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Use of chitosan scaffolds for repairing rat sciatic nerve defects

Maria J. Simões[a,1], Sandra Amado[b,1], Andrea Gärtner[a], Paulo A.S. Armada-da-Silva[b], Stefania Raimondo[c], Marcia Vieira[a], Ana L. Luís[a,d], Yuki Shirosaki[g], António P. Veloso[b], José D. Santos[e], Artur S.P. Varejão[f], Stefano Geuna[c] and Ana C. Maurício[a,d,2]

[a] Centro de Estudos de Ciência Animal (CECA), Instituto de Ciências e Tecnologias Agrárias e Agro-Alimentares (ICETA), Universidade do Porto (UP), Portugal.
[b] Faculdade de Motricidade Humana (FMH), Universidade Técnica de Lisboa (UTL), Portugal.
[c] Department of Clinical and Biological Sciences, University of Turin, Italy.
[d] Departamento de Clínicas Veterinárias, Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Universidade do Porto (UP), Portugal.
[e] Faculdade de Engenharia da Universidade do Porto (FEUP), Porto, Portugal.
[f] Departamento de Ciências Veterinárias, Universidade de Trás-os-Montes e Alto Douro (UTAD), Portugal.
[g] Graduate School of Natural Science and Technology, Okayama University, Japan.

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Summary

Neurotmesis must be surgically treated by direct end-to-end suture of the two nerve stumps or by a nerve graft harvested from elsewhere in the body in case of tissue loss. To avoid secondary damage due to harvesting of the nerve graft, a tube-guide can be used to bridge the nerve gap. Previously, our group developed and tested hybrid chitosan membranes for peripheral nerve tubulization and showed that freeze-dried chitosan type III membranes were particularly effective for improving peripheral nerve functional recovery after axonotmesis. Chitosan type III membranes have about 110 μm pores and about 90% of porosity, due to the employment of freeze-drying technique. The present study aimed to verify if chitosan type III membranes can be successfully used also for improving peripheral nerve functional recovery after neurotmesis of the rat sciatic nerve. Sasco Sprague-Dawley adult rats were divided into 6 groups: Group 1: end-to-end neurorrhaphy enwrapped by chitosan membrane type III (*End-to-EndChitIII*); Group 2: 10mm-nerve gap bridged by an autologous nerve graft enwrapped by chitosan membrane type III (*Graft180°ChitIII*); Group 3: 10mm-nerve gap bridged by chitosan type III tube-guides (*GapChitIII*); These 3 experimental groups were compared with 3 control groups, respectively: Group 4: 10mm-nerve gap bridged by an autologous nerve graft (*Graft180°*); Group 5: 10mm-nerve gap bridged by PLGA 90:10 tube-guides (*PLGA*); Group 6: end-to-end neurorrhaphy alone (*End-to-End*). Motor and sensory functional recovery were evaluated throughout a healing period of 20 weeks using extensor postural thrust (EPT), withdrawal reflex latency (WRL) and ankle kinematics. Regenerated nerves withdrawn at the end of the experiment were analysed histologically. Results showed that nerve regeneration was successful in all experimental and control groups and that chitosan type III tubulization induced a significantly better nerve regeneration and functional recovery in comparison to PLGA tubulization control. Further investigation is needed to explore the mechanisms at the basis of the positive effects of chitosan type III on axonal regeneration.

Key words

Nerve regeneration, biomaterials, chitosan, stem cells, kinematics, rat

[1] These authors contributed equally to this work.
[2] Correspondence author: Campus Agrário de Vairão, Rua Padre Armando Quintas; 4485-661 Vairão VCD, Portugal. Telephone: +351252660412, Mobile: +351919071286, Fax: +351252661780; E-mail: ana.colette@mail.icav.up.pt; ana.colette@hotmail.com

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Introduction

Functional recovery after peripheral nerve injury and repair is rarely satisfactory (Millesi, 1990; Madison et al., 1992; Kline, 2000; Lundborg, 2002; Hoke, 2006; Siemionow and Brzezicki, 2009). In case of loss of substance a nerve autograft procedure, using commonly expendable sensory nerves, is required (Matsuyama et al., 2000; Lundborg, 2003; Siemionow and Brzezicki, 2009). However, nerve autograft leads to donor site morbidity and secondary sensory deficit (May, 1983). Alternatives to peripheral nerve grafts include cadaver nerve segments allografts, end-to-side neurorraphy and entubulation by means of autologous non nervous tissues such as vein and muscles (Doolabh et al., 1996; Schmidt and Leach, 2003; Jensen et al., 2004; Lundborg, 2004; Chen et al., 2006b; Battiston et al., 2009). Therefore one of the scientist's challenges over the last thirty years has been to find an alternative to the autologous nerve graft (Battiston et al., 2009). Employment of a nerve conduit (i.e. a tubular structure designed to bridge the gap of a sectioned nerve, protects the nerve from the surrounding tissue, and guides the regenerating axons into the distal nerve stump) is the most popular alternative to nerve autografts; yet, conduits can also play an important role as a vehicle for neurotrophic factors and cellular systems (Doolabh et al., 1996; den Dunnen et al., 1997; Schmidt and Leach, 2003; Jensen et al., 2004; Lundborg, 2004; Chen et al., 2006b; Geuna et al., 2006; Amado et al., 2008; Luís et al., 2008). Comparative clinical studies between autograft and conduit in relatively short gaps revealed similar functional outcomes with the autografts and the collagen or polyglycolide tube-guides (Archibald et al., 1995; Battiston et al., 2005). Most natural biomaterials used in clinical applications (e.g. hyaluronic acid, collagen, and gelatine) are derived from animal sources, thus bearing the inherent risk of transfer of viral diseases which is absent when synthetic biomaterials are used (Dubey et al., 1999; Meek et al., 2004; Meek et al., 2004a; Kannan et al., 2005; Luís et al., 2007).

Entubulation nerve repair has been investigated extensively by our research group over the last years (Varejão et al., 2003a,b; Battiston et al., 2005; Luís et al., 2008). Nerve guides can be made of biological or synthetic materials and, among the latter, both non-absorbable (e.g. silicon) and biodegradable tubes have been used (Schmidt and Leach, 2003). Biodegradable nerve guides must be preferred since no foreign body material will be left in the host after the device has fulfilled its task. Our group has recently tested both *in vitro* and *in vivo* the efficacy of synthetic biodegradable tubes made of PLGA in a 90:10 proportion of the two polymers, Poly(L-lactide):Poly(glycolide) (PLA:PLG) and membranes made of hybrid chitosan (Rodrigues et al., 2005a,b; Luís et al., 2007, 2008; Amado et al., 2008). In particular, chitosan has recently attracted particular attention because of its biocompatibility, biodegradability, low toxicity, low cost, enhancement of wound-healing and antibacterial effects and its potential usefulness in nerve regeneration have been demonstrated both *in vitro* and *in vivo* (Chandy and Sharma, 1990; Shirosaki. et al., 2005). Chitosan is a partially deacetylated polymer of acetyl glucosamine obtained after the alkaline deacetylation of chitin (Senel and McClure, 2004). While chitosan matrices have low mechanical strength under physiological conditions and are unable to maintain a predefined shape after transplantation, their mechanical properties can be improved by modification with a silane agent, namely γ-glycidoxypropyltrimethoxysi lane (GPTMS), one of the silane-coupling agents which has epoxy and methoxysilane

groups. The epoxy group reacts with the amino groups of chitosan molecules, while the methoxysilane groups are hydrolyzed and form silanol groups, and the silanol groups are subjected to the construction of a siloxane network due to the condensation. Thus, the mechanical strength of chitosan can be improved by the cross-linking between chitosan and GPTMS and siloxane network. We have previously obtained, by adding GPTMS, chitosan type III membranes (hybrid chitosan membranes) which have about 110 μm pores and about 90% of porosity, due to the employment of freeze-drying technique, and which were successful in improving sciatic nerve regeneration after axonotmesis (Amado et al., 2008). Wettability of the material surfaces is one of the key factors for protein adsorption, cell attachment and migration (Hench and Ethridge, 1982; Amado et al., 2008). The addition of GPTMS improves the wettability of chitosan surfaces, and therefore chitosan type III membranes are more hydrophilic than chitosan membranes without GPTMS (Shirosaki et al., 2005). In addition, chitosan type III was developed to be more porous, with a larger surface to volume ratio, and to preserve mechanical strength and ability to adapt to different shapes. Significant differences in water uptake between commonly used chitosan and our hybrid chitosan type III were previously reported and it has been shown that they retain about two times as much biological fluid (Tateishi et al., 2002). In the present study, we aimed to use the same rat sciatic nerve model for investigating the effects of chitosan type III membranes in surgical neurotmesis repair either by direct suture, or autograft or tubulization.

Material and methods

Tube-guides design and preparation

Poly (dl-lactide-co-glycolide) copolymers with ratio of 90PLA:10PLG were obtained from their cyclic dimers, dl-lactide and glycolide. Non-woven constructs were used to prepare tube guides 16 mm long, internal diameter of 2.0 mm and thickness wall of 1.5mm. These fully synthetic non-woven materials are extremely flexible, biologically safe and are able to sustain the compressive forces due to body movement after implantation. They have also some degree of porosity to allow for influx of low molecular nutrients required for nerve regeneration. The non-woven structure allowed the tube-guide to hold suture without difficulties, however greater care had to be taken in order to ensure its integrity. These tube-guides of PLGA are expected to degrade to lactic and glycolic acids through hydrolysis of the ester bonds (Luís et al., 2007, 2008).

Chitosan (high molecular weight, Aldrich®, USA) was dissolved in 0.25M acetic acid aqueous solution to a concentration of 2% (w/v). To obtain type III membranes, GPTMS (Aldrich®, USA) was also added to the chitosan solution and stirred at room temperature for 1h. The drying process for type III chitosan membrane was as follows: the solutions were frozen for 24h at -20°C and then transferred to the freeze-dryer, where they were left 12h to complete dryness. The chitosan type III membranes were soaked in 0.25N sodium hydroxide aqueous solution to neutralize remaining acetic acid, washed well with distilled water, and freeze dried (Amado et al., 2008). All membranes were sterilized with ethylene oxide gas, considered by

some authors the most suitable method of sterilization for chitosan membranes (Marreco et al., 2004). Prior to their use *in vivo*, membranes were kept 1 week at room temperature to clear any ethylene oxide gas remnants (Amado et al., 2008).

Surgical procedure

Adult male Sasco Sprague-Dawley rats (Charles River Laboratories, Barcelona, Spain) weighing 300-350 g, divided in 6 groups of 6 animals each (except Group 5 or *PLGA* group, with 10 animals and Group 6 or *End-to-end* group with 7 animals), were used. All animals were housed in a temperature and humidity controlled room with 12-12 hours light / dark cycles, two animals per cage (Makrolon type 4, Tecniplast, VA, Italy), and were allowed normal cage activities under standard laboratory conditions. The animals were fed with standard chow and water *ad libitum*. Adequate measures were taken to minimize pain and discomfort taking in account human endpoints for animal suffering and distress. Animals were housed for two weeks before entering the experiment.

For surgery, rats were placed prone under sterile conditions and the skin from the clipped lateral right thigh scrubbed in a routine fashion with antiseptic solution. The surgeries were performed under an M-650 operating microscope (Leica Microsystems, Wetzlar, Germany). Under deep anaesthesia (ketamine 9 mg/100 g; xylazine 1.25 mg/100 g, atropine 0.025 mg/100 body weight, intramuscular), the right sciatic nerve was exposed through a skin incision extending from the greater trochanter to the distal mid-half followed by a muscle splitting incision. After nerve mobilisation, a transection injury was performed (neurotmesis) using straight microsurgical scissors. The nerve was injured at a level as low as possible, in general, immediately above the terminal nerve ramification. In Group 1 (*End-to-EndChitIII*), immediate cooptation with 7/0 monofilament nylon sutures of the 2 injured nerve endings was immediately performed under magnification and involved with a chitosan type III membrane. In Group 2 (*Graft180°ChitIII*) the sciatic nerve was transected immediately above the terminal nerve ramification and in a 10 mm distal point. The nerve graft obtained, with a length of 10 mm, was inverted 180° and sutured with 7/0 monofilament nylon. The sutured graft was involved afterwards with a chitosan type III membrane. In Group 3 (*GapChitIII*) the proximal and distal nerve stumps were inserted 3 mm into the chitosan type III tube-guides and held in place, maintaining a nerve gap of 10 mm, with two epineurial sutures using 7/0 monofilament nylon, respectively. These groups were compared with three control groups, concerning the following experimental conditions: in Group 4 (*Graft180°*) the sciatic nerve was transected immediately above the terminal nerve ramification and in a 10 mm distal point. The nerve graft obtained, with a length of 10 mm, was inverted 180° and sutured with 7/0 monofilament nylon; in Group 5 (*PLGA*) the proximal and distal nerve stumps were inserted 3 mm into the PLGA tube-guides and held in place, maintaining a nerve gap of 10 mm, with two epineurial sutures using 7/0 monofilament nylon, respectively; and in Group 6 (*End-to-End*) immediate cooptation with 7/0 monofilament nylon sutures of the 2 injured nerve endings was immediately performed under magnification. Opposite leg and sciatic nerve were left intact in all groups and served as control for normal nerves. To prevent autotomy a deterrent substance was applied to rats' right foot (Kerns et al., 1991; Sporel-Ozakat et al., 1991). The animals were intensively examined for signs of

autotomy and contracture during the post-operative period and none presented severe wounds, infections or contractures. All procedures were performed with the approval of the Veterinary Authorities of Portugal in accordance with the European Communities Council Directive of November 1986 (86/609/EEC).

Evaluation of motor performance (EPT) and nociceptive function (WRL)

Motor performance and nociceptive function were evaluated by measuring extensor postural thrust (EPT) and withdrawal reflex latency (WRL), respectively. The animals were tested pre-operatively (week-0), at weeks 1, 2, and every two weeks thereafter until week-20. The animals were gently handled, and tested in a quiet environment to minimize stress levels. The EPT was originally proposed by Thalhammer and collaborators, (Thalhammer et al., 1995) as a part of the neurological recovery evaluation in the rat after sciatic nerve injury. For this test, the entire body of the rat, excepting the hind-limbs, was wrapped in a surgical towel. Supporting the animal by the thorax and lowering the affected hind-limb towards the platform of a digital balance, elicits the EPT. As the animal is lowered to the platform, it extends the hind-limb, anticipating the contact made by the distal metatarsus and digits. The force in grams (g) applied to the digital platform balance (model TM 560; Gibertini, Milan, Italy) was recorded. The same procedure was applied to the contra-lateral, unaffected limb. Each EPT test was repeated 3 times and the average result was considered.The normal (unaffected limb) EPT (NEPT) and experimental EPT (EEPT) values were incorporated into a equation (Equation 1) to derive the percentage of functional deficit, as described by Koka and Hadlock (2001).

$$\text{Motor Deficit} = (\text{NEPT} - \text{EEPT}) \,/\, \text{NEPT} \quad \text{(Equation 1)}$$

To assess the nociceptive withdrawal reflex (WRL), the hotplate test was modified as described by Masters et al. (1993). The rat was wrapped in a surgical towel above its waist and then positioned to stand with the affected hind paw on a hot plate at 56ºC (model 35-D, IITC Life Science Instruments, Woodland Hill, CA). WRL is defined as the time elapsed from the onset of hotplate contact to withdrawal of the hind paw and measured with a stopwatch. Normal rats withdraw their paws from the hotplate within 4.3 s or less (Hu et al., 1997). The affected limbs were tested 3 times, with an interval of 2 min between consecutive tests to prevent sensitization, and the three latencies were averaged to obtain a final result (Campbell, 2001; Shir et al., 2001). If there was no paw withdrawal after 12 s, the heat stimulus was removed to prevent tissue damage, and the animal was assigned the maximal WRL of 12 s (Varejão et al., 2003b,c).

Morphological analysis

At the end of the experiments, the rats were sacrificed under deep anaesthesia and, from two animals from each experimental group, a 10-mm-long segment of the sciatic nerve distal to the site of lesion was removed. The specimens were fixed by immediate immersion in 2.5% purified glutaraldehyde and 0.5% sucrose in 0.1M Sorensen phosphate buffer for 6-8 hours. Nerves were then washed in a solution con-

taining 1.5% sucrose in 0.1M Sorensen phosphate buffer, post-fixed in 1% osmium tetroxide, dehydrated and embedded in resin. From each specimen, 2-μm thick series of semi-thin transverse sections were cut, starting from the distal stump, using an Ultracut UCT ultramicrotome (Leica Microsystems, Wetzlar, Germany). Finally, sections were stained using Toluidine blue and analyzed with a DM4000B microscope equipped with a DFC320 digital camera and an IM50 image manager system (Leica Microsystems, Wetzlar, Germany).

Kinematic Analysis

Ankle kinematics analysis was carried out prior nerve injury, at week-2 and every 4 weeks during the 20-week follow-up time. Animals walked on a Perspex track with length, width and height of respectively 120, 12 and 15 cm. Motion capture was performed by 2 digital high speed infrared cameras (200 images/sec) (Oqus, Qualysis®), and Qualisys Track Manager software (QTM, Qualysis®). This motion capture system is extremely precise in locating the markers. The system was calibrated using a 15 cm-long wand with two reflective markers in each end. The system precision was adjusted as to keep the standard deviation of wand length measurements. Four reflective markers were attached to the rat right hindlimb at the proximal end of the tibia, the lateral malleolus, the fifth metatarsal head, and the tip of second toe. 2-D movement of the ankle joint (sagittal plan) was performed with Visual3D software (C-Motion®, Inc). The perpendicular position between the leg and foot was considered the neutral position (0º degrees) with dorsiflexion and plantarflexion assuming positive and negative angle values, respectively. For each step cycle the following time points were identified: midswing, midstance, initial contact (IC) and toe-off (TO) (Dijkstra et al., 2000; Varejão et al., 2002, 2003a; Cappozzo et al., 2005; Goulermas et al., 2005; Johnson et al., 2008) and normalized in time of step cycle. Angular velocity of the ankle joint was also determined with negative and positive velocity values corresponding to dorsiflexion and plantarflexion, respectively. A total of six walking cycles for each animal with stance phases lasting between 150 and 400 ms (walking velocity between 20-60 m/s) was averaged and considered for analysis (Varejão et al., 2003a,b).

Statistical analysis

Two-way mixed factorial ANOVA was used to test for the effect of time and experimental group. The sphericity assumption was evaluated by the Mauchly's test and whenever this test was not computed or sphericity could not be assured, the degrees of freedom were corrected by using the more conservative Greenhouse-Geiser's epsilon. In the cases two-way ANOVA revealed the existence of a significant main effect of time (within subjects' factor), simple planned contrasts (General Linear Model, simple contrasts) were then employed to compare the different time points during recovery to baseline data with groups collapsed. If two-way mixed factorial ANOVA analysis identified a significant effect of experimental group (between subjects factor), the post hoc HSD Tukey's test was applied for paired comparisons. All statistical procedures were performed by using the statistical package SPSS (version 17.0, SPSS, Inc).

Results

Motor deficit and Nociception function

Motor deficit (EPT)

Table 1 presents the results of Motor Deficit derived from EPT measures. Severe loss of the operated hindlimb extensor response was observed in all groups after nerve injury suggesting deep paralysis of affected muscles. From week-2 post-operatively, the hindlimb extensor response initiated recovery which proceeded until week-20 to a similar extent in all groups, excepting *PLGA* group (Group 5) with EPT data significantly different from *End-to-End* group (Group 6) ($p = 0.03$).

Nociception function (WRL)

Table 2 depicts the data for the WRL tests. In the first week post surgery all animals presented severe loss of sensory function and all tests had to be interrupted at the selected cut off time of 12 s that remained identical during week-2 except in the *End-to-End* group (Group 6). The withdrawal response recovered progressively in the course of the 20 weeks but at different rates depending on treatment [main effect of group $F_{(6,30)} = 22.243$; $p < 0.001$]. As expected, recovery of noxious thermal sensation was faster and more complete in the *End-to-End* (Group 6) and *End-to-EndChitIII* (Group 1) groups with no differences between these two groups ($p = 0.312$). No differences in WRL data were observed for the two groups treated with reversed sciatic nerve autografts covered (*Graft180ºChitIII* or Group 2) or not with a chitosan membrane (*Graft180º* or Group 4) although the group with chitosan membrane (*Graft180ºChitIII* or Group 2) tended to show a slower rate of recovery ($p = 0.056$). WRL values were significant different between the groups treated with nerve conduits fabricated with chitosan and PLGA ($p<0.001$). Significant differences were also observed between the group treated with tube-guides and the reversed autograft group ($p <0.001$). No differences existed between the later group and the one treated with PLGA-made nerve conduits.

Kinematics Analysis

Sciatic nerve neurotmesis caused a relative increase in the duration of the swing phase (33% and 43% of gait cycle duration before and after injury; $p=0.001$). Sciatic nerve injury also affected severely the motion of the ankle joint during the swing phase with loss of the normal dorsiflexion movement (Figure 1) confirmed by decreased value of the joint angle at midswing (Table 3).

Ankle joint velocity at midswing was also significantly affected by sciatic injury [$F_{(6,84)} = 9.819$; $p = 0.000$] with contrast analysis showing that differences from pre-injury values were significant from week-4 until week-20. Peak velocities during the swing phase were significantly lower for both plantarflexion [$F_{(6,84)} = 33.320$; $p = 0.000$] and dorsiflexion [$F_{(6,84)} = 10.378$; $p = 0.000$] (Table 4a) with no complete recovery during the 20-weeks follow up.

Ankle joint motion during the stance phase of the gait cycle was also affected by sciatic injury at midstance and TO events (Figure 2) but not at IC [$F_{(6,84)} = 2.112$; $p = 0.060$] (Table 3) with no differences between groups (Tables 3 and 4).

Table 1 – Values of Motor Deficit were obtained performing Extensor Postural Thrust (EPT) test. This test has been performed preoperatively (week-0), at week-1 and every 2 weeks after the surgical procedure until week-20, when the animals were sacrificed for morphological analysis. Results are presented as mean and standard error of the mean (SEM). N corresponds to the number of rats within the experimental group. Group 1: end-to-end reconstruction involved with a chitosan membrane type III (*End-to-EndChitI-II*); Group 2: gap reconstructed using an autologous nerve graft involved with a chitosan membrane type III (*Graft180°ChitIII*); and Group 3: gap reconstructed with chitosan type III tube-guides (*GapChitIII*); These 3 groups were compared with 3 control groups, concerning, Group 4: gap reconstructed using an autologous nerve graft (*Graft180°*); Group 5: gap reconstructed with PLGA 90:10 tube-guides (*PLGA*); and Group 6: end-to-end reconstruction (*End-to-End*).

Week	0	1	2	4	6	8
End-to-end chit III Mean±SEM N =6	1.29±0.50	93.55±0.43	91.96±0.63	90.35±1.04	88.01±1.32	80.69±3.33
Graft 180° chit III Mean±SEM N = 6	1.41±0.42	92.48±0.59	91.03±0.40	88.97±1.65	85.67±1.83	75.18±2.29
Gap chit III Mean±SEM N = 6	2.38±0.24	92.71±0.39	90.00±0.58	88.92±0.71	85.82±1.57	81.09±2.96
Graft Mean±SEM N = 6	5.33±4.18	90.00±3.70	89.00±3.30	85.00±5.30	77.00±1.20	72.00±6.20
PLGA Mean±SEM N = 10	8.00±1.40	93.00±5.80	86.00±10.70	86.00±8.50	85.00±5.40	84.00±8.40
End-to-end Mean±SEM N = 7	7.29±0.95	89.14±6.20	87.29±5.62	78.29±7.52	74.43±8.08	67.71±8.67

Week	10	12	14	16	18	20
End-to-end chit III Mean±SEM N =6	73.04±4.13	63.10±3.42	53.91±3.28	41.78±1.90	32.17±1.75	29.20±1.64
Graft 180° chit III Mean±SEM N = 6	68.89±3.60	61.01±4.13	49.71±3.06	39.12±2.21	30.45±2.21	23.40±1.53
Gap chit III Mean±SEM N = 6	71.69±5.48	64.97±6.99	53.25±5.52	43.97±4.02	39.15±4.11	35.16±4.31
Graft Mean±SEM N = 6	64.00±5.30	66.00±3.40	60.00±8.70	53.00±7.70	54.00±3.10	52.00±3.20
PLGA Mean±SEM N = 10	81.00±7.40	79.00±5.40	71.00±5.2	70.00±10.80	67.00±11.70	55.00±6.90
End-to-end Mean±SEM N = 7	58.43±6.27	49.00±5.83	51.71±7.18	39.86±4.85	38.86±2.79	42.71±4.99

Table 2 – Values in seconds (s) were obtained performing Withdrawal Reflex Latency (WRL) test to evaluate the nociceptive function. This test has been performed pre-operatively (week-0), at week-1 and every 2 weeks after the surgical procedure until week-20, when the animals were sacrificed for morphological analysis. Results are presented as mean and standard error of the mean (SEM). N corresponds to the number of rats within the experimental group. Group 1: end-to-end reconstruction involved with a chitosan membrane type III (*End-to-EndChitIII*); Group 2: gap reconstructed using an autologous nerve graft involved with a chitosan membrane type III (*Graft180ºChitIII*); and Group 3: gap reconstructed with chitosan type III tube-guides (*GapChitIII*); These 3 groups were compared with 3 control groups, concerning, Group 4: gap reconstructed using an autologous nerve graft (*Graft180º*); Group 5: gap reconstructed with PLGA 90:10 tube-guides (*PLGA*); and Group 6: end-to-end reconstruction (*End-to-End*).

Week	0	1	2	4	6	8
End-to-end chit III Mean±SEM N =6	1.17±0.17	12.00±0.00	12.00±0.00	12.00±0.00	12.00±¨0.00	12.00±0.00
Graft 180º chit III Mean±SEM N = 6	1.00±0.00	12.00±0.00	12.00±0.00	12.00±0.00	12.00±0.00	12.00±0.00
Gap chit III Mean±SEM N = 6	1.17±0.17	12.00±0.00	12.00±0.00	12.00±0.00	12.00±0.00	12.00±0.00
Graft Mean±SEM N = 6	1.80±0.41	12.00±0.00	12.00±0.00	12.00±0.00	12.00±0.00	12.00±0.00
PLGA Mean±SEM N = 10	1.70±0.52	12.00±0.00	12.00±0.00	8.50±4.46	8.80±4.22	8.80±3.71
End-to-end Mean±SEM N = 7	2.30±0.95	12.00±0.00	7.60±4.31	7.00±4.12	8.00±3.20	3.40±1.27

Week	10	12	14	16	18	20
End-to-end chit III Mean±SEM N =6	7.50±0.43	4.67±0.56	3.83±0.54	3.17±0.54	2.83±0.17	2.50±0.34
Graft 180º chit III Mean±SEM N = 6	11.67±0.33	11.83±0.17	12.00±0.00	7.00±1.21	5.50±1.43	4.33±1.61
Gap chit III Mean±SEM N = 6	12.00±0.00	12.00±0.00	12.00±0.00	12.00±0.00	11.17±0.83	10.5±0.96
Graft Mean±SEM N = 6	6.70±2.94	5.50±2.35	4.00±1.55	3.80±1.60	3.33±0.42	3.33±1.21
PLGA Mean±SEM N = 10	5.50±4.46	3.70±3.08	6.70±3.33	5.80±4.12	5.30±1.86	3.80±1.72
End-to-end Mean±SEM N = 7	4.90±3.08	4.40±3.64	3.60±1.13	3.60±1.81	3.00±1.30	2.70±0.95

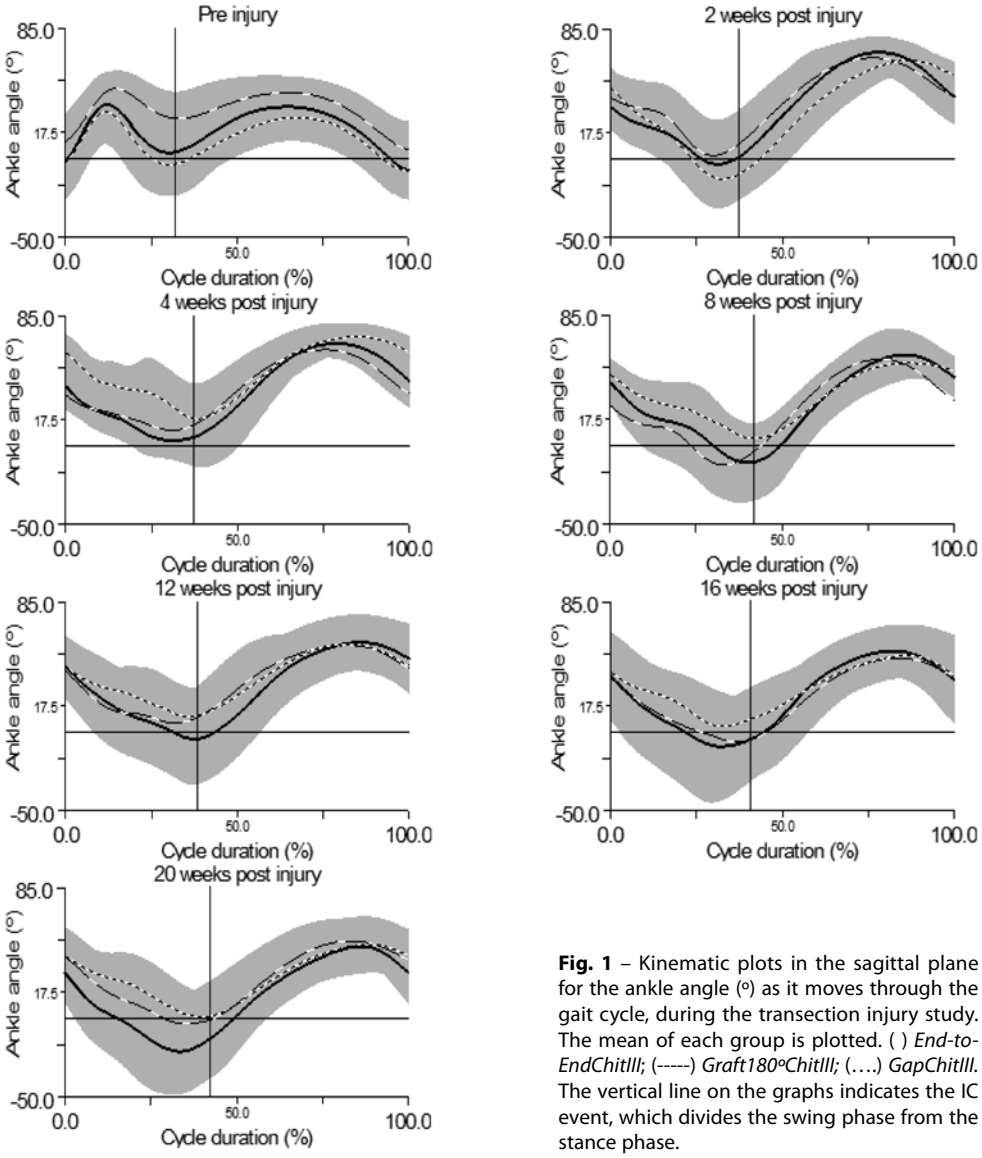

Fig. 1 – Kinematic plots in the sagittal plane for the ankle (°) as it moves through the gait cycle, during the transection injury study. The mean of each group is plotted. ( ) *End-to-EndChitIII*; (-----) *Graft180°ChitIII;* (….) *GapChitIII.* The vertical line on the graphs indicates the IC event, which divides the swing phase from the stance phase.

Peak velocities of ankle joint motion during stance were affected by sciatic injury in both plantarflexion [$F_{(6,84)} = 2.773$; $p = 0.016$] and dorsiflexion [$F_{(6,84)} = 8.867$; $p = 0.00$] directions. Peak plantarflexion velocity during stance recovered its normal values after 4 weeks of recovery while dorsiflexion peak velocity showed only incomplete recovery along the 20 weeks recovery (-395.2 ± 20.2°/s, -411.4 ± 19.1°/s, -450.3 ± 40.0°/s at week-0, for *End-to-EndChitIII*, *Graft180ChitIII* and *GapChitIII* groups, and -347.0 ± 58.6°/s, -217.6 ± 41.9°/s, -158.4 ± 24.6°/s at week-20 for *End-to-EndChitIII*,

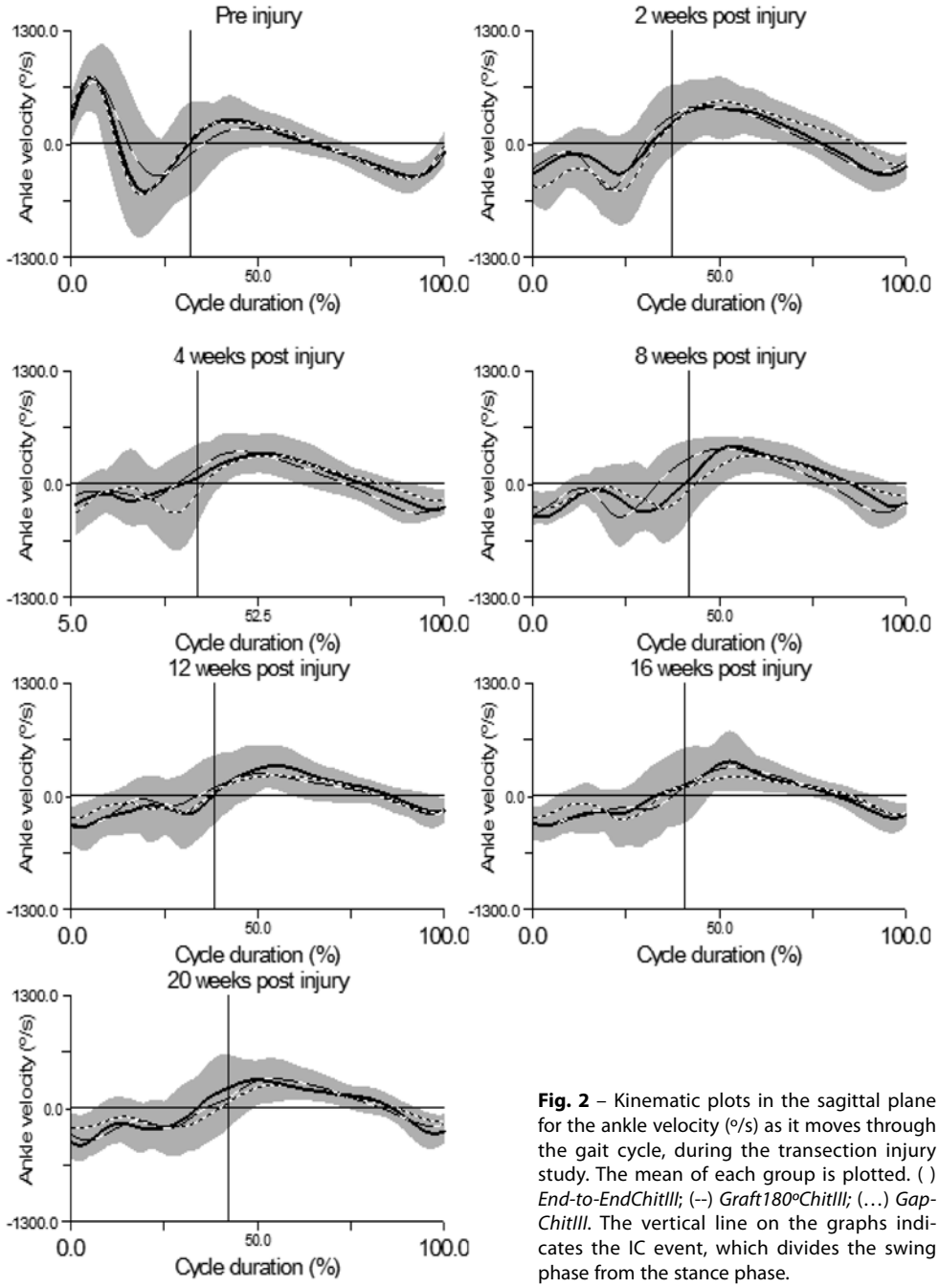

Fig. 2 – Kinematic plots in the sagittal plane for the ankle velocity (º/s) as it moves through the gait cycle, during the transection injury study. The mean of each group is plotted. ( ) *End-to-EndChitIII*; (--) *Graft180ºChitIII;* (…) *Gap-ChitIII*. The vertical line on the graphs indicates the IC event, which divides the swing phase from the stance phase.

Table 3 – Ankle kinematics and stance duration analysis were carried out prior to nerve injury (week-0), at week-2, and every 4 weeks during the 20-week follow-up period. Values of the ankle angular position (º) at initial contact (IC) and toe-off (TO) of the stance phase; midswing, and midstance. Results are presented as mean and standard error of the mean (SEM). All groups N = 6.

Temporal Parameter		Week 0	Week 2	Week 4	Week 8	Week 12	week 16	Week 20
IC	End-to-EndChitIII	-3.2±2.7	-10.1±7.1	-2.9±4.0	-10.3±7.8	-6.7±10.1	-11.0±10.5	-24.1±8.5
	Graft180ºChitIII	18.3±5.1	6.4±7.4	7.7±4.8	-9.5±3.5	0.7±3.9	-1.0±7.3	1.9±4.4
	GapChitIII	-8.6±6.4	-6.1±5.5	16.0±8.0	-2.1±11.9	3.4±3.6	-1.7±5.3	-1.4±4.6
TO	End-to-EndChitIII	-6.9±3.4	41.1±5.0	42.6±3.8	44.9±5.4	48.1±9.5	34.6±12.0	30.3±8.5
	Graft180ºChitIII	6.5±4.3	40.9±7.1	34.3±2.7	31.7±8.3	41.6±5.5	36.3±6.5	38.2±7.9
	GapChitIII	-9.6±7.0	54.4±2.9	61.2±3.9	47.3±11.0	43.5±6.8	37.7±3.9	41.3±7.3
Midswing	End-to-EndChitIII	40.3±3.8	19.6±6.3	20.7±4.8	14.7±12.6	17.2±12.2	4.0±14.4	-1.0±13.1
	Graft180ºChitIII	51.3±2.9	25.3±9.2	21.7±3.5	12.1±6.8	14.1±6.2	7.6±6.8	10.1±8.4
	GapChitIII	27.4±7.6	16.1±7.1	44.3±5.0	29.2±9.1	29.0±5.9	25.3±4.0	21.8±8.2
Midstance	End-to-EndChitIII	34.2±2.4	64.3±4.0	63.3±1.3	49.1±7.8	50.2±8.9	46.9±6.6	34.1±5.8
	Graft180ºChitIII	43.2±2.9	63.5±3.2	61.6±1.9	52.9±2.9	54.9±3.2	44.1±6.6	46.5±5.6
	GapChitIII	28.0±5.7	53.1±5.9	65.2±5.1	46.0±10.1	52.2±7.0	41.8±6.0	39.9±6.4

Table 4a – Ankle kinematics and stance duration analysis were carried out prior to nerve injury (week-0), at week-2, and every 4 weeks during the 20-week follow-up period. Values of the ankle angular velocity (º/sec) at initial contact (IC) and toe-off (TO) of the stance phase; midswing, peak value during dorsiflexion during swing phase; peak value during plantarflexion during swing phase. Results are presented as mean and standard error of the mean (SEM). N corresponds to the number of rats within the experimental group. All groups N = 6.

Temporal Parameter		Week 0	Week 2	Week 4	Week 8	Week 12	week 16	Week 20
IC	End-to-EndChitIII	90.2±67.5	354.6±108.5	230.5±132.0	314.1±166.6	266.5±157.2	470.1±196.5	350.9±167.9
	Graft180ºChitIII	99.3±33.9	429.9±113.0	274.8±41.6	351.6±55.9	338.9±56.3	489.6±45.9	476.0±30.3
	GapChitIII	156.9±25.7	424.7±39.9	248.7±56.0	167.4±69.7	207.0±53.2	244.8±65.7	266.3±69.4
TO	End-to-EndChitIII	-84.3±23.4	-257.1±39.7	-263.2±19.6	-218.0±60.3	-159.3±55.0	-208.1±50.5	-261.9±52.2
	Graft180ºChitIII	-91.1±27.0	-202.2±21.0	-223.4±21.2	-221.3±45.4	-177.3±37.4	-188.2±31.4	-184.3±36.0
	GapChitIII	-29.2±39.8	-241.0±19.9	-167.7±33.3	-121.9±30.0	-162.7±24.3	-186.2±35.8	-141.8±22.4
Midswing	End-to-EndChitIII	-479.9±172.4	-212.1±82.8	-72.0±75.4	-21.8±66.8	-110.0±79.0	-120.6±101.2	-160.5±89.9
	Graft180ºChitIII	-259.3±163.7	-400.3±111.8	-136.2±55.9	-127.6±33.4	-29.5±54.7	-132.3±56.7	-184.0±48.3
	GapChitIII	-634.8±54.5	-493.7±75.9	-91.7±57.2	11.3±56.7	-58.5±88.3	-114.6±43.5	-96.0±54.3
Peak value during dorsiflexion (-) during swing phase	End-to-EndChitIII	-1016.4±71.4	-821.9±57.7	-616.6±47.3	-702.5±101.7	-652.6±78.9	-543.9±56.3	-663.1±66.7
	Graft180ºChitIII	-786.4±55.9	-769.1±54.9	-521.7±53.6	-698.4±65.1	-576.4±60.1	-548.7±44.2	-588.3±79.5
	GapChitIII	-866.5±55.1	-922.5±54.8	-820.8±63.1	-712.8±94.0	-692.1±109.2	-636.7±78.1	-597.8±78.4
Peak value during plantarflexion (+) during swing phase	End-to-EndChitIII	954.2±50.1	307.9±66.6	288.1±88.3	401.6±128.7	367.3±95.9	519.4±152.9	417.6±93.0
	Graft180ºChitIII	912.1±82.3	396.6±112.4	235.4±38.5	321.6±53.6	320.1±54.1	480.2±44.3	442.6±37.5
	GapChitIII	865.5±45.3	405.3±32.8	339.8±87.0	206.2±56.1	281.2±28.3	271.0±50.6	265.6±66.5

Table 4b – Ankle kinematics and stance duration analysis were carried out prior to nerve injury (week-0), at week-2, and every 4 weeks during the 20-week follow-up period. Values of the ankle angular velocity (º/sec) at Midstance, peak value during dorsiflexion during stance phase; peak value during plantarflexion during stance phase. Results are presented as mean and standard error of the mean (SEM). N corresponds to the number of rats within the experimental group. All groups N = 6.

Temporal Parameter		Week 0	Week 2	Week 4	Week 8	Week 12	week 16	Week 20
Midstance	End-to-EndChitIII	-18.5±15.5	172.5±24.9	166.7±22.0	226.0±34.8	165.8±27.1	140.0±24.0	171.9±15.1
	Graft180ºChitIII	-67.4±26.3	96.9±42.1	65.7±29.0	119.4±24.6	100.2±40.7	120.9±30.8	115.4±45.2
	GapChitIII	-23.1±25.5	239.8±34.1	129.6±39.9	175.3±33.0	160.7±43.7	136.2±27.2	151.2±35.5
peak value during dorsiflexion (-) during stance phase	End-to-EndChitIII	-395.2±20.2	-437.2±59.8	-329.4±19.9	-285.9±57.4	-218.8±61.9	-278.7±71.5	-347.0±58.6
	Graft180ºChitIII	-411.5±19.1	-369.3±58.2	-371.0±39.1	-336.1±68.5	-227.7±37.6	-224.9±42.8	-217.6±41.9
	GapChitIII	-450.3±40.0	-256.6±22.1	-214.1±47.3	-211.7±51.0	-245.4±24.8	-224.6±44.7	-158.5±24.6
peak value during plantarflexion (+) during stance phase	End-to-EndChitIII	422.3±34.4	665.8±118.5	529.6±76.5	535.3±90.0	512.1±97.5	606.2±137.1	519.1±96.1
	Graft180ºChitIII	414.8±29.4	579.6±77.6	542.3±65.3	496.1±35.5	448.7±36.6	502.4±41.6	522.4±37.1
	GapChitIII	431.5±56.0	593.9±39.0	430.4±41.1	451.0±58.6	383.0±40.4	392.1±52.4	383.2±44.4

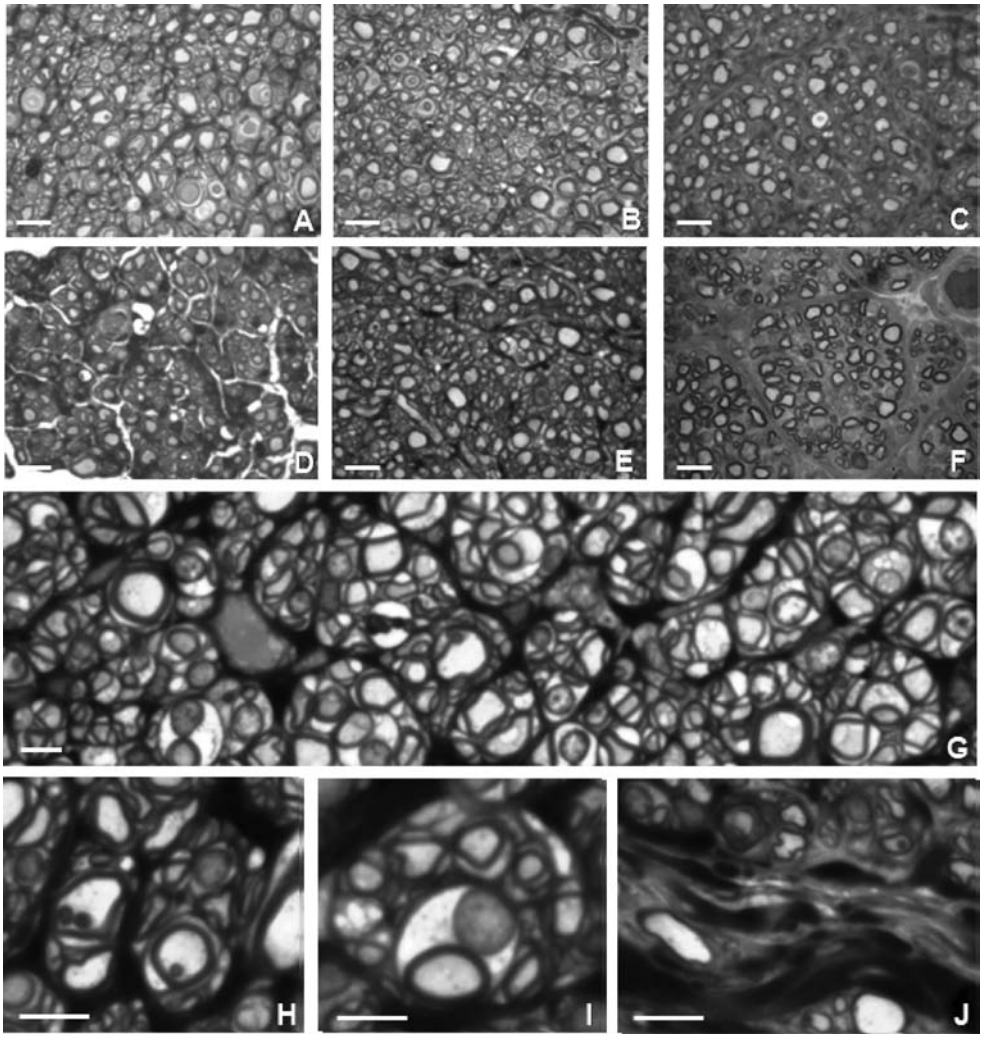

Fig. 3 –Light micrographs of Toluidine blue-stained sciatic nerve semi-thin sections from the 6 different experimental groups: *End-to-End* group (A) *Graft180º* (B), *EndChitIII* (C), *Graft180ºChitIII* (D), *GapChitIII* (E), *PLGA* (F). Figures G-J show higher resolution light micrographs of nerve fiber regeneration along chitosan type III tube guides disclosing the presence of a rich perineural connective architecture which contributes to axonal fasciculation. Scale bars: A-F = 10υm; G-J = 5υm.

Graft180ChitIII and *GapChitIII* groups) (Table 4b). No differences between groups were observed in peak velocity data.

Morphological Analysis

Representative high resolution light micrographs of semithin sections of regenerated sciatic nerves of the six groups are shown in Figures 3 A-F. A: *End-to-End;* B:

Graft180°; C: *End-to-EndChitIII*; D: *Graft180°ChitIII*; E: *GapChitIII*; F: *PLGA*. Axon regeneration occurred in all experimental groups with a good regeneration pattern with the presence of microfascicles typical of regenerated nerves. As expected, when comparing nerve fiber regeneration in the groups where a nerve gap was repaired with conduits, morphological analysis revealed a better pattern of regeneration in the *GapChitIII* (E) than in the *PLGA* (F) group, with the presence of larger axons and with a better anatomical organization of the nerve fascicles. Higher resolution light micrographs of nerve fiber regeneration along chitosan type III tube guides disclosed the presence of a rich perineurial connective architecture which contributes to nerve compartimentalization and axonal fasciculation. The formation of an adequate stroma inside the nerve regenerated along a non-nervous conduit is a positive predictor of successful recovery and is suitable to be attributed to the interaction of the chitosan matrix with the regenerating autologous tissues, especially the connective component.

Discussion

In the present study, we have used the rat sciatic nerve model for investigating the effects of chitosan type III membranes after neurotmesis followed by surgical repair either by direct suture, or autograft or tubulization. Since successful nerve regeneration should be paralleled by satisfactory functional recovery, we assessed thermal nociceptive function and motor function using specific behavioral tests. Both these functions showed good degree of recovery during the 20 weeks after the sciatic nerve transection in all experimental groups. Acutely after sciatic nerve transection there was a complete loss of both motor and thermal sensory function. Sensory and motor deficit then progressively decreased along the post-operative. Overall inter-group statistical comparison showed that, in comparison to the gold standard end-to-end direct suture, PLGA conduit group only presented worse recovery. In addition, we also performed kinematic analysis of the rat walk which is a more sensitive behavioral test and which is increasingly being used to assess functional recovery in peripheral nerve research because of its higher accuracy and better relationship with histological outcome (Luís et al., 2007, 2008). Locomotion is also of higher functional relevance since it involves integrated function of both the motor and sensory systems and their respective components, such as skeletal muscles, sensory endings, efferent and afferent nerve fibers and integrative centers within the central nervous system. Muscles innervated by sciatic nerve branches include both dorsiflexors and plantarflexors and, although in previous studies we focused our kinematic analysis only in the stance phase, we now prefer to include analysis of the ankle joint motion also during the swing phase in order to provide additional information. Almost all the kinematics parameters of ankle motion during the whole phase of the rat walk that we have analyzed in this study, revealed a persistent abnormal pattern, independently of type of treatment. Normal and abnormal patterns of ankle joint motion are clearly depicted in Figure 1. Acute changes are particularly evident during push off phase during stance, reflected in increased dorsiflexion at TO. During the swing phase, the normal biphasic motion pattern of dorsiflexion followed by plantarflexion is totally lost. In replace, the ankle seems to perform a steady plantarflexion movement throughout the whole swing phase time. With time, there is a limited recovery towards normality during stance phase with less pronounced dor-

siflexion at TO, suggesting that extensor muscles regain the ability to partially perform the push off action. However, the pattern of movement during the swing phase progressed without any evidence of improvement even until the end of the experiments at week-20. It should be noticed that walking requires fine coordination of limbs motion and definitely quadruped animals have many movement strategies to compensate for deficit in one of the hindlimbs. Through plasticity of integrative structures, animals may develop adaptive patterns that persist even if significant reinnervation takes place. Also, no direct relation exists between more simple tests of muscle strength or of sensory function and a complex action such as walking.

From a morphological point of view, our results showed that nerve regeneration occurred in all experimental groups and that, at time of withdrawal, Wallerian degeneration was almost completed and substituted by re-growing axons and the accompanying Schwann cells. Qualitative comparison between the different experiment groups showed that the axon regeneration pattern was similar in all groups with the exclusion of the *PLGA* where the regeneration pattern was worse.

The synergistic effect of a more favourable porous microstructure and physicochemical properties (more wettable and higher water uptake level) of chitosan type III compared to common chitosan, as well as the presence of silica ions, may be responsible for the good results in promoting post-traumatic nerve regeneration (Amado et al., 2008) suggesting that this material may not just work as a simple mechanical scaffold but instead may work as an inducer of nerve regeneration (Amado et al., 2008). The neuroregenerative property of chitosan type III might be explained by a direct stimulation of Schwann cell proliferation, axon elongation and myelination (Yuan et al., 2004; Shirosaki et al., 2005). Yet, the expression of established myelin genes such as PMP22, PO and MBP (Kuhn et al., 1993; Pietak et al., 2007) may be influenced by the presence of silica ions which exert an effect on several glycoprotein expression (Kuhn et al., 1993; Shirosaki et al., 2005; Pietak et al., 2007). Taken together, the results of this study support the view that hybrid chitosan type III membranes can be a valuable tool for fashioning nerve guides aimed to bridge nerve defects.

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