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Abstract

Objective. The objective was to study the effectiveness of a commercially available collagen conduit filled with fibrin-agarose hydrogels alone or with fibrin-agarose hydrogels containing autologous adipose-derived mesenchymal stem cells (ADMSCs) in a rat sciatic nerve injury model. *Approach*. A 10 mm gap was created in the sciatic nerve of 48 rats and repaired using saline-filled collagen conduits or collagen conduits filled with fibrin-agarose hydrogels alone (acellular conduits) or with hydrogels containing ADMSCs (ADMSC conduits). Nerve regeneration was assessed in clinical, electrophysiological and histological studies. *Main results*. Clinical and electrophysiological outcomes were more favorable with ADMSC conduits than with the acellular or saline conduits, evidencing a significant recovery of sensory and motor functions. Histological analysis showed that ADMSC conduits produce more effective nerve regeneration by Schwann cells, with higher remyelination and properly oriented axonal growth that reached the distal areas of the grafted conduits, and with intensely positive expressions of S100, neurofilament and laminin. Extracellular matrix was also more abundant and better organized around regenerated nerve tissues with ADMSC conduits than those with acellular or saline conduits. *Significance*. Clinical, electrophysiological and histological improvements obtained with tissue-engineered ADMSC conduits may contribute to enhancing axonal regeneration by Schwann cells.

(Some figures may appear in colour only in the online journal)

Introduction

The peripheral nervous system largely comprises peripheral nerves, which are complex histological structures mainly composed of neuron axons, Schwann cell-synthesized myelin sheaths and an organized extracellular matrix (ECM) (Geuna *et al* 2009).

Peripheral nerves connect the central nervous system to peripheral target organs of the motor and sensory pathways (Geuna *et al* 2009). These organs can be affected by various conditions and neuropathies that produce different degrees of axonal degeneration and demyelination (Geuna *et al* 2009), and their large extension means that they are vulnerable to damage from traumatic injury at any anatomic site. Surgery is the preferred treatment in short nerve defects, and autologous

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nerve grafting is the gold standard for more severe injuries. However, nerve autografts are not always clinically available, and they require a second operative procedure that may result in sensory loss at the donor site, where there is also a potential for neuroma formation and dysesthesia or anesthesia, besides scarring (Gu *et al* 2011, Kehoe *et al* 2012, Whitlock *et al* 2009, Wangensteen and Kalliainen 2010, Weber *et al* 2000). Furthermore, animal studies have found that motor regeneration is less well supported by sensory nerve autografts than by mixed sensorimotor nerves (Brenner *et al* 2006, Nichols *et al* 2004, Whitlock *et al* 2009).

Various types of nerve conduits based on different biomaterials have been shown to improve the outcomes of peripheral nerve defect repair surgery (Bell and Haycock 2012, Daly *et al* 2012, Gu *et al* 2011, Kehoe *et al* 2012, Nectow *et al* 2012, Subramanian *et al* 2009). In animal studies, these artificial nerve conduits enhanced and accelerated nerve regeneration and prevented excessive sprouting and the resulting development of neuroma (Belkas *et al* 2004, Bell and Haycock 2012, Daly *et al* 2012, Gu *et al* 2011, Nakayama *et al* 2007, Nectow *et al* 2012, Siemionow *et al* 2010).

Silicone is one of the most widely used synthetic conduit materials and can provide tubular support for nerve regeneration (Lundborg 1982, Penna *et al* 2011, Siemionow *et al* 2010). However, silicon conduits cannot be degraded or absorbed *in vivo* and can therefore trigger a foreign body reaction, fibrosis and nerve compression, requiring new surgery (Belkas *et al* 2004, Siemionow *et al* 2010). Various researchers have proposed the utilization of conduits based on synthetic biodegradable biomaterials (polyglycol acid, poly-lactic-co-glycolic acid, or polycaprolacton conduits), which prevent compression of the regenerating nerve, exert no adverse effect on axon regeneration and are effective for the repair of short nerve defects (Battiston *et al* 2005, Bell and Haycock 2012, Gu *et al* 2011, Siemionow *et al* 2010). Conduits formed of natural biomaterials, such as veins, arteries and skeletal muscle (Geuna *et al* 2003, Gu *et al* 2011, Siemionow *et al* 2010), have achieved less satisfactory outcomes than those obtained with autologous nerve grafting (Belkas *et al* 2004, Penna *et al* 2011, Nectow *et al* 2012). One of the most widely used nerve guides in the clinical setting is the saline-filled lyophilized type I collagen conduit (Kehoe *et al* 2012), which proved effective in rat and monkey models of nerve repair and is approved by the FDA for use in humans (Kehoe *et al* 2012, Wangensteen and Kalliainen 2010). They are porous, biocompatible, absorbable and safe, and a retrospective study showed that treatment with NeuraGen® (NG) conduits composed of purified bovine type I collagen was effective in 43% of cases (Wangensteen and Kalliainen 2010). However, reports on their effectiveness have been contradictory, and not all patients have experienced a good functional recovery, especially those with long or complex nerve defects (Bushnell *et al* 2008, Lohmeyer *et al* 2007, Pabari *et al* 2010, Wangensteen and Kalliainen 2010, Weber *et al* 2000).

Fibrin hydrogels are natural and biodegradable biomaterials that are widely used in tissue engineering and peripheral nerve regeneration (Kalbermatten *et al* 2009, Martin *et al* 2011, Nakayama *et al* 2007, Pettersson *et al* 2010). However, fibrin biomaterial has a low consistency and high compressibility, and surgical handling of the autologous-based scaffold is difficult (di Summa *et al* 2010, Ronfard *et al* 2000). Our group recently developed a novel biomaterial based on a mixture of agarose and human fibrin that proved effective for the tissue-engineered construction of artificial human corneas, oral mucosa and skin in the laboratory. Its good biocompatibility, biodegradability and physical properties have allowed its surgical implantation and *in vivo* analysis in animals (Alaminos *et al* 2006, 2007, Carriel *et al* 2012, Garzon *et al* 2009a, Martin *et al* 2011, Rodriguez *et al* 2012). The natural origin of this biomaterial provides an excellent environment for the growth of different cell types, including adipose tissue-derived stem cells, which could be differentiated *ex vivo* into different cell types, including those of neural lineage (Nieto-Aguilar *et al* 2011). However, the potential usefulness of this biomaterial for peripheral nerve regeneration has yet to be tested.

Given the variable outcomes of peripheral nerve regeneration with the use of biomaterials alone (Bell and Haycock 2012, Gu *et al* 2011), cell transplantation in a bioengineered conduit has been proposed as an alternative strategy (Bell and Haycock 2012, Daly *et al* 2012, Gu *et al* 2011, Nectow *et al* 2012). Promising results were obtained with the utilization of adipose-derived mesenchymal stem cells (ADMSCs) for this purpose (di Summa *et al* 2010, Lopatina *et al* 2011). The objective of the present study was to determine the usefulness of fibrin-agarose biomaterials and ADMSCs for peripheral nerve regeneration in a rat sciatic nerve injury model.

Materials and methods

Laboratory animals

Forty-eight 12-week-old male Wistar rats weighing 250–300 g at the beginning of the study were obtained from and maintained in the Experimental Unit of the Virgen de las Nieves University Hospital in Granada (Spain). They were housed in a temperature-controlled room (21 ± 1 °C) on a 12 h light/dark cycle with *ad libitum* access to tap water and standard rat chow. After the 12-week study period, animals were euthanatized under general anesthesia. The study was approved by the ethics committee of the University of Granada, Spain.

Experimental study groups

Animals were anesthetized with the intraperitoneal injection of a mixture of acepromazine (Calmo-Neosan® 0.001 mg per g of weight of the animal), ketamine (Imalgene 1000® 0.15 mg per g of weight) and atropine (0.05 μg of per g of weight). A 10 mm segment of the left sciatic nerve was then surgically excised from each animal, while the right sciatic nerve served as non-operated control. The animals were then randomly assigned to one of the following four groups ($n = 12$ in each): a negative control group (N-CTR), in which the nerve gap created was not surgically repaired; a positive

control group (P-CTR), in which microsurgical repair of the nerve defect was performed with a saline-filled commercially available absorbable collagen guide conduit (NeuraGen®, Integra® LifeSciences Corporation, Plainsboro, NJ, USA) widely used to repair peripheral nerve lesions (Wangensteen and Kalliainen 2010); an acellular fibrin-agarose hydrogel group (A-FAH), in which microsurgical repair was performed using a NeuraGen® collagen nerve conduit filled with acellular fibrin-agarose hydrogels; and a cellular fibrin-agarose hydrogel group (C-FAH), in which microsurgical repair was performed with a NeuraGen® collagen nerve conduit filled with fibrin-agarose hydrogels containing ADMSCs.

After the surgery and wound closure, all rats were kept in individual cages and received metamizole in the drinking water as analgesia. Animals were kept for 12 weeks before euthanasia. No antibiotics were used.

Primary cultures of ADMSCs

A 1 cm^3 biopsy of the inguinal pad fat was obtained from each animal under general anesthesia. In the laboratory, adipose tissue biopsies were washed in phosphate buffered saline (PBS) and mechanically fragmented into small pieces. Then, the tissue samples were enzymatically digested in 0.3% type I collagenase solution (Gibco BRL Life Technologies, Karlsruhe, Germany) for 8 h at 37 °C. Finally, isolated ADMSCs were harvested by centrifugation and cultured in basal medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Sigma-Aldrich Co.) and antibiotics-antimycotics (100 U mL^{-1} of penicillin G, 100 μg mL^{-1} of streptomycin and 0.25 μg mL^{-1} of B amphotericin). The culture medium was renewed every three days, and the cells were subcultured until passage 3.

Generation of fibrin-agarose hydrogel collagen conduits for nerve repair

Collagen conduits filled with fibrin-agarose hydrogels (for the A-FAH and C-FAH groups) were prepared as previously described (Alaminos *et al* 2006, 2007, Cardona Jde *et al* 2011, Carriel *et al* 2012, Garzon *et al* 2009a, 2009b, Gonzalez-Andrades *et al* 2009, Ionescu *et al* 2011, Martin *et al* 2011, Nieto-Aguilar *et al* 2011, Rodriguez *et al* 2012, Sanchez-Quevedo *et al* 2007). Briefly, 1 mL of mixture was prepared by adding 760 μL of human donor plasma to 125 μL DMEM, and 15 μL tranexamic acid (Amchafibrin, Fides-Ecofarma, Valencia, Spain) to avoid spontaneous fibrinolysis. Then, 50 μL 2% $CaCl_2$ were added to the solution to precipitate the polymerization reaction of the hydrogels and, in a final step, 50 μL of previously melted type VII-agarose (Sigma-Aldrich, Steinheim, Germany) dissolved in PBS were added. For the hydrogels containing cells (C-FAH group), the 125 μL DMEM added in step 2 contained 10 000 previously trypsinized autologous ADMSCs that always corresponded to passage 3.

Commercially available sterile NeuraGen® collagen guides (Integra® LifeSciences Corporation, Plainsboro, NJ, USA) were rapidly filled with the sterile hydrogels (with ADMSCs in C-FAH group and without in A-FAH group) and polymerized at 37 °C in 5% carbon dioxide for 24 h.

Clinical assessment

All animals in each experimental group were evaluated for sensory and motor functions by analyzing: (1) the presence of self-amputation of the toes of the operated leg (lack of 1–4 toes); (2) the presence of neurotrophic ulcers and their size ($\leqslant$2 mm/>2 mm); (3) the results of the pinch test of sensory recovery (also known as pinprick test); (4) the length of the plantar foot (in mm), an indicator of the neurogenic retraction of muscles innervated by the sciatic nerve; and (5) the results of the toe-spread test, an indicator of motor recovery.

For the pinch test of sensory recovery, standard forceps were used to apply a mild pinching stimulus to the skin of the left hind limb of each animal, from the toe to the knee joint, until a withdrawal reaction was observed. Care was taken not to damage the skin. The reaction to the painful stimulus perceived by the rat was graded on a four-point scale as previously described (Lubiatowski *et al* 2008, Siemionow *et al* 2011): 0 = no withdrawal response, 1 = response to stimulus above the ankle, 2 = response to stimulation distal to the ankle in the heel/plantar region, and 3 = response to stimulation in the metatarsal region.

Plantar foot length was measured by staining the control right hind foot with blue ink and the operated left hind foot with red ink so that the animals left distinct imprints when walking on white paper. The length was measured as the distance between the most proximal and the most distal part of each foot imprint (i.e. from tip of longest toe to heel).

The toe-spread test was performed as previously described (Siemionow *et al* 2011). Briefly, each animal was suspended by the tail and the extension and abduction reaction of the toes was examined. The results were graded on a four-point scale: 0 = no toe movement, 1 = some sign of toe movement, 2 = toe abduction, and 3 = toe abduction with extension.

All analyses were carried out by a single researcher (JGG) blinded to the group allocation of the animals.

Electrophysiological evaluation

At 12 weeks after the grafting, all animals underwent electrophysiological evaluation under mild anesthesia with ketamine and acepromazine to determine the spontaneous electrical activity of each muscle group. For each rat, the biceps femoralis, gastrocnemius, tibialis anterior and extensor digitorum brevis muscle groups were analyzed with concentric-needle single-fiber electromyography using a 1″ × 30 G needle and Topas 4-channel electromyograph (Schwarzer GmbH®, Munich, Germany) with band-pass filter settings of 5–5000 Hz. For each muscle group, three different measurements were made in three areas of each muscle group, scoring denervation and reinnervation signs on a four-point scale as 0 = absent (no signs in any of the 3 muscle areas), 1 = mild (signs in 1 of the 3 areas), 2 = moderate (signs in 2 areas), or 3 = severe (signs in all 3 areas). All analyses were carried out by a single researcher (JAS) blinded to the group allocation of the animals.

Table 1. Clinical results for each experimental group. The incidence of self-amputations, the presence of neurotrophic ulcers and the incidence of large neurotrophic ulcers (> 2 mm) are expressed as percentages. Foot length results are expressed as mean ± standard deviation (in millimeters), and pinch test of sensory recovery and toe spread test results as mean ± standard deviation (from 0 to 3).

Group	Self-amputations	Neurotrophic ulcers (presence)	Neurotrophic ulcers (>2 mm)	Foot length	Pinch test	Toe spread
Native ($n = 12$)	0.00%	0.00%	0.00%	36.56 ± 0.12 mm	3.00 ± 0.00	3.00 ± 0.00
N-CTR ($n = 12$)	50.00%	100.00%	25.00%	2.93 ± 0.23 mm	0.00 ± 0.00	0.00 ± 0.00
P-CTR ($n = 12$)	16.67%	58.33%	16.67%	14.86 ± 0.77 mm	0.33 ± 0.49	0.58 ± 0.51
A-FAH ($n = 12$)	8.33%[a]	33.33%[a,b]	8.33%[a]	14.93 ± 2.87 mm[a]	0.63 ± 0.50	1.27 ± 0.47[a,b]
C-FAH ($n = 12$)	0.00%[a,b]	8.33%[a,b]	0.00%[a,b]	27.12 ± 0.94 mm[a,b]	1.70 ± 0.67[a,b]	2.00 ± 0.47[a,b]

[a] significant difference with N-CTR group.

[b] significant difference with P-CTR group. Native = non-operated (right) sciatic nerve; N-CTR = negative control, P-CTR = positive control. A-FAH = acellular fibrin-agarose hydrogels, C-FAH = cellular fibrin-agarose hydrogels.

Histology

Normal sciatic nerves and grafted conduits were fixed in 10% buffered formalin for 24 h and embedded in paraffin. All samples were cut in 5 μm thick sections for morphological, histochemical and immunohistochemical analyses. Morphological pattern, myelin sheath and stromal collagen fibers were simultaneously evaluated by using the MCOLL histochemical method as previously described (Carriel *et al* 2011a). Samples from each study group were analyzed in cross sections ($n = 4$) and longitudinal sections ($n = 4$).

For immunohistochemical analysis, paraffin-embedded tissue sections were dewaxed, hydrated and heat-treated at 95 °C for 25 min in citrate buffer (pH 6) for antigenic unmasking; for laminin detection, samples were pretreated with pepsin solution (DakoCytomation, Glostrup, Denmark) for 5 min at 37 °C after the heat treatment. For the blockade of endogenous peroxidase activity, all sections were incubated with 3% (v/v) H_2O_2 in 0.1 M PBS for 10 min. Nonspecific staining was blocked with Cas-Block solution (Invitrogen, Carlsbad, CA, USA) for 15 min. Samples were incubated with the primary antibodies diluted in PBS for 1 h at room temperature, rinsed in PBS and then incubated for 30 min with secondary anti-mouse or anti-rabbit antibody. The peroxidase reaction was visualized using AEC and DAB (Vector, Burlingame, CA, USA) and contrasted with Harris hematoxylin. For each immunohistochemical reaction, controls were performed by omitting the primary antibody. The antibodies used in this study were rabbit polyclonal anti-S100 at 1/400 dilution (DakoCytomation, Glostrup, Denmark), mouse monoclonal anti-neurofilament 160/200 clone RMdO20 at 1/500 dilution, and mouse monoclonal anti-laminin clone LAM-89 at 1/1000 dilution (Sigma-Aldrich, Steinheim, Germany). Horse polyclonal anti-mouse IgG and anti-rabbit IgG were used as secondary antibodies (Vector Laboratories, Burlingame, CA, USA).

For the study of semithin tissue sections, samples ($n = 4$ from each group) were fixed in 2.5% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) at 4 °C for 6 h and then washed twice in 0.05 M cacodylate buffer (pH 7.2) at 4 °C. Next, the tissues were embedded in epoxy resin, and 1 μm semithin sections were obtained using an Ultracut Reichter. Sections were then stained with toluidine blue for myelin evaluation by light microscopy.

In each group of samples, the intensity of the histochemical reactions (for collagen fibers and myelin sheath) and the immunohistochemical reactions (expression of S-100, neurofilament and laminin) was quantified in red, green and blue (RGB) channels with Image J software (National Institute of Health, USA), as previously described (Carriel *et al* 2011b). Briefly, in each histological section, 40 points were randomly selected in regions with positive reactions for each method, and the reaction intensity was determined in the RGB channels and corrected by subtracting the blank level. Finally, the mean intensity value was calculated for each variable and each group of samples. In addition, for each histochemical and immunohistochemical technique, the area fraction corresponding to the positive signal was determined by using Image J software.

Statistical analysis

Fisher's exact test was used to compare the percentages of animals showing self-amputation or neurotrophic ulcer, and the Mann–Whitney non-parametric test was used to compare foot length, pinch test of sensory recovery, toe-spread test and histological (intensity and area fraction) results among the study groups. SPSS 16.00 was used for all data analyses. $P < 0.05$ was considered statistically significant in two-tailed tests.

Results

Clinical results

Clinical results are given in table 1. Self-amputations were recorded in 50% of the N-CTR group (no repair treatment) <17% of the P-CTR group (saline-filled conduit), <10% of the A-FAH group (conduit with acellular fibrin-agarose hydrogels), and none of the animals in the C-FAH group (conduit with cellular fibrin-agarose hydrogels containing AMSCs). Statistically significant differences were found between N-CTR and A-FAH groups, N-CTR and C-FAH groups, P-CTR and C-FAH groups, and A-FAH and C-FAH groups but not between P-CTR and A-FAH groups.

Neurotrophic ulcers were observed in 100% of the N-CTR group and in 8.33% of the C-FAH group. Significant differences in the presence of ulcers were found between N-CTR and A-FAH groups, N-CTR and C-FAH groups ($p < 0.001$ for both comparisons), P-CTR and A-FAH groups,

Table 2. Electrophysiological analysis results for each experimental group, including the percentage of animals with specific denervation or reinnervation signs (scored as 0, 1, 2 or 3) in each of the four muscle groups. In each experimental group ($n = 12$), three independent measurements per muscle group were performed in each animal ($n = 36$ determinations per muscle group).

Group	Muscles	Denervation 0	1	2	3	Reinnervation 0	1	2	3
N-CTR ($n = 12$)	Biceps femoralis	0.0	0.0	75.0	25.0	75.0	25.0	0.0	0.0
	Gastrocnemius	0.0	0.0	0.0	100.0	100.0	0.0	0.0	0.0
	Tibialis anterior	0.0	0.0	0.0	100.0	100.0	0.0	0.0	0.0
	Extensor digitorum brevis	0.0	0.0	0.0	100.0	100.0	0.0	0.0	0.0
P-CTR ($n = 12$)	Biceps femoralis	0.0	50.0	41.7	8.3	0.0	8.3	91.7	0.0
	Gastrocnemius	0.0	16.7	75.0	8.3	41.7	58.3	0.0	0.0
	Tibialis anterior	0.0	8.3	50.0	41.7	100.0	0.0	0.0	0.0
	Extensor digitorum brevis	0.0	0.0	8.3	91.7	100.0	0.0	0.0	0.0
A-FAH ($n = 12$)	Biceps femoralis[a,c,d]	18.2	63.6	18.2	0.0	0.0	9.1	90.9	0.0
	Gastrocnemius[a,b,d,e]	0.0	81.8	18.2	0.0	0.0	63.6	36.4	0.0
	Tibialis anterior[a]	0.0	9.1	72.7	18.2	63.6	36.4	0.0	0.0
	Extensor digitorum brevis	0.0	0.0	9.1	90.9	100.0	0.0	0.0	0.0
C-FAH ($n = 12$)	Biceps femoralis[a,b,c,d,e]	100.0	0.0	0.0	0.0	0.0	10.0	80.0	10.0
	Gastrocnemius[a,b,d,e]	30.0	60.0	10.0	0.0	0.0	70.0	30.0	0.0
	Tibialis anterior[a]	0.0	30.0	50.0	20.0	70.0	20.0	10.0	0.0
	Extensor digitorum brevis	0.0	0.0	30.0	70.0	90.0	10.0	0.0	0.0

[a] significant difference in denervation results with N-CTR group.
[b] significant difference in denervation results with P-CTR group.
[c] significant difference in denervation results between A-FAH and C-FAH groups.
[d] significant difference in reinnervation results with N-CTR group.
[e] significant difference in reinnervation results with P-CTR group. No significant differences in reinnervation were found between A-FAH and C-FAH groups.

P-CTR and C-FAH groups, and A-FAH and C-FAH groups ($p < 0.05$ for all). Large neurotrophic ulcers ($\geqslant 2$ mm) were found in 25% of the N-CTR group, whereas all ulcers were <2 mm in the C-FAH group.

Foot length was significantly shorter in the N-CTR group than in the A-FAH and C-FAH groups ($p < 0.05$, Mann–Whitney test) and significantly shorter in the P-CTR group than in the C-FAH group ($p < 0.05$), but it was similar between the P-CTR and A-FAH groups ($p > 0.05$). The mean length was significantly longer in the untreated group (36.56 ± 0.12 mm) than in any of the treated groups.

Lowest scores for the pinch test of sensory recovery and the toe spread tests were found in the N-CTR group (0 for both tests) and the highest in the C-FAH group, while the mean scores were higher in the A-FAH group than in the P-CTR group. Statistically significant differences were found for toe-spread results in the comparisons of N-CTR with A-FAH and C-FAH groups, P-CTR with A-FAH and C-FAH groups, and A-FAH with C-FAH groups, and for the pinch test of sensory recovery results in the comparisons of the C-FAH group with the N-CTR, P-CTR and A-FAH groups.

Electrophysiological analysis

Electromyography results showed a wide variation in denervation and reinnervation signs among the muscle groups, with higher muscle reinnervation and lower denervation potentials in groups more proximal to the lesion site. As shown in table 2, moderate or severe levels of muscle denervation were observed in 100% of the N-CTR group, with slight or absent signs of reinnervation in the most proximal muscle (biceps femoralis), and severe denervation and absence of

reinnervation in all distal muscles (gastrocnemius, tibialis anterior and extensor digitorum brevis). Some signs of muscle reinnervation were observed in the P-CTR group, which showed lesser denervation than in the N-CTR group, and the differences between these groups were significant for the biceps femoralis and gastrocnemius muscles but not for the extensor digitorum brevis muscle. More than 18% of the A-FAH group showed no denervation signs in the biceps femoralis, and more than 90% showed moderate signs of reinnervation signs in this muscle, and the differences with the N-CTR results for this muscle were statistically significant. No denervation potentials were observed in the biceps femoralis muscle of the C-FAH group (score of 0 in all animals), and scores of 0 or 1 were recorded in the gastrocnemius muscle of 90% of the group, with a higher denervation level in the more distal muscle groups. Intense reinnervation was observed in the biceps femoralis of 90% of the C-FAH group and in the gastrocnemius muscle of 30% of this group (scores 2 and 3). These results were significantly superior to those obtained in the N-CTR group for both muscles and to those obtained in the A-FAH group for the biceps femoralis muscle. No intergroup differences were found in values for the extensor digitorum brevis muscle (table 2).

Histological analyses

According to the histochemical analysis, nerve regeneration characteristics varied widely among the study groups. *In the normal control nerve* (right foot), the MCOLL histochemical study showed a highly organized axonal pattern, with a typical undulated and parallel organization of nerve fibers

surrounded by a clearly identifiable myelin sheath and thin collagen endoneural tube (figure 1(A)). *In the P-CTR group*, it revealed a regeneration cone (composed of several fascicles of cells and an ECM resembling bands of Büngner) in the proximal area that did not reach the central or distal areas of the conduits (figure 1(B)); some fascicles were observed in the central area, but there were considerably fewer in the distal area. The regeneration process was highly disorganized in this group, with a random distribution of regenerated nerve fascicles in the conduit that were surrounded by disorganized collagen fibers under both light and polarized microscopy (figure 1(B)). The intensity of the myelin histochemical reaction in the fascicles was very weak in the regeneration cone and in central and distal areas of the grafted conduit (figure 1(B)). These results are consistent with the images obtained from semi-thin cross-sections stained with toluidine blue (figure 2(A)). *In the A-FAH group,* the nerve regeneration was more abundant and organized than in the P-CTR group; the regeneration process reached the central area of the conduit, and numerous regenerated fascicles were observed in the distal area (figure 1(C)). Furthermore, the collagen fiber reaction was significantly more intense and organized than in the P-CTR group (figure 1(C) and table 3). The remyelination was more abundant and more widely distributed than in the P-CTR group according to the myelin-positive area fraction, although the difference in the intensity of the reaction was not significant (table 3). *In the C-FAH group*, the neural regeneration reached the distal area of the conduit, where numerous regenerated nerve fascicles were observed. The structure and organization of the regeneration process was more organized, compact and uniform than in any other study group (figure 1(D)). Light and polarized microscopy studies of the ECM showed a significantly higher collagen fiber reaction intensity in the C-FAH group than in the P-CTR and A-FAH groups (table 3); the fibers were correctly oriented and highly organized around the regenerated nerve fascicles in the proximal, central and distal areas of the conduits (figure 1(D)). Histochemical study revealed a markedly higher remyelination in the C-FAH group than in the other groups, and the area fraction of the positive histochemical reaction for myelin was significantly larger in longitudinal (figure 1(D)) and cross-sectional evaluations (figure 2) in comparison to the P-CTR group. The histological analysis also revealed an evident revascularization (composed of capillaries, arterioles, venules and veins) closely associated with the peripheral nerve regeneration in the proximal, central and distal areas of these conduits. In each study group, the revascularization findings were directly related to the degree of nerve regeneration (figures 1 and 2).

Immunohistochemical S100 expression results varied among the groups: *the normal control nerve* showed intense positivity with a regular pattern for Schwann cells surrounding the axons (figure 3(A)); *in the P-CTR grou*p, the expression was positive for fascicles in proximal (regeneration cone) and central areas but markedly lower in the distal area of the graft (figure 3(B)); *in the A-FAH group,* S100 protein expression was slightly but not significantly more intense and organized than in the P-CTR group in the proximal, central and distal

areas of the conduit (figure 3(C)); *in the C-FAH group*, the expression showed a regular and organized pattern and was significantly more intense and abundant (higher intensity and area fraction) than in the other study groups, and the intensity and organization of expression in the central and distal areas were similar to those in the proximal area (figure 3(D)).

Immunohistochemical neurofilament expression results were as follows: *in the normal control nerve*, positive expression was detected in all axons, with a regular distribution (figure 4(A)); *in the P-CTR group*, positive expression was observed for nerve fascicles in the proximal area but was markedly reduced in the central and distal areas (figure 4(B)); *in the A-FAH group*, expression was moderately positive in the proximal area, with a slight decrease in the central and distal areas (figure 4(C)), and the differences with the P-CTR group were statistically significant (table 3); and *in the C-FAH group*, an intense and abundant positive expression was found in the fascicles, revealing a highly organized linear and parallel pattern that was not observed in the P-CTR or A-FAH groups. The immunostaining was significantly more abundant and intense in the C-FAH group than in the other study groups, and no differences were found among the proximal, central and distal areas of the conduit (figure 4(D) and table 3).

Laminin expression results were as follows: *the normal control nerve*, specific staining of the thin basal lamina (endoneural tube) was observed, with a regular and parallel distribution (figure 5(A)); *in the P-CTR group*, positive expression was detected in the few fascicles observed in the proximal and central portions of the graft and was markedly reduced in the distal area (figure 5(B)); *in the A-FAH group*, laminin expression was significantly more abundant than in the P-CTR group in the proximal, central and distal areas, showing the same pattern as observed in the histological analysis (figure 5(C)); and *in the C-FAH group*, the expression of laminin was significantly more abundant, intense, regular and organized in all fascicles in comparison to the other study groups (figure 5(D)).

Discussion

Peripheral nerve injuries are attracting increasing research interest due to their high incidence and the limited capability of the human nerve to spontaneously regenerate. Injuries producing a long gap between nerve ends are very difficult to treat. The limitations of available therapies, including nerve autografting, the gold standard, are driving research into the development of alternative approaches for peripheral nerve repair (Kehoe *et al* 2012).

This study tested the capability of two new nerve guide conduits to repair a 10 mm gap in a sciatic nerve rat model. Clinical, functional and histological outcomes at 12 weeks were superior with the use of collagen guides containing biocompatible fibrin-agarose hydrogels (A-FAH group) to those obtained with collagen guides containing heparinized saline (P-CTR), especially when the fibrin-agarose hydrogels were impregnated with ADMSCs (C-FAH).

Both groups in which fibrin-agarose hydrogels were used (A-FAH and C-FAH) had a significantly lower rate
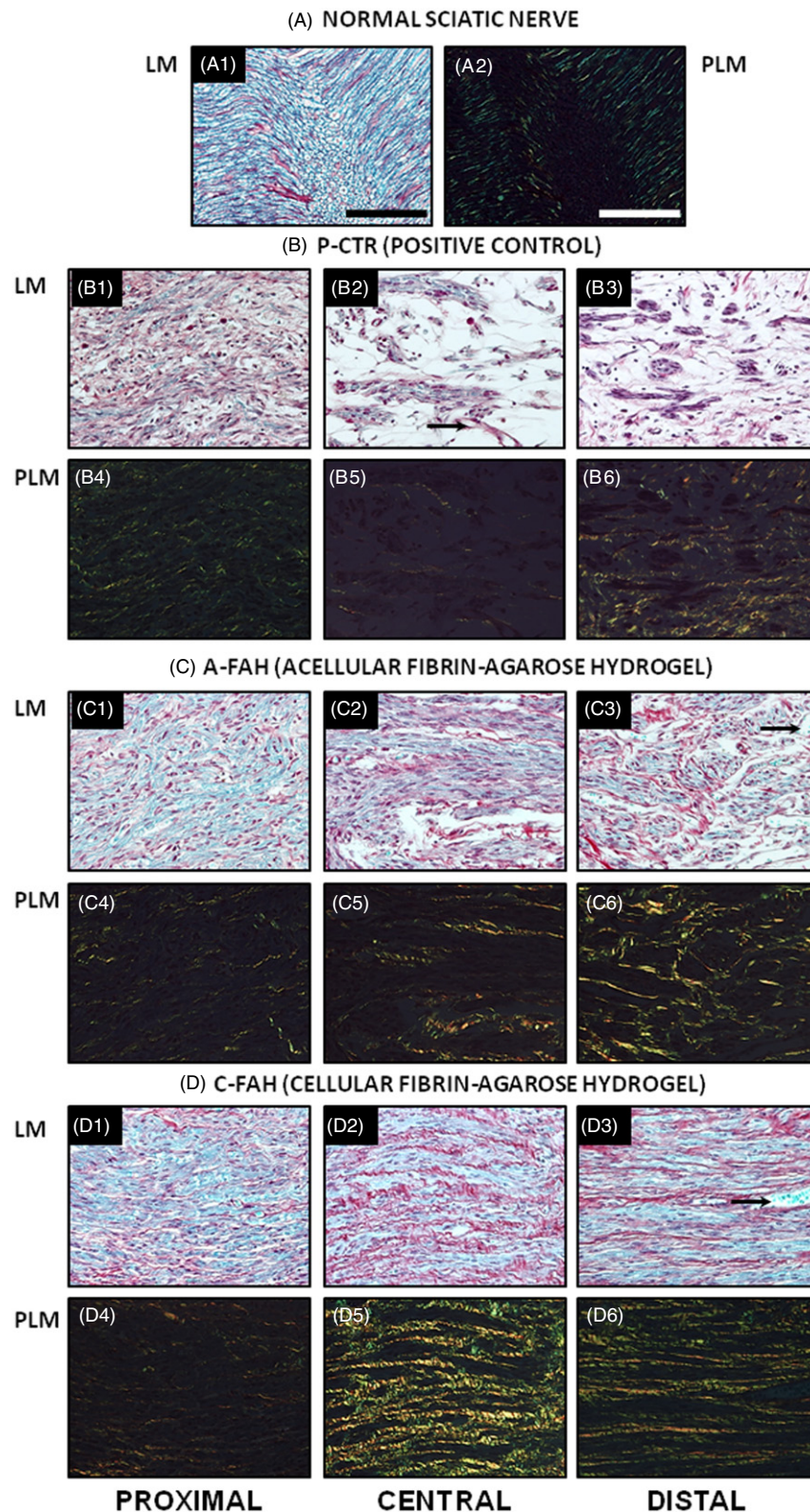
Figure 1. Longitudinal evaluation of samples stained with MCOLL histochemical method. (A) Normal sciatic nerve (unoperated) with intense histochemical reaction for myelin (in blue) surrounded by organized thin collagen fibers (in red) (A1), with a weak birefringence under polarized microscopy (A2). (B) Longitudinal evaluation of positive control group (P-CTR), showing a decrease in histochemical reaction for myelin and collagen fibers from the proximal to distal areas of the conduit. (C) Acellular fibrin-agarose hydrogel group (A-FAH), showing a positive histochemical reaction for myelin and collagen fibers in randomly organized fascicles throughout the grafted conduit. (D) Cellular fibrin-agarose hydrogel group (C-FAH), where it is possible to observe the abundant and organized nerve regeneration from the proximal to the distal areas of the conduit. LM = light microscopy; PLM = polarized light microscopy. Arrows indicate some blood vessels. Scale bar = 100 μm.
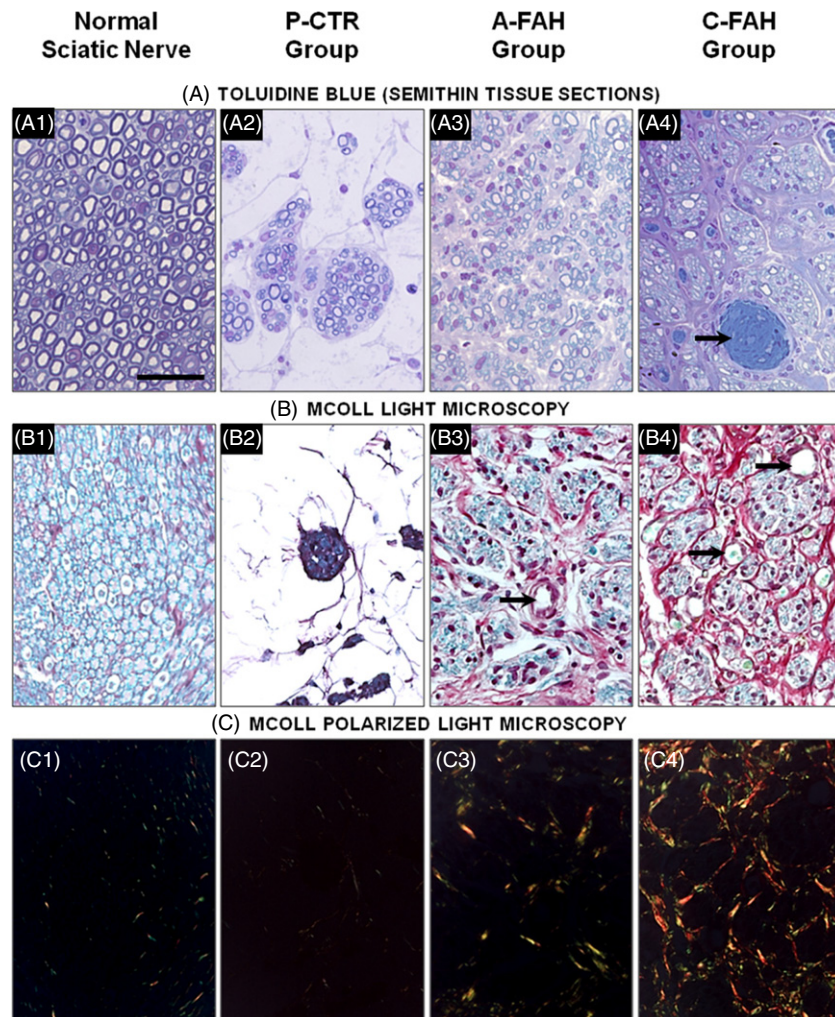
Figure 2. Cross evaluation of samples stained with toluidine blue and MCOLL. Semithin sections stained with toluidine blue (A) and histological sections stained with MCOLL histochemical method under light (B) and polarized microscopy (C), taken from the normal sciatic nerve (A1, B1, C1), and from the middle portion of the conduits corresponding to the P-CTR group (A2, B2, C2), A-FAH group (A3, B3, C3) and C-FAH group (A4, B4, C4). Both methods (A, B) show higher density of the remyelinated nerve fibers in the C-FAH and A-FAH groups in comparison to the P-CTR group. Note the intense histochemical reaction (B4) and birefringence (C4) of the collagen fibers around the regenerated nerve fascicles in the C-FAH group. Arrows indicate some blood vessels. Scale bar = 50 μm.

of self-amputations and neurotrophic ulcers and showed a better response to the pinch test of sensory recovery (lower frequency of sensory alterations in the affected limb) in comparison to the group treated with saline-filled conduits. The improvement was even greater when the fibrin-agarose hydrogels contained ADMSCs (C-FAH group). These findings suggest that the conduits with hydrogels, especially those containing ADMSCs, may be of potential clinical value for improving the sensory functioning of patients with this type of nerve injury.

Analysis of the motor function of the animals showed significantly superior foot length and toe-spread test results when the hydrogels with ADMSCs were used to fill the conduit rather than heparinized saline. Sciatic nerve lesions are associated with muscle dysfunction, limb deformities and alterations in the use of limbs, hampering walking and reflected in foot length alterations (Kim *et al* 2007). Although the length of the unoperated control right foot was not achieved, these results indicate that the motor dysfunction

and neurogenic muscle retraction were reduced in the C-FAH group. Moreover, electrophysiological analyses revealed a markedly lower degree of muscle denervation and an enhanced reinnervation with the use of autologous ADMSCs. This finding indicates the ability of the grafted cellular biomaterial to prevent muscle denervation after nerve injury and to induce rapid reinnervation. These clinical results are in agreement with previous *in vivo* findings on the positive impact of conduits filled with intra-luminal biomaterials and/or cells in comparison to hollow conduits (Huang *et al* 2012, Koh *et al* 2010, Rutkowski *et al* 2004, Wei *et al* 2011). In some cases, the results of these novel strategies have been comparable to those obtained with autografts (Koh *et al* 2010, Xu *et al* 2011).

Histological studies are essential to evaluate the presence of axonal sprouting, remyelination and extracellular matrix remodeling in peripheral nerve injuries and associated regeneration (Carriel *et al* 2011a, Geuna *et al* 2009, Vleggeert-Lankamp 2007). In the present investigation, they revealed wide variations among our study groups in the morphology

Table 3. Quantitative histological analysis of the nerve conduits implanted *in vivo*. For each histochemical and immunohistochemical method, the intensity and the area fraction corresponding to the positive signal are shown as mean $\pm$ standard deviation.

| | Immunohistochemistry | | | | | | Histochemistry | | | |
| | S-100 | | Neurofilament | | Laminin | | Collagen | | Myelin | |
Groups	Intensity	Area fraction	Intensity	Area fraction	Intensity	Area fraction	Intensity	Area fraction	Intensity	Area fraction
P-CTR ($n = 8$)	108.83 ± 29.33	14.24 ± 6.88	115.56 ± 28.27	5.43 ± 3.01	110.59 ± 26.62	10.91 ± 0.4	83.78 ± 0.27	1.90 ± 2.21	80.77 ± 19.3	4.03 ± 3.88
A-FAH ($n = 8$)	110.49 ± 29.00^b	18.31 ± 7.51^b	$142.43 \pm 28.52^{a,b}$	5.01 ± 2.02^b	116.49 ± 27.22^b	$15.68 \pm 2.63^{a,b}$	$104.70 \pm 30.27^{a,b}$	7.87 ± 6.00	77.23 ± 19.53	20.66 ± 20.02^a
C-FAH ($n = 8$)	$143.74 \pm 28.19^{a,b}$	$25.72 \pm 6.76^{a,b}$	$162.48 \pm 28.48^{a,b}$	$22.69 \pm 2.01^{a,b}$	$159.23 \pm 26.54^{a,b}$	$25.74 \pm 3.52^{a,b}$	$152.76 \pm 30.36^{a,b}$	14.63 ± 4.29	79.53 ± 19.62	33.53 ± 19.53^a

[a] significant difference with P-CTR group.

[b] significant difference between A-FAH and C-FAH groups.
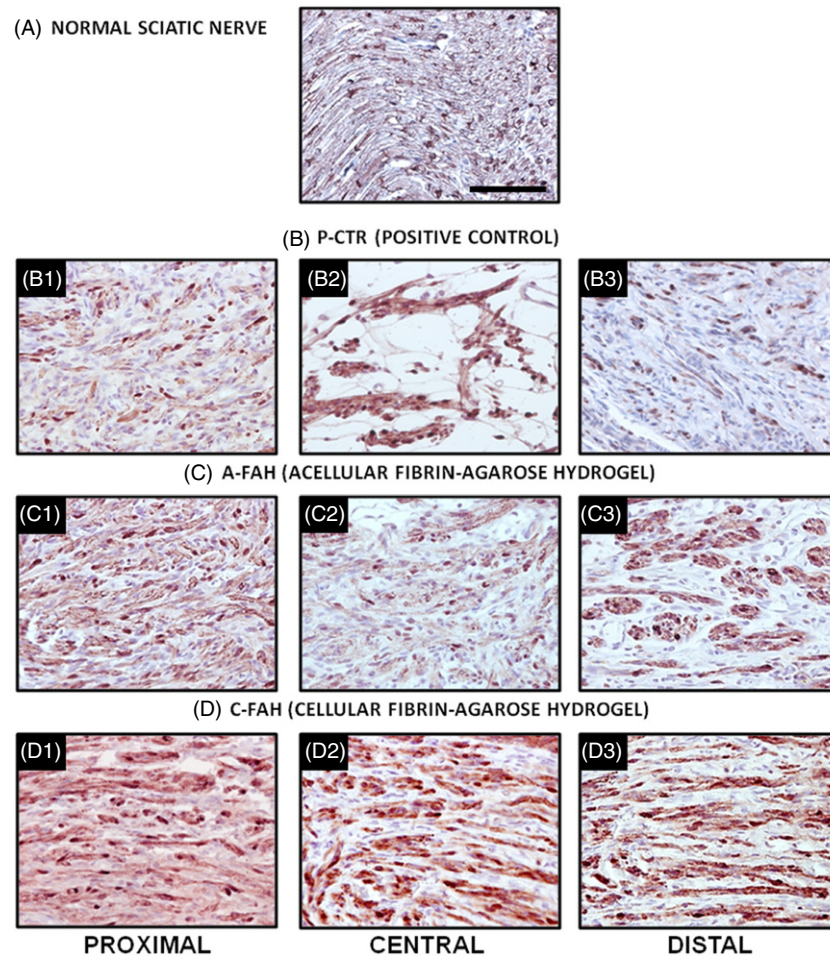
Figure 3. Light microscopy analysis of Schwann cells by S-100 immunostaining. (A) Normal sciatic nerve, (B) P-CTR group, (C) A-FAH group and (D) C-FAH. 1: Proximal area, 2: central area and 3: distal area of the grafted conduits. Scale bar = 100 μm.

and organization of the regenerating tissues. Although there was a certain degree of nerve regeneration at the 10-mm-long sciatic nerve defect in all groups, it was significantly greater in the groups repaired with fibrin-agarose biomaterials and even more so when these materials were combined with autologous ADMSCs (C-FAH group). These histological results were consistent with the clinical findings.

The ECM plays an important role during peripheral nerve development (Chernousov *et al* 2008), and artificial conduits filled with biomaterials that mimic the structure and function of the native ECM promote peripheral nerve repair (Bell and Haycock 2012, Daly *et al* 2012, Gu *et al* 2011, Nakayama *et al* 2007, Nectow *et al* 2012, Siemionow *et al* 2010). *In vitro* and *in vivo* studies have suggested that the use of highly aligned biomaterials may help to guide the axonal outgrowth and accelerate nerve regeneration (Bell and Haycock 2012, Cao *et al* 2011, Huang *et al* 2012, Nectow *et al* 2012, Wang *et al* 2009). In the present study, collagen conduits filled with heparinized saline (P-CTR group) demonstrated regeneration potential in proximal areas of the grafted material, but few fascicles reached central and distal areas. The histology study revealed that the regenerating tissues were not properly organized in this group, with a significantly lower area fraction of myelinated tissue in comparison to the other groups and

lesser positive expression of S100 protein, neurofilament and laminin. The ECM of regenerating tissues in this group (P-CTR) was characterized by a poor and disorganized collagen mesh, confirming the incomplete nature of the regeneration process. The worse clinical outcomes obtained in the P-CTR group support the conclusions of other authors that hollow conduits offer poorer support for nerve regeneration in comparison to those filled with biomaterials (Cao *et al* 2011, Koh *et al* 2010, Yao *et al* 2010).

Some authors suggested that multichannel collagen conduits have a more favorable impact on nerve regeneration in comparison to hollow collagen conduits (Yao *et al* 2010). Other types of conduits made of synthetic biomaterials, such as poly-glycolic acid or poly-lactic acid, did not improve on the results of type-I collagen conduits (Hernandez-Cortes *et al* 2010, Meek and Coert 2008, Pabari *et al* 2010, Shin *et al* 2009). It was recently demonstrated that the use of novel electrospun PCL/collagen conduits (poly ε-caprolactone/collagen type I) may support nerve regeneration and functional recovery at 20 weeks after surgery (Lee *et al* 2012). However, the therapeutic usefulness of bioengineered nerve conduits remains controversial (Bell and Haycock 2012, Daly *et al* 2012, Kehoe *et al* 2012, Yao *et al* 2010).
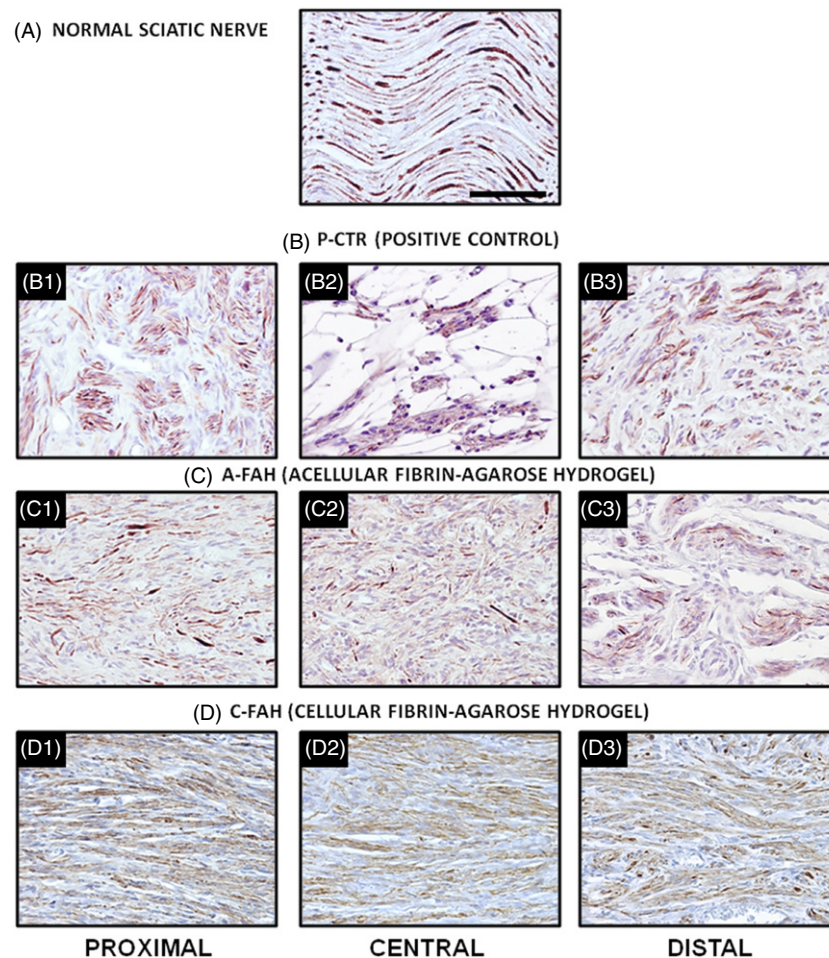
Figure 4. Light microscopy analysis of axons by neurofilament immunostaining. (A) Normal sciatic nerve, (B) P-CTR group, (C) A-FAH group and (D) C-FAH. 1: Proximal area, 2: central area and 3: distal area of the grafted conduits. Scale bar = 100 μm.

Biomaterials should offer low antigenicity, revascularization support and porosity for oxygen diffusion, and they should not cause nerve compression (Siemionow *et al* 2010). Various studies have found the use of natural biodegradable biomaterials to be preferable (Bell and Haycock 2012, Daly *et al* 2012, Gu *et al* 2011, Siemionow *et al* 2010). Fibrin-agarose hydrogels are highly biocompatible and biodegradable, with adequate consistence, transparency and rheological properties (Alaminos *et al* 2006, Cardona Jde *et al* 2011, Carriel *et al* 2012, Garzon *et al* 2009a, Gonzalez-Andrades *et al* 2009, Ionescu *et al* 2011, Rodriguez *et al* 2012). The present results indicate the fibrin-agarose hydrogels incorporated in collagen conduits (A-FAH group) are highly biocompatible and permeable, supporting cell migration and revascularization throughout the grafted conduits. The nerve regeneration process in the A-FAH group was more intense and organized in comparison to the P-CTR group, and the histology study revealed a more effective regeneration in central and distal areas, with an increase in the synthesis and reorganization of the collagen fibers. All of these results demonstrate that the incorporation of the biomaterial was able to promote and favor cell migration from the proximal nerve area. Once the cells migrated to the regenerating nerve tissues, the *in vivo* synthesis and remodeling of the ECM, including collagen fibers and laminin at this level, was able to promote axonal growth from proximal areas.

The combination of biomaterials with Schwann or stem cells has achieved promising outcomes in nerve repair (Bell and Haycock 2012, Daly *et al* 2012, Gu *et al* 2011, Lopatina *et al* 2011, Nectow *et al* 2012), secreting growth factors and ECM molecules to facilitate nerve regeneration (Bell and Haycock 2012, Daly *et al* 2012, Gu *et al* 2011). Although we obtained good outcomes with the use of fibrin-agarose hydrogels alone (A-FAH group), these results were improved when autologous ADMSCs were used within fibrin-agarose scaffolds. With regard to their potential in the clinical setting, human ADMSCs are very abundant, easy to obtain under local anesthesia, and are preferred for tissue engineering applications in comparison to other sources of mesenchymal stem cells (di Summa *et al* 2010, Lopatina *et al* 2011). Histological analysis of the C-FAH group revealed that the regeneration process reached distal areas and was associated with a larger area fraction and greater intensity of properly oriented nerve fascicles (versus control and acellular groups), whose structure and organization was similar to observations in the control native sciatic nerve. These fascicles showed a larger area of remyelination, accompanied by a significantly higher positive expression of S100, neurofilament and laminin (versus control and acellular groups), with a homogeneous pattern from the proximal to the distal areas of the implanted conduits. In relation to the ECM, we found an increment in the synthesis
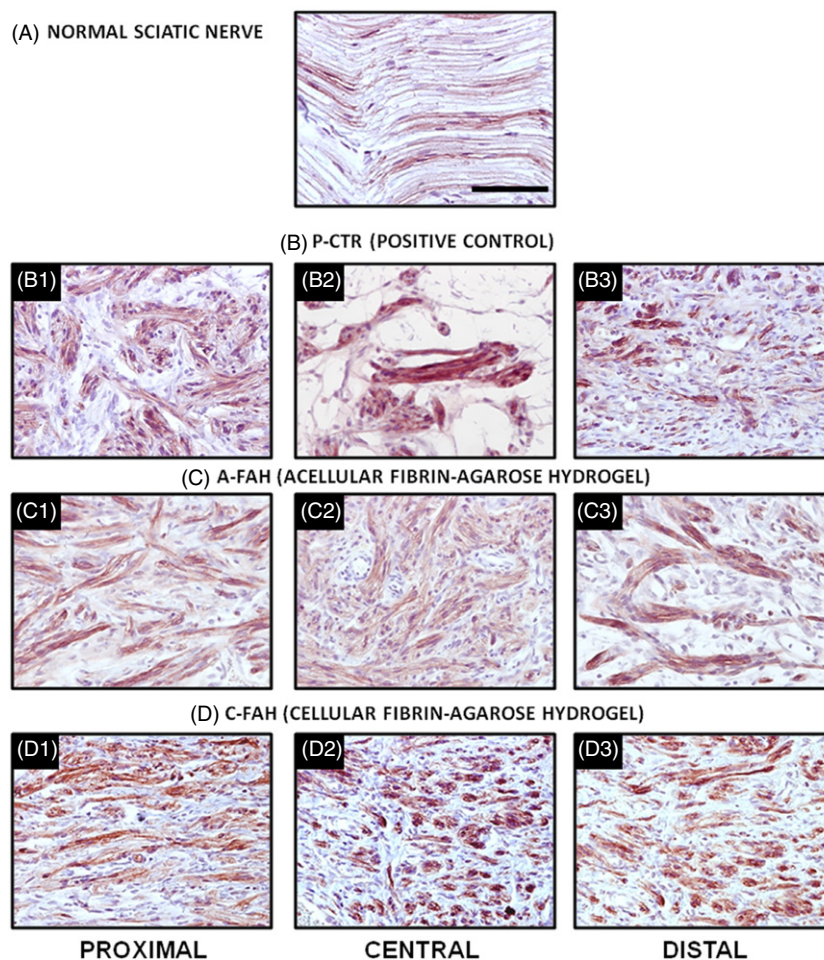
Figure 5. Light microscopy analysis of laminin expression. (A) Normal sciatic nerve, (B) P-CTR group, (C) A-FAH group and (D) C-FAH. 1: Proximal area, 2: central area, and 3: distal area of the grafted conduits. Scale bar = 100 μm.

and organization of collagen fibers and laminin in comparison to the other groups. These collagen fibers were abundant and properly oriented in the regenerated tissue. Collagen and laminin play essential roles in the myelination and remyelination process of axons by Schwann cells (Chernousov *et al* 2008, Geuna *et al* 2009). Collagen fibers favor Schwann cell migration, whilst laminin promotes higher Schwann cell proliferation (Bell and Haycock 2012, Chernousov *et al* 2008). Therefore, the use of fibrin-agarose hydrogels may provide structural support for Schwann cell migration through the conduit and favor the spread of oxygen and growth factors between the proximal and distal stumps. Moreover, the incorporation of ADMSCs to the fibrin-agarose hydrogels may add neurotropic factors that promote the synthesis and proper orientation of the ECM within the regenerated nerve tissue and increase the chemotactic attraction of the regeneration cone. The combination of these components would stimulate Schwann cell migration and organization from the proximal to the distal nerve stumps and therefore generate the cellular events involved in nerve regeneration.

Our results are in agreement with previous findings of a significant clinical and histological improvements in nerve regeneration with the utilization of stem cells embedded in the biomaterials filling the nerve conduit (di Summa *et al* 2010, Frattini *et al* 2012, Rutkowski *et al* 2004, Sasaki *et al* 2011, Wei *et al* 2011, Xu *et al* 2012). Other authors have also demonstrated that ADMSCs, like Schwann cells, can supply neurotropic factors to injured nerves, thereby enhancing nerve regeneration (Lopatina *et al* 2011). Nevertheless, the fate of the transplanted ADMSCs remains unknown, and further histological research with labeled cells is required to determine the destiny and survival rate of the implanted cells and their role in the regeneration process. These stem cells could support regeneration by synthesizing major ECM proteins and releasing neurotrophic and angiogenic factors, which could promote peripheral nerve regeneration along the grafted conduits by Schwann cells (di Summa *et al* 2012, 2010, Lopatina *et al* 2011).

According to the present results, collagen conduits filled with cellular fibrin-agarose hydrogels may offer an effective alternative for the repair of a 10-mm-gap lesion when the use of an autograft nerve is not possible. Future studies are warranted to compare the effectiveness of this approach with that of autografting and to determine its usefulness for repairing longer gaps (>10 mm). The advantages of our novel strategy, based on tubes filled with natural biodegradable biomaterials, include its simplicity and the fact that the procedure is rapid to perform and inexpensive in comparison to other types of conduits prepared using more complex technology.

Conclusions

Superior clinical, electrophysiological and histological results were obtained in a rat sciatic nerve model at 12 weeks after repair of a 10-mm-nerve defect with collagen nerve guide conduits containing a natural fibrin-agarose material than with collagen conduits containing heparinized saline. Even better outcomes were achieved when the fibrin-agarose material was impregnated with autologous adipose-derived mesenchymal stem cells. Both strategies avoid the additional donor site morbidity associated with autografts. The combination of a scaffold of agarose and fibrin, a natural material that can be autologously obtained, with autologous adipose-derived mesenchymal stem cells offers a simple and inexpensive procedure. Molecular studies and clinical trials are warranted to determine the potential usefulness of this approach in the treatment of patients with peripheral nerve injuries, especially when surgery or autograft procedures are not available.

Acknowledgments

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