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Human Mesenchymal Stem Cells Enhance Nerve Regeneration in Nerve Gap Repair with Human Epineural Conduit of a Large - Unmatched Diameter

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Abstract: Different therapies have been reported to support nerve gap regeneration following traumatic nerve injuries. Clinically, lack of the donor nerve or nerve conduit match is challenging and may have a negative impact on nerve regeneration and functional outcomes. This study introduces an innovative approach for management of nerve gaps in the cases of nerve conduit mismatch by applying the unmatched human epineural conduit (hEC) of large diameter supported with human mesenchymal stem cells (hMSC). Following resection of 20mm of a sciatic nerve in the athymic nude rat model (Crl: NIH-Foxn1rnu): 24 animals were assessed in four experimental groups of n=6 rats each: Group 1- no repair control, Group 2- nerve autograft repair, Group 3- hEC filled with 1mL of saline, Group 4- hEC filled with 3×10^6 of hMSC. We performed functional tests of: toe-spread and pinprick, Gastrocnemius Muscle Index (GMI) and muscle fiber area ratio, immunofluorescence staining for vWF, VEGF, S-100, GFAP, Laminin B, NGF for assessment of nerve regeneration, assessment of human origin of the MSC by HLA-1, HLA-DR and human MSC labeling with PKH26 dye, and Toluidine blue staining of nerve cross sections for histomorphometric analysis of the myelin thickness, axonal density, fiber diameter, and percentage of the myelinated nerve fibers. The hEC supported with human MSC group was second to regain best recovery, following the autograft group, regarding functional assessments of toe-spread and pinprick ($p = 0.0032$ and $p = 0.0079$ respectively). Gastrocnemius Muscle Index analysis revealed comparable results between the autograft and hEC supported with hMSC and significantly better results when compared with no gap repair ($p < 0.0001$ and $p = 0.0092$ respectively). Significantly increased number of myelinated fibers was observed in the large diameter hEC enhanced with hMSC when compared with the control group of large diameters hEC filled with saline. The myelin sheath thickness, fiber diameters, axonal density and percentage of myelinated fibers analysis results were comparable between the large diameter hEC enhanced with hMSC and the control group of large diameters hEC filled with saline. We confirmed the supportive role of hMSC in the improvement of nerve regeneration after nerve gap repair with hEC of large-unmatched diameter. At 12-weeks we demonstrated comparable functional recovery, histomorphometric parameters and growth factors expression between the autograft repair and the hEC enhanced with hMSC, whereas significantly worse recovery was noted in the control group of hEC filled with saline, further confirming regenerative potential of MSC.

Keywords: Human Epineural Conduit, Conduit Diameter Mismatch, Mesenchymal Stem Cells, Peripheral Nerve Regeneration

1. Introduction

Incidence of peripheral nerve injuries among the population is estimated to be between 10-20 cases per 100,000 persons per year [1, 2]. Severe nerve injuries after trauma often create gaps which cannot be repaired by the direct end-to-end nerve repair and require application of the nerve grafts or nerve conduits [3-5]. Nerve regeneration across nerve gaps is a multifaceted process resulting in Wallerian degeneration to the distal stump, with a significant role of specific proteins produced by the cell body, Schwann cells (SCs) and extracellular matrix [6, 7]. In injuries with large nerve gaps regenerating axons fail to reinnervate target muscles due to irreversible denervation atrophy with ultimate fat replacement which leads to limited functional recovery [8, 9]. Furthermore, limited sensory recovery is often observed due to sensory axons failing to reach the skin, cross-reinnervation, and possibly degeneration of sensory receptors [10]. When the distance between the proximal and distal nerve stump exceeds 2-3 cm, the tension-free repair is not feasible and application of nerve grafts or conduits is required [4, 11]. The golden standard in bridging the nerve gap is autologous nerve graft, although this management may affect sensory and motor loss at the donor sites, moreover availability of nerve autografts is limited specifically in cases of traumatic injuries [12]. Alternative methods of treatment include application of conduits, both biological and synthetic, as an element connecting the transected nerve stumps. The conduits' essential purpose is to assure axon pathfinding, maintain mechanical isolation, avoid development of inflammation, fibrosis or nerve compression, as well as prevent the diffusion of neurotrophic factors produced by the distal nerve stump [13-15].

Neuroprotective properties of human mesenchymal stem cells (hMSC) originate from their potential to differentiate into neural tissue cells, as well as from the ability to produce neurotrophic factors that improve nerve fiber regeneration such as: Brain-derived neurotrophic factor (BDNF), Glial fibrillary acidic protein (GFAP) and Nerve growth factor (NGF) [16-19]. It is also proved, that MSCs express multiple pro-angiogenic cytokines, such as: Vascular endothelial growth factor (VEGF), Insulin-like growth factor 1 (IGF-1), or Fibroblast growth factor (FGF), that promote new blood vessels formation [20, 21]. Moreover, hMSC are easily accessible and may be collected from different types of tissues including fat tissue, bone marrow or umbilical cord blood cells [17].

We have previously confirmed encouraging outcomes after application of human epineural tube or sheet for enhancement of nerve regeneration [22-25]. In our previous study, repair of 20 mm sciatic nerve gap with matched human epineural conduit (hEC) filled with hMSC demonstrated comparable functional outcomes with the autograft repair [26]. The current study examines the human epineural conduit of large-unmatched diameter supported with human mesenchymal stem cells as a novel approach for enhancement of nerve gap management in cases where there is no access to nerve conduits with the matched diameter.

2. Materials and Methods

2.1. Experimental Animals

In this study 24 male athymic homozygous nude rats (Crl: NIH-*Foxn1rnu, Charles River Laboratories, USA)* weighing between 150 and 250g were used. The rats were housed in pairs in room temperature hooded cages. A light-dark schedule of 14/10 was used, with no limitation of food or water. Following 'Principles of Laboratory Animal Care' formulated by the National Society for Medical Research and the 'Guide for the Care and Use of Laboratory Animal Resources', all animals received humane care. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of University of Illinois at Chicago, which is approved by the American Association for the Accreditation of Laboratory Animal Care (AAALAC).

2.2. Culture, Labeling and Phenotype Analysis of Human Mesenchymal Stem Cells

Mesenchymal stem cells (MSC, purchased from Lonza, Inc., Switzerland) were cultured in MSC growth media supplemented with MSC growth supplement, L-glutamine, and Gentamicin-Amphotericin-B (Lonza, Inc., Switzerland). After 5-8 passages using 0.25% trypsin-EDTA (Gibco-Thermo Fisher, USA), cultured MSCs were harvested at 60-70% confluency. Using PKH26 fluorescent dye (Sigma-Aldrich, UK), MSC were labeled and analyzed by confocal microscopy at the study endpoint. Analysis was done using Zeiss Meta confocal microscope and ZEN software (Zeiss, Germany). hMSC cell viability was tested using 0.4% Trypan Blue before and after PKH26 labeling.

Mesenchymal Stem Cell Phenotype Analysis:

The phenotype of MSC was confirmed prior to cell delivery by flow cytometry. Cells suspended in staining buffer containing 1% BSA and 0.05% sodium azide in D-PBS were incubated with Rat BD Fc Block (BD Biosciences, USA) for 5 min., and later with fluorochrome-conjugated antibodies: APC anti-human CD29, FITC anti-human CD44, BV421 anti-human CD90, APC-anti-human CD105, BV421 anti-human CD73 (BD Biosciences, USA), BV570 anti-human CD45 (Biolegend, USA), APC mouse anti-human CD34, APC mouse anti-human CD14 (BD Biosciences) for 40 min. Cells were fixed with 1% neutral buffered formalin overnight and resuspended in 1% BSA. The samples were assessed using BD LSR II analyzer (Becton Dickinson, USA).

2.3. Creation of Human Epineural Conduit (hEC)

Sterile frozen human sciatic nerves delivered from The Musculoskeletal Transplant Foundation (NJ, USA) were defrosted using a warm water circulating heating pad (T/Pump, Gaymar Industries, USA) at 38 °C. In aseptic conditions, resection of the sciatic nerve in 3-4 cm long sections without side branches was done in preparation for nerve epineural conduits. The fascicles were removed from

the sciatic nerve under 36x magnification of surgical microscope (Wild 691, Leica Microsystems, Germany) and microsurgical instruments. Following fascicle removal, an empty epineural conduit was created and was carefully examined for any damage before being used for implantation. The conduit was then cut into 2 cm long segments and placed in saline before implantation into the sciatic nerve gap. A large diameter hEC was defined as a conduit with a diameter at least five times larger than the diameter of the rat's sciatic nerve.

2.4. Surgical Procedure and Experimental Design

Prior to surgery, the weight of each rat was recorded using a triple beam scale (700/800 series, OHAUS®, USA). Subcutaneous injection of Buprenorphine SR (1.2 mg/kg) was given 15 minutes before the surgery for pain management. The animal was anesthetized with Isoflurane (Terrell Isoflurane, Piramal Critical Care Inc., USA) inhalation (induction 5% until unconscious, maintenance 1.5–2.5%) through the SurgiVet Vaporizer (Smiths Medical, USA). Hair was removed from the rat's right hind limb by shaving and using hair remover lotion (Nair, Church & Dwight Co., USA) for 3-5 minutes. The surgical site was prepped with a 5% povidone-iodine solution (Betadine, Purdue Products L. P., USA). The procedure was performed at room temperature where the animal was placed on a warm water circulating heating pad before the surgery. A 3 cm oblique surgical incision was performed to expose the right sciatic nerve by dissecting the right gluteal area, gluteus superficialis and biceps femoris. An intact 20 mm segment of the sciatic nerve, from the sciatic notch to the bifurcation into terminal branches was resected and the control group wound was closed without repair. In the autograft control group, the resected 20 mm fragment was reversed 180 degrees and sutured using eight epineural sutures (10-0 Vicryl). For the conduit groups, the 20 mm nerve gap was created and repaired with a 20 mm long human epineural conduit (hEC) filled with saline or with hMSC and was implanted to fill the 20 mm nerve gap using eight epineural sutures under 36X magnification of the microsurgical operating microscope (Wild 691, Leica Microsystems, Germany) (Figure 1). The surgery was performed by one surgeon using an aseptic technique. Next, the gluteus superficialis and biceps femoris muscles were sutured using 4-0 interrupted vicryl suture. The skin was closed using interrupted 5-0 monocryl sutures (Ethicon, USA) and an antibiotic cream (Neosporin, Johnson & Johnson, USA) was applied. The animals were monitored for 24 hours post-surgery. In this study, twenty-four nude rats (Crl: NIH-*Foxn1rnu*) (n=6) were evaluated in four experimental groups. All animals were assigned randomly to each experimental group. Group 1: represented the no repair control where no repair was made after resection of the sciatic nerve. Group 2: Nerve gap was repaired with a 20 mm segment of the sciatic nerve autograft. Group 3: 20 mm long human epineural conduit filled with 1 mL of saline was implanted between proximal and distal nerve stumps. Group 4: 20 mm human epineural conduit filled with 3×10^6 hMSC suspended in 1 mL of saline was implanted to the created nerve gap. Functional assessments were conducted at 1, 3, 6, 9 and 12-weeks post-gap repair.

Postsurgical Animal Care:

Each rat was quarantined individually with a collar to provide protection against wound biting for the first 24 hours post intervention. The following day the collar was taken off and the rats were returned to their cage. Buprenorphine (0.1mg/kg) was given twice a day for the first two days to help with postoperative pain. The first 14 days post-surgery, the animals were inspected daily. The veterinary team at University of Illinois at Chicago inspected each rat once a week as well.

2.5. Functional Assessment Tests and Histomorphometrical Analysis

Functional recovery measured by pinprick and toe-spread tests was evaluated at 1, 3, 6, 9 and 12-weeks post nerve gap repair. Animals were euthanized at 12-weeks using euthanasia solution SomnaSol (Henry Schein, Inc., USA). The sciatic nerve and gastrocnemius muscles were collected for histological and immunological examination.

2.5.1. The Toe-Spread Test for Assessment of Motor Function Recovery

To evaluate motor recovery the toe-spread test was conducted as described before [26]. Briefly, voluntary response of the rat's toes was observed after raising the animal by the tail. The toe-spread test was graded between 0 and 3 points as follows: full toe extension and abduction (a normal reaction) was assigned 3 points; abduction of the toes was graded with 2 points; any sign of movement of the toes was assigned 1 point; no reaction was given 0.

2.5.2. The Pinprick Test for Assessment of Sensory Recovery

To evaluate sensory recovery the pinprick test was performed as described before [26]. Pressure was applied to the skin of the right hind limb using Adson's toothed forceps. Looking for retraction of the limb and/or a vocal response, pressure was applied starting from the toe to the knee level. Pinching of the deep tissues and periosteum of the limb was avoided. The pinprick test was graded 0: no sensation, 1: the rat has a withdrawal response between the knee and the ankle, 2: the rat has a withdrawal response between the ankle and toes, and 3: the rat has a withdrawal response to the pinch of the toes. The test was done in triplicates to prevent false-positive results.

2.5.3. Evaluation of the hEC Integrity at the 12-Weeks Study Endpoint

After euthanasia, a 3 cm incision was made at the gluteal region of the right hindlimb to visualize the sciatic nerve. The following assessments of the sciatic nerve repair site were made presence of adhesions with the surrounding tissues, local signs of inflammation, human conduit structure, shape and integrity, presence of the fascicle-like structures inside the conduit, and assessment of vascularization of the conduit.

2.5.4. Assessment of Muscle Denervation Atrophy

Gastrocnemius muscle index (GMI) was used to assess muscle denervation atrophy. The muscle was collected from both hindlimbs at the 12-week end point. Using a digital scale (Ohaus Precision Standard, Germany) the gastrocnemius muscles' (GM) weight was measured. The wet weight of the right GM was related to the left GM, and the GMI was calculated. The percentage value of the GMI represents recovery of the denervation atrophy of the GM, where 100% indicates full recovery.

2.5.5. Muscle Fiber Area Ratio

After GMI was calculated, the gastrocnemius muscles were fixed in formalin and hematoxylin and eosin (H&E) stainings were done to cross-sections of the muscle samples. Six non-overlapping areas were chosen, with three hundred muscle fibers analyzed in total. Images were taken using a Leica DM4000B Compound Microscope (Leica Microsystems, Germany) with a Qimaging Retiga 2000R Color Digital Camera (Teledyne Photometrics, USA), and analysis was done using Image-Pro Plus, Ver 6.3.0.512 (Media Cybernetics, USA). The average muscle fiber area was compared to the right and left hindlimb and values were expressed as a R/L ratio.

2.5.6. Toluidine Blue Staining for Histomorphometric Assessment of the hEC

Samples of the conduit on the proximal and distal stump of the nerve were collected and fixed in 2.5% glutaraldehyde, 2.5% paraformaldehyde and 0.1M Cacodylate buffer. Then, the samples were post-fixed using 4% aqueous osmium tetroxide and embedded in Araldite 502. Toluidine blue stain was used on 1 μm thick cross-sections for light microscope analysis of histological samples. Six non-overlapping cross-sections were selected from each nerve. Images were taken using a Leica DM5500B Compound Microscope with a Leica DFC290 Color Digital Camera (Leica Microsystems, Germany). Using Image-Pro Plus, Ver 6.3.0.512 (Media Cybernetics, USA), each image was assessed for myelin thickness, axonal density, fiber diameter, and percentage of the myelinated nerve fibers.

2.5.7. Assessment of Human Origin of the Epineural Conduit and MSC

At 12-weeks after nerve gap repair with hEC, rats were euthanized using SomnaSol (Henry Schein, Inc., USA) and samples of sciatic nerve were collected for PKH staining and immunofluorescence staining of HLA-1 and HLA-DR. The proximal and distal end of the nerve conduit was dissected, and samples were suspended and frozen in O. C. T compound and cut for 5 μm thick slides and fixed for 10 minutes in acetone. For immunofluorescence staining the slide-sections were washed in Tris Buffered Saline (TBS, Agilent Technologies, Inc., USA) and incubated with mouse anti-human HLA-1 and HLA-DR (Abcam, Inc., UK) monoclonal antibodies for 30 min. incubation with goat anti-mouse IgG Cross-Absorbed Alexa Fluor 488 (Thermo Fisher Scientific, USA) secondary antibody. Then all slides for PKH and Immunofluorescence analysis were stained with DAPI and analyzed using Leica DM4000B Compound Microscope (Leica Microsystems, Germany) with a Qimaging Retiga 2000R Color Digital Camera (Teledyne Photometrics, USA). Assessment of images was completed using Image-Pro Plus, Ver 6.3.0.512 (Media Cybernetics, USA) and staining intensity (PKH26) or immunoreactivity was graded as: 0: no staining, 1: weak, 2: moderate, and 3: strong.

2.5.8. Immunostaining Assessment of Neurogenic and Angiogenic Markers

The proximal and distal ends of the hEC were dissected at study endpoint and the samples were suspended and frozen in O. C. T compound, cut for 5 μm slides and fixed for 10 minutes in acetone. Prior immunofluorescence staining the slides were washed in Tris Buffered Saline (TBS, Agilent Technologies, Inc., USA) and incubated with monoclonal mouse anti-rat S-100 (Abcam, Inc., UK), rabbit anti-rat GFAP (Thermo Fisher Scientific, USA), Laminin B and NGF (Abcam, Inc., UK), mouse anti-rat vWF, VEGF (Thermo Fisher Scientific, USA) antibodies for 30 min. Incubation with goat anti-mouse or goat anti-rabbit IgG Cross-Absorbed Alexa Fluor 488 (Thermo Fisher Scientific, USA) secondary antibodies was done. Slides were stained with DAPI and analyzed using Leica DM4000B Compound Microscope (Leica Microsystems, Germany) with a Qimaging Retiga 2000R Color Digital Camera (Teledyne Photometrics, USA). Assessment of images were completed using Image-Pro Plus, Ver 6.3.0.512 (Media Cybernetics, USA) and immunoreactivity was graded as: 0: no staining, 1: weak, 2: moderate, and 3: strong.

2.6. Statistical Analysis

Data are expressed as mean ± SEM (standard error of the mean). GraphPad Prism (ver. 9.2.1) software was used to perform statistical analysis. One-way or two-way ANOVA with post-hoc Tukey's test were used for group comparisons to define statistical significance. Results were considered statistically significant when $p < 0.05$.

3. Results

3.1. Confirmation of Lack of Side Effects at 12-Weeks After Implantation of Human Epineural Conduit into the Sciatic Nerve Gap Macroscopic Evaluation of the hEC

At the 12-weeks study endpoint each conduit was assessed macroscopically before harvesting. There were no signs of adhesions with surrounding tissues or local inflammation around the conduits. Well-preserved structure, shape and integrity with good vascularization of the graft were maintained. Moreover, fascicle-like structures were present inside the conduits.

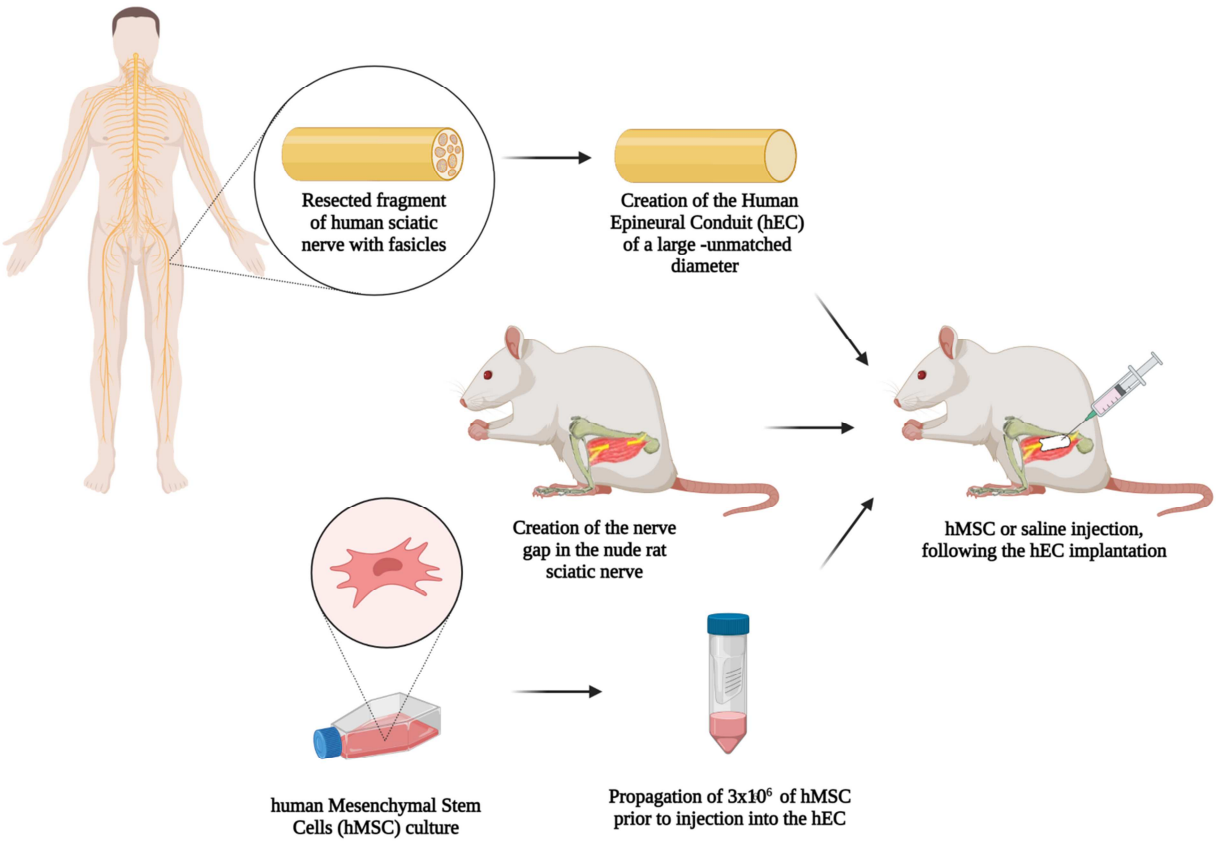

Figure 1. *Study design of creation of a large diameter - unmatched human Epineural Conduit (hEC) from the human sciatic nerve and application of the large hEC supported with human MSC or saline for repair of the sciatic nerve gap in the nude rat model.*

3.2. Confirmation of Sciatic Nerve Recovery by Functional Tests at 12-Weeks After Nerve Gap Repair with hEC Supported with MSC

Improvement of Motor Recovery Assessed by Toe-spread test at 12-weeks after Nerve gap repair with hEC supported with MSC

The toe-spread test was conducted at 1, 3, 6, 9 and 12-weeks after the nerve repair in all experimental groups. The hEC with hMSC group, the hEC with saline group, as well as the autograft group presented improvement in motor function starting from the 3-weeks follow up. There was no functional recovery observed in the no repair control over the entire follow-up period. At 6-weeks follow-up no significant differences were observed between the experimental groups, moreover the hEC with hMSC group presented the highest value of toe-spread test (0.33±0.25) when compared with the autograft and the hEC enhanced with hMSC groups. At 9-weeks follow-up the hEC with hMSC group reached the second highest motor recovery level (0.50±0.22), following the autograft group (1.67±0.21). Significant differences were observed when the autograft group was compared with the hEC enriched with hMSC group ($p = 0.0007$), the hEC with saline group (0.17±0.17; $p < 0.0001$), as well as the no repair group (0±0; $p < 0.0001$). No significant difference was observed between the hEC with hMSC group (0.50±0.22) and hEC with saline group (0.17±0.17). At 12-weeks follow-up the hEC with hMSC group maintained the second highest return of motor function (0.50±0.22), exceeding the hEC with saline group (0.33±0.33), although the values were not significantly different. As expected, the autograft group presented the highest level of functional recuperation (1.83±0.17), with significant differences when compared with the hEC enhanced with hMSC group (0.50±0.22; $p = 0.0032$), the hEC with saline group (0.33±0.33; $p = 0.0010$), as well as the no repair group (0.17±0.17; $p = 0.0003$) (Figure 2A).

Improvement of Sensory Recovery Assessed by Pinprick Test at 12-Weeks After Nerve Gap Repair with hEC Supported with MSC

The pinprick test was performed at 1, 3, 6, 9 and 12-weeks after the nerve repair in all experimental groups. Only the autograft group fully regained sensory function at 12-weeks after nerve gap repairy. The group repaired with hEC with hMSC, as well as the group of hEC with saline showed improvement in sensory recovery by the pinprick test as early as at 3-weeks follow up. At 6-weeks follow-up the hEC with hMSC group presented the second highest value of pinprick response (0.83±0.32), following the autograft group (1.67±0.42). Moreover, no significant difference was noted between the hEC supported with hMSC and the autograft group regarding sensory recovery. The comparison between the autograft group (1.67±0.42) and the hEC with saline group (0.33±0.21; $p = 0.0155$), as well as the no repair group (0±0; $p = 0.0024$) proved significant differences. At 9-weeks follow-up the hEC with hMSC group maintained the second

highest level of sensory response (0.83±0.17), exceeding the hEC with saline group (0.67±0.33), with no significant differences between the groups. The comparison between the autograft group (2.33±0.33) and the hEC with hMSC group (0.83±0.17; p = 0.0044), the hEC with saline group (0.67±0.33; p = 0.0017), as well as the no repair group (0.40±0.24; p = 0.0002) proved significant difference. At 12-weeks timepoint the hEC with hMSC group reached the second highest value of sensory recovery (1.50±0.22), following the autograft group (3.00±0), however the difference between these groups was statistically significant (p = 0.0001). Moreover, the hEC with hMSC group presented significantly higher value of pinprick response when compared to the hEC with saline group (0.50±0.22; p = 0.0079), as well as to the no repair group (0.50±0.22; p = 0.0079). Other than that, we found significant difference when the autograft group (3.00±0) was compared to the hEC with saline group (0.50±0.22; p < 0.0001) and the no repair group (0.50±0.22; p < 0.0001) (Figure 2B).

3.3. Confirmation of Improved Muscle Recovery by Gastrocnemius Muscle Index (GMI) at 12-Weeks After Nerve Gap Repair with hEC Supported with MSC

Assessment of Gastrocnemius muscle atrophy expressed by GMI was performed at 12-weeks after the repair of sciatic nerve gap. The hEC with hMSC group presented the second highest level of muscle denervation atrophy index (0.21±0.01), following the autograft group (0.32±0.01). Moreover, the hEC enriched with hMSC group reached a significantly higher GMI level when compared with the no repair group (0.16±0.01; p = 0.0092). Comparison between the hEC with hMSC group (0.21±0.01) and the hEC with saline group (0.19±0.01) did not reveal significant advantage of the hEC supported with hMSC. Analysis revealed significant differences when the autograft group (0.32±0.01) was compared with hEC with hMSC (0.21±0.01; p < 0.0001), hEC with saline (0.19±0.01; p<0.0001) and no repair group (0.16±0.01; p < 0.0001) (Figure 2C).

3.4. Muscle Fiber Area Ratio

The second highest Muscle Fiber Area Ratio was reached by the hEC enriched with hMSC group (0.21±0.05), as assessed at 12-weeks follow-up. There was no significant difference between the hEC with hMSC group and the hEC with saline group (0.19±0.02), however the hEC with hMSC group reached a greater value of muscle fiber area ratio. Expectedly, the autograft group reached the highest ratio of muscle fiber area, with significant difference when compared with the hEC with hMSC group (0.21±0.05; p = 0.0007), hEC with saline group (0.19±0.02; p = 0.0003), as well as no repair group (0.11±0.01; p < 0.0001) (Figure 2D).

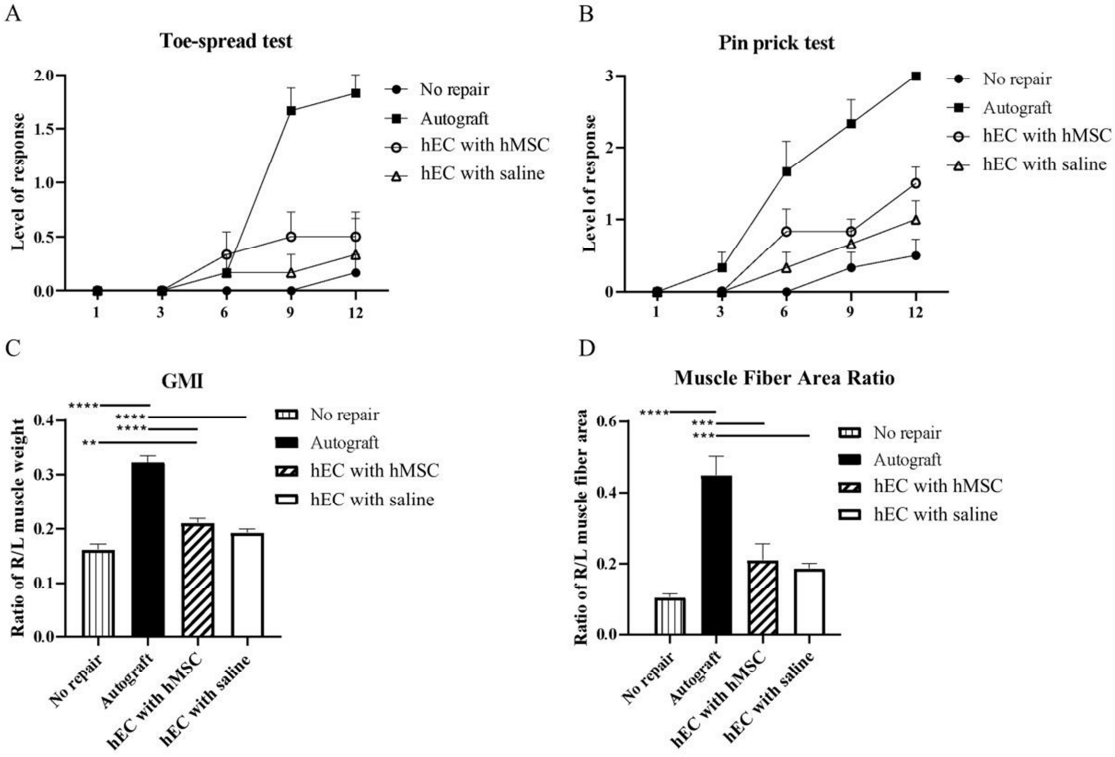

Figure 2. *Functional and histomorphometric assessment of nerve regeneration at 12-weeks after the repair of sciatic nerve gap. (A) Recovery of motor function by the hEC with hMSC group measured by the toe-spread test at 1, 3, 6, 9 and 12 weeks' time-point. (B) Improvement in sensory recovery of the hEC enriched with hMSC group measured by the pinprick test at 1, 3, 6, 9 and 12-weeks follow up. (C) Gastrocnemius Muscle Index (GMI) improvement after hEC application. The hEC with hMSC group presented the second highest GMI level, significantly higher when it was compared with the no repair group. (D) Increase in Muscle Fiber Area Ratio by the hEC with hMSC group at 12 weeks post-repair. The highest results were reached by the autograft group, with significant differences revealed when compared with the hEC with hMSC group, hEC with saline group as well as no repair group. The hEC with hMSC group obtained the second highest value of the muscle fiber area ratio. All data is presented as (mean ± SEM), One-way ANOVA, ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05.*

3.5. Histomorphometric Assessment of the Sciatic Nerve

Proximal myelin thickness, fiber diameter, axonal density and percentage of myelinated fibers were assessed in the hEC with saline, the hEC enhanced with hMSC and in the autograft groups at 12-weeks study checkpoint. The hEC with hMSC group presented a greater value than hEC with saline group regarding myelin thickness (Figure 3A), fiber diameter (Figure 3B), axonal density (Figure 3C), as well as percentage of myelinated fibers (Figure 3D), however without any significant differences between these groups. Expectedly, the autograft group reached the highest value of myelin thickness, fiber diameter and axonal density, however without any significant advantage over the remaining study groups. Regarding the percentage of myelinated fibers, we found a significant difference when the autograft group (84.67±1.31) was compared with the hEC with hMSC group (67.50±4.16; p = 0.0026) and hEC with saline group (61.33±2.72; p = 0.0002) (Figure 3D).

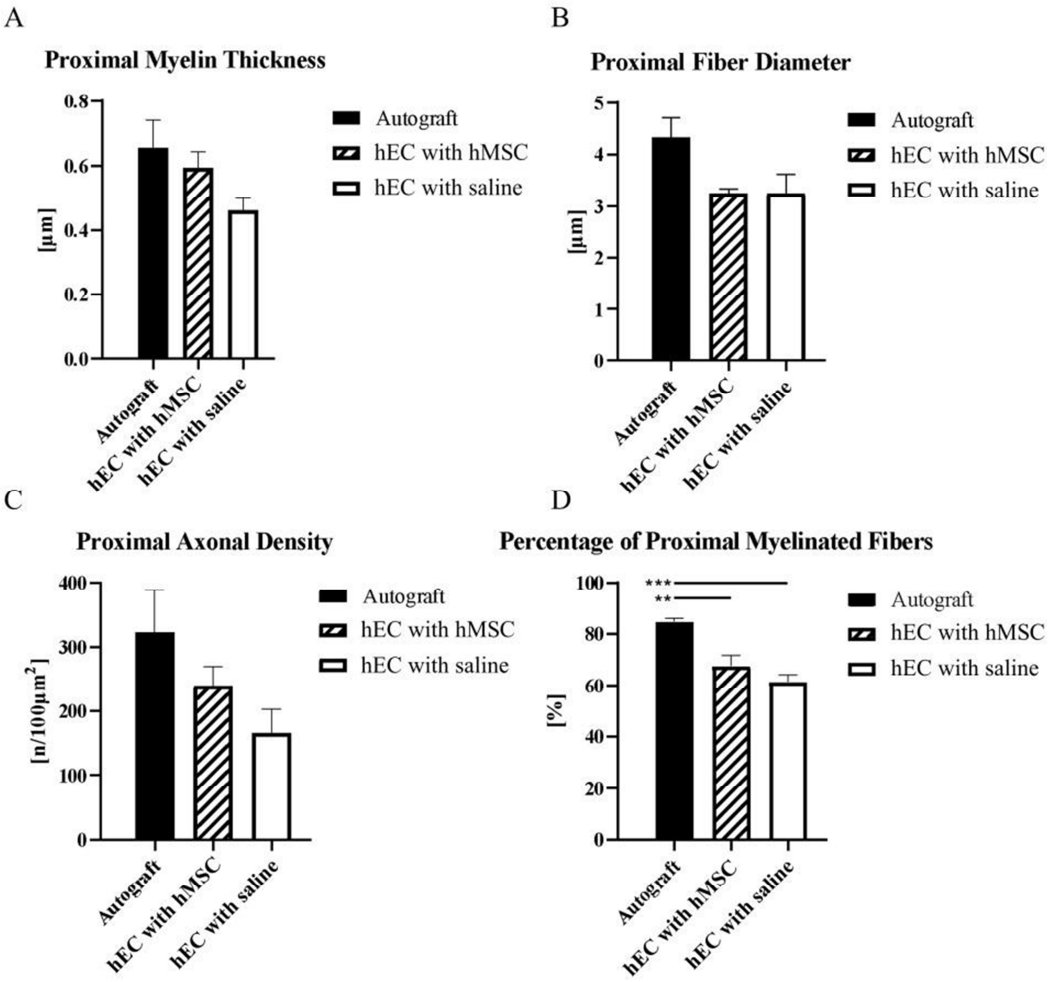

Figure 3. *Histological evaluation of the proximal end of the conduit at 12-weeks after nerve gap repair. (A) Myelin thickness assessment revealed no significantly different values between the autograft, the hEC with hMSC and hEC with saline groups. The hEC enhanced with hMSC group reached the second highest value, following the autograft group (B) No significant difference was revealed in fiber diameters evaluation between the autograft, the hEC with hMSC and hEC with saline groups. The greatest fiber diameter value was presented by the autograft group, followed then by the hEC with saline and hEC with hMSC group. (C) No significant difference was detected for axonal density. The hEC with hMSC group presented the second greatest axonal density, following the autograft group. (D) Percentage of myelinated fibers analysis revealed a significant difference, when the autograft group was compared with both hEC with hMSC group, as well as hEC with saline group. The hEC with hMSC group presented the second highest percentage of myelinated fibers, however without any other significant statistical differences observed. All data is presented as (mean ± SEM), One-way ANOVA, ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05.*

Distal myelin thickness, fiber diameter, axonal density and percentage of myelinated fibers were assessed in the hEC with saline, the hEC with hMSC and in the autograft groups at 12-weeks study checkpoint. The hEC with hMSC group reached the second highest value of myelin thickness followed by the hEC with saline group, however without any significant difference between these groups. As expected, the autograft group presented significantly higher myelin thickness value (0.63±0.04) when it was compared to the hEC with hMSC group (0.38±0.02; p = 0.0009), as well as to the hEC with saline group (0.37±0.02; p = 0.0001) (Figure 4A). The hEC with hMSC group (3.08±0.21) reached a lower

value of fiber diameter than the hEC with saline group (3.13±0.19), however the difference between these groups was not significant and was equal to approximately 0.04 μm (Figure 4B). Further, the hEC with hMSC group reached the second highest value of axonal density (106.50±13.56) with the hEC with saline group next to follow (71.00±15.05), however the difference between these groups was not significant. The autograft group (321.83±45.87) revealed a significantly higher value of axonal density when compared with the hEC with hMSC group (106.50±13.56; $p = 0.0003$), as well as with the hEC with saline group (71.00±15.05; $p < 0.0001$) (Figure 4C). Considering the percentage of myelinated fibers, the hEC with hMSC group (65.67±3.98) presented a significantly higher value than the hEC with saline group (53.33±3.51), therefore determining the supportive role of hMSC on nerve regeneration. Other than that, significant differences were found when the autograft group (82.67±1.09) was compared with the hEC with hMSC group (65.67±3.98; $p = 0.0043$), as well as with the hEC with saline group (53.33±3.51; $p < 0.0001$) (Figure 4D).

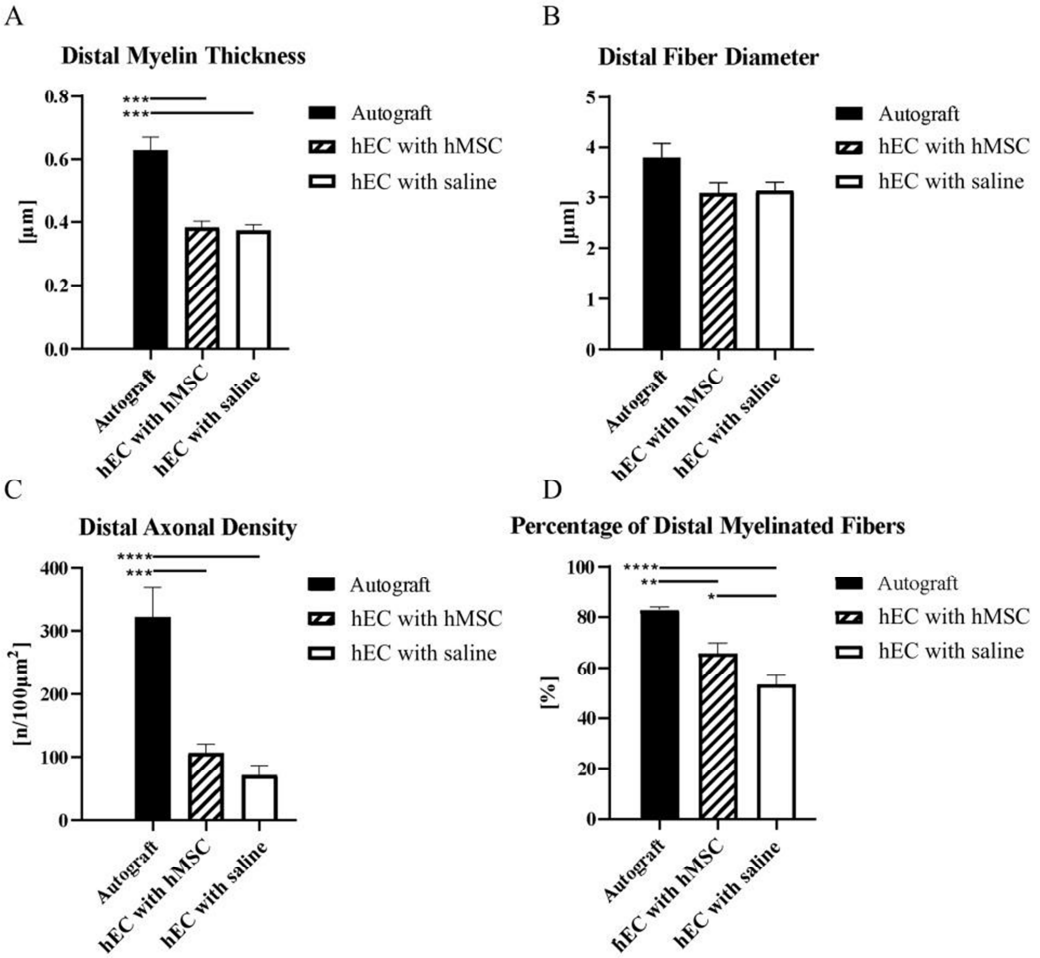

Figure 4. *Histological evaluation of the distal end of the conduit at 12-weeks after nerve gap repair. (A) Analysis of the myelin thickness revealed a significantly higher value in the autograft group after comparison with both hEC with saline and hEC with hMSC group. (B) Fiber diameter analysis revealed no significant differences between the autograft, the hEC with hMSC and the hEC with saline groups. (C) Analysis of axonal density presented a significant difference when the autograft group was compared with the hEC with hMSC group, as well as with the hEC with saline group. The hEC with hMSC group presented the second highest axonal density value, following the autograft group. (D) Percentage of myelinated fibers assessment revealed significant differences after comparison between the autograft group and e hEC with saline group, as well as the hEC with hMSC group. Moreover, significant improvement in percentage of myelinated fibers was observed for the hEC with hMSC group (65.67±3.98) when it was compared with the hEC with the saline group. All data is presented as (mean ± SEM), One-way ANOVA, ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05.*

3.6. Confirmation of Human Origin of MSC by PKH Labeling and hEC by HLA-ABC Immunostaining

PKH26 staining confirmed presence of human MSC only in the hEC with hMSC group, within both proximal and distal conduit end (Figure 5A). As expected, no PKH26 dye signal was detected in autograft and hEC with saline within both conduit ends. Expression of HLA-1 was detected in the hEC with hMSC within both proximal and distal conduit end, therefore confirming the human origin and presence of hEC at 12-weeks post nerve repair. Consequently, no HLA-1 expression was detected in the autograft and the hEC with saline group. The expression level of HLA-1 in the hEC with hMSC group reached a higher value within the proximal conduit end when compared with the distal (Figure 5B). HLA-DR expression was detected in the hEC with hMSC

group within both proximal and distal conduit end. No HLA-DR signal was presented in the autograft and the hEC with saline group (Figure 5C).

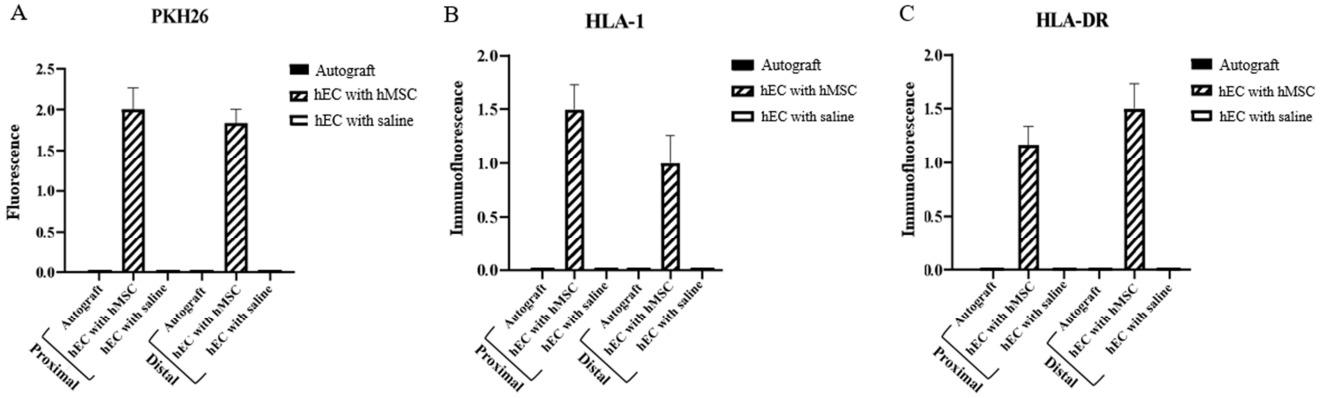

Figure 5. *Presence of PKH26 assessed by fluorescence staining and expression of HLA-1 and HLA-DR evaluated by immunofluorescence staining to confirm the human origin of the MSC and epineural conduit within the proximal and distal end of the conduit at 12-weeks after the repair of sciatic nerve gap. (A) PKH26 staining confirmed MSC presence in hEC injected with hMSC group within both proximal and distal nerve conduit end, in favor of the former. Expectedly, no PKH26 fluorescence signal was detected in the remaining experimental groups within both conduit ends. (B-C) High HLA-1 and HLA-DR immunofluorescence levels in the hEC injected with hMSC group was detected, confirming the presence of human conduit at 12-weeks post nerve gap repair within both nerve conduit ends. Consequently, no HLA-1 expression was detected within the remaining experimental groups. All data is presented as (mean ± SEM), One-way ANOVA, ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05.*

3.7. Confirmation of Neurogenic Markers (Laminin B, S-100, GFAP, NGF) Expression by Immunostaining Analysis at 12-weeks After Nerve Gap Repair

Expression of Laminin B, an axonal growth promoting marker was detected in all experimental groups within the proximal and distal conduit ends. The hEC with hMSC group reached the second highest value of Laminin B expression (1.83±0.21) followed by the hEC with saline group (1.33±0.21) (Figure 6A), no significant differences were detected between these groups. The highest level of Laminin B expression was revealed in the autograft group (2.33±0.21) with significant difference when compared with the hEC with saline group (1.33±0.21; p = 0.0072). No statistical differences were observed between the hEC with hMSC and the autograft, as the groups presented similar expression levels of the growth promoting marker.

Expression of Schwann cells marker, S-100 was detected in both proximal and distal conduit end. The hEC supported with hMSC group presented the second highest S-100 expression of all experimental groups within the proximal end of the conduit (Figure 6B). Only the comparison between the autograft group (1.50±0.22) and the hEC with saline group (0.50±0.22) proved to be statistically significant (p = 0.0355).

Expression of GFAP, a marker produced by Schwann cells, was low or not detected in most of the experimental groups. However, rats which nerve was repaired with autograft (1.17±0.31) presented a significantly higher expression of GFAP in the proximal end of the conduit in comparison with the hEC with saline group (0.20±0.20; p = 0.0230) within the adequate conduit end. Within the distal end of the conduit no significant differences were observed between the groups, as only the autograft group presented mild GFAP expression. The hEC injected with hMSC group presented the second highest expression level of all experimental groups within the proximal conduit end (Figure 6C). The second highest expression of NGF, a neurite outgrowth promotor, was presented within both proximal and distal conduit end by the group of rats treated with hEC with hMSC without any statistical differences detected after comparison with the two remaining experimental groups (Figure 6D). Analysis of the NGF immunofluorescence in the proximal end of the conduit revealed a significantly higher values for the autograft group (2.17±0.17) when compared with the hEC with saline group (0.50±0.22; p = 0.0008) as well as within the distal conduit end for which we found a significant difference between the autograft (2.00±0.26) and hEC with saline group (0.83±0.17; p = 0.0170). The same pattern for hEC supported with hMSC was observed in all neurogenic markers, which revealed comparable results for autograft standard and hEC with hMSC treatment group.

3.8. Confirmation of Angiogenic Markers (VEGF, vWF) Expression by Immunostaining Analysis at 12-Weeks After Nerve Gap Repair

Significantly higher expression of VEGF, an endothelial cell mitogen marker, was detected in the hEC with hMSC group in the proximal conduit end (2.00±0.26) when compared with the hEC with saline group (0.33±0.21; p = 0.0004). Moreover, the comparison between the autograft group (2.50±0.22) and the hEC with saline group (0.33±0.21) within the same conduit end showed significant differences (p < 0.0001). Considering the distal end of the conduit, the hEC with hMSC group and the autograft group presented the same level of VEGF expression (both 1.33±0.21), therefore concluding that the hEC injected with hMSC was as effective as the autograft application regarding an endothelial cell

mitogen marker expression. The results for hEC with MSC and autograft (both 1.33±0.21) revealed significant differences between the hEC with saline group (0.17±0.17; p = 0.0022), (Figure 6E).

Low expression levels of vWF, an angiogenesis regulator marker, in all experimental groups were similar to each other and presented the same value of the mean (0.33). No significant differences were detected in both ends of the conduit between three experimental groups (Figure 6F).

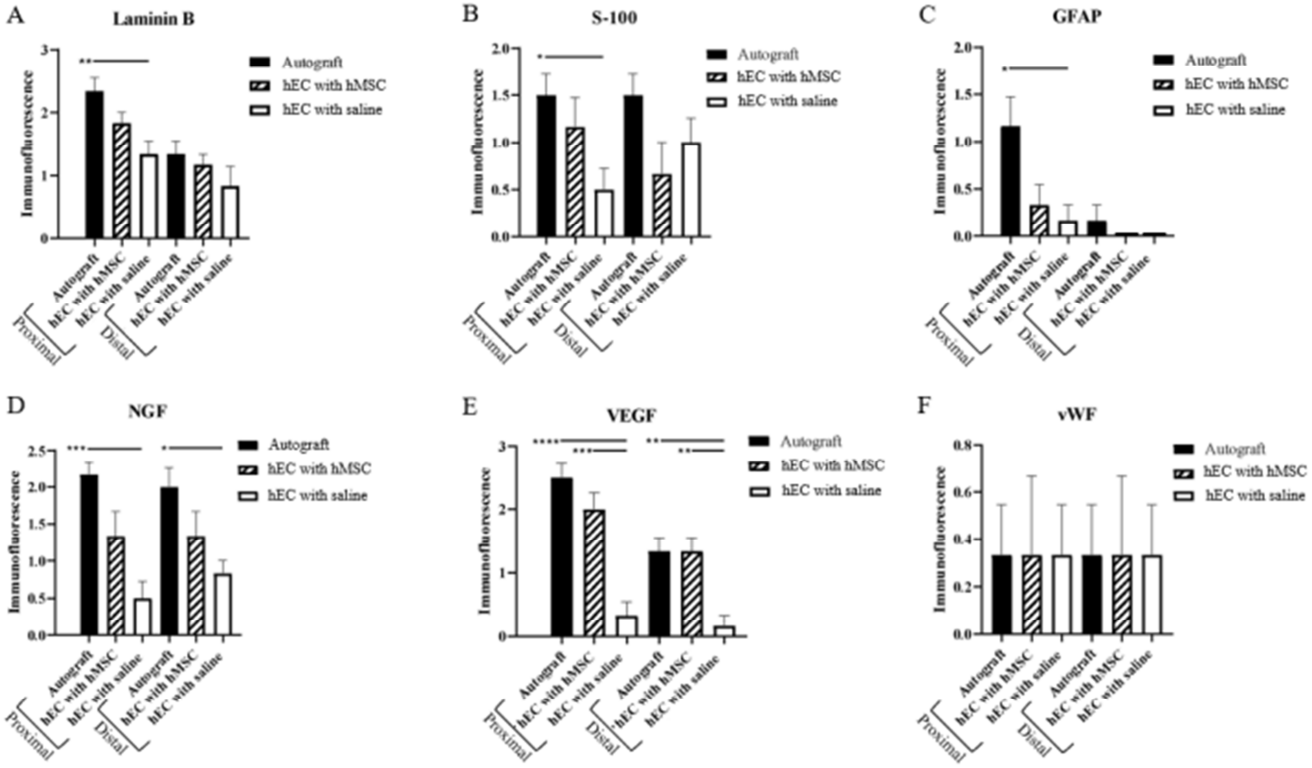

Figure 6. *Expression of neurogenic markers and angiogenic markers in the proximal and distal end of the conduit at 12-weeks after nerve gap repair. (A) Within both conduit ends, the second highest level of Laminin B expression was reached by the hEC with hMSC group, following the autograft group, however with significant difference only between the autograft and the hEC with saline group in the proximal conduit end. (B) The S-100 immunofluorescence level in the hEC with hMSC group was higher within the proximal than within the distal conduit end, however no significant differences were found after comparison with the remaining experimental groups. (C) GFAP expression in the hEC with hMSC group was low within the proximal conduit end and undetectable within the distal conduit end. (D) NGF expression reached the second highest level in the hEC injected with hMSC group within both nerve conduit ends, with significant differences only between the autograft and the hEC with saline groups. (E) The VEGF expression reached the second highest value in the hEC with hMSC group and was significantly higher when compared with the hEC with saline group within both conduit ends. (F) No significant differences were observed regarding vWF expression, when the immunofluorescence was evaluated in both proximal and distal conduit end. All data is presented as (mean±SEM), One-way ANOVA, ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05.*

4. Discussion

Autologous nerve grafting is the golden standard for peripheral nerve gap repair, when direct epineurorrhaphy is not applicable, however nerve grafting has its challenges such as – insufficient availability of the graft material, donor site morbidity due to nerve harvesting, the motor and sensory loss, neuroma formation and potential for infection, inflammation and wound healing problems. In recent years researchers employed conduits as an alternative method to nerve autografts [3, 27-30]. The optimal conduit provides a mechanical scaffold for the sprouting of nerve fibers, restricts inflammatory response and fibrosis, and reduces possibility of neuroma formation [31]. Furthermore, conduits should maintain proper microenvironment, which is essential during regeneration of damaged nerves. Currently, clinically available biological conduits include the veins, muscle lamina and tendons. The veins' efficacy as a conduit was confirmed in clinical studies, however only for repair of small, under 3 cm nerve gaps in the hand and forearm [32, 33]. As an alternative, Fawcett et al. applied muscle basal lamina grafts into sciatic nerve gaps and obtained a comparable nerve regeneration to the autograft repair. It was suggested that favorable results were related to the longitudinally oriented basal lamina of the skeletal muscles promoting cell adhesion to the extracellular matrix [34].

Synthetic materials, being readily available, have also been investigated as a possible alternative in peripheral nerve gap repair. Many years of research on neuroregenerative properties of silicone conduits constructed from non-degradable materials have proven their capacity to promote nerve recovery. However, synthetic conduits' regenerative potential is hindered as they lack a supportive matrix [35].

In the current study we have chosen the human epineurial conduit as a novel strategy for nerve regeneration. Epineurium is a naturally occurring tissue covering the nerve fibers, with high laminin expression, that promotes SCs attachment. SCs play a crucial role in peripheral nerve regeneration due to their ability to produce and secrete growth factors and structural molecules that create the favorable microenvironment for nerve regeneration [36]. The epineurial sheath conduit's efficacy to restore a 6 cm nerve defect in the sheep median nerve injury model was confirmed by Siemionow Laboratory [24]. Only a few studies have investigated the influence of the diameter discrepancy in the application of conduits as a substitute for the nerve autograft. Giusti et al. stated that a better size-matched nerve collagen conduit presented better motor recovery after segmented nerve repair in the rat model [37]. Our previous study [26], demonstrated comparable results between the human epineural conduit of matched diameter supported with hMSC and the autograft in repair of 20 mm sciatic nerve defect. In the current study, we examined the human epineural conduit of large-unmatched diameter for nerve gap management which will be applicable in cases of motor vehicle accidents, multilevel extremity trauma, military injuries or war condition where there is a limited access to the autologous nerves or nerve conduits with the matched diameters.

Severe nerve injuries that result in the large gaps between the nerve stumps, may require combined therapies for enhancement of nerve regeneration and improved clinical outcomes [38]. Cellular therapies have been extensively investigated as a supportive modality for surgical management of peripheral nerves after trauma. Previous studies have proved the mesenchymal stem cells' efficacy in stimulating endogenous nerve regeneration, as they are able to differentiate into SCs. More interestingly MSCs improve the efficacy of guidance conduits by producing pro-regenerative agents, reducing inflammation, oxidative stress and apoptosis resulting from nerve injury [39]. Cui et al. investigated collagen conduits enhanced with human mesenchymal stem cells applied to the nerve gap after sciatic nerve transection in dogs. Their results strongly suggested better functional recovery in conduits filled with MSCs when compared with the conduit alone [40].

Although, as expected, at 12-weeks observation the functional outcomes, measured by the toe-spread and pin-prick tests revealed the highest values for the autograft group, it was the hEC supported with hMSC group that reached the second highest values of the assessed functional tests. Moreover, a significant difference was observed for the pin-prick test in hEC with hMSC, when compared to hEC with saline group, suggesting the potential advantage of mesenchymal stem cells addition to the conduit. Similar pattern was observed for muscle regeneration, assessed by the gastrocnemius muscle index and muscle-fiber area ratio, where hEC with hMSC group reached the second highest values, following the autograft group. Immunofluorescent staining revealed no significant differences in expression of Laminin B, S-100, GFAP and NGF within the proximal nerve stump between the autograft control and hEC supported with hMSC, whereas significantly increased values were found in the autograft group, when compared with the group where hEC was filled with saline. Additionally, VEGF expression in both proximal and distal nerve end was significantly higher in the hEC supported with hMSC, when compared to the hEC filled with saline, therefore confirming the regenerative potential of human mesenchymal stem cells. Histological assessment revealed comparable results between the autograft and hEC with hMSC group in the values of proximal myelin thickness, fiber diameter and axonal density as well as in the distal end fiber diameter. The percentage of myelinated fibers in distal stumps was significantly higher in hEC with hMSC, when compared to the control group of hEC filled with saline.

At 12-weeks after the nerve gap repair with unmatched-large hEC we demonstrated comparable results of hEC supported with hMSC and the autograft repair, confirming the supportive role of hMSC in enhancement of nerve regeneration after nerve gap repair with hEC of large-unmatched nerve diameters.

5. Conclusions

Although literature reports on the influence of conduit diameter on nerve regeneration are limited, our findings indicate that hEC of the unmatched diameter supported with hMSC is a valuable alternative to the autograft technique in the management of nerve gap repair in the cases of complex traumatic nerve injuries.

Author Contributions

K. K. analyzed data, wrote the manuscript, M. M. S. performed experiments, collected data, K. R. analyzed data, performed graphs, figures and edited manuscript, S. B. analyzed the data, performed statistical analysis, figures, wrote part of results and edited manuscript, A. L. participated in manuscript editing, M. S. designed and supervised the project, reviewed the article. All authors edited and approved the manuscript.

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Conflict of Financial Interest

All the authors do not have any possible conflicts of financial interest.

Conflict of Non-Financial Interest

M. S. is the inventor of the patent application related to Methods of Engineering of Neural Tissue (US/2012/171172A1) and holds a patent on the use of

epineural sheath grafts for neural regeneration and protection (WO/2009/124170A1). All the authors do not have any possible conflicts of non-financial interest.

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