PERIPHERAL NERVE RECONSTRUCTION WITH COLLAGEN TUBES FILLED WITH DENATURED AUTOLOGOUS MUSCLE TISSUE IN THE RAT MODEL

ULF DORNSEIFER, M.D.,[1] ANDREAS M. FICHTER, M.D., D.D.S.,[2] STEFAN LEICHTLE, M.D.,[2] ANDREW WILSON, M.D.,[2] ANGELIKA RUPP, D.V.M.,[3] KARSTEN RODENACKER, M.Sc.,[4] MILOMIR NINKOVIC, M.D., Ph.D.,[1] EDGAR BIEMER, M.D., Ph.D.,[2] HANS-GÜNTHER MACHENS, M.D., Ph.D.,[2] KASPAR MATIASEK, D.V.M., Ph.D.,[3] and NIKOLAOS A. PAPADOPULOS, M.D., Ph.D.[2*]

Conventional nerve conduits lack cellular and extracellular guidance structures critical for bridging larger defects. In this study, an exogenous matrix for axonal regeneration was provided by pretreated muscle tissue. In 24 rats, 14-mm sciatic nerve segments were resected and surgically reconstructed using one of the following methods: autograft (AG); bovine type I collagen conduit; (MDM) collagen tube filled with modified denatured autologous muscle tissue. For 8 weeks, functional regeneration was evaluated by footprint and video gait analysis. Evaluation was complemented by electrophysiology, as well as qualitative and quantitative structural assessment of nerves and target muscles. Group AG was superior both structurally and functionally, showing higher axon counts, a more normal gait pattern, and less severe muscle atrophy. Fiber quality (fiber size and myelin thickness) was highest in group MDM, possibly related to the myelin-producing effect of muscular laminin. However, axon count was lowest in this group, and ultrastructural analysis of the denatured muscle tissue showed areas of incomplete denaturation that had acted as a mechanical barrier for regenerating axons. In light of these results, the often advocated use of muscular exogenous matrix for peripheral nerve reconstruction is reviewed in the literature, and its clinical application is critically discussed. In conclusion, combined muscle tubes may have a positive influence on nerve fiber maturation. However, muscle pretreatment is not without risks, and denaturation processes need to be further refined. © 2011 Wiley Periodicals, Inc. Microsurgery 31:632–641, 2011.

Peripheral nerve damage often occurs in young, otherwise healthy individuals and can be the cause of high-grade disability and lifelong morbidity.[1] Today, artificial nerve conduits are considered a promising new approach to peripheral nerve reconstruction. Unlike autologous nerve grafts—the current standard therapy for substantial nerve defects[2]—artificial conduits are readily available in all required sizes, and their application does not require the harvesting of a healthy donor nerve. However, conventional conduits fail to bridge larger nerve defects[3] due to a lack of physical guidance and trophic support.[4] Although schwann cells have been shown to migrate into conventional conduits,[5] their number is too low to effectively support axonal regeneration. Addition of cultured schwann cells or growth factors can increase the regenerative potential,[6] but this technique is still in its infancy, technically challanging and associated with high costs and unpredictable risks. Alternatively (or additionally), axonal regeneration along artificial conduits can be improved by filling the tubes with an endoluminal matrix that serves as a guiding structure for regenerating axons. A promising biological matrix is skeletal muscle,[7–14] because it has been shown that schwann cells migrate, colonize,[15] and proliferate[9] inside muscle scaffolds at an early time and are able to survive there for a long time without axon contact.[16] Moreover, muscle tissue is rich in laminin and collagen type IV, two extracellular matrix molecules known to promote nerve growth.[17,18] Muscle tissue can be pretreated to gain a more open extracellular structure that decreases tissue resistence[19] and enables schwann cells and axons to migrate at an even earlier time.[20] As with conventional tubes, however, the major drawback of pure muscle guides is the limited gap length that can be successfully bridged.[8] One reason for this limitation is a tendency of nerve fibers to grow out of muscle guides, promoting neuroma formation and the risk of ingrowth of surrounding fibrous tissues.[21] A misdirected growth of nerve fibers might be overcome by inserting the muscle tissue into a vein or an artificial nerve conduit.

In this study, a combination of muscle tissue and collagen tube was hoped to overcome the drawbacks of each method used separately, providing a three-dimensional neurotropic guidance structure, while at the same time prohibiting a misdirected nerve fiber outgrowth and fibrous tissue ingrowth. Today's knowledge about muscle tube combinations for nerve repair is mainly based on clinical studies using muscle vein grafts, with a majority of studies evaluating only sensory nerve recovery.[22,24,25] More comprehensive investigations are limited to a small number of animal studies using polylactic acid tubes.[19,26,27]

[1]Department of Plastic, Reconstructive, Hand and Burn Surgery, Academic Hospital Bogenhausen, Munich 81925, Germany

[2]Department of Plastic Surgery & Hand Surgery, University Hospital Rechts der Isar, Technical University Munich, Munich 81675, Germany

[3]Institute of Veterinary Pathology, Ludwig-Maximilians University, Munich 80539, Germany

[4]Institute of Biomathematics and Biometry, Helmholtz Zentrum München, Research Center for Health & Environment, Neuherberg 85764, Germany

Ulf Dornseifer and Andreas M. Fichter contributed equally to this work.

*Correspondence to: Nikolaos A. Papadopulos, Department of Plastic Surgery & Hand Surgery, University Hospital Rechts der Isar, Technical University Munich, Munich 81675, Germany. E-mail: n.papadopulos@lrz.tum.de

Received 13 December 2010; Revision accepted 24 April 2011; Accepted 29 April 2011

Published online 24 August 2011 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/micr.20926

The purpose of our study was to evaluate the regenerative potential of a combination of denatured autologous muscle tissue and collagen conduits (CCs) for the reconstruction of substantial peripheral nerve defects in the rat model compared with conventional CCs and autologous nerve grafts. We chose collagen tubes, because this material has proven superior to other nonresorbable[28] and bioresorbable materials[29] commonly used for the production of artificial nerve guidance tubes. Moreover, collagen tubes have shown promising results in the reconstruction of short nerve defects[30] and are FDA- and EU-approved for clinical application. So far, the combination of collagen tube and pretreated muscle tissue for peripheral nerve reconstruction has only been described for short (10 mm) peroneal nerve defects.[23]

MATERIALS AND METHODS

Surgical Procedure and Experimental Design

Animal studies were approved by the local animal care committee. The current German regulations for animal welfare and the institutional research guidelines for care and use of laboratory animals were followed. All surgical procedures were performed under general anesthesia (2 mg/kg midazolam, 150 μg/kg medetomidine, and 5 μg/kg fentanyl) and aseptic conditions. In 24 male Lewis rats (310–340 g), the left sciatic nerve was exposed, a 14-mm nerve segment resected and subsequently repaired using one of the following methods: autologous nerve graft (group AG, $n = 8$); collagen type I conduit (group CC, $n = 8$); CC filled with modified denatured autologous muscle tissue (group MDM, $n = 8$). In group MDM, muscle tissue was harvested from the latissimus dorsi muscle two days before the nerve surgery, pretreated using a series of thermal and chemical processes as described in detail by Meek et al.[19,26] and stored in phosphate-buffered saline for further use. In group AG, the nerve defect was bridged using the reversed nerve segment as an autologous nerve graft. In groups CC and MDM, a collagen tube (20-mm length, 2-mm inner diameter, purified bovine type I collagen, NeuraGen™, Integra LifeSciences Corporation, Plainsboro, NJ) was used to reconstruct the defect. In the MDM group, predegenerated muscle tissue was precut to pieces of $14 \times 3 \times 3$ mm^2 using microscissors to fit into the tube lumen, and the collagen tubes were prefilled with these pieces of muscle using microinstruments (Fig. 1). Following nerve repair, the biceps femoris muscle was carefully sutured back into place, the skin incision was closed and the anesthesia reversed (0.75 mg/kg atipamezole, 200 μg/kg flumazenile, and 120 μg/kg naloxone). For postoperative analgesia, the rats received metamizol (200 mg/kg p.o.) directly after waking up, and buprenorphine (50 μg/kg s.c.) every 12 hours for 3 days.

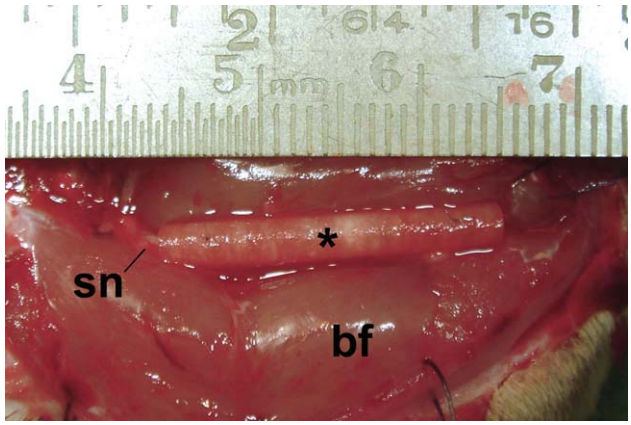

Figure 1. In vivo nerve repair using a muscle-filled conduit. bf = M. biceps femoris; sn = sciatic nerve; (*) collagen tube filled with autologuous denatured muscle tissue. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Functional rehabilitation was regularly assessed over an 8-week postoperative period. These eveluations were complemented by electrophysiological, histological, and histomorphometric assessment of nerve and target organs (muscle) after 8 weeks. For functional evaluations, preoperative base values served as control, whereas for electrophysiological and histomorphometric evaluations, the contralateral, unoperated side was used as control.

Functional Evaluation

Footprint analysis was performed once a week using aquired static footprints and prints taken during walking, as described in more detail by Rupp et al.[11] For evaluation, three footprints for each hind limb were chosen and measured as described by Varejão et al.[31] and used to calculate the sciatic function index (SFI)[32] and the static sciatic index (SSI).[33] SFI and SSI scores of approximately −100 indicate total impairment of the sciatic nerve, whereas scores around 0 can only be achieved in rats with complete function.

Ankle kinematics were perfomed preoperatively and, at weeks 3 and 8, to quantify the amount of the returning function. For that a special floorless wooden cage with a glass front was designed that could be mounted on a conventional treadmill for large animals. A dividing wall separated the construction into two chambers only connected by a small "loophole." The smaller chamber was painted black and served as a suggested hide-out. Rats were gently lifted into the larger chamber, and the treadmill was started at low speed. Speed was then gradually increased until the rats ran with steady (nonhesistent) speed toward the loophole. Movement was recorded with a video camera mounted at a standardized distance in right angle to the running direction of the rat. The procedure was stopped, as soon as five complete gait cycles

had been recorded on tape. The recordings were then digitized, and five still images per animal were captured with Adobe Premiere® CS3 (Adobe Systems, San Jose, CA) at two predefined points: in mid-swing phase and in mid-stance phase. Subsequently, ankle kinematics of these images were performed with ImageJ.[34] Therefore, the ankle angle was defined as the angle between the two straight lines formed by (1) the tibia shaft and (2) the connection between the lateral malleolus and the fourth metatarsal. For each of the two time points, the ankle angle was calculated as the average of five individual measurements. These angles corresponded to the mid-swing angle (AMS)[35] and the ankle stance angle.[36]

Electrophysiologic Assessment

Eight weeks after surgery, the rats were anesthetized as described above, and the severed sciatic nerve was re-exposed to determine nerve conduction velocities (NCVs) across the surgical site. Care was taken to ensure that the rats' core temperature was maintained between 36 and 38°C. In both the hind legs, the sciatic/tibial nerve was stimulated with two monopolar needle electrodes (length 12 mm; diameter 0.3 mm; Viasys Healthcare Supplies 2003 Catalogue No.: 019-404700; Madison, WI) positioned directly on the nerve. The stimulation sites lay proximal and distal to the interposition graft on the operated hind limb. On the unoperated hind limb, stimulation sites were at mid-thigh level and at the medial malleolus. The recording electrodes (monopolar needle electrodes) were placed with the cathode within the gastrocnemic muscle (GM) and the anode subcutaneously over the GM. The ground electrode was positioned subcutaneously between the stimulating and recording electrodes, with the tip pointing toward the heart. One hertz single pulse with duration of 0.2 ms was delivered with increasing intensities until a potential could be recorded from the relevant muscles. On the unoperated leg, a stimulation intensity of 1.9 mA was found to provide supramaximal stimulation. All stimulations and recordings were performed with a Viking Quest electrodiagnostic unit and associated software (Viasys Healthcare; Madison, WI). The compound muscle action potential (CMAP) latencies, defined as the lag between stimulus and onset of the first deflection of the action potential from the baseline, were measured at supramaximal stimulation intensities, and the NCV was subsequently calculated from these values.

Evaluation of Muscle Atrophy

After completion of the electrophysiologic examinations, the rats were euthanized by intraperitoneal lethal injection (2 mL per animal) of a combination of Embutramid (200 mg), Mebezonium (50 mg), and Tetracain (5 mg/mL). The soleus and gastrocnemic muscles of both hind limbs were harvested. Muscle volume was assessed by water displacement. Subsequently, muscles were cut into small samples and immersed in 2.5% glutaraldehyde in Soerensen's phosphate buffer (pH 7.4) for 1 hour. After fixation, samples were rinsed with Soerensen's phosphate buffer and fixed with 1% osmium solution (1.0 g OsO_4 in 50 mL aqua dest., Caulfield 1957) for 2 hours at room temperature, followed by repeated buffer rinses and a graded alcohol series before being embedded in epoxy resin. Subsequently, semithin sections (0.5 μm) were cut with a microtome, dried, flame-fixed, and stained with Azur II and Safranin O for histological appraisal. Of these sections, 10 representative areas (88,864 μm^2) were captured for each animal both on the operated and on the control side with a Zeiss Axiovert 100® light microscope (Zeiss, Jena, Germany) equipped with a NEOPLAN oil immersion objective (40×, n.A. 1.25), a CCD camera (2592 × 1944 px), and a motorized stage. ImageJ[34] was used to assess the cross-sectional area of the muscle fibers. Therefore, a total of 150 muscle fibers per animal per side were manually circumscribed with the help of a graphic tablet and pen, and the cross-sectional area was calculated with ImageJ.

Histologic and Histomorphometric Assessment

The sciatic nerve was harvested from both hind legs from its most proximal accessible point near the major trochanter along with its tibial branch to the middle of the plantar side of the foot. Whole trunk samples containing the area at the mid-graft level and the area at the same level of the sciatic nerve on the contralateral side were fixed, embedded, and cut as described above. Semithin sections (0.5 μm) were mounted on triethoxysilane-coated slides and stained with hematoxylin-eosin for evaluation of the denatured muscle tissue in vivo and with *p*-phenylene diamine for morphometric evaluations, respectively. Cross sections of the denatured muscle tissue were assessed with a Zeiss Axiovert 100® light microscope equipped with a NEOPLAN oil immersion objective (40×, n.A. 1.25). Morphometry was performed on cross sections of the sciatic nerve at mid-graft level. Photographs were taken of these sections with a Zeiss Axiovert 100® light microscope equipped with a NEOPLAN oil immersion objective (40×, n.A. 1.25), a CCD camera (2592 × 1944 px), and a motorized stage. The pictures were then assembled semiautomatically, and image processing was performed by MT_O_P,[37] an IDL-routine (ITT Visual Information Solutions, Boulder, CO) specifically programmed for this purpose. Morphometric parameters comprised total fiber counts (TFCs), fiber and axon diameter, myelin thickness, and *G*-ratio. Electron microscopic assessments were performed on segments of the denatured muscle tissue at mid-scaffold level and of segments of the sciatic and tibial nerve (mid-graft level and 0.5 cm distal to the calcaneus). Slices with a thickness of

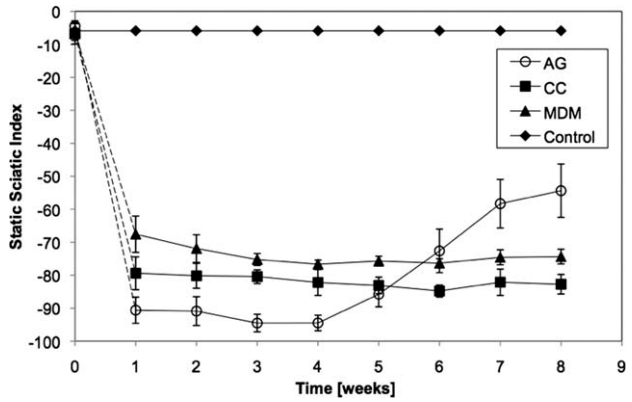

Figure 2. Changes in the SSI score during the 8-week evaluation period. A significant improvement of the static sciatic index can only be observed in the autograft group. AG = Autograft; CC = Collagen conduit; MDM = Modified denatured muscle; (*) Statistically significant difference (P < 0.05).

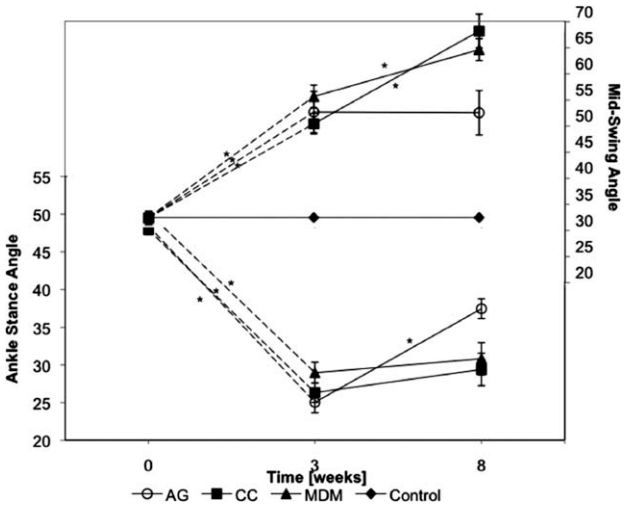

Figure 3. Ankle stance and mid-swing angles. Between weeks 3 and 8, the ankle stance angle shows a significant improvement only in the autograft group. At the same time, the mid-swing angle shows stable values in the autograft group but a significant progressive deterioration in both collagen groups. AG = Autograft; CC = Collagen conduit; MDM = Modified denatured muscle; (*) Statistically significant difference (P < 0.05).

80 nm were contrasted with uranyl acetate and lead citrate, and then examined under a Zeiss-EM10 (Zeiss, Jena, Germany).

Statistics

The SPSS software package (SPSS 15.0, SPSS, Chicago, IL) was used for statistical analysis. For functional, electrophysiologic, and morphometric data, the Kuskal–Wallis test was chosen to determine significant differences ($P < 0.05$) between groups. If a significant difference was detected, the Mann–Whitney *U*-test was performed to compare the groups in pairs. Wilcoxon's test was used to compare parameters inside a study group. For the analysis of muscle atrophy, generalized estimation equation models were used, because this method best resembles the structure of repetitive data and takes correlations within a single individuum into account. All data are presented as mean ± standard deviation.

RESULTS

Functional Recovery

Postoperatively, SFI and SSI scores tended toward −100, a clear sign of complete loss of function. After 8 weeks, only group AG showed a significant increase of SFI and SSI scores to −86 ± 11 ($P = 0.017$) and −54 ± 23 ($P = 0.012$), respectively. In the tube groups, neither SFI (CC: −95 ± 4, $P = 0.575$; MDM: −98 ± 4, $P = 0.600$) nor SSI scores (CC: −82 ± 6, $P = 0.674$; MDM: −74 ± 8, $P = 0.499$) showed a significant change after 8 weeks. The difference between group AG and the tube groups was statistically significant ($P < 0.05$), whereas no statistically significant difference was observed between groups CC and MDM (SFI: $P = 0.61$; SSI: $P = 0.14$; Fig. 2).

Postoperative examination of knee and ankle movements showed a complete loss of function of both flexor and extensor muscles of the shank. Preoperatively, the average ankle stance angle was 48.9 ± 2.9° in all groups and decreased significantly after surgery (26.8 ± 4.0° after 3 weeks, $P < 0.05$). After 8 weeks, a statistically significant increase could only be observed in group AG (37.5 ± 3.7°, $P = 0.011$). The difference between group AG and both tube groups (CC: 29.4 ± 6.1, $P = 0.010$; MDM: 30.8 ± 6.1, $P = 0.021$) after 8 weeks was statistically significant, whereas no difference was found between the two conduit groups ($P = 0.645$, Fig. 3). Evaluation of the AMS showed a significant postoperative increase from 27.4 ± 2.7° (preoperative base value) to 47.9 ± 8.0° (after 3 weeks) in all groups ($P < 0.05$). After 8 weeks, AMS remained unchanged in group A (47.5 ± 12.0°, $P = 0.483$), whereas in groups CC (63.1 ± 9.2°, $P = 0.012$) and MDM (59.6 ± 5.9°, $P = 0.025$), a progressive increase was observed. Again, the difference between group AG and both conduit groups was statistically significant (CC: $P = 0.010$; MDM: $P = 0.005$) but not between groups CC and MDM ($P = 0.493$, Fig. 3).

Electrophysiological Assessment

In none of the rats, CMAPs could be recorded in the interosseus muscles of the operated hind limbs. Therefore, on the operated side, CMAPs recorded from the GM had to be used to calculate the NCVs. The mean stimulation intensity required to elicit CMAPs after stim-

ulation of the proximal stump was 3.1 and 5.6 mA after stimulation of the distal stump. The mean NCV calculated from CMAP latencies was 35.9 ± 6.6 m/s in group AG, 31.5 ± 5.1 m/s in group CC and 33.0 ± 3.4 m/s in group MDM. On the contralateral, unoperated leg all rats exhibited clearly distinct physiological CMAPs after supramaximal stimulation with an intensity of 1.9 mA. The mean conduction velocity on this side was 54.3 ± 6.9 m/s. Statistically, NCVs between operated and control side were significant ($P = 0.008$). On the operated side, the autograft showed a higher NCV in tendency, but the differences between the surgical groups were not statistically significant ($P = 0.39$, Table 1).

Assessment of Muscle Atrophy

Macroscopically, all animals showed a significant muscle atrophy on the operated leg with compensatory hypertrophy of the contralateral leg after 8 weeks. The atrophy, however, was significantly less severe in group AG with a muscle volume of 38% of the unoperated leg when compared with 21% in group CC and 25% in group MDM. The difference between conventional and modified collagen tubes was not significant (Table 1). Evaluation of the muscle fiber area demonstrated corresponding results, with group AG (1662 ± 864 μm^2, control: 2941 ± 119 μm^2) showing significantly higher mean muscle fiber areas compared with group CC (339 ± 88 μm^2) and group MDM (423 ± 147 μm^2). Again, no significant difference between groups CC and MDM was observed (Table 1).

Table 1. Muscle Volume, Fiber Area, and Nerve Conduction Velocity

	Muscle		Nerve
Group	Volume (mL)	Fiber area (μm^2)	Conduction velocity (m/s)
AG	0.85 ± 0.19	1,662 ± 864	35.9 ± 6.6
CC	0.50 ± 0.12	339 ± 88	31.5 ± 5.1
MDM	0.58 ± 0.12	423 ± 147	33.0 ± 3.4
Control	2.3 ± 0.1	2,941 ± 119	54.3 ± 6.8
		P-values	
AG vs. CC	0.001*	<0.001*	
AG vs. MDM	0.01*	<0.001*	0.39
CC vs. MDM	0.16	0.251	
Control	<0.05*	<0.05*	<0.05*

Histomorphometric Assessment

On the control side, TFCs did not differ significantly between the groups. On the operated hind limb, however, differences between the study groups were highly significant with highest fiber counts found in the autograft group, followed by conventional collagen tubes with significantly less nerve fibers. Lowest fiber counts were observed inside muscle-filled conduits, where fibers were predominantly found in the narrow space between inner tube wall and denatured muscle tissue. Only few nerve fibers were detected inside the basal lamina of the muscle tissue (Fig. 4 and Table 2).

In contrast to these observations, mean fiber diameter and myelin thickness were largest in the MDM group (Figs. 5 and 6, Table 2) with 3.7 ± 0.5 μm and 0.5 ± 0.06 μm, respectively. However, only the myelin thickness showed a statistically significant difference ($P = 0.005$) between muscle-filled conduits and the other study groups (AG: 0.43 ± 0.03; CC: 0.41 ± 0.03). On the unoperated side, mean fiber diameter and myelin thick-

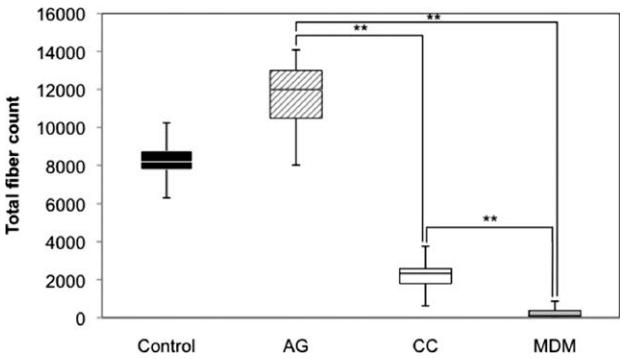

Figure 4. Total fiber count. Total fiber count was significantly higher in the autograft group than in the collagen tube groups. Differences between conventional and muscle-filled collage tubes was also statistically significant with higher total fiber counts in the conventional tube group. AG = Autograft; CC = Collagen conduit; MDM = Modified denatured muscle; (**) Statistically highly significant difference (P < 0.005).

Table 2. Morphometric Assessment at Mid-graft Level

Group	Total fiber count	Fiber diameter	Myelin thickness	*G*-ratio
AG	11,649 ± 2,013	3.33 ± 0.21	0.43 ± 0.03	0.54 ± 0.01
CC	2,248 ± 1,284	3.31 ± 0.19	0.40 ± 0.03	0.58 ± 0.03
MDM	256 ± 325	3.73 ± 0.53	0.49 ± 0.06	0.52 ± 0.08
Control	1,805 ± 324	5.16 ± 0.40	1.27 ± 0.08	0.55 ± 0.04
		P-values		
AG vs. CC	0.001*		0.141	0.005*
AG vs. MDM	0.001*	0.112	0.016*	0.753
CC vs. MDM	0.002*		0.005*	0.115

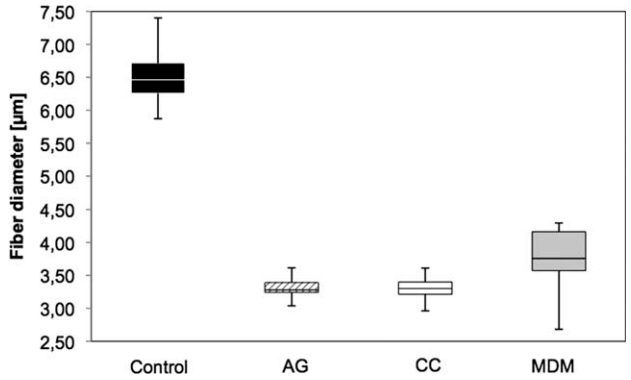

Figure 5. Fiber diameter (µm). By trend, fiber diameter was larger in the modified collagen tube group. In comparison with the other groups, however, this difference was not statistically significant. AG = Autograft; CC = Collagen conduit; MDM = Modified denatured muscle.

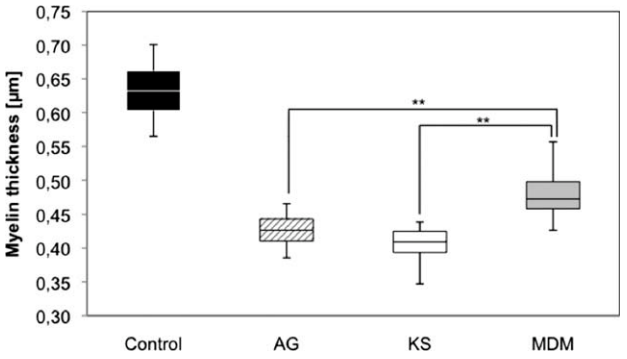

Figure 6. Myelinsheath Thickness [µm]. In comparison to the other study groups, myelin thickness was significantly higher in the modified collagen tube group. AG = Autograft; CC = Collagen conduit; MDM = Modified denatured muscle; (**) Statistically highly significant difference (P < 0.005).

ness of all groups were 6.5 ± 0.4 and 0.64 ± 0.05 µm, respectively. The relative myelin thickness (*G*-ratio), expressed by the ratio of total absolute axon diameters and absolute fiber diameters, was 0.55 ± 0.04 in the control nerve (Table 2). Eight weeks post surgery, the *G*-ratio in groups AG (0.54 ± 0.01) and MDM (0.52 ± 0.08) was smaller than in the control nerve, whereas group CC showed an increased *G*-ratio (0.58 ± 0.03). Statistically, differences between the study groups were only significant between groups AG and CC ($P = 0.005$).

Histological Assessment of the Denatured Muscle Tissue

Excessive evaluation of the pretreated muscle tissue using light microscopy, immunohistochemistry, and cryo-scanning electron microscopy has already been performed elsewhere.[19] Therefore, our evaluation concentrated on light and electron microscopic asessment of the pretreated muscle tissue 8 weeks after in vivo implantation. The muscle tissue showed a loss of the typical myofibril structure with loss of the typical striation of skeletal muscles in most parts. However, sections of the muscle tissue showed signs of incomplete denaturation, like identifiable myofibril structure with swollen myofibrils and remnants of muscle cells (Fig. 7). Only few nerve fibers had penetrated the dense muscle tissue in these areas and had regenerated inside the muscular basal membrane (Figs. 7D and 8).

DISCUSSION

In our study, pretreated muscle tissue was used in combination with conventional collagen tubes for the reconstruction of large sciatic nerve defects in the rat model. This combination has so far only been used for the reconstruction of short rat peroneal nerve defects.[23] Muscle tissue has been successfully used as a physical guidance structure for peripheral nerve regeneration both in animals and humans.[12–14] Pretreatment is intended to provide an open extracellular network of coaxially alligned muscle basal lamina tubes to serve as a temporary scaffold supporting guided axonal growth. In comparison with untreated muscle, migrated schwann cells and regenerating axons can be found inside predegenerated muscle tissue at an earlier point in time.[12,20] Furthermore, pretreated muscle tissue is nonimmunogenic and can be stored indefinitely, theoretically allowing the use as allograft and providing numerous bio- and tissue-engineering approaches.[38] Countless different methods to predegenerate muscle tissue have been proposed in the past, including physical,[39–41] chemical,[42] or a combination of both procedures.[19] Considering the shortcomings of each approach used alone, we decided to use a combination of chemical and physical techniques that had been previously described in the literature[19]: Meek et al. had evaluated the influence of different denaturation techniques on the ultrastructure and molecular structure of muscle tissue and observed the best results after applying a combination of freeze-thaw-cycles, vaccum treatment, and acetic acid. In an in situ pilot study, polylactide tubes filled with muscle tissue pretreated in the described way successfully supported axonal regeneration accross 15-mm rat sciatic nerve gaps. In accordance with Meek's findings, ultrastructural analysis of the pretreated muscle tissue in our study confirmed the existence of an open three-dimensional extracellular structure with intact basal lamina. In some areas, however, degeneration was incomplete and after 8 weeks, only few axons had successfully regenerated through the muscle basal lamina tubes, while most axons were found in the periphery between muscle tissue and inner tube wall in cross sections. In our study, the TFC was significantly lower in modified collagen

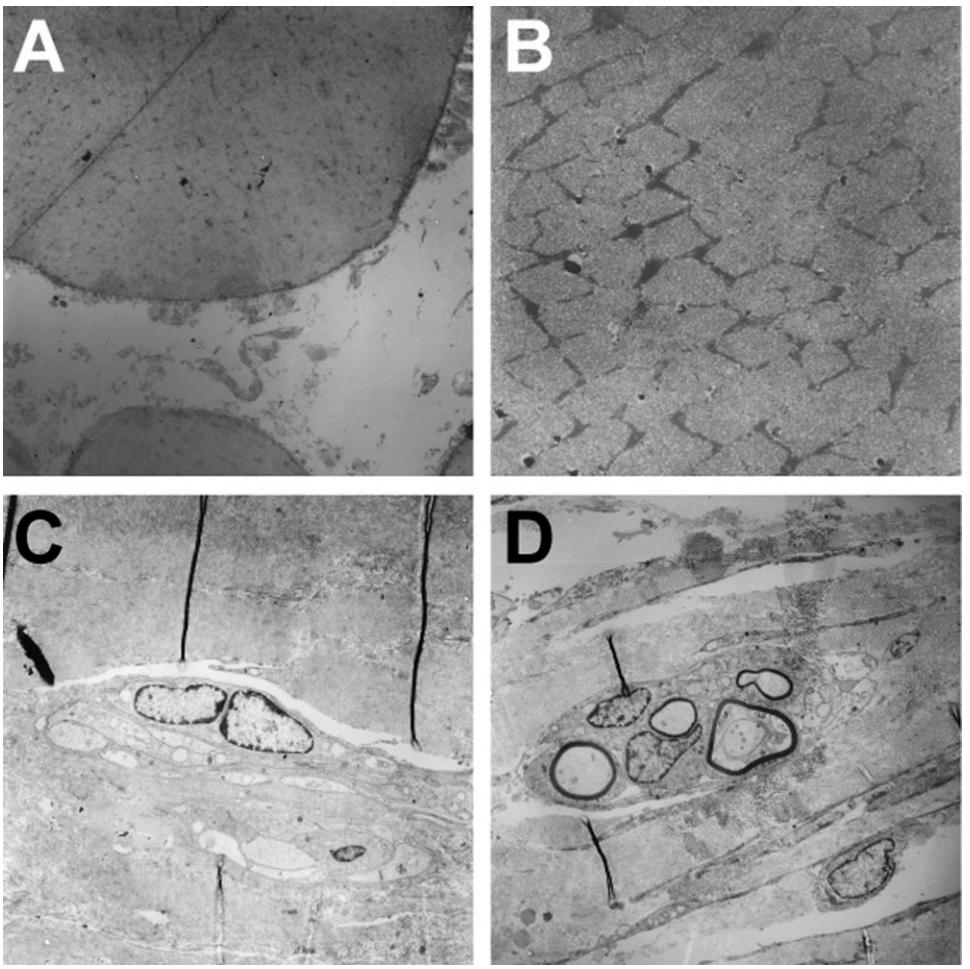

*Figure 7. Electronmicroscopic Assessment of the Denatured Muscle Tissue. (**A**) Degenerated muscle fibers inside the collagen conduit, Magnification 1250×; (**B**) Muscle filaments, Magnification 6300×; (**C**) Fibrous material within the denatured muscle tissue, incomplete denaturation, Magnification 2000×; (**D**) Minifascicle with weakly myelinated schwann cells within the denatured muscle tissue, Magnification 1600×.*

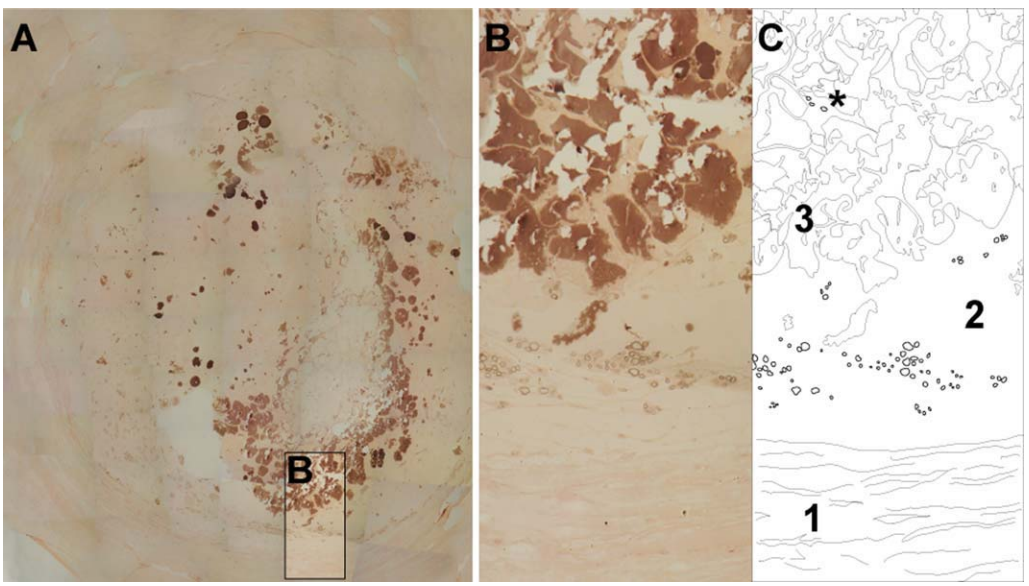

Figure 8. Cross section of a muscle-filled nerve conduit. Staining method: p*-phenylene diamine. (**A**) Cross section of a muscle-filled conduit at mid-graft level (mosaic of 9 × 11 digitally assembled frames). (**B**) Detail of the same image, magnification 400×. (**C**) Schematic view. Regenerating nerve fibers (2) are situated in the space between inner tube wall (1) and muscle tissue (3). Only few solitary fibers (*) are located within the muscle tissue. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]*

tubes than in the autograft and even in conventional collagen tubes. This finding suggests that instead of promoting axonal growth, the muscle tissue acted as a mechanical barrier impeding axonal regeneration. Interestingly, analysis of fiber qualities in our study showed larger fiber diameters and a higher myelin thickness in muscle-filled tubes than in both other study groups. We suspect that muscular laminin, which is well known for its neurite growth-promoting and myelin-producing effect,[17,18] had a positive influence on myelinization. However, considering the small number of axons found inside muscle-filled tubes, it is possible that the effort that might under normal circumstances be used for the regeneration of countless axon sprouts, was spent on only a small number of axons, providing them with excellent fiber qualities. Apart from improved fiber qualities, muscle-filled tubes yielded no significant advantages over conventional tubes in our study. Assessment of muscle atrophy, as well as electrophysiological and functional analyses, confirms the morphometric results. As expected, empty collagen tubes did not improve nerve regeneration in this experimental setting, confirming observations of other authors that beyond the critical defect a satisfactory regeneration through conventional tubes is not possible.[3,43] Compared with the autograft group, both conventional and modified collagen tube groups showed significantly higher levels of muscle atrophy, lower NCVs, and poor functional recovery after 8 weeks. Only in the autograft group, a significant improvement of function with more normal foot print indices and ankle angles after 8 weeks was seen. In stark contrast to our results, Meek et al.[19,26] had observed the first signs of functional recovery 3 weeks after reconstruction of 15-mm sciatic nerve defects with muscle-filled tubes. After 12 weeks, the SFI had improved to 60% of the original nerve function. The choice of guidance tube materials alone (polylactide vs. collagen) does not explain these discrepancies, particularly because collagen tubes have proven superior to other biodegradable materials used for peripheral nerve reconstruction.[29] Despite closely following Meek et al.'s denaturation process, methodical discrepancies associated with predegeneration may have been the cause for the inconsistent results between our two studies: For one thing, Meek et al. make no comment on size of the harvested muscle samples, amount of muscle tissue placed into the tubes, and whether or not denaturation was complete in all areas of their specimens. In our study, the entire tube lumen was filled with muscular exogenous matrix. For another thing, the inner tube diameter in our study was larger in comparison with Meek et al. (2.0 mm vs. 1.5 mm), requiring a larger amount of predegenerated muscle tissue. It is possible that the denaturation process recommended by Meek et al. only works with small specimens and fails if larger muscle bulks are used. From a surgical point of view, another consideration can explain the different results between Meek's and our study. In contrast to Meek et al., the bridged defect length matched the length of the originally created defect in our experimental setup. Meek et al. on the other hand, like most other researchers, removed a small nerve segment (12 mm), let the severed nerve stumps retract several millimeters and consequently bridged a comparably larger nerve defect (15 mm). This popular approach is based on the assumption that not the length of the removed segment but solely the resulting gap length is crucial for the effectiveness of peripheral nerve regeneration. Although this may be true in theory, this approach neglects the aspect of tension at the suture line. Letting the severed nerve stumps retract reduces the stress at the suture lines compared with our method. As even minor tension can have a negative impact on axonal regeneration,[44] this methodical difference might explain the better results reported by Meek et al. This assumption is substantiated by the fact that Meek et al. found no improvement of the SFI in their autograft group after 12 weeks,[26] where the surgically created nerve defect was reconstructed with the resected nerve segment: Although the distance between the two suture lines was shorter in comparison with the other surgical groups (12 vs. 15 mm), the tension at the suture lines became considerably higher due to retraction of nerve stumps and resected nerve segment itself. In view of these considerations, it must be pointed out that a comparative study between autograft and other reconstructive methods is only significant if the reconstructed defect length is equal in all study groups.

Against the background of the poor performance of muscle-filled tubes in our study, an extensive review of the literature was performed. Surprisingly, an incomplete denaturation is not an uncommon problem associated with both physical[13] and chemical[39] pretreatment processes. In accordance with our results, the review of the literature also revealed that denatured muscle tissue does not in all cases improve the outcome of peripheral nerve reconstruction but can in fact impede axonal regeneration.[12,13,23,39–42,45] The reasons for this are versatile and depend on the denaturation process used: Physical techniques alone usually yield unsatisfactory results. In practice, muscle tissue pretreated by repetitive freeze-thaw-cycles gets brittle, hard to handle, is subject to tissue shrinkage, and neural regeneration through such nerve guides is often poor.[13,40] Freezing the tissue for too long leads to a complete disruption of the muscle structure with fragmentation of the basal lamina.[45] Heating the tissue to 60°C reduces tissue shrinkage and the risk of fracture[40] but leads to an increased immune response, thereby impeding axonal regeneration.[41] Regeneration also fails after heating the tissue to 80°C, which is partly explainable by the thermosensitivity of laminin.[40] Chemically

extracted muscle tissue maintains its original elasticity, making its handling much easier. However, as a more recent study has demonstrated, muscle tissue pretreated by chemical techniques alone is prone to early resorption and therefore unsuited for the reconstruction of larger nerve defects.[42] In another recent study, Alluin et al.[23] blame a misorientation of the muscular exogenous matrix for the poor results of muscle-filled collagen tubes that had led to a mechanical obstruction. They evaluated the reconstruction of 10-mm rat peroneal nerve defects with collagen type I/III tubes filled with muscle tissue that had previously been degenerated by repeated freeze-thawing. In accordance with our findings, animals treated with muscle-filled tubes showed an incomplete functional recovery, increased muscle atrophy, as well as a significantly higher loss of fibers between proximal and distal nerve stump compared with conventional collagen tubes and autografts. The significance of an unobstructed regeneration passage is corroborated by histological and electrophysiological evaluations of nerves regenerating through diversely oriented muscle matrices.[8,12]

As the results of our study and the review of the literature show, pretreatment of muscle tissue is a complex and risky process. Brittleness and fracture of tissue,[13,40] distruction of basal lamina or important proteins,[40,45] increased immune response,[41] early resorption,[42] wrong orientation of muscular basal lamina,[12,23] or—as in our case—an incomplete denaturation[13,39] can impede axonal regeneration. Considering these associated risks, some authors have questioned the necessity of pretreatment in principle. In a recent comparative study, Tos et al.[20] used vein grafts filled with either fresh or denatured muscle tissue (freeze-thaw-cycles with liquid nitrogen) to bridge 10-mm rat median nerve defects. Morphological and biomolecular assessment showed a higher schwann cell infiltration and gene expression in pretreated muscle tissue after 5 days but not after 30 days. The authors conclude that pretreatment is not prerequisite for the production of effective muscle-based nerve tubes. However, their assumptions are based exclusively on qualitative observations. As neither histoquantitative (i.e., morphometry) nor functional assessments were performed, no reliable statement can be made as to what extend the observed differences between the groups after 5 days will effect the final regenerative outcome. After all, the cellular and biomolecular processes that dominated in the pretreated muscle group were observed in the critical early stages of peripheral nerve regeneration.

Taking all these considerations into account, it becomes clear that despite considerable scientific effort in the field of muscle-guided nerve regeneration, many basic questions remain unanswered. As our study suggests, collagen tubes filled with pretreated muscle tissue may have a positive effect on myelinization attributable to muscular laminin. However, further research is needed to address the issues, we and other researchers experienced with the process of denaturation, which in our case has led to an incomplete axonal regeneration.

CONCLUSIONS

Combined muscle tubes have several advantages over autologous nerve grafts, such as no necessity to harvest a sensible nerve, no donor site morbidity, infinite availability of custom-sized material, and excellent preconditions for further modification by schwann-cell, growth factor or stem cell application. In our study, denatured muscle tissue was used in combination with conventional collagen tubes for the reconstruction of large rat sciatic nerve defects. Despite some evidence of improved fiber myelinization, axonal regeneration through muscle-filled tubes was obstructed by an incomplete denaturation of the muscle tissue. Critical questions on application form (fresh or pretreated) and proper pretreatment processes need to be addressed in further studies.

ACKNOWLEDGMENTS

The authors wish to thank Integra™ LifeSciences Corp. for generously providing NeuraGen™. Dipl.-Stat. A. Hapfelmeier of the Institut für Medizinische Statistik und Epidemiologie, University Hospital Rechts der Isar, Technical University Munich, Munich, Germany, Dr. B. Bockstahler, Movement Science Group Vienna and Ambulance for Physiotherapy, Department of Small Animals and Horses, University of Veterinary Medicine, Vienna, Austria and S.J. Archibald, Ph.D., Integra™ LifeSciences Corporation, Plainsboro, NJ, USA, are thanked for their discussion and for the review of this manuscript.

REFERENCES

1. Noble J, Munro CA, Prasad VS, Midha R. Analysis of upper and lower extremity peripheral nerve injuries in a population of patients with multiple injuries. J Trauma 1998;45:116–122.
2. Bellamkonda RV. Peripheral nerve regeneration: An opinion on channels, scaffolds and anisotropy. Biomaterials 2006;27:3515–3518.
3. Meek MF, van der Werff JF, Klok F, Robinson PH, Nicolai JP, Gramsbergen A. Functional nerve recovery after bridging a 15 mm gap in rat sciatic nerve with a biodegradable nerve guide. Scand J Plast Reconstr Surg Hand Surg 2003;37:258–265.
4. Gulati AK, Rai DR, Ali AM. The influence of cultured Schwann cells on regeneration through acellular basal lamina grafts. Brain Res 1995;705:118–124.
5. Williams LR, Longo FM, Powell HC, Lundborg G, Varon S. Spatial-temporal progress of peripheral nerve regeneration within a silicone chamber: Parameters for a bioassay. J Comp Neurol 1983;218:460–470.
6. Ansselin AD, Fink T, Davey DF. Peripheral nerve regeneration through nerve guides seeded with adult Schwann cells. Neuropathol Appl Neurobiol 1997;23:387–398.

7. Brunelli GA, Battiston B, Vigasio A, Brunelli G, Marocolo D. Bridging nerve defects with combined skeletal muscle and vein conduits. Microsurgery 1993;14:247–251.
8. Meek MF, Varejao AS, Geuna S. Use of skeletal muscle tissue in peripheral nerve repair: Review of the literature. Tissue Eng 2004;10:1027–1036.
9. Geuna S, Raimondo S, Nicolino S, Boux E, Fornaro M, Tos P, Battiston B, Perroteau I. Schwann-cell proliferation in muscle-vein combined conduits for bridging rat sciatic nerve defects. J Reconstr Microsurg 2003;19:119–123; Discussion 124.
10. Rupp A, Dornseifer U, Fischer A, Schmahl W, Rodenacker K, Jutting U, Gais P, Biemer E, Papadopulos N, Matiasek K. Electrophysiologic assessment of sciatic nerve regeneration in the rat: Surrounding limb muscles feature strongly in recordings from the gastrocnemius muscle. J Neurosci Methods 2007;166: 266–277.
11. Rupp A, Dornseifer U, Rodenacker K, Fichter A, Jutting U, Gais P, Papadopulos N, Matiasek K. Temporal progression and extent of the return of sensation in the foot provided by the saphenous nerve after sciatic nerve transection and repair in the rat—Implications for nociceptive assessments. Somatosens Mot Res 2007;24: 1–13.
12. Vincenzi B, Santini D, Russo A, Gavasci M, Battistoni F, Dicuonzo G, Rocci L, Rosaria VM, Gebbia N, Tonini G. Circulating VEGF reduction, response and outcome in advanced colorectal cancer patients treated with cetuximab plus irinotecan. Pharmacogenomics 2007;8:319–327.
13. Calder JS, Norris RW. Repair of mixed peripheral nerves using muscle autografts: A preliminary communication. Br J Plast Surg 1993; 46:557–564.
14. Pereira JH, Palande DD, Narayanakumar TS, Subramanian AS, Gschmeissner S, Wilkinson M. Nerve repair by denatured muscle autografts promotes sustained sensory recovery in leprosy. J Bone Joint Surg Br 2008;90:220–224.
15. Fornaro M, Tos P, Geuna S, Giacobini-Robecchi MG, Battiston B. Confocal imaging of Schwann-cell migration along muscle-vein combined grafts used to bridge nerve defects in the rat. Microsurgery 2001;21:153–155.
16. Raimondo S, Nicolino S, Tos P, Battiston B, Giacobini-Robecchi MG, Perroteau I, Geuna S. Schwann cell behavior after nerve repair by means of tissue-engineered muscle-vein combined guides. J Comp Neurol 2005;489:249–259.
17. Baron-Van Evercooren A, Kleinman HK, Ohno S, Marangos P, Schwartz JP, Dubois-Dalcq ME. Nerve growth factor, laminin, and fibronectin promote neurite growth in human fetal sensory ganglia cultures. J Neurosci Res 1982;8:179–193.
18. Kleinman HK, Weeks BS. Laminin: Structure, functions and receptors. Curr Opin Cell Biol 1989;1:964–967.
19. Meek MF, den Dunnen WF, Schakenraad JM, Robinson PH. Evaluation of several techniques to modify denatured muscle tissue to obtain a scaffold for peripheral nerve regeneration. Biomaterials 1999;20:401–408.
20. Tos P, Battiston B, Nicolino S, Raimondo S, Fornaro M, Lee JM, Chirila L, Geuna S, Perroteau I. Comparison of fresh and predegenerated muscle-vein-combined guides for the repair of rat median nerve. Microsurgery 2007;27:48–55.
21. Hems TE, Glasby MA. The limit of graft length in the experimental use of muscle grafts for nerve repair. J Hand SurgBr 1993;18: 165–170.
22. Battiston B, Tos P, Conforti LG, Geuna S. Alternative techniques for peripheral nerve repair: Conduits and end-to-side neurorrhaphy. Acta Neurochir Suppl 2007;100:43–50.
23. Alluin O, Wittmann C, Marqueste T, Chabas JF, Garcia S, Lavaut MN, Guinard D, Feron F, Decherchi P. Functional recovery after peripheral nerve injury and implantation of a collagen guide. Biomaterials 2009;30:363–373.
24. Battiston B, Tos P, Cushway TR, Geuna S. Nerve repair by means of vein filled with muscle grafts I. Clinical results. Microsurgery 2000;20:32–36.
25. Marcoccio I, Vigasio A. Muscle-in-vein nerve guide for secondary reconstruction in digital nerve lesions. J Hand SurgAm 2010;35:1418–1426.
26. Meek MF, Dijkstra JR, Den Dunnen WF, Ijkema-Paassen J, Schakenraad JM, Gramsbergen A, Robinson PH. Functional assessment of sciatic nerve reconstruction: Biodegradable poly (DLLA-epsilon-CL) nerve guides versus autologous nerve grafts. Microsurgery 1999;19:381–388.
27. Meek MF, Robinson PH, Stokroos I, Blaauw EH, Kors G, den Dunnen WF. Electronmicroscopical evaluation of short-term nerve regeneration through a thin-walled biodegradable poly(DLLA-epsilon-CL) nerve guide filled with modified denatured muscle tissue. Biomaterials 2001;22:1177–1185.
28. Kemp SW, Syed S, Walsh SK, Zochodne DW, Midha R. Collagen nerve conduits promote enhanced axonal regeneration, Schwann cell association, and neovascularization compared to silicone conduits. Tissue Eng Part A 2009;15:1975–1988.
29. Waitayawinyu T, Parisi DM, Miller B, Luria S, Morton HJ, Chin SH, Trumble TE. A comparison of polyglycolic acid versus type 1 collagen bioabsorbable nerve conduits in a rat model: An alternative to autografting. J Hand Surg Am 2007;32:1521–1529.
30. Archibald SJ, Shefner J, Krarup C, Madison RD. Monkey median nerve repaired by nerve graft or collagen nerve guide tube. J Neurosci 1995;15(5 Pt 2):4109–4123.
31. Varejao AS, Melo-Pinto P, Meek MF, Filipe VM, Bulas-Cruz J. Methods for the experimental functional assessment of rat sciatic nerve regeneration. Neurol Res 2004;26:186–194.
32. Bain JR, Mackinnon SE, Hunter DA. Functional evaluation of complete sciatic, peroneal, and posterior tibial nerve lesions in the rat. Plast Reconstr Surg 1989;83:129–138.
33. Bervar M. Video analysis of standing—An alternative footprint analysis to assess functional loss following injury to the rat sciatic nerve. J Neurosci Methods 2000;102:109–116.
34. Rasband WS. ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, 1997–2009.
35. Yu P, Matloub HS, Sanger JR, Narini P. Gait analysis in rats with peripheral nerve injury. Muscle Nerve 2001;24:231–239.
36. Lin FM, Pan YC, Hom C, Sabbahi M, Shenaq S. Ankle stance angle: A functional index for the evaluation of sciatic nerve recovery after complete transection. J Reconstr Microsurg 1996;12:173–177.
37. Rodenacker K, Bengtsson E. A feature set for cytometry on digitized microscopic images. Anal Cell Pathol 2003;25:1–36.
38. Keilhoff G, Goihl A, Stang F, Wolf G, Fansa H. Peripheral nerve tissue engineering: Autologous Schwann cells vs. transdifferentiated mesenchymal stem cells. Tissue Eng 2006;12:1451–1465.
39. Keynes RJ, Hopkins WG, Huang LH. Regeneration of mouse peripheral nerves in degenerating skeletal muscle: Guidance by residual muscle fibre basement membrane. Brain Res 1984;295:275–281.
40. Hall SM, Enver K. Axonal regeneration through heat pretreated muscle autografts. An immunohistochemical and electron microscopic study. J Hand Surg Br 1994;19:444–451.
41. Whitworth IH, Dore C, Hall S, Green CJ, Terenghi G. Different muscle graft denaturing methods and their use for nerve repair. Br J Plast Surg 1995;48:492–499.
42. Keilhoff G, Pratsch F, Wolf G, Fansa H. Bridging extra large defects of peripheral nerves: Possibilities and limitations of alternative biological grafts from acellular muscle and Schwann cells. Tissue Eng 2005;11:1004–1014.
43. Lundborg G, Dahlin LB, Danielsen N, Gelberman RH, Longo FM, Powell HC, Varon S. Nerve regeneration in silicone chambers: Influence of gap length and of distal stump components. Exp Neurol 1982;76:361–375.
44. Krarup C, Archibald SJ, Madison RD. Factors that influence peripheral nerve regeneration: An electrophysiological study of the monkey median nerve. Ann Neurol 2002;51:69–81.
45. Roganovic Z, Ilic S, Savic M. Radial nerve repair using an autologous denatured muscle graft: Comparison with outcomes of nerve graft repair. Acta Neurochir (Wien) 2007;149:1033–1038; Discussion1038–1039.