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Abstract

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: (A) autograft; (B) bovine type I collagen tube; (C) collagen tube filled with autologous denatured 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 A was superior both structurally and functionally, showing higher axon counts, a more normal gait pattern and less severe muscle atrophy. Fiber qualitiy (fiber size, myelin thickness) was highest in group C, 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.

Keywords: Nerve regeneration · Nerve guide · Collagen · Scaffold · Muscle · ECM (extracellular matrix) · Laminin · Animal model

1. Introduction

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 standart 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. Adding cultured schwann cells can increase the regenerative potential [6], but this technique is still in its infancy, technically challanging and associated with high costs. Axonal regeneration along artificial conduits can further 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 [7-11], since it has been shown that schwann cells migrate, colonize [12] and proliferate [9] inside muscle interonates at an early time and are able to survive there for a long time without axon contact [13]. Muscle tissue can be pretreated to gain a more open extracellular structure that decreases tissue resistence [14] and enables schwann cells and axons to migrate at an even earlier time [15]. In light of these observations, the aim of this study was to compare muscle-filled collagen tubes with conventional collagen tubes and autologous nerve grafts for the repair of rat sciatic nerve defects using functional, electrophysiological and morphometrical techniques.

2. Materials and Methods

2.1. Surgical procedure

All surgical procedures were performed under general anesthesia [2 mg/kg midazolam (DORMICUM®, Roche, Grezach-Wyhlen, Germany), 150 µg/kg medetomidine (DORMITOR®, Pfitzer, Karlsruhe, Germany) and 5 µg/kg fentanyl (FENTANYL®, Curamed, Nürnberg, Germany) i.p.] and aseptic conditions. In 24 male Lewis rats 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); collagen conduit 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 prior to the nerve surgery, pretreated using a series of thermal and chemical processes as described in detail by Meek *et al.* [14,16] and stored in phosphate-buffered saline (PBS) for further use. In group AG, the reversed nerve segment was reimplanted between the two nerve stumps 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, the collagen tubes were prefilled with denatured muscle tissue prior to implantation using micro instruments (Fig. 1). Following the implantation of the interponate, the biceps femoris muscle was carefully sutured back into place and the anesthesia was reversed [0.75 mg/kg atipamezole (ANTISEDAN®, Pfitzer, Karlsruhe, Germany), 200 µg/kg flumazenile (ANEXATE®, Roche, Grenzach-Wyhlen, Germany) and 120 µg/kg naloxone (NARCANTI®, Pharmaselect, Ladenburg, Germany) s.c.]. For postoperative analgesia, the rats received metamizol (200 mg/kg p.o.; corresponds to 3 drops of NOVALGIN®;

Ratiopharm, Germany) directly after waking up, and buprenorphine (50 µg/kg s.c.; TEMGESIC®; Essex Pharma, Germany) every 12 hours for 3 days.

2.2. Functional Evaluation

Footprint analysis was performed both from static footprints and from prints taken during walking, as described in more detail by Rupp *et al.* [11]. For evaluation, 3 footprints for each hind limb were chosen and measured as described by Varejão *et al.* [17] and used to calculate the sciatic function index (SFI) [18] and the static sciatic index (SSI) [19]. 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.

For ankle kinematics, 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 „loop hole“. 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 (non-hesistent) speed towards the „loop-hole“. 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 5 complete gait cycles had been recorded on tape. The recordings were then digitized and 5 still images per animal were captured with Adobe Premiere® CS3 (Adobe Systems Inc, San Jose, CA, USA) at two predefined points: in mid-swing phase and in mid-stance phase. Subsequently, ankle kinematics of these images were performed with ImageJ [20]. 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 5 individual measurements. These angles corresponded to the mid-swing angle (AMS) [21] and the ankle stance angle (ASA) [22]. Ankle kinematics were performed preoperatively, as well as 3 and 8 weeks postoperatively in order to quantify the amount of the returning function.

2.3. Electrophysiologic assessment

Eight weeks after surgery, the rats were anesthetized as described above and the severed sciatic nerve was re-exposed in order to determine nerve conduction velocities (NCV) across the surgical site. Care was taken to ensure that the rats' core temperature was maintained at between 36℃ and 38℃. In both 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, USA) positioned directly on the nerve. The stimulation sites lay proximal and distal to the interponate 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 towards the heart. All stimulations and recordings were performed with a Viking Quest electrodiagnostic unit and associated software (Viasys Healthcare; Madison, WI, USA). The 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.

2.4. *Evaluation of Muscle Atrophy*

After completion of the electrophysiologic examinations the rats were euthanised by intraperitoneal lethal injection [2 ml per animal] of a combination of 200 mg Embutramid, 50 mg Mebezonium and 5 mg Tetracain per ml (T61®, Intervet, Unterschleißheim, Germany). 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 (Glutaraldehyd 25%, AppliChem GmbH, Darmstadt, Germany) 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 (88864 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 (40x, n.A. 1.25), a CCD camera (2592 x 1944 px) and a motorized stage. ImageJ [20] 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.

2.5. *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-interponate 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 p-phenylene diamine for morphometric evaluations. Morphometry was performed on cross-sections of the sciatic nerve at mid-interponate level. Photographs were taken of these sections with a Zeiss Axiovert 100® light microscope equipped with a NEOPLAN oil immersion objective (40x, n.A. 1.25), a CCD camera (2592 x 1944 px) and a motorized stage. The pictures were then assembled semi-automatically and image processing was performed by MT_O_P [23], an IDL-routine (ITT Visual Information Solutions, Boulder, CO, USA) 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 tibial nerve taken from the left, operated hind limb (mid-interponate level and 0.5 cm distal to the calcaneus). Slices with a thickness of 80 nm were contrasted with uranyl acetate and lead citrate, and then examined under a Zeiss-EM10 (Zeiss, Jena, Germany).

2.6. Statistics

The SPSS software package (SPSS 15.0, SPSS Inc. Chicago, IL, USA) 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 analysis of muscle atrophy, Generalized estimation equation models were used, since this method best resembles the

structure of repetitive data and takes correlations within a single individuum into account.

3. Results

3.1. Functional recovery

Postoperatively, SFI and SSI scores tended towards -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 and -54 ± 23, respectively. In the tube groups, neither SFI (CC: -95 ± 4; MDM: -98 ± 4) nor SSI scores (CC: -82 ± 6; MDM: -74 ± 8) showed a significant change after 8 weeks (Table 1). The difference between group AG and the tube groups was statistically significant, whereas no significant difference was observed between groups CC and MDM (Fig. 2).

Postoperative examination of knee and ankle movement showed a complete loss of function of both flexor and extensor muscles of the shank. Preoperatively, the average Ankle stance angle (ASA) was 48.9 ± 2.9° in all groups and decreased significantly after surgery (26.8 ± 4.0° after 3 weeks). 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; MDM: 30.8 ± 6.1) after 8 weeks was also statistically significant, whereas no difference was found between the two conduit groups (Fig. 3, Table 1). Evaluation of the Mid-swing angle (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. After 8 weeks, AMS remained unchanged in group A (47.5 ± 12.0°, p=0483), whereas in groups CC (63.1 ± 9.2°) and MDM (59.6 ± 5.9°, $p<0.05$) a progressive increase ($p<0.05$) was observed.

Again, the difference between group AG and both conduit groups was statistically significant, but not between groups CC and MDM (Fig. 3, Table 1).

3.2. 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 nerve conduction velocities. The mean stimulation intensity required to elicit CMAPs after stimulation of the proximal stump was 3.1 mA and 5.6 mA after stimulation of the distal stump. The mean nerve conduction velocity 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 4).

3.3. 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 as compared to 21% in group CC and 25% in group MDM. The difference between conventional and modified collagen tubes was not significant (Fig. 5, Table 3). 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 (Fig. 6, Table 3).

3.4. Histomorphometric assessment

On the control side, total fiber counts (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. 7, Table 4).

In contrast to these observations, mean fiber diameter and myelin thickness were largest in the MDM group (Fig. 8 and 9, Table 4) 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 thickness of all groups were 6.5 ± 0.4 µm 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 4). 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).

3.5. 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 [14]. 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. 10). Only few nerve fibers had penetrated the dense muscle tissue in these areas and had regenerated inside the muscular basal membrane (Fig. 10D, Fig. 11).

4. Discussion

Muscle tissue has been successfully used as a physical guidance structure for peripheral nerve regeneration both in animals and humans [24-26]. Schwann cells colonize [12] and proliferate [9] inside muscle tissue at an early stage and are able to survive for a long time, even without axon contact [13]. Moreover, muscle tissue is rich in laminin and collagen type IV, two extracellular matrix molecules known to promote nerve growth [27,28]. Again, 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 [29]. A misdirected growing out of nerve fibers might be overcome by positioning the muscle tissue inside a vein [7,9,30] or an artificial nerve conduit [8,31]. The combination of muscle tissue and tube is 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.

In our study, predegenerated muscle tissue was positioned inside a conventional collagen type I tube. Collagen tubes were chosen because these tubes have shown promising results in the reconstruction of short nerve defects [32] and are FDA- and EU-approved for clinical application. Moreover, collagen has proven superior to other non-resorbable [33] and bioresorbable materials [34] commonly used for the production of artificial nerve guidance tubes.

Pretreatment of muscle tissue is intended to provide an open extracellular network of coaxially alligned muscle basal lamina tubes to serve as a temporary scaffold to support guided axonal growth. In comparison to untreated muscle, migrated schwann cells and regenerating axons can be found inside predegenerated muscle tissue at an earlier point in time [15,24]. Furthermore, pretreated muscle tissue is non-immunogenic and can be stored indefinitely, theoretically allowing the use as allograft and providing numerous bio- and tissue-engineering approaches [35]. Countless different methods to predegenerate muscle tissue have been evaluated in the past, including physical [36-38], chemical [39] or a combination of both procedures [14]. 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 [25,37]. Freezing the tissue for too long leads to a complete disruption of the muscle structure with fragmentation of the basal lamina [40]. Heating the tissue to 60°C reduces tissue shrinkage and the risk of fracture [37], but leads to an increased immune response, thereby impeding axonal regeneration [38]. Regeneration also fails after heating the tissue to 80°C, which is partly explainable by the thermosensitivity of laminin [37]. Mligiliche *et al.* [41] were the first to promote a detergent-based technique

(triton X-100, sodium-dodecylsulfate) to degenerate skelettal muscle tissue for production of effective nerve guides. Their technique was able to completely remove myoplasm and cellular components, while keeping the basal lamina intact, thus facilitating the successful regeneration across a 10 mm rat sciatic nerve gap. Chemically extracted muscle tissue maintaines 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 [39]. Considering the shortcomings of each approach used alone, we decided to use a combination of chemical and physical techniques described in the literature [14]. Meek *et al.* [14] had evaluated the influence of different denaturation techniques on the ultrastructure and molecular structure of muscle tissue. Best results were seen after applying a combination of freeze-thaw-cycles, vaccum treatment and acetic acid. Muscle tissue treated with this method showed an open extracellular structure with intact basal lamina that could successfully support axonal regeneration accross 15 mm rat sciatic nerve gaps in an *in situ* pilot study.

In accordance with Meek's findings, ultrastructural analysis of the pretreated muscle tissue in our study confirmed the existence of an open three-dimensional 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 total fiber count was significantly lower in modified collagen 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 [27,28], 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, however, muscle-filled tubes yielded no significant advantages over conventional tubes in our study. Assessment of muscle atrophy, as well as electrophysiological and functional analysis confirm 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 [3,42]. Compared with the autograft group, both conventional and modified collagen tube groups showed significantly higher levels of muscle atrophy, lower nerve conduction velocities, and poor functional recovery after 8 weeks. Only in the autograft group a significant improvement of function with more normal foot print indeces and ankle angles after 8 weeks was seen.

In stark contrast to our results, Meek *et al.* [14,16] 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 sciatic function index 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 since collagen tubes have proven superior to other biodegradable materials used for peripheral nerve reconstruction [34]. Despite closely following Meek *et al.*'s denaturation process, methodical discrepancies associated with predegeneration may have occured and

ultimately have been the cause for the impaired nerve regeneration in our study. For instance, 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. Moreover, the inner tube diameter in our study was larger in comparison to 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. Notably, an incomplete denaturation is not an uncommon problem associated with both physical [25] and chemical [36] pretreatment processes.

From a surgical point of view, another consideration would explain the poor performance of muscle-filled tubes in our study. In contrast to Meek *et al.*, the bridged defect length matched the length of the originally created defect 1:1 in our experimental setup. Meek *et al.* on the other hand, like most other researchers, removed a smaller nerve segment (12 mm), let the severed nerve stumps retract several millimeters and consequently bridged a comparably larger nerve defect (15 mm). Thereby, tension to the suture lines was reduced compared with our method. Since even minor tension can have a negative impact on axonal regeneration [43], 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 sciatic function index in their autograft group after 12 weeks [16]. In this group, the surgically created nerve defect was reconstructed with the resected nerve segment. As a consequence, the distance that axons had to cross between the two suture lines was in fact shorter in comparison with their other surgical groups (12 *vs* 15 mm). However, due to retraction of nerve stumps and resected nerve segment itself, tension at the suture lines became considerably higher, possibly accounting for the

poor performance of the autograft group in Meek *et al.*'s study. 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 lenght is equal in all study groups.

Alluin *et al.* [31] give yet another explanation for bad performance of muscle-filled tubes. In a recent study, 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. In their discussion, the authors blame a misorientation of the muscular exogenous matrix that had led to a mechanical obstruction. The significance of an unobstructed regeneration passage is corroborated by histological and electrophysiological evaluations of nerves regenerating through diversely oriented muscle matrices [8,24].

In accordance with our results, a review of the literature has shown that denatured muscle tissue does not in all cases improve the outcome of peripheral nerve reconstruction but can in fact impede axonal regeneration [24,25,31,36-40]. The reasons for this are versatile and depend on the denaturation process used: brittleness and fracture of tissue [25,37], distruction of basal lamina or important proteins [37,40], increased immune response [38], early resorption [39], wrong orientation of muscular basal lamina [24,31] or – as in our case – an incomplete denaturation [25,36]. Considering these associated risks, the question arises whether or not muscle tissue should be pretreated at all. Recently, some authors have questioned the necessity of pretreatment in principle. In a comparative study, Tos *et al.* [15] 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 conlcude that pretreatment is no prerequisite for the production of effective muscle-based nerve tubes. However, their assumptions are based exclusively on qualitative observations. Since neither histo-quantitative (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.

5. Conclusions

Combined muscle-tubes have several advantages over autologous nerve grafts, like 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. However, critical questions on application form (fresh or pretreated) and proper pretreatment processes still need to be addressed in further studies.

6. Conflict of Interest

The authors have no conflict of interest.

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Appendix

Tables

Table 1: SFI and SSI scores, Ankle Angles

	Group	W0	W1	W2	W3	W4	W5	W6	W7	W8
SFI	AG	-10.3 ±3.9	-99.1 ±9.2	-98.7 ±4.6	-97.4 ±3.2	-92.8 ±4.3	-89.3 ±7.3	-87.6 ±10.8	-87.1 ±10.6	-86.7 ±11.0
	CC	-7.4 ±7.1	-93.9 ±6.3	-93.5 ±3.2	-95.4 ±4.5	-94.4 ±4.9	-94.1 ±5.4	-96.5 ±7.1	-97.2 ±5.8	-95.4 ±4.1
	MDM	-13.9 ±4.1	-91.4 ±11.6	-94.1 ±10.8	-96.1 ±4.5	-96.6 ±5.3	-96.1 ±7.2	-96.0 ±6.5	-94.5 ±8.1	-97.6 ±3.6
SSI	AG	-4.4 ±4.5	-90.6 ±11.3	-90.9 ±12.4	-94.5 ±7.6	-94.5 ±6.7	-85.8 ±10.7	-72.6 ±18.7	-58.3 ±20.9	-54.4 ±22.9
	CC	-7.1 ±8.6	-78.5 ±17.5	-78.0 ±11.6	-79.2 ±5.1	-82.2 ±4.1	-83.7 ±4.3	-85.0 ±4.3	-82.0 ±7.2	-81.7 ±6.2
	MDM	-6.1 ±8.7	-67.6 ±14.1	-71.9 ±10.7	-75.2 ±5.9	-76.7 ±11.1	-75.7 ±7.1	-76.3 ±5.1	-74.5 ±11.3	-74.3 ±8.3
ASA	AG	48,5 ±3.4			25.0 ±3.9					37.5 ±3.7
	CC	47.9 ±1.8			26.3 ±3.7					29.4 ±6.1
	MDM	50.2 ±3.1			28.9 ±3.9					30.8 ±6.1
AMS	AG	27.4 ±3.6			47.6 ±11.2					47.5 ±12.0
	CC	27.3 ±1.0			45.4 ±5.6					63.1 ±9.2
	MDM	27.4 ±3.2			50.6 ±6.2					59.6 ±5.9

Table 2: SFI, SSI and Ankle angles - p-values

		Footprint analysis		Ankle kinematics	
Week	**Group**	**SFI**	**SSI**	**ASA**	**AMS**
W3 *vs* W8	AG	0.017*	0.012*	0.011*	0.483
	CC	0.575	0.674	0.482	0.012*
	MDM	0.600	0.499	0.395	0.025*
W8	AG *vs* CC	0.03*	0.01*	0.010*	0.010*
	AG *vs* MDM	0.03*	0.04*	0.021*	0.005*
	CC *vs* MDM	0.61	0.14	0.645	0.493

Table 3: 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	1662 ± 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	2941 ± 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*

Table 4: Morphometric assessment at mid-interponate 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 and Table Captions

Fig. 1: In vivo implantation of a muscle-filled conduit

bf = M. biceps femoris; sn= sciatic nerve; (*) collagen tube filled with autologuous denatured muscle tissue.

Fig. 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. 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 ($p<0.05$).

Fig. 3: Ankle Stance and Mid-Swing Angles

AG = Autograft; CC = Collagen conduit; MDM = Modified denatured muscle; (*) Statistically significant difference ($p<0.05$).

Fig. 4: Nerve conduction velocity [m/s]

After 8 weeks, the nerve conduction velocity is higher in the autograft group by trend, but the difference is not statistically significant. AG = Autograft; CC = Collagen conduit; MDM = Modified denatured muscle.

Fig. 5: Gastrocnemic muscle volume [ml]

In comparison with conventional and modified collagen tubes, gastrocnemic muscle volume is significantly higher in the autograft group after 8 weeks. AG = Autograft;

CC = Collagen conduit; MDM = Modified denatured muscle; (**) Statistically highly significant difference ($p<0.005$).

Fig. 6: Muscle fiber area [µm^2]

The mean muscle fiber area from cross sections of the gastrognemic muscle was significantly higher in the autograft group as compared to both collagen groups. AG = Autograft; CC = Collagen conduit; MDM = Modified denatured muscle; (***) Statistically highly significant difference ($p<0.001$).

Fig. 7: 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 ($p<0.005$).

Fig. 8: 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.

Fig. 9: 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 ($p<0.005$).

Fig. 10: Electronmicroscopic Assessment of the denatured muscle tissue

(A) Degenerated muscle fibers inside the collagen conduit, Magnification 1250x; (B) Muscle filaments, Magnification 6300x; (C) Fibrous material within the denatured muscle tissue, incomplete denaturation, Magnification 2000x; (D) Minifascicle with weakly myelinated schwann cells within the denatured muscle tissue, Magnification 1600x.

Fig. 11: Cross-section of a muscle-filled nerve conduit

Staining method: p-phenylene diamine. (A) Cross-section of a muscle-filled conduit at mid-interponate level (mosaic of 9x11 digitally assembled frames). (B) detail of the same image, magnification 400x. (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.

Table 1: SFI and SSI scores, Ankle Angles

AG = Autograft; CC = Collagen conduit; MDM = Modified denatured muscle; SFI = Sciatic function index; SSI = Static sciatic index; ASA = Ankle stance angle; AMS = Ankle angle in mid-swing; W0 = preoperative base value, W1-W8 = postoperative weeks 1-8; Data is shown as mean ± standard deviation.

Table 2: SFI, SSI and Ankle angles - p-values

AG = Autograft; CC = Collagen conduit; MDM = Modified denatured muscle; SFI = Sciatic function index; SSI = Static sciatic index; ASA = Ankle stance angle; AMS = Ankle angle in mid-swing; (*) Statistically significant difference ($p<0.05$).

Table 3: Muscle volume, fiber area and nerve conduction velocity

AG = Autograft; CC = Collagen conduit; MDM = Modified denatured muscle; (*) Statistically significant difference ($p<0.05$).

Table 4: Morphometric assessment at mid-interponate level

AG = Autograft; CC = Collagen conduit; MDM = Modified denatured muscle; (*) Statistically significant difference ($p<0.05$)

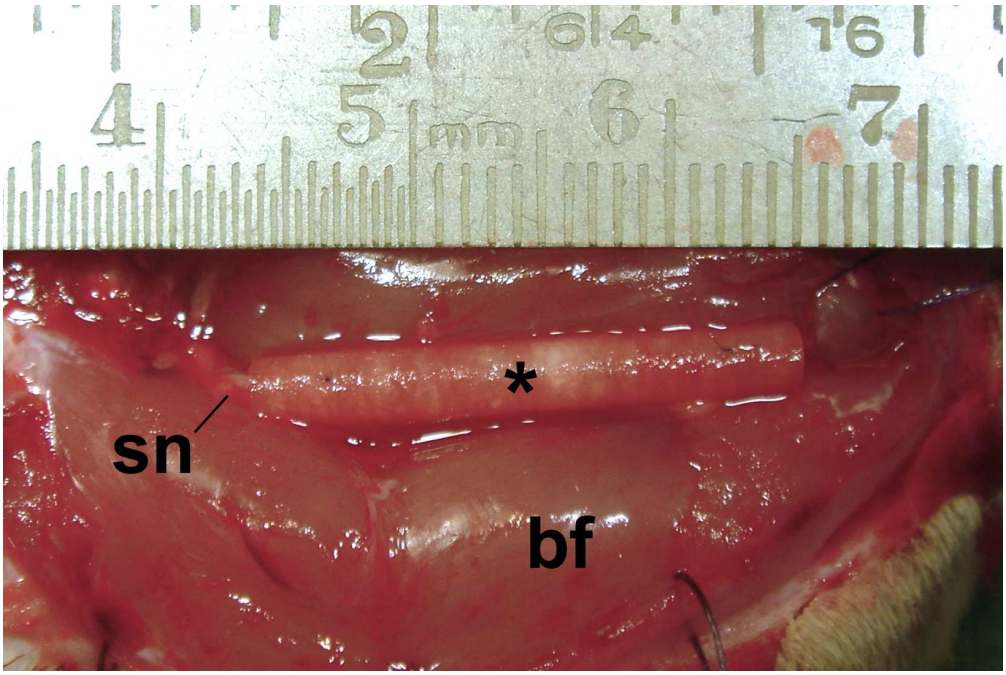

Fig. 1: In vivo implantation of a muscle-filled conduit
bf = M. biceps femoris; sn= sciatic nerve; (*) collagen tube filled with autologuous denatured muscle tissue.
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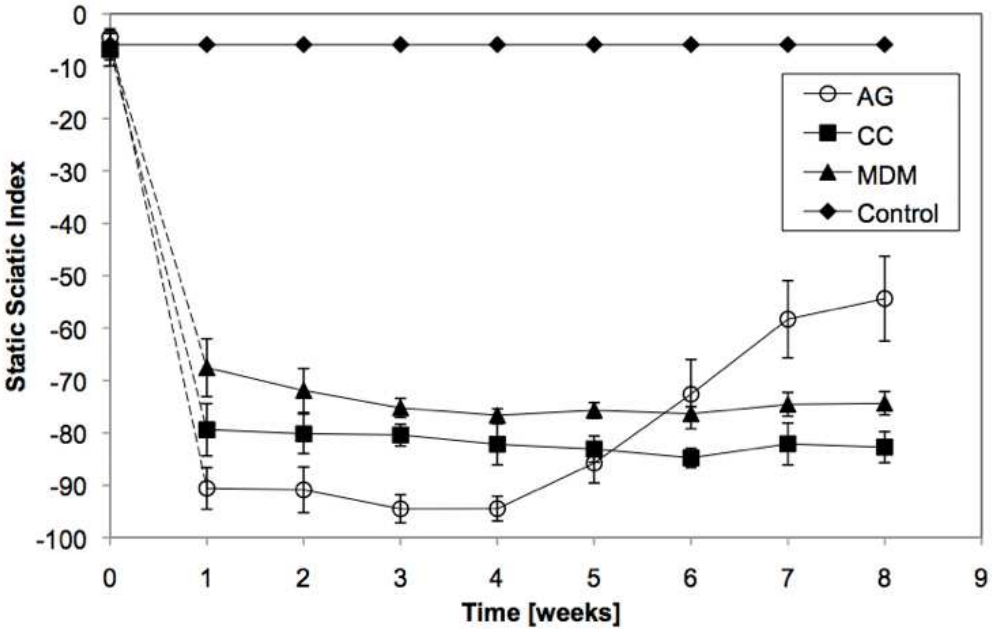

Fig. 2: Changes in the SSI score during the 8 week evaluation period
A significant improvement of the static sciatic index can only be observed in the autograft group. AG = Autograft; CC = Collagen conduit; MDM = Modified denatured muscle. Between weeks 3 and 8, the ankle stance angle shows a significant 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 ($p<0.05$).
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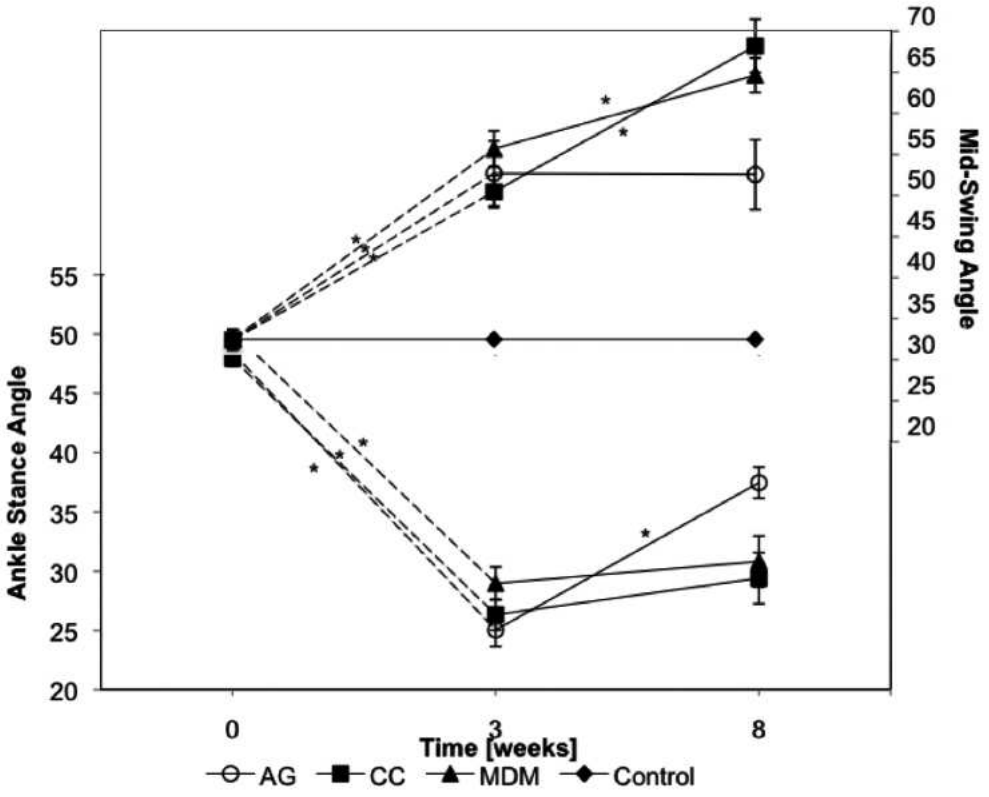

Fig. 3: Ankle Stance and Mid-Swing Angles
AG = Autograft; CC = Collagen conduit; MDM = Modified denatured muscle; (*) Statistically significant difference ($p<0.05$).
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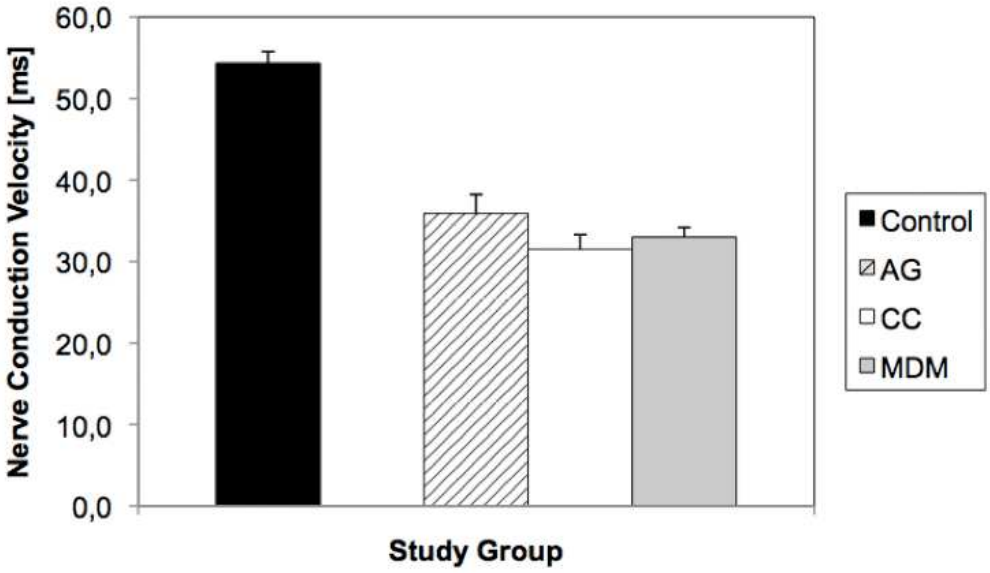

Fig. 4: Nerve conduction velocity [m/s]
After 8 weeks, the nerve conduction velocity is higher in the autograft group by trend, but the difference is not statistically significant. AG = Autograft; CC = Collagen conduit; MDM = Modified denatured muscle.
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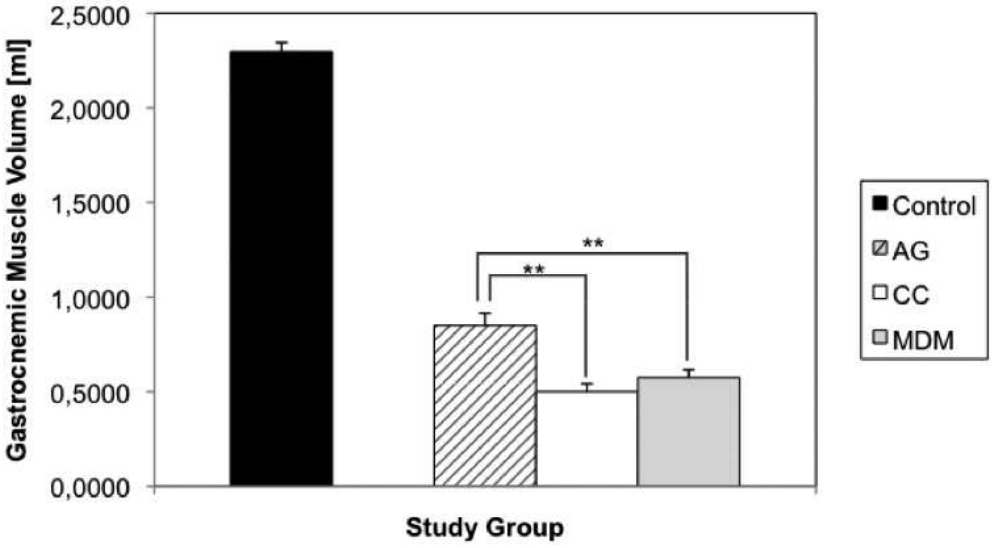

Fig. 5: Gastrocnemic muscle volume [ml]
In comparison with conventional and modified collagen tubes, gastrocnemic muscle volume is significantly higher in the autograft group after 8 weeks. AG = Autograft; CC = Collagen conduit; MDM = Modified denatured muscle; (**) Statistically highly significant difference ($p<0.005$).
64x36mm (300 x 300 DPI)

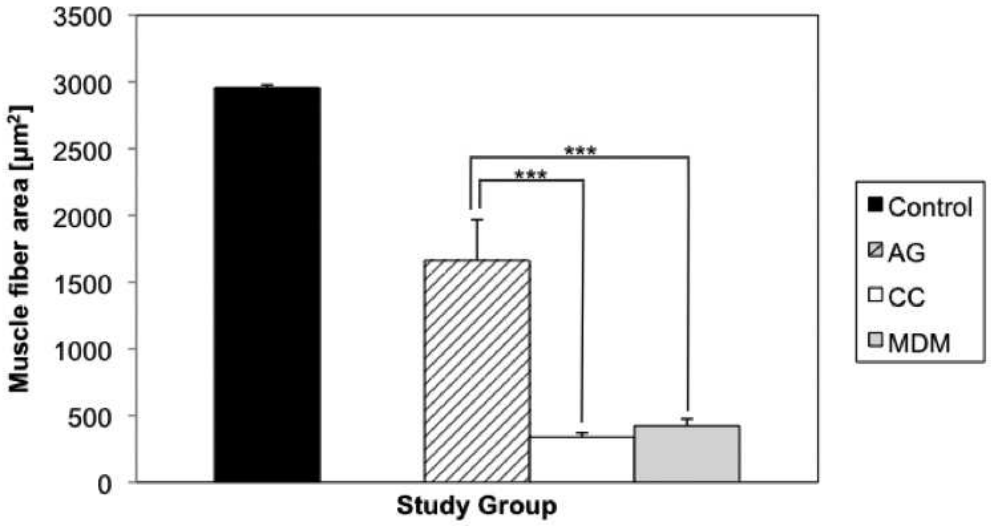

Fig. 6: Muscle fiber area [µm2]
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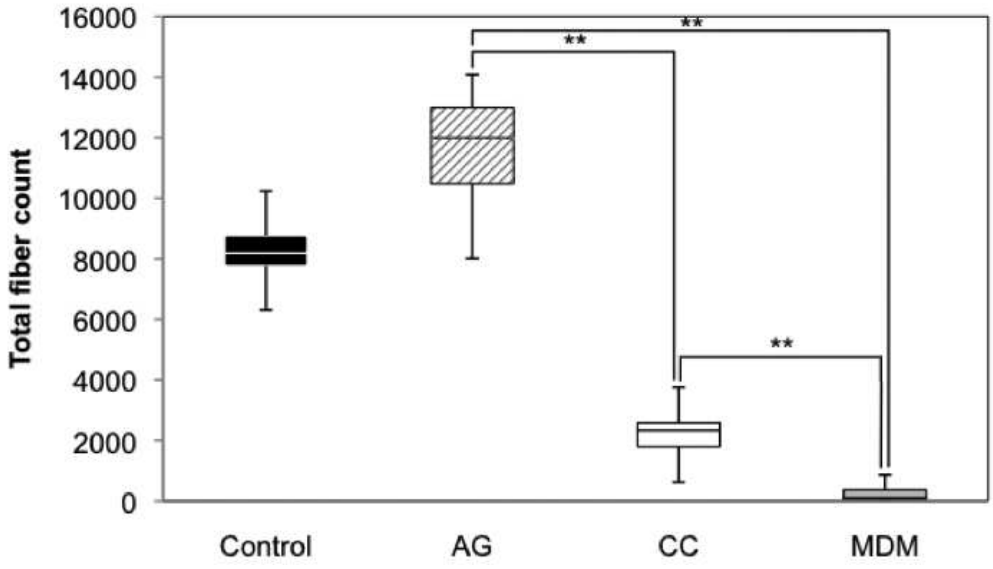

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Total fiber count was significantly higher in the autograft group than in the collagen tube groups. Differences between conventional and muscle-filled collage tubes was also statistically significant with higher total fiber counts in the conventional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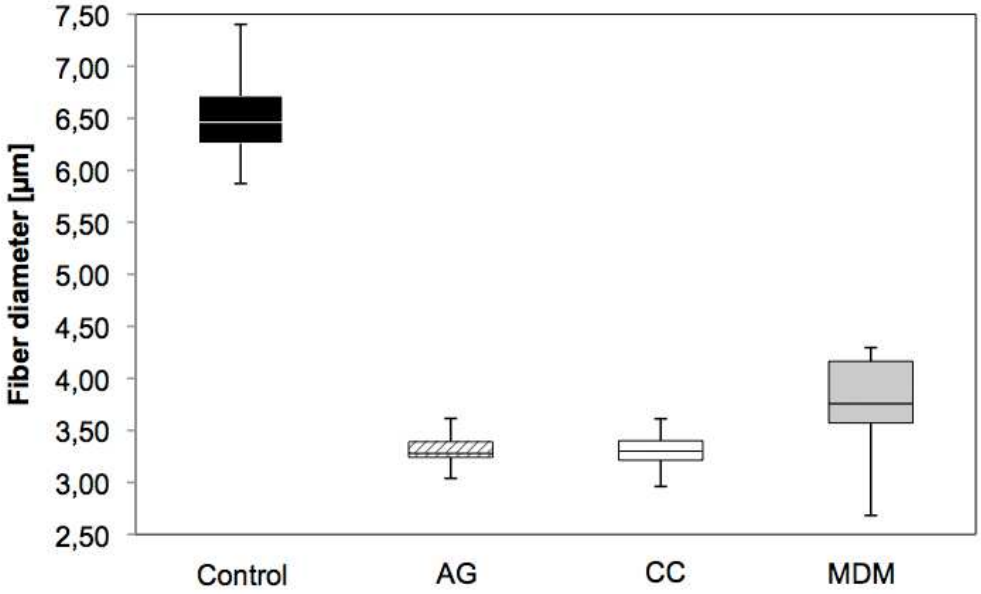

Fig. 8: Fiber diameter [µm]
By trend, fiber diameter was larger in the modified collagen tube group. In comparison with the other groups, however, this difference was not statistically significant. AG = Autograft; CC = Collagen conduit; MDM = Modified denatured muscle.
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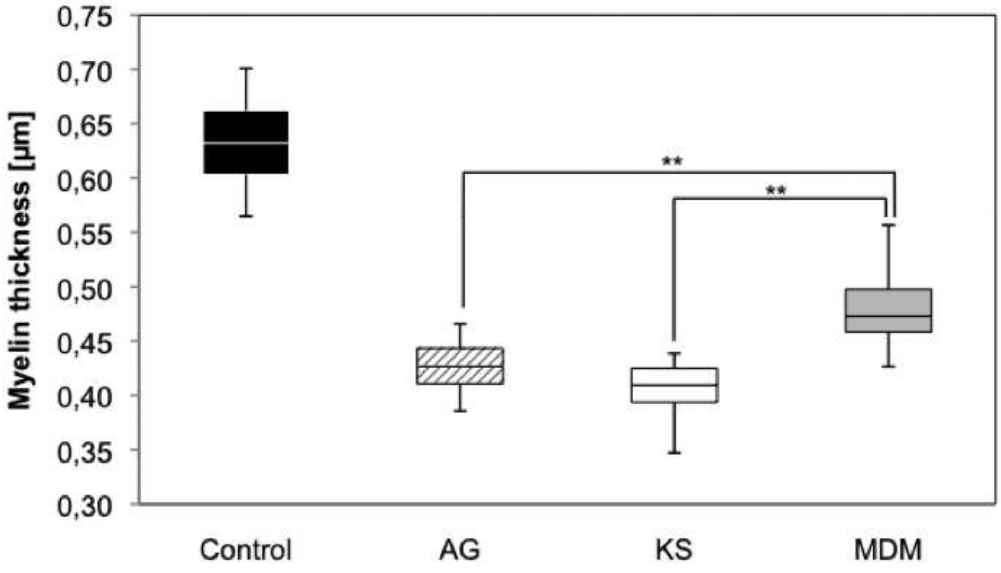

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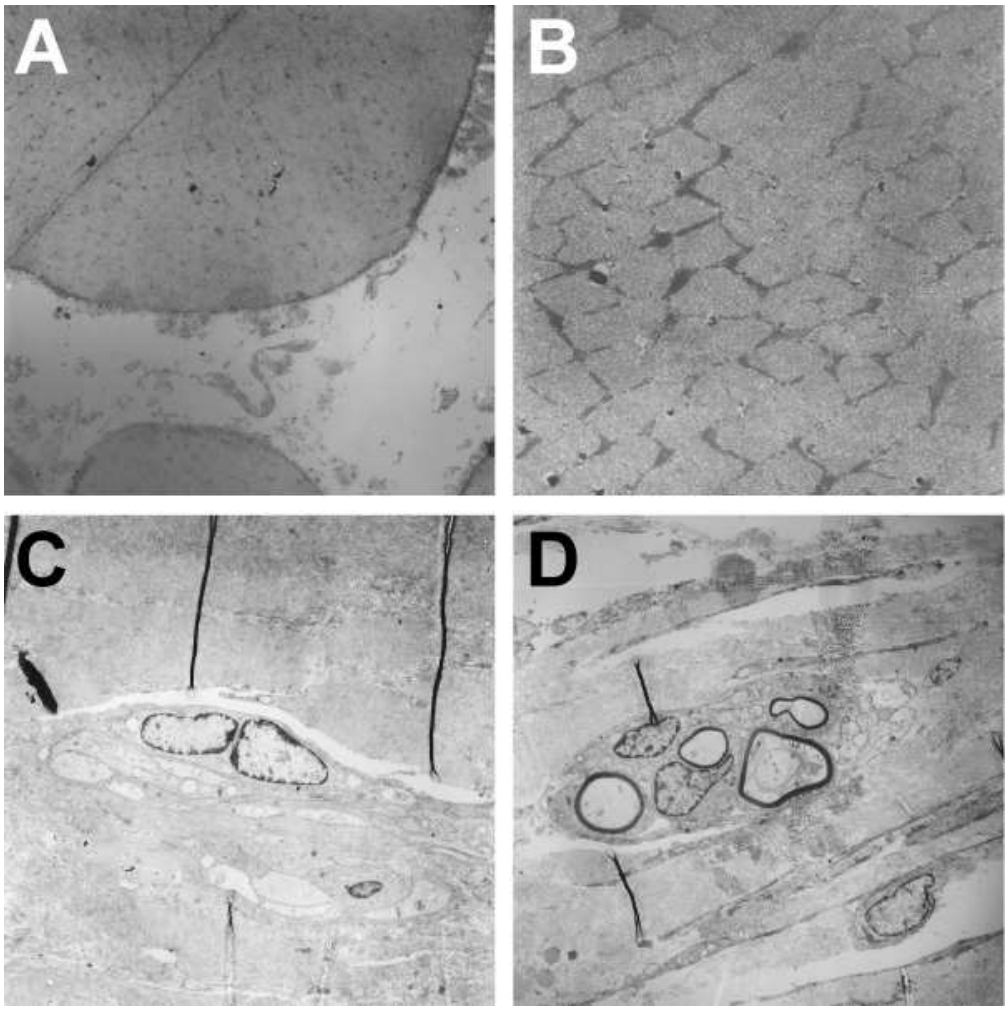

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(A) Degenerated muscle fibers inside the collagen conduit, Magnification 1250x; (B) Muscle filaments, Magnification 6300x; (C) Fibrous material within the denatured muscle tissue, incomplete denaturation, Magnification 2000x; (D) Minifascicle with weakly myelinated schwann cells within the denatured muscle tissue, Magnification 1600x.
50x50mm (300 x 300 DPI)

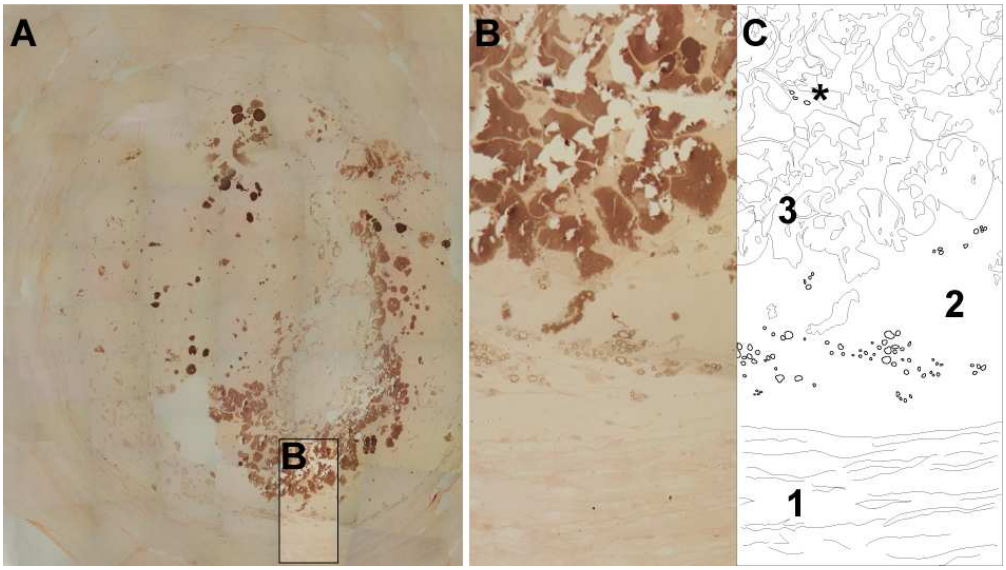

Fig. 11: Cross-section of a muscle-filled nerve conduit
Staining method: p-phenylene diamine. (A) Cross-section of a muscle-filled conduit at mid-interponate level (mosaic of 9x11 digitally assembled frames). (B) detail of the same image, magnification 400x. (C) schematic view. Regenerating nerve fibers (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.
80x45mm (300 x 300 DPI)