IN VITRO CHARACTERIZATION OF A NANOSTRUCTURED FIBRIN-AGAROSE BIOARTIFICIAL NERVE SUBSTITUTE

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Abstract:

Neural tissue engineering is focused on the design of novel biocompatible substitutes to repair peripheral nerve injuries. In this study, we describe a nanostructured fibrin-agarose bioartificial nerve substitute (NFABNS) based on nanostructured fibrin-agarose hydrogels (FAH) with human adipose-derived mesenchymal stem cells (HADMSC). These NFABNS were mechanically characterized and HADMSC behavior was evaluated by using histological and ultrastructural techniques. Mechanical characterization showed that the NFABNS were resistant, flexible and elastic, with a high deformation capability. Histological analyses carried out *in vitro* during 16 days revealed that the number of HADMSC decreased overtime with a significant increase after 16 days. HADMSC formed cell clusters and degraded the surrounding scaffold along the time. Additionally, HADMSC showed active cell proliferation and cytoskeletal remodeling with a progressive synthesis of extracellular matrix molecules. Finally, this study demonstrate that it is possible to generate biologically active and mechanically stable tissue-like substitutes with specific dimensions based on the use of HADMSC, FAH and nanostructuration technique. However, *in vivo* analyses are needed to demonstrate their potential usefulness in peripheral nerve repair.

Key words: neural tissue engineering, biomimetic nerve substitute, fibrin-agarose hydrogels, nanostructured biomaterials, adipose derived mesenchymal stem cells, extracellular matrix, cell-biomaterial interaction.

1. INTRODUCTION:

Peripheral nerves (PN) are essential organs for the normal function of the body at the motor and sensory levels. Their structure and function is often affected by several conditions with different degrees of structural damage and/or dysfunction (Carriel *et al.* 2014a). Depending on the severity of the nerve injury, these lesions can be clinically managed by several surgical techniques. In cases of incomplete or short nerve gaps, direct nerve repair or end-to-end neurorrhaphy is the most preferred and effective method, while nerve autografting is the current gold standard to bridge critical nerve gaps (Daly *et al.* 2012; Carriel *et al.* 2014a). Nerve autograft provides an appropriate extracellular matrix (ECM), functional Schwann cells (SC) and essential growth factors to the regenerative microenvironment, but functional recovery is found only in 50% of these cases (Pabari *et al.* 2010). In the case of the nerve allografts, it is similar, but it has less successful and more clinical complications (Mackinnon *et al.* 2001; Daly *et al.* 2012; Kehoe *et al.* 2012; Carriel *et al.* 2014a).

Nowadays, it is clinically possible to bridge nerve gaps using nerve conduits (NC) based on natural or synthetic biomaterials by a technique called tubulization (Carriel *et al.* 2014a; Gu *et al.* 2014). They were used to bridge short and critical nerve gaps, and acceptable results were obtained just in short nerve defects (Moore *et al.* 2009; Wangensteen and Kalliainen 2010; Carriel *et al.* 2014a). These NC lack the supportive structures to promote appropriate proliferation and migration of the SC, especially in critical nerve gaps, where a significant reduction of essential growth factors occur with the failure of the nerve regeneration (Webber and Zochodne 2010). Currently, it is accepted that tubulization technique is a safe and effective alternative to repair sensory nerves with defects with a maximum length of 3 cm (Moore *et al.* 2009; Wangensteen and Kalliainen 2010; Carriel *et al.* 2014a).

Despite these advances, nerve autografting remains as the gold standard technique to bridge critical nerve gaps. For this reason, the current research has the aim to develop more functional substitutes, which provide the essential elements to the regenerative microenvironment and mimic the three-dimensional (3D) structure of PN (Carriel et al. 2014a). In this sense, the *in vitro* studies play an important role in testing and characterizing different biomaterials for their application in nerve repair (Wang *et al.* 2009; Wrobel *et al.* 2014). In the case of the *in vivo* studies, a range of strategies were tested, including the use of micropatterned structures, aligned biomaterials and the incorporation of cellular intraluminal fillers (Rutkowski *et al.* 2004; Xu *et al.* 2011; Daly *et al.* 2012; Carriel *et al.* 2013; Georgiou *et al.* 2013; Carriel *et al.* 2014a). These strategies offer physical support for SC proliferation and migration, whilst the transplanted cells (SC or mesenchymal stem cells) could release a range of growth factors and extracellular matrix precursors which enhance nerve regeneration (Lopatina *et al.* 2011; Carriel *et al.* 2013; Carriel *et al.* 2014a).

Recently, we developed a novel fibrin and type VII agarose hydrogels (FAH), which was successfully used to create biocompatible models of cornea (Alaminos *et al.* 2006; Garzon *et al.* 2014), skin (Carriel *et al.* 2012), oral mucosa (Sanchez-Quevedo *et al.* 2007) and peripheral nerves

(Carriel *et al.* 2013; Carriel *et al.* 2014c). In the model of nerve regeneration, FAH was combined with undifferentiated autologous adipose-derived mesenchymal stem cells (ADMSC) and injected into the lumen of biodegradable collagen conduits. This strategy promoted a significant improvement of nerve regeneration at the clinical, functional and histological levels (Carriel *et al.* 2013; Carriel *et al.* 2014c). The intraluminal FAH used was composed by randomly oriented fibers with low density and poor mechanical properties. Currently, both properties can be easily improved with the use of the plastic compression technique (PCT), which allows regulating fiber density and orientation in a controlled manner (Cheema and Brown 2013). PCT was successfully applied to improve the mechanical properties of collagen (Cheema and Brown 2013) and FAH (Ionescu *et al.* 2011; Scionti *et al.* 2013). PCT produces nanoscale molecular bonds among the fibers of the biomaterials, and for this reason, it is also known as nanostructuration (Ionescu *et al.* 2011; Scionti *et al.* 2013).

This study was designed based on the promising results obtained with the use of FAH containing ADMSC in a model or nerve regeneration (Carriel *et al.* 2013; Carriel *et al.* 2014c) and, on the other hand, on the possibilities to elaborate mechanically stable substitutes by applying PCT to the FAH. For these reasons, the objective of this *in vitro* study was to develop and to characterize a novel nanostructured fibrin-agarose bioartificial nerve substitute (NFABNS) based on the use of nanostructured FAH in combination with human adipose-derived mesenchymal stem cells (HADMSC).

2. MATERIALS AND METHODS:

2.1 Cell culture:

The HADMSC used in this study were isolated, cultured under GMP-like conditions and characterized by flow cytometry in the HISTOCELL S.L. laboratories, Bilbao, Spain. Briefly, HADMSC were isolated from healthy adipose tissue from patients subjected to surgical procedures and they were isolated and cultured following previously described protocols (Nieto-Aguilar *et al.* 2011; Carriel *et al.* 2013). In the laboratory, adipose tissue biopsies were washed in sterile phosphate buffered saline (PBS), mechanically fragmented into small pieces and digested with 0.3% type I collagenase solution -Gibco BRL Life Technologies, Karlsruhe, Germany- for 8 h at 37ºC. After that, HADMSC were harvested by centrifugation and cultured in basal medium (Dulbecco's modified Eagle's medium -DMEM- supplemented with 10% fetal bovine serum -Sigma Aldrich Co.- and antibiotic-antimycotic cocktail solution -100 U mL^{-1} of penicillin G, 100 $\mu g\ mL^{-1}$ of streptomycin and 0.25 $\mu g\ mL^{-1}$ of B amphotericin, Sigma Aldrich Co., Steinheim, Germany-) at 37ºC and 5% carbon dioxide. In addition, HADMSC were characterized by flow cytometry as follows: positive for the markers CD90, CD13, CD105, CD44 and negative for CD45, CD34, CD14, HLA-DR. The culture medium was renewed every three days and the clinical-grade HADMSC were expanded until passages 4-6 and used for the fabrication of hydrogels.

2.2 Preparation of FAH:

In this study, FAH were elaborated by following previously described protocols (Alaminos *et al.* 2006; Carriel *et al.* 2012; Carriel *et al.* 2013; Scionti *et al.* 2013; Garzon *et al.* 2014). In order to prepare 10 mL of FA hydrogel, 7.6 mL of Human plasma, 1.25 mL of DMEM containing or not HADMSC (with a cellular density of 50,000 cells/mL), and 0.15 mL of tranexamic acid (Amchafibrin, Fides-Ecofarma, Valencia, Spain) were used. The solution was mixed and 1 ml of 2% $CaCl_2$ (to promote the gelation) and 0.5 mL of melted 2% type VII-agarose were added, carefully mixed and kept at 37ºC in 60 mm Petri dishes until its complete gelation. As a result, we obtained cellular and acellular FAH with a volume of 10 ml, 5 mm of thickness and 6 cm of diameter (Fig. 1 A).

Acellular FAH were kept with basal medium for 24 h at 37ºC and then used to fabricate acellular NFABNS (a-NFABNS) for mechanical characterization. In the case of the cellular FAH, these were kept in culture conditions with basal medium for 5 days in order to favor cell adaptation prior to the fabrication of cellular NFABNS (c-NFABNS).

2.3 Fabrication of NFABNS:

To fabricate cellular and acellular NFABNS, the FAH were subjected to PCT following previously described and standardized procedures (Ionescu *et al.* 2011; Scionti *et al.* 2013). Briefly, we placed squares of FAH (3 x 3 x 0.5 cm) between a couple of nylon filter membranes with 0.22 µm pore size (Merck-Millipore, Darmstadt, Germany), and we compressed them between a pair of sterile Whatman 3-mm absorbent pieces of paper below a flat glass surface. We applied uniform mechanical pressure (500 g homogeneously distributed) for 2.5 min (Fig. 1 B), and as result, we obtained a high density nanostructured FAH (NFAH) with approximately 80% of hydration and 50-60 µm of thickness (a compression of 100 times approximately) (Scionti et al. 2013). Finally, NFAH were used to fabricate cellular or acellular NFABNS with a length and diameter comparable to the adult rat sciatic nerve. For this purpose, NFAH were rolled to form multilayered cylinders of 3 cm of length and ~1.5 mm of diameter (Fig. 1 C).

2.4 Experimental groups:

In this study, the novel NFABNS were mechanically and microscopically characterized. For the mechanical characterization, c-NFABNS and a-NFABNS were subjected to tensile test at 0, 8 and 16 days of *in vitro* development (IVD). In addition, native rat sciatic nerves were used as control group (N-CTR). For the microscopical analyses, c-NFABNS and a-NFABNS were cut in segments of 1 cm of length and placed in six-well plates with 3 mL of basal culture medium and kept in static culture conditions during 16 days. Additionally, cellular FAH (uncompressed) were kept in culture and used as control group. The culture medium was renewed every three days and samples were harvested for histological and ultrastructural evaluation at 0, 2, 4, 8 and 16 days of IVD.

2.5 Tensile test:

N-CTR, c-NFABNS and a-NFABNS (all of 3 cm of length and ~1.5 mm of diameter) were subjected to tensile tests (n=10 each) by using an electromechanical material testing instrument (Instron, Model 3345-K3327) as previously described (Scionti et al. 2013). For this test, samples were oriented with their length along the direction of tension and clamped at each end leaving a constant distance of 1 cm between the clamps (in all cases 1 cm of the sample was gripped between each clamp). The tests were run at a constant strain rate of 5 mm/min. at room temperature. The Young's modulus was calculated as the tangent modulus of the initial, linear portion of the stress-strain curve of each experimental run, while the stress at break (σ break) and the strain at break (Ɛ break) values were determined by selecting the point of the stress-strain curve where the fracture occurred. A 500N Instron load cell sampling at 10Hz was used to obtain the data for the stress-strain curves. Calculation of the average value and standard deviation of the results for each experimental run was operated automatically using the Instron BlueHill2 Material Testing software.

The native rat sciatic nerves were obtained from five adult Wistar rats from the Experimental Unit of the Virgen de las Nieves University Hospital in Granada (Spain). These animals were housed in a temperature-controlled room (21±1°C) on a 12h light/dark cycle with *ad libitum* access to tap water and standard rat chow. The animals were euthanatized under general anesthesia and 3 cm of both sciatic nerves were carefully dissected before the distal bifurcation of the nerve. These studies were performed according to the European Union and Spanish Government guidelines for the ethical care of animals (EU Directive 63/2010, RD 53/2013), and this project was approved by the ethical committee of Granada (FIS PI14/01343).

2.6 Histological evaluation:

For the histological and immunohistochemical analyses, c-NFABNS, a-NFABNS (harvested and cut in fragments of 0.5 cm) and FAH were fixed in 10% buffered formalin for 24h at room temperature. Fixed samples were dehydrated, embedded in paraffin and transversal 5 µm sections were obtained from their central part. Sections were stained with hematoxylin-eosin (HE) and observed with a light and fluorescence microscopy (Texas Red Ex 540-580 nm/DM 595 nm/ BA 600-660). Acid proteoglycans were histochemically identified with alcian blue staining (AB) and contrasted with nuclear fast red as previously used (Carriel *et al.* 2012; Oliveira *et al.* 2013).

To study the cell behavior, different cellular processes were analyzed. In order to determine the number of cells, tissue sections were stained with 4',6-diamidino-2-phenylindole (DAPI). The immunohistochemical detection of the proliferating cell nuclear antigen (PCNA) was used to identify proliferating cells along the IVD (Martin-Piedra *et al.* 2014). The active actin cytoskeletal remodeling (associated to lamellipodia formation or cell migration) was evaluated by the identification of cortactin (cortical actin binding protein) (Ren *et al.* 2009). The capability of cells to synthesize the

three main components of the ECM (fibers, glycoproteins and proteoglycans) was analyzed by the immunohistochemical identification of collagen type I, laminin and decorin proteoglycan.

For immunohistochemistry, tissue sections were dewaxed, hydrated and pretreated for antigenic unmasking. In the case of laminin, we followed a previously described procedure (Carriel *et al.* 2013; Carriel *et al.* 2014b). Endogenous peroxidase activity was blocked with 3% (v/v) H_2O_2 in 0.1 M PBS for 10 min, whilst the nonspecific binding of the primary antibody was blocked using Cas-Block solution (Invitrogen, Carlsbad, CA, USA) for 15 min. Then, sections were incubated with the primary antibodies at room temperature, rinsed in PBS and then incubated with secondary antibody conjugated with peroxidase molecules. Finally, the antigen-antibody reaction was visualized with diaminobenzidine (DAB) (Vector, Burlingame, CA, USA) and contrasted with Mayer hematoxylin. For each immunohistochemical reaction, negative controls were used by omitting the primary antibody. In addition, human skin was used as external positive control, due to the well-known immunoreactivity of the skin for all the antibodies used (pictures not shown). All these procedures were performed at the same time with the same conditions to ensure reproducibility of the results. All the technical information related to the immunohistochemical markers is summarized in Table 1.

2.7 Structural and ultrastructural evaluation:

For the structural and ultrastructural evaluation of FAH, c-NFABNS, a-NFABNS, samples were fixed in 2.5% glutaraldehyde in 0.05M cacodylate buffer (pH 7.2) at 4ºC for 6 h. Then, samples were washed three times in 0.05M cacodylate buffer (pH 7.2) at 4ºC and randomly assigned to scanning electron microscopy (SEM) or semithin and transmission electron microscopy (TEM).

For SEM, samples were dehydrated in increasing concentrations of acetone (30 to 100%), subjected to the critical point method and covered with gold. The analyses and pictures were carried out by using a FEI Quanta 200 environmental scanning electron microscope (FEI Europe, Eindhoven, Netherlands).

For the semithin and ultrastructural analyses by TEM, samples were post-fixed with 1% osmium tetroxide for 1h at room temperature and washed. After that, samples were placed in 0.15% tannic acid (mordant), washed and en bloc stained with 2% aqueous uranyl acetate for 2h. Finally, the samples were dehydrated in increasing concentrations of alcohol and embedded in epoxy resin. Semithin sections of 1 µm thickness were obtained and then stained with a modified HE protocol. Briefly, epoxy resin was removed with NaOH saturated alcoholic solution for 1h, hydrated and stained with Mayer hematoxylin for 20 min. at 30ºC, differentiated and contrasted with acidified eosin-phloxine during 2 min. Stained slides were dehydrated, diaphanized and covered for light microscopy.

For the ultrastructural analysis of the c-NFABNS, ultrathin sections of 50-nm-thickness were stained with 2% uracil acetate, lead citrate and transferred to mesh grids. The analysis and pictures were carried out by using a JEOL JEM 1200 EX II transmission electron microscope (JEOL LTD., Akishima Tokyo, Japan).

2.8 Quantitative and statistical analyses:

To perform statistical analysis, we first confirmed the normality of the distribution of each variable by using the Kolmogorov-Smirnov statistical test. The tensile test results were non-normally distributed. Therefore, Mann-Whitney non-parametric test was used to compare the Young's modulus, the stress at break and tensile strain at break between the CTR, a-NFABNS and c-NFABNS.

The number of cells and cell proliferation index in the FAH and c-NFABNS at each day of IVD were determined in histological sections stained with DAPI and PCNA, respectively. For both analyses, sections corresponding to three different samples at each day were evaluated, and six images were taken at x200 magnification (area of 122.500 μm^2) from each group (n=18 in each). For the number of cells, images were converted to binary color and the number of cells was determined by using the "analyze particles" function (particle size=0-500 $pixels^2$) of the Image J software (National Institute of Health, USA) as previously described (Oliveira *et al.* 2013; Martin-Piedra *et al.* 2014). The number of cells was normally distributed and the ANOVA was used to determine statistical significance of the number of cells in the FAH and c-NFABNS along the 16 days of IVD. For the cell proliferation index, the number of PCNA positive cells was calculated from the total cells per analyzed area and expressed in percentage.

In this study, the results of each variable were expressed as mean and standard deviation (SD) values, and data were analyzed with the SPSS 16.00 software. The values $p < 0.05$ were considered as statistically significant in two-tailed tests.

3. RESULTS:

3.1 Mechanical characterization of acellular NFABNS:

Tensile tests of N-CTR showed average values of tensile stress at fracture of 2.14 MPa, Young's modulus of 8.56 MPa and 49% of deformation capability (strain at fracture). Tensile tests of NFABNS at day 0 showed significantly lower values of tensile stress at fracture (0.42 MPa, $p<0.05$) and Young's modulus (0.30 MPa, $p<0.05$). However, the deformation capability of the NFABNS (169%, $p<0.05$) was significantly higher than the strain at fracture of the N-CTR (48.3%) (Table 2). Tensile tests of c-NFABNS after 8 and 16 days of IVD revealed a significant decrease of tensile stress at fracture, Young's modulus and strain at fracture values as compared to the NFABNS at 0 day ($p<0.05$). In addition, when we compared the tensile test results between 8 and 16 days of IVD, a significant decrease of the average values of tensile stress at fracture and Young's modulus were found ($p<0.05$), but not for the strain at fracture values ($p=0.36$). Although there was a significant decrease of the tensile properties with the incorporation of the HADMSC into the NFABNS over the time, all NFABNS showed significantly higher deformation capability than the N-CTR ($p<0.05$) (Table 2).

3.2 Histological analysis:

The histological analysis carried out on semithin sections stained with HE allowed us to identify the HADMSC in both c-NFABNS and FAH (CTR group) (Fig. 2). In the CTR group, the cells were found immersed in a loose FAH with a random distribution and irregular morphology from 0 to 16 days of IVD. In addition, signs of degradation of the hydrogels were observed at 8 and 16 days of IVD (representative pictures are shown in Fig. 2). During the first hours after c-NFABNS fabrication, it was possible to observe that the HADMSC were properly integrated into the nanostructured FAH layers, which were evidently denser than the control group. Interestingly, following nanostructuration, the cells acquired a flat morphology and followed the concentric orientation of the nanostructured biomaterials, which differs from the cellular morphology and distribution observed in FAH group (Fig. 2). After 2 days, it was possible to observe that the cells began forming small cell clusters with a slight displacement of the surrounding scaffold. However, this activity was less evident after 4 days of IVD, when the size of cells and clusters was slightly decreased (Fig. 2).

After 8 days of IVD, we observed a clear change in the cell behavior with an impact in the surrounding scaffold. The cells formed cell clusters, from which cells degraded the dense NFAH biomaterial with the consequent formation of small and irregular cavities. Interestingly, the cells inside the cavities were immersed in a loose FAH network and they preserved the concentric orientation of the NFAH layers. Morphologically, the cells increased their size, adopted an elongated phenotype and the nucleus and nucleolus were more prominent. After 16 days of IVD, these changes were more evident and bigger cell clusters and interconnected cavities comprising several NFAH layers were observed, which were similar to the degradation observed in the FAH group. Morphologically, the cells were elongated with a prominent nucleus and nucleolus, immersed in a loose network, and they preserved the concentric orientation (Fig. 2).

3.3 Histochemical and immunohistochemical analyses:

The analysis revealed that nanostructuration of the FAH had an impact on cellularity, cell behavior and ECM deposition in comparison to uncompressed FAH (control) (Fig. 3). DAPI fluorescent staining showed that the cells were homogeneously distributed in the c-NFABNS and FAH. In addition, DAPI histological and quantitative analysis revealed that the nanostructuration technique induced a significant increase ($p<0.05$) of the cell concentration in the c-NFABNS as compared to the FAH in the same analyzed area (Fig. 3, 0-IVD; Fig. 4). The DAPI analysis of the c-NFABNS confirmed the progressive reorganization of the cells, which formed several cell clusters. When we quantitatively determined the cellularity of the c-NFABNS, we found a progressive and significant decrease ($p< 0.05$) of the number of cells along the 16 days of IVD in comparison to the c-NFABNS at day 0. However, we observed a significant increase ($p< 0.05$) of the number of cells from 8 day onward (Fig. 3 and 4). In contrast, a significant and progressive increase of the cellularity was observed in FAH along the time ($p< 0.05$), which was significantly higher than the c-NFABNS at the 8 and 16 days of IVD ($p<0.05$) (Fig. 3 and 4).

In relation with the cell proliferation, the immunohistochemical identification of PCNA (Fig. 3) and the cell proliferation index (Fig. 4) revealed the presence and percentage of cells with active proliferation in both c-NFABNS and FAH during IVD. The PCNA analysis of the c-NFABNS showed a decrease of the PCNA staining at day 2 with a cell proliferation index of 21.1%. However, cell proliferation was reactivated from day 4, reaching to a cell proliferation index of 68.7% after 16 days of IVD (Fig. 3 and 4). In the FAH groups, this index varied along the analyzed periods, but it was always over the 50%, and reached 80% after 16 days of IVD (Fig. 3 and 4).

The actin cytoskeletal remodeling of the cells was evaluated by the immunohistochemical identification of cortactin. This analysis demonstrated that cells were highly active along the analyzed periods in the FAH group (Fig. 3). In contrast, the cells were positive for cortactin in c-NFABNS group, but this expression decreased at day 2, and it was practically negative after 4 days of IVD. However, the cells showed an intense positive reaction for cortactin from day 8 onward (Fig.3).

The comparative analysis of DAPI, PCNA and cortactin between c-NFABNS and FAH showed differences that demonstrated that the HADMSC were functionally affected by PCT. However, these cells were able to degrade the scaffold (Fig. 3), proliferate and form interconnected cavities inside the NFABNS (Fig. 5 and 6).

The histochemical and immunohistochemical analyses of the ECM demonstrated that the HADMSC has the capacity to synthesize some ECM molecules in the c-NFABNS and FAH (Fig. 5 and 6). Alcian blue histochemical analyses of both c-NFABNS and FAH revealed a progressive synthesis of acid proteoglycans from the cells, especially after 8 and 16 days of IVD (Fig. 5). The immunohistochemical analysis of laminin glycoprotein revealed differences between both groups. In the FAH group, laminin was observed in the cytoplasm of some cells and its expression decreased along the time. In contrast, in the c-NFABNS laminin was first cytoplasmic and progressively extracellular, and its expression did increase after 16 days (Fig. 5). The immunohistochemical analysis of decorin in both groups revealed a weak cytoplasmic positive reaction at the first two days, but it became negative from day 4 onward (Fig. 6). The immunohistochemical analysis of collagen type I revealed a positive reaction in both groups. In the FAH group, this protein was first cytoplasmic and then pericellular, but it tended to decrease after 16 days. Surprisingly, an important increase of the extracellular collagen content was found in the c-NFABNS after 16 days (Fig. 6).

3.4 Structural and ultrastructural analyses:

The optical and SEM analyses revealed structural differences between the c-NFABNS, a-NFABNS and FAH over the time (Fig. 7). These analyses showed the characteristic multilayered structure of the c-NFABNS, a-NFABNS, and a random fiber orientation in FAH (Fig. 7). The multilayered pattern and dimensions of both NFABNS were stable over the time and some structural changes were observed in the c-NFABNS (Fig. 7). Interestingly, the structural changes observed in the c-NFABNS were strictly related to the HADMSC. These cells progressively degraded the scaffold and created

tridimensional and interconnected cavities around the central core of the substitute. These cavities were clearly identified after 8 days of IVD, but they were evidently larger and more abundant after 16 days of IVD. In contrast, the a-NFABNS were structurally uniform with a compact morphology, and we did not observe any sign of degradation during the analyzed period. In the case of the FAH, it was possible to observe an increase of the cells which progressively covered the surface of the construct and acquired an elongated and irregular morphology with a random orientation (Fig. 7).

TEM analysis c-NFABNS showed the intra and extracellular ultrastructural changes that took place during the IVD (Fig. 8). The analysis at the second day revealed that most HADMSC had a metabolically active profile characterized by prominent nuclei, lax chromatin, the presence of prominent nucleoli, a well-developed rough endoplasmic reticulum (RER) and an intact plasma membrane. Besides, these cells were observed with an elongated shape and completely surrounded by a highly dense FA hydrogel. After 8 days, it was possible to identify several active cells interacting with the surrounding fibers. However, some cells showed irreversible damage, which was characterized by cytoplasmic vacuolization, rupture of organelles and discontinuous plasma membrane (Fig. 8). Finally, TEM analysis at the 16 days showed predominance of metabolically active cells forming clusters inside the cavities. Interestingly, it was possible to observe the presence of a non-fibrillar ECM material around the cell surface and among the FA hydrogel fibers (Fig. 8). This result suggests an active synthesis of ECM molecules from the HADMSC, but it was not possible to confirm the synthesis and assembly of the mature collagen fibrils, which are characterized by the presence of periodically oriented bands.

4. DISCUSSION:

In this *in vitro* study, we described the fabrication and characterization of a novel FA tissue-like substitute called NFABNS that could be used for neural applications. This novel NFABNS was mechanically characterized and the behavior of the HADMSCs within the substitute was evaluated during 16 days of IVD. Over the recent years, uncompressed FAH were successfully used in tissue engineering for the generation of several tissue models (Alaminos *et al.* 2006; Carriel *et al.* 2012; San Martin *et al.* 2013; Garzon *et al.* 2014), including peripheral nerve repair (Carriel *et al.* 2013; Carriel *et al.* 2014c). Unfortunately, FAH have poor mechanical stability to direct bridge nerve gaps, which limits their potential use for the reconstruction of peripheral nerves. Recently, it was demonstrated that nanostructuration significantly improves the mechanical properties of FAH in a controlled manner and according to specific needs (Scionti et al. 2013). For this reason, NFABNS were generated based on NFAH which are characterized by their mechanical stability, high fiber density and structural homogeneity. In this study, NFAH were successfully rolled for the generation of mechanically stable cellular or acellular NFABNS with comparable diameter and length to the rat native sciatic nerve.

The tensile analysis of NFABNS demonstrated that these substitutes showed comparable Young's modulus with the previously characterized NFAH (Scionti et al. 2013). Interestingly, the stress at

break values demonstrated that these nerve substitutes were more resistant than the simple sheet of NFAH previously described (Scionti et al. 2013). When we compared the NFABNS vs. native rat sciatic nerves we found that our NFABNS did not have the high elasticity and resistance of the control nerve under tensile stress, but they showed a significantly higher deformation capability than this organ. These differences could be explained by the high organization and complex composition of the peripheral nerve stroma, especially the perineurial sheath around each nerve fascicle (Carriel et al. 2014a). Although our NFABNS did not have the tensile properties of the rat sciatic nerve, we hypothesize that these substitutes have enough resistance and deformation capability for use in peripheral nerve repair.

Natural and biodegradable hydrogels are considered as useful biomaterials for peripheral nerve repair (Daly *et al.* 2012; Carriel *et al.* 2014a). Previous biomechanical studies demonstrated that PCT is able to significantly improve the tensile properties of acellular and cellular collagen hydrogels (HOS TE85, ATCC cell line) (Bitar *et al.* 2007). Interestingly, these properties decreased in function of the time in culture (10 days *in vitro*), and the cells did not show a significant impact on these results (Bitar *et al.* 2007). In our case, the incorporation of HADMSC into the NFABNS significantly decreased the tensile mechanical properties of the NFABNS over the time. However, c-NFABNS preserved their mechanical stability, high deformation capability and structure. These differences with the tensile properties of the collagen hydrogels could be related technical differences between both studies (construct dimensions, parameters analyzed, equipments and cells used, etc) and, especially, to the analyzed periods of time.

The histological and ultrastructural analyses revealed structural modifications of the substitutes by the HADMSC and the impact of the nanostructuration method on cell behavior as compared to uncompressed FAH. In this study, the HADMSC grew in cell clusters, progressively degraded the surrounding scaffold and created interconnected cavities inside the NFABNS. In contrast, these cells did not grow in cell clusters in uncompressed FAH, but they also actively degraded the scaffold. In a previous study carried out by our group, *in vitro* degradation of the fibrin and FAH was also observed (San Martin *et al.* 2013). These results demonstrated a progressive recovery of the HADMSC metabolism in NFABNS, and suggest the activation of proteolytic enzymes in response to the surrounding scaffold, as previously observed (Alaminos *et al.* 2007). Finally, we observed a correlation between the progressive formation of interconnected cavities and the decrease of the tensile properties of the c-NFABNS, and both processes took place in function of the presence of cells and *in vitro* time.

The effect of nanostructuration on cell behavior was confirmed by the significant decrease of the cellularity of c-NFABNS and by the significant and progressive increase of this parameter in FAH. Interestingly, c-NFABNS revealed a significant increase of the cellularity between 8 and 16 days, which was correlated with the increase of the cell proliferation (PCNA quantitative analysis), the reappearance of cortactin immunohistochemical expression and cluster formation. Furthermore, TEM analysis revealed that some cells were negatively affected, while others progressively

acquired a metabolically active phenotype with a positive cell-biomaterial interaction. These results are in agreement with previous studies demonstrating that uncompressed FAH supported cell proliferation, migration and differentiation of different cells types (Alaminos *et al.* 2006; Cardona Jde *et al.* 2011; Carriel *et al.* 2012; San Martin *et al.* 2013; Garzon *et al.* 2014). In addition, the impact of nanostructuration on cell behavior found in this work is supported by studies carried out with nanostructured collagen hydrogels, demonstrating that a decrease of oxygen diffusion may be associated to PCT ant this would have an impact on cell function and viability (Cheema *et al.* 2008; Cheema *et al.* 2010). Although this hypoxic microenvironment is an unwanted effect, our results along with the previous studies demonstrate that cells have high capacity to recover their function, reactivate their metabolism, proliferate and migrate. As previously suggested, the angiogenic factors released by cells could stimulate *in vivo* vascularization of the tissue substitutes once grafted (Bitar *et al.* 2007; Cheema *et al.* 2008; Cheema *et al.* 2010). In addition, the natural formation of interconnected cavities in our NFABNS may have favored cell function recovery, likely due to the increase of the oxygenation and nutrient diffusion, as previously observed in channeled collagen tissue-engineered constructs (Cheema *et al.* 2010).

Natural ECM plays an important role in supporting and guiding peripheral nerve regeneration (Chernousov *et al.* 2008; Carriel *et al.* 2013; Carriel *et al.* 2014a). In this sense, engineered cellular substitutes based on natural ECM molecules able to support the *in vitro* synthesis of ECM could be promising alternatives for several tissue engineering applications. Unfortunately, the resident cells could take weeks/months (if ever) to synthesize ECM molecules within the scaffold (Cheema and Brown 2013). In the case of uncompressed fibrin and FAH, it was demonstrated that different cell types were able to synthesize the three main elements of the ECM (fibers, proteoglycans and glycoproteins) over the time (Carriel *et al.* 2012; San Martin *et al.* 2013; Garzon *et al.* 2014). Interestingly, the ECM synthesis was affected by the content of agarose, thus demonstrating that the biomaterials had an impact on ECM synthesis (Ionescu et al. 2011). In this study, we used several histological techniques to demonstrate if HADMSC were able to synthesize the three main components of the ECM into the NFABNS and FAH. In the case of the acid proteoglycans (alcian blue staining) and laminin, HADMSC progressively synthesized both elements with a clear extracellular pattern that filled the cavities created around the cells in both groups, especially in c-NFABNS. When we analyzed decorin and collagen type I, we found that decorin was cytoplasmatic at the beginning of the study in both groups, but its expression decreased and became negative after 16 days. Conversely, collagen type I was initially cytoplasmic and progressively pericellular and finally extracellular in c-NFABNS. The analysis of collagen type I in FAH, revealed a slight increase of this protein, but it was mainly cytoplasmic and pericellular, and did not reach high levels of positivity and distribution, according to our previous studies carried out with uncompressed FAH (Carriel *et al.* 2012; San Martin *et al.* 2013; Garzon *et al.* 2014). Strikingly, we identified immunohistochemically the presence of collagen type I, but the TEM analysis revealed that HADMSC produced a non-fibrillar ECM material and it was not possible to confirm the synthesis of

mature collagen fibrils. These results could suggest that the collagen type I identified by immunohistochemistry corresponded to pro-collagen or even collagen with low levels of extracellular assembly. Collagen fibrillogenesis is a complex and multi step process where decorin, biglycan and others proteoglycans regulate collagen assembly and reorganization at the extracellular level (San Martin et al. 2013). More recently, an interesting *in vitro* study demonstrated that the incorporation of decorin during collagen type I fibrillogenesis resulted in an increase and better organization of the collagen hydrogels, suggesting that decorin could play a key role in the extracellular reorganization of collagen type I (Reese *et al.* 2013). In this regard, the lack of decorin expression could explain the absence of mature and well organized collagens in c-NFABNS. However, our results demonstrate that HADMSC were able to produce the three main elements of the ECM in c-NFABNS, thus enriching the molecular composition of our tissue-like substitute.

Currently, several studies support the hypothesis that the combination of natural biomaterials and cells is an efficient strategy to increase the degree of peripheral nerve regeneration and functional recovery (Daly *et al.* 2012; Carriel *et al.* 2014a). In this regard, ADMSC are considered as one of the most promising alternatives in peripheral nerve repair (Faroni *et al.* 2013; Carriel *et al.* 2014a). Indeed, undifferentiated and differentiated to SC-like cells were used in peripheral nerve repair and a significant improvement of the regeneration and functional recovery was achieved (Lopatina *et al.* 2011; Carriel *et al.* 2013; Faroni *et al.* 2013; Carriel *et al.* 2014a; Carriel *et al.* 2014c). These previous results along with the success obtained with the combination of FAH and ADMSC in nerve repair support the use of undifferentiated HADMSC in our NFABNS. In relation to the design of the substitute, these were elaborated with a characteristic cylindrical and multilayered structure based on the peripheral nerve dimensions (length and diameter). It is especially important because these results demonstrate that substitutes with different lengths and diameters can be elaborated according to the clinical needs. Multilayered cylindrical structures were previously described in peripheral nerve repair, and it was demonstrated that multilayered decellularized scaffolds seeded with SC supported the peripheral nerve regeneration in rats (Hadlock *et al.* 2001). Similarly, multilayered collagen-based hydrogels containing SC were used to fill biodegradable conduits and successfully to repair critical nerve gaps in rats (Georgiou et al. 2013). These studies support the hypothesis that our NFABNS could be useful for neural regeneration applications, although *in vivo* studies are in need.

5. **CONCLUSION**:

These results demonstrate that it is possible to generate biomimetic and biodegradable three-dimensional tissue substitutes by using cellular nanostructured FAH. These tissue-like substitutes can be generated under a controlled manner and according to the physical, geometrical and biological needs. These substitutes showed to be highly flexible and elastic, and from a biological standpoint they allowed the HADMSC to proliferate, migrate and actively synthesize different ECM molecules. All these results suggest that these bioengineered tissue-like structures could be

potentially used for several tissue engineering applications, including peripheral nerve repair. Finally, further *in vivo* preclinical studies are still required to demonstrate the potential use of NFABNS in peripheral nerve repair.

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REFERENCES:

Alaminos, M, M Del Carmen Sanchez-Quevedo, JI Munoz-Avila, D Serrano, S Medialdea, I Carreras and A Campos (2006). "Construction of a complete rabbit cornea substitute using a fibrin-agarose scaffold." Invest Ophthalmol Vis Sci 47(8): 3311-3317.

Alaminos, M, I Garzon, MC Sanchez-Quevedo, G Moreu, M Gonzalez-Andrades, A Fernandez-Montoya and A Campos (2007). "Time-course study of histological and genetic patterns of differentiation in human engineered oral mucosa." J Tissue Eng Regen Med 1(5): 350-359.

Bitar, M, V Salih, RA Brown and SN Nazhat (2007). "Effect of multiple unconfined compression on cellular dense collagen scaffolds for bone tissue engineering." J Mater Sci Mater Med 18(2): 237-244.

Cardona Jde, L, AM Ionescu, R Gomez-Sotomayor, M Gonzalez-Andrades, A Campos, M Alaminos and M Perez Mdel (2011). "Transparency in a fibrin and fibrin-agarose corneal stroma substitute generated by tissue engineering." Cornea 30(12): 1428-1435.

Carriel, V, M Alaminos, I Garzon, A Campos and M Cornelissen (2014a). "Tissue engineering of the peripheral nervous system." Expert Rev Neurother 14(3): 301-318.

Carriel, V, J Aneiros-Fernandez, M Ruyffelaert, S Arias-Santiago, V Riady, F Izquierdo-Martinez, O Roda, M Cornelissen, A Campos and M Alaminos (2014b). "Histological and immunohistochemical study of an unusual type of gallbladder duplication." Histol Histopathol.

Carriel, V, J Garrido-Gomez, P Hernandez-Cortes, I Garzon, S Garcia-Garcia, JA Saez-Moreno, M Del Carmen Sanchez-Quevedo, A Campos and M Alaminos (2013). "Combination of fibrin-agarose hydrogels and adipose-derived mesenchymal stem cells for peripheral nerve regeneration." J Neural Eng 10(2): 026022.

Carriel, V, I Garzon, A Campos, M Cornelissen and M Alaminos (2014c). "Differential expression of GAP-43 and neurofilament during peripheral nerve regeneration through bio-artificial conduits." J Tissue Eng Regen Med.

Carriel, V, I Garzon, JM Jimenez, AC Oliveira, S Arias-Santiago, A Campos, MC Sanchez-Quevedo and M Alaminos (2012). "Epithelial and stromal developmental patterns in a novel substitute of the human skin generated with fibrin-agarose biomaterials." Cells Tissues Organs 196(1): 1-12.

Cheema, U, T Alekseeva, EA Abou-Neel and RA Brown (2010). "Switching off angiogenic signalling: creating channelled constructs for adequate oxygen delivery in tissue engineered constructs." Eur Cell Mater 20: 274-280; discussion 280-271.

Cheema, U and RA Brown (2013). "Rapid Fabrication of Living Tissue Models by Collagen Plastic Compression: Understanding Three-Dimensional Cell Matrix Repair." Adv Wound Care (New Rochelle) 2(4): 176-184.

Cheema, U, RA Brown, B Alp and AJ MacRobert (2008). "Spatially defined oxygen gradients and vascular endothelial growth factor expression in an engineered 3D cell model." Cell Mol Life Sci 65(1): 177-186.

Chernousov, MA, WM Yu, ZL Chen, DJ Carey and S Strickland (2008). "Regulation of Schwann cell function by the extracellular matrix." Glia 56(14): 1498-1507.

Daly, W, L Yao, D Zeugolis, A Windebank and A Pandit (2012). "A biomaterials approach to peripheral nerve regeneration: bridging the peripheral nerve gap and enhancing functional recovery." J R Soc Interface 9(67): 202-221.

Faroni, A, G Terenghi and AJ Reid (2013). "Adipose-derived stem cells and nerve regeneration: promises and pitfalls." Int Rev Neurobiol 108: 121-136.

Garzon, I, MA Martin-Piedra, C Alfonso-Rodriguez, M Gonzalez-Andrades, V Carriel, C Martinez-Gomez, A Campos and M Alaminos (2014). "Generation of a Biomimetic Human Artificial Cornea Model Using Wharton's Jelly Mesenchymal Stem Cells." Invest Ophthalmol Vis Sci 55(7): 4073-4083.

Georgiou, M, SC Bunting, HA Davies, AJ Loughlin, JP Golding and JB Phillips (2013). "Engineered neural tissue for peripheral nerve repair." Biomaterials 34(30): 7335-7343.

Gu, X, F Ding and DF Williams (2014). "Neural tissue engineering options for peripheral nerve regeneration." Biomaterials 35(24): 6143-6156.

Hadlock, TA, CA Sundback, DA Hunter, JP Vacanti and ML Cheney (2001). "A new artificial nerve graft containing rolled Schwann cell monolayers." Microsurgery 21(3): 96-101.

Ionescu, AM, M Alaminos, J de la Cruz Cardona, J de Dios Garcia-Lopez Duran, M Gonzalez-Andrades, R Ghinea, A Campos, E Hita and M del Mar Perez (2011). "Investigating a novel nanostructured fibrin-agarose biomaterial for human cornea tissue engineering: rheological properties." J Mech Behav Biomed Mater 4(8): 1963-1973.

Kehoe, S, XF Zhang and D Boyd (2012). "FDA approved guidance conduits and wraps for peripheral nerve injury: a review of materials and efficacy." Injury 43(5): 553-572.

Lopatina, T, N Kalinina, M Karagyaur, D Stambolsky, K Rubina, A Revischin, G Pavlova, Y Parfyonova and V Tkachuk (2011). "Adipose-derived stem cells stimulate regeneration of peripheral nerves: BDNF secreted by these cells promotes nerve healing and axon growth de novo." PLoS One 6(3): e17899.

Mackinnon, SE, VB Doolabh, CB Novak and EP Trulock (2001). "Clinical outcome following nerve allograft transplantation." Plast Reconstr Surg 107(6): 1419-1429.

Martin-Piedra, MA, I Garzon, AC Oliveira, CA Alfonso-Rodriguez, V Carriel, G Scionti and M Alaminos (2014). "Cell viability and proliferation capability of long-term human dental pulp stem cell cultures." Cytotherapy 16(2): 266-277.

Moore, AM, R Kasukurthi, CK Magill, HF Farhadi, GH Borschel and SE Mackinnon (2009). "Limitations of conduits in peripheral nerve repairs." Hand (N Y) 4(2): 180-186.

Nieto-Aguilar, R, D Serrato, I Garzon, A Campos and M Alaminos (2011). "Pluripotential differentiation capability of human adipose-derived stem cells in a novel fibrin-agarose scaffold." J Biomater Appl 25(7): 743-768.

Oliveira, AC, I Garzon, AM Ionescu, V Carriel, L Cardona Jde, M Gonzalez-Andrades, M Perez Mdel, M Alaminos and A Campos (2013). "Evaluation of small intestine grafts decellularization methods for corneal tissue engineering." PLoS One 8(6): e66538.

Pabari, A, SY Yang, AM Seifalian and A Mosahebi (2010). "Modern surgical management of peripheral nerve gap." J Plast Reconstr Aesthet Surg 63(12): 1941-1948.

Reese, SP, CJ Underwood and JA Weiss (2013). "Effects of decorin proteoglycan on fibrillogenesis, ultrastructure, and mechanics of type I collagen gels." Matrix Biol 32(7-8): 414-423.

Ren, G, MS Crampton and AS Yap (2009). "Cortactin: Coordinating adhesion and the actin cytoskeleton at cellular protrusions." Cell Motil Cytoskeleton 66(10): 865-873.

Rutkowski, GE, CA Miller, S Jeftinija and SK Mallapragada (2004). "Synergistic effects of micropatterned biodegradable conduits and Schwann cells on sciatic nerve regeneration." J Neural Eng 1(3): 151-157.

San Martin, S, M Alaminos, TM Zorn, MC Sanchez-Quevedo, I Garzon, IA Rodriguez and A Campos (2013). "The effects of fibrin and fibrin-agarose on the extracellular matrix profile of bioengineered oral mucosa." J Tissue Eng Regen Med 7(1): 10-19.

Sanchez-Quevedo, MC, M Alaminos, LM Capitan, G Moreu, I Garzon, PV Crespo and A Campos (2007). "Histological and histochemical evaluation of human oral mucosa constructs developed by tissue engineering." Histol Histopathol 22(6): 631-640.

Scionti, G, M Moral, M Toledano, R Osorio, JD Duran, M Alaminos, A Campos and MT Lopez-Lopez (2013). "Effect of the hydration on the biomechanical properties in a fibrin-agarose tissue-like model." J Biomed Mater Res A.

Wang, HB, ME Mullins, JM Cregg, A Hurtado, M Oudega, MT Trombley and RJ Gilbert (2009). "Creation of highly aligned electrospun poly-L-lactic acid fibers for nerve regeneration applications." J Neural Eng 6(1): 016001.

Wangensteen, KJ and LK Kalliainen (2010). "Collagen tube conduits in peripheral nerve repair: a retrospective analysis." Hand (N Y) 5(3): 273-277.

Webber, C and D Zochodne (2010). "The nerve regenerative microenvironment: early behavior and partnership of axons and Schwann cells." Exp Neurol 223(1): 51-59.

Wrobel, S, SC Serra, S Ribeiro-Samy, N Sousa, C Heimann, C Barwig, C Grothe, AJ Salgado and K Haastert-Talini (2014). "In vitro evaluation of cell-seeded chitosan films for peripheral nerve tissue engineering." Tissue Eng Part A 20(17-18): 2339-2349.

Xu, H, Y Yan and S Li (2011). "PDLLA/chondroitin sulfate/chitosan/NGF conduits for peripheral nerve regeneration." Biomaterials 32(20): 4506-4516.

Table 1. Antibodies used for the immunohistochemical and immunofluorescence analysis.

ANTIBODIES	DILUTION/ INCUBATION	PRETREATMENT	REFERENCE
Mouse anti-Laminin (Clone LAM-89)	1:1000 1 h.	Citrate buffer ph 6 25 min. at 95ºC. Pepsin 5 min.	Sigma Aldrich. Steinheim. Germany. Cat. nº L8271
Rabbit anti-Collagen type I	1:400 1.5 h.	Pepsin 10 min.	Acris Antibodies GMBH. Germany. Cat. nº R1038
Goat anti-Decorin	1:400 1 h.	Chondroitinase ABC 1 h. at 37ºC.	R&D System. Inc. MN. USA Cat. nº AF143
Mouse anti-PCNA (Clone PC10)	1:1000 Over night at 4ºC	Citrate buffer ph 6 25 min. at 95ºC.	Sigma Aldrich. Steinheim. Germany Cat. nº P8825
Rabbit anti-Cortactin	1:100 1 h.	EDTA ph 8 25 min. at 95ºC.	Abcam plc. Cambridge. UK Cat nº EP1922Y
Horse Anti-Mouse IgG (peroxidase)	R.T.U. 30 min.	-	Vector Laboratories. CA. USA. Cat. nº MP 7402
Horse Anti-Rabbit IgG (peroxidase)	R.T.U. 30 min.	-	Vector Laboratories. CA. USA. Cat. nº MP-7401
Horse Anti-Goat IgG (peroxidase)	R.T.U. 30 min.	-	Vector Laboratories. CA. USA. Cat. nº PI 9500

Table 2. Tensile mechanical results and statistical analysis.

GROUPS	YOUNG'S MODULUS (MPa)	STREES AT FRACTURE σ break (MPa)	STRAIN AT FRACTURE Ɛ break (%)
N-CTR	8.5 ± 2.48	2.14 ± 0.49	48.3 ± 11.96
a-NFABNS 0-IVD	0.30 ± 0.04 a	0.42 ± 0.03 a	169.6 ± 9.85 a
c-NFABNS 8-IVD	0.26 ± 0.05 a,b	0.30 ± 0.14 a,b	138.46 ± 42.47 a,b
c-NFABNS 16-IVD	0.16 ± 0.05 a,b,c	0.15 ± 0.04 a,b,c	117.98 ± 28.05 a,b

Quantitative tensile test results of the normal rat sciatic nerve (N-CTR), a-NFABNS and c-NFABNS. Results are shown as mean ± standard deviation and the comparison between the groups and times were carried out by using the Mann-Whitney test. [a] Differences vs. N-CTR were statistically significant; [b] Differences vs. a-NFABNS at day 0 of *in vitro* development (0-IVD) were statistically significant; [c] Differences between both c-NFABNS samples (8 vs. 16 days of *in vitro* development) were statistically significant.