

Human Dental Pulp Cells: A New Source of Cell Therapy in a Mouse Model of Compressive Spinal Cord Injury

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Abstract

Strategies aimed at improving spinal cord regeneration after trauma are still challenging neurologists and neuroscientists throughout the world. Many cell-based therapies have been tested, with limited success in terms of functional outcome. In this study, we investigated the effects of human dental pulp cells (HDPCs) in a mouse model of compressive spinal cord injury (SCI). These cells present some advantages, such as the ease of the extraction process, and expression of trophic factors and embryonic markers from both ecto-mesenchymal and mesenchymal components. Young adult female C57/BL6 mice were subjected to laminectomy at T9 and compression of the spinal cord with a vascular clip for 1 min. The cells were transplanted 7 days or 28 days after the lesion, in order to compare the recovery when treatment is applied in a subacute or chronic phase. We performed quantitative analyses of white-matter preservation, trophic-factor expression and quantification, and ultrastructural and functional analysis. Our results for the HDPC-transplanted animals showed better white-matter preservation than the DMEM groups, higher levels of trophic-factor expression in the tissue, better tissue organization, and the presence of many axons being myelinated by either Schwann cells or oligodendrocytes, in addition to the presence of some healthy-appearing intact neurons with synapse contacts on their cell bodies. We also demonstrated that HDPCs were able to express some glial markers such as GFAP and S-100. The functional analysis also showed locomotor improvement in these animals. Based on these findings, we propose that HDPCs may be feasible candidates for therapeutic intervention after SCI and central nervous system disorders in humans.

Key words: Basso Mouse Scale; electron microscopy; global mobility test; human dental pulp cell; spinal cord injury; trophic factors

Introduction

AFTER INJURY, the spinal cord exhibits limited endogenous repair and poor functional recovery. Several factors contribute to this, including secondary damage that occurs after the initial event, a limited ability to remyelinate spared demyelinated axons, failure of axons to overcome local expression of myelin-associated inhibitory molecules such as Nogo, OMgp, and MAG, a lack of neurotrophic factors to support axonal growth, and reactive astrocytic scarring with

large amounts of chondroitin sulfate proteoglycans that are also inhibitory to axonal regeneration (Deumens et al., 2005; Dietz and Curt, 2006; Harel and Strittmatter, 2006; Yiu and He, 2006).

Many therapeutic strategies for central nervous system (CNS) lesions have been proposed, including administration of trophic factors (Piantino et al., 2006; Yin et al., 2006), neuroprotective molecules (Arataki et al., 2005; Couto et al., 2004), and cell transplantation. Recently, numerous studies have demonstrated that transplantation of Schwann cells (Takami

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et al., 2002), pre-differentiated embryonic stem cells (Keirstead et al., 2005; Marques et al., 2010), and mesenchymal stem cells can enhance regeneration. Even though there are many potential sources of stem cells, little attention has been given to human dental pulp cells (HDPCs) as a source of cells for treatment of CNS disorders. In this study, we investigated the effects of these cells in a compression model of spinal cord injury (SCI) in mice.

An interesting characteristic of HDPCs is their embryogenesis, because dental pulp is made of ecto-mesenchymal components with neural crest-derived cells, which display plasticity and multipotential capabilities (D'Aquino et al., 2009; Sinanan et al., 2004). HDPCs present some other advantages, such as the extraction process, which is very easy and simple. They also show rapid expansion and proliferation *in vitro*, and there is no need for an invasive process to collect them. In addition, when these cells are exposed to chemically-defined culture conditions, they are able to differentiate into smooth and skeletal muscles, neural-like cells, cartilage, and bone (Kerkis et al., 2006), which confirms that these cells can be considered very promising in the treatment of CNS disorders.

Based on all this evidence, the aim of this study was to investigate the efficacy of HDPCs when transplanted at two different time points, 7 and 28 days after a compression lesion in mice. Our results suggest that HDPC transplantation into the spinal cord improves locomotor recovery and may contribute to myelin preservation and the regenerative process.

Methods

Spinal cord injury

Adult female mice 6–8 weeks old were anesthetized with ketamine and xylazine (100 and 15 mg/kg, respectively) and subjected to a compression injury for 1 min at the T9 level, by means of a 30-g vascular clip (Kent Scientific Corporation, Torrington, CT), as previously described by Marques and coworkers (2009). After surgery, the animals were allowed to recover on a warm pad and received 1 mL of saline solution to compensate for dehydration and loss of blood, and enrobaytril injections (2.5 mg/kg/d SC). The bladders were manually expressed twice a day until spontaneous urinary function returned.

Human dental pulp cell preparation

HDPCs from the DL2 lineage were cultured in 150 cm² flasks in a monolayer, with Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 supplemented with 15% fetal bovine serum (FBS; HyClone, Logan, UT), 2 mM glutamine (Gibco, Carlsbad, CA), 50 µg/mL gentamicin sulfate (Schering-Plough, Whitehouse Station, NJ), and 1% nonessential amino acid (Gibco). The medium was refreshed every 2 days, and the culture was maintained semiconfluent. All cultures were incubated at 37°C in a 5% CO₂ high-humidity environment. The cells were dissociated with TrypLE Express (Invitrogen Corp., Carlsbad, CA), centrifuged, and counted using a Neubauer chamber, to obtain the desired amount of cells (8 × 10⁵ cells in 4 µL). Before transplantation, the cells were labeled with Cell Tracer, a fluorescent marker, in order to follow them in the host tissue. To investigate the HDPC morphology, these cells were cultured on coverslips for 4

days, fixed with 4% paraformaldehyde, and stained by hematoxylin and eosin (H&E). In addition, some samples were dehydrated in acetone and embedded in epoxy resin for ultrastructural analysis.

RT-PCR

Total RNA was extracted from dental pulp stem cell cultures with Trizol (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized utilizing the ImProm-IITM Reverse Transcription System (Promega Corp., Madison, WI), with Oligo(dT)₁₅, and a control reaction without the reverse transcriptase was also performed. PCR consisted of 35 cycles of 45 sec at 94°C, 45 sec at 55°C, and 1 min at 72°C, with the following primer pairs: BDNF-F (5'-AGA GGC TTG ACA TCA TTG GCT G-3'), BDNF-R (5'-CAA AGG CAC TTG ACT ACT GAG CAT C-3'); GDNF-F (5'-CAC CAG ATA AAC AAA TGG CAG TGC-3'), GDNF-R (5'-CGA CAG GTC ATC ATC AAA GGC G-3'); NGF-β-F (5'-ATA CAG GCG GAA CCA CAC TCA G-3'), NGF-β-R (5'-GTC CAC AGT AAT GTT GCG GGT C-3'); NT-3-F (5'-TGG GGG AGA CTT TGA ATG AC-3'), NT-3-R (5'-CTG GCA AAC TCC TTT GAT CC-3'); NT-4/5-F (5'-AGG AGG CAC TGG GTA TCT GA-3'), NT-4/5-R (5'-ATC CCT GAG GTC TCT CAG CA-3'); IL6-F (5'-TAC CCC CAG GAG AAG ATT CC-3'), IL6-R (5'-GCC ATC TTT GGA AGG TTC AG-3'). Reactions with no reverse transcriptase and without template were performed as negative controls for absence of amplification from genomic DNA, and for absence of contamination, respectively. Amplicons have, respectively, 147 bp (human brain-derived neurotrophic factor; BDNF), 335 bp (human glial cell derived neurotrophic factor; GDNF), 174 bp (human nerve growth factor, beta polypeptide; NGF-β), 201 bp (human neurotrophin 3; NT-3), 198 bp (human neurotrophin 4/5; NT-4/5), and 199 bp (human interleukin-6; IL-6).

Cell transplantation

The cells were transplanted at two different time points, in order to compare the recovery between a subacute and a chronic lesion. The subacute group received treatment 7 days after the injury: injection of the medium in a total volume of 4 µL (subacute + DMEM, *n* = 8) or HDPCs (8 × 10⁵ in 4 µL, *n* = 8, subacute + HDPC). The chronic group received the medium 28 days after the lesion (chronic + DMEM, *n* = 8) or HDPC (chronic + HDPC, *n* = 8) with the same concentration and volume. In all groups, the cells were injected into the epicenter of the lesion, using a 10-µL Hamilton syringe. We also had a sham group that received no cells but were subjected to all surgical procedures.

White-matter sparing

Twenty-five animals (5 per group) were perfused intracardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The spinal cords were extracted and divided at the lesion epicenter into two different regions: caudal and rostral. These samples were cryoprotected with 10%, 20%, or 30% sucrose, embedded in embedding medium, and 10-µm-thick coronal sections were obtained with a cryostat. We made serial sections, and one of every eight slides, with 6 sections spaced 50 µm apart, rostral and caudal to the epicenter, was stained with luxol fast blue, which stains myelin, over a total

of 2.4 mm of the sample. The spared white matter was calculated as the total coronal section area minus the non-stained area, and reported as a percentage of total area. For this quantification we used JAVA software. These quantifications were performed using ImageJ free software. For statistical analysis, data were analyzed with GraphPad Prism 4.0 (GraphPad Software, San Diego, CA). We used a one-way analysis of variance (ANOVA) and Tukey's test for all *post-hoc* comparisons for white matter analysis.

Immunohistochemistry

To evaluate the contribution of transplanted cells in the host tissue, we performed immunohistochemistry for glial fibrillary acidic protein (GFAP), an astrocyte marker, Gal-C, an oligodendrocyte marker, and S-100, a Schwann cell marker. After dissection and post-fixation, the spinal cord segments were cryoprotected in increasing concentrations of sucrose up to 30%, left in this solution overnight, and then ice-cold embedded in mounting medium. Ten-micrometer-thick sections were obtained with a cryostat and collected on gelatin-coated glass slides. The slides were then incubated with 0.06% potassium permanganate for 15 min to avoid spinal cord autofluorescence, washed in PBS, incubated with a blocking solution containing 10% NGS in PBS and 0.3% Triton for 1 h at room temperature, washed in PBS 0.3% Triton, and incubated in primary antibody rabbit anti-GFAP (1:200; Sigma-Aldrich, St. Louis, MO), rabbit anti Gal-C (1:100; Chemicon, Temecula, CA), or rabbit anti S-100 (1:200; Sigma-Aldrich) overnight. After this, the slides were washed for 30 min and incubated with secondary antibody Alexa 488 goat anti-rabbit (1:600; Sigma-Aldrich) for 2 h, followed by three washes of 10 min each, counterstained with 4,6-diamino-2-phenylindole (DAPI) nuclear label, and cover-slipped with Fluoromount (Sigma-Aldrich). Primary antibodies were omitted for negative controls. The sections were observed under confocal microscopy.

Trophic factor quantification

We evaluated the quantity of trophic factors in the tissue by performing an immunofluorescence analysis for BDNF, NGF- β , NT-3, and NT-4 on the spinal cord sections. We used the same protocol described above and the primary antibodies used were: rabbit anti-human BDNF (1:100; PreproTech, Rocky Hill, NJ), rabbit anti-human NGF (1:100; PreproTech), goat anti-mouse NT-3 (1:100; PreproTech), or goat anti-mouse NT-4 (1:100; PreproTech). After primary incubation, the slides were washed and incubated with the appropriate antibodies: Alexa 488 goat anti-rabbit (1:600; Sigma-Aldrich) or Alexa 488 rabbit anti-goat (1:600; Sigma-Aldrich) for 2 h at room temperature, followed by three washes, and cover-slipped with Fluoromount (Sigma-Aldrich). For quantification studies 12 animals (3 per group) were used. The immunofluorescence images were analyzed with Image-Pro Plus (version 6.0), by evaluating the ratio between the stained area and the total field area. We used six sections from each animal, and these sections were 100 μ m apart. From each section we took 2 photos using the 10 \times objective in order to encompass the whole spinal cord cross-section. After that, the trophic factors BDNF, NGF- β , NT-3, and NT-4 were quantified using these images. The data were statistically analyzed using GraphPad Prism software, and we used one-way ANOVA and Tukey's

test as a post-test. Data are presented as mean \pm standard error of the mean (SEM).

Electron microscopy

Eight weeks after the animals received the cell transplantation, they were anesthetized with ketamine and xylazine as described above, and perfused intracardially with a solution of 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The spinal cords were extracted, divided into three different segments (epicenter of the lesion, and rostral and caudal to it), and post-fixed by immersion in 1% osmium tetroxide in cacodylate buffer with 0.8% potassium ferrocyanide for 6 h at room temperature. The samples were washed three times with 0.1 M phosphate buffer (pH 7.4), dehydrated with a graded acetone series, embedded in resin, and polymerized for 48 h at 60°C. Semi-thin (500 nm) and ultra-thin sections were made using an RMC ultramicrotome. The semi-thin sections were stained with toluidine blue and observed under a Zeiss microscope (Axioscop 2 Plus), and the pictures were acquired using the Axiovision Program version 4.5 (Zeiss). The ultra-thin sections were collected on copper grids, stained with uranyl acetate and lead citrate, and observed in a Zeiss 900 transmission electron microscope.

Behavioral analysis

The animals were evaluated before injury, 1 d after injury, and weekly up to 8 weeks after cell transplantation. To analyze the recovery, we used the global mobility test described by Marques and coworkers (2009), which evaluates locomotor improvement by recording the animals in an open field for 1 min with a webcam (5 frames per sec). After that, we measured the velocity (cm/sec) that the animals reached during this 1-min period using ImageJ software. We also performed the Basso Mouse Scale (BMS) test (Basso et al., 2006), which is a 9-point scale that indicates the locomotor ability and locomotion features such as ankle movement, paw position, weight support, plantar steps, hindlimb and forelimb coordination, and trunk stability. For statistical analysis, we used the mean of the scores obtained from the left and right hindlimb (HL) to establish a single score per animal. We used one-way ANOVA and Tukey's test for *post-hoc* comparisons.

Statistical analysis

All the statistical analyses were performed with Graph Pad Prism 4.0. Results are expressed as mean \pm SEM, and *p* values \leq 0.05 were considered significant.

Results

Human dental pulp cell morphology and trophic factor expression in vitro

H&E staining (Fig. 1A and B) showed that *in vitro* these cells are large and showed an elongated morphology with many processes. The ultrastructural analysis (Fig. 1C and D) showed a prominent rough endoplasmic reticulum and an irregular nucleus rich in euchromatin (Fig. 1D), characteristics typical of cells that are in the process of protein synthesis.

NGF- β , BDNF, GDNF, NT-3, and NT-4/5 mRNA transcripts were expressed by cultures of HDPCs (Fig. 1E),

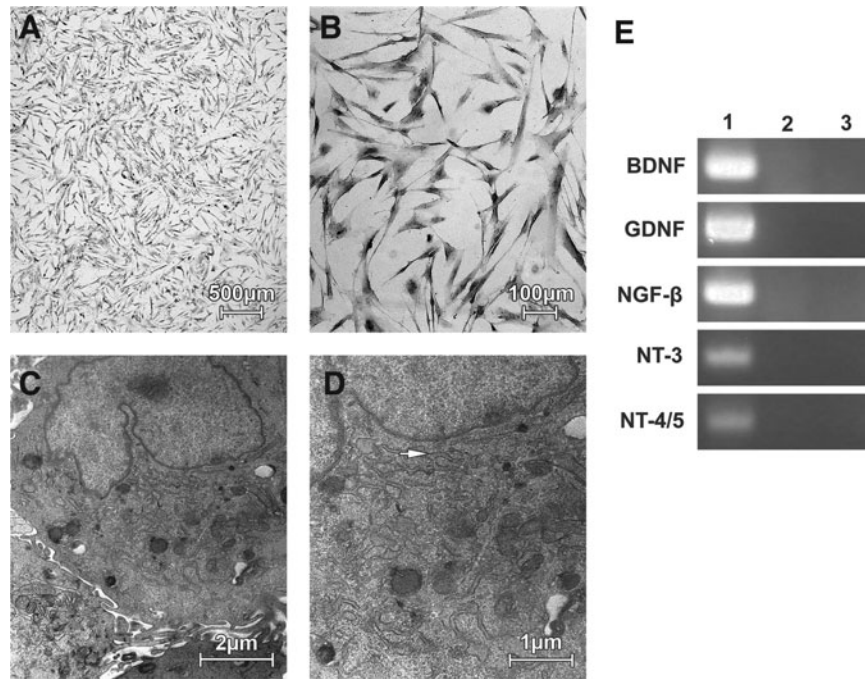


FIG. 1. Light and electron microscopy of human dental pulp cells (HDPCs). (A and B) Elongated morphology of cells *in vitro*. (C and D) Ultrastructural images showing a prominent granular endoplasmic reticulum (arrow), and euchromatin-rich nuclei. (E) HDPCs express neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), glial cell derived neurotrophic factor (GDNF), nerve growth factor, beta polypeptide (NGF- β), neurotrophin 3 (NT-3), and neurotrophin 4/5 (NT-4/NT-5), as detected by reverse transcriptase polymerase chain reaction (1, cDNA template; 2, negative control, no reverse transcriptase; 3, negative control, no template).

indicating that these cells are able to produce neurotrophic factors *in vitro*.

White matter preservation

The analysis of this quantification showed a better preservation of white matter areas in the HDPC groups than in the DMEM groups (Fig. 2). The sham group exhibited normal and superior values ($63.23 \pm 1.2\%$) in comparison to the lesion groups, with normal distribution. The HDPC animals showed values around $46.85 \pm 1.97\%$ for the subacute and $43.49 \pm 2.89\%$ for the chronic group, while the animals that received only DMEM as treatment showed lower values, about $38.03 \pm 4.28\%$ and $39.00 \pm 2.54\%$ for the subacute and chronic groups, respectively. The white-matter preservation was calculated in terms of the percentage of spared white matter in the injured area. All the values are illustrated in Figure 2, which also shows a schematic view of the injury site and the spinal cord samples used for this quantification (Fig. 2D and E).

Immunofluorescence and trophic factor quantification in the tissue

Since RT-PCR results showed the expression of trophic factors we decided to investigate the amounts of some of them in the tissue. So we performed immunofluorescence for BDNF, NGF- β , NT-3, and NT-4 on the spinal cord sections, followed by immunoreactivity quantification per area (Fig. 3). Although trophic-factor staining showed low immunoreactivity because they are soluble and therefore spread very

quickly in the tissue, we could observe a difference in the staining pattern among the groups. In general, the HDPC groups showed an increased immunoreaction per area compared to the DMEM groups, especially the subacute group, which showed higher levels of all analyzed trophic factors. For the chronic group we only observed differences between HDPCs and DMEM for BDNF and NGF- β quantification.

We also performed immunohistochemistry in both groups for several glial cell markers to evaluate the differentiation of cell tracer-positive transplanted HDPCs in the spinal cord sections. All animals used for this analysis showed cell tracer-positive cells. The results were the same in both groups, and Figure 4 illustrates what we observed in the subacute group. The triple-labeled sections with cell tracer, DAPI, and glial markers showed that many HDPCs found in the spinal cord expressed GFAP (Fig. 4B) and S-100 (Fig. 4D) immunoreactivity, indicating that some of the transplanted cells were able to differentiate into astrocytes and Schwann cells, respectively. However, we did not find co-localization of HDPCs and Gal-C (Fig. 4C), suggesting that the oligodendrocytes detected in the spinal cord were host-derived.

Electron microscopy

Figures 5 and 6 show semi-thin and ultra-thin sections from all groups. We observed that in general, the ultrastructural analysis of the animals that received DMEM in the subacute phase (Fig. 5A–C) or in the chronic phase (Fig. 6A, C, and E) revealed tissue disorganization, with many cavitations (small red arrows in Fig. 5A, and asterisks in Fig. 5C and 6C), and strong astrocytosis (arrowhead in Fig. 5C), few preserved

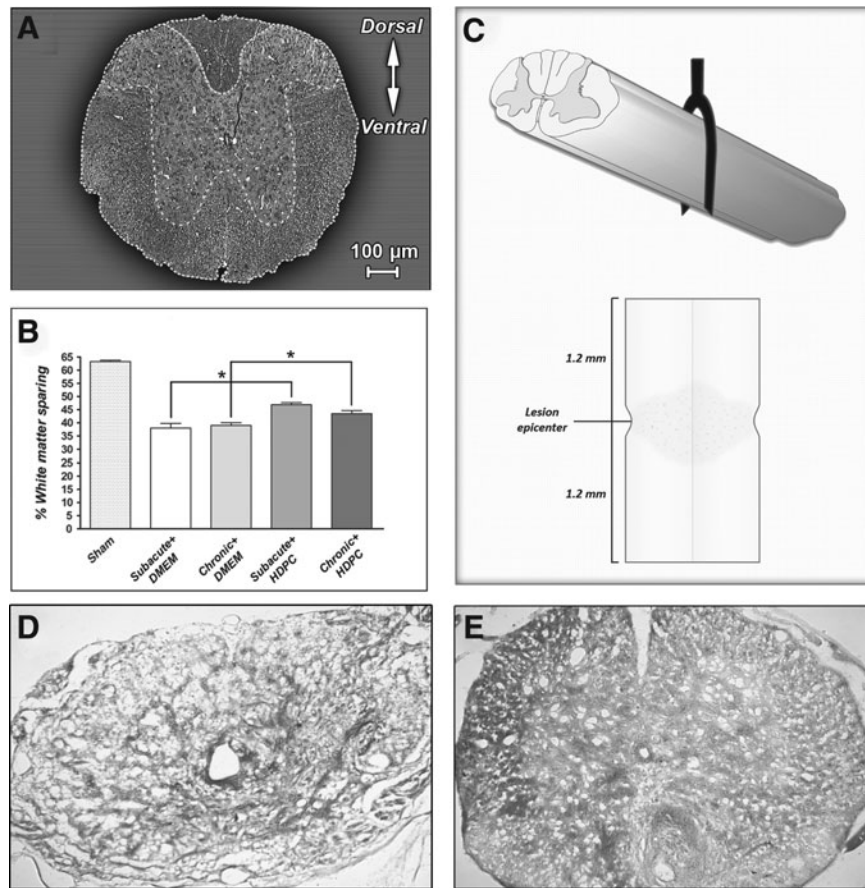


FIG. 2. Quantification of spared white matter. (A) Luxol fast blue (LFB)-stained area used for white-matter quantification. (B) The graph represents the mean values of spared white matter, showing higher values in the human dental pulp cell (HDPC) groups ($*p < 0.05$). (C) Diagram of spinal cord injury compression at the T9 level (upper part of figure), and the segment of spinal cord used for quantification of the serial sections (below). (D and E) Images illustrating spinal cord sections stained by LFB used for quantification of spared white matter from the subacute+DMEM (D) and subacute+HDPC (E) groups (DMEM, Dulbecco's modified Eagle's medium).

fibers (Figs. 5B and 6C), and some myelinating Schwann cells with their typical basal laminae (solid arrow in Fig. 5B, and star in Fig. 6C). Both the subacute and chronic groups that received HDPC transplantation showed better tissue preservation. In the HDPC subacute group (Fig. 5D–I), we could observe a large number of preserved fibers inside the white matter (large arrows in Fig. 5E), and a notable number of macrophages with many cytoplasmic inclusions, myelin debris, and lipids in their interior (black arrow in Fig. 5E). Intact Schwann cells (solid arrow in Fig. 5F), and oligodendrocytes remyelinating axons (black arrow in Fig. 5F); regenerative islands (Fig. 5G), and some preserved neurons were also observed. Some of these neurons showed several preserved synapse contacts (artificially colored portions in Fig. 5H). The ultrastructural analysis of the HDPC chronic group (Fig. 6) showed many preserved fibers (red arrows in Fig. 6B), some Schwann cells myelinating axons (arrows in Fig. 6D), and preserved neurons with synapse contacts (rectangle in Fig. 6F).

Functional analysis

Global mobility test. This test was previously described by our group (Marques et al., 2009), and evaluates the speed

that the animals walk in the open field for 1 min. The sham animals did not show any alteration in their locomotion, and exhibited normal values around 16.21 ± 1.73 cm/sec. The subacute group that received HDPCs (6.94 ± 1.85 cm/sec) showed higher values compared to the DMEM group (3.71 ± 0.95 cm/sec), and the improvement in locomotion began 7 days after cell transplantation (i.e., 14 days after SCI), and was maintained throughout the subsequent weeks of the study. The chronic animals showed a slight improvement 7 days after the treatment. However, 14 days after cell transplantation, they exhibited higher locomotion speed, with an average around 6.6 ± 2.03 cm/sec, versus 4.15 ± 1.07 cm/sec in the DMEM group. In both groups that received the HDPCs, the animals did not reach normal rates, but they showed a better exploratory pattern, walking much more and crossing the open field several times during the test. Figure 7 (A–F) illustrates the displacement of these animals 35 days after HDPC transplantation, and the velocity measurements made for 8 weeks after treatment.

In relation to the BMS, the sham animals exhibited normal scores during the entire assessment period. Immediately after lesion, all injured animals showed paralysis with no hindlimb movement, and generally maintained this pattern 1 day

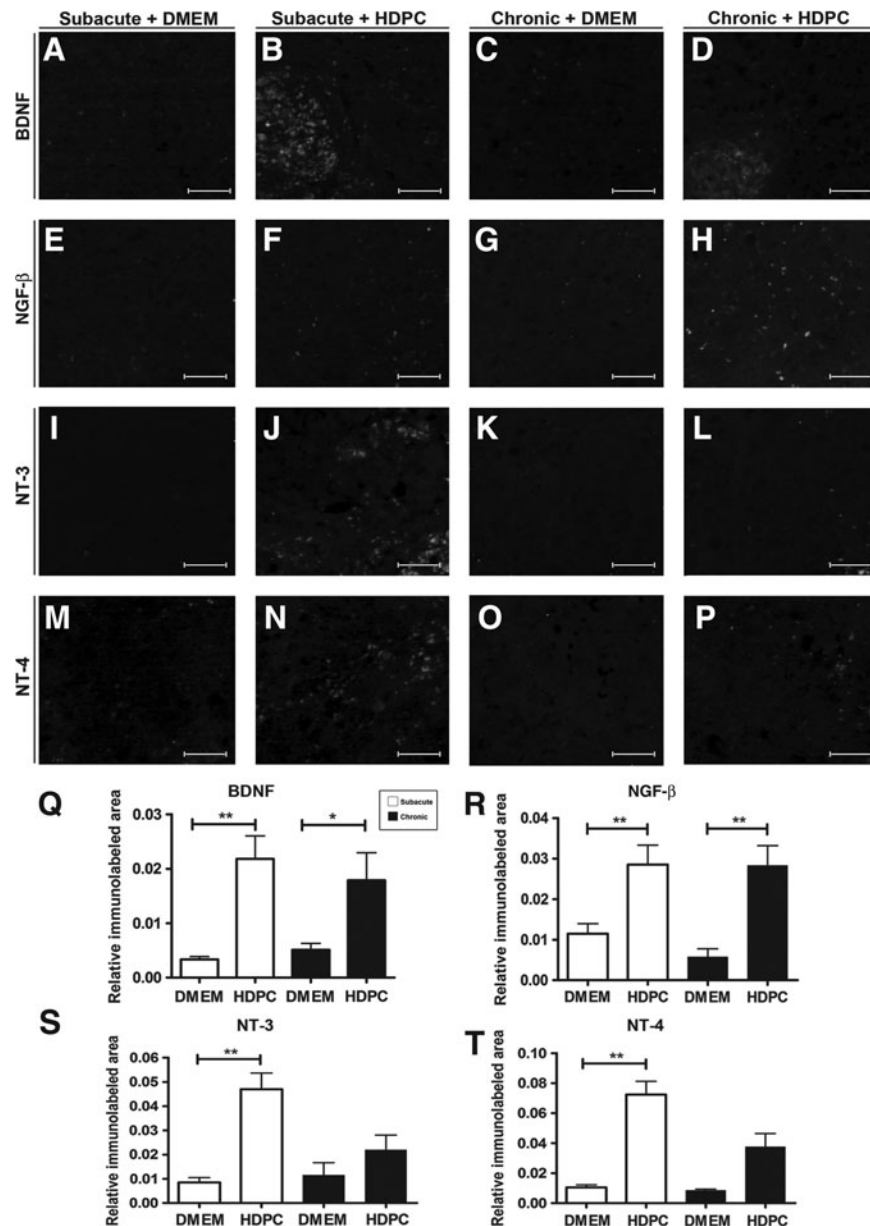


FIG. 3. Trophic factor immunofluorescence and immunolabeled area quantification. (A–D) Brain-derived neurotrophic factor (BDNF) staining. (E–H) nerve growth factor, beta polypeptide (NGF- β) staining. (I–L) Neurotrophin 3 (NT-3) staining. (M–P) Neurotrophin 4 (NT-4) staining of subacute and chronic groups that received human dental pulp cell (HDPC) transplantation or Dulbecco's modified Eagle's medium (DMEM). (Q–T) These graphs illustrate trophic factor quantification. Note the stronger staining in HDPC-treated animals for all analyzed trophic factors in comparison to DMEM-treated animals, especially BDNF and NGF- β , which showed more strongly immunolabeled areas both in the subacute and chronic groups (* $p < 0.05$; ** $p < 0.01$; scale bars = 50 μ m).

post-surgery with a BMS score of 0. The subacute DMEM animals only improved to an initial phase of recovery (2.08 ± 0.15), with no perceptible further improvement, while the subacute HDPC group reached the intermediate phase of recovery (3.7 ± 0.25). Some of these animals were also able to perform occasional plantar steps (BMS score 4), which is a very good sign in terms of functional recovery. The DMEM chronic animals showed lower BMS scores (1.5 ± 0.18) than the HDPC animals (3.2 ± 0.12). Interestingly, these animals began to improve their BMS scores 21 days after cell transplantation, and this locomotor improvement increased until 42 days after treatment. After that,

the animals only maintained the functional behavior. These results are illustrated in Figure 7 (G and H).

Discussion

Although there is not yet a fully restorative treatment for spinal cord injury, several molecular and cellular therapies have been tested, with some success in animal models. Cell transplantation has great therapeutic potential for spinal cord functional repair because of its ability to bridge cysts and cavities, to replace dead cells, and to create an appropriate

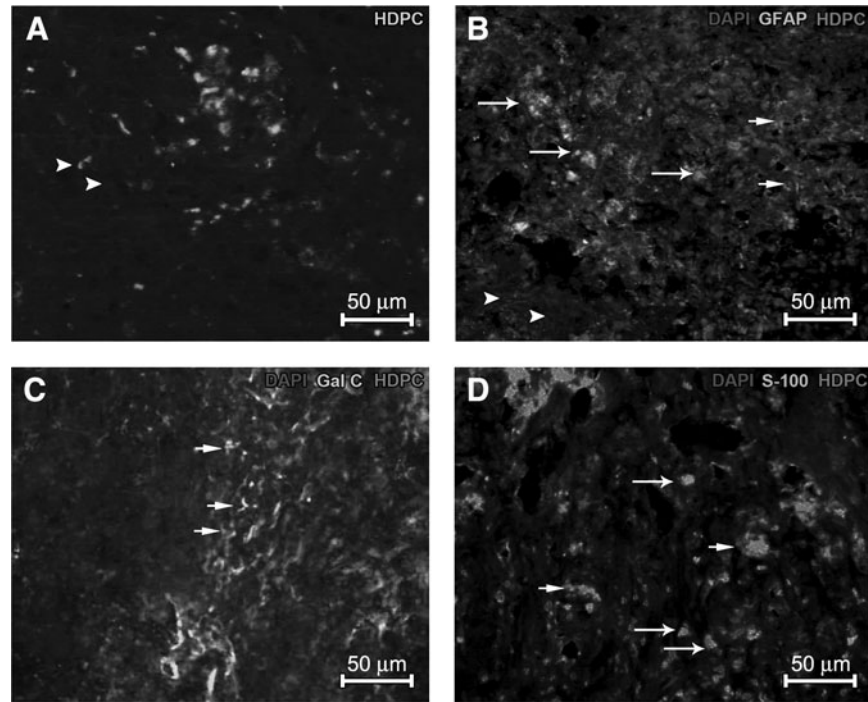


FIG. 4. Cell tracer positive cells (arrowheads in **A**) and immunohistochemistry for glial fibrillary acidic protein (GFAP; **B**), Gal-C (**C**), and S-100 (**D**) of spinal cord sections taken from transplanted animals. Note that human dental pulp cells (HDPCs) pre-labeled with cell tracer (red) are distributed in the white matter co-localized with GFAP (arrows in **B**), and S-100 (arrows in **D**). However, we did not observe any co-localization of HDPCs and Gal-C (**C**). We also observed that there were some host cells in the tissue, like astrocytes (short arrows in **B**), oligodendrocytes (short arrows in **C**), and Schwann cells (short arrows in **D**). We can also see some cell tracer-positive HDPCs in **B** (arrowheads; DAPI, 4,6-diamino-2-phenylindole).

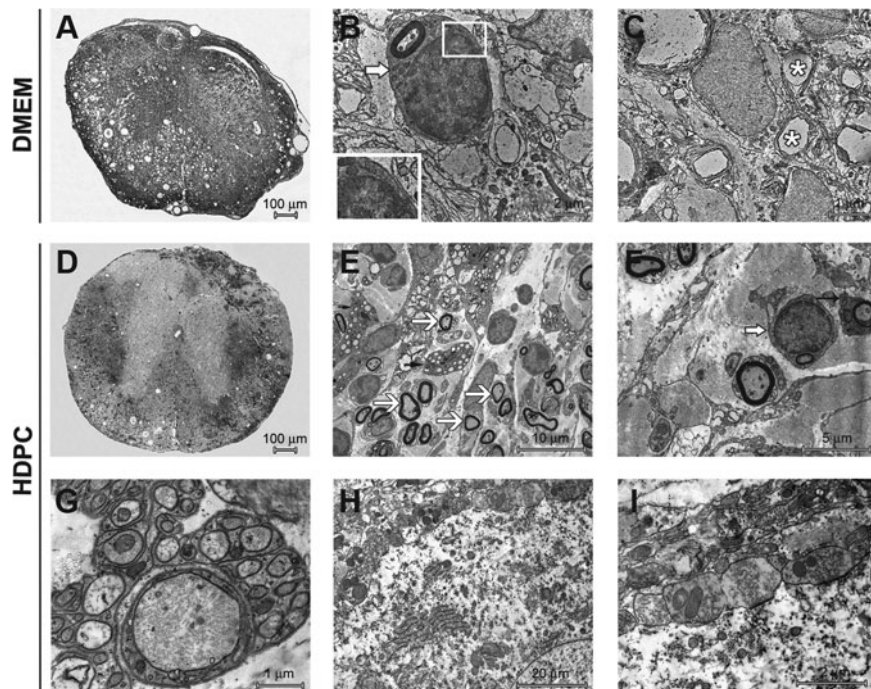


FIG. 5. Semi-thin and ultra-thin sections of spinal cords from subacute DMEM-treated animals (**A–C**) and HDPC animals (**D–I**). Note in **A**, a semithin section, that there are several cavitations (short red arrows). Note in **B** a Schwann cell (solid white arrow) with its typical basal laminae (inset), and in **C** astrocyte processes (arrowhead), and several microcavitations (asterisks). **D** shows a semi-thin section from an HDPC-treated animal with more preserved morphology. **E** shows many preserved fibers (long white arrows) and macrophages (black short arrows). We can see in **F** Schwann cells (solid arrows), and oligodendrocytes (long black arrow) myelinating axons. **G** shows an example of regenerative islands, and **H** represents a preserved healthy-appearing neuron with intact terminals making synaptic contacts on its cell body, which are shown at higher magnification in **I** (DMEM, Dulbecco's modified Eagle's medium; HDPC, human dental pulp cell).

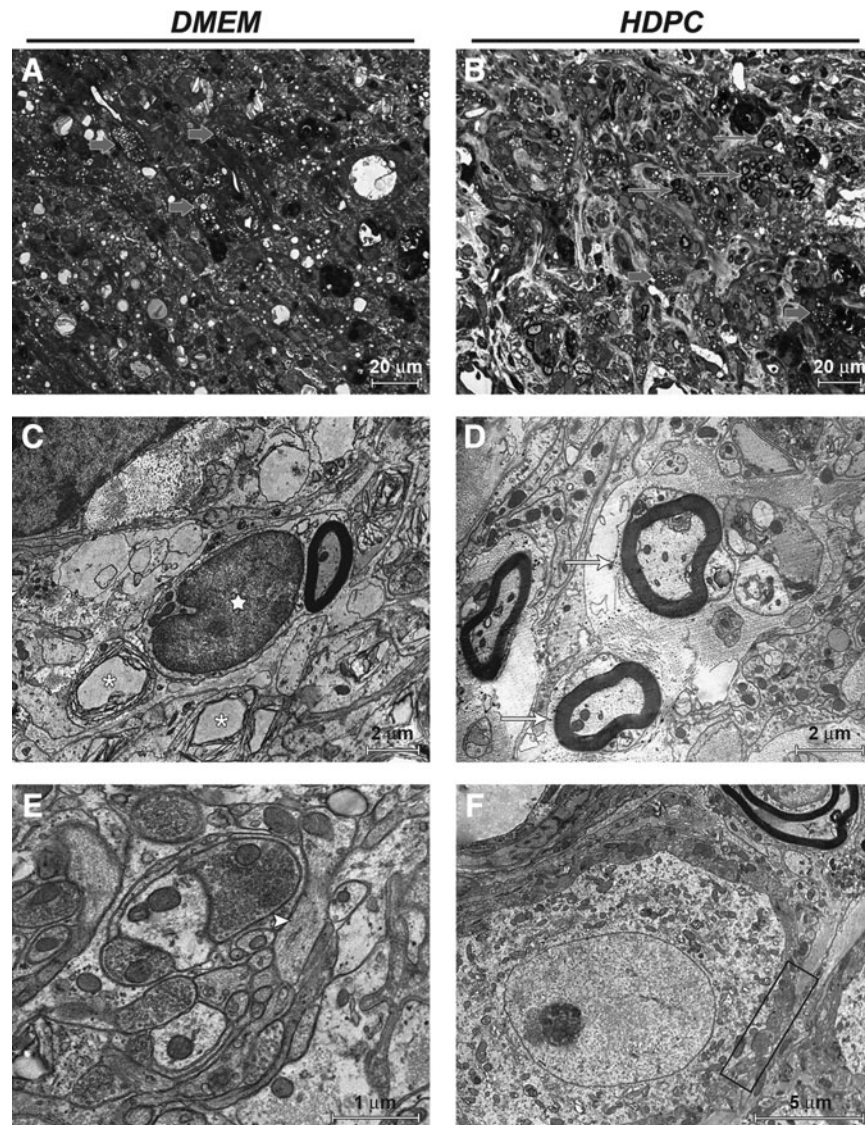


FIG. 6. Semi-thin and ultra-thin sections of spinal cords from chronic DMEM- (A, C, and E), and HDPC-treated (B, D, and F) animals. (A) Macrophages (red solid arrows). (B) Preserved fibers in white matter (long arrows), and macrophages (red short arrows). (C) Schwann cells (star) and many cavitations (asterisks). (D) Some preserved fibers (white arrows). (E) Intense astrocytosis (arrowhead) typical of a chronic lesion. (F) Preserved neuron cell body with many synaptic contacts terminating on it (rectangle area; DMEM, Dulbecco's modified Eagle's medium; HDPC, human dental pulp cell).

environment for axon regeneration (Thuret et al., 2006). In the present study, we transplanted HDPCs into the epicenter of the spinal cord lesion, and demonstrated that this strategy promoted overall better tissue organization, larger areas of white-matter preservation, release of trophic factors, and a significantly better functional outcome.

HDPCs can be considered a very promising cell type for the treatment of CNS diseases because of their embryologic origin. Dental pulp is made of ecto-mesenchymal components, containing neural crest-derived cells. Corroborating this idea is the expression of nestin, an ectodermal protein, and vimentin, a mesodermal protein (Kerkis et al., 2006). It has also been proposed that HDPCs show characteristics of both embryonic and mesenchymal stem cells, and are considered in an intermediate position between these two sources of stem cells (Kerkis et al., 2006).

Among the cells that have been tested for spinal cord injury treatment, HDPCs have received comparatively less attention. This is somewhat surprising, since these cells exhibit several advantages that make them excellent candidates for human trials in the future. First of all, they are obtained from teeth that are normally discharged, and therefore there is no need for an invasive procedure for their extraction (Miura et al., 2003). Another advantage is that the extraction process is itself a very simple and easy technique (Kerkis et al., 2006). Also, there are no ethical or rejection issues related to the use of these cells, since they can be obtained from young teeth, multiplied in culture, and frozen for later use, if necessary. The risk of rejection after transplantation is diminished because these cells reduce the proliferation and activity of T-lymphocyte cells, promoting better immunosuppression than other cells such as mesenchymal stem cells (Pierdomenico et al., 2005).

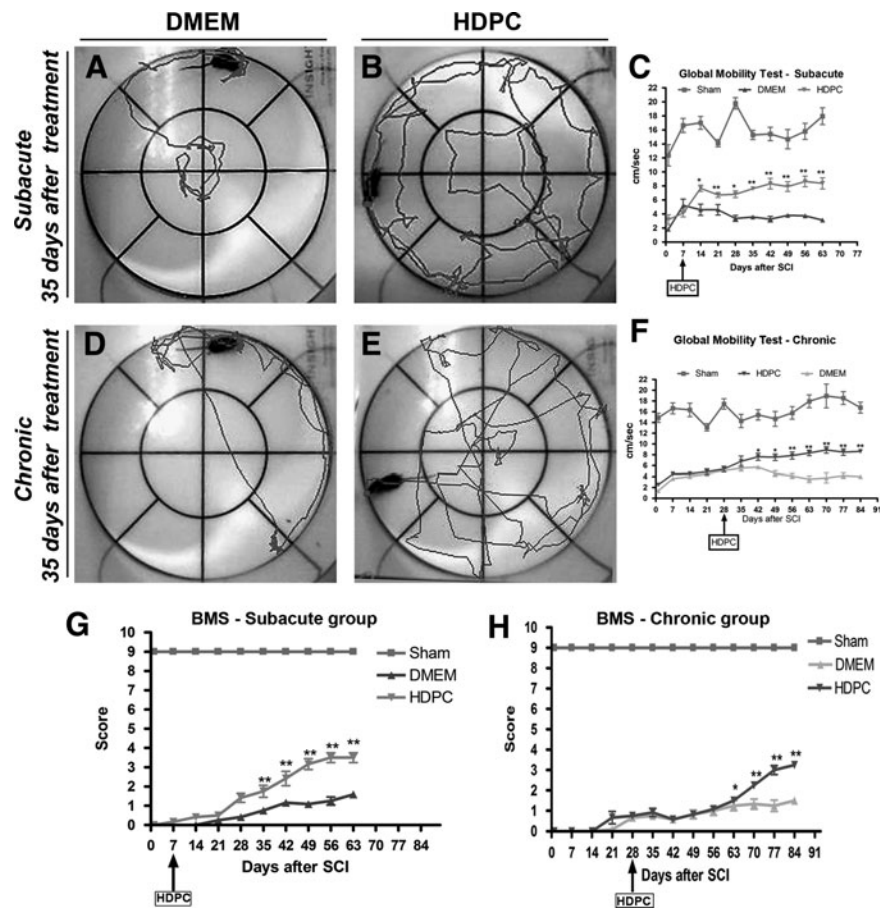


FIG. 7. Functional analysis of the DMEM and HDPC groups. The global mobility test showed a progression of global mobility of the subacute and chronic groups 35 days after treatment. The HDPC-treated animals (**B** and **E**) walked longer distances (blue lines) in the open field than did the DMEM-treated animals (**A** and **D**), and also showed superior speed (**C** and **F**) during the assessment period. **G** and **H** show the comparison of BMS functional analysis between the subacute (**A**) and chronic (**B**) groups (* $p < 0.05$, ** $p < 0.01$). In both phases, the HDPC-treated animals scored higher than the DMEM-treated animals, reaching the intermediate phase of functional recovery (DMEM, Dulbecco's modified Eagle's medium; HDPC, human dental pulp cell; BMS, Basso Mouse Scale).

There are several ways to morphologically analyze the results of the treatment in spinal cord models of injury. We can, for example, count the number of nerve fibers and neurons, measure the amount of spared white matter, and search for the presence of intact neurons with typical synapses, among others. For the present study, we quantified the spared white matter and scanned ultra-thin sections looking for remaining neurons and the presence of intact synapses. Quantification of luxol fast blue-stained areas showed larger areas of preserved white matter in the HDPC groups. The ultrastructural analysis revealed large numbers of normal fibers, many axons being remyelinated by either Schwann cells or oligodendrocytes, and preserved neurons exhibiting intact synapses on their cell bodies. This better preservation may be attributed to trophic-factor secretion by transplanted cells because we demonstrated *in vitro* that HDPCs express NGF- β , BDNF, GDNF, NT-3, and NT-4/5 mRNAs. We also confirmed this result *in vivo*, because we found higher levels of BDNF, NGF- β , NT-3, and NT-4 in the spinal cord of animals that received HDPC transplantation, than in the group that received only DMEM. These results are in agreement with those of Nosrat and coworkers (2001), who demonstrated that dental pulp

cells produce trophic factors and prevent the death of motor neurons after SCI. The beneficial effects of trophic factors in spinal cord injury models have also been pointed out by several authors (Piantino et al., 2006; Pisati et al., 2007; Sharma, 2009). For example, BDNF and NT-4 promote rubrospinal axonal regeneration (Kobayashi et al., 1997), reduce necrotic area, and maintain motor-neuron survival after SCI (Novikova et al., 1996). Therefore, the local release of trophic factors, as seen in the present study, might be an important finding in terms of the better results that were obtained in HDPC-treated animals.

The functional improvement shown by HDPC-treated animals can also be attributed to the release of neurotrophic factors by these cells. This may act by stimulating collateral sprouting, which may bypass the lesion area and make new synaptic contacts, enhancing the functional outcome (Cumings et al., 2005). This may be a plausible explanation, because we found many healthy neurons, intact synapse contacts, and preserved axons in the ultrastructural analysis.

Another interesting characteristic of these cells is that they survive and express some neural-associated markers when transplanted into the rodent brain (Miura et al., 2003; Nosrat et al., 2004). These results corroborate our findings because 8

weeks after transplantation, some cells were able to survive and expressed some glial staining, such as S-100 and GFAP, Schwann cell and astrocyte markers. These results are very similar to those for the embryonic stem cells. Our group has already demonstrated that after spinal cord injury and cell transplantation, these cells also expressed GFAP and S-100 markers (Marques et al., 2010).

Better morphological preservation after trauma must be accompanied by improvement in functional locomotor performance. Therefore, the functional outcome must be tested by the use of methods that can be properly measured. In our study, we used the BMS and the global mobility test to assess motor performance. The BMS test, first described by Basso and coworkers in 2006, is specific for evaluating mice locomotor behavior, and is considered sensitive and reliable in detecting slight differences between groups. The global mobility test, which was described by our group (Marques et al., 2009), evaluates the distance that the animal is able to walk in an open field, and also calculates the speed that it reaches after 1 minute of observation. The fact that the global mobility test allows an objective evaluation makes it ideal to further test locomotor performance, and also to confirm the results obtained by the BMS, which is based on subjective evaluation. The results of the functional analysis revealed that the HDPC-treated animals showed better locomotor performance in both functional tests, and exhibited higher speeds in comparison to the DMEM groups, especially the subacute animals that received cell transplantation 7 days after injury. These results are similar to those reported by previous researchers that also used cell transplantation as a treatment strategy for CNS injuries (Cizkova et al., 2006; Zurita and Vaquero, 2006). This improvement in performance may be attributed to large amounts of Schwann cells found in the HDPC spinal cords, which can also contribute to white-matter preservation. Our results support this idea because we observed several cells that were positive for Schwann markers. It is important to remember that electrophysiological studies have previously shown that axons remyelinated by either oligodendrocytes or Schwann cells can conduct typical action potentials (Akiyama et al., 2002).

Chronic SCI is a challenging area of restorative medicine. One of the issues that have not received adequate experimental attention is the best time window for cell transplantation in animal injury models. One of the aims of this study was to compare results obtained from animals treated in the subacute phase with those in the chronic phase. Our study showed better results when the cells were transplanted 7 days after the lesion; however, we also found positive effects when the treatment was applied in the chronic phase, when there is a very hostile environment with astroglial scarring and tissue loss. These results suggest that these cells can be successfully applied in SCI treatments in both periods.

Here we demonstrated an important therapeutic potential of HDPC transplantation, as applied in both subacute and chronic stages of a mouse compressive SCI. We conclude that HDPCs are excellent candidates for further evaluation of stem-cell-based treatments, to be used either in other animal injury models, or perhaps in human trials in the near future.

Author Disclosure Statement

No competing financial interests exist.

References

- Akiyama, Y., Radtke, C., Honmou, O., and Kocsis, J.D. (2002). Remyelination of the spinal cord following intravenous delivery of bone marrow cells. *Glia* 39, 229–236.
- Arataki, S., Tomizawa, K., Moriwaki, A., Nishida, K., Matsushita, M., Ozaki, T., Kunisada, T., Yoshida, A., Inoue, H., and Matsui, H. (2005). Calpain inhibitors prevent neuronal cell death and ameliorate motor disturbances after compression-induced spinal cord injuries in rats. *J. Neurotrauma* 22, 398–406.
- Basso, D.M., Fisher, L.C., Anderson, A.J., Jakeman, L.B., McTiague, D.M., and Popovich, P.G. (2006). Basso Mouse Scale for locomotion detects differences in recovery after spinal cord injury in five common mouse strains. *J. Neurotrauma* 23, 635–659.
- Cizková, D., Rosocha, J., Vanický, I., and Jergová, S. (2006). Transplants of human mesenchymal stem cells improve functional recovery after spinal cord injury in the rat. *Cell Mol. Neurobiol.* 26, 1167–1180.
- Cummings, B.J., Uchida, N., Tamaki, S.J., Salazar, D.L., Hooshmand, M., Summers, R., Gage, F.H., and Anderson, A.J. (2005). Human neural stem cells differentiate and promote locomotor recovery in spinal cord-injured mice. *PNAS* 39, 14069–14074.
- Couto, L.A., Narciso, M.S., Hokoç, J.N., and Martinez, A.M.B. (2004). Calpain inhibitor 2 prevents axonal degeneration of opossum optic nerve fibers. *J. Neurosci. Res.* 77, 410–419.
- D'Aquino, R., De Rosa, A., Laino, G., Caruso, F., Guida, L., Rullo, R., Checchi, V., Laino, L., Tirino, V., and Papaccio, G. (2009). Human dental pulp stem cells: from biology to clinical applications. *J. Exp. Zool. B Mol. Dev. Evol.* 312, 408–415.
- Deumens R., Koopmans, G.C., and Joosten, E.A. (2005). Regeneration of descending axon tracts after spinal cord injury. *Prog. Neurobiol.* 77, 57–89.
- Dietz, V., and Curt, A. (2006). Neurological aspects of spinal-cord repair: promises and challenges. *Lancet Neurol.* 5, 688–694.
- Harel, N.Y., and Strittmatter, S.M. (2006). Can regenerating axons recapitulate developmental guidance during recovery from spinal cord injury? *Nat. Rev. Neurosci.* 7, 603–616.
- Keirstead, H.S., Nistor, G., Bernal, G., Totoiu, M., Cloutier, F., Sharp, K., and Steward, O. (2005). Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants remyelinate and restore locomotion after spinal cord injury. *J. Neurosci.* 25, 4694–4705.
- Kerkis, I., Kerkis, A., Dozortsev, D., Stukart-Parsons, G.C., Gomes Massironi, S.M., Pereira, L.V., Caplan, A.I., and Ceruti, H.F. (2006). Isolation and characterization of a population of immature dental pulp stem cells expressing OCT-4 and other embryonic stem cell markers. *Cells Tissues Organs* 184, 105–116.
- Kobayashi, N.R., Fan, D.P., Giehl, K.M., Bedard, A.M., Wiegand, S.J., and Tetzlaff, W. (1997). BDNF and NT-4/5 prevent atrophy of rat rubrospinal neurons after cervical axotomy, stimulate GAP-43 and Talphal1-tubulin mRNA expression, and promote axonal regeneration. *J. Neurosci.* 17, 9583–9595.
- Marques, S.A., Almeida, F.M., Fernandes, A.M., Souza, C.S., Cadilhe, D.V., Rehen, S.K., and Martinez, A.M.B. (2010). Pre-differentiated embryonic stem cells promote functional recovery after spinal cord compressive injury. *Brain Res.* 1349, 115–128.
- Marques, S.A., Garcez, V.F., Del Bel, E.A., and Martinez, A.M. (2009). A simple, inexpensive and easily reproducible model of spinal cord injury in mice: morphological and functional assessment. *J. Neurosci. Methods* 177, 183–193.

- Miura, M., Gronthos, S., Zhao, M., Lu, B., Fisher, L.W., Robey, P.G., and Shi, S. (2003). SHED: Stem cells from human exfoliated deciduous teeth. *Proc. Natl. Acad. Sci. USA* 100, 5807–5812.
- Nosrat, I.V., Smith, C.A., Mullally, P., Olson, L., and Nosrat, C.A. (2004). Dental pulp cells provide neurotrophic support for dopaminergic neurons and differentiate into neurons in vitro: implications for tissue engineering and repair in the nervous system. *Eur. J. Neurosci.* 19, 2388–2398.
- Nosrat, I.V., Widenfalk, J., Olson, L., and Nosrat, C.A. (2001). Dental pulp cells produce neurotrophic factors, interact with trigeminal neurons in vitro, and rescue motoneurons after spinal cord injury. *Dev. Biol.* 238, 120–132.
- Novikova, L., Novikov, L., and Kellerth, J.O. (1996). Brain-derived neurotrophic factor reduces necrotic zone and supports neuronal survival after spinal cord hemisection in adult rats. *Neurosci. Lett.* 220, 203–206.
- Piantino, J., Burdick, J.A., Goldberg, D., Langer, R., and Benowitz, L.I. (2006). An injectable, biodegradable hydrogel for trophic factor delivery enhances axonal rewiring and improves performance after spinal cord injury. *Exp. Neurol.* 201, 359–367.
- Pierdomenico, L., Bonsi, L., Calvitti, M., Rondelli, D., Arpinati, M., Chirumbolo, G., Becchetti, E., Marchionni, C., Alviano, F., Fossati, V., Staffolani, N., Franchina, M., Grossi, A., and Bagnara, G.P. (2005). Multipotent mesenchymal stem cells with immunosuppressive activity can be easily isolated from dental pulp. *Transplantation* 80, 836–842.
- Pisati, F., Bossolasco, P., Meregalli, M., Cova, L., Belicchi, M., Gavina, M., Marchesi, C., Calzarossa, C., Soligo, D., Lambertenghi-Deliliers, G., Bresolin, N., Silani, V., Torrente, Y., and Polli, E. (2007). Induction of neurotrophin expression via human adult mesenchymal stem cells: implication for cell therapy in neurodegenerative diseases. *Cell Transplant* 16, 41–55.
- Sharma, H.S. (2010). Selected combination of neurotrophins potentiate neuroprotection and functional recovery following spinal cord injury in the rat. *Acta Neurochir. Suppl.* 106, 295–300.
- Sinanan, A.C., Hunt, N.P., and Lewis, M.P. (2004). Human adult craniofacial muscle-derived cells: neural-cell adhesion-molecule (NCAM; CD56)-expressing cells appear to contain multipotential stem cells. *Biotechnol. Appl. Biochem.* 40, 25–34.
- Takami, T., Oudega, M., Bates, M.L., Wood, P.M., Kleitman, N., and Bunge, M.B. (2002). Schwann cell but not olfactory ensheathing glia transplants improve hindlimb locomotor performance in the moderately contused adult rat thoracic spinal cord. *J. Neurosci.* 22, 6670–6681.
- Thuret, S., Moon, L.D.F., and Gage, F.H. (2006). Therapeutic interventions after spinal cord injury. *Nature Rev. Neurosci.* 7, 628–643.
- Yin, Y., Henzl, M.T., Lorber, B., Nakazawa, T., Thomas, T.T., Jiang, F., Langer, R., and Benowitz, L.I. (2006). Oncomodulin is a macrophage-derived signal for axon regeneration in retinal ganglion cells. *Nature Rev. Neurosci.* 9, 843, 852.
- Yiu, G., and He, Z. (2006). Glial inhibition of CNS axon regeneration. *Nature Rev. Neurosci.* 7, 617–627.
- Zurita, M., and Vaquero, J. (2006). Bone marrow stromal cells can achieve cure of chronic paraplegic rats: functional and morphological outcome one year after transplantation. *J. Neurosci. Lett.* 402, 51–56.

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