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From Cell Biology to Tissue Engineering

A modified chemical protocol of decellularization of rat sciatic nerve and its recellularization with mesenchymal differentiated Schwann-Like cells: Morphological and functional assessments

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Summary. The functional reconstruction of large neural defects usually requires the use of peripheral nerve autografts, though these have certain limitations. As a result, interest in new alternatives for autograft development has risen. The acellular peripheral nerve graft is an alternative for peripheral nerve injury repair, but to date there is not a standardized chemical decellularization method widely accepted. The objective of this study was to propose a modified chemical protocol of decellularization of rat sciatic nerve and its recellularization *in vitro* with mesenchymal differentiated Schwann-like cells. After the transplantation, an evaluation of its regeneration was performed using morphological and functional tests. The study consisted of two phases; in phase 1, different concentrations and times of exposure of rat sciatic nerves to detergents were tested, to establish a modified chemical protocol for nerve decellularization. The chemical treatment with 3% triton X-100 and 4% sodium deoxycholate for 15 days allowed a complete decellularization whilst conserving the extracellular matrix of the harvested nerve. In phase 2, the decellularized and recellularized alografts were compared against autografts. The morphological analysis showed a higher positivity to specific myelin antibodies in the recellularized group compared to the autograft. There were no differences in this parameter between the control limb and the experimental limb (recellularized group). The functional analysis showed no statistical differences at week 15 in the Sciatic Function Index in the autograft group vs the other groups. This study sets the morphological and functional bases for posterior studies about nerve defects regeneration in humans.

Key words: Acellular graft, Decellularization, Peripheral nerve, Schwann-like cells, Sciatic nerve

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Introduction

Peripheral nerve lesions are a relevant clinical problem with a prevalence of functional loss in 2.8% of trauma patients and an incidence between 13-23 cases per 100,000 people every year (Noble et al., 1998). In the United States, about 50,000 annual surgical procedures are performed to repair peripheral nerves, with an estimated cost of seven billion dollars (Asplund et al., 2009).

In cases with simple defects in peripheral nerves, an adequate functional recovery may be achieved by

surgically joining both nerve endings. In contrast, functional reconstruction of large neural defects usually requires the interposition of nerve autografts for completing the repair. Although the use of autografts is considered the "gold standard" treatment for handling complex peripheral nerve injuries, this treatment has several limitations (Moore et al., 2011). Some of these are: a greater operating time, a limited number of sites for autograft harvesting, morbidity in the surgical site (pain, scarring, formation of a neuroma, sensory loss in the area where autograft is obtained and an increase in the risk of surgical site infections) (Rappaport et al., 1993). As a result, more attention has been given to the development of new and more effective alternatives to autografts for the management of peripheral nerve injury (Moore et al., 2011).

An alternative to nerve grafts is the use of fresh cadaveric allografts. Allografts have the advantage of being readily available and provide a wide selection of graft size and length. However, the use of cadaveric donor allografts requires the use of systemic immunosuppression in the patient for approximately 18 months. Immunosuppression allows axons and Schwann cells to regenerate through the allograft scaffold, but leaves the patient vulnerable to opportunistic infections and tumor formation, and the appearance of toxicity related to drugs used for achieving immunosuppression (Mackinnon et al., 2001).

Several synthetic nerve conduits have been approved by the FDA and are marketed in the United States for the restoration of short severed nerve gaps (Zhang et al., 2010). Still, these nerve conduits processed from biomaterials do not contain certain features of regeneration, such as the natural structure of a peripheral nerve and nerve growth factors, which are necessary for axonal elongation. Therefore successful repair using these conduits is limited when the nerve defect is large (Zhang et al., 2010).

For these reasons, decellularized human nerve allografts have become an attractive alternative because they are able to function temporarily as a viable scaffold for axonal regeneration, avoiding the need for immunosuppression (Mackinnon et al., 2001). The chemical decellularization of peripheral nerves, not only reduces the cellular and humoral immune response to Schwann cells and myelin from the donor, but also keeps the internal structure and components of the nerve extracellular matrix, which are necessary for nerve regeneration (Totey and Pal, 2009). Acellular nerve grafting is an alternative to nerve autografts for short nerve injury repairs that has already been studied (Kim et al., 2004). However, the results seem inconsistent (Walsh et al., 2009), and to date there is not a standardized chemical decellularization method widely accepted.

Mesenchymal stem cells (MSCs) are pluripotent stem cells located in the stromal cells of the bone marrow (Phinney and Prockop, 2007). Experimental studies have shown that MSCs injected into damaged nerves can improve the growth and myelination of regenerating axons (Goel et al., 2009; Ribeiro-Resende et al., 2009). Recent studies have shown that the stem cells derived from adipose tissue cells (ADSCs) have similar characteristics to MSCs from bone marrow, which have multipotential differentiation properties. The ADSCs also have other advantages such as: easy accessibility, rapid growth in cell cultures and successful integration into the host cells without generating immunogenicity (Zhang et al, 2010). Recent studies have found that nerve conduits supplemented with Schwann cells represent an effective method for nerve damage treatment (Rodriguez et al., 2000).

The aim of this study was to propose a modified chemical protocol of decellularization of rat sciatic nerve and its recellularization *in vitro* with mesenchymal differentiated Schwann-like cells, for transplantation in rats and evaluating their regeneration by morphological and functional methods.

Materials and methods

Study design

This is a morphological, experimental, cross-sectional, descriptive and comparative study.

Ethical consideration

This study was approved by Research Ethics Committee, Research Committee and the Institutional Committee for Care and Use of Laboratory Animals. The animals used for experimentation were handled according to current international regulations and to the Mexican Official Norm for the handling of experimentation animals (NOM-062-ZOO-1999). The animals were kept in constant light and dark cycle of 12 hours. The food and access to water was *ad libitum*. The temperature and humidity were maintained at a constant level (22°C and 60% respectively). Every effort was made to minimize animal suffering and pain, as well as the necessary number of animals used to produce reliable scientific data. The authors declared no conflict of interest for the production of this study.

Phases of the study

This study consisted of two phases of experimentation: in phase 1, different concentrations and exposure times of sciatic nerves of Wistar rats to chemical detergents were tested to develop a modified chemical protocol of decellularization with a posterior morphological evaluation. In phase 2, the decellularized nerves were recellularized with ADSCs differentiated to Schwann-like cells and were implanted in Wistar rats. A group of rats with implanted allografts was included. Functional assessments were performed against the gold

standard treatment for peripheral nerve injuries (autograft). Morphological and morphometric analysis were also performed in implanted nerves after 15 weeks.

Phase 1. Development of a modified chemical protocol to decellularize rat sciatic nerves and their posterior morphological analysis

Decellularization of rat sciatic nerves

For phase 1 of this study, 48 sciatic nerves of adult Wistar rats (24 rats) were extracted with a measurement of 15 mm length and were divided into three groups equally (n=16). All the samples were immersed in distilled water, which was replaced every 2 hours during 10 hours. Then, the nerves were exposed to Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) overnight at concentrations of 1%, 2% and 3% for groups 1, 2 and 3, respectively. This was followed by 24 hours of stirring (500 rpm) in a solution of sodium deoxycholate (SDC) (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 1% for group 1, 2.5% for group 2 and 4% for group 3. This process was repeated for 5, 9, 12 and 15 days for all groups (4 nerves for each group). The process was conducted under sterile conditions and at room temperature (RT). Finally, a final wash was performed in distilled water and the nerves were stored in 1X PBS buffer, pH 7.2 at 4°C until they were processed for morphological analysis. Similarly, 6 sciatic nerves of 3 rats were extracted and used as healthy controls.

Morphological analysis of decellularized nerves

To verify successful decellularization of nerves, these were fixed with 2.5% glutaraldehyde for 24 hours; then the samples were processed with conventional histological technique and embedded in paraffin blocks. Longitudinal and transversal histological sections (5 μm thick) were stained with H&E to determine which of the groups with different days of exposure and concentrations, showed absence of nuclei and/or cellular or nuclear remnants, which was considered a successful decellularization.

Subsequently, only in the group in which it was possible to achieve the absence of visible cellular elements observed in the H&E staining, the following stains were applied: Masson trichrome to identify the extracellular matrix (ECM) and collagen, and two histochemical methods: Klüver–Barrera (KB) stain that shows affinity for lipids in the myelin sheath (Klüver and Barrera, 1953), and the silver impregnation of Marsland-Glees and Erickson (M-G-E) which identify axons (Marsland et al., 1954), all the experimental samples were compared with healthy nerve controls.

Furthermore, to determine alterations in axons and to identify myelin debris, 4 μm thick histological sections were incubated with monoclonal antibodies anti-Neurofilaments (NF) ready to use (dilution 1:1) and anti-Myelin Basic protein (MBP) (dilution 1:200). The Envision® detection system was used and the positivity was identified with 3,3'diaminobenzidine (DAB), nuclei were contrasted with Gill's hematoxylin. As a negative control, the monoclonal antibody was omitted. NF, MBP antibodies and the Envision® detection system were purchased from Dako Cytomation, Inc® (Carpinteria, Ca; USA).

The samples were analyzed by light microscopy with a Nikon Eclipse 50i microscope and the image analysis software NIS-Elements Advanced Research Digital Sight DDS-2Mu. The morphological analysis was performed by two specialists with a PhD in Morphology, which confirmed the absence of cellular elements, alterations of the ECM and the organization of the collagen fibers, the absence of myelin and the integrity of axons, and results were compared with healthy controls.

Phase 2. In vitro nerves recellularization with ADSCs differentiated to Schwann-like cells and in vivo functional and morphological assessments

Isolation of ADSCs

ADSCs were obtained from Wistar rat adipose tissue. The tissue was placed in sterile PBS 1X with gentamicin, 50 mg/mL (Son's Chemical Laboratories LTD, Puebla, México), and amphotericin B, 0.25 mg/mL (GIBCO®/Invitrogen™ , Grand Island, NY, USA) (PBS 1X-GA). The tissue was minced to small pieces and sequential washes with PBS 1X-GA were performed by mix and left to stand so the separation in phases could occur. The upper phase was removed and the process was repeated until the lower phase was clear.

Then, a digestion with collagenase type I, 200 U/mL (GIBCO®/ Invitrogen™) in PBS 1X-GA was performed. The sample was incubated at 37°C with gentle stirring for 1 hour, until a homogeneous and thicker mix was observed. Upon completion of the digestion process, PBS 1X-GA was added, mixed gently and then centrifuged at 2000 rpm for 5 minutes. The precipitate had a small amount of adipocytes and ADSCs. The supernatant was discarded and the cell pellet was resuspended in PBS 1X-GA and centrifuged again; this process was repeated 2 more times. Finally, the cells were suspended in MEM–Alpha+Gluta-max™–I (GIBCO®/Invitrogen™) medium, supplemented with 10% fetal bovine serum (FBS) (GIBCO®/Invitrogen™) and antibiotic/antimycotic. Cells were plated in 25 cm^2 culture bottles and incubated at 37°C with 5% CO_2. After 3 days the medium was replaced and the culture was allowed to expand. When the culture reached 80-90% confluence, the cells were harvested using 0.25% trypsin (GIBCO®/ Invitrogen™). The obtained cells were then checked for CD-105 marker (1:10), which is specific for ADSCs. Positivity was identified with the Envision® system and DAB, the nuclei were contrasted

with Gill's hematoxylin. As a negative control, the monoclonal antibody was omitted.

Differentiation of ADSCs to Schwann-like cells with differentiation factors

Differentiation of ADSCs to Schwann-like cell (ADSCs-SC) was performed in the third pass of cell culture. Cells were washed with PBS and MEM-Alpha+Glutamax™-I medium with 1 mM β-mercapto-ethanol (Sigma-Aldrich, St. Louis, MO, USA) was added. After 24 hours the medium was changed by MEM-Alpha+Glutamax™-I with 10% FBS and 0.1mM transretinoic acid (ATRA) (Calbiochem®, MerkKGaA, Darmstadt, Germany), this medium was left for 72 h. Finally, the medium MEM-Alpha+Glutamax™-I supplemented with 10% FBS, 5 mM forskolin (Calbiochem®), 10 ng/mL of recombinant human fibroblast growth factor-b (bFGF) (Peprotech, INC., Rocky Hill. NJ, USA), 10 ng/mL human Platelet-Derived Growth Factor-AA (PDGF-AA)(Peprotech, INC.) and 200 ng/mL recombinant human heregulin β-1 (Peprotech, INC.) was added. This latter medium was preserved from 2 to 8 days, renewed according to the growth of cells.

Identification of ADSCs-SC was performed by the immunohistochemistry analysis described above to detect the positivity to Glial Fibrillary Acidic Protein (GFAP) (1:200) and S-100 protein (1:1500) respectively, which are expressed by Schwann-like cells derived from mesenchymal stem cells (Tohill et al., 2004). Both antibodies were purchased from Dako Cytomation, Inc®, (Carpinteria, Ca; USA).

In vitro nerve recellularization with ADSCs-SC

In vitro nerve recellularization was performed following the method described by Badylak et al., (2011): the decellularized nerves were immersed in a suspension of $1x10^5$ ADSCs-SC and the mixtures were centrifuged at 3000 rpm for 1 minute. Then, they were incubated for 48 hrs at 37°C and subsequently were transplanted into recipient rats.

Surgical procedure and study groups

24 male Wistar rats, young adults (age at the beginning of the protocol between 3-6 months and 200-500 grs of weight); skeletally mature and clinically healthy were included. Animals were divided in 4 groups (n=6): group 1 (autograft), group 2 (decellularized), group 3 (recellularized), and group 4 (sham). In all of these groups, peripheral nerve surgery was performed according to the respective treatment. The group 0 (Control) corresponds to the first evaluation of all experimental groups (before injury of each experimental group), so we ensure that each one had an index sciatic normal function.

All surgeries were performed by the same plastic surgeon specialized in peripheral nerve microsurgery. Experimental animals were anesthetized with intraperitoneal ketamine (90 mg/kg) and xylazine (10 mg/kg); after the trichotomy of the posterior thigh, an incision of 2 cm in a craniocaudal direction in the posterior thigh to half the distance between the femoral head and the ischium in the rat right hind limb was performed, along the longitudinal axis of the femur. After dissecting the subcutaneous tissue, the gluteal muscles and ischiotibial muscles were identified, followed by blunt dissection of these muscle groups to expose the sciatic nerve throughout the incision's axis.

In group 1 (autograft), the nerve was cut into a 15 mm length segment and this segment was inverted and the proximal and distal ends were microsurgically sutured using a 10.0 gauge suture. In group 2 (decellularized), a nerve segment was removed and replaced by a decellularized nerve segment, group 3 (recellularized) received a recellularized nerve with ADSCs-SC. Finally, group 4 (Sham) only underwent incision and nerve manipulation, but no section of the nerve was performed. Once the intervention was finished, the rats were monitored during their recovery period from the anesthetic under heating lamps, and were left to feed and drink *ad libitum*.

Functional assessment: Measuring the Sciatic Function Index (SFI)

The rats were evaluated functionally by applying the SFI 3 times per week for a period of 15 weeks (the SFI of the week corresponds to the average of Monday, Wednesday and Friday), for which the limbs of the rats were inked and left to pass through a dark tunnel from one end to another on a sheet of white paper. Once the prints were obtained, the following measurements on the paper were performed using a digital vernier caliper with millimetric accuracy of 0.01 mm (Mitutoyo Digimatic Encoders w/500 series, Mitutoyo Corp., Kawasaki, Japan):

1. Length of the footprint: distance from heel to toe along the middle axis.
2. External length: is the distance of the total separation of the fingers, i.e. the distance between first and fifth finger.
3. Internal length: is the intermediate distance of the fingers, i.e. the distance between the second and fourth finger.
4. Total distance to the opposite foot: distance between the most anterior point of a footprint with the most anterior point of the next.

After obtaining the parameters described in both limbs (injured and normal) these were incorporated into the formula described by Bain et al. (1989) for the SFI on a scale of +11 to -100 where +11 to -11 corresponds to a normal state and a -100 corresponds to complete damage. The data were collected in a data collection

sheet prepared for purposes of this study.

Morphological and microdensitometric analysis

After functional assessment (15 weeks), the rats were euthanatized with sodium pentobarbital (100 mg/kg, intraperitoneally) and perfused through the heart with 200 mL of 4% paraformaldehyde in 0.1 M PBS pH 7.2-7.4. The dissection of the nerves of the 4 groups was taken about 5 mm before both sutures in the proximal end (closest to the spinal cord), and the distal end (farthest from the spinal cord). Samples were fixed in 2.5% glutaraldehyde for 24 hrs. Subsequently the methods, techniques and staining previously described for phase 1 study were applied to all nerves. Also for the histochemistry evaluation of the ECM the periodic acid of Schiff (PAS) method was applied (Bancroft and Gamble, 2002).

Additionally, a microdensitometric analysis was performed to quantify the positivity to MBP in the samples after 15 weeks. This was performed in 10 consecutive high-resolution digital images with a high power objective (40x) from the stained slides. The parameters of color, distribution of tonality, saturation and luminance were established in the data software and were the same for all the images obtained. Subsequently the images were analyzed with Image J 1.49V (National Institutes of Health) software to determine the intensity of the density (Int Dent) in each sample, this was performed in triplicate.

Statistical analysis

The statistical analysis was performed using the computer program SPSS 19.00 version for Windows XP (SPSS, Chicago, IL). The mean and standard deviation for the results of the SFI after15 weeks were obtained. Comparisons of the results within the groups of SFI and the results of the microdensitometric analysis of histological sections, were performed using tests for parametric data (ANOVA of one factor) to determine the significance of differences when comparing all the groups. Afterward, t-student post hoc Turkey test for independent variables was performed to make individual comparisons between the two groups. In both analyses a $p \leq 0.05$ value was considered as statistically significant.

Results

Phase 1

Sciatic nerve decellularization and ECM preservation

To decellularize the nerve, different concentrations of the detergent Triton X-100 + SDC were tested as well as different treatment periods. The histological test with H&E was done once the process of decellularization was completed. In the stained slides, it was observed that as the concentration of detergents and exposure time increase the nuclei lose their integrity, and are positioned at the periphery of the nerve. It was noticed that at the 15th day and with a concentration of 3% triton X-100 + 4% SDC the nerve was completely decellularized (Fig. 1).

To verify that the ECM was not affected with a concentration of 3% Triton X-100 + 4% SDC for 15 days, a Masson trichrome staining was performed. In all the samples of this treated group it was observed that the collagen fibers showed no significant morphological changes (Fig. 2D) compared with control nerves (Fig. 2A), indicating that the decellularization protocol proposed does not significantly affect the ECM at these concentrations.

The M-G-E staining protocol achieves a specific silver impregnation in the axons. The control nerves showed presence of well-preserved and uniform axons (Fig. 2B); nerves treated with 3% Triton X-100 + 4%SDC for 15 days showed morphological changes such as the presence of variations in diameter axons (some thicker and others thinner), as well as the presence of vacuoles that interrupt the continuity of the axons (Fig. 2E). The control nerve that was used to identify NF showed well defined axons (Fig. 2C) while nerves treated with 3% triton X-100 + 4% SDC for 15 days were negative in all samples (Fig. 2F).

The KB staining and the immunolabeling for MBP were used to verify that there were no myelin residues in the treated nerve. The KB staining has affinity for lipids in the myelin sheath staining it in blue color in the control group (Fig. 3A). In samples from sciatic nerve treated with 3% triton X-100 + 4% SDC for 15 days, there was no evidence of the presence of myelin in any case (Fig. 3B).

In the control nerve, treated with immunolabeling against MBP, the positivity of myelin was seen as brown staining (Fig. 3C); while in the slides of nerves treated with triton 3% X-100 + 4% SDC for 15 days antibody negativity was observed in all slides (Fig. 3D).

Phase 2

In vitro nerve recellularization with ADSCs-SC

To ensure the correct isolation of ADSCs, these cells were characterized by looking for the CD-105 biomarker, which is specific for mesenchymal stem cells. Positivity for this marker was found in slides that appear brown after the staining procedure (Fig. 4B), which did not happen in the negative control slide (Fig. 4A). Then, the aforementioned cells were differentiated into Schwann-like cells by using differentiation factors. Fig. 4D,E show positivity for differentiated cells when looking for the GFAP and S-100 markers, respectively, which are specific for ADSCs-SC (Tohill et al., 2004) compared with the respective negative control (Fig. 4C). These ADSCs-SC cells were implanted in the decellularized nerves (Fig. 4F) after incubation all of the

samples showed the presence of nuclei ensuring they were recellularized (Fig. 4G).

Harvesting and decellularization of autograft to recellularizate and transplant in receptive rats

All surgical procedures were performed without a hitch in the 4 experimental groups, autografts and decellularized allografts were implanted for nerve defect repair. No adverse effects or complications were reported in either the surgical procedures or in the postoperative period.

Functional evaluation of the experimental groups by calculation of the SFI

To functionally evaluate the sciatic nerve, the SFI for each rat at the 15 week mark was calculated. The SFI has values between +11 to -11 and -100, with +11 to -11 being the most favorable outcome (equivalent to a normal outcome) and -100 is the least favorable (equivalent to a complete nerve damage).

The Sham group had SFI normal results throughout the 15-week study, indicating that surgical manipulation of the nerve has very little effect on the function of the

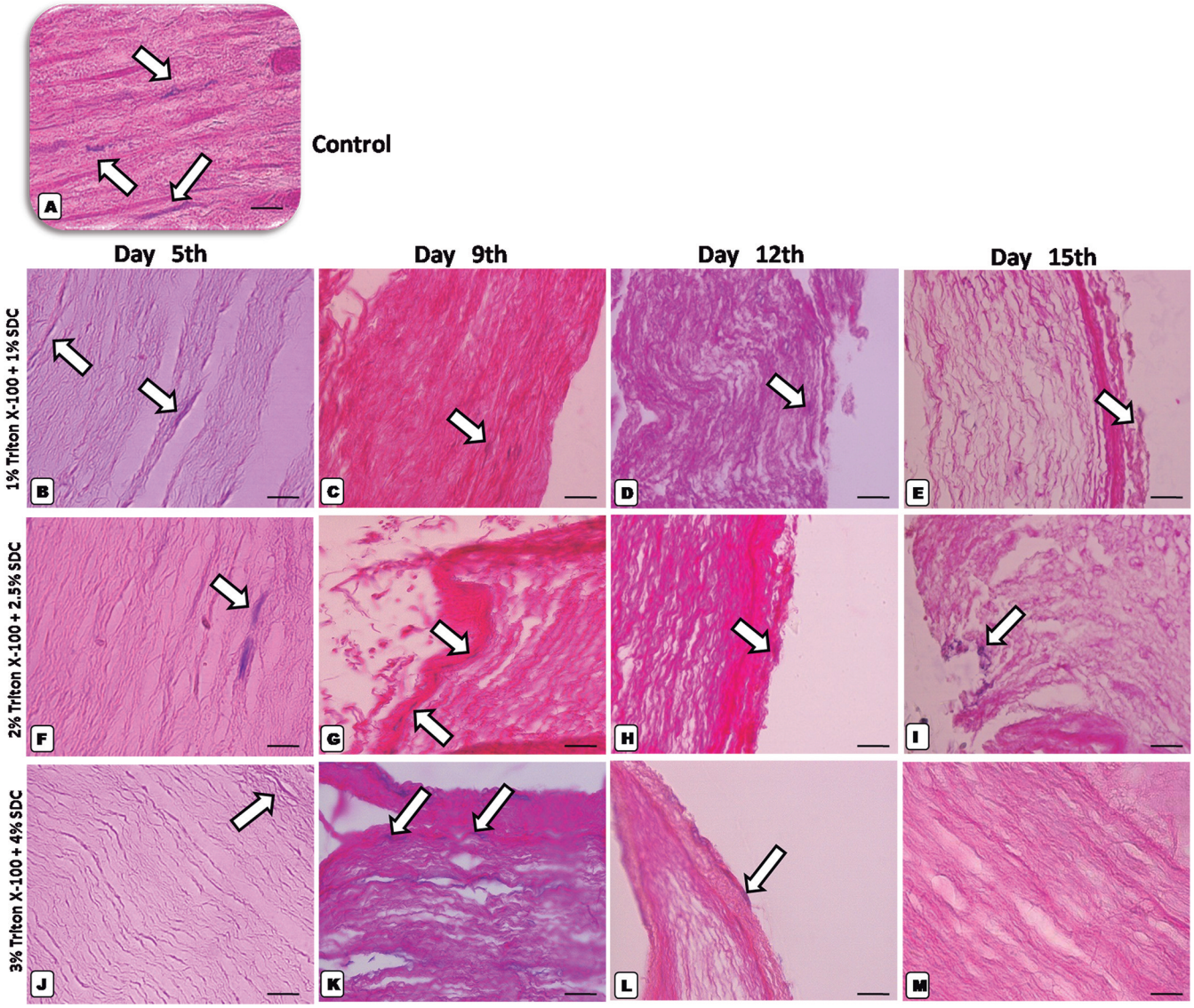

Fig. 1. Development of the method to decellularizate rat sciatic nerve. Segments of sciatic nerve treated with different concentrations of Triton X-100 + SDC and incubated for 5 **(B, F, J)**, 9 **(C, G, K)**, 12 **(D, H, L)** and 15 days **(E, I, M)** compared with control nerve **(A)**. The white arrows show the nuclei or nuclei remnants, observe that with 3%Triton X-100 + 4% SDC no nuclei are observed. H&E stain. Paraffin embedded, light microscopy. Bar: 100 μm.

sciatic nerve. It's also noticeable that the Autograft, Decellularized and the Recellularized groups, throughout the15 weeks of observation, had values below -50 with a trend towards improvement in the Decellularization and Recellularization groups. The results obtained from the Recellularized group at week 15 of the study showed that it was the only one that manages to hold values over -50 (Fig. 5).

The average result of SFI was -8.29±1.67 for the Sham group, -76.05±5.58 for the Autograft group, -71.14±10.97 for Decellularized group and -60.84±6.14 for Recellularized group. The average results for each week are shown in Table 1. Significant differences were observed between the average results of the Sham group vs Autograft, Decellularized and Recellularized groups at the end of 15 weeks, shown by performing the ANOVA test (p=0,03). When performing individual group analyses using the Tukey's method, statistically significant differences were found when comparing the

Table1. SFI behavior in the 4 experimental groups throughout the 15 weeks of the study.

Week	Sham	Autograft	Decellularized	Recellularized
0	-7.81	-7.94	-8.72	-9.24
1	-5.29	-83.82	-87.64	-68.19
2	-10.54	-76.85	-82.40	-60.82
3	-9.17	-69.69	-74.06	-72.34
4	-8.06	-68.01	-82.12	-65.91
5	-8.80	-75.16	-88.10	-59.50
6	-8.80	-71.48	-83.71	-61.69
7	-6.74	-67.09	-71.98	-70.00
8	-6.77	-75.99	-63.90	-64.14
9	-9.73	-75.06	-64.66	-55.01
10	-10.62	-78.95	-62.68	-54.62
11	-9.46	-81.63	-61.64	-57.08
12	-8.31	-74.00	-59.60	-55.36
13	-8.80	-86.02	-58.12	-57.82
14	-8.47	-80.94	-67.02	-58.63
15	-4.75	-76.05	-59.52	-51.48

Table 2. Statistical comparison between the mean results of Sham vs experimental groups throughout the 15 weeks of the study.

Comparison	P value
Sham vs Decellularized	0.002*
Sham vs Autograft	0.000*
Sham vs Recellularized	0.002*

*Statistically significant differences.

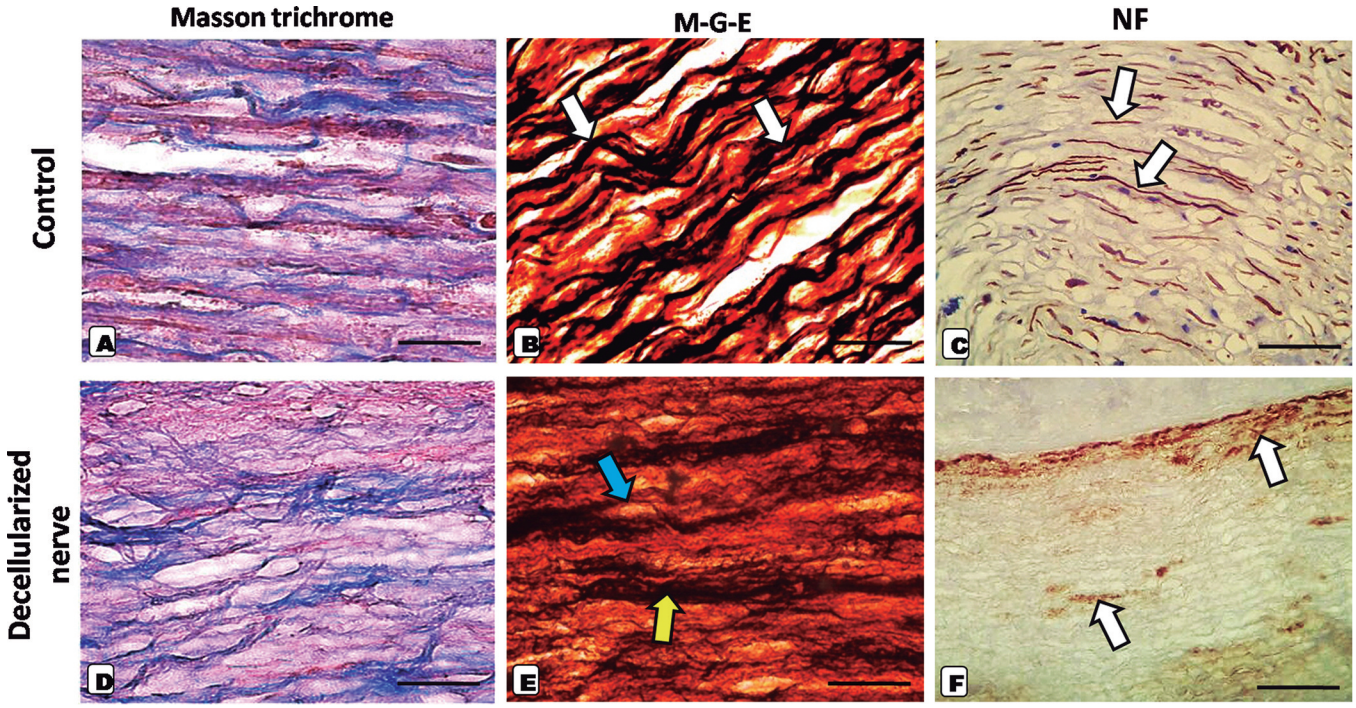

Fig. 2. Proposed method of decellularization decreased the presence of axons without affecting nerve collagen fibers after 15 days of treatment with detergents. **A.** Untreated control nerve. **D.** Treated nerve, collagen fibers in the treated group showed no significant morphological changes compared with control nerve. **B.** White arrows indicate the normal structure of axons. **E.** Shows alterations in the structure of the axon as vacuoles and thinning (blue arrow) as well as thickening (yellow arrow). **C.** Arrows indicate the normal positive signal for NF. **F.** White arrows show the decreasing signal for NF in the nerve after treatment. A and D: Masson trichrome; B and E: M-G-E silver impregnation technique; C and F: immunohistochemistry for NF identification. Paraffin embedded, light microscopy. Scale bar: 100 μm.

average results of the groups, as shown in Table 2. One important point was that comparing the group with autograft (considered the "gold standard" treatment for handling complex peripheral nerve injuries) vs Decelullarized , and Autograft vs Recellularized groups, no statistically significant differences were found (table 3).

Morphological and microdensitometric analysis

The nerve transplanted after 15 weeks of recovery was dissected taken about 5 mm before the both sutures in the proximal end (closest to the spinal cord), and the distal end (farthest from the spinal cord), to make the corresponding analysis. In Fig. 6 we can see the morphological analysis with representative images of each group. In the slides stained with H&E, it was shown that the nerves of the Decellularized group had uniform recellularization at 15 weeks of recovery (Fig.

Table 3. Statistical comparison between the mean results of Autograft vs experimental groups throughout the 15 weeks of the study.

Comparison	P value
Autograft vs Decellularized	0.147
Autograft vs Recellularized	0.07
Decellularized vs Recellularized	0.968

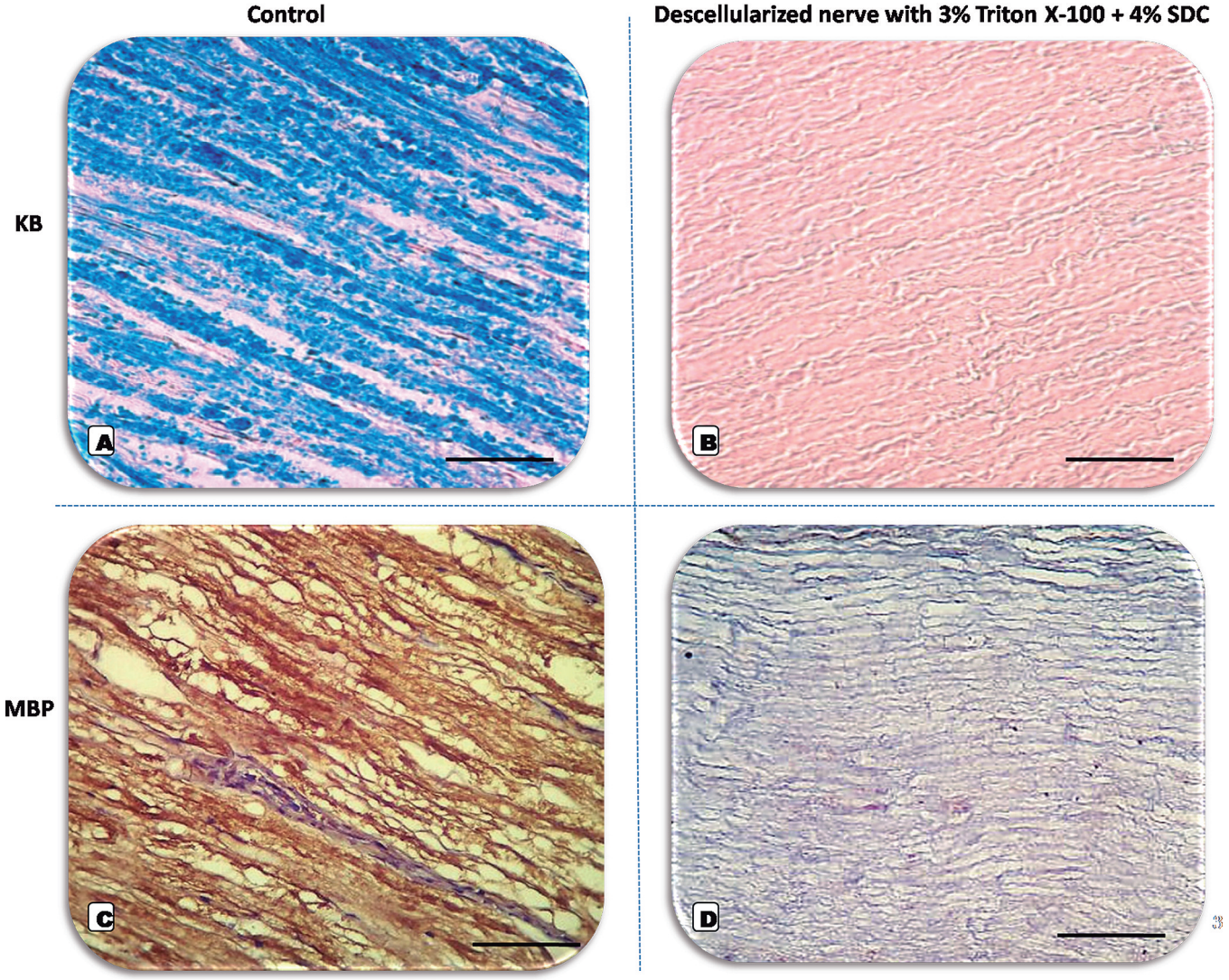

Fig. 3. Remotion of myelin in decellularized nerves. **A and C** are control nerves, **B and D** are nerves treated with 3% triton X-100 + 4% SDC for 15 days. Note the absence of myelin in decelullarized nerves. A and B: KB staining; C and D: immunohistochemistry for MBP identification. Paraffin embedded, light microscopy. Scale bar: 50 μm.

6C), which is very similar to the Sham group (that also showed increased collagen fiber disorganization without architecture loss or fiber disruption) (Fig. 6A), and the Autograft group (in which an increased cellularity and a decrease in the extracellular component was observed) (Fig. 6B). However, the slides in the Recellularized

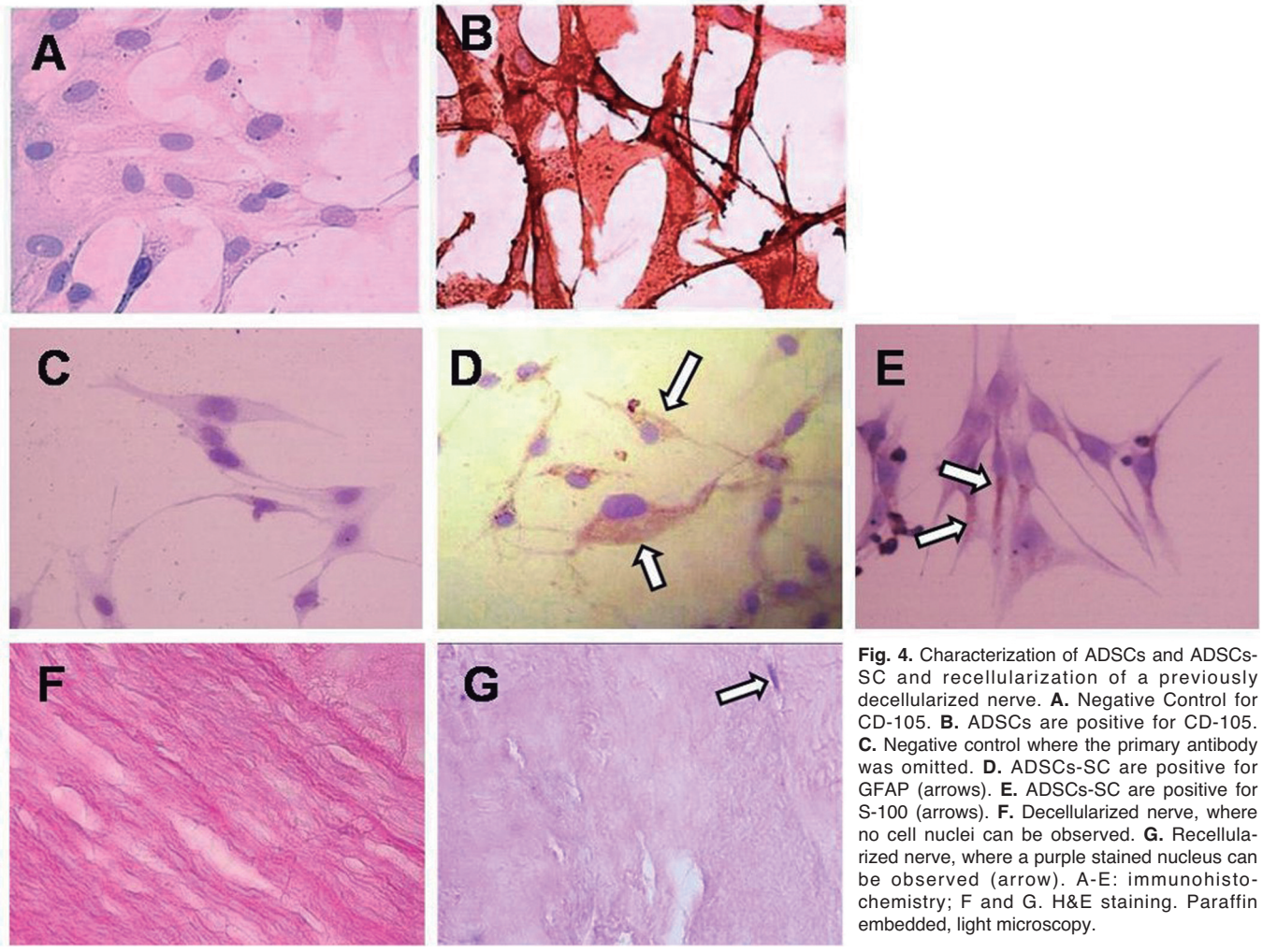

Fig. 4. Characterization of ADSCs and ADSCs-SC and recellularization of a previously decellularized nerve. **A.** Negative Control for CD-105. **B.** ADSCs are positive for CD-105. **C.** Negative control where the primary antibody was omitted. **D.** ADSCs-SC are positive for GFAP (arrows). **E.** ADSCs-SC are positive for S-100 (arrows). **F.** Decellularized nerve, where no cell nuclei can be observed. **G.** Recellularized nerve, where a purple stained nucleus can be observed (arrow). A-E: immunohistochemistry; F and G. H&E staining. Paraffin embedded, light microscopy.

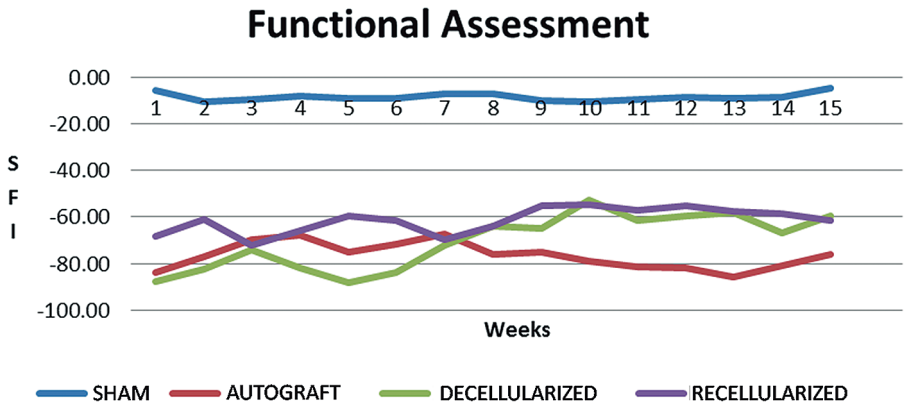

Fig. 5. SFI behavior patterns throughout the 15-week study.

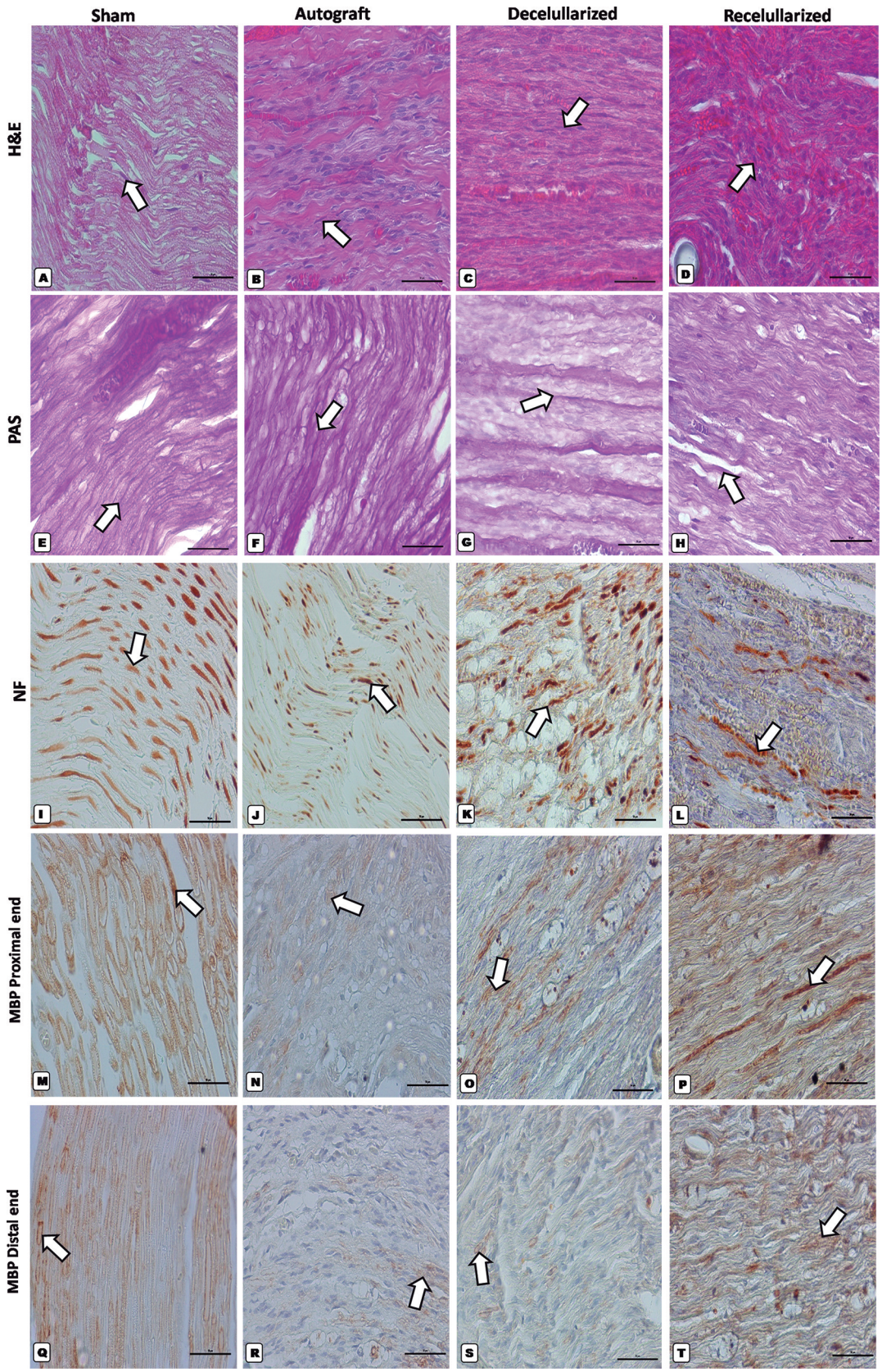

Fig. 6. Morphological analysis of nerves retrieved 15 weeks after grafting in rats. Representative images of slides each group stained with H&E are shown, the Decellularized group **(C)** presents a similar recellularization to the Sham group **(A)** and the Autograft recellularization **(B)**; however, the Recellularized group **(D)** shows disorganization of nerve fibers with greater number of nuclei (arrows). Integrity of ECM: In the identification of ECM with PAS method all groups **(E-H)** showed the integrity of the external lamina (arrows). Recovery of integrity of axons: The immunolabeling for NF shows that the integrity in axons is recovered after 15 weeks in the Decellularized nerve **(K)** and Recellularized nerve groups **(L)**. Autograft group showed alterations in axons linearity **(J)**, and the Sham group showed non damaged axons **(I)**. Positivity to NF (arrows). Remyelination of nerve in experimental groups: Immunodetection of MBP in the proximal end shows that the Decellularized group **(O)** and the Recellularized group **(P)** showed higher remyelination in the nerve after 15 weeks. Autograft group had lower remyelinization **(N)** compared with the Sham group **(M)**. In the distal end, Autograft **(R)** and Decellularized **(S)** groups showed low remyelination; however in the Recellularized nerve the positivity is remarkable **(T)**. Sham group showed extensive positivity to MBP antibody **(Q)**. Positivity to MBP (arrows). A-D: H&E staining; E-H: histochemistry with PAS method; I-T: immunohistochemistry for NF and MBP. Paraffin embedded, light microscopy. Scale bar: 50 μm.

group with ADSCs-SC clearly had an increased cellularity as well as an alteration in the overall morphological structure compared to the other three groups (Fig. 6D).

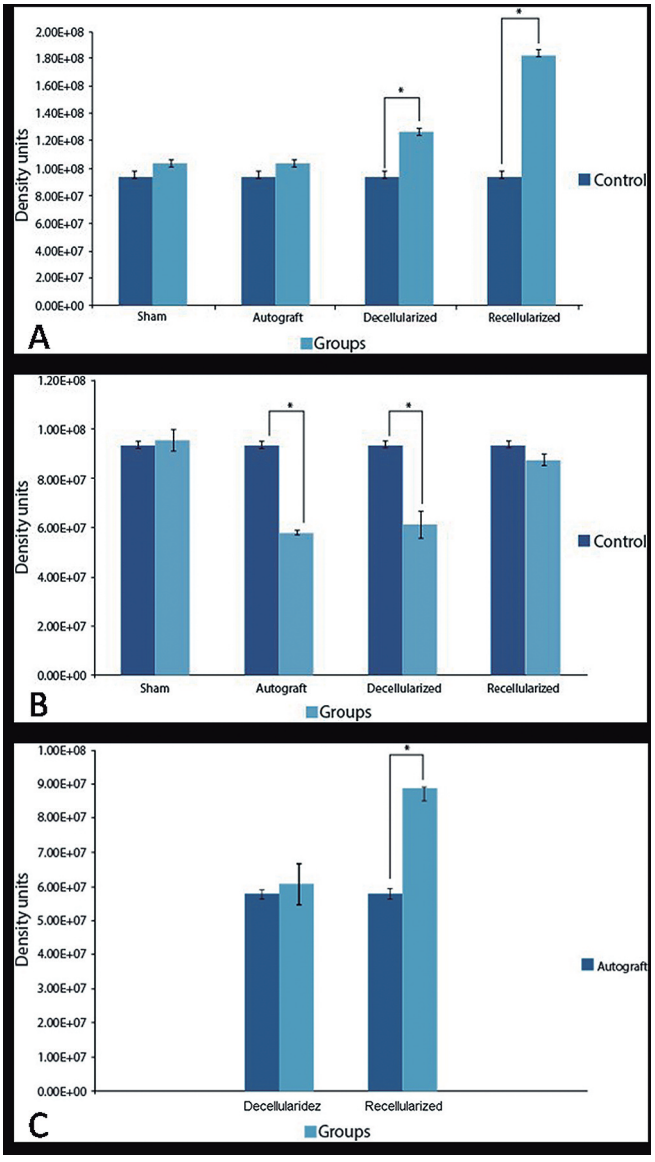

Fig. 7. A. Quantification of the MBP signal in the proximal end. The Image J program was used to measure units of density corresponding to the positivity to MBP expressed in the proximal end after 15 weeks of recovery for each group. The Decellularized and Recellularized groups have higher remyelination compared to Sham and Autograft group. p≤0,05. **B.** Quantification of the MBP signal in the distal end. Positivity expressed in the distal end at 15 weeks of recovery for each of the groups. The Autograft and Decellularized groups have a lower remyelination compared to the control group, whilst the difference in the Recellularized group vs control nerve is not statistically significant. p≤0,05. **C.** Comparison of the MBP signal at the distal end of Decellularized and Recellularized groups vs the gold standard (Autograft). The Recellularized group shows increased remyelination in the distal end compared to Autograft group. p≤0,05.

In samples of all the study groups positivity to PAS method was observed which demonstrates the integrity of the ECM elements (Fig. 6E-H).

In the decellularized nerve, after the procedure with detergents the axons lost their integrity and showed significant alterations (Fig. 2E,F). However after 15 weeks of implantation, it is clear that the nerves in the Decellularized group and the Recellularized group had recovered axons integrity in both cases (Fig. 6K,L) compared with Autograf that showed alterations in axons linearity (Fig. 6J), and with the Sham group that showed non damaged axons (Fig. 6I).

Axonal regeneration is unidirectional and is from the proximal end until the distal end. In the detection of MBP in the portion of the proximal end it was found that the Decellularized group and the Recellularized group showed higher remyelination in the nerve after 15 weeks (Fig. 6O,P). The remyelinization in the Autograft group was lower (Fig. 6N), when compared with the Sham group that showed normal positivity to MBP antibodies (Fig. 6M).

In the portion of the distal end, it was observed that in nerves of Autograft and Decellularized groups the remyelination has not yet reached that area (Fig. 6R,S); however in the Recellularized nerve the positivity is remarkable (Fig. 6T). Sham group showed normal positivity to MBP antibody (Fig. 6Q).

Using the ImageJ software, a quantification of the positivity to MBP in the proximal end was performed, and it was observed that the Decellularized and Recellularized nerves had an increased amount of positivity compared with slides with undamaged control nerves (Fig. 7A). Also, it can be observed that quantitatively, there is a lower positivity in the Autograft and Decellularized groups relative to the control nerves in the distal segment (Fig. 7B). In the Sham and Recellularized groups no statistically significant differences were observed when they were compared with control nerves. However, comparing the Decellularized group and Recellularized group with the Autograft group (Fig. 7C), which represents the gold standard treatment for the management of complex peripheral nerve injuries, there is increased remyelination in the first two groups, specifically in the Recellularized nerve group, where it showed a statistically significant increase in myelin.

Discussion

The best procedure currently available for the treatment of complex peripheral nerve injury is an autograft, however, this method has certain disadvantages such as: the shortage of material for grafting, requirement of a second site of surgery, sensory loss in the donor site and potential risk of neuroma formation. Because of these drawbacks, efforts to replace this procedure have focused on the development of an acellular nerve allograft to serve as a scaffold in the

repair of these lesions and present some advantages such as: easy availability, no immunogenicity and conservation of the ECM (Ducic et al., 2012).

Several methods of decellularization have been described for the grafting of an acellular peripheral nerve alograft one of which is the use of detergents. Sondell et al. (1998) showed that using a combination of Triton X-100 and SDC it is possible to achieve decellularization of a Sprague-Dawley rat sciatic nerve. In this paper, the combination of these detergents used for decellularization which gave the best results was 3% triton X-100 + 4% SDC for a period of 15 days. By H&E staining and immunohistochemistry to detect MBP it was possible to ensure that the complete removal of cellular material was achieved. Olausson et al., (2012) noted that the removal of the cellular component in any decellularized allograft is crucial to avoid an immune reaction. In this case, Olausson made a decellularized allograft vein transplant, in which the patient did not have any immune reaction.

Axonal regeneration in the central nervous system (CNS) is much slower than in the peripheral nervous system (PNS), the main cause of this could be the delayed clearance of myelin debris, as they contain several inhibitory factors, therefore, their presence could hinder axonal regeneration (Brown et al., 2004). By eliminating the decellularized nerve myelin, we are eliminating this inhibitory effect and encouraging a more efficient axonal regeneration.

The decellularized nerve graft provides a scaffold for regenerating axons and is a support structure for Schwann cells, therefore its ECM should be kept in good condition and not be affected by treatment with detergents. Staining with Masson trichrome structure allowed for the observation of the collagen fibers of the treated nerves, which were similar to the control nerves, noting that the treatment used in this study to decellularize nerves did not affect the ECM. Wallis et al., (2012) reported that it is essential to maintain the integrity of the ECM to achieve adequate recellularization.

In the decellularized nerve, it can be seen that the structure of the axons itself was affected by the decellularization process. However, this integrity was recovered 15 weeks after transplantation. Salazar et al., (2006) reported that when there is damage caused by a neuropathy, axons show disturbances caused by demyelination but can be restored at the stage of recovery if the right conditions are present. Regarding the nerve myelination, it is possible to observe that in the case of the Autograft and Decellularized groups, nerve remyelination was yet to reach the distal stump; this may be because the recovery time was not enough to allow that process. However, remyelination in recellularized nerve is significantly higher in the proximal end compared to the control group and showed no significant differences with the distal end. The Recellularized group showed significant differences compared to the Autograft group, which is the gold standard in peripheral nerve repair. This may be due to the addition of SC, since they produce growth factors that attract new axonal sprouts growing from the proximal to the distal end and also provide structural orientation guiding the regenerating axons in the right direction (Chang et al., 2013).

The results of the SFI, showed considerably better results in the Recellularized nerve group when compared to Autograft and Decellularized groups without any statistically difference among them. This resulted in improved clinical progress, a loss of external rotation of the limb and better flexion and abduction of the fingers. Further studies are needed that focus specifically on orthopedic and neurological assessment for functionality testing of the affected limb.

The results of the SFI in the Autograft group showed worse results compared to the other groups. In previous studies it was shown that after 12 weeks, the SFI had results around -70 to -75, and in our studies these remained around -80, this could be because previous studies have used neural defects ten millimeters in length, whereas in our study neural defects 15 millimeters in length (which is more like what occurs in clinical context) were used. It is well known that the greater the size of the nerve defect the more difficult will be the restoration of nerve function (Liu et al., 2011; Nagao et al., 2011). Currently nerve reconstruction is limited in length. There is a relationship between the length of the nerve gap and the level of recovery following nerve repair. Two large clinical studies have divided three different gap lengths: 5-14 mm, 15-29 mm and 30-50 mm nerve gaps. After adjustment for technical failures. The nerve grafts of 5-14 mm had a 100% meaningful level of functional recovery in both studies. For nerve grafts above 15 mm in both studies a meaningful recovery around 80% is seen (Brooks et al., 2012; Cho et al., 2012).

Multiple studies have evaluated the clinical efficacy of decellularized nerve allografts in humans (Tang and Chauha, 2015). Karabekmez et al., (2009) reported on the clinical outcomes after allograft transplantation in eight patients with digital nerve injuries and two patients with dorsal ulnar sensory branch injuries. The average length of the allograft was 2.15 cm. The two-point static and moving discrimination were 5.5 and 4.4 mm, respectively. There were no rejections and all patients had excellent or good results based on the Mackinnon scale. Guo et al., (2013) reported outcomes for five patients with an average defect of 23 mm treated with digital nerve repair using decellularized nerve allografts. The authors reported no transplant rejections, an average two point discrimination of 6 mm at an average follow-up of 13 months, and an average Semmes-Weinstein monofilament test score of 4.3.

Conclusion

The treatment with 3% Triton X-100 + 4% SDC for a period of 15 days allowed for decellularization with

retention of the nerve ECM in good condition, for subsequent use as an efficient allograft for treatment of peripheral nerve lesion in a murine model. Increased remyelination was observed in *in vitro* recellularized nerve with ADSCs-SC with differentiation factors.

The morphological analysis showed increased MBP signal intensity in the Recellularized group when compared to the Autograft and Decellularized groups. At 15 weeks there was no difference in this parameter between the experimental limb and the control limb of the Recellularized group. Functional tests yielded no significant differences at 15 weeks in the SFI between the Recellularized group and the Autograft and Decellularized group.

The present study is the first to use adipose derived mesenchymal and differentiation to Schwann-like cells by using growth factors in nerves previously decellularized with the proposed technique. This study provides morphological and functional bases for further similar studies in neural defects in humans.

Disclosure of potential conflicts of interest. The authors declare no conflict of interest.

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