Neuroscience 170 (2010) 1295–1303

MESENCHYMAL STEM CELLS IN A POLYCAPROLACTONE CONDUIT ENHANCE MEDIAN-NERVE REGENERATION, PREVENT DECREASE OF CREATINE PHOSPHOKINASE LEVELS IN MUSCLE, AND IMPROVE FUNCTIONAL RECOVERY IN MICE

J. T. OLIVEIRA,[a] F. M. ALMEIDA,[a] A. BIANCALANA,[a] A. F. BAPTISTA,[b] M. A. TOMAZ,[c] P. A. MELO[c] AND A. M. B. MARTINEZ[a]*

[a]*Programa de Neurociência Básica e Clínica, Instituto de Ciências Biomédicas, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil*

[b]*Departamento de Biomorfologia, Instituto de Ciências da Saúde, Universidade Federal da Bahia, Salvador, Bahia, Brazil*

[c]*Departamento de Farmacologia Básica e Clínica, Instituto de Ciências Biomédicas, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil*

Abstract—Although the majority of peripheral-nerve regeneration studies are carried out on the sciatic nerve, lesions of the upper extremities are more common in humans and usually lead to significant physical disabilities. The present study was driven by the hypothesis that a combination of strategies, namely grafts of mesenchymal stem cells (MSC) and resorbable polycaprolactone (PCL) conduits would improve median-nerve regeneration after transection. Mouse median nerves were transected and sutured to PCL tubes that were filled with either green fluorescent protein (GFP^+) MSC in DMEM or with DMEM alone. During the post-operative period, animals were tested weekly for flexor digitorum muscle function by means of the grasping test. After 8 weeks, the proximal and middle portions of the PCL tube and the regenerating nerves were harvested and processed for light and electron microscopy. The flexor digitorum muscle was weighed and subjected to biochemical analysis for creatine phosphokinase (CK) levels. Scanning electron microscopy of the PCL tube 8 weeks after implantation showed clear signs of wall disintegration. MSC-treated animals showed significantly larger numbers of myelinated and unmyelinated nerve fibers and blood vessels compared with DMEM-treated animals. The flexor digitorum muscle CK levels were significantly higher in the MSC-treated animals, but muscle weight values did not differ between the groups. Compared with the DMEM-treated group, MSC-treated animals showed, by the grasping test, improved functional performance throughout the period analyzed. Immunofluorescence for S-100 and GFP showed, in a few cases, double-labeled cells, suggesting that transplanted cells may occasionally transdifferentiate into Schwann cells. Our data demonstrate that the polycaprolactone conduit filled with MSC is capable of significantly improving the median-nerve regeneration after a traumatic lesion.

*Corresponding author. Tel: +55-21-2562-6431; fax: +55-21-2562-6431.
E-mail address: martinez@histo.ufrj.br (A. M. B. Martinez).
Abbreviations: CK, creatine phosphokinase; MSC, mesenchymal stem cells; PCL, polycaprolactone; VEGF, vascular endothelial growth factor.

Key words: cell therapy, mesenchymal cells, median nerve, polycaprolactone conduit, nerve regeneration, grasping test.

After an injury, the peripheral nerve undergoes a process called Wallerian degeneration, where the distal stump degenerates while the proximal stump survives and promotes regeneration (Waller, 1850). The peripheral nervous system is provided with an intrinsic growth capacity and a favorable microenvironment that guides and facilitates the elongation of the growth cone after a minor lesion. In spite of these peripheral nervous system features, sometimes, such as after a complete lesion, the growth cone may not reach the correct target organ, making functional recovery unlikely (Lundborg, 2000; Gordon et al., 2003). Therefore, therapeutic strategies must be applied to the damaged nervous tissue to enhance the regenerative process.

After traumatic nerve lesions, the damaged tissue must be surgically repaired, such as by direct suturing, autograft, allograft, or tubulization. When there is tissue loss, direct suturing is not possible because it would produce a secondary lesion by stretching the nerve stumps. Autograft and allograft both require the removal of healthy tissue to fill the gap left by the lesioned nerve, and therefore both techniques can lead to morbidity. Furthermore, when the donor is not the recipient, the allograft can trigger a pronounced inflammatory response. The tubulization technique is based on the use of a cylindrical conduit made of biological or synthetic material. This therapeutic strategy protects the lesion site from external negative influences, keeps trophic factors inside the conduit, and functions as a guide for the elongation of the growth cone (Lundborg et al., 1982; Chamberlain et al., 1998).

Several kinds of conduits have been used in experimental and clinical trials (Johnson and Soucacos, 2008). The synthetic polycaprolactone (PCL) conduit is biodegradable, and its biocompatibility has been proven (Den Dunnen et al., 1993; Chang, 2009). This biopolymer has also been assessed for the ability to support the adhesion, survival, differentiation, and migration of Schwann cells, and has proved to be an adequate substrate for these purposes (Schnell et al., 2007; Chiono et al., 2009).

The PCL conduit may give promising results when associated with cell therapy. Stem cells are capable of self-renewal and differentiation into several specialized cells. Bone-marrow mesenchymal stem cells (MSC) are multipotent adult stem cells that can differentiate into mesodermal lineages, such as adipocytes, condrocytes, os-

0306-4522/10 $ - see front matter
doi:10.1016/j.neuroscience.2010.08.042

teoblasts, muscle cells, or tendons (Muraglia et al., 2000). Previous studies have demonstrated their capacity to differentiate into endodermal (hepatocyte) and ectodermal (neuron and glia) lineages (Woodbury et al., 2000; Kim et al., 2002; Bossolasco et al., 2005; Krampera et al., 2006). MSC secrete several trophic factors, supporting neuritogenesis and neurite growth *in vitro* (Gu et al., 2009) and survival and elongation of the growth cone *in vivo* (Pereira Lopes et al., 2006; Chen et al., 2007; Pan et al., 2007; Ribeiro-Resende et al., 2009). Furthermore, these cells are easily obtained, isolated, and expanded *in vitro*; they have paracrine effects and migratory behavior; and unlike embryonic cells, no ethical issues impede their use (Brooke et al., 2007) and there is no risk of tumorigenesis. In addition, there is the possibility of an autologous transplant, avoiding tissue-rejection effects.

The majority of peripheral-nerve regeneration studies use the sciatic-nerve model, perhaps because of the large size of this nerve. Clinically, however, upper-extremity lesions are more common and can lead to disorders with emotional, social, work-related, and economic aspects (Lundborg and Dahlin, 1996; Kouyoumdjian, 2006). Recent studies have used the mouse median nerve as a model of peripheral-nerve injury (Bontioti et al., 2003; Tos et al., 2008) to investigate therapeutic strategies such as tubulization and neurorrhaphy. However, until now, cell therapy has not been applied in this nerve model.

In the present study, we investigated the regeneration of the mouse median nerve after a transection lesion followed by tubulization with a PCL conduit, filled or not with MSC. The combination of these two strategies has proved to be very encouraging, since it resulted in regenerated nerves with linear and organized nerve fiber growth. In addition, the nerves treated with MSC showed a significant increase in the number of myelinated fibers and blood vessels. Moreover, this group showed higher levels of CK in the muscle innervated by the median nerve, accompanied by improved functional recovery.

EXPERIMENTAL PROCEDURES

Animals

For this study, we used 32 C57/Black6 mice, weighing between 25 and 30 g. The animals were housed in cages with food and water *ad libitum* and a 12/12-h light/dark cycle. All experiments were performed in accordance with the Ethics Committee for the Use of Experimental Animals of the Universidade Federal do Rio de Janeiro (Protocol DHE003). All efforts were taken in order to minimize number of animals used and their suffering.

The animals were divided into three groups according to the surgical procedure performed: Normal (neither nerve lesion nor treatment, $n=10$), DMEM (nerve lesion followed by tubulization with DMEM, $n=10$), and MSC-treated [nerve lesion followed by tubulization with MSC in DMEM, $n=11$ (10 for all analyses performed and one for immunohistochemistry)].

Surgical procedure

The animals were deeply anesthetized with ketamine (100 mg/kg) and xylazine (15 mg/kg, União Química Farmacêutica S/A, Brazil). Their right median nerve was exposed from the axilla to the cubital fossa and then was transected at the middle portion of the *brachialis* muscle. The proximal stump was sutured through the epineurium to the PCL conduit using a 10.0 suture, and the MSC and/or DMEM (2×10^6/2 μL) were injected into the conduit by a microsyringe (Hamilton), and finally, the distal stump was sutured to the other end of the PCL conduit in the same way as the proximal stump, leaving a 3-mm gap between them. The left median nerve was exposed and also transected, but its distal stump was sutured to the *pectoralis major* muscle, preventing its regeneration, in order to neutralize this forepaw through the subsequent period of functional analysis. PCL conduits were kindly provided by Dr. Langone from Universidade Estadual de Campinas, Brazil.

Eight weeks after surgery, the animals were deeply anesthetized and submitted to transcardiac perfusion with a fixative solution (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for immunohistochemistry, or with 2% glutaraldehyde in 0.1 sodium cacodylate buffer, pH 7.4, for light and electron microscopy, 35 mL/animal). The right median nerve was dissected and two 2-mm-long segments were harvested, corresponding to the proximal and middle portions of the conduit.

Cell culture

The MSCs were obtained from bone marrow of C57/black 6 GFP mice. Expression of GFP allowed the identification of transplanted cells. Marrow was flushed with Hank's buffered saline solution (HBSS) from femurs and tibias of donor mice and centrifuged at 2000 rpm for 5 min. Cells were seeded into 25 cm^2 culture flasks at a density of 5×10^7 containing DMEM supplemented with 20% FBS and 100 U/mL penicillin. The cells were incubated at 37 °C in 5% CO_2 for 3 days. At this time, the nonadherent cells were removed from the flasks and the adherent cells were washed once with HBSS and fed with supplemented medium. These adherent cells were cultured and passaged three times until the final use. The culture medium was replaced three times per week. Before transplantation, the cells were counted, re-suspended at a density of 10^6 cells in 2 μL, and then injected into the conduit at the time of surgery. After surgery, a sample of the remaining cells was plated overnight, to assess the cell viability.

Fluorescence microscopy

We investigated the presence of GFP^+ cells 4 weeks after transplantation. To evaluate the expression of S-100 (a Schwann cell marker) by the GFP transplanted cells, we also performed an immunofluorescence analysis of these proteins. Nerve segments were washed twice in 0.1 M phosphate buffer (pH 7.4), cryoprotected in graded sucrose (10, 20, and 30%), and mounted in Tissue-Tek OCT compound (Sakura, USA). Frozen cross sections were cut at 10 μm on a cryostat (Leica CM 1850) and mounted on gelatin pre-coated slides. The sections were permeabilized and blocked with 0.03% Triton-X 100, 10% normal goat serum (NGS, Invitrogen) and 1% bovine serum albumin (BSA, Sigma) in PBS (pH 7.4) for 1 h at room temperature. Then, except for the negative control sample, sections were incubated with primary antibody (S-100, 1:200, rabbit anti-mouse, SIGMA, USA) overnight at 4°C followed by 2 washes, each one for 15 min, with 0.03% Triton-X 100. Then, samples were incubated with Alexa Fluor 546 red goat anti-rabbit IgG secondary antibody (1:600, Invitrogen, USA) for 1 h at room temperature and again washed three times. The nuclei were stained with DAPI for 5 min (1:100), washed two times, and finally the sections were mounted on slides with an aqueous gel montage medium with anti-fading agents.

Scanning electron microscopy

To investigate the ultrastructural features of the PCL conduit before and after 8 and 12 weeks of the implant, its proximal and middle portions were fixed by immersion, then were washed in 0.1 M phosphate buffer (pH 7.4) followed by 0.1 M cacodylate buffer

(pH 7.4) and postfixed for 2 h in 1% osmium tetroxide containing 0.8% potassium ferrocyanide and 5 nM calcium chloride in 0.1 M cacodylate buffer (pH 7.4). Then, they were washed in 0.1 M cacodylate buffer (pH 7.4) and dehydrated in an ethanol series. Samples were gold sputtered (FL-9496 Balzers, Union Coater) and observed and photographed in a Jeol JSM-5310 scanning electron microscope.

Transmission electron microscopy

After nerve segments corresponding to the conduit middle portion were fixed by immersion in 2.5% glutaraldehyde for 2 h, they were washed in 0.1 M phosphate buffer (pH 7.4) followed by 0.1 M cacodylate buffer (pH 7.4), and postfixed for 2 h in 1% osmium tetroxide containing 0.8% potassium ferrocyanide and 5 nM calcium chloride in 0.1 M cacodylate buffer (pH 7.4). The segments were washed in 0.1 M cacodylate buffer (pH 7.4) and distilled water and stained in 1% uranyl acetate overnight, dehydrated in graded acetone, infiltrated with Poly/Bed 812 resin (Polysciences Inc., Washington, PA, USA) and polymerized at 60°C for 48 h. Semithin cross-sections (500 nm) were obtained and stained with Toluidine Blue for light microscopy analysis. Ultrathin cross-sections (70 nm) were collected on copper grids and contrasted in uranyl acetate and lead citrate. Microscopy analysis and documentation were carried out using a Zeiss 900 transmission electron microscope operated at 80 Kv.

Histomorphometry analysis

Quantitative analysis was performed on the segment of the median nerve corresponding to the middle portion of the conduit. For the quantification procedure, we photographed the total nerve semithin cross section at a magnification of 20× under the light microscope. Then, we counted the total number of myelinated nerve fibers and blood vessel profiles. Unmyelinated nerve fibers were counted by using images obtained from the transmission electron microscope. For this purpose, 10 systematic fields in each nerve cross section were sampled and photographed at a magnification of 3000×, and in each field all unmyelinated nerve fibers were counted. All quantitative analysis were carried out by Image Java Software.

Flexor digitorum muscle weight and CK activity

This analysis was carried out as described by Melo and Ownby (1996). Just before fixation by perfusion, the flexor digitorum muscle was isolated and harvested, freed from fat and tendon, dried with absorbent filter paper, and weighed. The muscles were kept in 2 mL physiological saline solution (135 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 15 mM $NaHCO_3$, 1 mM NaH_2PO_4, and 11 mM glucose) containing 0.1% albumin. Next, they were cut into several pieces and homogenized. After 1:10 dilution, CK activity was determined using a diagnostic kit (Procedure No. 47 UV, Sigma, Chemical Company, USA) by light spectrophotometer (UV 1240, Shimazu). Values were expressed in units per gram of muscle tissue per hour (U/g/h), in which 1 U corresponds to the amount of enzyme that catalyzes the transformation of 1 μmol of substrate at 25°C.

Functional analysis

All animals were subjected weekly to a postoperative grasping test analysis, based on the protocol described by Bertelli and Mira (1995) and adapted for mice by Tos et al. (2008). The functional test apparatus was designed and constructed by our group, and consists of a grid (made of a stainless-steel wire screen with a 5-mm^2 mesh size, wire diameter 1 mm) supported at two points on a digital balance (Fig. 6A). The grid was kept on top of the balance by placing a 300-g weight on it. The weight was zeroed by pressing the tare button, then the animals were held by the tail and allowed to grasp the grid, and at the moment that they released their grip, the traction force exerted was recorded by the observer as a negative value. This test was repeated three times, and the mean value was used for the analysis (Tos et al., 2008). The force in grams was evaluated as an absolute value.

Statistical analysis

The statistical analyses were performed by a descriptive analysis, assessing the mean and error deviation. In the functional analysis, using the grasping test, we performed a paired and unpaired analysis, and in the other analyses we performed an unpaired analysis. Paired inferences were made by Student's *t*-test (between two groups) or one-way ANOVA (among three groups), followed by Tukey post hoc analysis when necessary. The confidence interval was 95%, with an accepted alpha value of 5% ($P<0.05$). The analyses were carried out using the Prism Graph Pad 4.0 statistical software.

RESULTS

PCL in association with MSC is a suitable substrate for the regenerating nerve

We observed the ultrastructural features of the proximal and middle portions of the PCL conduit, prior to surgery (Fig. 1) and 8 and 12 weeks after surgery (Fig. 2) by scanning electron microscopy. We did not observe any tube fragments at the surgical site, or an exacerbated inflammatory response. The ultrastructural analysis of the PCL tube prior to implantation showed the presence of prominent pores on the outer surface (Fig. 1A′, B, B′), and the inner surface was smoother (Fig. 1A′, C, C′). This attribute enables the nutrients to diffuse through the conduit wall while allowing the cells to remain inside it. At 8 and 12 weeks after surgery, we could observe the regenerated median nerve growing inside the conduit in a linear and organized way (Fig. 2B–D). At 12 weeks postsurgery, we observed that the PCL conduit was still present *in vivo* and it showed signs of the initial process of degradation with a less-porous surface and evident disorganization of the tube wall (Fig. 2C, C′). The thickness of the tube wall was 70 μm before surgery (Fig. 2A, A′), and was reduced to 65 and 60 μm by 8 and 12 weeks after implantation, respectively (Fig. 2B′, C′).

MSC increase the total number of myelinated nerve fibers and blood vessels

Eight weeks after the surgery, the total numbers of myelinated fibers and blood vessels were quantified by light microscopy, and the number of unmyelinated fibers was counted by electron microscopy as described above. All quantitative analyses were performed in the middle segment of the nerve.

Normal median nerves show the usual characteristics of a peripheral nerve (Fig. 3A, A′). Many clusters of regenerating nerve fibers were observed in the MSC-treated and DMEM nerves (Fig. 3B′, C′). We quantified the total number of myelinated nerve fibers in the normal group (1655 ± 70) and we observed that it was statistically different from both MSC-Treated ($P<0.05$) and DMEM ($P<0.001$)

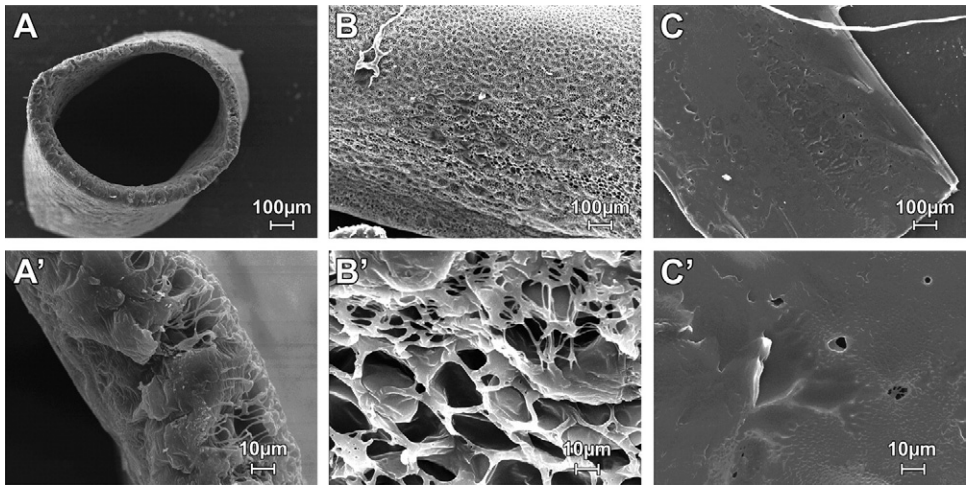

Fig. 1. Scanning electron micrographs of the PCL conduit before implant. (A) PCL conduit; (A′) PCL conduit wall; (B, B′) external surface; (C, C′) internal surface. Scale bars: (A–C: 100 μm); (A′–C′: 10 μm).

groups. The total number of myelinated fibers was higher in the MSC-treated group (1236±133) compared with the DMEM group (857±84) (Fig. 3D), and this difference was significant (Student's *t*-test, $P<0.05$). The number of blood vessels was significantly higher in the MSC-treated group (13.75±2.93) compared with the DMEM group (7.6±1.63) (Fig. 3E; Student's *t*-test, $P<0.05$).

Regarding the number of unmyelinated fibers, there was a significant difference between the MSC-treated group (19±1.34) and the DMEM group (13.94±0.65; $P<0.01$) (Fig. 4C).

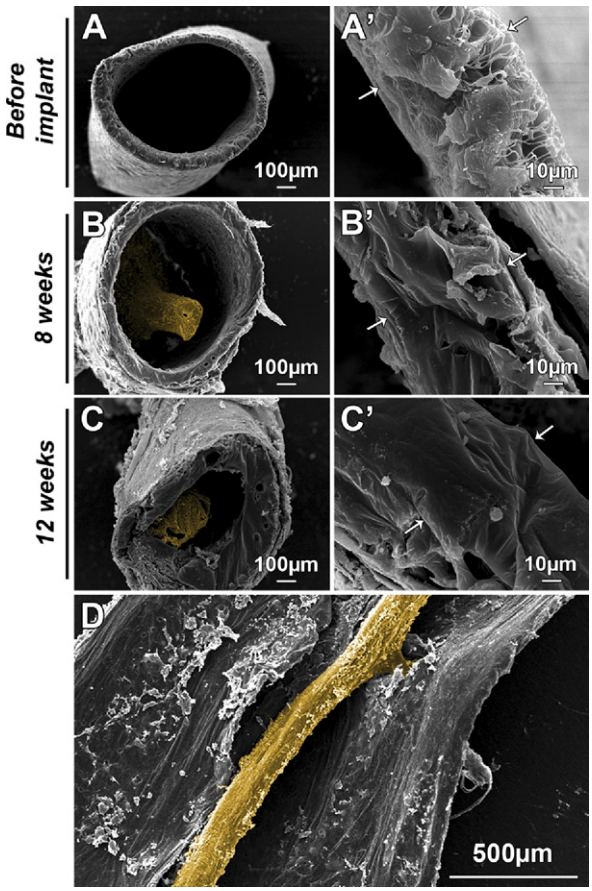

Fig. 2. Scanning electron micrographs of the PCL conduit before surgery (A, A′) and after 8 (B, B′) and 12 (C, C′) weeks of surgery. (D) Longitudinal view of the regenerated nerve inside the conduit 12 wk post-surgery. Areas between arrows represent the thickness of the tube wall. Scale bars: (A–C: 100 μm); (A′–C′: 10 μm); (D: 500 μm). For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

MSC prevented muscle-weight reduction and decrease of CK levels

Eight weeks after surgery, the flexor digitorum muscle trophism and its CK levels were determined by muscle weight analysis and by spectrophotometry, using a CK diagnostic Kit (Procedure No. 47 UV, Sigma, Chemical Company, USA).

As expected, the normal group (4.52±0.23 mg) showed the greatest flexor digitorum muscle weight compared with both the MSC-treated and DMEM groups, although this difference was not statistically significant. Despite of the greater weight of the flexor digitorum muscle in the MSC-treated group (3.81±0.36 mg) than in the DMEM group (3.23±0.37 mg), these values were not statistically significant either (Fig. 5A).

The DMEM group (336.33±87.65 U/g/h) showed lower CK levels compared with the normal (614.75±69.76 U/g/h) and MSC-treated (593.33±32.63 U/g/h) groups, and this difference was significant for both comparisons (One-way ANOVA, $P<0.05$). The value for the MSC-treated group was closer to that for the normal group, and there was no difference between them (Fig. 5B).

Functional recovery was improved in MSC-treated animals

Functional analysis was performed weekly by the grasping test. Until the fourth and fifth weeks, DMEM and MSC-

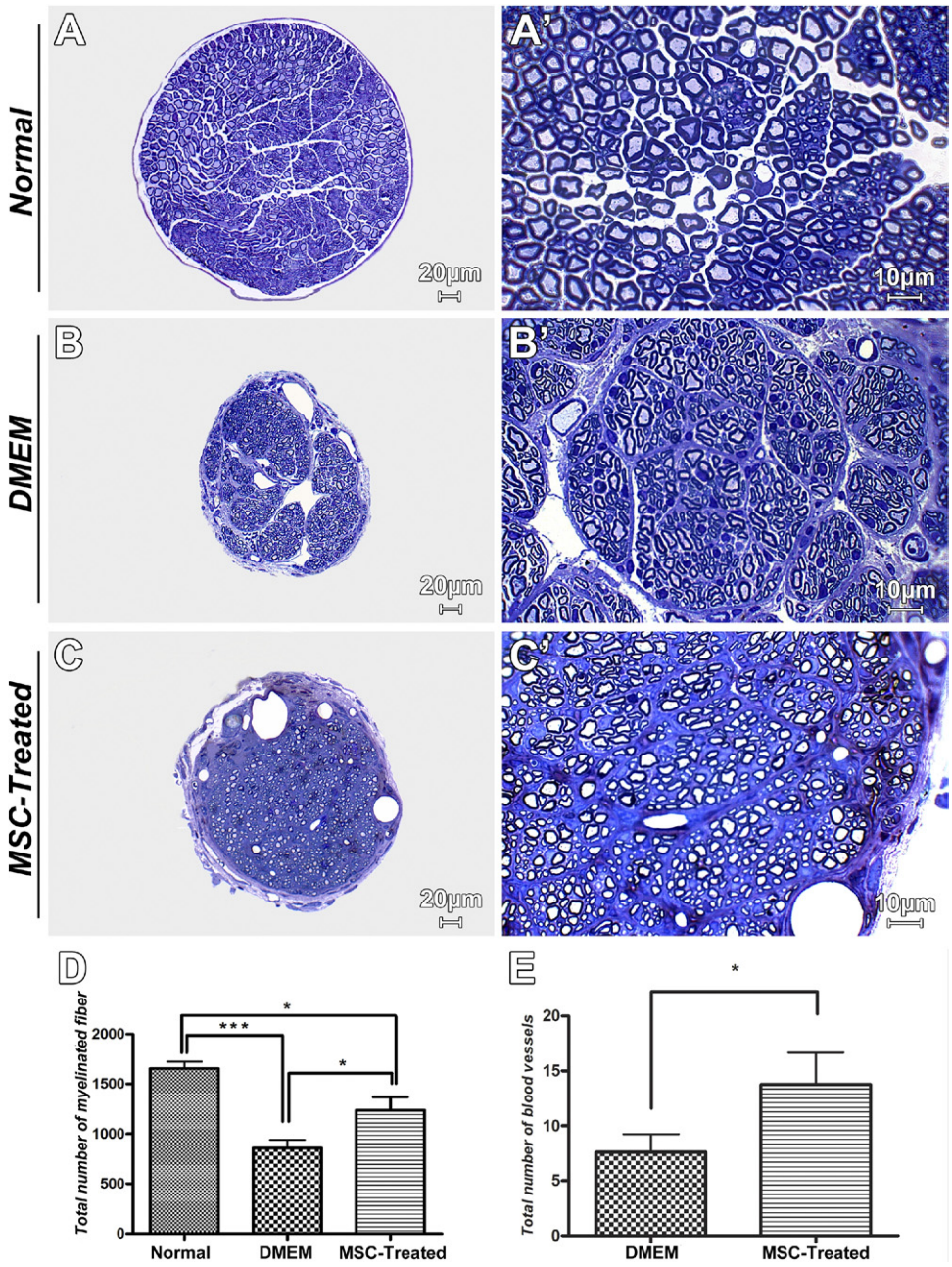

Fig. 3. Semithin cross sections of regenerating median nerve in the conduit middle portion (Toluidine Blue stain) 8 wk after surgery. (A, A′) Normal median nerve; (B, B′) DMEM nerve; (C, C′) MSC-treated nerve. (B′, C′) many clusters of regenerating nerve fibers; (C′) several blood vessels are seen in the MSC-treated nerve. (D) Total number of myelinated fibers; (E) Total number of blood vessels. Scale bars: (A–C: 20 μm); (A′–C′: 10 μm). * $P<0.05$; *** $P<0.001$. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

treated animals showed no flexor digitorum muscle activity, these values being equal to zero. From this time on, they began to show muscle activity, which increased progressively in both groups until the eighth week. The MSC-treated group showed a trend towards improved functional recovery compared to the DMEM group throughout the period analyzed, and this improvement was statistically significant at the sixth week (Student's *t*-test, $P=0.0013$) (Fig. 6B).

MSC differentiated, in a few cases, into Schwann cells *in vivo*

The immunohistochemistry for S-100 and GFP showed few positive areas for both proteins, as seen in Fig. 7.

DISCUSSION

Lesions in the nerves of the upper extremities are very common in humans, frequently leading to limiting and dis-

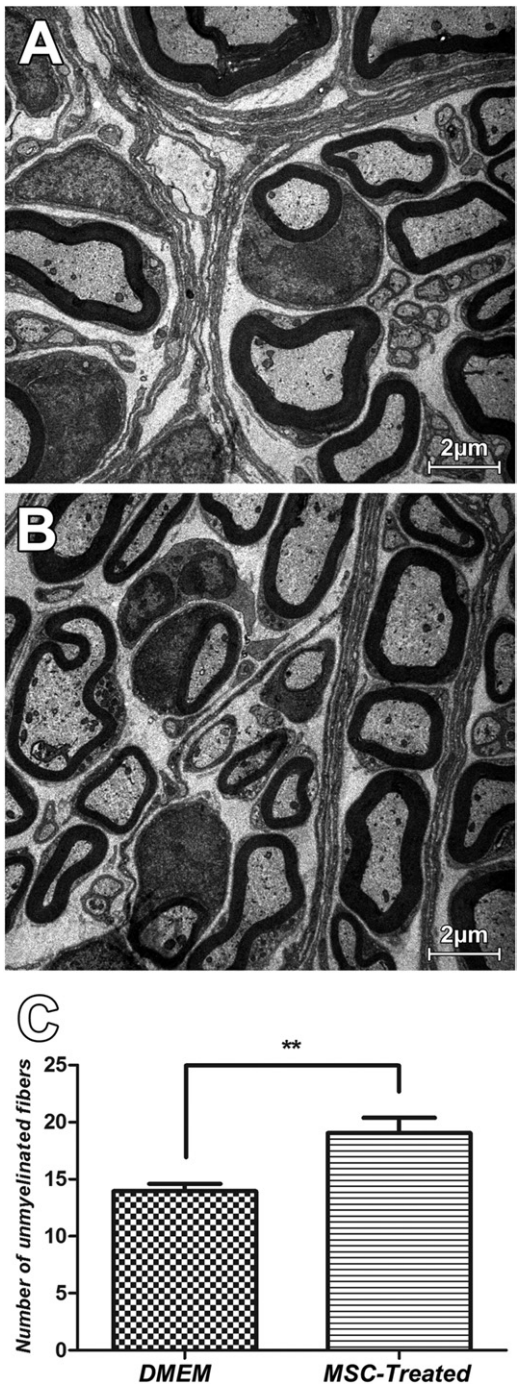

Fig. 4. Ultrathin cross sections of regenerating median nerve in the conduit middle portion 8 wk after surgery. (A) DMEM nerve, (B) MSC-treated nerve showing regenerating clusters and a trend towards larger numbers of fibers in the MSC-treated nerve. (C) Number of unmyelinated fibers. ** $P<0.01$. Scale bar: (A, B: 2 μm).

abling pathologies, decreasing the quality of life of those affected. After a peripheral-nerve injury, there is an orchestrated sequence of events conducted by Schwann cells (trophic support), neurons (regenerative capacity), and the extracellular matrix (Boyd and Gordon, 2003; Gantus et al., 2006). Schwann cells are activated and proliferate, while immune cells are recruited to the lesion site and contribute to debris clearance. Genes related to the regenerative process are upregulated, aiming toward cytoskeleton reorganization; trophic factors are released and the expression of cytokines and hormones is increased to support elongation of the growth cone (Navarro et al., 2007). Despite this intrinsic growth capacity and the permissive microenvironment, full functional recovery is seldom restored. Therefore, therapeutic strategies must be applied in order to potentiate the regenerative process and improve the motor-function outcome.

Mesenchymal stem cells have many advantages that make them the subject of studies aiming to improve nervous system regeneration. Besides the capability to secrete several trophic factors, and to promote neuritogenesis *in vitro* (Gu et al., 2009) and survival and elongation of the growth cone *in vivo* (Pereira Lopes et al., 2006; Chen et al., 2007; Pan et al., 2007; Ribeiro-Resende et al., 2009), these cells are easily obtained, isolated, and expanded in culture; they have paracrine effects and migratory behavior; also, their use avoids difficult ethical questions (Brooke et al., 2007) and does not involve a risk of tumorigenesis. In addition, there is the possibility of autologous transplant, avoiding tissue-rejection effects. All these advantages make them one of the favorite subjects for translational medicine.

The present study was driven by the hypothesis that MSC seeded into resorbable PCL tubes would improve median-nerve regeneration after transection. The combination of the two strategies enhanced nerve regeneration and functional recovery, as evidenced by morphological, biochemical, and functional analyses. The PCL conduit used in this study proved to be a suitable substrate for adhesion and elongation of the growing nerve fibers. Ultrastructural analysis of the PCL tubes before implantation revealed structural features of the tube wall that favored its association with the implanted cells, enabling nutrients to diffuse through the conduit wall while allowing cells to remain within it, exerting their functions at the lesion site. At 12 weeks post-surgery, we observed that the PCL conduit was still present *in vivo* but it showed clear signs of an initial process of degradation, with an apparently less-porous surface and structural disorganization of the wall. In agreement with our observations, other investigators have also reported the presence of the PCL conduit some time after surgical implantation (Panseri et al., 2008).

Functional analysis of peripheral nerves is usually performed by the walking-track test, as described by De Medinaceli et al. (1982). This test can also be applied to upper-extremity lesions, but is not indicated when only the median nerve is injured. Simultaneous injury to both the median and ulnar nerves is required to cause a decrease in the toe-spread value (Bontioti et al., 2003), which is a paw print parameter analyzed by the walking-track test. Therefore, the grasping test was chosen for use in this study, based on the protocol described by Bertelli and Mira (1995) and adapted for mice by Tos et al. (2008). This analysis is a simple behavioral method for objective quantitative assessment of the median-nerve regeneration, as it

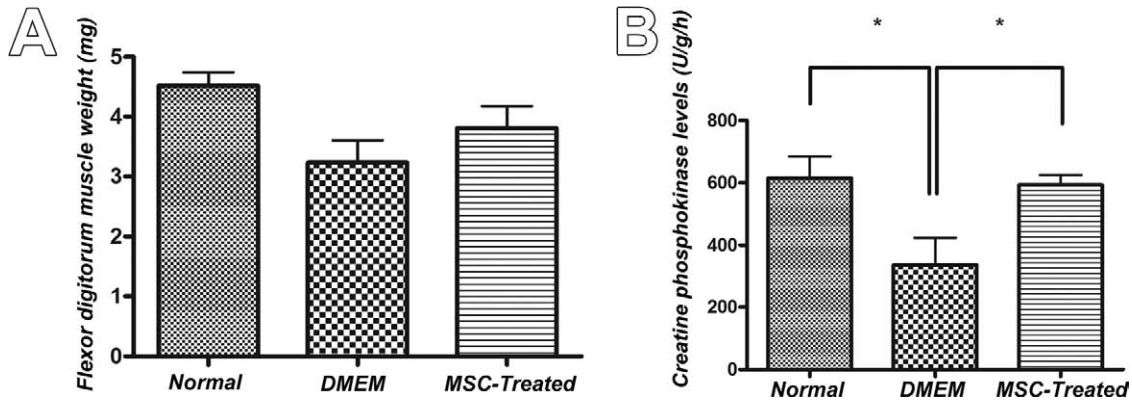

Fig. 5. *Flexor digitorum* muscle analyses 8 wk post-surgery. (A) Muscle weight; (B) Quantitative analysis of creatine phosphokinase levels. * $P<0.05$.

evaluates the force in grams exerted by the flexor digitorum muscle, which in rodents is innervated exclusively by the median nerve. To measure this force, we constructed an apparatus by fixing a grid on top of an ordinary balance (see Experimental procedures and Fig. 6A). MSC-treated animals showed an improved functional recovery by the grasping-test analysis compared to untreated animals, and this improvement was statistically significant 6 weeks after surgery. Previous studies have shown that MSC are able to improve locomotor function after a sciatic or spinal cord lesion in rodents (Cizková et al., 2006; Pereira Lopes et al., 2006). This finding indicates that the MSC had a positive effect on the regenerative process of the median nerve. This improved functional recovery may be due to the larger amounts of myelinated and unmyelinated nerve fibers and blood vessels in the MSC-treated animals, compared with the DMEM group. It is possible that many unmyelinated fibers found in the MSC-treated nerve would become myelinated fibers at a later stage of regeneration. Enhanced myelination is usually accompanied by faster action potentials and consequently better muscle performance.

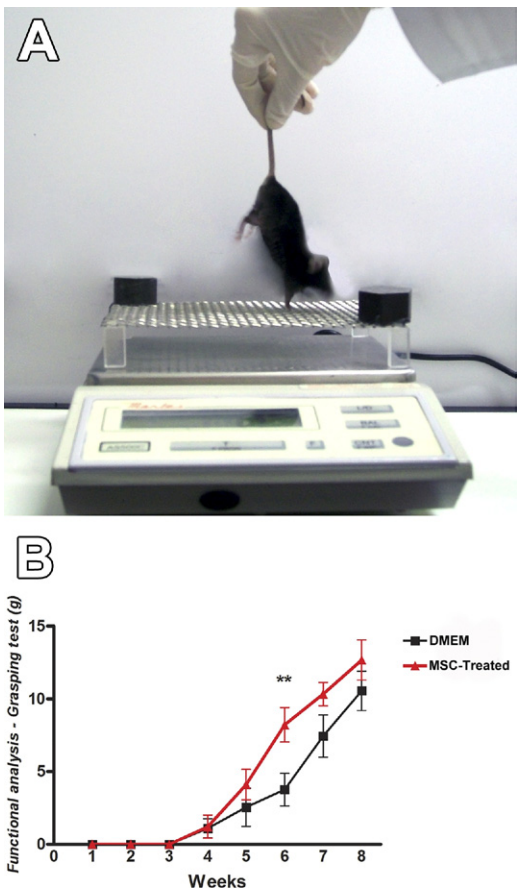

Fig. 6. Functional analysis by the grasping test. (A) Functional apparatus designed and constructed by our group, which consists of a grid with two supporting points on a digital balance. (B) Quantitative analysis by the grasping test, showing the force in grams exerted by the animal. ** $P<0.01$. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

In addition, as seen in this and previous reports, MSC are able to differentiate *in vivo* into Schwann cells, although in small amounts. It is therefore possible that the increased myelination observed in the present study is partly due to larger amounts of Schwann cells. Alternatively, MSC could act favorably by releasing trophic factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), glial cell line-neurotrophic factor (GDNF), and neurotrophin-3 (NT-3) (Pan et al., 2007) that induce Schwann-cell activation and endogenous repair by remyelination. Akiyama et al. (2002) injected bone-marrow stromal cells systemically in rats after a spinal cord injury, and found improved remyelination of the spinal tract and, therefore, faster nerve conduction in the treated animals. It was suggested that these cells induced or accelerated endogenous repair by recruiting or inducting resident cells to form myelin. Observations from several studies are in agreement with these findings, showing that MSC are able to act in tissue repair through the remyelinating process (Cuevas et al., 2002; Choi et al., 2005; Cizková et al., 2006; Pereira Lopes et al., 2006).

An interesting finding in this study was the presence of a larger number of blood vessels in the MSC-treated group compared to the DMEM group. Since pericytes and smooth-muscle cells originate from mesoderm, it is possible that the implanted MSC differentiated into these cells, as previously demonstrated by Shi and Gronthos (2003). These mural cells may provide nutrition and increased

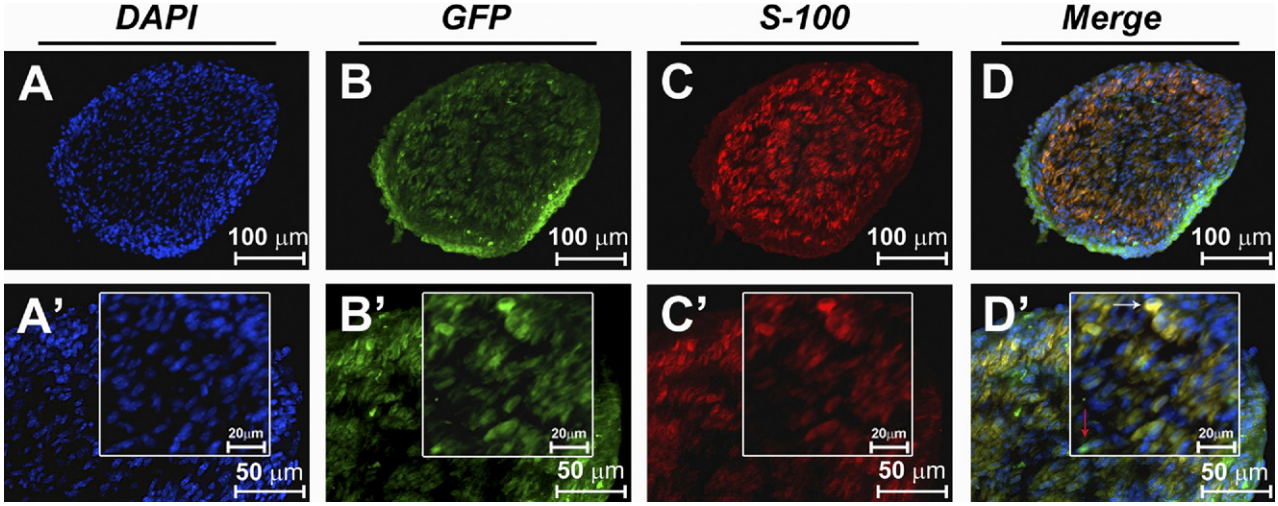

Fig. 7. Fluorescence micrographs of the regenerating MSC-treated nerve 4 wk after surgery. (A, A′) DAPI; (B, B′) GFP immunostaining; (C, C′) S-100 immunostaining; (D, D′) GFP merged in a few cases with S-100 protein (white arrow). Few GFP positive cells did not show S-100 staining (red arrow). Scale bar: (A–D: 100 μm), (A′–D′: 50 μm), inserts (20 μm). For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

structural support, contributing to peripheral-nerve regeneration. In addition, it is known that MSC may differentiate into endothelial cells which can release vascular endothelial growth factor (VEGF), promoting vasculogenesis. Furthermore, it has been postulated that MSC are also capable of releasing VEGF (Zisa et al., 2009). This growth factor acts on both the nervous system and blood vessels, as they are closely related during neurogenesis and neuroregeneration. In addition to the capability of VEGF to extend the capillary network and support the growth cone, it prevents neuronal death by stimulating neuroprotection (Bearden and Segal, 2004; Ruiz de Almodovar et al., 2009). Another mechanism whereby VEGF may exert its therapeutic effects is by affecting the Schwann cells. This growth factor stimulates chemotaxis and proliferation of Schwann cells *in vitro* and also induces phosphorylation of its receptor (VEGFR-2) on the Schwann cell membrane, indicating that VEGFR-2 binding sites are functionally active in these cells (Schratzberger et al., 2000).

Denervated muscle deteriorates progressively, leading to atrophy, weight loss, adipose and fibrous tissue deposition, and decrease of CK levels (Goldspink, 1976; Rochkind et al., 2009). The phosphocreatine molecule is phosphorylated by the CK enzyme, and it can anaerobically donate a phosphate group to ADP to form ATP following a neuronal effort, and this reaction is reversible. Creatine phosphokinase plays a particularly important role in tissues that have high energy demands, such as muscle and nerve, and the levels of CK may be an indicator of tissue activity. Biochemical changes induced by denervation may be prevented by trophic signals that could induce an increase of phosphokinase enzyme levels, preserving the high-energy phosphate reservoir and rapid ATP resynthesis. We observed that MSC-treated animals showed higher CK levels compared with the DMEM group, closer to the values of the normal group, suggesting that the cell treatment prevented a decrease of enzyme levels in the flexor digitorum muscle. Because MSC can express and release several trophic factors such as NGF, BDNF, CNTF, and NT-3 (Pereira Lopes et al., 2006; Chen et al., 2007; Pan et al., 2007; Gu et al., 2009; Ribeiro-Rezende et al., 2009), it is possible that they could improve nerve regeneration through this mechanism.

In the present study, we observed that MSC grafted to PCL conduits have the capability to improve the regeneration and function of the median nerve after a traumatic lesion. This report provides important results in terms of the beneficial effects of cell therapy in rodent forepaw lesions, as previously reported for rodent hind-limb injuries.

Acknowledgments—We are grateful to Jorge Luís da Silva for excellent technical assistance and to Klauss Mostacada for help with image processing. This study was supported by FAPERJ, CNPq, INNT-MCT, CAPES, and FUJB.

REFERENCES

Akiyama Y, Radtke C, Honmou O, Kocsis JD (2002) Remyelination of the spinal cord following intravenous delivery of bone marrow cells. Glia 39(3):229–236.

Bearden SE, Segal SS (2004) Microvessels promote motor nerve survival and regeneration through local VEGF release following ectopic reattachment. Microcirculation 11(8):633–644.

Bertelli JA, Mira JC (1995) The grasping test: a simple behavioral method for objective quantitative assessment of peripheral nerve regeneration in the rat. J Neurosci Methods 58:151–155.

Bontioti EN, Kanje M, Dahlin LB (2003) Regeneration and functional recovery in the upper extremity of rats after various types of nerve injuries. J Peripher Nerv Syst 8:159–168.

Bossolasco P, Cova L, Calzarossa C, Rimoldi SG, Borsotti C, Deliliers GL, Silani V, Soligo D, Polli E (2005) Neuro-glial differentiation of human bone marrow stem cells *in vitro*. Exp Neurol 193(2):312–325.

Boyd JG, Gordon T (2003) Neurotrophic factors and their receptors in axonal regeneration and functional recovery after peripheral nerve injury. Mol Neurobiol 27(3):277–324.

Brooke G, Cook M, Blair C, Han R, Heazlewood C, Jones B, Kambouris M, Kollar K, McTaggart S, Pelekanos R, Rice A, Rossetti T, Atkinson K (2007) Therapeutic applications of mesenchymal stromal cells. Semin Cell Dev Biol 18(6):846–858.

Chamberlain LJ, Yannas IV, Arrizabalaga A, Hsu HP, Norregaard TV, Spector M (1998) Early peripheral nerve healing in collagen and silicone tube implants: myofibroblasts and cellular response. Biomaterials 19:1393–1403.

Chang CJ (2009) The effect of pulse-released nerve growth factor from genipin-crosslinked gelatin in Schwann cell-seeded polycaprolactone conduits on large-gap peripheral nerve regeneration. Tissue Eng Part A 15(3):547–557.

Chen CJ, Ou YC, Liao SL, Chen WY, Chen SY, Wu CW, Wang WY, Huang YS, Hsu SH (2007) Transplantation of bone marrow stromal cells for peripheral nerve repair. Exp Neurol 204(1):443–453.

Chiono V, Vozzi G, Vozzi F, Salvadori C, Dini F, Carlucci F, Arsipici M, Burchielli S, Di Scipio F, Geuna S, Fornaro M, Tos P, Nicolino S, Audisio C, Perroteau I, Chiaravalloti A, Domenici C, Giusti P, Ciardelli D (2009) Melt-extruded guides for peripheral nerve regeneration. Biomed Microdevices 11(5):1037–1050.

Choi BH, Zhu SJ, Kim BY, Huh JY, Lee SH, Jung JH (2005) Transplantation of cultured bone marrow stromal cells to improve peripheral nerve regeneration. Int J Oral Maxillofac Surg 34(5):537–542.

Cizková D, Rosocha J, Vanicky I, Jergová S, Cizek M (2006) Transplants of human mesenchymal stem cells improve functional recovery after spinal cord injury in the rat. Cell Mol Neurobiol 26(7–8):1167–1180.

Cuevas P, Carceller F, Dujoyny M, Garcia-Gómez L, Cuevas B, González-Corrochano R, Diaz-González D, Reimers D (2002) Peripheral nerve regeneration by bone marrow stromal cells. Neurol Res 24(7):634–638.

De Medinaceli L, Freed WJ, Wyatt RJ (1982) An index of the functional condition of rat sciatic nerve based on measurements made from walking tracks. Exp Neurol 77(3):634–643.

Den Dunnen WF, Van Der Lei B, Schakenraad JM, Blaauw EH, Stokroos I, Pennings AJ, Robinson PH (1993) Long-term evaluation of nerve regeneration in a biodegradable nerve guide. Microsurgery 14(8):508–515.

Gantus MA, Nasciutti LE, Cruz CM, Persechini PM, Martinez AM (2006) Modulation of extracellular matrix components by metalloproteinases and their tissue inhibitors during degeneration and regeneration of rat sural nerve. Brain Res 1122(1):36–46.

Goldspink DF (1976) The effects of denervation on protein turnover of rat skeletal muscle. Biochem J 156(1):71–80.

Gordon T, Sulaiman O, Boyd JG (2003) Experimental strategies to promote functional recovery after peripheral nerve injuries. J Peripher Nerv Syst 8:236–250.

Gu Y, Wang J, Ding F, Hu N, Wang Y, Gu X (2009) Neurotrophic actions of bone marrow stromal cells on primary culture of dorsal root ganglion tissues and neurons. J Mol Neurosci 40(3):332–341.

Johnson EO, Soucacos PN (2008) Nerve repair: experimental and clinical evaluation of biodegradable artificial nerve guides. Injury 395:530–536.

Kim BJ, Seo JH, Bubien JK, Oh YS (2002) Differentiation of adult bone marrow stem cells into neuroprogenitor cells *in vitro*. Neuroreport 13:1185–1188.

Kouyoumdjian JA (2006) Peripheral nerve injuries: a retrospective survey of 456 cases. Muscle Nerve 34(6):785–788.

Krampera M, Marconi S, Pasini A, Gali M, Rigotti G, Mosna F, Tinelli M, Lovato L, Anghileri E, Andreini A, Pizzolo G, Sbarbati A, Bonetti B (2006) Induction of neural-like differentiation in human mesenchymal stem cells derived from bone marrow, fat, spleen and thymus. Bone 40:382–390.

Lundborg G (2000) A 25-year perspective of peripheral nerve surgery: evolving neuroscientific concepts and clinical significance. J Hand Surg Am 25(3):391–414.

Lundborg G, Dahlin LB (1996) Anatomy, function and pathophysiology of peripheral nerves and nerve compression. Hand Clin 12(2):185–193.

Lundborg G, Gelberman RH, Longo FM, Powell HC, Varon S (1982) *In vivo* regeneration of cut nerves encased in silicone tubes. J Neuropathol Exp Neurol 41:412–422.

Melo PA, Ownby CL (1996) Different sensitivity of fast- and slow-twitch muscles to some snake venoms and myotoxins. Toxicon 34(6):653–669.

Muraglia A, Cacedda R, Quarto R (2000) Clonal mesenchymal progenitors from human bone marrow differentiate *in vitro* according to a hierarchical model. J Cell Sci 113:1161–1166.

Navarro X, Vivó M, Valero-Cabré A (2007) Neural plasticity after peripheral nerve injury and regeneration. Prog Neurobiol 82(4):163–201.

Pan HC, Chen CJ, Lai SZ, Yang DY, Chang MH, Ho SP (2007) Post-injury regeneration in the rat sciatic nerve facilitated by neurotrophic factors secreted by amniotic fluid mesenchymal stem cells. J Clin Neurosci 14(11):1089–1098.

Panseri S, Cunha C, Lowery J, Del Carro U, Taraballi F, Amadio S, Vescovi A, Gelain F (2008) Electrospun micro- and nanofiber tuber for functional nervous regeneration in sciatic nerve transections. BMC Biotechnol 8:1–12.

Pereira Lopes FR, Camargo De Moura Campos L, Dias Corrêa J Jr, Balduino A, Lora S, Langone F, Borojevic R, Martinez AMB (2006) Bone marrow stromal cells and resorbable collagen guidance tubes enhance sciatic nerve regeneration in mice. Exp Neurol 198:457–468.

Ribeiro-Resende VT, Pimentel-Coelho PM, Mesentier-Louro LA, Mendez RM, Mello-Silva JP, Cabral-Da-Silva MC, De Mello FG, De Melo Reis RA, Mendez-Otero R (2009) Trophic activity derived from bone marrow mononuclear cells increases peripheral nerve regeneration by acting on both neuronal and glial cell populations. Neuroscience 159(2):540–549.

Rochkind S, Geuna S, Shainberg A (2009) Chapter 25: phototherapy in peripheral nerve injury: effects on muscle preservation and nerve regeneration. Int Rev Neurobiol 87:445–464.

Ruiz de Almodovar C, Lambrechts D, Mazzone M, Carmeliet P (2009) Role and therapeutic potential of VEGF in the nervous system. Physiol Rev 89(2):607–648.

Schnell E, Klinkhammer K, Balzer S, Brook G, Klee D, Dalton P, Mey J (2007) Guidance of glial cell migration and axonal growth on electrospun nanofibers of poly-epsilon-caprolactone and a collagen/ply-epsilon-caprolactone blend. Biomaterials 28:3012–3025.

Schratzberger P, Schratzberger G, Silver M, Curry C, Kearney M, Magner M, Alroy J, Adelman LS, Weinberg DH, Ropper AH, Isner JM (2000) Favorable effect of VEGF gene transfer on ischemic peripheral neuropathy. Nat Med 6(4):405–413.

Shi S, Gronthos S (2003) Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. J Bone Miner Res 18(4):696–704.

Tos P, Ronchi G, Nicolino S, Audisio C, Raimondo S, Fornaro M, Battiston B, Graziani A, Perroteau I, Geuna S (2008) Employment of the mouse median nerve model for the experimental assessment of peripheral nerve regeneration. J Neurosci Methods 169:119–127.

Waller A (1850) Experiments on the section of the glossopharyngeal and hypoglossal nerves of the frog, and observations of the alterations produced thereby in the structure of their fibers. Phil Trans R Soc Lond 140:423–429.

Woodbury D, Schwarz EJ, Prockop DJ, Black IB (2000) Adult rat and human bone marrow stromal cells differentiate into neurons. J Neurosci Res 61:364–370.

Zisa D, Shabbir A, Suzuki G, Lee T (2009) Vascular endothelial growth factor (VEGF) as a key therapeutic trophic factor in bone marrow mesenchymal stem cell-mediated cardiac repair. Biocheml Biophys Res Commun 390(3):834–838.

(Accepted 18 August 2010)
(Available online 26 August 2010)