Adipose Derived Stem Cells Reduce Fibrosis and Promote Nerve Regeneration in Rats

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ABSTRACT

Peripheral nerve regeneration is critical and challenging in the adult humans. High level of collagen infiltration (i.e., scar tissue), in the niche of injury, impedes axonal regeneration and path finding. Unfortunately, studies focusing on the modulation of scar tissue in the nerves are scarce. To address part of this problem, we have evaluated the differentiated adipose derived stem cells (dASCs) for their antifibrotic and regenerative effects in a 10 mm nerve gap model in rats. Three different animal groups (N = 5) were treated with fibrin nerve conduits (empty), or seeded with dASCs (F + dASCs) and autograft, respectively. Histological analysis of regenerated nerves, at 12 weeks postoperatively, reveled the high levels of collagen infiltration (i.e., 21.5% ± 6.1% and 24.1% ± 2.9%) in the middle and distal segment of empty conduit groups in comparison with stem cells treated (16.6% ± 2.1% and 12.1% ± 2.9%) and autograft (15.0% ± 1.7% and 12.8% ± 1.0%) animals. Thus, the dASCs treatment resulted in significant reduction of fibrotic tissue formation. Consequently, enhanced axonal regeneration and remyelination was found in the animals treated with dASCs. Interestingly, these effects of dASCs appeared to be equivalent to that of autograft treatment. Thus, the dASCs hold great potential for preventing the scar tissue formation and for promoting nerve regeneration in the adult organisms. Future experiments will focus on the validation of these findings in a critical nerve injurymodel. Anat Rec, 301:1714–1721, 2018. © 2018 The Authors. *The Anatomical Record* published by Wiley Periodicals, Inc. on behalf of American Association of Anatomists

Key words: adipose stem cells; axonal regeneration; collagen infiltration; fibrotic tissue; scar tissue; remyelination

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INTRODUCTION

Traumatic injuries to peripheral nerves are a frequent finding after hand trauma. High morbidity after nerve injuries mainly affects the younger and working population, with consequent decrease in life quality and productivity. When nerve loss of substance is present (Ngeow, 2010) current treatment consists in repair with an autologous nerve graft, with further non-negligible donor site morbidity. A spontaneous regeneration potential differentiates the peripheral nervous system from the central nervous system. However, even after direct nerve repair and microsurgical nerve coaptation, regeneration is often suboptimal with incomplete target reinnervation. Suboptimal outcome is attributed to axonal degeneration, fibrotic scar formation, and neuromas at the site of injury (Ngeow, 2010). Previous research has focused on the different cellular and structural components of the regenerating *niche* and managed to improve peripheral nerve regeneration through axonal guidance with neural conduits (Zhang et al., 2010; Wang et al., 2015), growth factor delivery (Madduri et al., 2010), cell therapy hydrogels (Masand et al., 2012) and extracellular matrix molecules (de Luca et al., 2014; de Luca et al., 2018).

Previous work of the group focused on fibrin conduits enriched with SC-like differentiated adipose derived stem cells (dASCs) in order to enhance the microenvironment and promote effective axonal regeneration (di Summa et al., 2011). Furthermore, Polydimethylsiloxane (PDMS) conduits enriched with adipose derived stem cells enhanced axonal regeneration over a critical distance of 15 mm (de Luca et al., 2018). Despite the vast literature reported on possible elements for improving nerve regeneration, studies on collagen infiltration and scar tissue formation in regenerating nerves are scarce. Nerve fibrosis and scarring have been detected when the nerve needs to grow through nonresorbable guides, especially in microchannel (FitzGerald et al., 2012) devices intended for electrophysiological recording (Lacour et al., 2009).

Microchannel interfaces have been proposed as a potential solution for applications such as prosthetic limb control or enhancing recovery after nerve injury. However, intra-channel fibrotic sheaths may become a limiting factor for its clinical applicability due to the fibrotic tissue mediated interruption of the electrical recording. Potential way to overcome this problem include modulation of the foreign body response *in vivo* to reduce the extent of fibrous tissue deposition, which may maintain the caliber of microchannel devices without impeding axonal growth (FitzGerald et al., 2012).

In fact extrinsic fibrosis due to contact to foreign bodies (e.g., synthetic or nonresorbable devices) may lead to nerve compression, hypoxia and ultimately cell death. Intrinsic fibrosis, due to the proliferation of fibroblasts in the nerve, epineurium, and surrounding matrix will eventually become a physical barrier preventing appropriate axonal regeneration (Que et al., 2013). Adipose derived stem cells have been generally appreciated for their use in clinical practice to treat scar and reduce fibrotic tissue deposition (Bruno et al., 2013; Klinger et al., 2013).

In this study, dASCs were evaluated for their ability in modulating the fibrotic tissue, that is, collagen tissue deposition, during the process of nerve regeneration. For this, fibrin conduits loaded with or without dASCs were implanted over 10 mm nerve gap injury for 3 months in rats. Extent of remyelination and collagen infiltration was analyzed as a measure of treatment effect in the animals. Animals treated with dASCs exhibited significant levels of reduction in the fibrotic tissue deposition showing the potential role of the dASCs in modulating the collagen tissue infiltration.

MATERIALS AND METHODS

Experimental Animals

Male Sprague–Dawley rats (Janvier, France) weighing about 250–300 g were used for this study. All animal protocols were approved by the local veterinary commission in Lausanne, Switzerland and were carried out in accordance with the European Community Council directive 86/609/ECC for the care and use of laboratory animals.

Cell Cultures and Differentiation

ASCs were harvested from Sprague–Dawley rats (Janvier, France) as described earlier(de Luca et al., 2018) and maintained in Modified Eagle Medium (α-MEM; Invitrogen, UK) containing 1% (v/v) streptomycin/penicillin solution and 10% (v/v) FBS. ASCs were differentiated at early passages, i.e., 4–6. When the cells attained the subconfluent sate, growth medium was replaced with fresh medium supplemented with 1 mM β-mercaptoethanol (Sigma-Aldrich, UK) for 24 h. Cells were then washed and fresh medium supplemented with 35 ng/mL all-transretinoic acid was added. 72 h later, cells were washed and differentiation medium, enriched with 5 ng/ml platelet-derived growth factor (PDGF; PeproTech, UK), 10 ng/mL basic fibroblast growth factor (bFGF; PeproTech, UK), 40 ng/mL of recombinant neuregulin-β1 (NRG1-β1), (R&D Systems, UK) and 14 μM forskolin, was added. Cells were incubated for 14 days under these conditions and differentiation growth medium was exchanged at an interval of 72 h. To confirm the effectiveness of the differentiation of stem cells, resulting SCs phenotype was confirmed by specific markers S100 and GFAP (Caddick et al., 2006; Kingham et al., 2007).

Fibrin Conduit Preparation

The fibrin conduit was prepared from two compound fibrin glue (Tisseel® Kit VH 1.0, Baxter, SA) as reported previously (di Summa et al., 2010). Fibrin glue was distributed in a silicone mold around a stainless steel core bar and pressed into shape for 5 min. This procedure allowed the creation of uniform conduits measuring 14 mm in length, with a 1 mm wall thickness and 2 mm lumen designed to bridge a 10 mm nerve gap injury.

Experimental Design and Surgical Procedure

Three experimental groups (each one formed by five animals) were included: fibrin conduit without cells (Empty), fibrin conduit seeded with dASCs (F + dASCs) and autograft control group. In all experimental groups, conduits were left in place for 12 weeks and then harvested together with the proximal and distal nerve stumps.

A few hours before implantation in the rats, cells were trypsinized and then centrifuged, 50 μL of growth medium was enriched with 2×10^6 cells and injected into the fibrin tubes as described previously (di Summa et al., 2010). In Empty group the fibrin tubes contained only growth

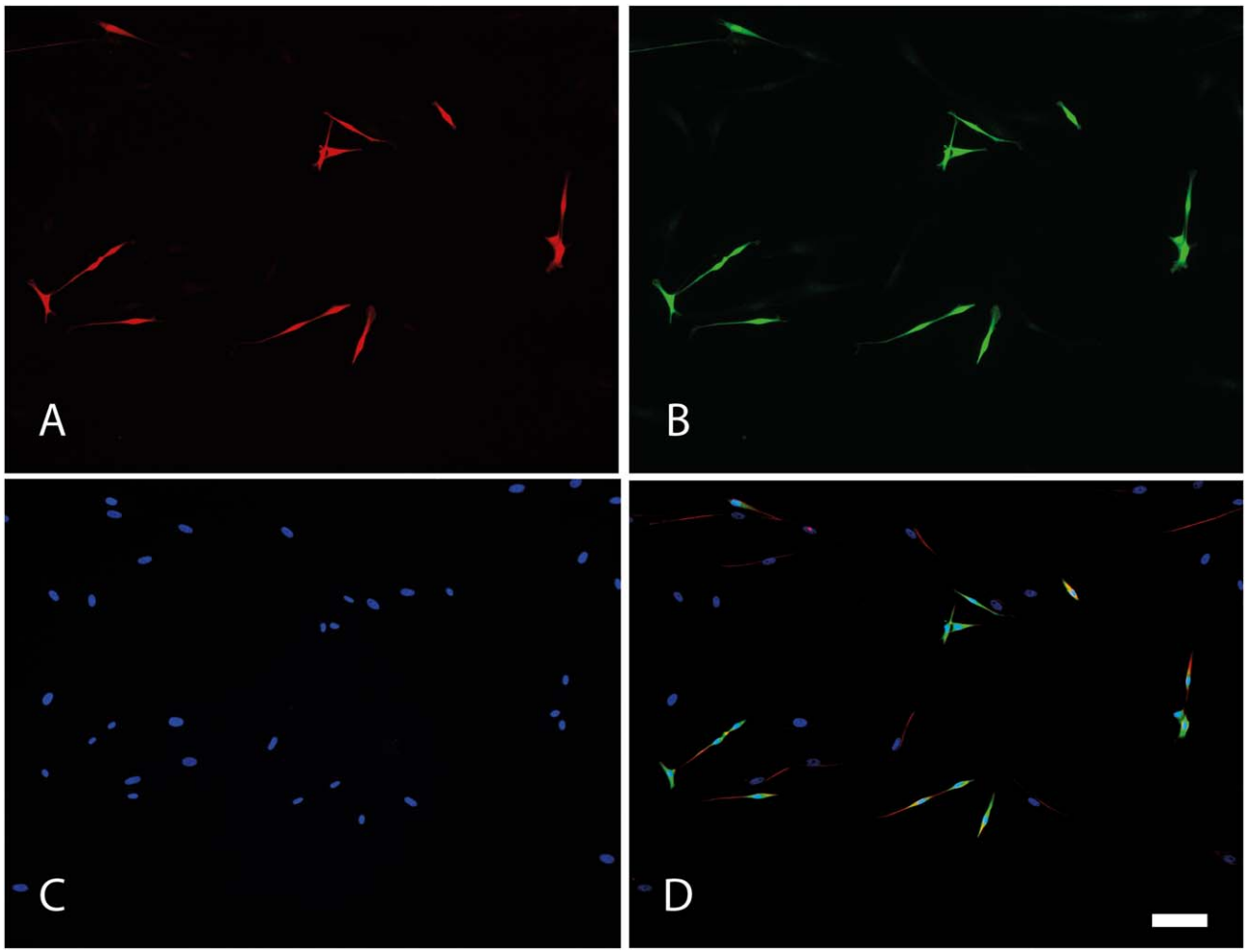

Fig. 1. Phenotype characterization of differentiated adipose stem cells (dASCs). Cells were stained for GFAP (**A**); S100 (**B**); DAPI (**C**); and merged for triple staining (**D**): scale bar = 50 μm.

medium. The operation was performed under aseptic conditions on the left sciatic nerve using a power focus surgical microscope (Carl Zeiss, Germany). An incision from the left hip to knee was made for exposing the underlying muscles, which were then retracted to expose the sciatic nerve. The sciatic nerve was gently divided which resulted in spreading of tibial and peroneal branches avoiding any nerve injury during the microdissection procedure. The peroneal segment was fully transected and nerve ends were secured to the conduit by a single epineural suture (9/0 Prolene, Ethicon, Germany). Proximal and distal nerve stumps were at 2 mm were secured into the fibrin conduit in order to leave a 10 mm gap. In the autograft group, after meticulous dissection of sciatic nerve, a 1 cm segment of peroneal nerve was excised, reversed, and sutured again on each side by the epineural sutures (Pettersson et al., 2010). Muscles and fascia layers were closed with an interrupted resorbable suture (4/0 Softcat, Braun, Germany) and the skin by an interrupted nonresorbable suture (4/0 Prolene, Ethicon, Germany). All experimental animals were housed on sawdust, one animal per cage with a 12 h light: 12 h dark cycle (lights on at 06.00 h) and received water and food *ad libitum*.

Tissue Harvesting and Processing

After 12 weeks of conduit implantation, the animals were deeply anaesthetized with an intraperitoneal injection of a mixture 1:5 of ketamine (Ketalar, Parke-Davis; 75 mg kg^{-1}) and xylazine (Rompun, Bayer; 10 mg kg^{-1}). The regenerated nerves were harvested under operating microscope with proximal and distal stump. Samples were fixed in PBS with 4% paraformaldehyde for 3 h in average and then washed and stored in 0.2 g glycine in 100 mL PBS before the embedding. On the embedding day, after a further PBS wash, nerves were immersed for 2 h in 2% osmium tetroxide (OT) (Sigma, St. Louis, MO) in the same PBS solution. The specimens were later dehydrated by an increasing alcohol series starting from 30% ethanol then the nerves were embedded in paraffin. Serial 5-μm thick cross sections were cut using a rotary ultramicrotome (Leica Mycrosystems, Wetzlar, Germany). One out of two slides were then stained with Masson's trichrome (TM) according to an established protocol (Di Scipio et al., 2008). The cross sections of nerves were collected starting 1 mm distally to the distal prolene suture (where the distal nerve stump had been originally sutured to the fibrin conduit) to allow

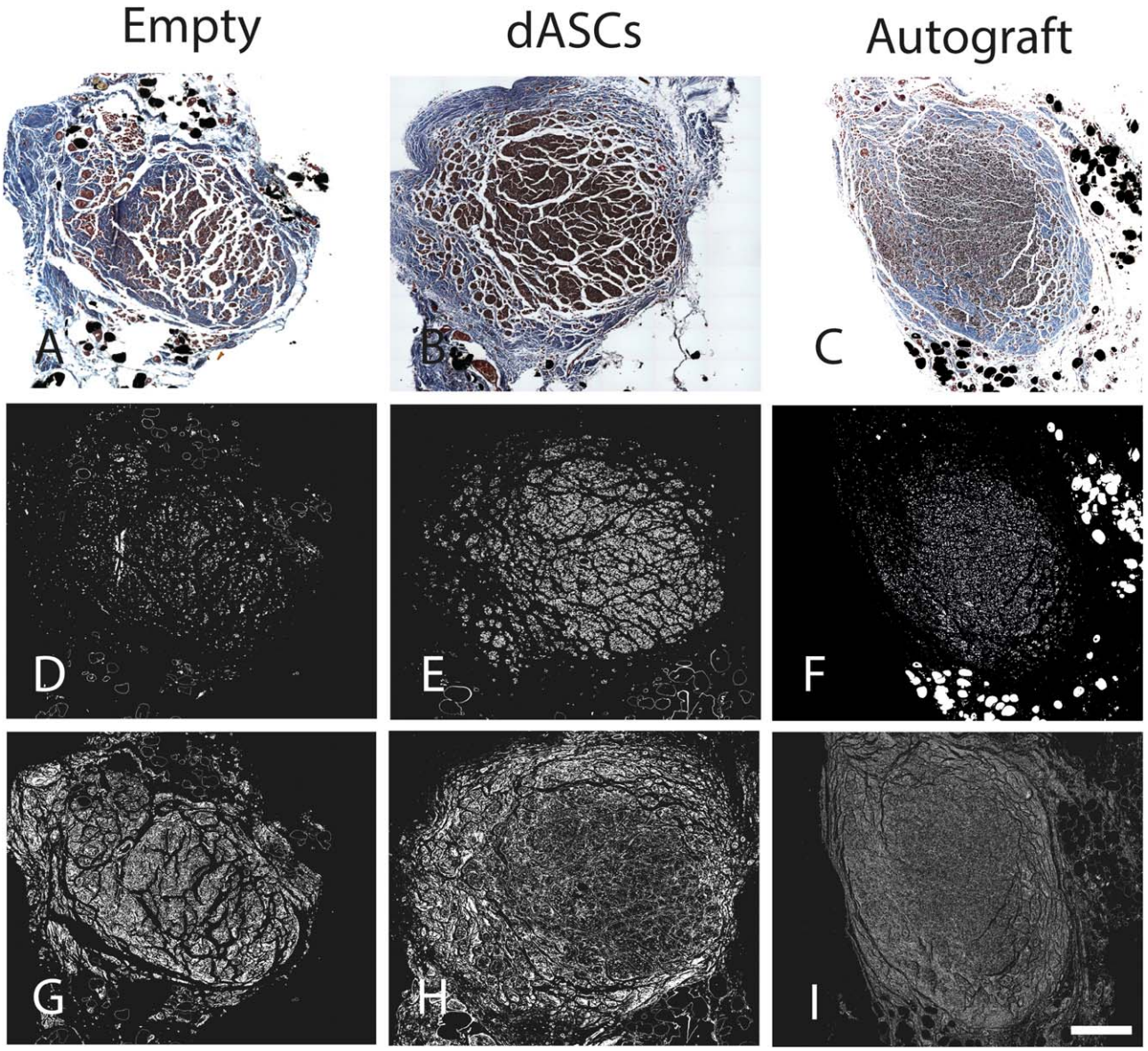

Fig. 2. Cross-sections of regenerated rat sciatic nerve from mid conduit, processed for TMB.staining (**A**–**C**); myelin surface threshold definition (**D**–**F**) and collagen-stained threshold (**G**–**I**): scale bar = 100 μm.

visualization of regenerated fibers entering the distal nerve stump. Similarly, sections from the mid part of the regenerated nerve were also obtained.

Collagen Infiltration and Myelinated Surface Analysis

Masson's trichrome (TM) stained cross-section were included in the collagen infiltration analysis process and standard osmium tetroxide (OT) stained cross section were included in the myelinated surface analysis process. All images were converted to an 8bit grey scale for software processing (Image Pro Plus, Media Cybernetics Manufacturing, Rockville, MD). By image processing of the area of interest it was possible to isolate either collagen or myelin areas according to the color selected; black color was chosen for myelin isolation in OT stained slides and blue for collagen isolation in TM stained slides. As previously reported (Gibbons et al., 2010; Huang et al., 2011) the software highlighted either myelin or collagen-infiltrated surface, quantifying the number of highlighted pixels within the area of interest. Once isolated, the software measured the processed area, which was expressed in percentage referring to the nerve cross-section area.

Data and Statistical Analysis

One-way analysis of variance (ANOVA) with Bonferroni multiple comparison test was used to statistically analyse histomorphometric data (GraphPad Prism 6.00, San Diego, USA). Significance was determined as

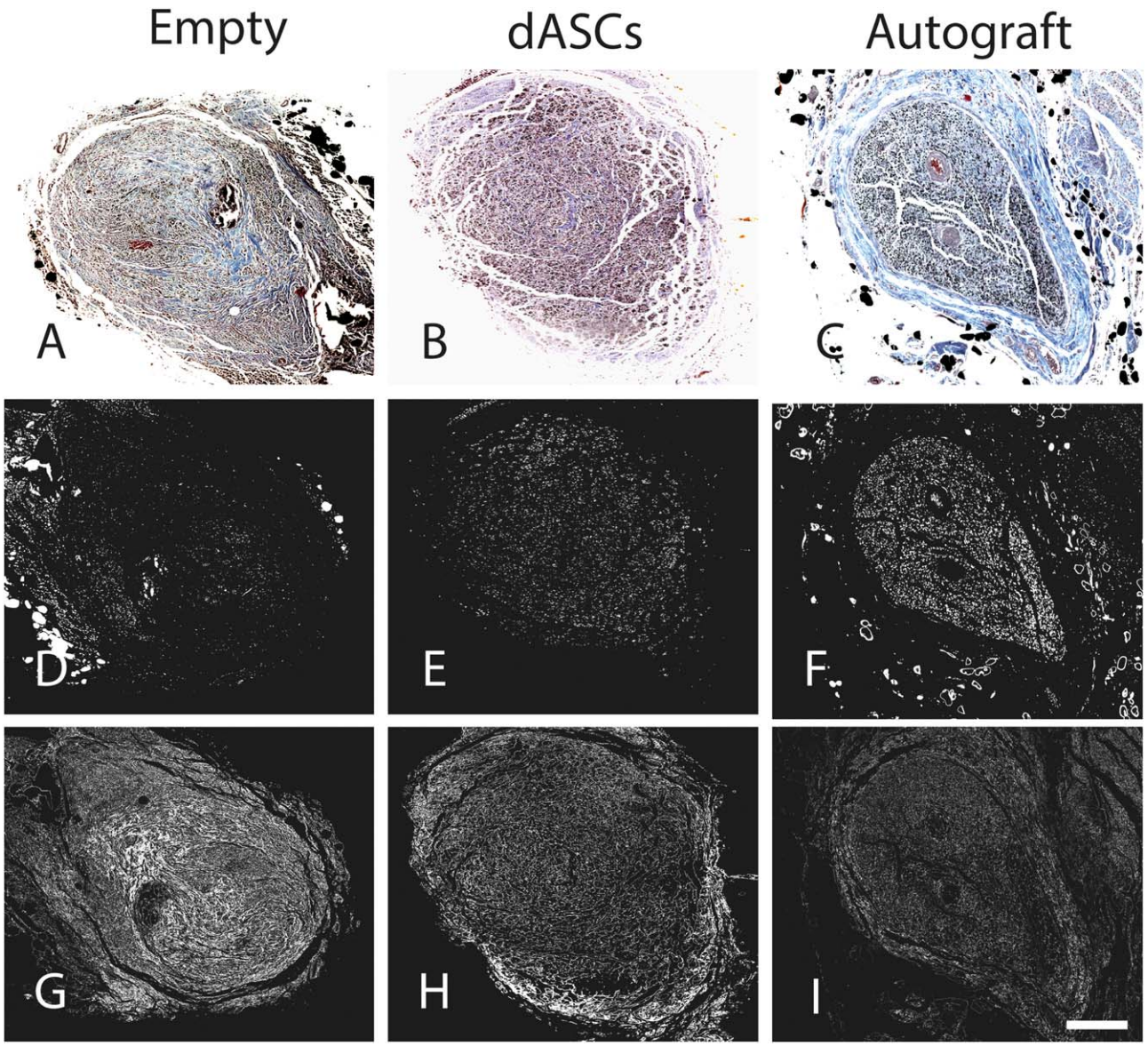

Fig. 3. Cross-sections of regenerated rat sciatic nerve from distal conduit, processed for TMB staining (**A–C**); myelin surface threshold definition (**D–F**) and collagen-stained threshold (**G–I**): scale bar = 100 μm.

$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and related to the comparison versus empty conduit group.

RESULTS

Adipose stem cells were successfully differentiated into Schwann cell like cells (dASCs), which were further confirmed by immunofluorescence staining (Fig. 1). Resulting dASCs were transplanted into animals via fibrin nerve conduits. All the animals recovered well. 12 week postsurgically, animals were sacrificed and regenerated nerve tissues were explanted and processed for histological analysis. Light microscopic photographs were used for the analysis of remyelination and extent of fibrotic tissue infiltration.

The software-detected, myelinated area was performed in both mid and distal sections (Figs. 2 and 3). All the animals exhibited slightly higher level of myelination in the middle region of regenerated nerve (Table 1), with an highest value 23.5% ± 1.1% found in autograft group (percentage of myelinated surface out of cross sectional area ± SEM). In groups treated with adipose stem cells (F + dASCs) and buffer (Empty) the myelinated area was amounted to 19.9% ± 1.7% and 8.0% ± 3.4%, respectively. Thus, there is a significant difference between these two groups. Adipose stem cells treatment appeared to promote myelination to a greater extent in comparison with control group (Table 1; Fig. 4). Further more, similar results were obtained from the distal nerve segments of Autograft, F + dASCs and Empty groups. Measured values for these groups were

TABLE 1. Quantitative measurements of processed nerve samples for myelin and collagen infiltration

	Empty		dASCs		Autograft	
Measurements	Mid	Distal	Mid	Distal	Mid	Distal
% Myelinated area ± SEM	8.0%±3.4%	5.8%±1.9%	19.9%±1.7%*	18.5%±2.3%**	23.5%±1.1%*	21.2%±1.2%**
% Collagen area ± SEM	21.5%±6.1%	24.1%±2.9%	16.6%±2.1%*	12.1%±2.9%*	15.0%±1.7%*	12.8%±1.0%*

All values are expressed as mean ± SEM. Significance values denoted with the * symbol compare samples with the empty fibrin conduit group (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

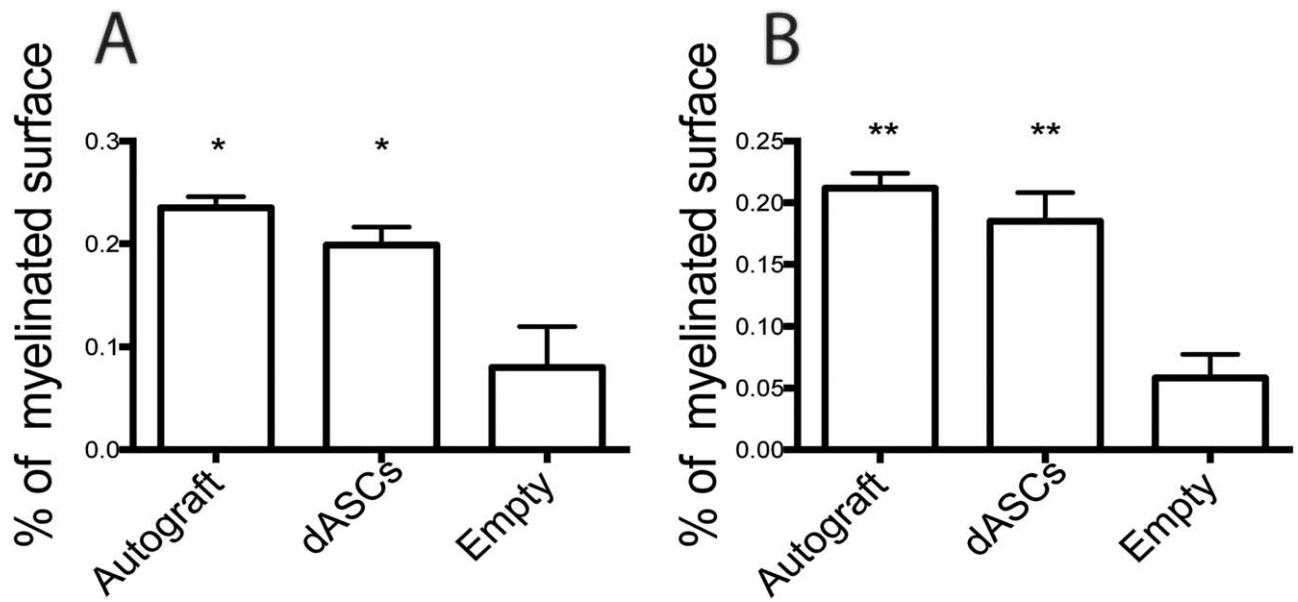

Fig. 4. Analysis of myelination in the regenerated sciatic nerve of middle (**A**) and distal (**B**) conduit region.

amounted to 21.2% ± 1.2%, 18.5% ± 2.3%, and 5.8% ± 1.9%, respectively (Table 1; Fig. 4).

Collagen stained area, in both mid and distal nerve sections was almost similar for Autograft and F + dASCs, i.e., 15.0% ± 1.7% and 12.8% ± 1.0%; 16.6% ± 2.1% and 12.1% ± 2.9%, respectively (Table 1 and Fig. 5). In contrast to later two groups, Empty group showed significantly higher level of collagen deposition amounting to 21.5% ± 6.1% and 24.1% ± 2.9%, respectively in the middle and distal nerve segments (Table 1; Fig. 5). Thus, the adipose stem cells treatment exhibited the great potential. Resulting outcome is comparable to the level of autograft treatment in reducing the collagen infiltration and promoting remyelination during the process of nerve regeneration.

DISCUSSION

Collagen VI is a three-chains protein abundantly deposited in the extra cellular matrix (ECM) of a variety of organ and tissues, such as skin (Keene et al., 1988), skeletal muscles (Bonnemann, 2011), and peripheral nerves (Allen et al., 2009). Immunofluorescence studies demonstrated that collagen VI is abundant in the endoneurium of rat sciatic nerve and it is located outside of myelin as demonstrated by labelling for myelin basic protein, a well-represented constituent of Schwann cell myelin sheaths and is a crucial component for the structural integrity of peripheral nerves function (Chen et al., 2014). The collagen IV production in peripheral nerves is supplied by Schwann cells (SCs) and macrophages (Vitale et al., 2001), but its role remains largely unknown.

In intact peripheral nerves the deposition of collagen VI in is mainly provided by SCs. Macrophages (the major type of resident immune cells in peripheral nerves) can also secret collagen VI but they are not activated under physiological conditions, suggesting their limited contribution on collagen VI deposition in a not-injured sciatic nerve. After nerve section, macrophages are pooled in the lesion site and activated by cytokines and inflammatory mediators and may be involved in the aberrant deposition of collagen IV.

The fibrotic scar containing type IV collagen formed in the lesion site is considered as an obstacle to axonal regeneration (Yoshioka et al., 2010). Limited number of studies made an attempt in reducing collagen infiltration in injured nerves. Recent attempts involve systemic (Xue et al., 2016) or local drug administration. Local drug delivery is based on elution properties of neural

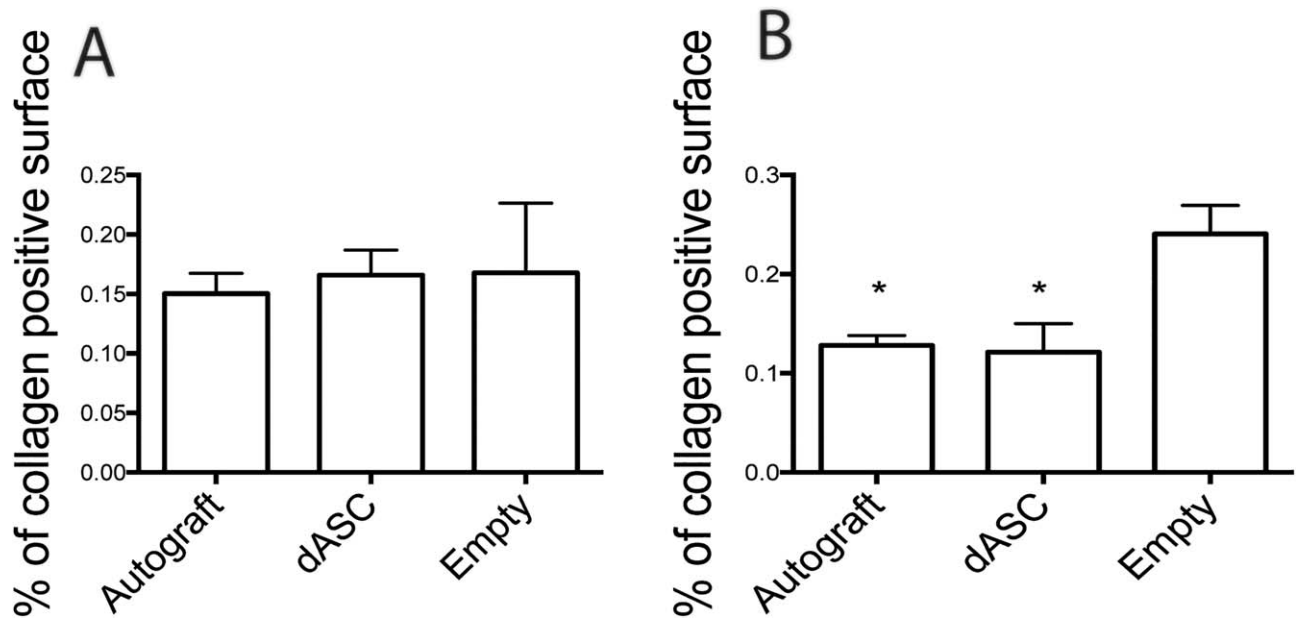

Fig. 5. Analysis of collagen infiltration in the regenerated sciatic nerve of middle (**A**) and distal (**B**) conduit region.

conduits (FitzGerald, 2016) ensuring sustained release. The neural tube composition also acts on scar formation; chitosan based conduits have proved to reduce collagen deposition in the site of neural regeneration but they are technically challenging to use (Marcol et al., 2011). Chitosan supposed antifibrotic role was attributed to its metabolite, i.e., chitooligosaccaride (COS) (Hou et al., 2016). This molecule can penetrate in fibroblast cytoplasm inhibiting their proliferation; adding COS in the growing medium was proved to be an effective method to reduce scar formation and enhance functional recovery in peripheral nerve regeneration (Hou et al., 2016).

Adipose-derived stem cells have been used in animal wound-healing models (Spiekman et al., 2017) to reduce severity of scarring, improve the wound-healing rate and reducing fibrotic areas after wound closure (Spiekman et al., 2017). Indeed, the skin is the tissue/organ in which regenerative and antifibrotic properties of ASCs have been studied the most, but in others animal models specifically designed to study others fibrotic disorder, such as Peyronie disease, ASCs showed their antifibrotic properties (Castiglione et al., 2013). ASCs appeared to have an effect of down regulating the gene expression of the pro-fibrotic markers α-smooth muscle actin and TGF-β1 (Uysal et al., 2014) and up regulating the gene of anti-fibrotic fibroblast growth factor and pro-angiogenic VEGF (Uysal et al., 2014). Despite deriving from the same mesodermal layer of myofibroblasts, dASCs appear to contrast their activity by remodeling the fibrotic matrix of a scar, tilting the balance between ECM deposition and ECM degradation in favour of degradation (Spiekman et al., 2017).

In line with the previous reports, the present work further evaluated the potential effect of dASCs on fibrotic protection in an *in vivo* nerve regeneration model and validated the correlation between remyelination and antifibrotic activity. The low percentage of surface occupied by collagen in F + dASCs group (12.1% ± 2.9%) suggests that the presence of dASCs can inhibit fibrosis formation in the lesion site and promote axonal regeneration, when compared with the empty fibrin conduits (24% ± 2.9%). This is similar to what happen in the control autograft group. In previous long-term studies (di Summa et al., 2011), despite the slightly reduced myelination when compared with autografts of primary Schwann cells, F + dASCs showed a better functional than morphological result. This may be related to the reduced scar tissue formation, which allowed the better functional nerve regeneration.

High levels of collagen invasion towards the distal stump in the empty conduits might have impeded the axonal regeneration, which may explain the poor level of remyelination. These findings suggest the direct correlation between the fibrotic tissue deposition and axonal regeneration. This pattern was not present either in dASCs or in autograft groups, with slight and nonsignificant reduction in both myelin and collagen components, suggesting a more even regeneration process along the conduit. These results reveal the existence of good balance between myelin and collagen components, and further suggest the good correlation for dASCs for possessing antifibrotic and remyelination activities.

CONCLUSION

Reduction of collagen infiltration and associated scar tissue development by using dASCs resulted in improved nerve regeneration. These results are consistent with previous knowledge and studies of ASCs, further explains their benefits when applied to regenerating nerves. The use of dASCs in peripheral nerve regeneration to prevent or diminish scar tissue while maintaining substantial regeneration makes adult stem cell therapy a concrete and clinically translatable option.

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