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Tubulization with chitosan guides for the repair of long gap peripheral nerve injury in the rat.

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Running title: Chitosan guides for nerve repair

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ABSTRACT

Biosynthetic guides can be an alternative to nerve grafts for reconstructing severely injured peripheral nerves. The aim of this study was to evaluate the regenerative capability of chitosan tubes to bridge critical nerve gaps (15 mm long) in the rat sciatic nerve compared with silicone tubes and nerve autografts. Twenty-eight Wistar Hannover rats were randomly distributed into four groups (n=7 each) in which the nerve was repaied by: silicone tube (SIL), chitosan guides of low (~2%, DAI) and medium (~5%, DAII) degree of acetylation, and autograft (AG). Electrophysiological and algesimetry tests were performed serially along 4 month follow-up, and histomorphometric analysis was performed at the end of the study. Both groups with chitosan tubes showed similar degree of functional recovery, and similar number of myelinated nerve fibers at mid tube after 4 months of implantation. The results with chitosan tubes were significantly better compared to SIL tubes ($P < 0.01$), but lower than with AG ($P < 0.01$). In contrast to AG, in which all the rats had effective regeneration and target reinnervation, chitosan tubes from DAI and DAII achieved 43% and 57% success respectively, whereas regeneration failed in all the animals repaired with silicone tubes. This study suggests that chitosan guides are promising conduits to construct artificial nerve grafts.

INTRODUCTION

Peripheral neurons have the ability to regenerate and reinnervate target organs when there is a suitable environment to do so, thus allowing some degree of recovery of the lost functions, depending upon severity of the injury and quality of the repair[1,2]. On the other hand, after complete nerve transection, surgical repair is mandatory to reunite the two stumps to facilitate regeneration. When the distance to be bridged does not allow direct suture between stumps, the interposition of a nerve graft is used as the gold standard technique in clinical practice. The use of autografts has some disadvantages, such as the sacrifice of a healthy nerve of the subject affected, the mismatch between the injured nerve and the grafts, and the limited source of donor nerves. As an alternative to autografts, the use of biogenic conduits[3,4] or artificial guides has been proposed[5,6]. However, the success of regeneration when using artificial nerves guides is limited by the length of the gap (less than 15 mm in the rat)[7]. Strategies focused on altering the characteristics of the guidance tubes to increase the ability to sustain axon regeneration, have been attempted to overcome the gap limitation[5,8].

The cross-sectional dimensions of the tube[9,10] and the materials in which the tubes are constructed are other factors that also affect the final outcome. Initial studies used non-degradable silicone tubes[11] but nowadays it is considered that the ideal material should be biocompatible, have sufficient mechanical stability, be flexible, be porous to facilitate the incorporation of nutrients, and degrade into non-toxic products to prevent long-term body reaction[12]. Indeed, nerve guides made from collagen (NeuragenTM)[13], polyglycolic acid (Neurotube^{TM})[14] and polylactide caprolactone (Neurolac^{TM})[15] have been approved for clinical use. However, for the moment nerve guides have been approved and tested in humans only for the repair of relatively short gap injuries.

Among the different materials experimentally tested to improve the results obtained by available nerve guides, chitosan is a promising alternative[16,17]. Chitosan is a polymer derived

from chitin, a molecule obtained from the exoskeleton of arthropods, shellfish and cell wall of fungi[18], and fulfills the characteristics above indicated to construct nerve guides. Some *in vivo* studies reported the benefits of chitosan scaffolds and guides in peripheral nerve repair. However, most of these studies combined the chitosan based guide with internal fillers, such as neurotrophic factors[19,20], molecules or peptides from the extracellular matrix[21,22] and supporting cells[23,24], in non-critical peripheral nerve gaps. Recent technological improvements overcame the poor mechanical strength of chitosan tubes, which was one of the main factors limiting their use as single material for nerve guides. In this study, we aimed to evaluate the capabilities of hollow chitosan tubes to sustain regeneration when used to repair a critical 15 mm sciatic nerve gap in rats, and compare their outcome to standard silicone tubes and the ideal nerve autograft. We tested two types of chitosan conduits with different degrees of acetylation, whose characteristics in terms of biocompatibility and adequacy for nerve repair have been recently described[17].

MATERIALS AND METHODS

Animals

Twenty-eight female Wistar Hannover rats, aged 3 months were used in the experiment. The animals were housed in plastic cages, maintained at 22ºC with a 12 h light/dark cycle and allowed free access to water and food. The experimental procedures were approved by the Ethical Committee of our institution and followed the rules of the European Communities Council Directive.

Experimental design and surgical procedure

Animals were randomly distributed into one of four experimental groups according to the type of repair: silicone (SIL) repaired animals (n=7), chitosan of low degree of acetylation (~2%) (DAI) (n=7), chitosan of medium degree of acetylation (~5%) (DAII) (n=7) and

autograft (AG) (n=7). The chitosan tubes manufacturing and characteristics were the same as reported in a previous study[17].

All surgical procedures were performed with aseptic operating conditions and under anesthesia with ketamine/xylazine (90mg/kg and 10mg/kg i.p., respectively). Chitosan tubes were immersed in saline solution 20 minutes before implantation to reduce the strength of the tube. Under a dissecting microscope the sciatic nerve was exposed and cut 6 mm distal to the exit of the gluteal nerve, and a nerve segment of 6 mm was resected. The distal and proximal stumps were fixed by two epineural 10-0 sutures into the ends of the implanted tube leaving a 15 mm gap (Fig. 1). All the tubes used had a length of 19 mm, and an internal diameter of 2 mm. Once implanted, the tubes were filled with sterile physiologic saline solution. For the AG group, the sciatic nerve was cut at the same level explained above and 15 mm distally. The nerve segment resected was flipped and sutured to bridge the gap with two 10-0 sutures at each side. The muscle plane was then sutured with re-absorbable 5-0 sutures, the skin with 2-0 silk sutures, and the wound was disinfected. Animals were treated with amitriptyline for preventing autotomy[25].

Electrophysiological tests

Functional reinnervation of target muscles was assessed at 7, 30, 60, 90 and 120 days post-operation (dpo). Animals were anesthetized with pentobarbital (40 mg/kg i.p.). The sciatic nerve was stimulated by transcutaneous electrodes placed at the sciatic notch, and the compound muscle action potential (CMAP) of tibialis anterior and plantar muscles was recorded using monopolar needle electrodes, placing the active one in the muscle belly and the reference in the fourth toe[26]. During the tests, the rat body temperature was maintained by means of a thermostated warming flat coil. The amplitude and the latency of the M wave were measured. The contralateral limb was used as control.

Functional evaluation of sensory recovery

The threshold of nociceptive responses to mechanical and thermal stimuli were evaluated on both hindpaws by means of algesimetry tests at 7, 21, 45, 60, 90, 92 and 120 dpo. For both tests, the lateral area (innervated by tibial and sural nerves, both being branches of the sciatic nerve) of the plantar surface was tested[27]. The contralateral paw of each rat was tested as control each day, to overcome possible variations between testing conditions. Sensibility to mechanical stimuli was measured by means of an electronic Von Frey algesimeter (Bioseb, Chaville, France). Rats were placed on a wire net platform in plastic chambers 30 min before the experiment for habituation. The mechanical nociceptive threshold was taken as the mean of three measurements per paw region, and expressed as the force (in grams) at which rats withdrew their paws in response to the stimulus. A cutoff force was set to 40 g at which stimulus lifted the paw with no response. Thermal sensibility was assessed by using a plantar algesimeter (Ugo Basile, Comerio, Italy). The beam of a projection lamp was focused onto the hindpaw plantar surface pointing at the lateral side. The thermal nociceptive threshold was taken as the mean of three trials, and expressed as the latency (in seconds) of paw withdrawal response. A cutoff time was set at 20 s to prevent tissue damage. All the values are presented as percentage of response with respect to the contralateral non-injured paw.

Histology and morphometry

Four months after the injury, animals were deeply anesthetized and perfused transcardially with 4% paraformaldehyde in phosphate-buffered saline solution (0.1M, pH=7.4). After perfusion, the regenerated nerves were harvested and postfixed in 3% paraformaldehyde - 3% glutaraldehyde phosphate-buffered solution. The nerves were postfixed in osmium tetroxide (2%, 2 h, 4°C), dehydrated through ascending series of ethanol, an embedded in Epon resin. Nerves were sectioned using an ultramicrotome (Leica). Semithin sections (0.5 μm) were stained with toluidine blue and examined by light microscopy. Images of the whole sciatic nerve were acquired at 10× with a digital camera, while sets of images chosen by systematic

random sampling of squares representing at least 30% of the nerve cross-sectional area were acquired at 100× from mid and distal parts of the tube or graft. Measurements of the cross-sectional area of the whole nerve, as well as counts of the number of myelinated fibers, were carried out by using Image software (National Institutes of Health). Morphometrical analysis was made using a protocol previously described[28] in order to obtain measurement of regenerated fibers and axons diameter, myelin thickness and the *g*-ratio.

Muscle weight

Once the animals were perfused, tibialis anterior and gastrocnemius muscles were dissected. The muscles were kept in a tube placed in an incubator at 37 ºC for two days to allow them to dry, and then weighted.

Statistics

Results are expressed as mean ± SEM. Statistical comparisons between groups and intervals for algesimetry and electrophysiological tests results were made by two-way ANOVA for repeated measurements, followed by Bonferroni post-hoc test. Statistical analysis of histological results was made by one-way ANOVA followed by Bonferroni post-test. Differences were considered significant if $p < 0.05$.

RESULTS

Muscle reinnervation

Nerve conduction tests performed one week after sciatic nerve injury demonstrated complete denervation of the hindlimb muscles. At 30 dpo, 4 of 6 rats in the AG group had evidence of reinnervation in the tibialis anterior muscle, whereas in the groups with chitosan tubes, the first CMAPs were recorded at 60 dpo in 2 of 7 animals. The CMAPs progressively increased in amplitude and were recorded in higher number of animals over time. At the end of follow up (120 dpo), reinnervation of the tibialis anterior muscle was observed in all the animals in

the AG group, in 3 of 7 rats repaired with the chitosan tubes and in none of the rats repaired with silicone tube. When comparing the mean CMAP amplitude between groups at the end of follow up (all the animals included in the analysis), the AG group showed a mean amplitude (29.90 ± 1.40 mV) significantly higher than SIL (0±0 mV; $P < 0.01$), DAI (10.50 ± 5.00 mV; $P < 0.01$) and DAII (9.54 ± 5.75 mV; $P < 0.01$) groups (Fig. 2A). Significant differences were also found between the two groups repaired with chitosan guides compared to SIL group ($P < 0.05$), but no differences ($P > 0.05$) were observed between DAI and DAII groups.

At the more distal interosseus plantar muscle, the first CMAPs were recorded as small polyphasic potentials at 60 dpo in all the animals of the AG group, and in two animals of the DAII group, whereas the first signs of plantar reinnervation appeared at 90 dpo in rats of the DAI group. After four months follow-up, plantar muscle reinnervation was detected in all the animals in the AG group, in 3 of 7 rats in group DAI, in 2 of 7 rats in group DAII, and in none of group SIL. Mean CMAP amplitude in group AG was significantly higher (2.65 ± 0.49 mV) compared to SIL (0 ± 0 mV, $P < 0.01$), DAI (0.212 ± 0.10 mV, $P < 0.01$) and DAII (0.716 ± 0.53mV, $P < 0.01$) groups (Fig. 2B). Significant differences were not observed within the tube-repaired groups ($P > 0.05$). When CMAPS were recorded, the latency of the waves was considerably longer than normal during the first stages of reinnervation and tended to shorten with time towards normal values. At the end of follow up latencies were 1.81 ± 0.03 ms in the AG group, 2.63 ± 0.31 ms in group DAI and 2.89 ± 1.38 ms in group DAII for the tibialis anterior muscle, and 3.69 ± 0.18 ms in group AG, 5.44 ± 0.89 ms in group DAI, and 4.43 ± 0.25 ms in group DAII for the plantar muscle (Fig. 3B).

Recovery of nociceptive sensibility

Withdrawal responses to mechanical stimuli, evaluated by means of the Von Frey test, revealed that animals had no responses in the denervated paw until 30 dpo, and therefore they were penalized with a cut off value of 40g. From 60 to 90 dpo most rats showed withdrawal

responses at lower stimulus intensity than in the contralateral side. After elimination of the saphenous nerve at 90 dpo, measurements made at120 dpo showed that all the rats of the AG group had withdrawal responses to mechanical stimuli (17.35 ± 7.16 g at 120 dpo) at lower intensity than in the contralateral paw (27.28 ± 1.76 g, $P<0.05$). Mean values in the chitosan tubes groups were higher (34.12 ± 4.34 g, $P<0.05$ for DAI; 31.45 ± 4.26 g, $P<0.05$ for DAII), due to failed regeneration of some animals that did not respond to mechanical stimuli. Values of the subset of rats that had reinnervated were similar to the values observed in AG rats. None of the animals of the SIL group responded (40.0 ± 0.0 g, $P<0.05$) indicating absence of sensory reinnervation of the hindpaw (Fig. 4A).

Withdrawal responses to heat stimulation in the plantar test showed similar results than the ones observed for the Von Frey test. Denervated paws did not respond to the hot stimuli on the sciatic lateral region until 45 dpo. At 120 days withdrawal latencies in the AG group (12.75 ± 1.38 s) were similar to the contralateral paw (12.36 ± 0.76 s, $P>0.05$). After saphenous nerve cut, some of the animals of the chitosan groups did not respond to heat stimuli, due to lack of reinnervation in the sole, thus resulting in slightly higher mean values (16.80 ± 2.19 s, $P>0.05$ for DAI; 16.87 ± 1.66 s, $P>0.05$ for DAII). None of the rats repaired with silicone tubes withdrew the paw when applying the heat stimuli (20.0 ± 0.0 s, $P<0.05$) (Fig. 4B).

Histological results

Macroscopic examination of the injured nerves after the 4 months follow-up showed that all the AG repaired nerves had good regeneration, whereas only 3 of the 7 rats in groups repaired with chitosan tubes presented a regenerative cable inside the tube. The regenerated nerve had a compact appearance and occupied the center of the tube lumen (see Fig. 1C). The nerves were surrounded by a thick, homogeneous connective layer, with no signs of inflammatory reaction. The size of the regenerated sciatic nerve found in the AG group was larger than the

regenerated cables found in both chitosan groups. There was no regenerative cable in any of the rats of the SIL group.

Transverse sections of the regenerated nerves taken at the midpoint of the graft of the tube and at the distal segment were analyzed under light microscopy (Figs. 5 and 6). In order to compare the absolute number of myelinated fibers, regenerated and non-regenerated rats were included in the statistical analysis. Non-regenerated animals were given null value. The mean number of myelinated fibers at the midpoint of the graft or the tube was higher in the AG group (14,409 ± 1,564; $P < 0.01$) compared to both chitosan tube groups (DAI: 2,275 ± 1,159; DAII: 2,265 ± 1,534). Significant differences were observed between the chitosan–repaired groups and the SIL group ($P < 0.01$) (Fig. 7A). These differences were also observed at 3 mm distal to the end of the graft or the tube, where the estimated number of myelinated fibers in the AG group (6,865 ± 295; $P < 0.01$) was significantly higher than in both chitosan tube groups (DAI: 1,971 ± 1,013; DAII: 1,644 ± 984). Significant differences were again observed between chitosan groups and the SIL group ($P < 0.01$) (Fig. 7B). When taking into account only the animals in which a regenerated nerve was found after 4 months post injury, the number of myelinated fibers was still higher in the AG group compared to both chitosan groups at the mid level (DAI: 5,308 ± 1,161; DAII: 6,218 ± 2,328) and at the distal level (DAI: 4,598 ± 1,070; DAII: 3,837 ± 1,602), but differences were not significant ($P > 0.05$).

The regenerated nerves at the mid level had a larger cross-sectional area in the AG group than in the chitosan tube repaired animals (AG: 0.427 ± 0.173 mm^2; DAI: 0.089 ± 0.056 mm^2; DAII: 0.149 ± 0.095 mm^2) ($P < 0.05$). However, the density of the myelinated fibers was similar in the three groups (AG: 40,714 ± 4,538 axons/mm^2; DAI: 51,886 ± 2,530 axons/mm^2; DAII: 38,877 ± 3,371 axons/mm^2) ($P > 0.05$). At the distal level, the AG group presented a higher density of myelinated fibers per section (32,805 ± 1,686 axons/mm^2) compared to both

chitosan groups (DAI: 16,865 ± 2,506 axons/mm^2; DAII: 18,399 ± 4,636 axons/mm^2) (P < 0.05).

Morphometrical analysis performed at the midpoint of the graft or tube showed that the mean values of the diameter of the regenerated axons were not significantly different (P > 0.05) between the AG group (2.12 ± 0.16) compared to DAI (1.9 ± 0.08) and DAII (2.27 ± 0.19) groups. The myelin thickness of the regenerated axons revealed no differences (P > 0.05) between the AG group (0.59 ± 0.03) and DAI (0.49 ± 0.01) and DAII (0.49 ± 0.03) groups. Regarding the g-ratio of the regenerated axons, no significant differences (P > 0.05) were observed between the AG group (0.63 ± 0.01) and the DAI (0.64 ± 0.01) and DAII (0.68 ± 0.03) groups (Fig. 8).

Muscle weight

Both tibialis anterior and gastrocnemius muscles of the AG group had higher weight (56.3 ± 4.66; 62.82 ± 3.57 g; P < 0.05) than in animals repaired with chitosan tubes of the two degrees of acetylation (DAI: 35.89 ± 1.56; 45.94 ± 0.72 g; DAII: 35.49 ± 4.37; 52.98 ± 3.79 g, respectively), corroborating a lower degree of reinnervation.

DISCUSSION

In this study, we have investigated the capability of hollow chitosan tubes to sustain axonal regeneration when used to repair a critical 15 mm gap resection of the sciatic nerve in rats, compared with repair by standard silicone tubes and the gold standard autologous graft. In contrast to autografts, in which all the cases presented effective regeneration and target reinnervation, only 3 of 7 and 4 of 7 animals of groups DAI and DAII respectively presented a regenerated nerve inside the guide at the end of follow up, whereas regeneration failed in all the animals repaired with silicone tubes. These failures were expected, since the main limitation of nerve guides is the distance between the stumps that may be bridged. As the

distance increases, regeneration and functional outcome decrease and eventually fail[29]. In rats, the limiting distance at which a simple nerve guide cannot sustain regeneration is considered 15 mm[7]. With silicone tubes, no axons reached the distal segment in a 15 mm defect, whereas axons readily crossed a gap up to 10 mm. Over time the characteristics and the quality of the nerve guides have been improved by research on biomaterials with the aim of sustaining regeneration over such critical long-gap[8]. The advantages of these guides are that, being artificial, there is no need to sacrifice a healthy donor nerve from the patient, and they reduce surgical time for repair[30]. In addition, nerve guides may be advantageous since they may reduce the fibrous entrapment of the injured nerve at the suture site[31, 32] and the problems related to non-correct alignment of nerve fascicles.

Among other biomaterials, chitosan has emerged as an interesting polymer for peripheral nerve bridging. Chitosan has been proved as a suitable biomaterial for medical and pharmaceutical applications because of its compatibility, non-toxicity and biodegradability[17, 33]. Furthermore, chitosan-based tubes are easy to handle and their transparency facilitates surgical manipulation and suturing of the nerve stumps in place. Chitosan has also been used as an scaffold, in the form of freeze-dried sponge[34] and micro/nanofiber mesh[35], serving as an internal filler of the lumen of the tube that can also be combined with other biomaterials or grafted cells[36]. Furthermore, chitosan tubes offer the possibility of modifying their inner surface to mimic the nerve-guiding basal lamina present in nerve grafts, by coupling small peptides derived from extracellular matrix components, such as laminin and fibronectin. By influencing cell adhesion and migration, axonal growth and vascularization of the regenerating cable[37], these extracellular matrix molecules can potentiate the role of tubes in repairing long peripheral nerve defects[38,39]. Chitosan-based materials have already been used for repairing long-gap nerve injuries in rats. In previous studies a mix of polypyrrole/chitosan composite[40] or the combination of chitosan tubes with cross-linked peptides[38] resulted in

enhancement of nerve regeneration, but the efficacy of the chitosan material alone to improve nerve regeneration has not been previously reported.

In the present study we used hollow chitosan tubes of two different degrees of acetylation, controlled during the manufacturing process that may affect the degradation process of the tube once implanted. We chose these degrees of acetylation since higher ones have been shown to be affected by faster degradation and lower mechanical stability[17]. Although, the success of regeneration was lower when using chitosan tubes than when using autografts, these tubes showed considerably better results than the standard control silicone tube. Indeed, the rate of successful regeneration and the levels of reinnervation achieved are among the highest reported for a hollow nerve guide alone over the critical 15 mm long gap in the rat sciatic nerve model.

To evaluate the success of regeneration and target reinnervation we used functional and electrophysiological techniques that allowed us to follow the evolution of motor and sensory recovery over time. By means of algesimetry tests, we evaluated the responses to mechanical and thermal stimuli of the denervated hind paw. Since confounding responses can be due to collateral sprouting of the intact saphenous nerve, we cut this nerve after tests performed at 90 dpo and repeated the measurements at 92 and 120 dpo to guarantee that the responses observed were exclusively due to reinnervation by the regenerated sciatic nerve[27]. We found withdrawal responses in all the animals of the AG group and some of the chitosan tube groups. In fact, all the animals with evidence of paw reinnervation displayed a mechanical withdrawal threshold lower than the control, indicative of hyperalgesia[41]. All the silicone tube repaired animals failed to respond to noxious stimuli. We also performed serial electrophysiological tests to evaluate the degree of muscle reinnervation by regenerating motor axons. Muscle reinnervation started earlier and achieved higher levels in the rats repaired by autograft than in those repaired with chitosan tubes. These differences are not

unexpected, since regeneration in tube repair depends on the initial formation of a new extracellular matrix bridge, over which fibroblasts and Schwann cells migrate and form a new nerve structure[42]. This implies a delay in onset of axonal elongation, and failure of regeneration if the nerve stumps do not provide enough promoting elements inside the tube, as occurred in the long gap repaired with silicone tubes. The histological study corroborated the functional findings explained above. Only in the animals with evidence of reinnervation a regenerated nerve was found inside the tube. The regenerated sciatic nerves in the AG group were larger and had a higher number of myelinated fibers than in the chitosan tubes groups. Although the mean size of the myelinated fibers was similar between animals repaired with AG and with the chitosan guides, the myelin sheath was slightly thicker in axons of the AG group. This could be due to the faster onset of regeneration in autografts compared to tubulization, where the formation of the fibrin cable slows the initial phase of regeneration[43].

CONCLUSION

The present study provides novel proof that chitosan-based tubes are good candidates for an artificial nerve guide, allowing nerve regeneration across a critical long gap in a significant number of cases.

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Author disclosure statement

No competing financial interests exist. The chitosan tubes used in the study were manufactured by Medovent GmbH.

References

1. Fu S, Gordon T. The cellular and molecular basis of peripheral nerve regeneration. Mol Neurobiol 1997;14:67–116.
2. Allodi I, Udina E, Navarro X. Specificity of peripheral nerve regeneration: interactions at the axon level. Prog Neurobiol 2012;98:16–37.
3. Riccio M, Pangrazzi PP, Parodi CC, Vaienti L, Marchesini A, Neuendorf AD, Bottegpmo C, Tos P, Geuna S. The amnion muscle combined graft (AMCG) conduits. Microsurgery 2014; (*In press),*
4. Manoli T, Schulz L, Stahl S, Jaminet P, Schaller HE. Evaluation of sensory recovery after reconstruction of digital nerves of the hand using muscle-in-vein conduits in comparison to nerve suture or nerve autograftin. Microsurgery 2014; *(In press).*
5. Gu X, Ding F, Yang Y, Liu J. Construction of tissue engineered nerve grafts and their application in peripheral nerve regeneration. Prog Neurobiol 2011;93:204–230.
6. Doolabh V, Hertl M, Mackinnon S. The role of conduits in nerve repair: a review. Rev Neurosci 1996;7:47–84.
7. 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.
8. Deumens R, Bozkurt A, Meek MF, Marcus M A, Joosten EA, Weis J, Brook GA. Repairing injured peripheral nerves: Bridging the gap. Prog Neurobiol 2010;92:245–276.
9. Butí M, Verdú E, Labrador R, Vilches J, Forés J, Navarro X. Influence of physical parameters of nerve chambers on peripheral nerve regeneration and reinnervation. Exp Neurol 1996;137:26–33.

10. Giusti G, Shin RH, Lee JY, Mattar TG, Bishop AT, Sjom AY. The influence of nerve conduits diameter in motor nerve recovery after segmental nerve repair. Microsurgery 2014(*In press)*.

11. Dahlin LB, Anagnostaki L, Lundborg G. Tissue response to silicone tubes used to repair human median and ulnar nerves. Scand J Plast Reconstr Surg 2001;35:29–34.

12. Chiono V, Tonda-Turo C, Ciardelli G. Artificial scaffolds for peripheral nerve reconstruction. Int Rev Neurobiol 2009;87:173–198.

13. Archibald SJ, Krarup C, Shefner J, Li ST, Madison R. A collagen-based nerve guide conduit for peripheral nerve repair: an electrophysiological study of nerve regeneration in rodents and nonhuman primates. J Comp Neurol 1991;306:685–696.

14. Weber RA, Bredenbach WE, Brown RE, Jabaley ME, Mass D. A randomized prospective study of polyglycolic acid conduits for digital nerve reconstruction in humans. Plast Reconstr Surg 2000;106:1036–1045.

15. Bertleff MJ, Meek MF, Nicolai J. A prospective clinical evaluation of biodegradable neurolac nerve guides for sensory nerve repair in the hand. J Hand Surg Am 2005;30:513–518.

16. Ao Q, Fung CK, Tsui AY, Cai S, Zuo HC, Chan YS, Shum DK. The regeneration of transected sciatic nerves of adult rats using chitosan nerve conduits seeded with bone marrow stromal cell-derived Schwann cells. Biomaterials 2011;32:787–796.

17. Haastert-Talini K, Geuna S, Dahlin LB, Meyer C, Stenberg L, Freier T, Heimann C, Barwig C, Pinto LF, Raimondo S, Gambarotta G, Samy SR, Sousa N, Salgado AJ, Ratzka A, Wrobel S, Grothe C.. Chitosan tubes of varying degrees of acetylation for bridging peripheral nerve defects. Biomaterials 2013;34:9886–9904.

18. Khor E, Lim LY. Implantable applications of chitin and chitosan. Biomaterials 2003;24:2339–2349.

19. Wang H, Zhao Q, Zhao W, Liu Q, Gu Z, Yang Y. Repairing rat sciatic nerve injury by a nerve-growth-factor-loaded, chitosan-based nerve conduit. Biotechnol Appl Biochem 2012;59:388–394.

20. Patel M, Mao L, Wu B, VandeVord P. GDNF–chitosan blended nerve guides: a functional study. J Tissue Eng Regen Med 2007;1:360–367.

21. Wang W, Itoh S, Matsuda A, Aizawa T, Demura M, Ichinose S, Shinomiya K, Tanaka J. Enhanced nerve regeneration through a bilayered chitosan tube: The effect of introduction of glycine spacer into the CYIGSR sequence. J Biomed Mater Res A 2008;85A:919–928.

22. Patel M, Vandevord PJ, Mattthew HW, De Silva S, Wu B, Wooley P. Collagen-Chitosan nerve guides for peripheral nerve repair: a histomorphometric study. J Biomater Appl 2008;23:101–121.

23. Hu N, Wu H, Xue C, Gong Y, Wu J, Xiao Z, Yang Y, Ding F, Gu X. Long-term outcome of the repair of 50 mm long median nerve defects in rhesus monkeys with marrow mesenchymal stem cells-containing, chitosan-based tissue engineered nerve grafts. Biomaterials 2011;34:100–111.

24. Ding F, Wu J, Yang Y, Hu W, Zhu Q, Tang X, Liu J, Gu X. Use of tissue-engineered nerve grafts consisting of a chitosan/poly(lactic-co-glycolic acid)-based scaffold included with bone marrow mesenchymal cells for bridging 50-mm dog sciatic nerve gaps. Tissue Eng Part A 2010;16:3779–3790.

25. Navarro X, Butí M, Verdú E. Autotomy prevention by amytriptiline after peripheral nerve section in different strains of mice. Restor Neurol Neurosci 1994;6:151-157.

26. Asensio-Pinilla E, Udina E, Jaramillo J, Navarro X. Electrical stimulation combined with exercise increase axonal regeneration after peripheral nerve injury. Exp Neurol 2009;219:258–265.

27. Cobianchi S, de Cruz J, Navarro X. Assessment of sensory thresholds and nociceptive fiber growth after sciatic nerve injury reveal the differential contribution of collateral reinnervation and nerve regeneration to neuropathic pain. Exp Neurol 2014;255:1–11.

28. Geuna S. Appreciating the difference between design-based and model-based sampling strategies in quantitative morphology of the nervous system. J Comp Neurol 2000;427:333–339.

29. Yannas IV, Hill BJ. Selection of biomaterials for peripheral nerve regeneration using data from the nerve chamber model. 2004;25:1593–1600.

30. Boeckstyns ME, Sørensen AI, Viñeta JF, Rosén B, Navarro X, Archibald SJ, Valss-Solé J, Moldovan M, Krarup C. Collagen conduit versus microsurgical neurorrhaphy: 2-year follow-up of a prospective, blinded clinical and electrophysiological multicenter randomized, controlled trial. J Hand Surg Am 2013;38:2405–2411.

31. Lee JY, Parisi TJ, Friederich PF, Bishop AT, Shin AY. Does the addtion of a nerve wrap to a motor nerve repair affect motor outcomes? Microsurgery 2014; 34:562-567.

32 Siemionow M, Uygur S, Ozturk C, Siemionow K. Techniques and materials for enhancement of peripheral nerve regeneration: a literature review. Microsurgery 2013;33:318-328.

33. Freier T, Koh HS, Kazazian K, Shoichet MS. Controlling cell adhesion and degradation of chitosan films by N-acetylation. Biomaterials 2005;26:5872–5878.

34. Ishikawa N, Suzuki Y, Ohta M, Cho H, Suzuki S, Dezawa M, Ide C. Peripheral nerve regeneration through the space formed by a chitosan gel sponge. J. Biomed Mater Res A 2007;83A:33–40.

35. Matsumoto I, Kaneko M, Odam M, Watanabe G. Repair of intra-thoracic autonomic nerves using chitosan tube. Interact Cardiovasc Thorac Surg 2009;10:498–501.

36. Zheng L, Cui HF. Use of chitosan conduit combined with bone marrow mesenchymal stem cells for promoting peripheral nerve regeneration. J Mater Sci Mater Med 2010 ;21:1713–1720.

37. Gnavi S, Barwig C, Freier T, Haastert-Talini K, Grothe C, Geuna S. The use of chitosan-based scaffolds to enhance regeneration in the nervous system. Int Rev Neurobiol 2013;109:1–50.

38. Itoh S, Yamaguchi I, Suzuki M, Ichonose S, Takakuda K, Kobayashi H, Shinomiya K, Tanaka J. Hydroxyapatite-coated tendon chitosan tubes with adsorbed laminin peptides facilitate nerve regeneration in vivo. Brain Res 2003;993:111–123.

39. Madison R, da Silva C, Dikkes P, Chiu TH, Sidman R. Increased rate of peripheral nerve regeneration using bioabsorbable nerve guides and a laminin-containing gel. Exp Neurol 1985;88:767–772.

40. Huang J, Lu L, Zhang J, Hu X, Zhang Y, Liang W, Wu S, Luo Z. Electrical stimulation to conductive scaffold promotes axonal regeneration and remyelination in a rat model of large nerve defect. PLoS One 2012;7.

41. Cobianchi S, Casals-Diaz L, Jaramillo J, Navarro X. Differential effects of activity dependent treatments on axonal regeneration and neuropathic pain after peripheral nerve injury. Exp Neurol 2013;240:157–167.

42. Williams L, Longo F, Powell H, 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.

43. Gómez N, Cuadras J, Butí M, Navarro X. Histologic assessment of sciatic nerve regeneration following resection and graft or tube repair in the mouse. Restor Neurol Neurosci 1996;10:187–196.

Figures

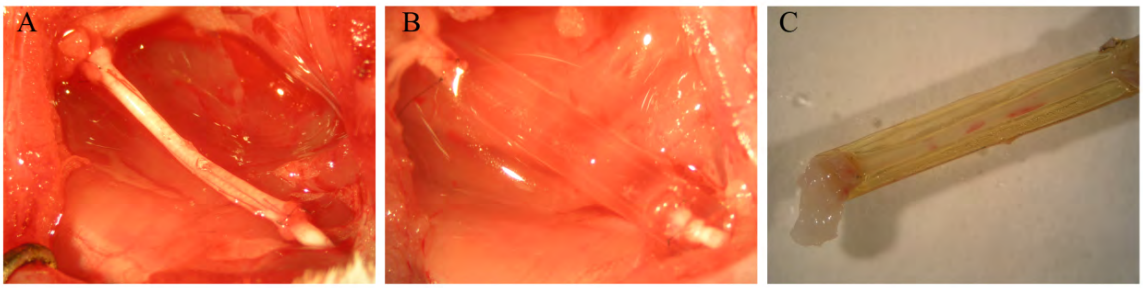

Figure 1: Representative images of a sciatic nerve resected and repaired with an autograft (AG) of 15 mm (A), or with a hollow DAII chitosan tube of 19 mm leaving a 15 mm gap (B). Regenerated nerve found 4 months after repairing the sciatic nerve with a hollow DAII chitosan tube (C).

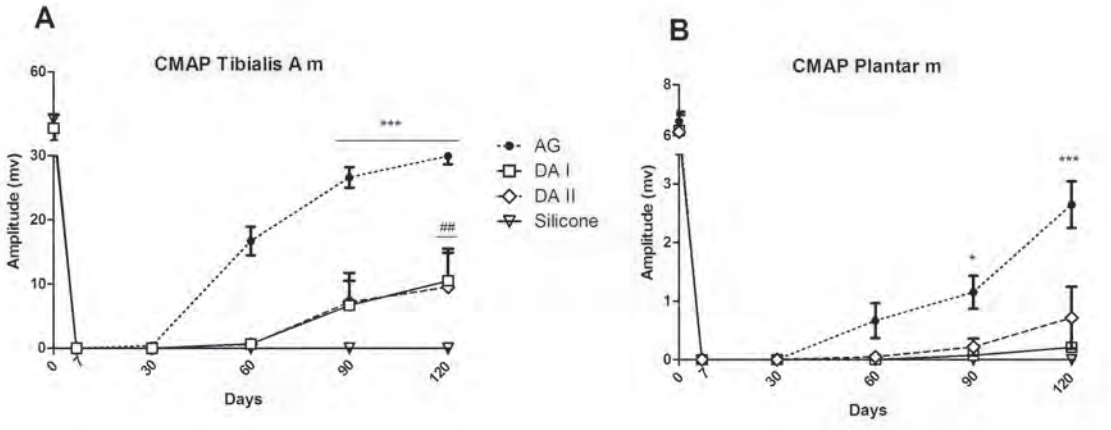

Figure 2: Mean amplitude of the compound muscle action potential (CMAP) of tibialis anterior (A) and plantar muscles (B) of the injured hind limb of the rats during 4 months after sciatic nerve lesion and repair. * $P < 0.05$ AG vs. DAI, DAII and SIL; * $P<0.05$ DAI and DAII vs. SIL.

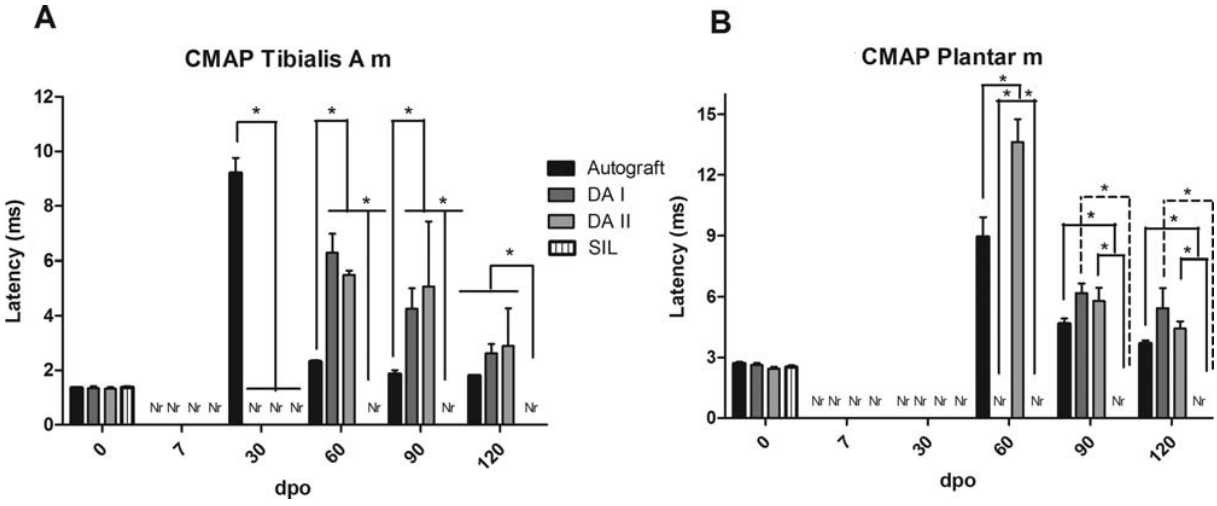

Figure 3**:** Mean latencies of the tibialis anterior (A) and plantar muscles (B) CMAP recorded in the regenerated rats during the 4 months follow-up. * P<0.05.

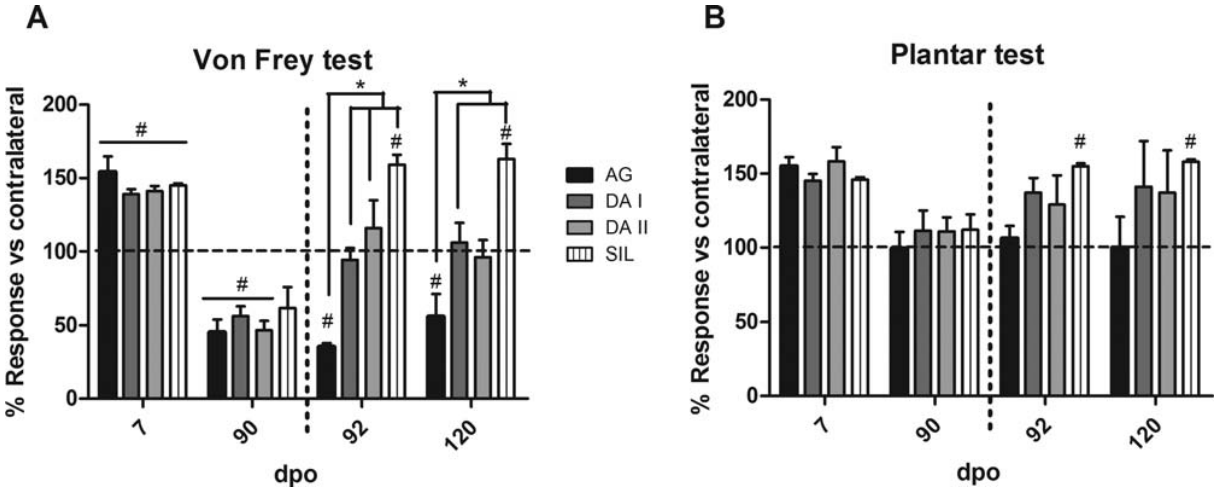

Figure 4: Mechanical (A) and thermal (B) algesimetry test results. Values were expressed as percentage of withdrawal response to mechanical stimulus (A) and thermal stimulus (B) applied to the lateral side of the injured paw vs. the withdrawal response in the uninjured paw. * P < 0.05 for differences between groups. # P < 0.05 for differences between groups and the baseline. Horizontal dotted lines represent the normalized baseline values. Vertical dotted lines indicate when the saphenous nerve was cut.

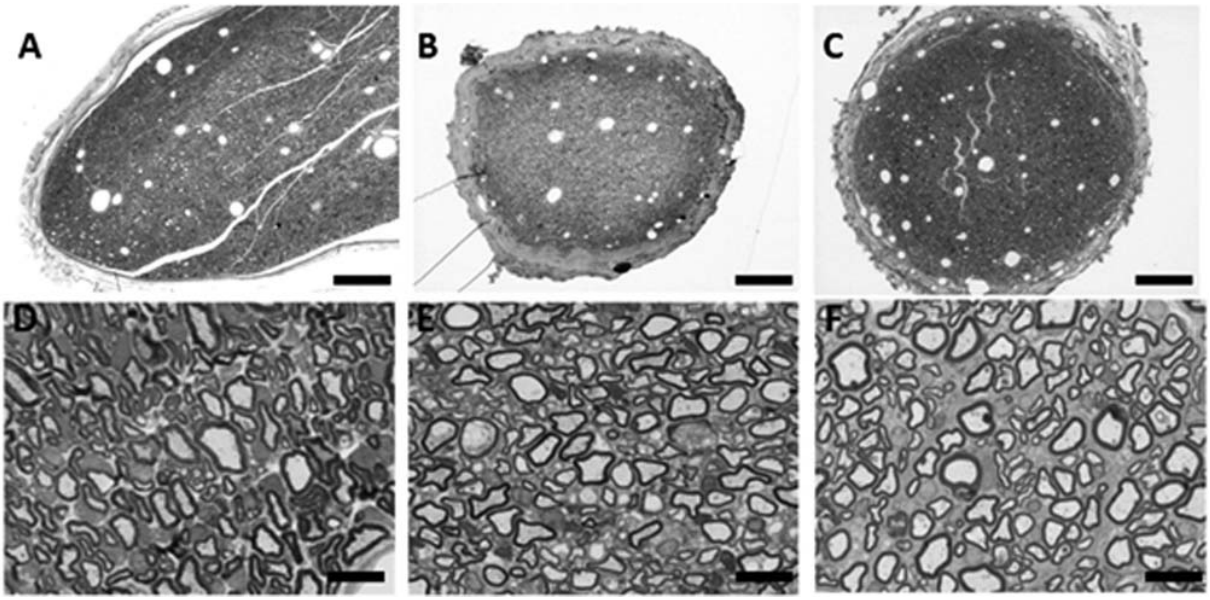

Figure 5: Micrographs of semithin sections of the regenerated nerve taken at the midpoint of the graft or tube 4 months after sciatic nerve resection and repair from a representative animal of group AG (A, D), and one of the animals that regenerated in group DAI (B, E) and DAII (C, F). General appearance of the regenerated nerves (A-C); bar =100 µm. Higher magnification of the regenerated nerves (D-F); bar =10 µm.

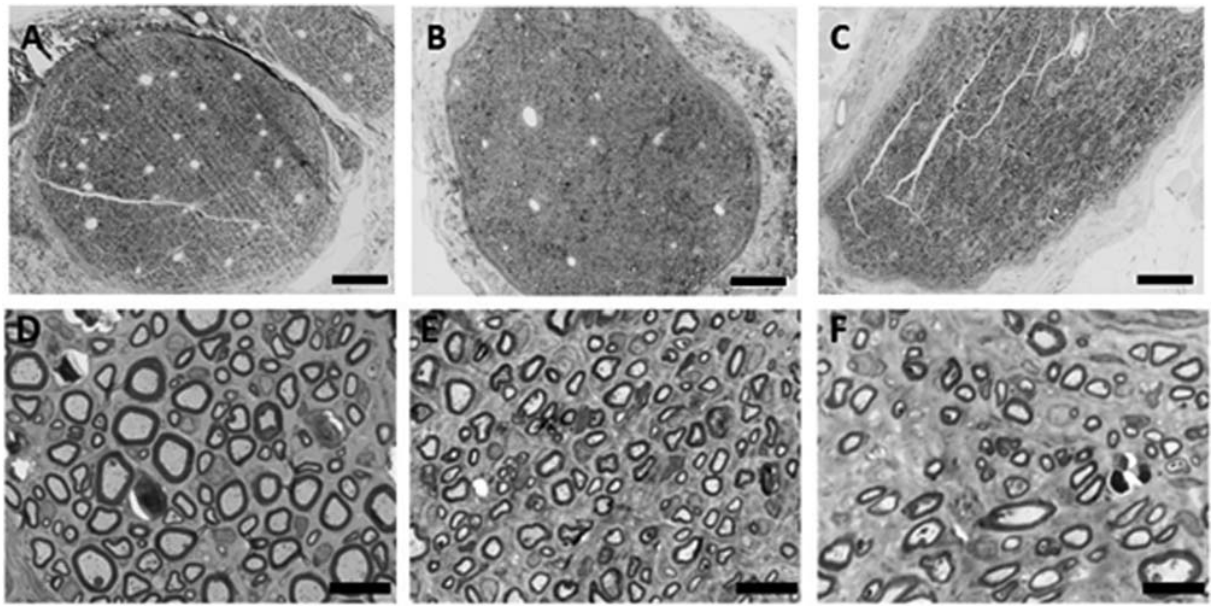

Figure 6: Micrographs of semithin sections of the regenerated nerve taken 3 mm distally to the graft or tube 4 months after sciatic nerve resection and repair from a representative animal of group AG (A, D), and one of the animals that regenerated in group DAI (B, E) and DAII (C, F).). General appearance of the regenerated nerves (A-C); bar =100 µm. Higher magnification of the regenerated nerves (D-F); bar =10 µm.

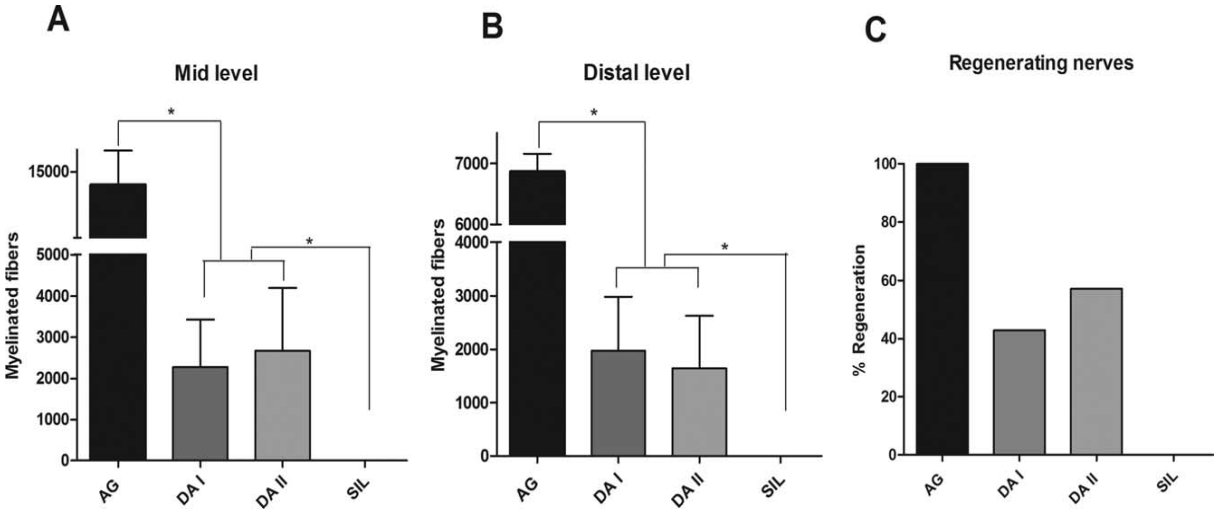

Figure 7: Number of regenerated myelinated fibers found in the tibial nerve at the mid-tube or graft (A) and 3 mm distal (B) in AG, DAI, DAII and SIL groups. Animals with no regenerated nerve were also included (with values of 0) in the calculation. * $P < 0.05$. Percentage of regenerated nerves found at 120 dpo (C).

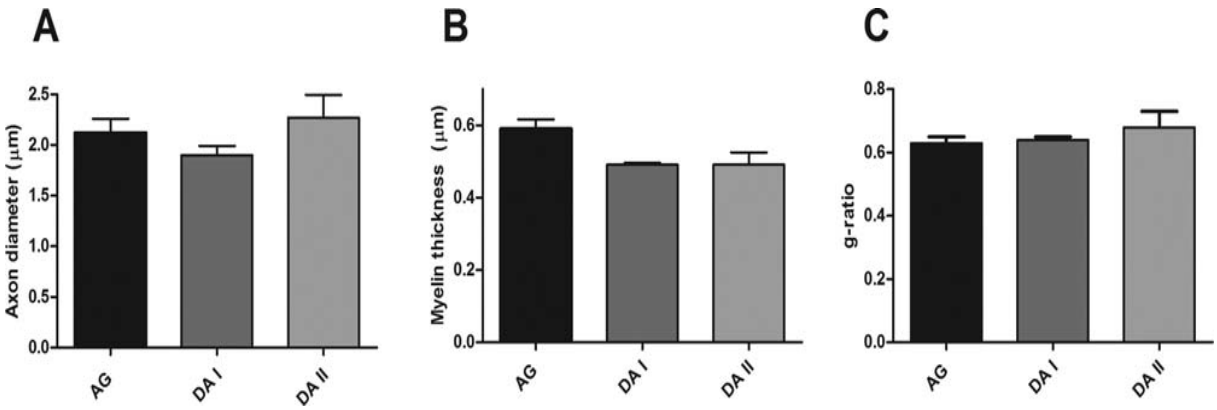

Figure 8: Morphometrical results of axon diameter (A), myelin thickness (B) and g-ratio (C) of the regenerated nerves found at the mid-point of the graft or the tube in groups AG, DAI and DAII. No significant differences were found between groups.