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Long-Term Effects of Fibrin Conduit with Human Mesenchymal Stem Cells and Immunosuppression after Peripheral Nerve Repair in a Xenogenic Model

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Abstract

Introduction: Previously we showed that a fibrin glue conduit with human mesenchymal stem cells (hMSCs) and cyclosporine A (CsA) enhanced early nerve regeneration. In this study long term effects of this conduit are investigated. **Methods:** In a rat model, the sciatic nerve was repaired with fibrin conduit containing fibrin matrix, fibrin conduit containing fibrin matrix with CsA treatment and fibrin conduit containing fibrin matrix with hMSCs and CsA treatment, and also with nerve graft as control. **Results:** At 12 weeks 34% of motoneurons of the control group regenerated axons through the fibrin conduit. CsA treatment alone or with hMSCs resulted in axon regeneration of 67% and 64% motoneurons respectively. The gastrocnemius muscle weight was reduced in the conduit with fibrin matrix. The treatment with CsA or CsA with hMSCs induced recovery of the muscle weight and size of fast type fibers towards the levels of the nerve graft group. **Discussion:** The transplantation of hMSCs for peripheral nerve injury should be optimized to demonstrate their beneficial effects. The CsA may have its own effect on nerve regeneration.

Keywords

peripheral nerve injury, spinal motoneurons, gastrocnemius muscle, human mesenchymal stem cells, immunosuppression

Introduction

Despite development of microsurgical technique and substantial experimental efforts, repair of a peripheral nerve gap remains a clinical and scientific challenge[1,2]. In search for a substitute to the nerve graft, repair with tubular conduits remains the most promising strategy[3] and has been widely researched using a variety of natural and synthetic materials[4,5]. A bioengineered nerve graft is expected to combine several elements[6]: a guidance channel, matrix with glial cells and/or growth factors to provide support to regenerating axons. To improve the properties of a tubular conduit, transplantation of different cells of various origins have been investigated. Among various types of cellular grafts tested experimentally[7], the most promising effects on axonal regeneration have been reported using cultured Schwann cells[8,9]. These cells provide a supportive environment and exert neuroprotective effects as they produce extracellular matrix molecules, integrins and neurotrophic factors[10]. However, with the ultimate goal of human application, as Schwann cells are difficult to obtain in large quantities, various types of stem cells have been suggested as alternatives which has included testing of mesenchymal stem cells (MSCs) in rat models[11]. Despite our own promising results

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showing short term benefits of human stem cells transplantation[12], obtaining a replacement for a nerve graft remains elusive in long term studies[13]. As the MSCs are postulated to be both immunomodulatory and uniquely immunotolerated, some authors report survival of transplanted mesenchymal stem cells of human origin in immunocompetent animals[14–16], while others advocate transplantation in nude animals with additional immunosuppresion to obtain cell survival. In the xenogenic mesenchymal stem cell transplantation model in the settings of nerve injury and repair with conduit, the role of immunosuppression is not clear. It is possible that Cyclosporine A (CsA) might have its own effect on nerve regeneration[12,17].

In our previous short-term study[12], we demonstrated that human MSCs transplanted in a fibrin conduit enhance regeneration at 3 weeks in a rat sciatic nerve injury model only if combined with CsA treatment. The aim of the present study was to test the long-term effects of a tubular fibrin conduit seeded with human mesenchymal stem cells (hMSCs) and supplemented with immunosuppressive treatment, on repair of a 10 mm long sciatic nerve gap defect in adult rats.

Materials and Methods

Experimental Procedures

Experimental animals. The experiments were performed on adult female inbred Fischer rats (n = 40; 10-12 weeks old, average weight of 170 g, Taconic Europe A/S, Denmark). The animal care and experimental procedures were carried out in accordance with the standards of the European Communities Council Directive (86/609/EEC) and the NIH Guide for Care and Use of Laboratory Animals (National Institutes of Health Publications No. 86-23, revised 1985). This study was approved by the Northern Swedish Regional Committee for Ethics and Animal Experiments (Dnr A127-10, A186-12). All animals received general anesthetic intraperitoneally using a mixture of ketamine (Ketalar®, Parke-Davies, 100 mg/kg) and xylazine (Rompun®, Bayer, 10 mg/kg).

Isolation and culture of mesenchymal stem cells. The procedures obtaining human cells were approved by the Local Ethical Committee for Clinical Research in Umeå University (Dnr 03-425). Human bone marrow was obtained from the iliac crest of two healthy donors (one female 28 years and one male 21 years old) during reconstructive surgery with written informed consent. The bone marrows were rinsed through each bone cavity with Minimum Essential Medium-alpha (α-MEM) containing 10% (v/v) foetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen Life Technologies, Fisher Scientific, Gothenburg, Sweden) using a 21G needle. The resulting cell suspension was triturated and centrifuged at 1500 rpm for 5 minutes. After resuspension of the pellet, the cells were filtered through a 70 μm cell strainer (Falcon brand Fisher Scientific, Gothenburg, Sweden) and plated in 75 cm^2 tissue culture flasks (Nunc brand, Fisher Scientific, Gothenburg, Sweden) and cultured in 37C 5% CO_2 until passage 2. After 24 h in culture the supernatant containing non-adherent cells was removed and discarded and fresh medium added. This was repeated for the following 2 days.

Characterization of hMSCs: Flow cytometry and immunostaining. MSCs at passage 3 were characterised by flow cytometry using antibodies against positive mesenchymal stem cell associated surface markers (CD73, CD90, CD105) and an antibody mix against surface markers which should not be expressed in MSCs (negative mix: CD11b, CD19, CD34, CD45 and HLA-DR). All antibodies were PE-conjugated (BD Biosciences, Stockholm, Sweden). Optimal concentrations of the antibodies were calculated (1:25 for all antibodies except for CD90 that was diluted at 1:33) and 20,000 events were analysed for each antibody. Corresponding isotype controls were also included (mouse IgG1, κ for the positive markers and mouse IgG2a, κ for the negative mix). Data were acquired using a BD Accuri C6 (BD Bioscience, Stockholm, Sweden) and plotted as histograms.

For immunostaining the cells were plated on Labtek® eight-well chamber slides (Fisher Scientific) at a density of 10 000 cells/well. After 72 hours, the cells were fixed with 4% w/v paraformaldehyde and stained with anti-CD14, anti-CD19, anti- H-CAM (CD44), according to the manufacturer's recommendations (Human Mesenchymal Stem Cell Characterisation Kit, EMDMillipore, Solna, Sweden). In addition, we used anti-CD54 (monoclonal, 1:20, Merck Millipore, Solna, Sweden), anti-collagen type IV (monoclonal, 1:200, Merck Millipore, Solna, Sweden) and anti- fibronectin (monoclonal, 1:500, Merck Millipore, Solna, Sweden) with secondary antibody Alexa Fluor 488 goat anti-mouse IgG (1:1000, Invitrogen, Fisher Scientific, Gothenburg, Sweden). The staining specificity was tested by the omission of primary antibodies. Expressions of the antigens were observed under a Nikon microscope and images were captured with an attached Nikon DXM1200 digital camera at 1280 × 1024 pixels.

Reverse transcription (RT)-PCR. The RNeasy™ mini kit (Qiagen Nordic, Sollentuna, Sweden) was used according to the manufacturer's protocol for the isolation of total RNA from the cell pellets of the human MSCs and then 1 ng RNA was incorporated into the One-Step RT-PCR kit (Qiagen Nordic, Sollentuna, Sweden) per reaction mix. Primers were manufactured by Sigma-Aldrich, Gillingham, UK (Table 1). A thermocycler (Biometra, Göttingen, Germany) was used with the following parameters: a reverse transcription step (50°C, 30 min), a nucleic acid denaturation/reverse transcriptase inactivation step (95°C, 15 min) followed by 35 cycles of denaturation (95°C, 30 sec) and annealing (30 sec, optimised per primer set as described in Table 1) and primer extension (72°C,1 min) followed by final extension incubation (72°C, 5 min). PCR amplicons were electrophoresed (50 V, 90 min) through a 1.5% (w/v) agarose gel and the size of the PCR products estimated using Hyperladder IV

Table 1. Primer Sequences for Reverse Transcription-PCR with Annealing Temperatures (°C).

Factor	Forward Primer (5′→3′)	Reverse Primer (5′→3′)	°C
NGF	ATACAGGCGGAACCACACTCAG	GTCCACAGTAATGTTGCGGGTC	65.1
BDNF	AGAGGCTTGACATCATTGGCTG	CAAAGGCACTTGACTACTGAGCATC	64.4
NT3	GGGAGATCAAAACGGGCAAC	ACAAGGCACACACACAGGAC	62.0
GDNF	CACCAGATAAACAAATGGCAGTGC	CGACAGGTCATCATCAAAGGCG	65.8
VEGF-A	TACCTCCACCATGCCAAGT	TGCATTCACATTTGTTGTGC	61.2
IGF-1	TGTCCTCCTCGCATCTCTTC	CACTCCCTCTACTTGCGTTC	60.4
Angiopoietin-1	CTTGACCGTGAATCTGGAGC	AGCAAGACATAACAGGGTGAG	59.7

(Bioline, Nordic Biosite AB, Täby, Sweden). Samples were visualised under UV illumination following GelRed™ nucleic acid stain (Biotium, Saveen & Werner, Limhamn, Sweden) incorporation into the agarose.

ELISA. Secreted neurotrophic and vasculogenic proteins were determined by ELISA. Stem cells were seeded at 6500 cells/cm^2 per 5 mL media in 25 cm^2 flasks. The media from the cells was changed after 4 days and replaced with fresh 5 ml media, which was then collected and analyzed at day 7 with BDNF, VEGF-A and angiopoietin-1 sandwich ELISA kits according to the manufacturer's protocols (RayBiotech, GA, USA). All samples were analysed in duplicate and the end-absorbance was measured at 450 nm (BioTek Synergy microplate reader). Quantity of secreted protein was calculated in pg/ml/10^4cells which was determined from data obtained from protein standard curves and final cell counts after 7 days of culture.

Conduit preparation. Tubular fibrin conduit was molded from two compound fibrin glue (Tisseel® Duo Quick, Baxter SA, Zurich, Switzerland). Fibrin glue contains; 70-110mg/ml of fibrinogen, 2-9mg/ml of plasma fibronection, 10-50 U/ml of factor XIII, 40-120 μg/ml of plasminogen, 3000 KIU/ml of aprotinin solution, 5 IU/ml of thrombin and 40mmol/l of calcium chloride. All components were mixed in sterile conditions and a silicone mold with centrally placed metal rod was used to prepare tubular 14-mm-long conduits with uniform 1-mm-thick walls and 2 mm lumen as described previously[18]. After glue polymerization, the rods and silicone mold were removed and fibrin glue conduits were stored in sterile Dulbecco's Minimum Eagle's Medium at room temperature. Fibrin conduits prepared in this manner degrade within 4 weeks after transplantation[18].

Preparation of matrix. Fibrin matrix was produced by modifying two component fibrin glue (Tisseel® Duo Quick, Baxter SA, Zurich, Switzerland). Both components of fibrin glue were diluted according to Bensaid et al[19]. To dilute the fibrinogen 1:5 a buffer consisting of 10 ml of de-ionised water with 73.5 mg of sodium citrate, 16.9 mg sodium chloride, 249.9 mg glycine, 30 000 KIU aprotinin (Sigma, A-3428, Sigma-Aldrich, Stockholm, Sweden) and 150 mg albumin serum (Sigma, A-3428, Sigma-Aldrich, Stockholm, Sweden). For dilution of thrombin, a solution of 10 ml of de-ionised water with 58.8 mg calcium chloride, 87.1 mg sodium chloride, 30.3 mg glycine and 500 mg serum albumin was prepared and diluted 1:5. The two solutions were then combined as explained below producing a relatively soft fibrin clot[19] with 18 mg of fibrinogen per ml and 100 IU of thrombin per ml generating the optimal solution of 1/100 dilution of thrombin and 1/10 dilution of fibrinogen. Fibrin matrix was used as a conduit filler and cell transplantation and no adverse effects were observed[18].

Incorporating cells into the matrix. Human mesenchymal stem cells were re-suspended with the diluted fibrinogen solution to a concentration of 80 x 10^6 per ml[20]. The diluted thrombin solution (25 μl) was injected into the lumen of a conduit and then immediately an equal volume of the cell/fibrinogen suspension was added. The matrix was allowed to polymerise for 10 minutes prior to surgical transplantation into the sciatic nerve defect. Conduits without cells were made as control.

Surgical procedure and experimental groups. A three cm long skin incision was made over the left gluteal area. Under an operating microscope (Zeiss, Carl Zeiss, Oberkochen, Germany), the sciatic nerve was exposed through splitting of gluteal and biceps muscle and then divided 5 mm below the exit point from sciatic notch and 7 mm of sciatic nerve distal to the division was removed creating 10 mm gap. The 14 mm long fibrin conduit was inserted in the gap, allowing for intubation of the nerve end 2 mm into the conduit, resulting again in 10 mm gap between proximal and distal sciatic stump. The conduit was fixed to the epineurium with one 10-0 Ethilon suture at each end. In the nerve graft repair group, a 10 mm-long sciatic nerve segment was removed, reversed and implanted back to re-connect proximal and distal nerve stumps using three interrupted 10-0 Ethilon epineural sutures. Tension was avoided and atraumatic handling and correct rotational alignment were employed throughout all procedures. The wound was then closed in layers. The animals were divided into the following experimental groups: (i) reversed nerve graft (n = 10), (ii) fibrin conduit with fibrin matrix (n = 10), (iii) fibrin conduit with fibrin matrix plus daily injections of Cyclosporine A (n = 10), and (iv) fibrin conduit with fibrin matrix supplemented with hMSCs plus daily injections of Cyclosporine A (n = 10). Operated animals were allowed to survive for 12 weeks.

Immunosuppressive treatment. Cyclosporine A (CsA; Sandimmun, Novartis Pharmaceuticals Corporation, East Hanover, New Jersey) was injected intraperitoneally at 1.5 mg per 100 g body weight starting from 24 hours before surgery. The weight of animals was checked at monthly intervals to ensure consistent delivery of the same concentration of CsA.

Retrograde labeling of spinal motoneurons. The medial gastrocnemius motoneuron pool was pre-labeled with 2% fluorescent tracer Fast Blue one week before sciatic nerve injury and repair as described previously[21]. In order to identify the number of spinal motoneurons which had regenerated axons through the nerve graft and fibrin conduits in the same animals the sciatic nerve was identified and transected 10 mm from the distal graft end at 11 weeks after transplantation. A small cube of Spongostan (Ethicon, Johnson & Johnson Medical, Sollentuna, Sweden) drenched in 10% aqueous solution of fluorescent tracer Fluoro-Ruby (Invitrogen, Fisher Scientific) was applied to the proximal stump of the transected nerve and isolated from the surrounding tissue by a well from mixture of silicone grease and Vaseline to prevent leakage. Two hours later the sponge was removed, the nerve was rinsed in normal saline and the wound closed in layers. The animals were left to survive for one more week to enable labeling of the neurons before the termination of the experiment.

Tissue processing. At the end of the survival period, the animals with retrogradely labeled spinal motoneurons were given an overdose of sodium pentobarbital (240 mg/kg, i.p., Apoteksbolaget AB, Umeå, Sweden) and transcardially perfused with Tyrode's solution (37°C) followed by cold 10% paraformaldehyde (PFA, pH 7.4). The spinal cord segments L4–L6 were harvested and post fixed overnight in the same fixative. The spinal cord segments were cut in serial 50 μm thick parasagittal sections on a Vibratome (Leica Biosystems, Triolab AB, Mölndal, Sweden), mounted onto gelatin-coated slides and coverslipped with DPX. In the remaining animals, the entire gastrocnemius muscles were dissected out using the operating microscope, dividing their tendinous origins and insertions flush with the bone. The muscles were weighed immediately after harvest and the left/right muscle weight ratios were calculated. The gastrocnemius muscles were embedded in OCT compound and snap frozen in liquid nitrogen.

Cell counts. The nuclei of the Fluoro-Ruby labeled spinal motoneurons were counted in all sections at x250 magnification in a Leitz Aristoplan microscope using filter block A. The total number of nuclear profiles was not corrected for split nuclei, since there was uniformity in nuclear size and the nuclear diameters were small in comparison with the section thickness.

Immunohistochemistry. Sixteen micron transverse sections of gastrocnemius muscles from operated (left) and contralateral (right) sides were cut on a cryostat, fixed with 4% paraformaldehyde for 15 min and blocked with normal serum. Sections were incubated with monoclonal primary antibodies raised against fast and slow myosin heavy chain protein (NCL-MHCf and NCL-MHCs, Novocastra, Peterborough, UK both 1:20 dilution) for 2 h at room temperature. Each slide was also co-incubated with rabbit anti-laminin antibody (Sigma, Poole, UK; 1:200 dilution). After rinsing in phosphate-buffered solution, secondary goat anti-rabbit and goat anti-mouse antibodies Alexa Fluor 488 and Alexa Fluor 568 (1:200; Invitrogen, Fisher Scientific, Gothenburg, Sweden) were applied for 1 h at room temperature in the dark. The slides were coverslipped with Prolong anti-fade mounting medium containing 4'-6-Diamidino- 2-phenylindole (DAPI; Invitrogen, Fisher Scientific, Gothenburg, Sweden). The staining specificity was confirmed by omission of primary antibodies.

Image processing. Preparations were photographed with a Nikon DXM1200 digital camera attached to a Leitz Aristoplan microscope. The captured images were resized, grouped into a single canvas and labeled using Adobe Photoshop CS3 software. The contrast and brightness were adjusted to provide optimal clarity.

Muscle analysis. Morphometric analysis of muscle sections was performed on coded slides without knowledge of their source. Five random fields were chosen (using the x16 objective) and images for the immunolocalisation of each myosin heavy chain type plus that for laminin were captured using the appropriate emission filters, and combined to provide dual-labeled images. Each image contained at least 25 individual muscle fibers for analysis. Image-Pro Plus software (Media Cybernetics, Rockville, MD, USA) was calibrated to calculate the mean area and diameter (in μm) for each muscle. The injured side was expressed relative to the contra-lateral control side and the relative mean % ± SEM calculated for each group.

Statistical analysis. One-way analysis of variance (ANOVA) followed by a post hoc Newman–Keuls test (Prism®, GraphPad Software, Inc.; San Diego, California, USA) was used to determine statistical differences between experimental groups.

Results

General Findings

During 12 weeks of observation period one animal from the group treated with fibrin conduit filled with matrix and receiving daily cyclosporine A injections died from sepsis related to intraperitoneal injections. There were no other minor or major complications related to intraperitoneal cyclosporine injections. The weight of experimental animals did not decrease during the observation period. Similarly, no

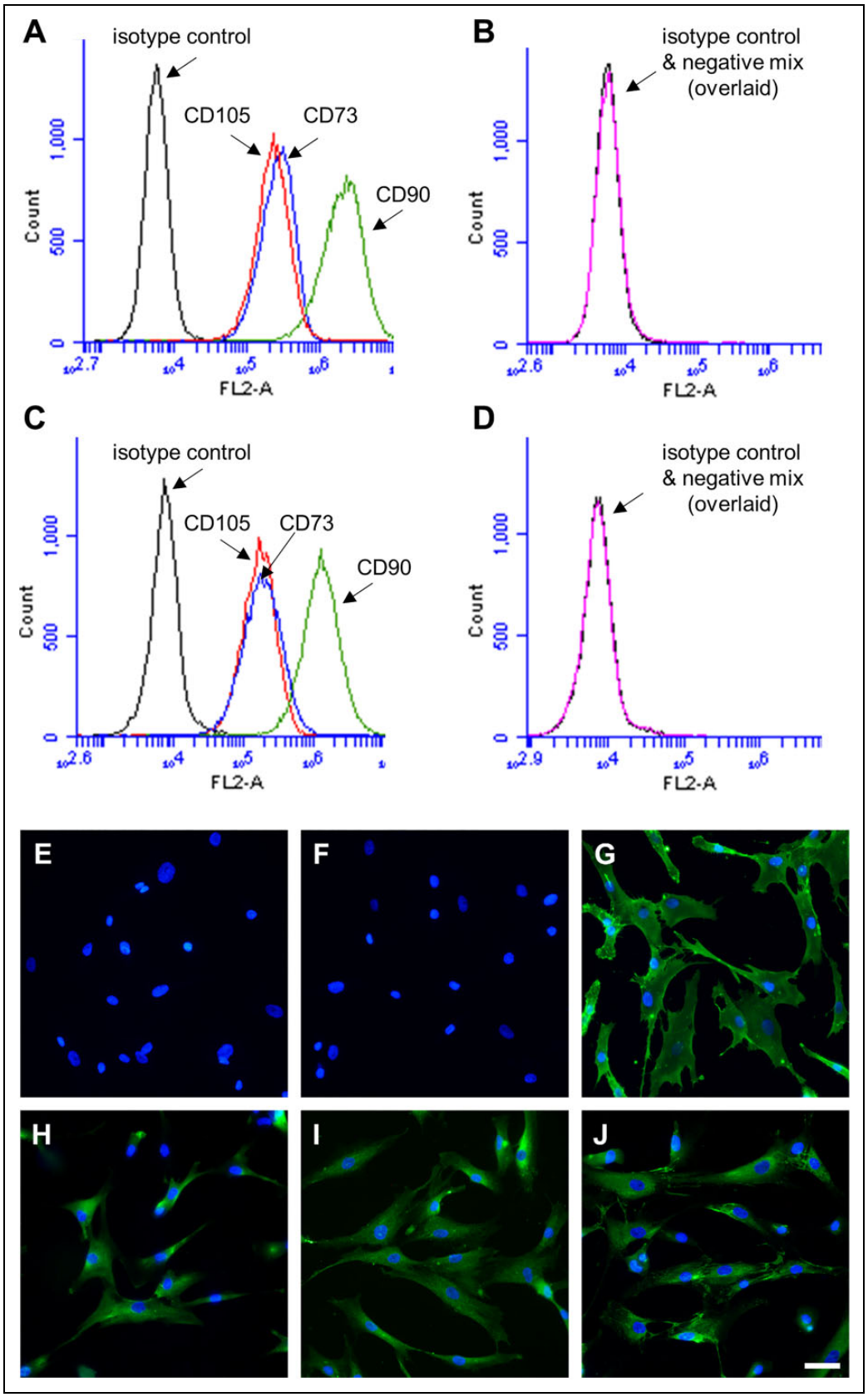

Fig. 1. Flow cytometry and immunostaining of stem cell markers expression in human MSCs. Flow cytometry: both patient samples (female donor in A and male donor in C) were stained for CD73, CD90, CD105 with corresponding isotype control. A mixture of antibodies against markers which should not be expressed in MSCs (negative mix: CD11b, CD19, CD34, CD45 and HLA-DR) was also used together with the respective isotype control for each patient shown in B and D. Immunostaining: cells were negative for leukocyte markers CD14 (E) and CD19 (F), positive for general mesenchymal stem cell markers H-CAM/CD44 (G), CD54 (H) and expressed extracellular matrix molecules collage type IV (I) and fibronectin (J). The nuclei were counterstained with DAPI. Scale bar 50 μm.

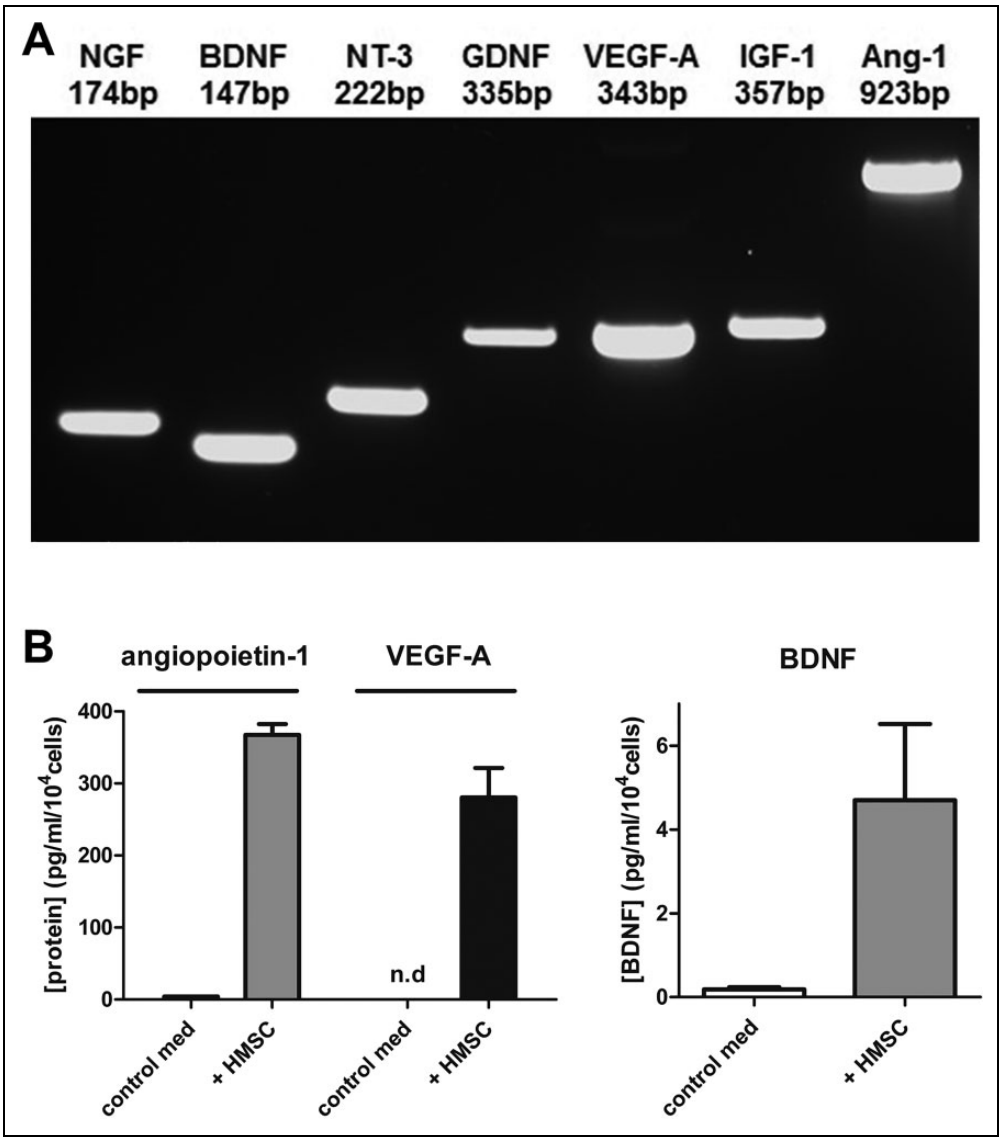

Fig. 2. Expression of neurotrophic and vasculogenic molecules in human MSCs. (A) Reverse transcription-PCR for the assessment of transcript levels of *NGF, GDNF, NT-3, BDNF, IGF-1, VEGF-A* and *angiopoietin-1* (ang-1). Amplicon size is shown in base pairs (bp). (B) ELISA analyses of cell culture supernatants. Data shown are mean ± SEM for both patient samples. n.d = not detected.

adverse effects of conduit implantation or cell transplantation were observed.

Characterization of MSCs

Flow cytometry revealed that hMSCs isolated from human bone marrow tissue stained positive for CD73, CD90, CD105 (Figs. 1A and 1C) and did not express a range of predicted negative surface markers (Figs. 1B and 1D). Immunostaining also showed that the hMSCs were negative for specific haematopoietic cell markers CD14 (Fig. 1E) and CD19 (Fig. 1F), and positive for other mesenchymal stem cell markers CD44 (Fig. 1G) and CD54 (Fig. 1H). The hMSCs also expressed collagen type IV (Fig. 1I) and fibronectin (Fig. 1 J). RT-PCR analysis revealed that hMSCs expressed transcripts for nerve growth factor (*NGF*), glial derived neurotrophic factor (*GDNF*), neurotrophin-3 (*NT-3*), brain derived neurotrophic factor (*BDNF*), insulin like growth factor-1 (*IGF-1*), vascular endothelial growth factor-A (*VEGF-A*) and *angiopoietin-1* (Fig. 2A). ELISA analyses (Fig. 2B) showed that the cells secreted detectable quantities of various vasculogenic and neurotrophic factors; angiopoietin-1 (368 ± 15 pg/ml/10^4 cells), VEGF-A (280 ± 41 pg/ml/10^4 cells) and BDNF (5 ± 2 pg/ml/10^4 cells).

Regeneration of Spinal Motoneuron Axons

To assess the number of spinal motoneurons that regenerated axons into the distal nerve stump, retrograde fluorescent tracer Fluoro-Ruby was used. At 12 weeks after nerve injury and repair with reversed nerve autograft (labeled as NG group in the graphs), 1770 ± 73 (mean ± SEM)

Table 2. Effects of Nerve Graft and Fibrin Conduit Supplemented with hMSCs and Daily Injections of Cyclosporine A on the Regeneration of Spinal Motor Neuron Axons and Recovery of the Gastrocnemius Muscle at 12 Weeks after Sciatic Nerve Repair.

Experiment	FR-labeled spinal motoneurons Mean ± SEM	% of control	Left/right gastrocnemius muscle weight ratio; Mean ± SEM, %
Nerve graft	1770 ± 73	100	62.20 ± 2.63
Fibrin conduit with matrix	609 ± 264	34**	21.40 ± 2.20***
Fibrin conduit with matrix + cyclosporine A	1186 ± 210	67	54.50 ± 1.32
Fibrin conduit with matrix + hMSC + cyclosporine A	1133 ± 250	64	58.00 ± 3.36

P < 0.001 is indicated by ** (Fibrin conduit with matrix vs Nerve graft).
P < 0.0001 is indicated by *** (Fibrin conduit with matrix vs Nerve graft, Fibrin conduit with matrix + cyclosporine A and Fibrin conduit with matrix + hMSC + cyclosporine A).

motoneurons were labeled with Fluoro-Ruby indicating that they had regenerated axons across the distal graft-nerve interface and extended for at least 10 mm into the distal stump of the sciatic nerve (Table 2, Fig. 3A). After transplantation of fibrin conduit with fibrin matrix, the number of motoneurons regenerating axons (Fluoro-Ruby labeled) was reduced to 34% when compared with the nerve graft group (P < 0.001; Table 2, Fig. 3B). Addition of the daily injections of CsA or combination of CsA treatment with transplantation of hMSCs resulted in axon regeneration of 67% and 64% of spinal motoneurons (as measured by Fluoro-Ruby counts), respectively (P > 0.05; Table 2, Figs. 3C and 3D). An interesting observation was the difference in appearance of retrogradely labeled dendrites in spinal motoneurons at 12 weeks after Fast Blue application to the medial gastrocnemius nerve (Fig. 3E-H). Following peripheral nerve grafting, second order and third order labeled dendrites were present in many studied motoneurons (Fig. 3E). In contrast, repair of the sciatic nerve with fibrin conduit preserved Fast Blue labeling mainly of the first order dendrites. Furthermore, Fast Blue was also found in small cells probably representing activated microglia and macrophages phagocytosing the retrogradely labeled degenerating motoneurons as previously described[22,23]. However, following CsA treatment or CsA treatment with hMSCs transplantation numerous spinal motoneurons demonstrated both first order and second order Fast Blue-labeled dendritic branches (Figs. 3G and 3 H).

Recovery of Medial Gastrocnemius Muscle Weight

The muscle weights of animals treated with reversed nerve graft recovered to 62% of the contra-lateral side (Table 2). Fibrin conduit with fibrin matrix reduced the muscle weight ratio to 21% (P < 0,001; Table 2) whereas daily CsA injections alone or in combination with hMSCs improved muscle weight ratio to 54% and 58%, respectively (Table 2). There was no statistically significant difference in muscle weight recovery between the nerve graft, fibrin conduit with daily injection CsA and fibrin conduit supplemented with hMSCs and CsA (P > 0.05; Table 2).

Morphological Analysis of Fast and Slow type Muscle Fibers

The gastrocnemius muscles were analysed further by immunostaining for fast and slow type muscle fibers (Fig. 4). The muscles from the nerve graft group showed few areas of gross atrophy and presented a characteristic mosaic pattern of muscle fibers resembling the contralateral unoperated side muscles (Fig. 4). In contrast the muscles from animals treated with fibrin conduit/fibrin matrix alone had large areas of atrophic, rounded small size muscle fibers (Fig. 4). The animals treated with CsA alone or CsA and hMSCs in the fibrin conduits showed morphology closer to the nerve graft groups, but the muscle fibers appeared to be smaller (Fig. 4). To assess this further, computerized image analysis of the muscle fiber size and area was performed (Fig. 5). The results revealed that the mean area of fast type fibers was significantly (P < 0.05) diminished in the fibrin conduit repaired group in comparison with nerve graft and CsA treated groups (Fig. 5A), however there was no statistical difference (P > 0.05) between the fibrin conduit group and fibrin conduit with hMSCs and CsA treatment. Analysis of mean diameter of fast type fibers demonstrated similar changes with significant differences (P < 0.05; Fig. 5B) between fibrin conduit and all other experimental groups (Fig. 5B). In contrast, the mean area and diameter occupied by slow type fibers were not significantly different between experimental groups (P > 0.05; Figs. 5C, D).

Discussion

The present study investigated the long-term growth-promoting effects of two component fibrin conduit seeded with human mesenchymal stem cells and combined with immunosuppressive treatment for peripheral nerve repair in adult rats. Similar to previous reports from our research group[24] and others laboratories[25] the results of the current study demonstrated that hMSCs express typical mesenchymal cell markers in addition to neurotrophic factors such as BDNF, NT-3, GDNF, NGF and VEGF[24,26,27]. It is well known that these neurotrophic factors can enhance survival of sensory dorsal root ganglion neurons and spinal motoneurons[28,29] and promote axonal regeneration after peripheral nerve injury[30,31]. In our previous short-term study[12] we demonstrated that hMSCs in the presence of CsA can enhance axonal regeneration across the fibrin glue conduit at 3 weeks postoperatively. By using the same experimental approach, we hypothesized that hMSCs could enhance the

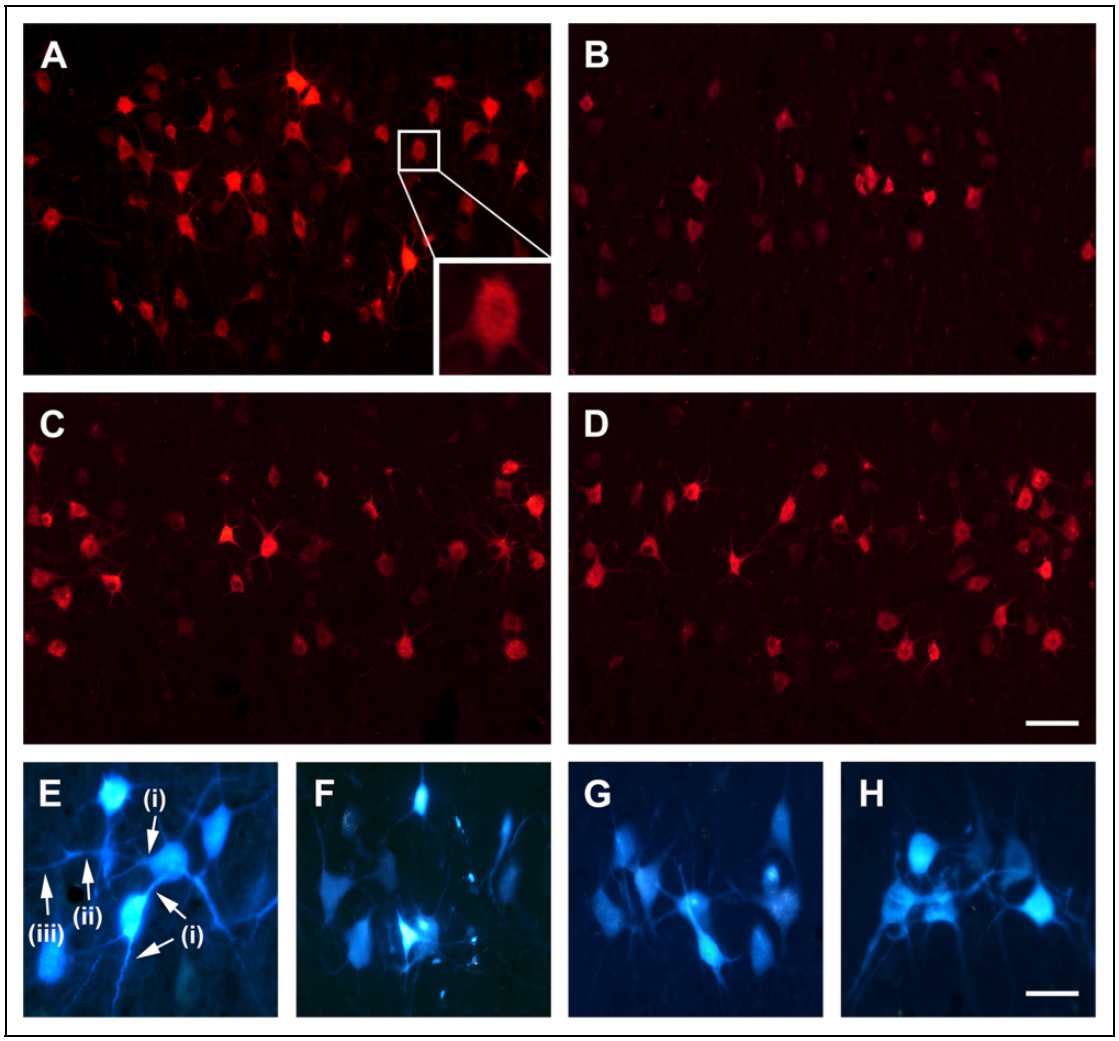

Fig. 3. Spinal motoneurons retrogradely labeled with Fluoro-Ruby (red) and Fast Blue (blue) at 12 weeks after sciatic nerve injury and followed by nerve repair with nerve graft A, E), fibrin conduit with fibrin matrix (B, F), fibrin conduit with matrix and CsA treatment (C, G) and fibrin conduit with matrix, hMSCs and CsA (D, H). Note the differences in dendritic appearance of Fast Blue-labeled motoneurons and the presence of microglia-like cells in the group repaired with fibrin conduit with matrix (E-H). The animals treated with nerve graft show clear first order (arrows i), second order (arrow ii) and third order (arrow iii) labeled dendrites (E). Scale bar: 100 μm (A-D) and 50 μm (E-H). Inset boxed area in (A) shows details of retrograde labeling with a nuclear profile.

regenerative response in long-term experiments. In this present study we have measured the number of motoneurons regenerating axons (as detected by Fluoro-Ruby labeling) and muscle weight (Table 2) and muscle morphology (Figs. 4 and 5) as parameters to assess the effects of hMSCs transplantation. In the short-term study[12] we measured area of the axonal staining within the conduits but because of axon sprouting the number of counted nerve fibers may not accurately reflect the effect of a treatment on regeneration. Therefore, in this present long-term study we decided to retrogradely label and count the number of motoneurons which had regenerated their axons. Using the results obtained from retrograde labeling and muscle analyses it was impossible to demonstrate potential beneficial effects of hMSC transplantation due to significant effects of the daily injections of Cyclosporine A.

The low impact of the hMSCs on long-term axonal regeneration and muscle recovery could also be due to increased cell death in the present xenogenic model when compared with transplantation of rat MSCs[32]. In addition, other factors such as poor blood supply, ischemia-reperfusion, inflammatory factors[33,34], conduit clogging as the density of transplanted cells was based on studies investigating Schwann cell transplantation[20] could modulate cell survival. Significant loss among transplanted cells has been also described in other injury models including cell transplantation into injured spinal cord[35,36]. Transplantation of human cells into the rat nervous system requires immunosupression which improves cell survival. It has been shown that CsA stabilizes mitochondrial membrane potential, up-regulates Bcl-2 and down-regulates bax expression[33]. As a result, several studies demonstrate that CsA can support the survival of transplanted fibroblasts[37],

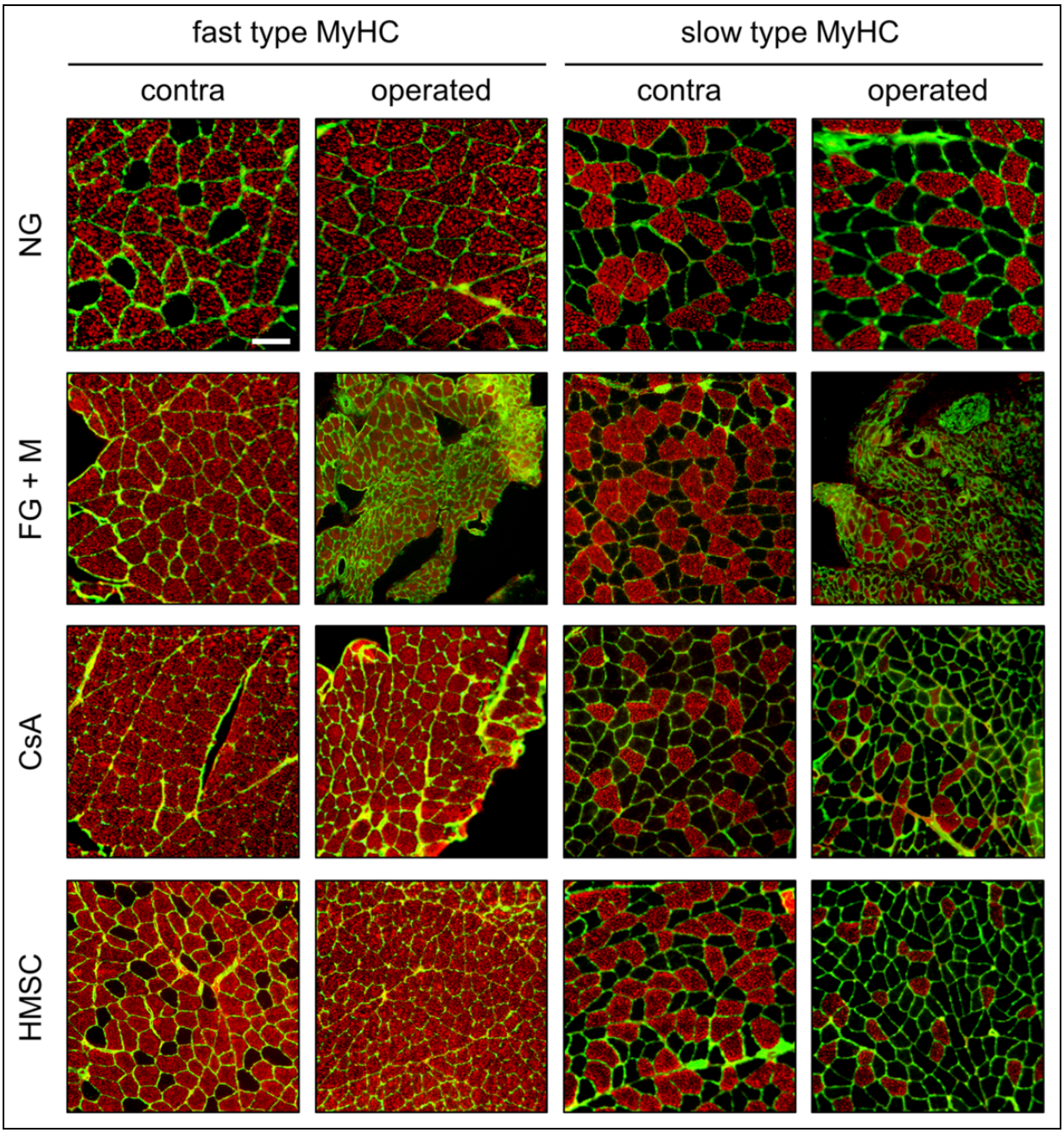

Fig. 4. Fast and slow type muscle fiber morphology. Transverse sections of medial gastrocnemius muscle stained with laminin antibody (green) and fast type or slow type myosin heavy chain (MyHC) protein antibody (red). Samples shown are 12 weeks following surgery from contra-lateral side muscles and muscles from the operated side of animals treated with nerve graft (NG), fibrin conduit with fibrin matrix (FG + M), fibrin conduit with fibrin matrix in the presence of cyclosporine A (CsA) and fibrin conduit with fibrin matrix with hMSCs in the presence of cyclosporine A (HMSC). Scale bar = 50 μm.

mesenchymal stem cells and adult neural precursor cells[38]. Our previous study[12] revealed that hMSCs re-suspended in fibrin matrix survived in fibrin conduit for at least 3 weeks if combined with CsA treatment. However, there are also reports that conventional immunosuppressive treatment with CsA is not sufficient to prevent death of hMSCs after transplantation in long-term experiments[26,39].

The minimal detected impact of the hMSCs in this present study is likely attributed in large part to the fact that the CsA "masks" the potential beneficial effects of hMSCs transplantation. For example, there are reports showing that CsA used in xenotransplant models promotes survival and axonal regeneration[40,41]. Treatment with CsA could be omitted if human cells are transplanted in the nervous system of athymic nude rats. It has been demonstrated that human mesenchymal precursor cells (Stro-1^{+}) transplanted into the injured spinal cord of nude rats can promote significant functional recovery[42]. The mechanisms of the

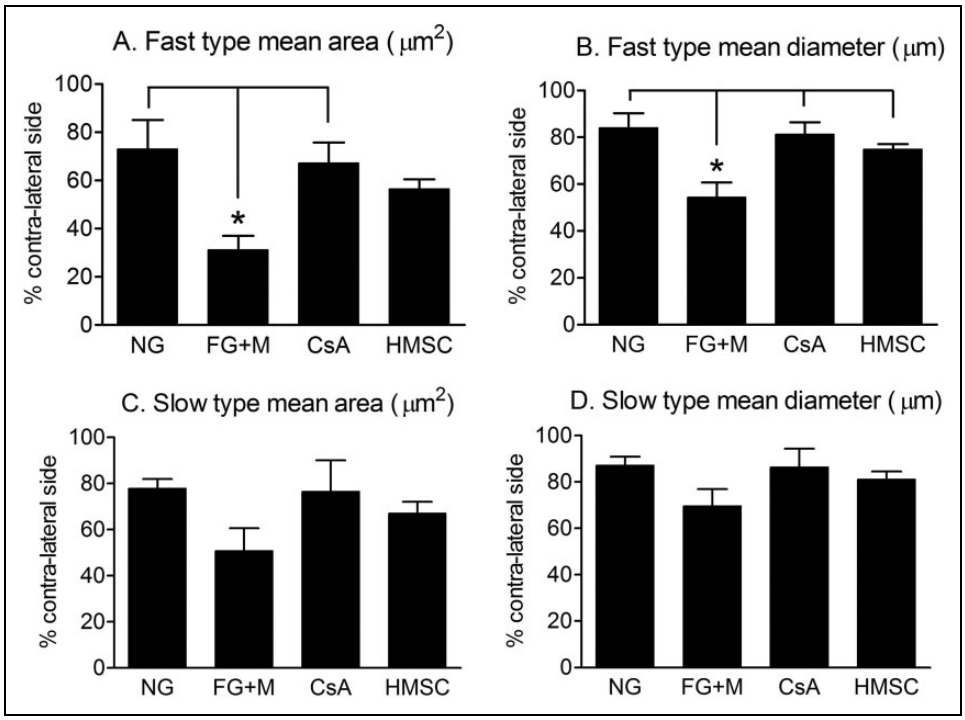

Fig. 5. Computerized image analysis was used to calculate the mean ± SEM area and diameter of fast (A, B) and slow (C, D) type fibers, respectively in muscle obtained from the contra-lateral and operated sides of animals repaired with nerve graft (NG); fibrin conduit with matrix (FG+M), fibrin conduit with matrix and CsA treatment (CA) and fibrin conduit with matrix, hMSCs and CsA treatment (HMSC) at 12 weeks postoperatively. Data are expressed as percentage of the contra-lateral side. $P < 0.05$ indicated by * (Fibrin conduit with matrix vs Nerve graft (A, B), Fibrin conduit with matrix + CsA (A, B) and Fibrin conduit with matrix + hMSC + Cs A (B).

neuroprotective effect of CsA could be due to down-regulation of nitric oxide, a well known neurotoxic agent[43] and direct inhibition of calcineurin, a potent regulator of muscle remodeling[44]. CsA has been considered as neuroprotective agent for treatment of acute traumatic brain injury in patients[45] since it can interrupt the endogenous mediators of secondary insult through inhibition of the mitochondrial permeability transition pore[46] and prevention of subsequent mitochondrial dysfunction[47–50]. A randomized clinical study evaluated safety and pharmacokinetics of a single iv infusion of CSA in 12 h following severe traumatic brain injury and observed increase in extracellular energy substrates and increase in lactate implicating higher glycolytic rate and hypermetabolism following the treatment[51,52]. Previous studies also show that CsA can support axonal regeneration after spinal cord injury[37,53], increase the regrowth of the retinal ganglion cells into the peripheral nerve graft[54] and accelerate the peripheral nerve regeneration[55]. In agreement with these observations, our previous short term study demonstrates that CsA treatment can induce significant axonal sprouting inside fibrin conduits[12].

Long-term CsA treatment could also have unwanted side effects such as weight loss and muscle weakness, possibly due to inhibition of calcineurin activity[56]. Although other studies have suggested that calcineurin inhibition by CsA modulates muscle phenotype rather than muscle mass[57] and results in an increase of type IIa MHC (fast fiber type) content at the expense of type I MHC (slow fiber type)[58]. Analysis of muscle morphology in the present study also revealed that CsA has positive effect on restoration of fast fiber type area and diameter. With respect to slow type muscle fibers, the mean area and diameter were not statistically different between experimental groups and this observation is in agreement with previous report that in denervated muscle a slow phenotype is triggered and maintained in a calcineurin- and nerve-independent manner[59].

In summary, due to the beneficial effects of CsA alone we were unable to prove that hMSCs transplantation could be a useful adjunct to the fibrin conduits for successful long-term nerve regeneration and reduction of muscle atrophy. Transplantation of human cells into rat models of peripheral nerve injury should be further optimized to demonstrate beneficial effects of human stem cell transplantation.

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Ethical Approval

This study was approved by the Northern Swedish Regional Committee for Ethics and Animal Experiments (Dnr A127-10, A186-12).The procedures obtaining human cells were approved by the

Local Ethical Committee for Clinical Research in Umeå University (Dnr 03-425).

Statement of Human and Animal Rights

The animal care and experimental procedures were carried out in accordance with the standards of the European Communities Council Directive (86/609/EEC) and the NIH Guide for Care and Use of Laboratory Animals (National Institutes of Health Publications No. 86-23, revised 1985). This study was approved by the Northern Swedish Regional Committee for Ethics and Animal Experiments (Dnr A127-10, A186-12).The procedures obtaining human cells were approved by the Local Ethical Committee for Clinical Research in Umeå University (Dnr 03-425).

Statement of Informed Consent

Written informed consent was obtained from the patients for their anonymized information to be published in this article.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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