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Intraspinal transplantation of motoneuron-like cell combined with delivery of polymer-based glial cell line-derived neurotrophic factor for repair of spinal cord contusion injury

Alireza Abdanipour, Taki Tiraihi, Taher Taheri

Shefa Neuroscience Research Center at Khatam Al-Anbia Hospital, Tehran, Iran

Corresponding author:
Taki Tiraihi, Department of Anatomical Sciences, Faculty of Medical Sciences, Tarbiat Modares University, Jalal El Ahmed express way, P.O. Box 14155-4838, Tehran, Iran, takialtr@modares.ac.ir; ttiraihi@gmail.com.

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Abstract

To evaluate the effects of glial cell line-derived neurotrophic factor transplantation combined with adipose-derived stem cells-transdifferentiated motoneuron delivery on spinal cord contusion injury, we developed rat models of spinal cord contusion injury, 7 days later, injected adipose-derived stem cells-transdifferentiated motoneurons into the epicenter, rostral and caudal regions of the impact site and simultaneously transplanted glial cell line-derived neurotrophic factor-gelfoam complex into the myelin sheath. Motoneuron-like cell transplantation combined with glial cell line-derived neurotrophic factor delivery reduced cavity formations and increased cell density in the transplantation site. The combined therapy exhibited superior promoting effects on recovery of motor function to transplantation of glial cell line-derived neurotrophic factor, adipose-derived stem cells or motoneurons alone. These findings suggest that motoneuron-like cell transplantation combined with glial cell line-derived neurotrophic factor delivery holds a great promise for repair of spinal cord injury.

Key Words: *nerve regeneration; spinal cord injury; adipose-derived stem cells; glial cell line-derived neurotrophic factor; motoneurons; cell transplantation; neurotrophic factor; spinal cord contusion injury; neural regeneration*

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Introduction

Traumatic spinal cord injury (SCI) can cause severe neurological damage (Onifer et al., 2011). Pathophysiology of SCI involves primary mechanical injury that leads to complex processes, such as disruption of axons and blood vessels and loss of neurons and glial cells (Taoka and Okajima, 1998). One of the approaches for the treatment of SCI is the replacement of the lost cells (Garbossa et al., 2012).

While obtaining neural stem cells is difficult, because of the ethical and the potential immunological rejection (Ruan et al., 2013), transplantation of the transdifferentiated cells could improve the locomotive ability of the injured spinal cord (Naghdi et al., 2009). Moreover, adult stem cells of autologous origin could differentiate into several neuronal types with low risk of tumorogenesis (Chen et al., 2006). Sources for adult stem cells could be olfactory ensheathing cells, cultured spinal cord stem cell, bone marrow-derived stem cells and dental pulp-derived stem cells (Schultz, 2005). Fetal neural stem cell transplantation was reported to improve the contused spinal cord (Tarasenko et al., 2007), and similar results were reported using the umbilical cord blood-derived mesenchymal stem cells (Park et al., 2012). Fetal forebrain-derived neural stem/progenitor cells could differentiate into spinal cord-type neurons, which are considered as alternative to spinal cord-derived neural stem/progenitor cells (Watanabe et al., 2004). Recovery of spinal cord injury using embryonic stem cells and their derivatives was reported (Yang et al., 2013). Allogeneic fetal and embryonic stem cells transplantation could result in immunological rejection (Luchetti et al., 2010) and tumorigenesis (Li et al., 2008). On the other hand, induced pluripotent stem cells were reported as another source for transplantation in spinal cord injury (Nakamura et al., 2012), however, they can cause tumor (Vitale et al., 2011). On the other hand, adipose-derived stem cells have recently been identified as an alternative source of adult stem cells (Wei et al., 2010), various experimental models using adipose-derived stem cells-transdifferentiated motoneuron grafting have shown that the cells could survive and integrate to some extent in the host cord (Nogradi et al., 2011). Besides adult stem cells

can be of an autologous source (Chen et al., 2006), however, *in vitro* adult stem cell culture is performed in an ideal growth environment, which is not available in the *in vivo* environment, and it can be a serious problem for their survival (Naghdi et al., 2009), therefore, the use of neuroprotective factors such as glial cell line-derived neurotrophic factor (GDNF) can boost their survival rate (Duarte et al., 2012).

In this study, we have evaluated the transplantation of motoneuron-like cells combined with exogenous delivery of GDNF in a rat model of spinal cord injury contusion injury.

Materials and Methods

Isolation of adipose-derived stem cells

Adipose-derived stem cell culture, pre-induction, and induction were performed according to previously described methods (Abdanipour et al., 2011; Abdanipour and Tiraihi, 2012). Briefly, adipose-derived stem cells were obtained from the pararenal fat of five female Sprague-Dawley rats (Razi institute, Tehran) (200–250 g) and treated with equal volumes of 0.075% collagenase type 1 (Sigma-Aldrich, St. Louis, MO, USA) at 37°C by continuous agitation for 1 hour. The resultant pellets were suspended, filtered through a 100-μm nylon mesh (Invitrogen, Eggenstein, Germany) and then incubated at 37°C under 5% CO_2 in Dulbecco's modified eagle's medium (DMEM; Gibco, Paisley, Scotland, UK) containing 10% fetal bovine serum (FBS; Gibco). The cells were immunostained with CD49d (a specific marker for fat cells), CD90 (a marker of mesenchymal stem cells), CD45 (a hematopoietic cell marker), CD31 (an endothelial cell marker) and CD106 (a marker of mesenchymal stem cells derived from bone marrow stromal cells) (all from Abcam, Cambridge, UK), then they were induced into lipogenic and osteogenic cells. In order to induce adipose-derived stem cells to differentiate towards osteogenic line, after the fourth passage, the cell culture medium was replaced with osteogenic maintenance medium containing 10 mmol/L β-glycerophosphate, 0.2 mmol/L ascorbic acid and 10^{-7} mol/L dexamethasone, which was kept for 21 days.

However, for differentiating adipose-derived stem cells into adipogenic line, after the fourth passage, the cell culture medium was refreshed with adipogenic maintenance medium containing 50 μg/mL indomethacin (Sigma-Aldrich), 50 μg/mL ascorbic acid and 100 nmol/L dexamethasone (Sigma-Aldrich), which was kept for 21 days (Eslaminejad et al., 2006).

Pre-induction and induction of adipose-derived stem cells

The sub-confluent culture of rat adipose-derived stem cells at passage 4 was maintained in serum-free induction medium containing DMEM and selegiline. The optimal concentration of selegiline (Sigma-Aldrich) was obtained by a dose-response experiment involving different concentrations (10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11} and 10^{-12} mmol/L) of selegiline matched with a time course experiment (3, 6, 12, 24 and 48 hours), and the results were evaluated using the percentages of the immunoreactive cells to nestin and neurofilament 68 (NF-68). The number of the cells immunoreactive to NF-68 divided by the total number of cells multiplied by 100. Then produced neuron-like cells were induced with B27 (1%; Gibco), basic fibroblast growth factor (20 ng/mL; Sigma-Aldrich), sonic hedgehog (1 μg/mL; Sigma-Aldrich) and retinoic acid (2×10^{-8} mol/L) (Sigma-Aldrich) for 2 days. The pre-induced cells were immunostained with NeuN (Abcam), microtubule-associated protein 2 (MAP-2; Abcam), Neuro D (Abcam) and choline acetyl transferase (Abcam), and then they were examined for expression of Islet-1 and Olig2 (both Millipore, Billerica, MA, USA). The quantitative real-time polymerase chain reaction (qRT-PCR) was performed on RNA extracted from the cells, using Purelink™ RNA mini kit (Invitrogen). The extracted total RNA was treated by DNase I (Invitrogen). We used 1,000 ng extracted RNA to synthesize 20 μL first-strand cDNA (Revert aid™ first strand cDNA synthesis: Fermentas, Vilnius, Lithuania) and 500 ng cDNA for PCR (PCR master mix, 2 ×: Fermentas). A negative control (without template DNA) with RNA from neonatal rat spinal cord was used. The size of the RT-PCR products was analyzed using 2% agarose gel electrophoresis (Nogradi et al., 2011), the primers used for evaluating brain-derived neurotrophic factor (BDNF), GDNF and neurotrophin-3 (NT-3) profiles are presented in **Table 1**. Real-time PCR was performed for 40 cycles using Applied Biosystems (Foster City, CA, USA). Pfaffl method was used for analyzing the relative changes in mRNA levels (Mocellin et al., 2003).

Functionality assay

Functionality assay was performed based on the fluorochrome uptake by the cells, resulting in fluorescence of the synaptic vesicles and fading of the fluorescence of the vesicles upon stimulation of the cells. The function of the differentiated motoneurons was demonstrated by staining motoneuron-like cells with FM1-43 (loading) and destaining (unloading) technique during KCl stimulation. The motoneuron-like cells were cultured in neurobasal medium on poly-L-lysine coated coverslips (Sigma, Steinheim, Germany). The culture medium of the adherent motoneuron-like cells was replaced with saline + solution (NaCl, 170 mmol/L; KCl, 3.5 mmol/L; KH_2PO_4, 0.4 mmol/L; $NaHCO_3$, 5 mmol/L; Na_2SO_4, 1.2 mmol/L; $MgCl_2$ ($6H_2O$), 1.2 mmol/L; $CaCl_2$ ($2H_2O$), 1.3 mmol/L; glucose, 5 mmol/L and tris buffer, 20 mmol/L) (all purchased from Sigma-Aldrich) for 10 minutes. The cells were perfused with another solution containing FM1-43 (molecular probes: 10 μmol/L) and KCl (saline + solution with 100 mmol/L KCl replacing 3.5 mmol/L), then were incubated with saline + solution containing FM1-43, and subsequently washed in saline + solution. FM1-43 was destained with saline + solution containing 100 mmol/L KCl, and then washed with saline + solution. Then several images were taken from one field for detecting vesicles using an inverted fluorescence microscope (Olympus IX71) for 10 minutes (one photograph each minute). A phase contrast image was obtained from the same field. The intensity of the fluorochrome in the motoneuron-like cells was measured by ImageJ software (NIH, Bethesda, MD, USA), and the data were plotted against the time schedule (Abdanipour et al., 2011; Abda-

Table 1 The BDNF, GDNF, NT-3 and β2m gene entry, forward and reverse primers and segment length of genes used for RT-PCR (β2m as a housekeeping gene (positive control))

Gene	Genbank accession No.	Sequence (5′ – 3′)	Fragment size (bp)
BDNF	NW_001084813	Sense: TGT ATC CGA CCC TCT CTG Antisense: CCT GGT GGA ACT TTA CG	165
GDNF	NM_019139.1	Sense: CTG ACC AGT GAC TCC AAT ATG C Antisense: GCC TCT GCG ACC TTT CCC	192
NT-3	NM_031073.2	Sense: CTT CTG CCA CGA TCT TAC Antisense: AAC ATC TAC CAT CTG CTT G	197
β2m	NM_004048	Sense: TGG CAC CTG CTG AGA TAC TG Antisense: AGG ATG CTA GGA CAG CAG GA	238

BDNF: Brain-derived neurotrophic factor; GDNF: glial cell line-derived neurotrophic factor; NT-3: neurotrophin-3; β2m: β2-microglobulin.

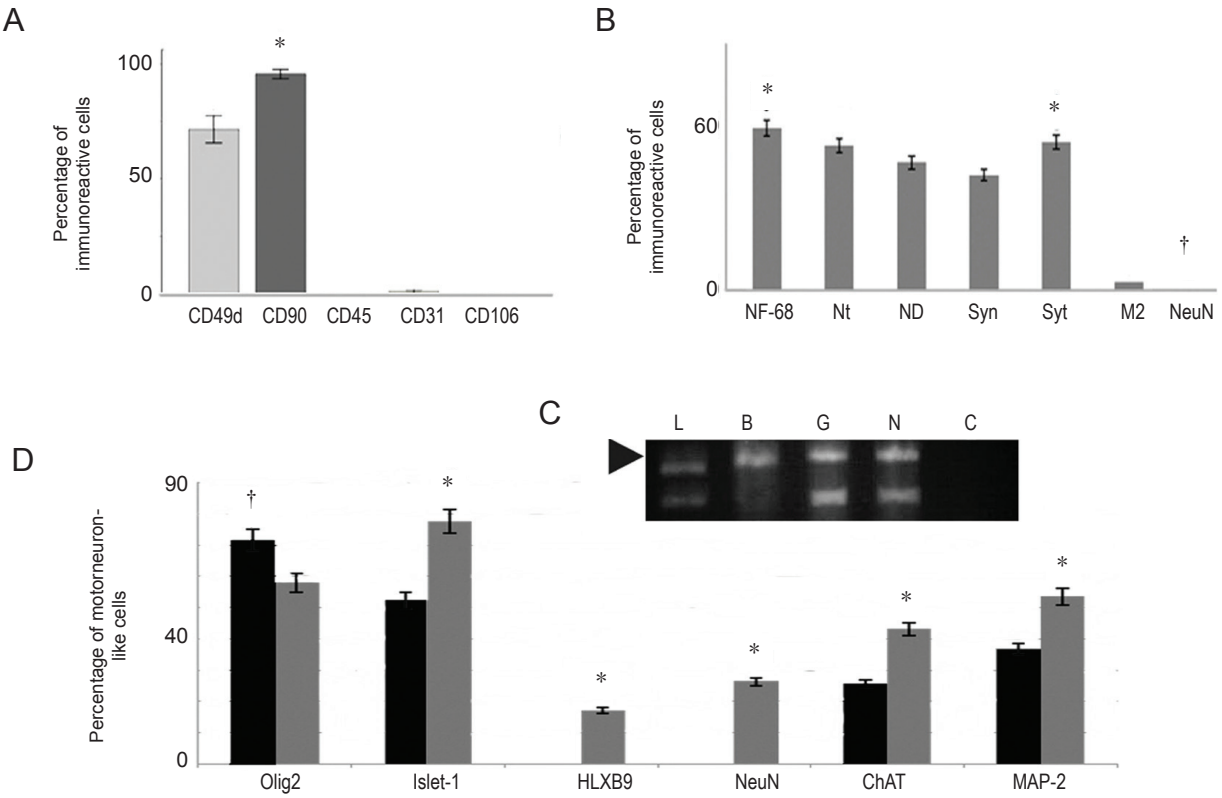

Figure 1 The percentage of immunoreactive cells to different markers of adipose-derived stem cells, pre-induced cells and motoneurons-like cells.

The data in the histogram were presented as mean ± SEM. Statistical analysis was done using one-way analysis of variance (ANOVA) and Tukey's test (A, B and D) and reverse transcriptase polymerase chain reaction of pre-induced cells (C). Each experiment was performed five times.

(A) A histogram representing the percentages of adipose-derived stem cells that were immunoreactive to different CD markers including CD 49d (a specific marker for fat cells), CD 90 (a marker of mesenchymal stem cells), CD 45 (a hematopoietic cell marker), CD 31 (an endothelial cell marker) and CD 106 (a marker of mesenchymal stem cells derived from bone marrow stromal cells). The "*" indicates that the expression is significantly higher than that of the other markers.

(B) A histogram representing the percentages of cells pre-induced with selegiline (10^{-9} mmol/L) that were immunoreactive to different markers including neurofilament 68 (NF-68: a proneural marker), nestin (Nt: a neural stem cell marker), Neuro D (ND: a proneural marker), synapsin (Syn: a neuronal marker), synaptophysin (Syt: a neuronal marker), microtubule-associated protein 2 (M2: a neuronal marker) and NeuN (a neuronal marker). The "*" indicates that the expression is significantly higher than that of the other markers; "†" indicates that the expression is significantly lower than that of the other markers.

(C) The electrophorogram of reverse transcriptase polymerase chain reaction of pre-induced cells for B (brain derived neurotrophic factor), G (glial cell derived neurotrophic factor, N (neurotrophin-3) and C (negative control). L is DNA ladder.

(D) A histogram representing the percentages of motoneuron-like cells at day 2 (solid black column) and day 7 (solid gray column) that were immunoreactive to different markers including Olig2 (a motoneuron lineage marker), Islet-1 (a motoneuron lineage marker), HLXB9 (a differentiated motoneuron lineage marker), neuronal nuclei (NeuN; a neuronal marker), choline acetyl transferase (ChAT: a cholinergic neuron marker) and microtubule-associated protein 2 (MAP-2; a neuronal marker). The "*" indicates that the expression is significantly higher on day 7 than on day 2.

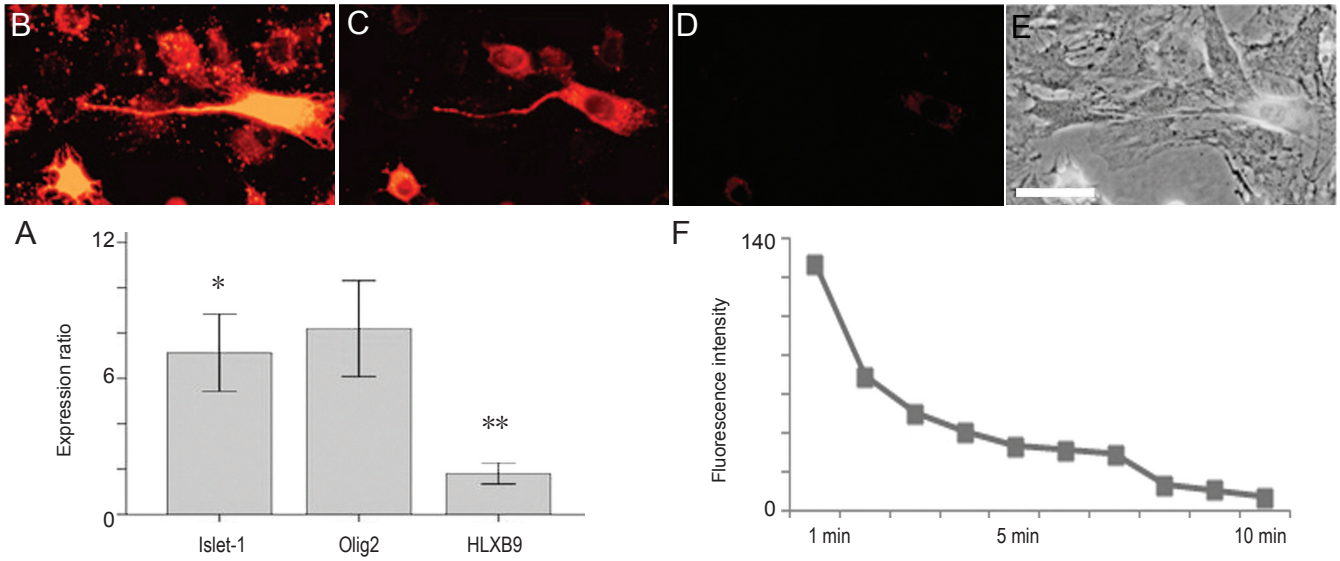

Figure 2 The cellular and molecular functionality of motoneuron-like cells.
(A) A histogram representing the fold change ratios of expression of Islet-1 (a motoneuron lineage marker), Olig2 (a motoneuron lineage marker) and HLXB9 (a differentiated motoneuron lineage marker). The mRNA was extracted from motoneuron-like cells derived from the adipose-derived stem cells using pre-induction (using selegiline) and induction (using 1 μg/mL sonic hedgehog and 2×10^{-8} mol/L all-trans retinoic acid for 2 days) steps. The fold change ratio was assessed using quantitative real-time polymerase chain reaction. The "*" indicates that difference with Islet 1 was not significant, and "**" indicates that the difference with the other genes was significant ($P < 0.05$). The data in the histogram were presented as mean ± SEM. Statistical analysis was done using one-way analysis of variance and Tukey's *post hoc* test. The data were obtained from three experiments. Functionality assay using staining and destaining of neuron-like cells differentiating into motoneuron-like cells exposed for 120 seconds to FM1-43 fluorochrome at 1 (B), 5 (C), and 10 minutes (min; D) after the start of the Potassium ion stimulation. (E) A phase-contrast image. Scale bar: 25 μm. (F) The total number of pixels in the time course (1–10 min) used in the study during which the motoneuron-like cells were stained with FM1-43 fluorochrome and then destained following the release of the synaptic vesicles. The bar graphs indicate the mean ± SEM.

nipour and Tiraihi, 2012). Also, motoneuron-like cells were co-cultured with myotube (Shin et al., 2007), and the latter was obtained by incubating C2C12 myoblast cell line (immunoreactive to MY5) in DMEM medium containing 2% horse serum for 4 days. When the myotubes formed, they were co-cultured with motoneuron-like cells for 4–10 days, and motoneuron-like cells formed contacts with the myotubes (Miles et al., 2004; Wada et al., 2009).

In vivo experimental design

Female Sprague-Dawley rats, weighing 200–250 g, were housed under standard condition; the experimental procedures were approved by the Ethical Committee for Laboratory Animal at Tarbiat Modares University, Iran. The rats were assigned to seven experimental groups ($n = 8$ for each group) as follows: sham operated (laminoectomy; SO); untreated spinal cord contusion injury (U); contused, injected with normal saline (S); GDNF-treated (G); adipose-derived stem cell-treated (A); motoneuron-like cell-treated (M); and motoneuron-like cell plus GDNF-treated ones (GM). In the S group, the contused animals were intraspinally injected with 9 μL normal saline at the epicenter, rostral and caudal regions of the impact site. In the GDNF-treated groups (G and GM), the rats received GDNF by biopolymer-based delivery, involving subthecal implantation of gelfoam soaked with 20 μg GDNF at 7 days after the injury (Otto et al., 1989). The cell transplantation and the sham operations were performed 7 days after injury.

Establishment of a rat model of spinal cord contusion injury

A standard surgical procedure was used (Bose et al., 2002). Briefly, the rats were anesthetized with an intraperitoneal injection of 80 mg/kg ketamine and 10 mg/kg xylazine. The rats were shaved, and dorsal laminectomy was performed at the T_{12}–L_1 level; the exposed spinal cord was contused by dropping a 10 g metal rod with a diameter of 2 mm, from a height of 25 mm (Ohta et al., 2004). The postoperative care in the first week after surgery included manual bladder expression and intramuscular injection of 50 mg/kg cefazoline twice daily (Jabir Ibn Hayyan, Tehran) (Khalatbary and Tiraihi, 2007).

Cell transplantation

Before cell transplantation, motoneuron-like cells and adipose-derived stem cells were harvested, stained with 1 μg/mL Hoechst 33342 (Sigma-Aldrich) for 1 minute at 37°C (Eliyahu et al., 2005) and then isolated using trypsin and ethylenediamine tetraacetic acid. Each animal in the cell transplantation groups (A, M, and GM) was intraspinally injected with 300,000 cells suspended in 9 μL normal saline at the epicenter, rostral and caudal regions of the impact site (each site was injected with 100,000 cells in 3 μL). The cell transplantation was performed with a 30 G needle and a microinjection

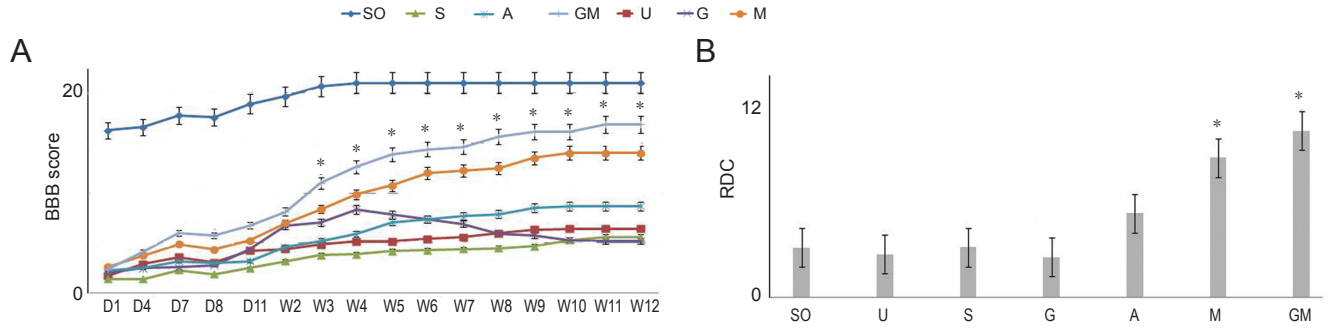

Figure 3 Results of open field behavioral test (BBB score test) and RDC of BBB score test.
(A) Represents the time course of BBB locomotor scores. The scores were recorded from the 1st day to the end of the 12th week post-injury. The histogram represents the sham operated (laminoectomy: SO), untreated spinal cord contusion injury (U), contused, injected with normal saline (S), GDNF-treated (G), ADSC-treated (A), MNLC-treated (M), and MNLC plus GDNF-treated ones (GM). Eight animals per group were used and the data were presented as mean ± SEM. Statistical analysis was done using repeated-measures analysis of variance and Tukey's *post hoc* test. BBB in the M and GM groups significantly differed from those of the other experimental groups. The "*" represents significant differences between the M and GM groups and the other groups ($P < 0.05$). (B) A histogram of the RDC in BBB scores between weeks 1 and 12 after injury in each group including the SO, U, G, A, M and GM groups. The data in the histogram were presented as mean ± SEM. One-way ANOVA followed by Tukey's post hoc test was used. The "*" represents significant differences between the GM group and the other groups, except the G group ($P < 0.05$). BBB: Basso-Beattie-Bresnahan; RDC: relative difference coefficient; GDNF: glial cell line-derived neurotrophic factor; ADSC: adipose-derived stem cell; MNLC: motoneuron-like cell.

pump (Stoelting Co., Wood Dale, IL, USA) (Naghdi et al., 2009). In the combined treatment (GM) group, a small piece of gelfoam was placed in the subdural space, and 20 μg GDNF in 4 μL saline (5 μg/μL) was injected into the gelfoam (Shibayama et al., 1998). The cell transplantation and the sham operations were performed 7 days after injury.

Behavioral assessment

The behavioral assessments were performed using Basso, Beattie, and Bresnahan (BBB) rating scale, from 0 (paralysis) to 21 points (normal gait) (Basso et al., 1995). The rats were individually videotaped for 4 minutes by four digital video cameras located inside a plastic container with a diameter of 110 cm and height of 50 cm. One week before surgery, the test was conducted for all of the animals (they were trained and handled for 10 days before the surgery) on days 1, 3, and 7 after surgery and then weekly until 12 weeks after surgery. During the evaluation, the animals were allowed to walk freely on an open-field surface. Two independent examiners, blinded to the treatment group, evaluated BBB score of the rats, and the mean value of both ratings was recorded.

Morphometric evaluation

Twelve weeks after surgery, the rats were killed, and the spinal cord at the injured area was removed and fixed in 4% paraformaldehyde for 12 hours. The harvested tissues were processed on an automatic processor (Leica TP 1020: Leica, Hamburg, Germany) and embedded in paraffin. Serial sections (thickness, 7 μm) were prepared, deparaffinized with xylol and stained with hematoxylin and eosin (Llewellyn, 2009). The cell density per area and percentage of cavitation in a 4,200-μm length of the lesion were determined using the Image J 1-44 software (NIH). The volume density of the spared gray matter and mean cavity percentage were calculated according to Cavalieri method (Parr et al., 2008). Serial summation of the spared tissue and cavity volumes yielded the total volume of the spared tissue. Any necrotic tissue within the cavities was counted as part of the lesion; the volume of cavities was also calculated according to the formula: $V_{sp} = a \times d$, where "a" is the measured area, and "d" is the intersection distance.

The neuroglial soma size was used for evaluating the cell density per area as follows: glial cells < 10 μm; interneurons, γ-motor neurons, and sensory neurons > 10 μm and α-motor neurons, > 25 μm (Sergeeva et al., 2007; Zhou et al., 2007; Wei et al., 2011).

Determination of the fate of the transplanted cells

In order to determine the fate of the transplanted cells in the cell therapy groups (A, M, and GM), double labeling was used. Hoechst 33342 was used as a supravital nuclear stain, and immunostaining of the cryosections with HLXB9, NF-200, O1 and GFAP markers (all from Abcam) and O1 (antibodies-online GmbH, Aachen, Germany) was performed. The cells immunoreactive to HLXB9 (mature motor neurons), NF-200 (neurons), O1 (oligodendrocytes), or GFAP (astrocytes) markers were evaluated using fifteen samples from each animal in a given group. In each sample, three regions were considered for evaluation (the epicenter, rostral and caudal regions of the lesion: 5 sections per region). From each region, 30-μm-thick cryosections were prepared, and both ventral horns (right and left), both dorsal horns and the central canal areas were assessed under 20 × magnification. Counts for each section were averaged for each animal, which were used for statistical comparison.

Statistical analysis

Statistical analysis was performed using SPSS 15.0 software (www.ibm.com). The significant level in BBB scores were analyzed using repeated-measures analysis of variance (ANOVA), followed by *post hoc* analysis with Tukey's *post hoc* test. In other statistical comparison of the multiple means in the groups,

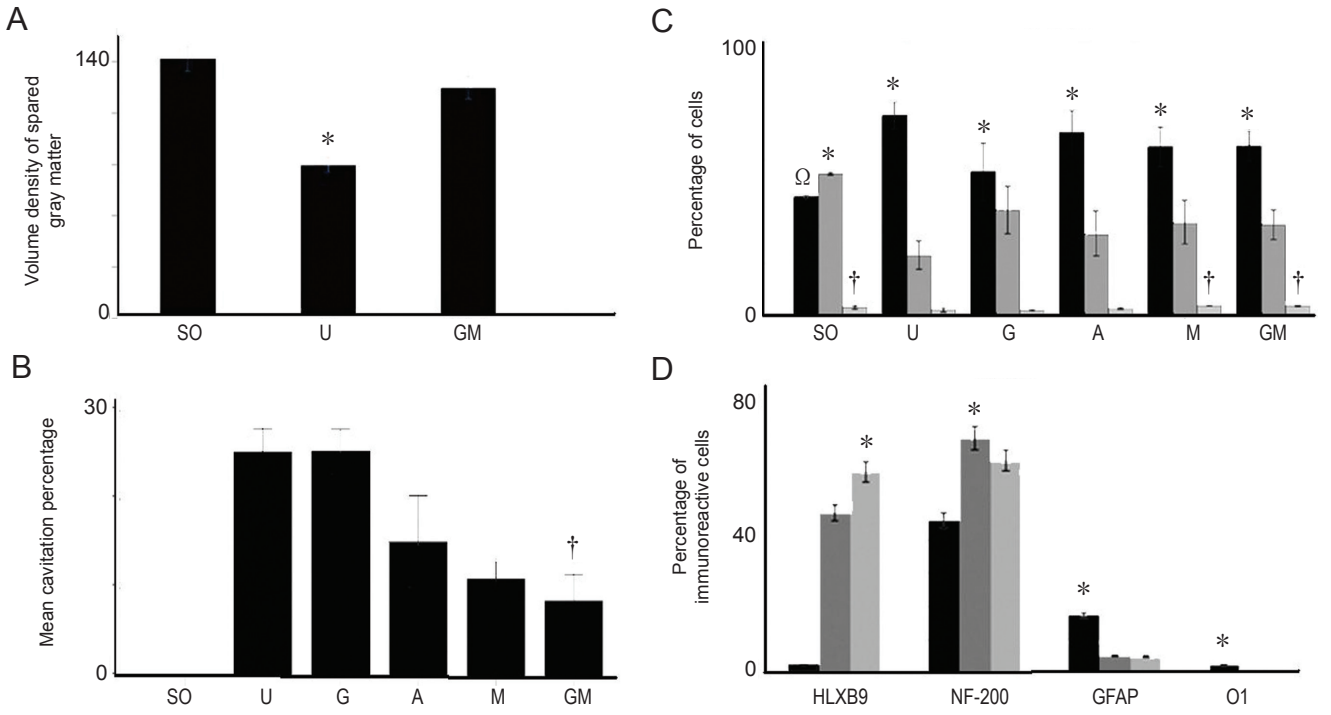

Figure 4 Morphometric analysis of spinal tissues.
Eight animals per group were used and the data were presented as mean ± SEM. Statistical analysis was performed using one-way analysis of variance and Tukey's *post hoc* test.
(A) The mean percentage of the spared gray matter in the sham-operated (SO), untreated (U) and MNLC plus GDNF-treated (GM) groups. The "∗" indicates that the percentage is significantly lower than that of the other groups.
(B) The mean cavitation percentages in the SO, U, GDNF-treated (G), ADSC-treated (A), MNLC-treated (M), and GM groups. The "†" indicates that the percentage in the GM group is significantly lower than that of the other groups, except the M group.
(C) The mean percentages of glial cells (solid black column), neurons (solid dark gray column), and MNLC (solid light gray column) in the SO, U, G, A, M and GM groups. The "∗" indicates that the percentage of the cells is significantly higher than that of the other cells in the same group ($P < 0.05$). The "†" indicates that the percentage of cells is significantly higher than that in the U, A and G groups ($P < 0.05$). The "Ω" indicates that the percentage of cells is significantly lower than that in the other groups, except the G group ($P < 0.05$).
(D) The mean percentage of the cells in the A (solid black column), M (solid dark gray column), and GM (solid light gray column) groups. The cells were immunoreactive to HLXB9 (a marker of differentiated motoneuron), NF-200 (a neuronal marker), glial fibrillary acidic protein (an astrocyte marker), and O1 (an oligodendrocyte marker). The "∗" indicates that the percentage of the immunoreactive cells in a given group is significantly higher than that of the other groups ($P < 0.05$).
GDNF: Glial cell line-derived neurotrophic factor; ADSC: adipose-derived stem cell; MNLC: motoneuron-like cell; NF-200: neurofilament-200.

one-way ANOVA followed by Tukey's *post hoc* test was used. The data in the histogram were presented as mean ± SEM. Values of $P < 0.05$ were considered statistically significant.

Results

Isolation, pre-induction, and induction of adipose-derived stem cells

The isolated adipose-derived stem cells differentiated into lipogenic and osteogenic cells (data not shown), which were immunostained with CD49d, CD90, CD45, CD31 and CD106 (data not shown). The percentage of CD90-positive cells was significantly higher than that of cells immunoreactive to the rest, and the percentage of CD49d positive cells was significantly higher than that of CD31, D106 or CD45 positive cells (**Figure 1A**). Pre-inducing the adipose-derived stem cells with 10^{-9} mmol/L selegiline for 24 hours significantly increased their viability, compared to the other experimental groups. The pre-induced adipose-derived stem cells were immunoreactive to nestin and NF-68. The percentages of the cells immunoreactive to nestin, Neuro D or synapsin were not significantly different from each other, but they were significantly different from those immunoreactive to synaptophysin, NF-68, MAP-2 and NeuN ($P < 0.05$; **Figure1B**). RT-PCR shows that the pre-induced cells expressed GDNF and NT-3 while BDNF was not expressed (**Figure 1C**). Induction was performed by incubating the pre-induced cells with 1 μg/mL sonic hedgehog and 2×10^{-8} mol/L retinoic acid. The percentage of cells immunoreactive to Olig2 on day 2 was significantly higher than the percentages of cells immunoreactive to other markers on the same day. The percentage of cells immunoreactive to Islet-1 on day 7 was significantly higher than the percentages of cells immunoreactive to other markers on the same day; however, HLXB9 and NeuN were not expressed on day 2 (**Figure 1D**). RT-PCR revealed expression of Islet-1, Olig2 and HLXB9 in the induced cells on day 2 (**Figure 2A**). During the functionality assay, motoneuron-like cells showed several puncta (synaptic vesicles) that released their contents upon stimulation (**Figure 2B–F**).

The myoblasts formed multinucleated myotubes following

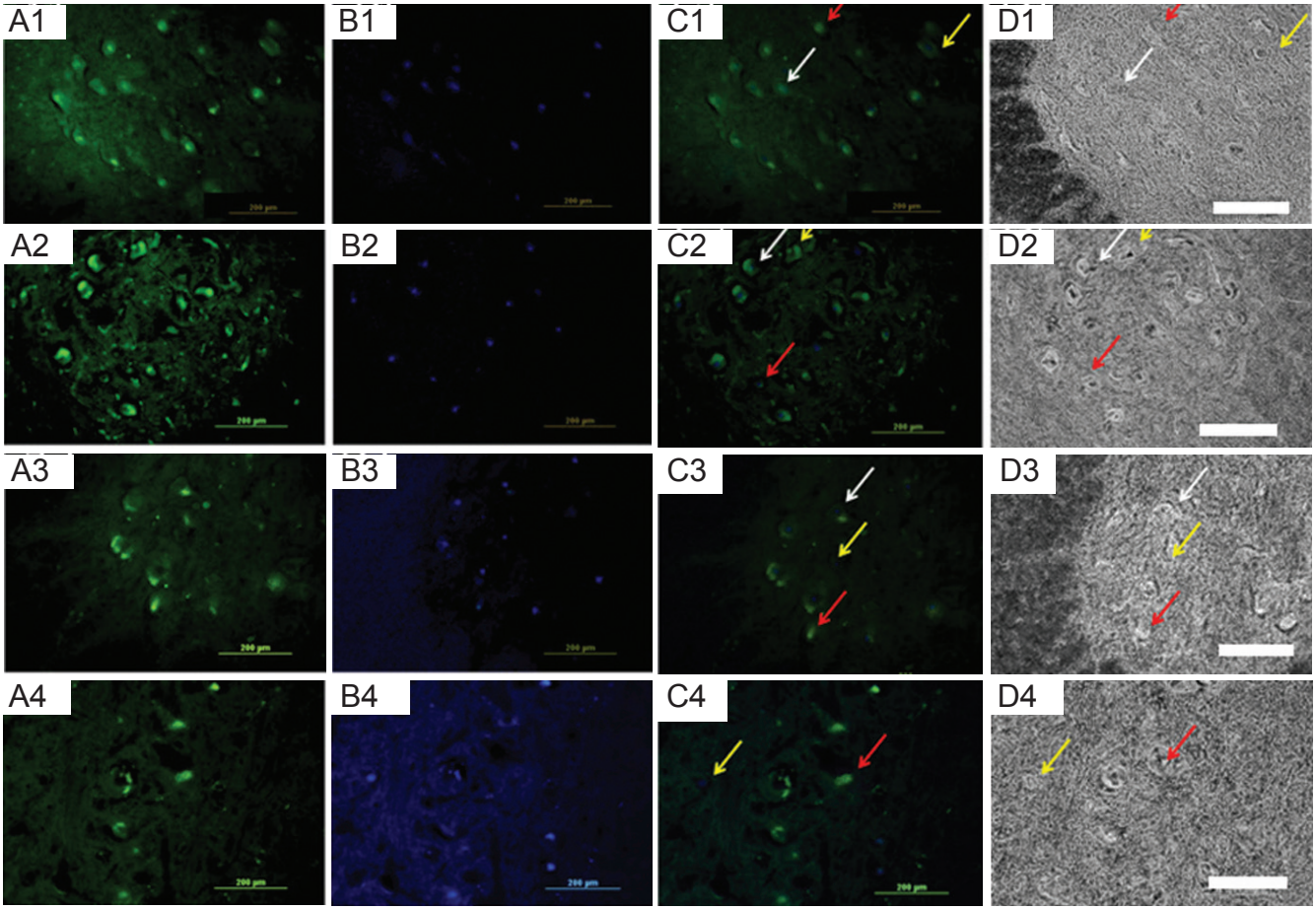

Figure 5 The fate of the transplanted cells in tissue sections from the injured spinal cord from the animal group treated with motoneuron-like cells with GDNF.
Photomicrographs represent the immunoreactivity of the transplanted cells to HLXB9 (A1, B1, C1 and D1), NF-200 (A2, B2, C2 abd D2), GFAP (A3, B3, C3 and D3) and O1 (A4, B4, C4 and D4) in the GM group. The cells were immunostained with relevant primary antibodies and labeled with FITC-conjugated secondary antibody (green) and Hoechst 33342 stain (a nuclear stain; blue). (A) Cells immunoreactive to HLXB9, NF-200, GFAP or O1 (A1, A2, A3 or A4, respectively). (B) Cells labeled with Hoechst 33342 stain (all panels). (C) A merged image of A and B (all panels), and a phase-contrast photomicrograph from the same field in A and B (all panels). The white, yellow, and red arrows indicate immunoreactive transplanted cells, non-immunoreactive transplanted cells, and immunoreactive host cells, respectively (scale bar: 200 μm). GDNF: Glial cell line-derived neurotrophic factor; NF-200: neurofilament-200; GFAP: glial fibrillary acidic protein; GM: motoneuron-like cell plus GDNF-treated.

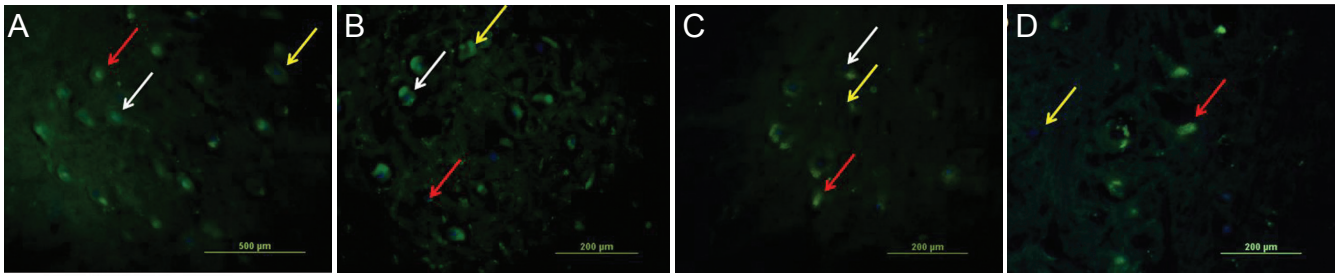

Figure 6 The fate of the transplanted cells in tissue sections from the injured spinal cord.
Merged photomicrographs represent the immunoreactivity of the transplanted cells to HLXB9 (A), NF-200 (B), GFAP (C), and O1 (D) in the GM group at higher magnification than figure 5. The cells were immunostained with relevant primary antibodies and labeled with FITC-conjugated secondary antibody (green) and Hoechst 33342 stain (a nuclear stain; blue). The white, yellow, and red arrows indicate immunoreactive transplanted, non-immunoreactive transplanted, and immunoreactive host cells, respectively (scale bars: 200 μm). NF-200: Neurofilament-200; GFAP: glial fibrillary acidic protein; GM: motoneuron-like cell plus GDNF-treated.

incubation in DMEM supplemented with horse serum, the myotubes were co-cultured with induced motoneuron-like cells and the myotubes were innervated by motoneuron-like cells (data not shown).

Motor function of rats with spinal cord contusion injury
The mean BBB scores of all groups are shown in **Figure 3A**. The mean scores gradually increased during 14 days after injury (G, M and GM), which significantly differed from

that of the motoneuron-like cell groups (M and GM) and the untreated group at the end of the 4th week. The statistical analysis at 12 weeks showed that the SO group scores were significantly higher than those of the experimental (G, A, M and GM) and negative control groups (U and S), while the M and GM groups scores were significantly higher than the scores of all of the other groups, except the SO group. Although the mean BBB score was higher in the GM group than in the M group, the difference was not significant. There were two main clusters of scores: high scores in the SO, M, and GM groups and low scores in the U, S, G and A groups. The increasing trend in the G group was similar to that of the M and GM ones during the first 4 weeks, but the G group scores abruptly declined thereafter, and became lower than the scores of the untreated group. Accordingly, the relative difference coefficients (RDC) between the G group scores and the scores of the other groups showed the same patterns; the least difference was with the U group score and the highest with the GM ones. The RDC in the cell therapy-only groups (A and M) was higher than the score in the GDNF-only group (G). No significant differences were observed in RDC among the U, S, G and A groups (**Figure 3B**).

Spared gray matter and cavitation analysis results

The volume density of the spared gray matter is presented in **Figure 4A**. It was significantly lower in the untreated group than that of the combined therapy one (GM), which was lower than the sham-operated group, however, the difference was not significant. In order to evaluate the therapeutic effects of stem cell and GDNF therapy, we performed histological assessments at 12 weeks after injury. The mean percentage of cavitation in a 4,200-μm length of the injured spinal cord was highest in the untreated group, and this value did not significantly differ from that of the G group. The GM group showed the lowest value, but there was no difference between the other cell therapy groups (A and M) (**Figure 4B**).

Percentage of different cells in the spinal cord

Figure 4C represents the morphometric evaluation of the percentage of cellular components in the spinal tissue. The mean percentage of glial cells was significantly higher in the untreated group than in the other groups, while that of the SO group was significantly lower than that in the experimental groups, including the untreated controls. The percentage of motoneuron-like cells was significantly lower in the U and G groups than in the other groups. The highest percentage of neurons was noted in the SO group, while the lowest was found in the U group. No significant differences were present among the G, A, M, and GM groups.

Fate of the transplanted cells

The mean percentage of the immunoreactive cells is presented in **Figure 4D**. The highest expression of HLXB9, NF-200, GFAP and O1 was in the GM, M and A groups, respectively. The statistical analysis shows that the expression of HLXB9, NF-200, and GFAP in group A significantly differed from the expression in the other treatment groups (M and GM), in particular, the percentage of cells immunoreactive to HLXB9 and NF-200, which was lowest in the cell therapy-only groups. No significant difference in NF-200 expression was found between the M and GM groups. A significantly higher proportion of cells in the GM group than in the M group was immunostained with HLXB9. The transplanted cells in the M and GM groups did not express the marker for mature oligodendrocytes (O1) (**Figure 4D**).

Figure 5 shows cryosections from the spinal cords of the rats in the GM group, after staining with Hoechst 33342 and immunostaining with HLXB9, NF-200, GFAP and O1, the results show that some of the transplanted cells differentiated into motoneuron-like cells and glial cells. The immunostaining of the GM group in **Figure 5** at higher magnification with HLXB9, NF-200, GFAP, and O1 is demonstrated in **Figure 6**.

Discussion

In the present study, intraspinal transplantation of motoneuron-like cells derived from adipose-derived stem cells combined with polymer-based GDNF delivery may contribute to reducing the locomotor deficit in a rat model of spinal cord contusion injury.

The mesenchymal lineage cells of adipose-derived stem cells were confirmed by inducing them into lipogenic and osteogenic phenotypes. They were also characterized by the expression of CD90 (a mesenchymal stromal cell marker) and CD49d (a specific adipose-derived stem cell marker), but not CD106 (a bone marrow stromal cell marker), CD31 (an endothelial cell marker) and CD45 (a hematopoietic cell marker). These results are consistent with those of other investigators (Pachon-Pena et al., 2011; Scherzed et al., 2011). It was reported that adipose-derived stem cells could be an alternative source for cell therapy in spinal cord injury because they were multipotent cells. Moreover, compared with bone marrow stromal cells, adipose-derived stem cells can be obtained by a less invasive method and in larger quantities (Fraser et al., 2006).

Selegiline has been used as a pre-inducer, because it is safer than the other chemicals used for pre-inducing bone marrow stromal cells (Ghorbanian et al., 2010). Selegiline is an efficient pre-inducer of adipose-derived stem cells that protects the neuron from apoptosis (Hobbenaghi and Tiraihi, 2003) and can switch on nestin NF-68 and neurotrophin genes (Abdanipour and Tiraihi, 2012). Esmaeili et al. (2006) also reported neurotrophin expression in embryonic stem cells incubated with selegiline. On the other hand, Qian et al. (2010) used chemicals (β-mercaptoethanol, dimethyl sulfoxide and butylated hydroxyanisole) in one of their protocols, which were shown to be teratogenic, carcinogenic, and mutagenic. In addition, other investigators considered bone marrow stromal cell transdifferentiation as an artifact (Lu et al., 2004). Qian et al. (2010) reported the generation of neural stem cells from neurosphere cells derived from adipose-derived stem cells, and at 4 weeks, the neuronal phenotype was approximately 50%, while the percentage of Islet-1 immunoreactive cells in this study was more than 70%.

Zhang et al. (2006) reported that the expression of Olig2 and HLXB9 genes could restrict the motor neuron lineage; in this investigation, we have confirmed the expression of olig-2 and HLXB9.

The Olig2 expressing cells (motor neuron progenitors) were generated by incubating the neuron-like cells with sonic hedgehog and retinoic acid in the early stages of induction, while HLBX9 was expressed later. Interestingly, RT-PCR shows that on the second day, HLXB9 was expressed, but no immunoreactivity was observed, which occurred possibly because HLXB9-proRNA was transcribed and processed, but not yet translated, or its expression was too low to be detected by fluorescent microscopy (Movaghar et al., 2008). Our PCR results are consistent with RT-PCR results reported previously (Abdanipour and Tiraihi, 2012) and another study that used sonic hedgehog and retinoic acid for the induction of mouse embryonic stem cells (Soundararajan et al., 2007). The motoneuron-like cells were immunoreactive to choline acetyl transferase and HLBX9 (characteristic of motor neurons) (Soundararajan et al., 2007), and the functionality assay showed that these cells could form synapses and release the contents of their synaptic vesicles upon stimulation, using FM1-43 fluorochrome as a marker for synaptic content release (Mareschi et al., 2009).

In vitro generation of motor neurons from embryonic stem cells and their transplantation for treating spinal cord injury was reported by Harper et al. (2004), who used sonic hedgehog and retinoic acid in a co-culture system for generating spinal motor neurons from embryonic stem cells. Considering the ethical issues, immunological rejection upon allograft transplantation and possibility of neoplastic growth associated with embryonic stem cells (Master et al., 2007), autologous adult stem cells could be an alternative source for cytotherapy (Moore et al., 2006).

In this study, GDNF was administered to the contused rats in order to reduce death of the spared neurons. GDNF shows neuroprotective effects during *in vitro* neuronal induction of embryonic stem cells and supports the survival of co-grafted cells into the brain (Emerich et al., 2003). In addition, Yang et al. (2001) reported that it had neuroprotective effects in Parkinson's disease and cerebral ischemia in rats (Yang et al., 2001). In our study, it has been used to protect the *in situ* degenerating neurons following the traumatic insult (Choi-Lundberg et al., 1997) and protect the transplanted cells. Before transplantation, the cells were maintained under *in vitro* conditions with optimal nutrient supply and oxygen tension. However, in the traumatized spinal cord, many blood vessels were damaged, and a number of surviving neurons died because of secondary events following the traumatic insult (Mautes et al., 2000).

Our results show that there was a significant improvement in BBB locomotor rating scores, which is consistent with the results of Cheng et al. (2002) who infused GDNF in the contused rat spinal cord. Deshpande et al. (2006) suggested that GDNF could salvage damaged neurons, while Ebert et al. (2010) documented that it maintained motor neuron function. It may play a role in promoting the survival and differentiation of grafted motoneuron-like cells in the GM group, the implanted motoneuron-like cells expressed HLXB9, a marker for differentiated motor neurons. Although the percentage of HLXB9-immunoreactive cells was higher in the GM group than in the M group, the RDC was not significant. This may be explained by the need for sustained GDNF release, which is required for synaptic maturation (Iannotti et al., 2004; Baudet et al., 2008). The decline in BBB score in the G group may be attributable to the dependency of the injured neurons on GDNF-mediated neuroprotection. Termination of GDNF diffusion from the gelfoam may have been followed by loss of the GDNF-dependent neurons and a subsequent decline in the behavioral test (Iannotti et al., 2004). Tai et al. (2003) reported that gene-based GDNF delivery resulted in functional recovery of the injured spinal cord, possibly due to sustained GDNF release, which resulted in axonal regeneration and ensheathment. Zhang et al. (2009) reported that BBB scores did not differ between contused rats treated with undifferentiated adipose-derived stem cells and those treated with neuronal differentiated adipose-derived stem cells. Our findings are not consistent with those of Zhang et al. (2009), however, this may be due to the use of different protocols. Alexanian et al. (2006) reported that after transplantation, the undifferentiated cells either remained undifferentiated or transformed into astrocytes, whereas the differentiated cells could integrate and differentiate into a chronically injured spinal cord after transplantation. The low BBB score in a study by Zhang et al. (2009) could be explained on the basis that the transplanted differentiated adipose-derived stem cells might not have been lineage restricted. Arboleda et al. (2011) reported that pre-differentiated adipose-derived stem cells gave slightly higher BBB score than undifferentiated ones, which is consistent with the results of this study.

The volume density of the spared gray matter was higher in the GM group than the untreated one, which is consistent with the finding of Rabchevsky et al. (2003) who used creatine; similar results were documented with the use of NIM811, a cyclosporine derivative (Ravikumar et al., 2007). Magnuson et al. (1999) reported that the improvement in the behavioral test correlated with the percentage of spared tissue, this was also emphasized by Scheff et al. (2003). On the other hand, the percentage of cavitation in the A, M and GM groups decreased, but the differences among the groups were not significant. This is consistent with the explanation of the integration of transplants in the A group; however, the cells were not functional, where the undifferentiated transplants resulted in a lower BBB score (Pedram et al., 2010). Moreover, the numerical density of motoneuron-like cells of the M and GM groups did not significantly differ from that of the SO one, this suggested that the transplanted cells were migrated and differentiated in the injured spinal cord, however, they did not functionally integrated.

BBB scores of the M and GM groups were not significantly different, indicating that intraspinal delivery of the motoneuron-like cells was an important factor in the recovery of motor activity after spinal cord trauma, this finding is in agreement with that of Nógrádi et al. (2011). However, the

difference at the end of the experiment (12th week) in BBB scores between the GM and M groups was 2.86, other investigators reported it to be 1.5 and considered it as an important improvement in the locomotive activities of the spinally injured animals (Yakovenko et al., 2007).

In conclusion, motoneuron-like cells can improve BBB score, especially when the score is evaluated using RDC and when combined therapy of GDNF and cell transplantation is used.

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