell mitosis.\textsuperscript{42,43} Although both the nerve graft and the MDMT contain basement membranes in a longitudinal fashion, regeneration through a nerve guide filled with MDMT is faster than in an autologous nerve graft. The differences in functional recovery in both groups might be explained by the open structure of MDMT. A nerve graft contains a dense structure, which may decrease growth of neurites. It should be considered that a nerve graft contains Schwann cells, whereas a nerve guide with MDMT does not. As soon as a regenerating axon makes contact with a Schwann cell, the speed of the regeneration of the axon slows down while the axon becomes myelinated.\textsuperscript{44} Nerve grafts contain proliferating Schwann cells, so the regenerating axons will make contact with Schwann cells in an earlier phase, compared to outgrowing axons inside the nerve guide. Empty nerve guides also showed faster nerve regeneration across a 10 mm nerve gap as compared to autologous nerve grafts.\textsuperscript{4} It is also known that poor vascularization of the nerve graft causes an increase in the formation of fibrosis, which in turn will decrease the speed of axonal regeneration. Another possible explanation for the differences in functional recovery is, that MDMT contains collagen IV, which is also known to positively influence the outgrowth of the damaged nerve fibers, because of their intrinsic neurite-promoting effect.\textsuperscript{45} When a nerve guide is filled with some kind of additive which is known to positively influence the nerve regeneration process, the additive in addition should contain a three-dimensional structure to guide the regenerating nerve fibers. The additive inside a nerve guide can be of special importance by acting as an enticing factor for the growth of nerve fibers towards the distal nerve stump. This is an important factor with regard to clinical application of denatured muscle tissue in patients with nerve injuries, especially for the reconstruction of larger (>15 mm) nerve defects. In summary, our results indicate that accelerated regrowth is favourable for functional nerve recovery.

It is remarkable that in our study, no severe automutilation was found in group A, whereas this was the case in rats from group B. Similar findings were reported by Zellem et al.\textsuperscript{46} They reproduced the results of de Medinaceli’s protocol and refined his technique, and showed self-mutilation of the limb supplied by the sciatic nerve in 39 of the 69 rats (57\%) after sciatic nerve repair. Inbal et al. reported that the extent of automutilation
(e.g. autotomy, self-mutilation) varies greatly in genetically different populations of rats. Autotomy is thought to reflect a sensory pathology, analogous to anesthesia dolorosa in humans. Wall and Gutnick hypothesized that following complete sciatic nerve transection, the functional territory of the intact saphenous nerve may expand by collaterals to innervate the majority of the sciatic territory except the lateral foot area. The abnormal painful impulse activity possibly is referred to the anesthetic area of the last two digits and by mutilation, the animal attempts to remove the offending stimulus.

In a previous study, we found automutilation in rats with collapsed nerve guides and concluded that the longer the regeneration process of sensory nerve fibers takes, the more likely it is that automutilation will occur. Den Dunnen et al. evaluated the effect of different tube dimensions on peripheral nerve regeneration. They found that nerve guides with a relatively small internal diameter showed nerve compression due to a pronounced swelling of the degrading tube. Parts of the toes of the operated hindpaws often had disappeared due to automutilation after an implantation time of 1 month. In contrast, nerve guides with a larger internal diameter and a thinner wall thickness did not result in automutilation.

The differences in automutilation in group A and B can only be explained by the different reconstruction techniques. According to the theory of Wall et al., the painful sensations in the anesthetic areas were earlier (partly) relieved (in group A) by new innervation from outgrowing sciatic nerve fibers. In group B, however, the formation of a neuroma-in-continuity is more likely to occur when using autologous nerve grafts, because the outgrowing nerve fibers have to pass two coaptation sites. Hall et al. also studied functional nerve recovery in the rat after reconstruction using nerve autografts. They found 50% automutilation and 40% recovery of the SFI, while the automutilated rats were excluded from the study. It is known that when nerve fibers regenerate across a restored nerve gap, 20-40% of the nerve fibers are lost at the suture line. Nerve regeneration through a nerve guide proceeds faster than the use of an autologous nerve graft, ultimately resulting in less automutilation.

In summary, we can conclude that both motor and sensory nerve recovery across a short nerve gap, after reconstruction with a thin-walled biodegradable nerve guide filled with MDMT, is faster and leads to less residual handicaps, as compared to nerve reconstruction using an autologous nerve graft.

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