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Immunomodulatory and neuroprotective effect of cryopreserved allogeneic mesenchymal stem cells on spinal cord injury in rats

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ABSTRACT. This study aimed to evaluate the immunomodulatory and neuroprotective effects of allogeneic and cryopreserved mesenchymal stem cells (MSCs) on spinal cord injury. A total of 120 rats were distributed into the following groups: negative control (NC) - without injury, positive control (PC) - with injury without treatment, and group

treated with MSC (GMSC) - with injury and treated. Motor function was evaluated by the BBB test at 24, 48, and 72 h and at 8 and 21 postoperative days. Spinal cords were evaluated by histopathology and immunohistochemistry to determine the expression of CD68, NeuN, and GFAP. IL-10, TNF-α, IL-1β, TGF-β, BDNF, GDNF, and VEGF expression was quantified by RT-PCR. The GMSC presented higher scores for motor function at 72 h and 8 and 21 days after injury, lower expression of CD68 at 8 days, and lower expression of GFAP at 21 days compared to the PC. In addition, higher expression of NeuN and lower degeneration of the white matter occurred at 21 days. The GMSC also showed higher expression of IL-10 24 h after injury, GDNF at 48 h and 8 days, and VEGF at 21 days. Moreover, lower expression of TNF-α was observed at 8 and 21 days and TGF-β at 24 h and 21 days. There were no differences in the expression of IL-1β and BDNF between the GMSC and PC. Thus, cryopreserved MSCs promote immunomodulatory and neuroprotective effects in rats with spinal cord injury by increasing IL-10, GDNF, and VEGF expression and reducing TNF-α and TGF-β expression.

Key words: Neurology; Cell therapy; Regenerative medicine; Inflammation

INTRODUCTION

Approximately 2.5 million individuals worldwide have suffered from spinal cord injury (SCI). Frequently, this type of lesion results in severe and permanent neurological deficits, causing affected individuals to require constant medical care and nursing (Sekhon and Fehlings, 2001). The neurological deficits caused by spinal cord injury result from the direct disruption of neuronal pathways and from progressive secondary changes causing lesion expansion (Brewer et al., 1999; Dasari et al., 2007).

There are three phases in the response to SCI. The acute phase occurs immediately after injury and extends until the seventh day after the lesion. In this phase, the release of pro-inflammatory cytokines is initiated, microglia are activated, and neutrophils and monocytes migrate to the lesion. From the seventh to the fourteenth day after the lesion is initiated, a subacute phase occurs, which is characterized by the persistence of the inflammatory reaction and by increased production of free radicals. These secondary changes cause lipid peroxidation and induce apoptosis of neurons and oligodendrocytes. In the chronic phase, which extends from 2 weeks to several months after injury, glial scar formation and degeneration of axons and myelin occur (Nandoe Tewarie et al., 2009). Therefore, an important characteristic of SCI pathogenesis is that the final lesion is much larger than the primary injury (Popovich et al., 1997). Thus, the rapid introduction of treatment is essential for stopping these secondary events and limiting tissue loss. Nevertheless, there are no effective therapies for this condition, although many studies have attempted to find effective therapeutic alternatives. Therapy with mesenchymal stem cells (MSCs) is considered a promising option, because these cells are multipotent and can easily be isolated from adult individuals and expanded *in vitro* (Osaka et al., 2010; Zhukareva et al., 2010; Nakajima et al., 2012; Dariolli et al., 2013; Zhou et al., 2013; Kingham et al., 2014; Lin et al., 2014).

The administration of MSCs during the acute or subacute phase of SCI shows better results than administration during the chronic phase (Dasari et al., 2007; Nandoe Tewarie et al., 2009; Osaka et al., 2010; Zhukareva et al., 2010; Hawryluk et al., 2012; Nakajima et al., 2012). These results support the hypothesis that MSCs can act in secondary events favoring neuronal survival and regeneration (Nakamura et al., 2003; Yune et al., 2003; Mead et al., 2014).

The isolation, cultivation, and expansion of MSCs require 2-3 weeks, limiting their early administration. This may be overcome by using cryopreserved MSCs (Dariolli et al., 2013; Davies et al., 2014).

SCI lesion models in rodents have proved to be useful in studies of pathogenesis and therapy (Popovich et al., 1997; Brewer et al., 1999; Nakamura et al., 2003; Yune et al., 2003; Dasari et al., 2007; Nandoe Tewarie et al., 2009).

Thus, the objective of the present study was to evaluate the immunomodulatory and neuroprotective effects of cryopreserved MSCs administered intravenously in rats subjected to SCI.

MATERIAL AND METHODS

The present study was developed in accordance with International Standards of Animal Welfare following approval by the Ethics Committee in Animal Experimentation at Universidade Federal de Minas Gerais (CEUA No. 41/2012). Animals were housed in individual cages and maintained at 23°C, 12-h light/dark cycle, fed with commercial chow and tap water *ad libitum.*

Isolation and cultivation of MSCs from adipose tissues of ten 4-week-old male GFP Lewis rats (LEW-Tg and GFP F455/Rrrc) weighing 200g were used for the isolation of MSCs. Retroperitoneal fat was harvested, and the MSCs were isolated as described by Carvalho et al. (2013).

Cell characterization

For their phenotype characterization, 5×10^6 MSCs were incubated with the following primary mouse anti-rat antibodies: CD45, CD54, CD73, CD90, CD11, MHCI, and MHCII (Abcam, Cambridge, UK) for 30 min at 4°C. The samples were then washed with PBS and incubated with secondary anti-mouse Alexa 488 antibody (Molecular Probes, Eugene, OR, USA) for 30 min at 4°C. Next, MSCs were washed and suspended in PBS. The quantification of the cells stained with each antibody was performed using a FACScan flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) with 30,000 events per sample and analyzed using the Cell Quest software (BD Biosciences). A sample of cells was incubated with only the secondary antibody to control non-specific reaction. Phenotypical characterization was performed before and after cell thawing.

MSCs were also evaluated for their differentiation potential before and after thawing. For osteogenic differentiation, MSCs were maintained in DMEM containing 10% fetal bovine serum (FBS), 0,1 μM dexamethasone, 50 mg/mL ascorbic acid-2-phosphate, and 10.0 mM β-glycerophosphate (Sigma, St. Louis, MO, USA) for 21 days at 37°C and 5% CO_2. On the 21st day, the cultures were stained as described by Von Kossa to assess the presence of mineralization nodules. For adipogenic differentiation, MSCs were maintained in DMEM containing 10% FBS, 1.0 μM f dexamethasone, 10.0 μL/mL insulin, 100 mM indomethacin, and 500 mM 3-isobutyl-1-methylxanthine (Sigma) for 21 days at 37°C and 5% CO_2. On

the 21st day, the cells were subjected to Oil Red O staining and assessed for deposition of intracellular lipid vesicles. Chondrogenic differentiation was performed in 3-D spheroid culture, with 1 x 10^6 cells maintained in DMEM containing 10% FBS, 0.0125 g/mL bovine serum albumin, 100 μM sodium pyruvate, 6.25 mg/mL insulin, 6.25 mg/mL transferrin, 50 μM ascorbic acid-2-phosphate, 100 nM dexamethasone, and 10.0 ng/mL transforming growth factor-beta 1 (TGF-b1), for 21 days at 37°C and 5% CO_2. On the 21st day, the cells were fixed in 4% paraformaldehyde and embedded in paraffin. Histological sections were prepared from pellets and proteoglycans were stained with Alcian blue.

Cryopreservation of MSCs

MSCs from the fourth passage were cryopreserved. For this, MSCs were detached from the flasks by trypsin, centrifuged at 1500 rpm for 10 min at 23°C, and pellets were diluted in cell cryopreservation medium (50% v/v DMEM + 40% v/v FBS + 10% v/v DMSO). MSCs (1 x 10^6) were stored in 2-mL cryogenic vials containing 1.5 mL medium. The cryotubes were placed in Mr. Frosty Cryo Freezing Container® filled with isopropyl alcohol in the lower compartment. Subsequently the container was kept for 24 h in freezer -70/80°C to gradual cooling until freezing, and then removed from the container and stored in the freezer at -70/80°C for 2-3 months.

Cellular analyses

Forty-eight hours before transplantation, the MSCs were thawed in a water bath at 37°C for 30 s. After thawing, MSCs were immediately washed in PBS, plated on DMEM, and maintained in an incubator at 37°C and 5% CO_2 until transplantation. The viability of MSCs was evaluated before and after cryopreservation, using trypan blue staining and the MTT viability test.

Surgical procedure

To study SCI, 120 male Wistar rats weighing 300 g each were used. Animals were pre-medicated with morphine (5.0 mg/kg, *sc*), and anesthetic induction and maintenance with isoflurane was provided through a facial mask in a semi-open system. Antibiotic therapy of cephalothin (60 mg/kg, *sc*) was administered for antisepsis of the surgical field and prophylaxis. Laminectomy of the tenth thoracic vertebra (T10) was performed using a pneumatic drill. After visualization of the spinal cord covered by intact dura mater, compressive SCI was induced using a French Fogarty No. 2 catheter. The catheter was introduced into the epidural space and conducted cranially to the T8-T9 segment; the catheter balloon was filled with 30 μL 0.9% saline solution, and pressure was maintained on the spinal cord for 5 min. During anesthetic recovery, the animals received oxygen therapy and were maintained at 37°C. During the postoperative period, the animals received morphine (5.0 mg/kg, *sc*) every 8 h for 3 consecutive days.

Treatment

The therapeutic protocol consisted of injection with 1 x 10^6 MSCs in 0.5 mL 0.15 M

PBS, administered in a single dose intravenously in the coccygeal lateral vein 3 h after SCI. Controls received only 0.15 M PBS by the same route 3 h after SCI or laminectomy.

Experimental groups

The animals were distributed into three experimental groups: NC (negative control) subjected to laminectomy and treated with placebo (N = 20); PC (positive control) subjected to SCI and treated with placebo (N = 50); GMSC (group treated with mesenchymal stem cell) subjected to SCI and treated with MSCs (N = 50). Each group was evaluated at 24, 48, and 72 h and at 8 and 21 days after the surgical procedure. Animals were allocated to experimental groups by randomization.

Histopathological analysis, immunohistochemistry, and reverse transcriptase-polymerase chain reaction (RT-PCR)

After the evaluation periods, the animals were sacrificed with an overdose of intraperitoneal sodium thiopental, and blood was collected by cardiac puncture.

Histopathological and immunohistochemical evaluations were carried out 8 and 21 days after the surgical procedures. The vertebral columns were removed and fixed in 4% buffered paraformaldehyde for 24 h and subsequently transferred to 70% alcohol and stored until they were embedded in paraffin. Next, 4-mm segments centered at the epicenter of the lesion were removed, embedded in paraffin, and cut into serial sections, which were stained with hematoxylin and eosin for optical microscopy evaluation. For histopathological analysis, two slides were selected per animal, containing focally extensive necrosis area in the dorsal funiculus, one from the cranial region and the other from the caudal region to the epicenter of the lesion. In NC group animals, histological sections from the epicenter region were used. Immunohistochemical evaluation of animals subjected to injury focused on the first histological section of the caudal region to the epicenter containing the focally extensive necrosis area in the dorsal funiculus. The primary antibodies used were: 1:500 anti-eGFP (Abcam), 1:200 anti-CD68 (Abcam), 1:500 anti-NeuN (Chemicon, Billerica, MA, USA), and 1:200 anti-GFAP (Abcam).

The spinal cord sections were deparaffinized and dehydrated using standardized protocols. Antigenic recovery was performed by heating the slides in 0.05% citric acid solution. Endogenous peroxidase was blocked in a solution of methanol and 3% H_2O_2 in a dark chamber, and non-specific epitopes in the protein (DAKO, Glostrup, Denmark) were blocked in a dark and humidified chamber. The histological sections were incubated overnight in the dark and humid chamber with the primary antibodies. Secondary antibody (DAKO) was added, and the sections were incubated under the same conditions for 45 min. Next, streptavidin-biotin peroxidase complex (DAKO) was added. The reactions were observed with 3-3'-diaminobenzidine peroxidase (DAKO), washed in water, and counterstained with Harris hematoxylin. Next, standardized dehydration and slides with cover glass and Canada balsam were prepared. The slides were photographed and analyzed using the ImageJ analysis software (NIH, Bethesda, MA, USA). For each image, the average integrated pixel density of the cells was subtracted from the background.

RT-PCR was performed for samples from 24, 48, and 72 h and 8 and 21 days after the surgical procedure. First, 1.5-cm segments of the spinal cord centered in the epicenter of the lesion were obtained, and the samples were placed in RNAase- and DNAase-free cryotubes containing

1.0 mL Trizol (Invitrogen, Carlsbad, CA, USA). The samples were frozen in liquid nitrogen (-196°C) and stored at -80°C. Total RNA was extracted, and RNA reverse transcription and RT-PCR were performed as described by de Lavor et al. (2015). Primer sequences are shown in Table 1. RT-PCR data were analyzed using the 7500 v.2.0.1 software from Applied Biosystems (Foster City, CA, USA) using the comparative cycle threshold method (Livak and Schmittgen, 2001). Gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method, where the results obtained for each group were compared quantitatively after normalization based on the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from *Rattus norvegicus*. The expression levels obtained in the NC were used as the standard for calculating the relative expression of each transcript.

Table 1. Genes with the primer sequences for real-time RT-PCR.

Gene	Accession No.	Oligonucleotide sequence (5'-3')	Product size
IL-10	NM_012854.2	F: CCCAGAAATCAAGGAGCATTT	64
		R: CAGCTGTATCCAGAGGGTCTTCA	
TNF-α	NM_012675.3	F: AACACATCTCCCTCCGGAAA	64
		R: CCAGTTCCACATCTCGGATCA	
IL-1β	M98820	F: TGACAGACCCCAAAAGATTAAGG	61
		R: CTCATCTGGACAGCCCAAGTC	
TGF-β	AY550025.1	F: GAAACGGAAGCGCATCGA	59
		R: TGGCGAGCCTTAGTTTGGA	
BDNF	NM_012513.3	F: CACAATGTTCCACCAGGTG	89
		R: TGGGCGCAGCCTTCAT	
GDNF	EU068472	F: GAGAGAGGAACCGGCAAGCT	59
		R: GCGACCTTTCCCTCTGGAAT	
VEGF	NC_013899.1	F: AAATCCTGGAGCGTTCACTGTG	106
		R: AACGCGAGTCTGTGTTTTTGC	
GAPDH	NC_005103.3	F: TGCACCACCAACTGCTTA	97
		R: GGATGCAGGGATGATGTTC	

Evaluation of motor function

Motor function was evaluated in an open field using the Basso, Beattie, and Bresnahan (BBB) test (Basso et al, 1996). The animals were filmed for 5 min, and the videos were evaluated by two observers who were unaware of the groups and treatments. Evaluations were performed 24 h before the surgical procedure, 24 h after, and weekly until 21 days after surgery.

Statistical analysis

The experiment was conducted in a completely randomized 3 x 5-factorial design (three experimental groups and five evaluation periods). Analysis of variance and calculation of the means, medians, and standard deviations was performed for each variable. The parametric data that did not present a normal distribution and homoscedasticity were submitted to logarithmic transformation $[x = \log(y + 1)]$. The means of gene expression and integrated density of pixels were compared by the non-paired *t*-test, and the scores of motor evaluation were compared by the non-parametric Mann-Whitney test. The level of significance was set to 5%.

RESULTS

Isolation, cultivation, and characterization of MSCs

The MSCs presented an elongated and fusiform morphology in culture. Phenotypic

characterization of MSCs by flow cytometry showed that the cells were similar before freezing and after thawing. High expression of the markers CD54 (89.71 ± 9.55%), CD73 (90.07 ± 5.25%), CD90 (91.28 ± 6.5%), and MHCI (92.69 ± 6.35%) was observed. The expression of hematopoietic cell markers was low: CD45 (4.93 ± 1.4%), CD34 (4.83 ± 0.62%), CD11b/c (5.37 ± 1.64%), and MHCII (9.1 ± 7.1%) (Figure 1).

MSCs grown in osteogenic medium acquired an osteoblast phenotype and presented mineralization of the extracellular matrix as demonstrated by Von Kossa staining. After cultivation in chondrogenic differentiation medium, the chondrocytes derived from MSCs produced and deposited proteoglycans in the extracellular matrix as observed by Alcian blue staining. Finally, after adipogenic induction, adipocytes derived from MSCs presented birefringent lipid vesicles within their cytoplasm, as observed by Oil Red O staining.
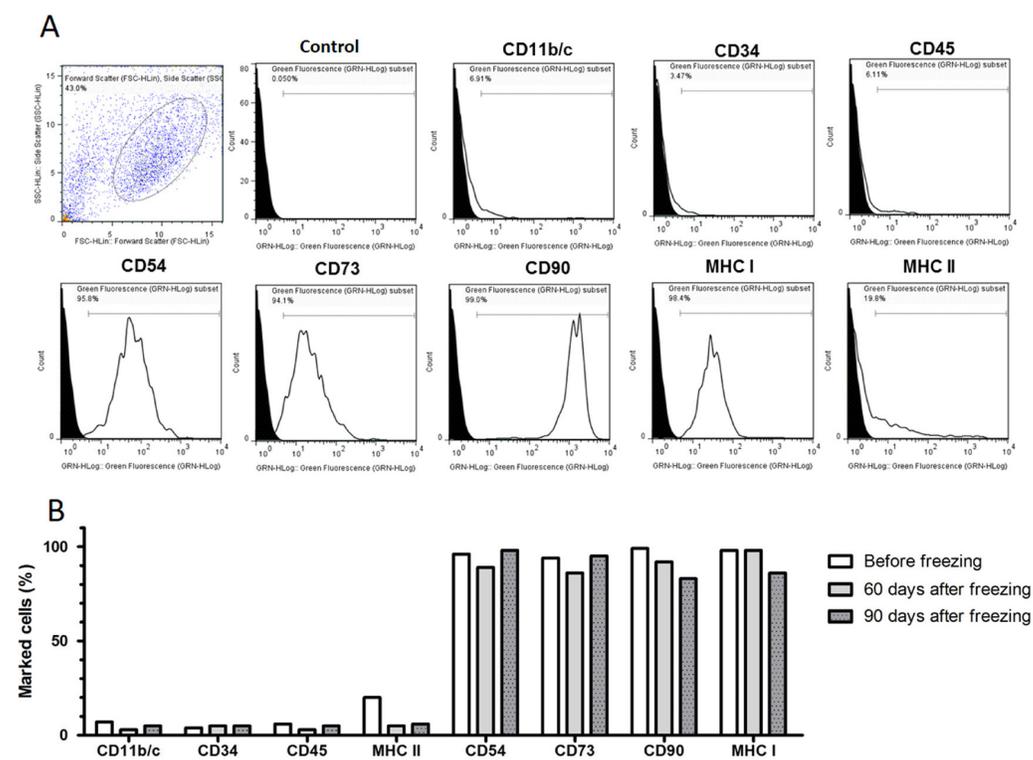

Figure 1. A. Histograms of the expression of CD11, CD34, CD45, CD54, CD73, CD90, MHCI, and MHCII by flow cytometry in cultures of MSCs of rat adipose tissue. The white graph represents the population of cells evaluated for a specific marker (offset to the right indicates the existence of staining). **B.** Percentage of cells expressing the markers CD11, CD34, CD45, CD54, CD73, CD90, MHCI, and MHCII before freezing and 60 and 90 days after freezing.

Assessment of cell viability by trypan blue and conversion of MTT into formazan crystals

The percentage of viable cells was 97.03 ± 0.25% before freezing. When frozen for 60 days, the cellular viability was 84.87 ± 0.81%, and after 90 days, this value was 81.83

± 3.72%. Thus, cell viability decreased after cryopreservation and thawing. There was no difference in cellular viability between 60 and 90 days of freezing (Figure 2A).

The conversion of MTT into formazan crystals was higher in MSCs after 90 days of freezing compared to that before freezing (Figure 2B).
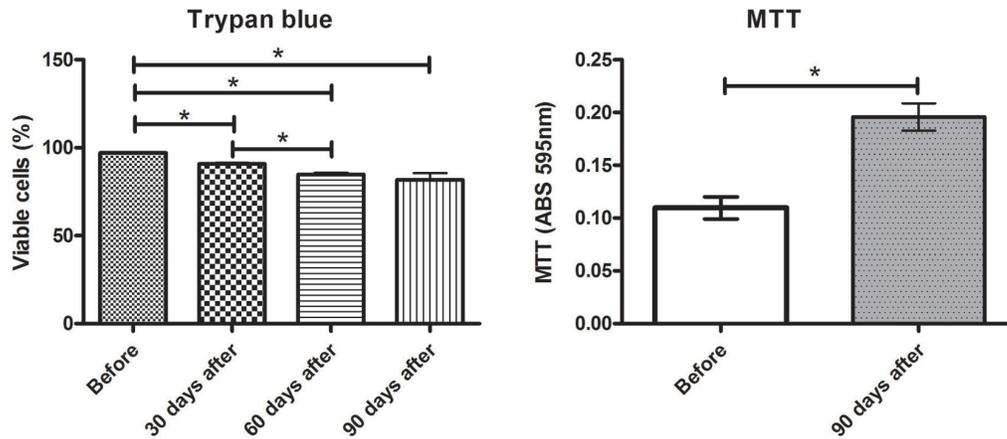
Figure 2. A. Means and standard deviations of the percentage of viable cells in MSCs cultured from rat adipose tissue before freezing and 60 and 90 days after freezing. **B.** Means and standard deviations of the conversion of MTT in MSC cultures before freezing and 90 days after freezing (*P < 0.05).

Histopathological analysis

The spinal cords of rats in the negative control group showed normal architecture (Figure 3A, B). However, the spinal cords of animals with injury showed uniform lesions. In the epicenter region, the spinal cords showed intense diffuse malacia affecting all of the white and gray matter (Figure 3C).

After 8 days, the histological sections from the cranial and caudal regions of the GMSC and PC presented focally extensive malacia areas, affecting the dorsal funiculus (Figure 3D-G), and degenerated neurons were observed in the gray matter (Figure 3H). Discreet axonal degeneration and myelin tumefaction occurred in the white matter (Figure 3I).

At 21 days, cranial and caudal segments of the cords of GMSC and PC animals presented areas of focally extensive malacia affecting the dorsal funiculus (Figure 4A-D). Evaluation of gray matter revealed some degenerated neurons. Discreet to moderate axonal degeneration and tumefaction of myelin occurred in the white matter. This finding was greater in the PC group than in the GMSC group (Figure 4E).
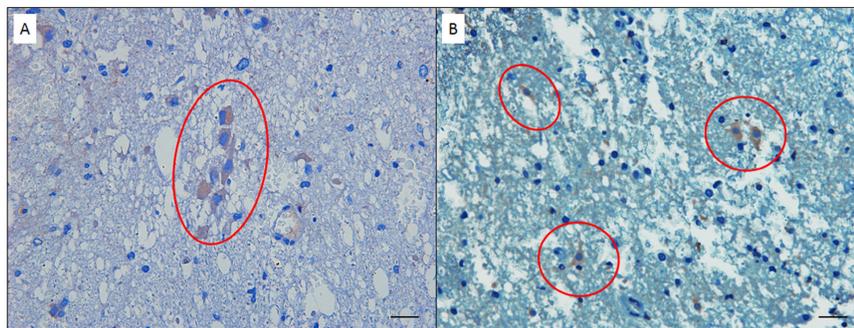
Immunohistochemistry analysis

Immunohistochemical staining with anti-eGFP antibody allowed for visualization of the MSCs in the spinal cord 8 and 21 days after administration. MSCs were identified in the gray matter in the caudal segments and in the malacia region at the lesion epicenter (Figure 5A, B).
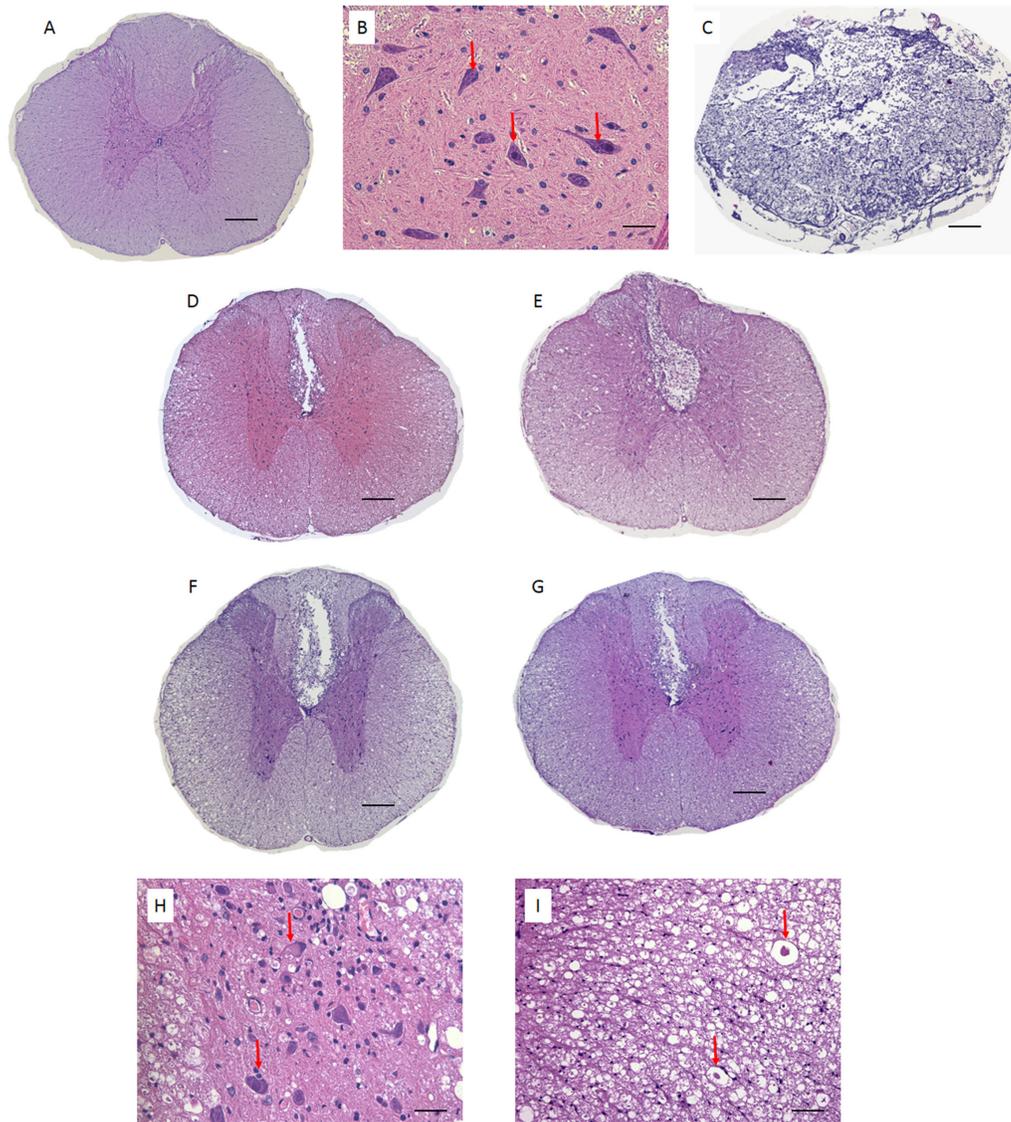
Figure 3. Photomicrographs of hematoxylin and eosin-stained spinal cords of rats 8 days after laminectomy and spinal cord injury. **A.** and **B.** Spinal cord of animal subjected only to laminectomy presenting normal tissue architecture (A) and intact neurons (arrows in B). **C.** Epicenter of the spinal medulla of animal subjected to injury presenting malacia affecting all white and gray matter. Cranial (**D**) and caudal regions (**E**) of the spinal cord in animal subjected to injury and treated with MSCs; note focally extensive areas of malacia affecting the dorsal funiculus and discreet axonal degeneration and tumefaction of myelin affecting the dorsal and lateral funiculi. Cranial (**F**) and caudal (**G**) regions of the spinal cords of animal subjected to injury without treatment; note focally extensive areas of malacia affecting the dorsal funiculus and discreet axonal degeneration and tumefaction of myelin affecting the dorsal and lateral funiculi. **H.** Degenerated neurons (arrows) in animals subjected to injury without treatment. **I.** Degeneration of white matter with tumefaction of axons and myelin sheath (arrows) in animal subjected to injury without treatment (A, C, D, E, F, G: bar = 285 mm, magnification 50X; B, H, I: bar = 35 mm, magnification 400X).
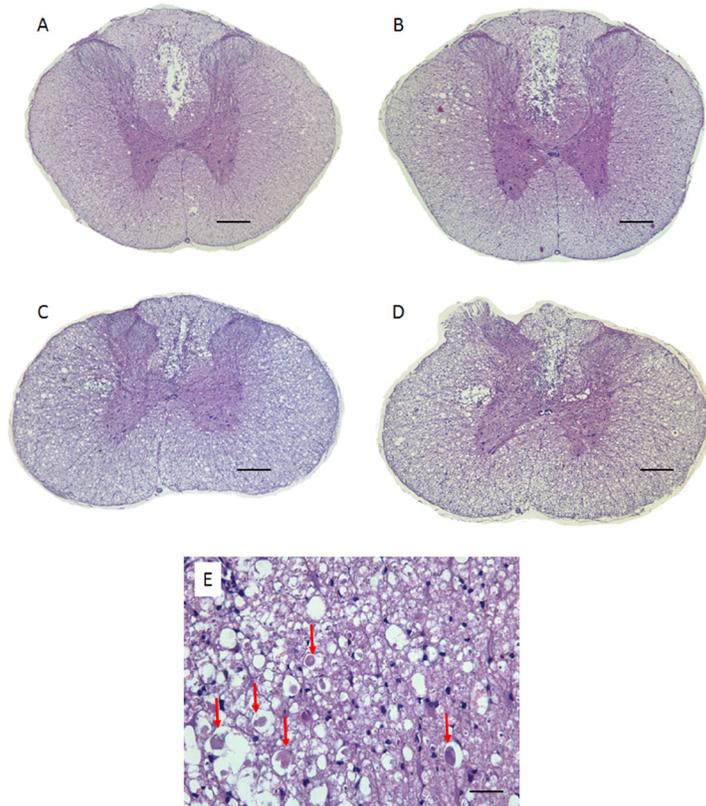
Figure 4. Photomicrographs of spinal cords of rats 21 days after laminectomy and spinal cord injury, HE staining: cranial (**A**) and caudal regions (**B**) of spinal cord of animal subjected to injury and treated with MSCs. Cranial (**C**) and caudal regions (**D**) of spinal cord of rats subjected to injury without treatment. Axonal and mild to moderate, diffuse myelin degeneration was less intense in the group treated with MSCs. **E.** White matter of the spinal cord of animal subjected to injury without treatment, displaying degeneration of axons and myelin sheath (arrows). (A, B, C, D: bar = 285 mm, magnification 50X; E: bar = 35 mm, magnification 400X).

Figure 5. Photomicrographs of spinal cords of rats subjected to injury and treated with MSCs. Evaluation by anti-eGFP immunohistochemistry. MSCs staining in gray matter (**A**) and in the epicenter region (**B**) (bar = 23 mm, magnification 400X).

There was no difference in the expression of CD68 in the dorsal funiculus between the PC ($12.91 \times 10^6 \pm 8.31 \times 10^6$) and GMSC ($16.91 \times 10^6 \pm 10.67 \times 10^6$) (Figure 6A). The expression of CD68 was significantly lower in the NC ($0.04 \times 10^6 \pm 0.01 \times 10^6$) compared to the GMSC and PC (Figure 6B, C, D). A difference was observed between groups regarding the morphological characteristics of stained cells. In the GMSC, the cells were larger and with foamy cytoplasm, while in the PC, they presented smaller and denser cytoplasm (Figure 6E, F).
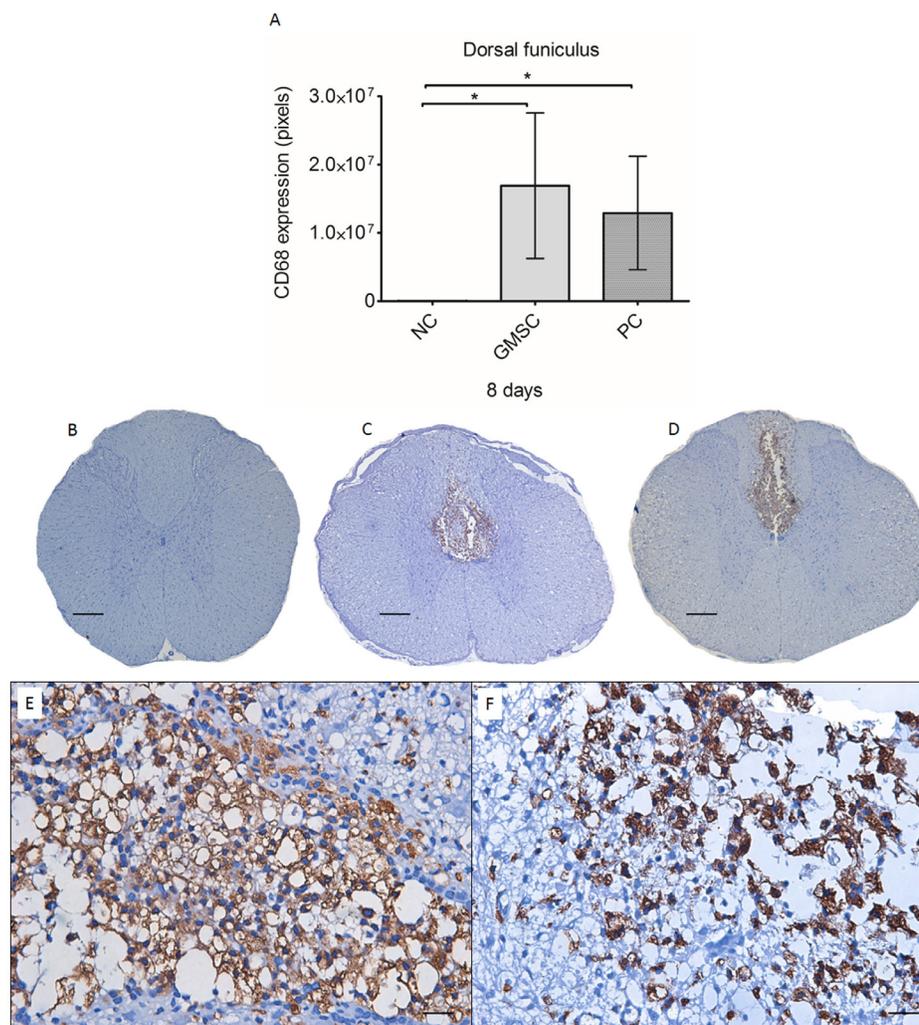
Figure 6. A. Means and standard deviations of the expression of CD68 in the region of the dorsal funiculus of cords in the negative control group (NC), group treated with mesenchymal stem cells (GMSC), and positive control group (PC) 8 days after laminectomy or injury (*P < 0.05). Photomicrographs of spinal cords evaluated by anti-CD68 immunohistochemistry 8 days after surgery (**C-F**). Discreet staining in the NC (**B**) and intense staining in the region of malacia in the dorsal funiculus of cords from the GMSC (**C**) and PC (**D**). Dorsal funiculus of spinal cord of GMSC (**E**) and PC (**F**). Stained cells in the GMSC (**E**) showed larger volumes and less dense cytoplasm compared to the PC (**F**) (B, C, D: bar = 285 mm, magnification 50X; E, F: bar = 35 mm, magnification 400X).

At 8 days, the expression of CD68 in the white matter was significantly lower in the NC ($0.05 \times 10^6 \pm 0.02 \times 10^6$) and GMSC ($0.56 \times 10^6 \pm 0.39 \times 10^6$) compared to in the PC ($2.92 \times 10^6 \pm 2.71 \times 10^6$) (Figure 7A, D, F, H).

At 8 days, the expression of CD68 in the gray matter was significantly lower in NC ($0.04 \times 10^6 \pm 0.01 \times 10^6$) and GMSC ($1.84 \times 10^6 \pm 1.58 \times 10^6$) compared to the PC ($5.54 \times 10^6 \pm 4.24 \times 10^6$) (Figure 7B, C, E, G).
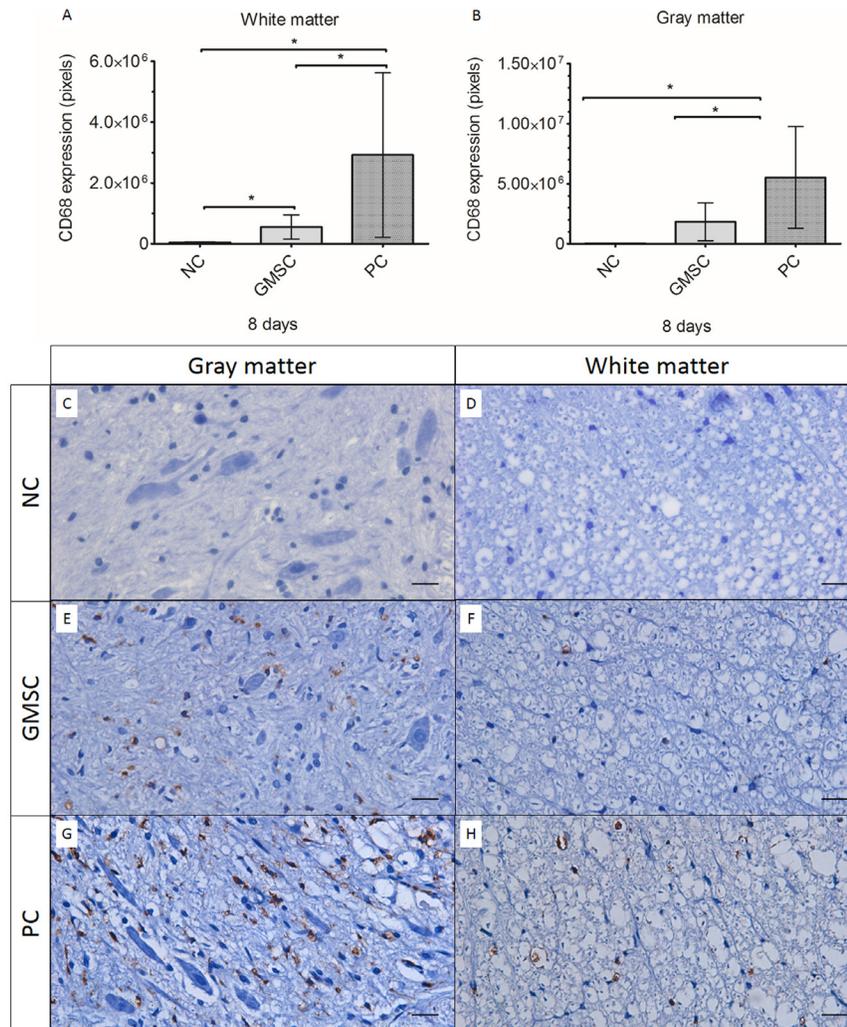
Figure 7. Means and standard deviations of CD68 expression in white (**A**) and gray matter (**B**) of spinal cords of rats in the negative control group (NC), group treated with mesenchymal stem cells (GMSC), and the positive control (PC) group evaluated 8 days after laminectomy or injury (*P < 0.05). Photomicrographs of spinal cords evaluated by anti-CD68 immunohistochemistry 8 days after surgery (**C**, **D**, **E**, **F**, **G**, **H**). Observe the insignificant staining in the white (**C**) and gray matter (**D**) of NC animals, discreet in the white (**E**) and gray matter (**F**) of the GMSC, and intense staining in the white matter (**G**) and gray matter (**H**) of animals in the PC (bar = 35 mm, magnification 400X).

At 21 days after injury, there was no difference in the expression of CD68 in the white and gray matter in GMSC ($3.73 \times 10^6 \pm 3.61 \times 10^6$; $2.15 \times 10^6 \pm 1.91 \times 10^6$) and PC ($2.66 \times 10^6 \pm 1.66 \times 10^6$; $1.64 \times 10^6 \pm 1.45 \times 10^6$). Staining in these groups (GMSC and PC) was higher than in the NC ($0.74 \times 10^6 \pm 0.58 \times 10^6$; $0.04 \times 10^6 \pm 0.01 \times 10^6$) (Figure 8A, B, C).
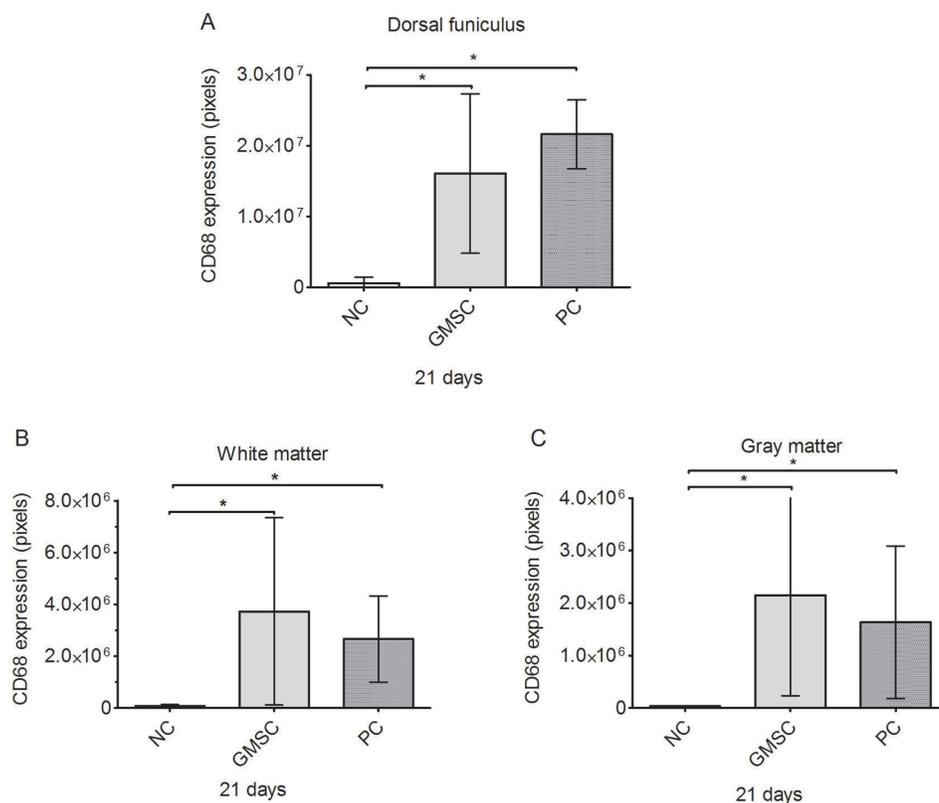
Figure 8. Means and standard deviations of CD68 expression in the dorsal funiculus (**A**), in white (**B**) and gray matter (**C**) of spinal cords of rats in the negative control (NC), treated with mesenchymal stem cells (MSC), and the positive control group (PC) 21 days after laminectomy or injury (*P < 0.05).

At 8 days after injury following staining with the anti-NeuN antibody, the spinal cords of animals in the NC and PC presented non-uniform staining of the cytoplasm and neuron nuclei; unstained neurons and other neurons showed different staining intensities (Figure 9C, E). In the GMSC, neurons presented only cytoplasmic staining (Figure 9D). NeuN expression was significantly higher in the NC ($1.67 \times 10^7 \pm 0.91 \times 10^7$) than in the PC ($0.98 \times 10^7 \pm 0.25 \times 10^7$) followed by the GMSC ($0.28 \times 10^7 \pm 0.20 \times 10^7$), which displayed less intense staining (Figure 9A).

In the assessments 21 days after injury, GMSC showed nuclear and cytoplasmic staining, similarly to in NC and PC (Figure 9F, G, H). NeuN expression was significantly higher in the NC ($1.67 \times 10^7 \pm 0.91 \times 10^7$) and GMSC ($1.69 \times 10^7 \pm 0.83 \times 10^7$) than in the PC ($0.80 \times 10^7 \pm 0.38 \times 10^7$) (Figure 9B).
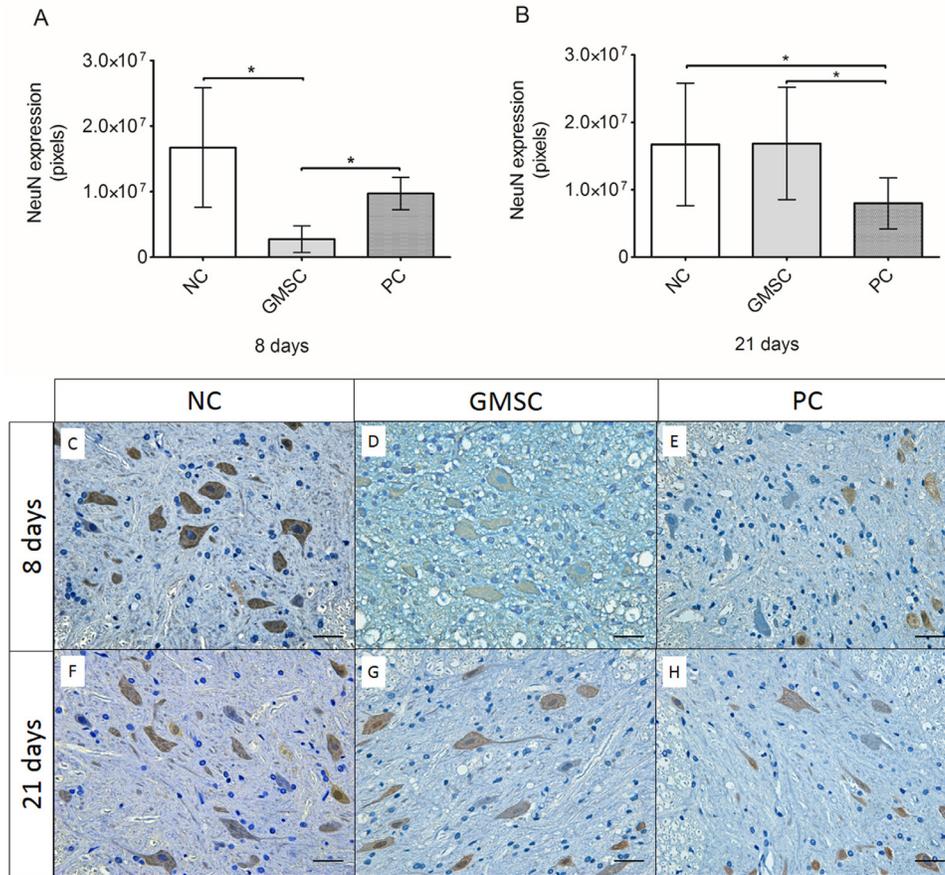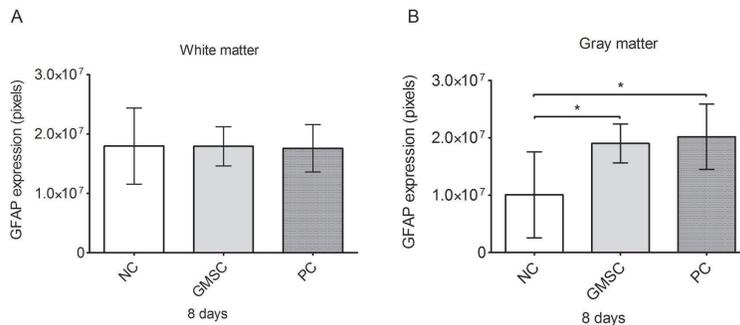
Figure 9. Means and standard deviations of NeuN expression in the spinal cords of rats in the negative control (NC) group treated with mesenchymal stem cells (GMSC), and the positive control group (PC) at 8 (**A**) and 21 (**B**) days after laminectomy or injury (*P < 0.05). Photomicrographs of spinal cords evaluated by anti-NeuN immunohistochemistry (C, D, E, F, G, H). Higher staining was observed in the NC (**C**), moderate staining in PC (**E**) and lower staining in the GMSC (**D**) at 8 days. At 21 days, higher staining was observed in NC (**F**) and GMSC (**G**) compared to the PC (**H**) (C, D, E, F, G, H: bar = 35 mm, magnification 400X).

Staining of astrocyte extensions with the GFAP antibody in the spinal cords from animals in the NC, GMSC, and PC, evaluated at 8 and 21 days, was cytoplasmic. This staining was not uniform, as the distribution of the stained extensions varied between white and gray matter (Figure 10A, B, C, D).

In the evaluation performed 8 days after injury, comparison of the means obtained only in the dorsal funiculus revealed higher expression in the PC ($1.34 \times 10^7 \pm 0.67 \times 10^7$) than in the NC ($0.52 \times 10^7 \pm 0.25 \times 10^7$) and GMSC ($0.14 \times 10^7 \pm 0.06 \times 10^7$). The GMSC displayed the lowest staining (Figure 10A, E, F, G). There was no difference in expression in the white and gray matter at 8 days between the NC ($1.80 \times 10^7 \pm 0.64 \times 10^7$; $1.01 \times 10^7 \pm 0.75 \times 10^7$), GMSC ($1.79 \times 10^7 \pm 0.33 \times 10^7$; $1.90 \times 10^7 \pm 0.34 \times 10^7$), and PC ($1.76 \times 10^7 \pm 0.40 \times 10^7$; $2.02 \times 10^7 \pm 0.57 \times 10^7$) (Figure 11A, B).
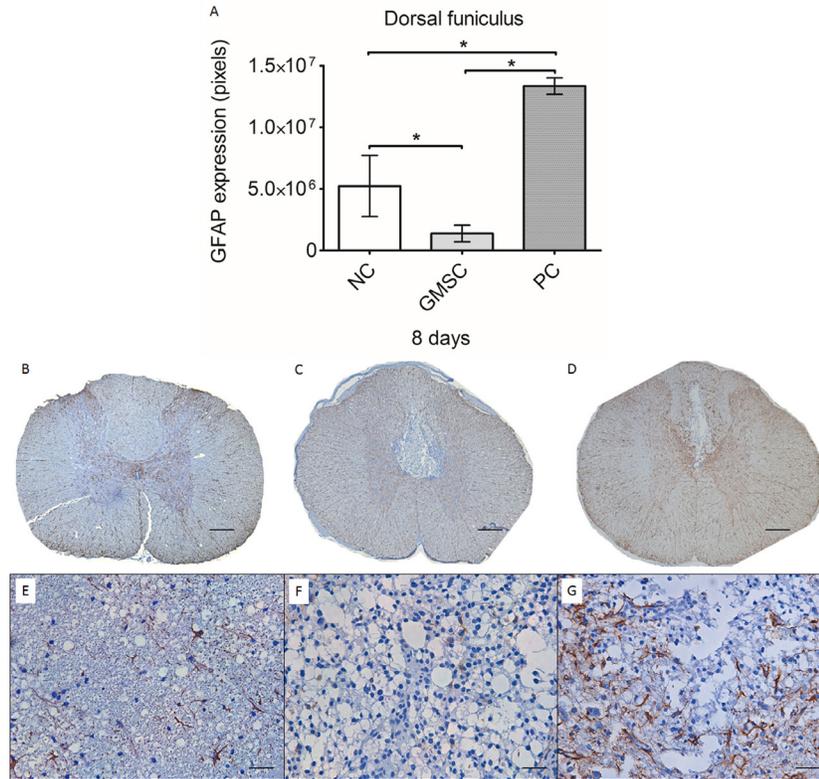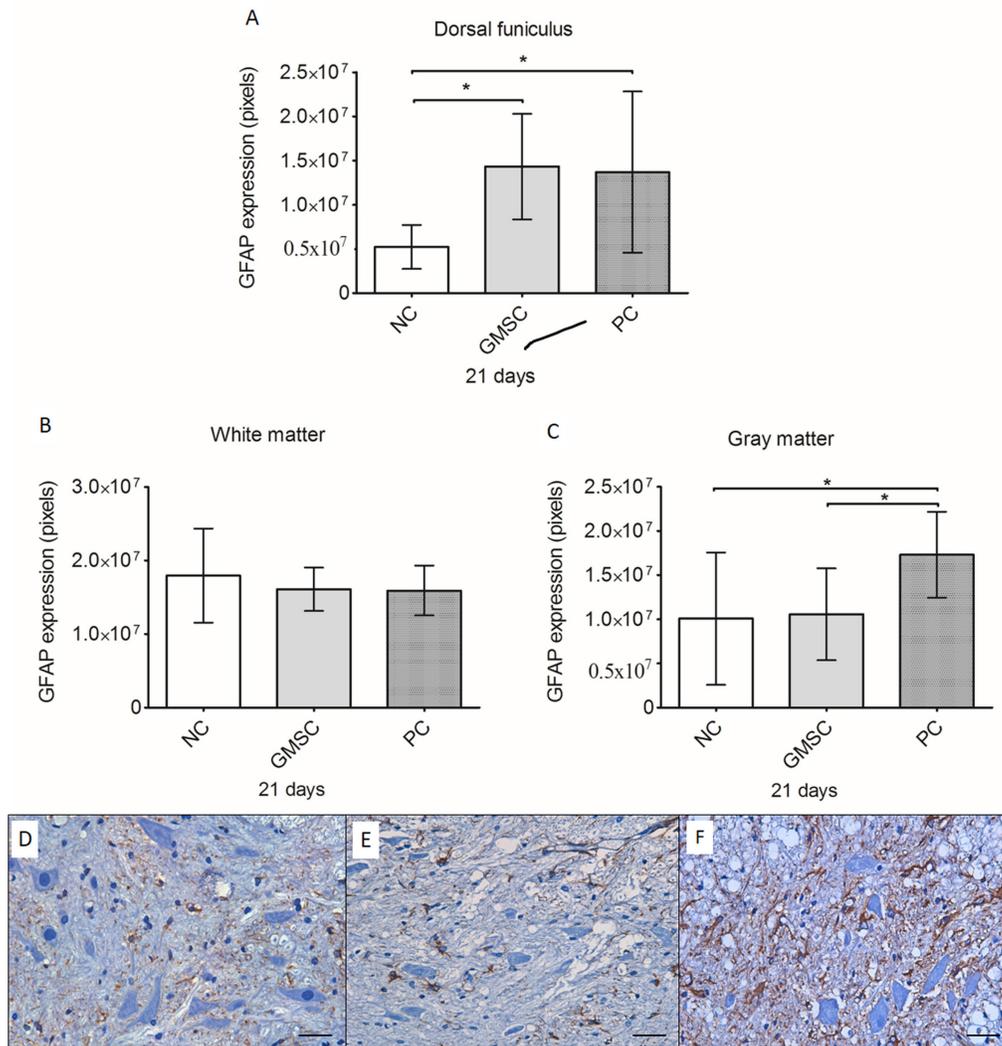
Figure 10. A. Means and standard deviations of GFAP expression in the region of the dorsal funiculus of spinal cords of rats in the negative control (NC), treated with mesenchymal stem cells (GMSC), and positive control group (PC) 8 days after laminectomy or injury (*P < 0.05). Photomicrographs of spinal cords evaluated by anti-GFAP immunohistochemistry (**B**, **C**, **D**, **E**, **F**, **G**). Staining of the extensions of astrocytes observed in white and gray matter of the spinal cords of animals in the NC (**B**), GMSC (**C**), and PC (**D**). In the regions of the dorsal funiculus, less intense staining was observed in the GMSC (**F**), intermediate in the NC (**E**), and intense in PC (**G**) (B, C, D: bar = 285 mm, magnification 50X; E, F, G: bar = 35 mm, magnification 400X).

Figure 11. Means and standard deviations of GFAP expression in white (**A**) and gray matter (**B**) of spinal cords of rats in the negative control (NC), treated with mesenchymal stem cells (GMSC), and positive control group (PC) evaluated 8 days after laminectomy or injury (*P < 0.05).

At 21 days, there was no difference in GFAP expression in the white matter between the NC ($1.80 \times 10^7 \pm 0.64 \times 10^7$), GMSC ($1.61 \times 10^7 \pm 0.29 \times 10^7$), and PC ($1.59 \times 10^7 \pm 0.34 \times 10^7$) (Figure 12A). Additionally, in the gray matter GFAP expression was higher in the PC ($1.73 \times 10^7 \pm 0.49 \times 10^7$) than in the GMSC ($1.06 \times 10^7 \pm 0.52 \times 10^7$) and NC ($1.01 \times 10^7 \pm 0.75 \times 10^7$) (Figure 12B, D, E, F). When only the dorsal funiculus was evaluated, expression was higher in the PC ($1.37 \times 10^7 \pm 0.91 \times 10^7$) and GMSC ($1.43 \times 10^7 \pm 0.60 \times 10^7$) than in the NC ($0.52 \times 10^7 \pm 0.25 \times 10^7$) (Figure 12C).
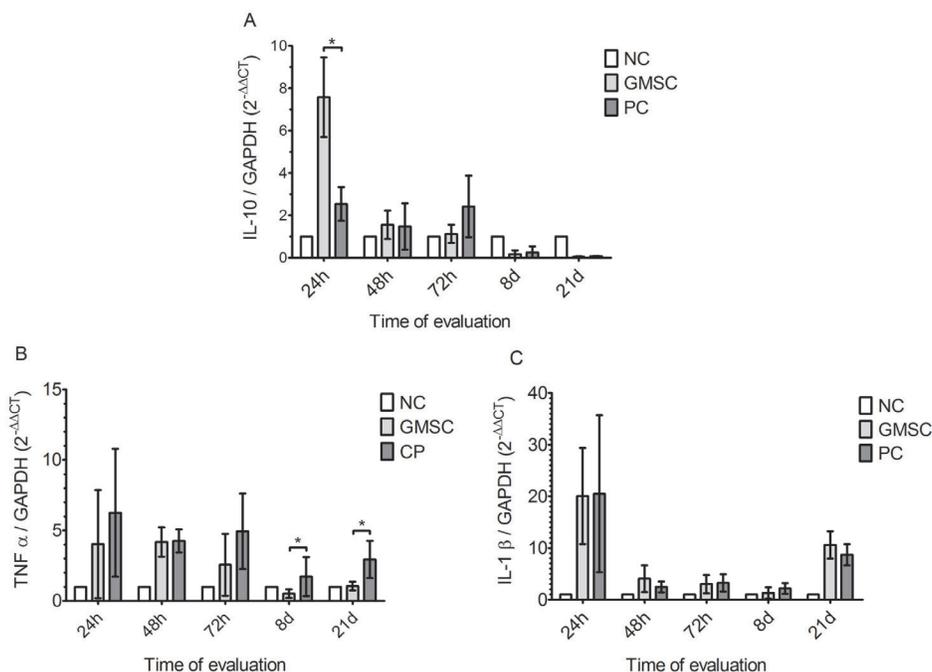

Figure 12. Means and standard deviations of GFAP expression in the dorsal funiculus (**A**) and white (**B**) and gray matter (**C**) of the spinal cords of rats in the negative control (NC), treated with mesenchymal stem cells (GMSC), and positive control group (PC) at 21 days after laminectomy or injury (*P < 0.05). Photomicrographs of spinal cords evaluated by anti-GFAP immunohistochemistry (D, E, F). Less intense staining was observed in the gray matter in the NC (**D**) and GMSC (**E**) and higher in the PC (**F**) (A, B, C: bar = 35 mm).

RT-PCR analysis

Relative expression of IL-10 was higher in the GMSC (7.57 ± 1.88) than in the PC (2.54 ± 0.79) at 24 h post-injury. No significant differences were observed between the GMSC and PC at 48 h (1.56 ± 0.67; 1.48 ± 1.09), 72 h (1.13 ± 0.43; 2.42 ± 1.45), 8 days (0.18 ± 0.17; 0.26 ± 0.28), and 21 days (0.04 ± 0.03; 0.08 ± 0.02) (Figure 13A).

Relative expression of TNF-α was lower in the GMSC than in the PC at 8 (0.51 ± 0.31; 1.72 ± 1.38) and 21 days (1.05 ± 0.31; 2.94 ± 1.32) ($P < 0.05$). There was no difference in the relative expression of TNF-α among groups at 24 h (4.03 ± 3.83; 6.25 ± 4.54), 48 h (4.17 ± 1.04; 4.25 ± 0.82), and 72 h (2.56 ± 2.20; 4.94 ± 2.68) after SCI (Figure 13B).

There was no difference in the relative expression of IL-1β between the GMSC and PC at 24 h (20.07 ± 9.28; 20.51 ± 15.19), 48 h (4.07 ± 2.58; 2.48 ± 1.04), 72 h (3.01 ± 1.76; 3.25 ± 1.67), 8 days (1.32 ± 1.09; 2.18 ± 1.03), and 21 days (10.60 ± 2.63; 8.70 ± 2.06) (Figure 13C).

Figure 13. Means and standard deviations of IL-10 (**A**), TNF-α (**B**), and IL-1β (**C**) expression in rats in the negative control (NC), treated with mesenchymal stem cells (GMSC), and positive control group (PC) at 24, 48, and 72 h and at 8 and 21 days after laminectomy or injury (*$P < 0.05$).

Higher expression of IL-10 was observed in the GMSC at 24 h after surgery and lower expression of TNF-α was observed at 8 and 21 days after surgery.

The relative expression of TGF-β was lower in the GMSC than in the PC at 24 h (2.53 ± 0.40; 10.31 ± 4.57) and 21 days (4.03 ± 2.34; 7.14 ± 1.58) after injury. There was no difference between the GMSC and PC at 48 h (3.90 ± 2.99; 2.89 ± 1.68), 72 h (2.90 ± 0.74; 3.92 ± 2.26), and 8 days (15.35 ± 12.88; 11.84 ± 7.58) after SCI (Figure 14A).

There was no difference between the relative expression of BDNF in the GMSC and PC at 24 h (1.09 ± 0.43; 0.87 ± 0.32), 48 h (0.71 ± 0.20; 0.60 ± 0.25), 72 h (0.25 ± 0.20; 0.16 ± 0.06), 8 days (0.11 ± 0.04; 0.10 ± 0.03) and 21 days (0.42 ± 0.33; 0.24 ± 0.15) after spinal cord injury (Figure 14B).

The relative expression of GDNF was higher in the GMSC than in the PC at 48 h (24.45 ± 15.38; 8.22 ± 4.19) and 8 days (1.42 ± 0.62; 0.38 ± 0.12) after SCI. There was no difference between the GMSC and PC at 24 h (9.78 ± 2.55; 5.87 ± 3.18), 72 h (2.21 ± 1.48; 2.48 ± 1.71), and 21 days (0.02 ± 0.01; 0.05 ± 0.02) SCI (Figure 14C).

The relative expression of VEGF was higher in the GMSC than in the PC at 21 days after SCI (1.78 ± 0.15; 0.95 ± 0.33). There was no difference in the expression of VEGF between the GMSC and PC at 24 h (1.20 ± 0.16; 0.98 ± 0.37), 48 h (0.13 ± 0.4; 0.11 ± 0.07), 72 h (0.11 ± 0.06; 0.07 ± 0.03), and 8 days (0.22 ± 0.15; 0.19 ± 0.06) (Figure 14D).
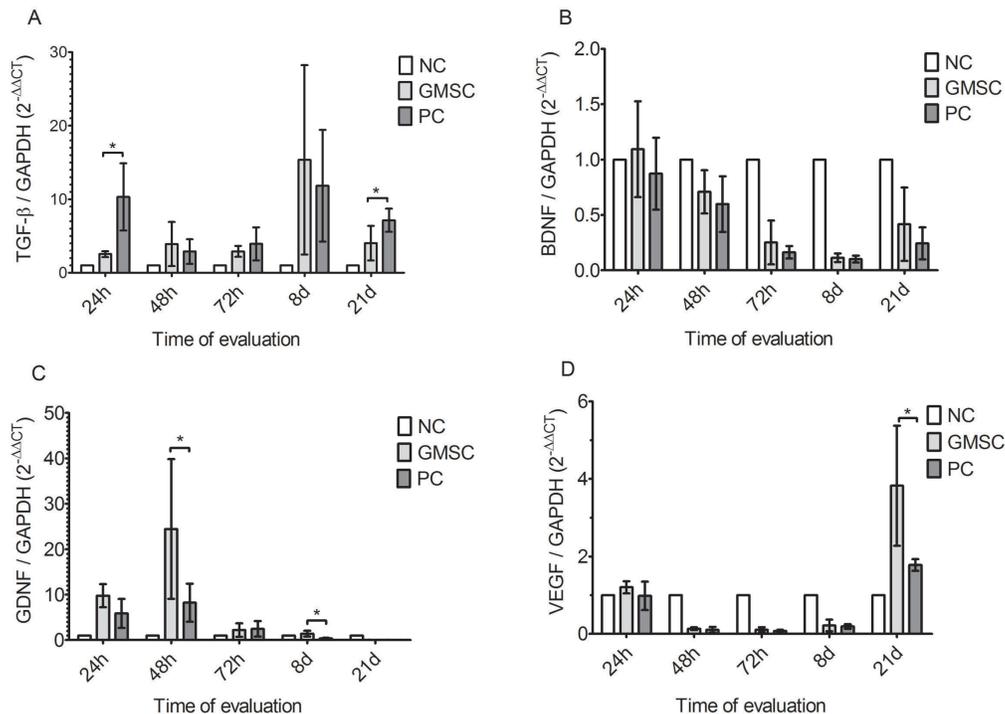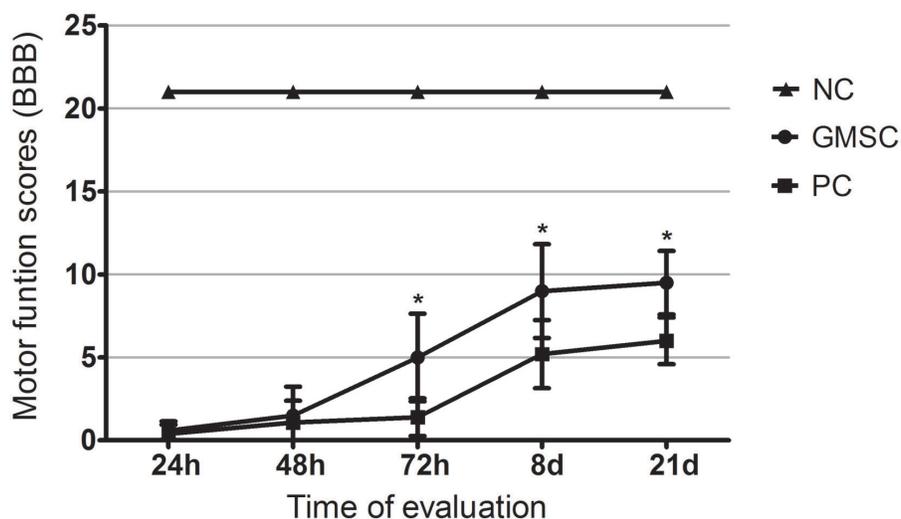

Figure 14. Means and standard deviations of TGF-β (**A**), BDNF (**B**), GDNF (**C**), and VEGF (**D**) expression in the negative control group (NC), group treated with mesenchymal stem cells (GMSC), and positive control group (PC) at 24, 48, and 72 h and at 8 and 21 days after spinal cord injury (*P < 0.05). In the GMSC, lower expression of TGF-β at 24 h and 21 days (A), higher expression of GDNF at 48 h and 21 days (C), and higher expression of VEGF 21 days after surgery (D) was observed.

Evaluation of motor function

Animals in the NC presented a score of 21 (maximum score) in all evaluation periods. The animals subjected to injury had a score of 0 or 1 in the BBB test at the first evaluation time (24 h). There was no significant difference between the scores obtained in the GMSC and PC

at 24 h (0.60 ± 0.55; 0.40 ± 0.55) and 48 h (1.50 ± 1.73; 1.10 ± 1.32). The scores were higher in the GMSC than in the PC at 72 h (5.00 ± 2.62; 1.4 ± 1.14), 8 days (9.00 ± 2.83; 5.20 ± 2.05), and 21 days (9.50 ± 1.92; 6.00 ± 1.41) (Figure 15).

Figure 15. Means and standard deviations of motor function scores in rats in the negative control group (NC), group treated with mesenchymal stem cells (GMSC), and positive control group (PC) at 24, 48, and 72 h and at 8 and 21 days after spinal cord injury. The mean scores for the GMSC were higher than those for the PC at 72 h and at 8 and 21 days (*$P < 0.05$).

DISCUSSION

The absence of significant variation in surface markers and differentiating potential after thawing indicate that the MSCs preserved their phenotypic and differentiation characteristics after cryopreservation. The reduction in the viability of MSCs after cryopreservation did not alter the number of transplanted viable cells; all samples were evaluated before transplantation, and the calculation of the number of cells to be transplanted was based on the number of viable cells. Higher conversion of MTT observed in the MSCs after 90 days of cryopreservation indicated an increase in mitochondrial activity in the cells after thawing. A recent study observed an increase in the expression of genes that favor cellular maintenance and pluripotency in MSCs of adipose tissue after cryopreservation, even with a significant reduction in viable cells (Davies et al., 2014). It is thought that cryopreservation selects for more resistant and functional cells.

The activated microglia/macrophages with rounded shape, voluminous cytoplasm, and abundant myelin debris are morphological characteristics of anti-inflammatory cells with high phagocytic activity (Wirjatijasa et al., 2002; Nakajima et al., 2012; Ishii et al., 2013). In the present study, the administration of MSCs favored the activation of microglia/macrophages with characteristic anti-inflammatory morphology and reduced the inflammatory infiltrate in the white and gray matter 8 days after spinal cord injury, which improved regeneration following injury. The reduction in neuron staining by the antibody anti-NeuN 8 days after

injury in the MSC group may not be associated with neuronal death, as these cells showed characteristics of intact neurons in histopathological evaluation. This may have occurred because of metabolic changes that reduced NeuN expression, but did not result in neuron death; at 21 days, neuron staining was higher in the same group (MSC). A study performed in ischemic brain neurons revealed a decrease in NeuN expression without neuronal death (Ünal-Çevik et al., 2004).

The reduction in activated astrocytes of the dorsal funiculus at 8 days and in the gray matter at 21 days, observed in the MSC group, may be related to the higher expression of IL-10 observed in the GMSC group at 24 h after injury, as MSCs can produce this cytokine (Nakano et al., 2010; Zhukareva et al., 2010).

Several studies have assessed the effects of IL-10 on spinal cord injury. This cytokine displayed anti-inflammatory action, either by direct administration or by the use of other treatments that lead to its increase (Bethea et al., 1999; Brewer et al., 1999; Jackson et al., 2005; Zhou et al., 2009). Some of the results include a reduction in the inflammatory infiltrate, activation of cells in the microglia with an anti-inflammatory phenotype (Wirjatijasa et al., 2002), increase in neuronal survival (Brewer et al., 1999, Jackson et al., 2005; Zhou et al., 2009), reduction in the activation of astrocytes (Bethea et al., 1999), and improvement of motor function (Bethea et al., 1999; Jackson et al., 2005; Zhou et al., 2009). In all of these studies, beneficial effects were observed when IL-10 was increased in the first 24 h after spinal cord injury (Bethea et al., 1999; Brewer et al., 1999; Jackson et al., 2005; Zhou et al., 2009), as observed in the present study. Therefore, IL-10 may be involved in reducing the inflammatory infiltrate by activating microglia/macrophage cells with an anti-inflammatory phenotype and reducing astrocyte activation.

The reduction in the expression of TNF-α at 8 and 21 days after SCI in the GMSC suggests that the inflammatory reaction was less intense in this group, as this cytokine is an indicator of the inflammatory process and the activation of microglia/macrophages and astrocytes (Bethea et al., 1999; Chen et al., 2015). Another factor demonstrating the reduction in the inflammatory response in the GMSC was the lower cell activation in the microglia/macrophages observed in this study.

The decreased TNF-α expression observed at 21 days in the GMSC is likely related to decreased activation of astrocytes; at this evaluation point, the activation of these cells was reduced in the gray matter. The reduction of TNF-α may have reduced degeneration of the white matter, which was also observed in this group and evaluation time, as TNF-α induces apoptosis of neurons and oligodendrocytes and demyelination (Lau and Yu, 2001; Nakamura et al., 2003; Yune et al., 2003). The reduced expression of TNF-α in animals subjected to spinal cord injury and treated with stem cells has been observed in previous studies (Hawryluk et al., 2012; Nakajima et al., 2012). Nevertheless, the mechanisms by which stem cells reduce the production of TNF-α had not been determined. In the present study, administration of MSCs appeared to reduce the inflammatory infiltrate and activation of astrocytes, and thus the production of TNF-α.

The absence of variation in the expression of IL-1β after treatment with MSCs may have occurred, because this cytokine is mostly produced by astrocytes that make up the blood brain barrier and not by the inflammatory infiltrate, as with TNF-α (Ledeboer et al., 2000; Lau and Yu, 2001). Disruption of the blood brain barrier induced by injury likely stimulated IL-1β production, and the MSCs were not able of halt this process.

TGF-β is a neurotrophic factor known for its role in the repair of tissue lesions,

and its expression increases in the first days after spinal cord injury (Nakamura et al., 2003; Hawryluk et al., 2012). The reduced expression of TGF-β observed at 24 h after injury in the GMSC suggests that the MSCs reduced the production of this protein. TGF-β opposes the action of IL-10, which is unfavorable for regeneration of the spinal cord, because initially, it increases the expression of adhesion molecules and activates microglia/macrophage cells with a pro-inflammatory phenotype (Wirjatijasa et al., 2002). Thus, the reduction in the expression of TGF-β at 24 h after injury in the GMSC may also have contributed to reduction of the inflammatory infiltrate and activation of cells with anti-inflammatory characteristics observed in this same group at 8 days after injury. The lower expression of TGF-β observed at 21 days in the GMSC can be attributed to the reduction in activated astrocytes observed in this group and time of evaluation, as astrocytes are the main producers of this neurotrophin (Wirjatijasa et al., 2002; Kohta et al., 2009). Furthermore, TGF-β has autocrine functions, inducing those of proliferation and activation of additional astrocytes that help form the glial scar, which functions as a physical barrier and prevents axon regeneration (Bethea and Dietrich, 2002). A previous study showed that blocking TGF-β improved motor function recovery after spinal cord injury (Kohta et al., 2009). Similarly, the reduced TGF-β expression observed at 21 days in the GMSC may also have decreased formation of the glial scar and stimulated the recovery of motor function in the present study.

As observed in previous studies (Hawryluk et al., 2012; Mead et al., 2014), MSCs cannot produce or stimulate the synthesis of BDNF. However, some authors reported increased expression of BDNF in animals subjected to injury and treated with MSCs (Osaka et al., 2010; Zhou et al., 2013; Kingham et al., 2014). This divergence in results may be related to the different times of MSC administration after injury. MSCs modify their secretion profile according to their microenvironment. In addition, there is variability in the production capacity of neurotrophins among MSCs from different individuals and tissues (Zhukareva et al., 2010; Mead et al., 2014).

The increase in GDNF expression observed at 72 h and 8 days in the GMSC indicates that these cells secreted or stimulated medullary parenchyma cells to produce GDNF. In the present study, the increased expression of GDNF was associated with a higher number of intact neurons, lower degeneration of white matter, and recovery of motor function in the GMSC. Other studies showed that GDNF could increase neuronal survival, reducing axonal retraction and stimulating axonal sprouting, thus promoting the recovery of motor function after SCI (Dolbeare and Houle, 2003; Hawryluk et al., 2012; Lin et al., 2014).

The increased expression of VEGF observed at 21 days after injury in the GMSC suggests that these cells produced VEGF or stimulated the medullary parenchyma cells to produce VEGF. The increase in VEGF occurred only during the chronic phase of spinal cord injury, i.e., in the period during which tissue repair begins. VEGF is known to stimulate angiogenesis, an important process that reduces ischemia and provides the blood supply needed for axon regeneration and motor function recovery (Figley et al., 2014; Li et al., 2014). A previous study found an increase in the concentration of VEGF in the chronic phase of animals with SCI treated with MSCs (Li et al., 2014). VEGF has direct neuroprotective and neurotrophic actions (Figley et al., 2014; Mead et al., 2014; Shinozaki et al., 2014); therefore, the increase in VEGF expression at 21 days after spinal cord injury in the GMSC may have contributed to a higher number of intact neurons, lower degeneration of white matter, and recovery of motor function.

In this study, the functional recovery observed in GMSC animals was associated with

reduction of the inflammatory infiltrate, lower activation of astrocytes, higher activation of cells in the microglia/macrophages with morphology typical of anti-inflammatory cells, and lower intensity in the degeneration of white matter. Therefore, in this study, cryopreserved allogeneic MSCs increased the expression of anti-inflammatory cytokine IL-10 (24 h post-injury) and the neurotrophic factors GDNF (72 h and 8 days post-injury) and VEGF (21 days post-injury) and reduced the expression of the pro-inflammatory cytokine TNF-α (8 days post-injury) and neurotrophic factor TGF-β (24 h and 21 days post-injury), stimulating an immunomodulatory and neuroprotective effect in rats subjected to spinal cord injury.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

REFERENCES

Basso DM, Beattie MS, Bresnahan JC, Anderson DK, et al. (1996). MASCIS evaluation of open field locomotor scores: effects of experience and teamwork on reliability. Multicenter Animal Spinal Cord Injury Study. *J. Neurotrauma* 13: 343-359. http://dx.doi.org/10.1089/neu.1996.13.343

Bethea JR and Dietrich WD (2002). Targeting the host inflammatory response in traumatic spinal cord injury. *Curr. Opin. Neurol.* 15: 355-360. http://dx.doi.org/10.1097/00019052-200206000-00021

Bethea JR, Nagashima H, Acosta MC, Briceno C, et al. (1999). Systemically administered interleukin-10 reduces tumor necrosis factor-alpha production and significantly improves functional recovery following traumatic spinal cord injury in rats. *J. Neurotrauma* 16: 851-863. http://dx.doi.org/10.1089/neu.1999.16.851

Brewer KL, Bethea JR and Yezierski RP (1999). Neuroprotective effects of interleukin-10 following excitotoxic spinal cord injury. *Exp. Neurol.* 159: 484-493. http://dx.doi.org/10.1006/exnr.1999.7173

Carvalho PH, Daibert APF, Monteiro BS, Okano BS, et al. (2013). Differentiation of adipose tissue-derived mesenchymal stem cells into cardiomyocytes. *Arq. Bras. Cardiol.* 100: 82-89. http://dx.doi.org/10.1590/S0066-782X2012005000114

Chen SH, Oyarzabal EA, Sung YF, Chu CH, et al. (2015). Microglial regulation of immunological and neuroprotective functions of astroglia. *Glia* 63: 118-131. http://dx.doi.org/10.1002/glia.22738

Dariolli R, Bassaneze V, Nakamuta JS, Omae SV, et al. (2013). Porcine adipose tissue-derived mesenchymal stem cells retain their proliferative characteristics, senescence, karyotype and plasticity after long-term cryopreservation. *PLoS One* 8: e67939. http://dx.doi.org/10.1371/journal.pone.0067939

Dasari VR, Spomar DG, Cady C, Gujrati M, et al. (2007). Mesenchymal stem cells from rat bone marrow downregulate caspase-3-mediated apoptotic pathway after spinal cord injury in rats. *Neurochem. Res.* 32: 2080-2093. http://dx.doi.org/10.1007/s11064-007-9368-z

Davies OG, Smith AJ, Cooper PR, Shelton RM, et al. (2014). The effects of cryopreservation on cells isolated from adipose, bone marrow and dental pulp tissues. *Cryobiology* 69: 342-347. http://dx.doi.org/10.1016/j.cryobiol.2014.08.003

de Lavor MS, Binda NS, Fukushima FB, Caldeira FMC, et al. (2015). Ischemia-reperfusion model in rat spinal cord: cell viability and apoptosis signaling study. *Int. J. Clin. Exp. Pathol.* 8: 9941-9949.

Dolbeare D and Houle JD (2003). Restriction of axonal retraction and promotion of axonal regeneration by chronically injured neurons after intraspinal treatment with glial cell line-derived neurotrophic factor (GDNF). *J. Neurotrauma* 20: 1251-1261. http://dx.doi.org/10.1089/089771503770802916

Figley SA, Liu Y, Karadimas SK, Satkunendrarajah K, et al. (2014). Delayed administration of a bio-engineered zinc-finger VEGF-A gene therapy is neuroprotective and attenuates allodynia following traumatic spinal cord injury. *PLoS One* 9: e96137. http://dx.doi.org/10.1371/journal.pone.0096137

Hawryluk GWJ, Mothe A, Wang J, Wang S, et al. (2012). An in vivo characterization of trophic factor production following neural precursor cell or bone marrow stromal cell transplantation for spinal cord injury. *Stem Cells Dev.* 21: 2222-2238. http://dx.doi.org/10.1089/scd.2011.0596

Ishii H, Tanabe S, Ueno M, Kubo T, et al. (2013). ifn-γ-dependent secretion of IL-10 from Th1 cells and microglia/macrophages contributes to functional recovery after spinal cord injury. *Cell Death Dis.* 4: e710. http://dx.doi.org/10.1038/cddis.2013.234

Jackson CA, Messinger J, Peduzzi JD, Ansardi DC, et al. (2005). Enhanced functional recovery from spinal cord injury following intrathecal or intramuscular administration of poliovirus replicons encoding IL-10. *Virology* 336: 173-183. http://dx.doi.org/10.1016/j.virol.2005.03.025

Kingham PJ, Kolar MK, Novikova LN, Novikov LN, et al. (2014). Stimulating the neurotrophic and angiogenic properties of human adipose-derived stem cells enhances nerve repair. *Stem Cells Dev.* 23: 741-754. http://dx.doi.org/10.1089/scd.2013.0396

Kohta M, Kohmura E and Yamashita T (2009). Inhibition of TGF-beta1 promotes functional recovery after spinal cord injury. *Neurosci. Res.* 65: 393-401. http://dx.doi.org/10.1016/j.neures.2009.08.017

Lau LT and Yu AC (2001). Astrocytes produce and release interleukin-1, interleukin-6, tumor necrosis factor alpha and interferon-gamma following traumatic and metabolic injury. *J. Neurotrauma* 18: 351-359. http://dx.doi.org/10.1089/08977150151071035

Ledeboer A, Brevé JJ, Poole S, Tilders FJ, et al. (2000). Interleukin-10, interleukin-4, and transforming growth factor-beta differentially regulate lipopolysaccharide-induced production of pro-inflammatory cytokines and nitric oxide in co-cultures of rat astroglial and microglial cells. *Glia* 30: 134-142. http://dx.doi.org/10.1002/(SICI)1098-1136(200004)30:2<134::AID-GLIA3>3.0.CO;2-3

Li Z, Guo GH, Wang GS, Guan CX, et al. (2014). Influence of neural stem cell transplantation on angiogenesis in rats with spinal cord injury. *Genet. Mol. Res.* 13: 6083-6092. http://dx.doi.org/10.4238/2014.August.7.23

Lin W, Li M, Li Y, Sun X, et al. (2014). Bone marrow stromal cells promote neurite outgrowth of spinal motor neurons by means of neurotrophic factors in vitro. *Neurol. Sci.* 35: 449-457. http://dx.doi.org/10.1007/s10072-013-1490-x

Livak KJ and Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25: 402-408. http://dx.doi.org/10.1006/meth.2001.1262

Mead B, Logan A, Berry M, Leadbeater W, et al. (2014). Paracrine-mediated neuroprotection and neuritogenesis of axotomised retinal ganglion cells by human dental pulp stem cells: comparison with human bone marrow and adipose-derived mesenchymal stem cells. *PLoS One* 9: e109305. http://dx.doi.org/10.1371/journal.pone.0109305

Nakajima H, Uchida K, Guerrero AR, Watanabe S, et al. (2012). Transplantation of mesenchymal stem cells promotes an alternative pathway of macrophage activation and functional recovery after spinal cord injury. *J. Neurotrauma* 29: 1614-1625. http://dx.doi.org/10.1089/neu.2011.2109

Nakamura M, Houghtling RA, MacArthur L, Bayer BM, et al. (2003). Differences in cytokine gene expression profile between acute and secondary injury in adult rat spinal cord. *Exp. Neurol.* 184: 313-325. http://dx.doi.org/10.1016/S0014-4886(03)00361-3

Nakano N, Nakai Y, Seo TB, Yamada Y, et al. (2010). Characterization of conditioned medium of cultured bone marrow stromal cells. *Neurosci. Lett.* 483: 57-61. http://dx.doi.org/10.1016/j.neulet.2010.07.062

Nandoe Tewarie RD, Hurtado A, Ritfeld GJ, Rahiem ST, et al. (2009). Bone marrow stromal cells elicit tissue sparing after acute but not delayed transplantation into the contused adult rat thoracic spinal cord. *J. Neurotrauma* 26: 2313-2322. http://dx.doi.org/10.1089/neu.2009.0987

Osaka M, Honmou O, Murakami T, Nonaka T, et al. (2010). Intravenous administration of mesenchymal stem cells derived from bone marrow after contusive spinal cord injury improves functional outcome. *Brain Res.* 1343: 226-235. http://dx.doi.org/10.1016/j.brainres.2010.05.011

Popovich PG, Wei P and Stokes BT (1997). Cellular inflammatory response after spinal cord injury in Sprague-Dawley and Lewis rats. *J. Comp. Neurol.* 377: 443-464. http://dx.doi.org/10.1002/(SICI)1096-9861(19970120)377:3<443::AID-CNE10>3.0.CO;2-S

Sekhon LHS and Fehlings MG (2001). Epidemiology, demographics, and pathophysiology of acute spinal cord injury. *Spine* 26 (Suppl): S2-S12. http://dx.doi.org/10.1097/00007632-200112151-00002

Shinozaki M, Nakamura M, Konomi T, Kobayashi Y, et al. (2014). Distinct roles of endogenous vascular endothelial factor receptor 1 and 2 in neural protection after spinal cord injury. *Neurosci. Res.* 78: 55-64. http://dx.doi.org/10.1016/j.neures.2013.09.011

Ünal-Çevik I, Kilinç M, Can A, Gürsoy-Ozdemir Y, et al. (2004). Apoptotic and necrotic death mechanisms are concomitantly activated in the same cell after cerebral ischemia. *Stroke* 35: 2189-2194. http://dx.doi.org/10.1161/01. STR.0000136149.81831.c5

Wirjatijasa F, Dehghani F, Blaheta RA, Korf HW, et al. (2002). Interleukin-4, interleukin-10, and interleukin-1-receptor antagonist but not transforming growth factor-beta induce ramification and reduce adhesion molecule expression of rat microglial cells. *J. Neurosci. Res.* 68: 579-587. http://dx.doi.org/10.1002/jnr.10254

Yune TY, Chang MJ, Kim SJ, Lee YB, et al. (2003). Increased production of tumor necrosis factor-alpha induces apoptosis after traumatic spinal cord injury in rats. *J. Neurotrauma* 20: 207-219. http://dx.doi.org/10.1089/08977150360547116

Zhou Z, Peng X, Insolera R, Fink DJ, et al. (2009). IL-10 promotes neuronal survival following spinal cord injury. *Exp. Neurol.* 220: 183-190. http://dx.doi.org/10.1016/j.expneurol.2009.08.018

Zhou Z, Chen Y, Zhang H, Min S, et al. (2013). Comparison of mesenchymal stromal cells from human bone marrow and adipose tissue for the treatment of spinal cord injury. *Cytotherapy* 15: 434-448. http://dx.doi.org/10.1016/j. jcyt.2012.11.015

Zhukareva V, Obrocka M, Houle JD, Fischer I, et al. (2010). Secretion profile of human bone marrow stromal cells: donor variability and response to inflammatory stimuli. *Cytokine* 50: 317-321. http://dx.doi.org/10.1016/j.cyto.2010.01.004