CHAPTER 5

Evaluation of functional nerve recovery after reconstruction with a poly(DL-lactide-ε-caprolactone) nerve guide, filled with modified denatured muscle tissue

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Abstract

The aim of this study was to compare the speed of functional nerve recovery after reconstruction with a biodegradable p(DLLA-ε-CL) nerve guide, as filled with either modified denatured muscle tissue (MDMT) or phosphate-buffered saline (PBS). To evaluate both motor and sensory nerve recovery, walking track analysis and electrostimulation tests were carried out after implantation periods, ranging from 3-15 weeks. Functional nerve recovery after reconstruction of a 15 mm nerve gap, with a biodegradable p(DLLA-ε-CL) nerve guide filled with modified denatured muscle tissue, was slightly faster, compared with nerve reconstruction of a 10 mm gap with a biodegradable p(DLLA-ε-CL) nerve guide filled with PBS. We conclude that our experiments have demonstrated that the use of MDMT increases the speed of recovery after reconstruction of a nerve gap with a p(DLLA-ε-CL) biodegradable nerve guide. Furthermore, the use of MDMT might open perspectives for repair of longer nerve gaps.

Introduction

The most widely-used technique for bridging nerve gaps is the use of autologous nerve grafts. This technique, however, has some disadvantages: harvesting of the graft causes sensory deficit at the donor site and the risk of neuroma formation there. To eliminate these problems, many alternative techniques, such as the use of biodurable nerve guides, have been developed to bridge a nerve gap. However, biodurable biomaterials remain in situ as a foreign body, potentially causing a chronic foreign body reaction with excessive scar tissue formation, resulting in constriction of the regenerating nerve, and ultimately limiting recovery of nerve function (e.g., secondary nerve impairment). Biodegradable nerve guides provide a successful alternative. The idea behind the use of biodegradable nerve guides is, that the nerve guide directs the outgrowing nerve fibers towards the distal nerve stump, while preventing neuroma formation and ingrowth of fibrous tissue into the nerve gap. After serving these functions, the nerve guide should gradually degrade, without inducing scar tissue formation. The use of a biodegradable nerve guide composed of an amorphous copolymer of DL-lactide and ε-caprolactone [p(DLLA-ε-CL)] has proven to be successful. This nerve guide degrades quickly and completely within 1 year, and nerve regeneration across a 1 cm nerve gap was faster and qualitatively better, compared with nerve regeneration through an autologous nerve graft. Moreover, reconstruction with this nerve guide showed good functional nerve recovery. However, in all these previous experiments, nerve gaps of 1 cm in the sciatic nerve of rats were bridged. To bridge a nerve gap of several centimeters (a more realistic point of view in the clinical situation), the addition of nerve growth stimulation factors will be necessary. The addition of one of these
factors can be of special importance in nerve regeneration through a nerve guide of several centimeters, since the maximum gap length that can be successfully reconstructed with a nerve guide in the rat is 2 cm. Several researchers have already studied the influence of growth factors, extracellular matrix molecules, and freeze-thawed muscle tissue on peripheral nerve regeneration. Most factors positively influence peripheral nerve regeneration. The use of denatured muscle tissue inside a nerve guide is very promising, since the longitudinally oriented basal lamina of the muscle tissue will direct the outgrowing nerve fibers towards the distal nerve stump by functioning as a scaffold for the outgrowing nerves. In addition, the basal lamina contains both collagen type IV and laminin, which are known to enhance the outgrowth and regeneration of peripheral nerve fibers. The nerve guide prevents neuroma formation and the ingrowth of fibrous tissue into the nerve gap. Due to the presence of muscle tissue inside the nerve guide, there is less tendency for the nerve guide to collapse. Recently we evaluated different preparation techniques of denatured muscle tissue, aiming at an open structure of the extracellular matrix (ECM) and an intact basement membrane. The aim of this study was to evaluate the speed of functional nerve recovery after reconstruction with a biodegradable \( p(DLLA-\varepsilon-CL) \) nerve guide, filled with modified denatured muscle tissue (MDMT).

**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% \( \varepsilon \)-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. 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 (PBS) at room temperature, and then filled with 0.1 M sterile PBS (group A), or with modified denatured muscle tissue (group B).

**Preparation of the Denatured Muscle Tissue**

Muscle tissue specimens were harvested from the back of male Wistar rats, stored at -20 °C for 24 hr, and then placed in demineralized 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 room temperature (RT) for 30 min, then 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 (Fig. 1). The preparation of modified denatured muscle tissue is described in more detail by Meek et al. This MDMT 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...
Fig. 1. Illustration (A) and cryoscanning electron micrographs (cry-SEM) (B) showing preparation of modified denatured muscle tissue obtained from the back of the rat. B1, B2: Untreated (normal) muscle tissue. B3, B4: Denatured muscle tissue shows that muscle fibers are swollen. B5, B6: Modified denatured muscle tissue shows that the original muscle-fiber shape has disappeared and that striations are no longer present. Furthermore, modified denatured muscle tissue shows a wider extracellular matrix. E, extracellular matrix; MFI, muscle fiber; My, myofibril. Bars in B1, B3 and B5, 4.5 µm. Bars in B2, B4 and B6, 0.3 µm.
p(DLLA-ε-CL) nerve guide filled with modified denatured muscle tissue
with atropine (0.25 mg/kg body weight) and anesthetized with 1% isoflurane (Forene®) and O$_2$/N$_2$O. The left sciatic nerve was exposed through a superficial gluteal muscle-splitting incision. In group A, 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. 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 9-0 nylon epineural suture [Auto Suture (ussc), MV 100-4 needle]. In group B, a 12 mm nerve segment was resected, leaving a gap of about 15 mm due to retraction of the nerve stumps. Continuity was reestablished by interposing an 18 mm nerve guide, filled with MDMT (Fig. 1A). 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 9-0 nylon epineural suture [Auto Suture (ussc), MV 100-4 needle]. Surgical procedures were performed under an operation microscope (Zeiss OPMI-6, Weesp, The Netherlands), and a sterile technique was used throughout the procedure.

We emphasize that in this study, we intentionally chose to compare two different gap lengths. We chose to reconstruct a 1.5 cm nerve gap with a nerve guide filled with MDMT, in order to evaluate whether a longer gap could be successfully bridged, and whether the results obtained after reconstruction using this technique are comparable to those after reconstruction of the “standard gap length” (i.e., 1 cm) without MDMT, which was evaluated in all previous studies. 8-11,16,20,22-25

In our opinion, the addition of MDMT inside a nerve guide (group B) would create a three-dimensional (3-D) structure, providing the possibility to reconstruct a longer nerve gap. Therefore, a 50% longer nerve gap was chosen.

After surgery, the animals were housed in a temperature- and humidity-controlled room with 12 hr light cycles and had access to standard rat food and water ad libitum. Good laboratory practice (GLP) was observed, according to the National Guidelines for Animal Welfare, comparable with the international rules for animal experimentation (International Guide on Animal Biomedical Research and Ethical Code for Animal Experimentation of the Council for International Organization of Medical Sciences).

**Walking Track Analysis**

After 3, 5, 8, 12, and 15 weeks of implantation, walking track analysis was carried out to evaluate motor-nerve recovery, as described in detail by Meek et al. 11 In brief, the 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 slightly thickened with glycerol. The rat was permitted to walk down the track, leaving its hind feet prints on the photographic paper. From the footprints, several measurements were obtained: distance from the heel to the third toe, the print length (PL); distance from the first to the fifth toe, the toe spread (TS); and distance from the second to the fourth toe, the intermediary toe spread (ITS). All three measurements were taken from the left operated foot as well as the contralateral nonoperated foot. As
a result, print length factor (PLF), toe spread factor (TSF), and intermediary toe spread factor (ITF) could be calculated. As a result, a sciatic function index (SFI) could be derived as follows:

\[ SFI = -38.3 \times PLF + 109.5 \times TSF + 13.3 \times ITF - 8.8 \]

An SFI of 0 is normal. An SFI of -100 indicates total impairment. To obtain significant data, several prints were measured for each rat. Sometimes several walks were required to obtain clear print marks.

**Electrostimulation Tests**

After 3, 5, 8, 12, and 15 weeks of implantation, electrostimulation tests to evaluate sensory-nerve recovery were carried out at three different places on the lateral side of the left (operated) foot-sole, as described in detail by Meek et al. In brief, an electrical stimulator with an adjustable current between 0-1.0 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. Furthermore, it should be mentioned that with electrostimulation tests, not only recovery of sensory-nerve function (e.g., afferent nerve fibers) is tested, but actually the reflex arc (e.g., afferent/sensory nerve fibers + efferent/motor nerve fibers) of a stimulus to the sensory receptor. Theoretically, it might be possible that sensory-nerve fibers are recovered while motor-nerve fibers are not. No withdrawal reflex is then observed.

**Statistical Analysis**

All data were submitted to linear regression analysis.

**Results**

**Walking Track Analysis**

Reproducible walking track patterns could be measured for all rats. Preoperative sciatic function indices for the test group did not differ from the control value (Fig. 2A). As a control, the SFI for a perfect functioning hindpaw (SFI = 0) was used. Group A: Nerve guides. The first signs of motor-nerve recovery were already observed after 5 weeks. After 12 weeks, approximately 60% of the motor-nerve function was recovered. Group B: Nerve guides + muscle. The first signs of motor-nerve recovery were observed after 3 weeks. During the evaluation period, the SFI increased with time. After 12 weeks the SFI was -43.4 (almost 60% recovery).

**Electrostimulation Tests**

The current (mA) necessary to cause a withdrawal reflex of the foot is outlined in Figure 2B. The threshold of the contralateral control-foot was 0.18 mA (= optimal value). Group A: Nerve guides. In the first 3 weeks after implantation of the nerve guide, the maximum current of 1.0 mA was not enough to cause the reflex. After 5 weeks, the first signs of sensory-nerve recovery could be observed. After 12 weeks, the threshold decreased to approximately 0.48 mA. Group B: Nerve guides + muscle. After 3 weeks, the first signs of sensory-nerve recovery could already be observed and the threshold decreased sharply to 0.71 mA at 5 weeks. After 12 weeks, the threshold decreased to 0.37 mA.

**Statistical Analysis**

Linear regression analysis was per-
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Discussion

This study demonstrates that functional nerve recovery after reconstruction of a 1.5 cm gap with a biodegradable p(DLLA-ε-CL) nerve guide filled with MDMT is slightly faster, compared with the reconstruction of a 1 cm gap with a biodegradable p(DLLA-ε-CL) nerve guide filled with PBS only.

The first signs of functional (motor and sensory) nerve recovery in group A (nerve guide + PBS) could be observed after 5 weeks of implantation, whereas the first signs of functional nerve recovery in group B (nerve guide + MDMT) were already observed after 3 weeks. The exact moment at which the first sign of functional nerve recovery of group B started must have been before our first evaluation period (at 3 weeks). Furthermore, it can be observed (Fig. 2) that the motor- and sensory-nerve recovery in group A was somewhat slower than in group B, although a 50% longer nerve gap was reconstructed in group B. Complete return of motor- and sensory-nerve function in both groups was not reached at the end of the evaluation period. However, since the return of nerve function started somewhat earlier in group B, it might be concluded that the addition of MDMT inside the nerve guides in group B enhanced the onset and speed of nerve regeneration. The results obtained in this study (recovery of functionality) can not be directly related to speed and quality of nerve regeneration. To evaluate this relationship more deeply, light-microscopy (LM), transmission-electron-microscopy (TEM), and morphometric analysis of the axon regeneration process will be necessary.

Fig. 2. A: Graph showing change in average sciatic function index (SFI) with time. Note that an SFI of 0 is normal, whereas an SFI of -100 indicates total impairment. An increase of SFI in group A can be observed after 5 weeks, whereas in group B, an increase of SFI can already be observed 3 weeks after implantation. B: Graph showing change in current, which is necessary to cause withdrawal reflex of the foot of the rat with time. The nonoperated contralateral foot-sole served as a control. A decrease of current in group A can be observed after 5 weeks, whereas in group B, a decrease of current can already be observed 3 weeks after implantation.

Group A, 10 mm nerve gap/12 mm nerve guide filled with PBS; group B, 15 mm nerve gap/18 mm nerve guide filled with modified denatured muscle tissue

formed, and showed that motor- and sensory-nerve recovery in both groups A and B were highly significant: in both cases $P < 0.001$. 
The use of denatured muscle grafts for peripheral nerve repair was studied by Glasby et al. They reconstructed 3 cm gaps in the ulnar nerve of primates. By 6 months, normal function of the hand had returned. Norris et al. studied the use of denatured muscle grafts in a series of human patients in order to span gaps in digital nerves. Nerve gaps of 15-25 mm were bridged. Evaluation of 3-11 months after reconstruction showed almost complete sensory recovery in 7 out of 8 patients. However, Stirrat et al. showed no motor recovery in 7 patients after reconstruction of major mixed nerves with degenerated skeletal muscle. This may be explained by the fact that by using only freeze-thawed denatured autologous muscle (without a nerve guide) to bridge a nerve gap, there is an increased risk that nerve fibers will grow out of the muscle tissue, thereby forming a neuroma-in-continuity. In our opinion, the risks as stated should not be taken, with regard to clinical application of denatured muscle tissue in patients with nerve injuries.

When autologous nerve grafts are used for the reconstruction of nerve gaps, regeneration of axons through these grafts is thought to be guided by endoneurial tubes (e.g., Schwann cell basement membranes). Modified denatured muscle tissue contains basement membranes that can function as a scaffold for regenerating axons. The major non-collagenous component of basement membranes is laminin, which has neurite-promoting activity, and stimulates Schwann cell mitosis. This might explain the better functional nerve recovery in group B. In a previous study, it was suggested that the idea behind the use of a thin-walled nerve guide (i.e., less biomaterial and less swelling) was correct; however, additional mechanical support is then needed. The nerve guide and basement membranes direct the outgrowing nerve fibers towards the distal stump, prevent the ingrowth of fibrous scar tissue, and provide an optimal environment enriched with factors to enhance and orient axonal regeneration. Furthermore, the risk that nerve fibers will grow out of the muscle tissue, thereby forming a neuroma-in-continuity, is prevented.

LM, TEM, and morphometric analysis of the axon regeneration process is now being performed to evaluate whether the addition of modified denatured muscle tissue inside nerve guides enhances the speed of nerve regeneration, as was suggested earlier. In the future, the effects of other additives (NGF, B50) inside biodegradable nerve guides on the speed and quality of functional nerve recovery and nerve regeneration will be evaluated. Appropriate applications of additives inside biodegradable nerve guides will not only improve peripheral nerve regeneration, but also allow a larger nerve gap to be reconstructed by nerve entubulation. The latter is of extreme importance with regard to the clinical application of biodegradable nerve guides, because in the clinical situation nerve gaps of several centimeters have to be bridged.

Finally, we conclude that our experiments have demonstrated that the use of MDMT increases the speed of recovery after reconstruction of a nerve gap with a p(DLLA-ε-CL) biodegradable nerve guide. Furthermore, the use of MDMT might open perspectives for repair of longer nerve gaps.
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