

Transplantation of mesenchymal stem cells promotes the functional recovery of the central nervous system following cerebral ischemia by inhibiting myelin-associated inhibitor expression and neural apoptosis

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Received December 18, 2014; Accepted January 14, 2016

DOI: 10.3892/etm.2016.3089

Abstract. Cerebral ischemia, which may lead to cerebral hypoxia and damage of the brain tissue, is a leading cause of human mortality and adult disability. Mesenchymal stem cells (MSCs) are a class of adult progenitor cells with the ability to differentiate into multiple cell types. The transplantation of bone marrow-derived MSCs is a potential therapeutic strategy for cerebral ischemia. However, the underlying mechanism has yet to be elucidated. In the present study, primary MSCs were isolated from healthy rats, labeled and transplanted into the brains of middle cerebral artery occlusion rat models. The location of the labeled MSCs in the rat brains were determined by fluorescent microscopy, and the neurological functions of the rats were scored. Immunohistochemical analyses demonstrated that the protein expression levels of myelin-associated inhibitors of regeneration, including Nogo-A, oligodendrocyte myelin glycoprotein and myelin-associated glycoprotein, were decreased following transplantation of the bone marrow-derived MSCs. Furthermore, the mRNA expression levels of Caspase-3 and B-cell lymphoma 2, as determined by reverse transcription-quantitative polymerase chain reactions, were downregulated and upregulated, respectively, in the MSC-transplanted rats; thus suggesting that neural apoptosis

was inhibited. The results of the present study suggested that the transplantation of bone marrow-derived MSCs was able to promote the functional recovery of the central nervous system following cerebral ischemia. Accordingly, inhibitors targeting myelin-associated inhibitors and apoptosis may be of clinical significance for cerebral ischemia in the future.

Introduction

Cerebral ischemia occurs as a result of insufficient blood flow to the brain (1,2), which leads to limited oxygen supply, or cerebral hypoxia, and may eventually result in the death of brain tissue, cerebral infarction or ischemic stroke (3). It is a leading cause of death and the predominant cause of adult disability (4). At present, there is no specific therapy for improving functional recovery.

Mesenchymal stem cells (MSCs) are a class of adult progenitor cells capable of differentiating into several cell types (5-8). Several previous studies have indicated that bone marrow derived MSCs may serve as a source of cells for cell transplantation therapy following cerebral ischemia and have been successfully used for the treatment of experimental stroke (9-11). In the aforementioned studies, bone marrow derived MSCs selectively targeted injured brain tissue and promoted functional recovery via various cell delivery routes. Although the transplantation of bone marrow derived MSCs has been revealed to provide therapeutic benefit to cerebral ischemia, the underlying mechanisms have yet to be elucidated.

The adult mammalian central nervous system (CNS) possesses a poor capability to regenerate axons following injury. The failure of CNS axons to regenerate after damage has significant consequences for brain ischemia (12). Several factors contributing to this regenerative failure include the intrinsic state of the injured neuron, the formation of the inhibitory glial scar and the presence of inhibitory myelin debris. A previous study demonstrated that myelin-associated neurite growth inhibitors had a crucial role in preventing CNS

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Key words: Caspase 3, B-cell lymphoma 2, cerebral ischemia, bone marrow stem cells, Nogo-A, oligodendrocyte myelin glycoprotein, myelin-associated glycoprotein

regeneration after brain injury (13). Three inhibitors of regeneration have been identified in myelin thus far, including: Nogo-A, oligodendrocyte myelin glycoprotein (OMgp) and myelin-associated glycoprotein (MAG). These molecules are membrane proteins of myelin and expressed predominately by oligodendrocytes in the adult mammalian CNS (12,14-17).

It has been previously reported that numerous brain neurons in ischemia penumbria undergo apoptosis following either global or focal ischemic insults (18). Apoptosis is a unique, gene-directed form of cell death, and is characterized by nuclear condensation and cytoplasmic fragmentation that contributes to physiological and pathological processes (19). The Caspases, in particular Caspase-3, perform a key role in the execution of apoptosis (20). B-cell lymphoma 2 (Bcl-2) is the crucial member of the Bcl-2 family of regulator proteins that regulate apoptosis, and is able to inhibit apoptosis without affecting cellular proliferation (21,22).

In the present study, the intraluminal model of transient middle cerebral artery occlusion (MCAO) was established in rats to investigate the effects of the transplantation of bone marrow derived MSCs on CNS functional recovery. It was determined in the present study that the transplantation of bone marrow derived MSCs promoted the functional recovery of the CNS following brain ischemia via the inhibition of the expression of Nogo-A, OMgp and MAG, as well as neuronal apoptosis.

Materials and methods

Rats. A total of 90 healthy adult male Sprague-Dawley (SD) rats weighing 220-280 g, and 15 healthy adult male SD rats weighing 80-120 g, were obtained from the Experimental Animal Center of the Second Affiliated Hospital of Harbin Medical University (Harbin, China). The rats were maintained in a room at $23\pm 1^\circ\text{C}$ under a 12-h light/dark cycle with *ad libitum* access to food and water. All experiments were performed with approval from the Ethics Committee of Harbin Medical University.

Rat MCAO model. Adult male SD rats ($n=90$; weight, 220-280 g) were used to establish the intraluminal model of transient MCAO. A transient (2-h) MCAO was induced using a modified version of the method described by Longa *et al* (23). Briefly, rats were anesthetized by intraperitoneal injection with 10% chloral hydrate (0.35 ml/100 g; Sigma-Aldrich, St. Louis, MO, USA) and placed in the supine position. The left common carotid artery (CCA), external carotid artery and internal carotid artery (ICA) were exposed and isolated through a carotid midline incision (2.0-2.5 cm). A surgical nylon suture 0.234 mm in diameter and wax-coated tip was advanced from the CCA into the lumen of the ICA until it blocked the origin of the middle cerebral artery. From the bifurcation of the CCA, 18-20 mm of the nylon suture was in the lumen and 1 cm was left outside. After 2 h occlusion, the suture was withdrawn to permit reperfusion until resistance was felt. Neurological examinations, as described by Longa *et al* (23), were performed subsequent to the onset of occlusion. The neurological results were scored using a 5-point scale as follows: A score of 0 indicated no neurological deficit; 1, (failure to extend left forepaw fully) indicated a mild focal neurological deficit; 2, (circling to the left) indicated a moderate focal neurological deficit and 3, (falling to the left)

indicated a severe focal deficit. Rats with a score of 4 did not walk unaided and had a decreased level of consciousness. A total of 90 rats with a score of 1-3 were considered as suitable models of MCAO and were used in further experiments.

Bone marrow-derived MSCs culture. MSCs were harvested from 15 SD rats (weight, 80-120 g). Primary MSCs were isolated from the bone marrow of the tibias and femurs of the aforementioned rats by their adherence to plastic *in vitro* culture. At 3 days following MCAO, the rats were sacrificed by cervical dislocation following anesthetization by intraperitoneal injection with 10% chloral hydrate (0.35 ml/100 g). Under sterile conditions, tibias and femurs were harvested, the adherent soft tissue was removed and the ends of the bones were excised toward the start of the marrow cavity. Fresh bone marrow was harvested aseptically by flushing the cavity of the bone with needles filled with Dulbecco's modified Eagle's medium-low glucose (DMEM-LG; HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone). A single cell suspension was prepared by gentle pipetting several times and passing the cell suspension through a 200 mesh metal strainer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The cells were seeded into each tissue culture flask at a density of 10^6 cells/ml and cultured in an incubator (Forma Scientific; Thermo Fisher Scientific, Inc.) containing 5% CO_2 at 37°C . After 48 h, non-adherent cells were removed and the medium renewed and changed every 2 days thereafter. Once cells reached ~90% confluency, as determined by phase contrast microscopy (Eclipse 80i; Nikon Corporation, Tokyo, Japan), they were passaged into culture flasks at a 1:2 ratio.

Cell labeling. In order to monitor the distribution of bone marrow-derived MSCs in rat brains, 5-(and 6)-carboxy-fluorescein diacetate succinimidyl ester (CFSE; Fluka, Buchs, Switzerland) was used to label the MSCs. Briefly, after trypsinization (Invitrogen; Thermo Fisher Scientific, Inc.), cells at passage 3 were washed twice and resuspended in 200 μl DMEM. The cell suspension was then mixed with 200 μl labeling solution in a tube for 10 min at 37°C , with periodical tapping of the tube. The reaction was terminated by the addition of FBS, and the CFSE labeled cells were washed twice and resuspended in 0.01 ml PBS. Cell viability was evaluated by Trypan blue staining (Sigma-Aldrich). Only single cell suspensions with 95% viability were used for implantation. Approximately 3×10^6 cells/ml of MSCs in 1 ml PBS were transplanted into each rat.

Experimental groups and transplantation. All rats ($n=90$) were randomly divided into 2 groups at 24 h following the induction of MCAO or sham operation: MCAO group ($n=40$), and MSCs group ($n=50$). Rats in the MCAO group received 1 ml 0.01 M PBS via an intravenous injection. Rats in the MSCs group received CFSE-labeled 3×10^6 MSCs in 1 ml 0.01 M PBS via an intravenous injection. All transplantation procedures were performed under aseptic conditions and rats that received transplantation were selected at random.

Distribution of CFSE-labeled bone marrow derived MSCs. Three days subsequent to the establishment of MCAO,

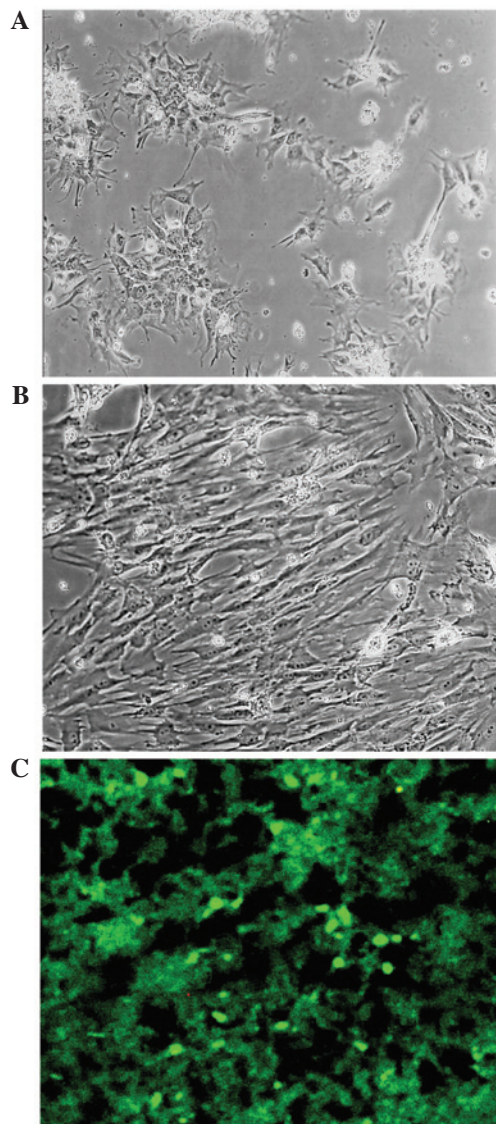


Figure 1. Morphology of primary and passage 3 cultures of bone marrow derived mesenchymal stem cells (MSCs). (A) MSCs formed colonies following 14 days of primary culture, at 70% confluence (magnification, x200). (B) MSCs at passage 3 with 80-90% confluence. Cells exhibited a fibroblast-like morphology (magnification, x200). (C) Distribution of 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled MSCs in an ischemic rat brain. CFSE-labeled MSCs migrated to rat brain and survived in the ischemic brain.

10 rats in the MSCs group were sacrificed, and their brains were removed and stored in liquid nitrogen. Coronal blocks (2 mm) containing brain tissues were cut anterior and posterior to the optic chiasm, and cryostat sections were prepared. Subsequently, the survival and distribution of CFSE-labelled MSCs were observed using an inverted fluorescence microscope (Eclipse 80i).

Behavioral testing. Modified neurological severity scores (mNSS) described by Chen *et al* (9) were employed to evaluate neurological deficits in rats. In all rats, a series of NSS tests was performed on days 3, 7 and 14 following the establishment of MCAO by 2 investigators who were blind to the experimental groups. Neurological function was scored on a scale of 0 to 11 (normal score, 0; maximal deficit score, 11). The

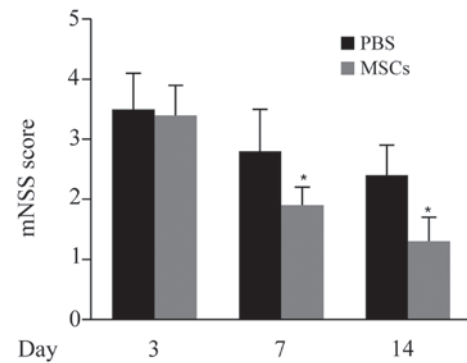


Figure 2. Assessment of cerebral ischemia-induced neurological deficits by modified neurological severity score (mNSS) test. Animals were either in the PBS or MSCs group and received 1 ml 0.01 M PBS, or were subjected to MCAO through the transplantation of cultured MSCs. At days 7 and 14, the mNSS scores of the MSCs group were significantly lower compared with those of the PBS group. The transplantation of MSCs promoted the functional recovery of the CNS following cerebral ischemia. *P<0.05 vs. PBS group.

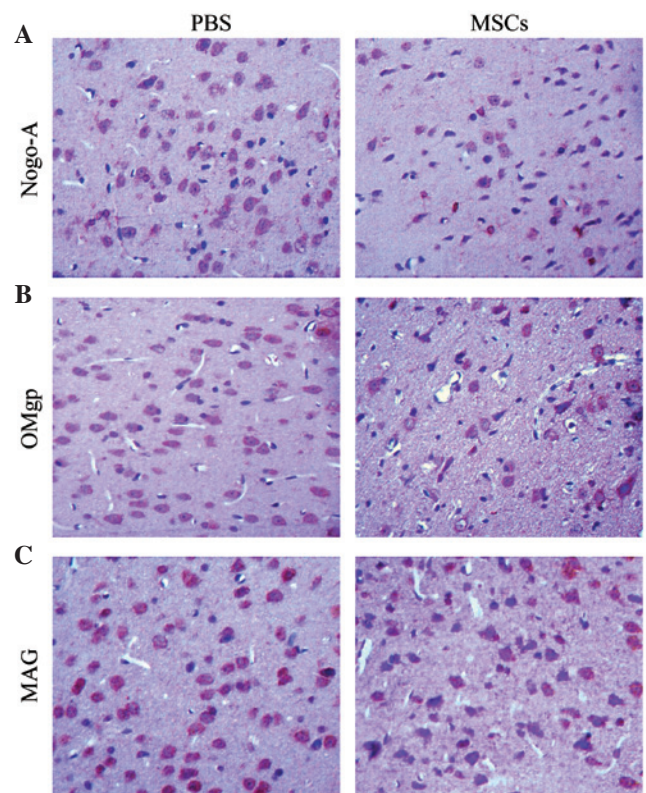


Figure 3. Expression levels of myelin associated inhibitors Nogo-A, OMgp and MAG in ischemic rat brains. Immunohistochemical staining of (A) Nogo-A, (B) OMgp and (C) MAG, in middle cerebral artery occlusion and MSCs groups at day 14 after MCAO (magnification, x200; n=10 per group). OMgp, Oligodendrocyte myelin glycoprotein; MAG, myelin-associated glycoprotein; PBS, phosphate-buffered saline; MSCs, mesenchymal stem cells.

severity of injury was scored by awarding 1 point each time the rat was unable to perform the assessment or for the lack of a tested reflex; thus, the higher the score, the more severe the injury. This modified version of mNSS is composite of the motor, sensory and reflex assessments.

Immunohistochemical assessment. Rats were anesthetized with 10% chloral hydrate (0.35 ml/100 g, intraperitoneal

injection). The rat brains were removed and were postfixed in 4% paraformaldehyde (Sigma-Aldrich) for 24 h, and embedded in paraffin. Sections were deparaffinized and incubated with 3% H₂O₂ for 10 min to block endogenous peroxidase activity. Brain sections were placed in citrate buffer (pH 6) at 80 kPa for 2 min. Tissues were treated with primary antibodies and incubated at 4°C for 12 h. Tissues were incubated at room temperature for 20 min with horseradish peroxidase-labeled goat anti-rat IgG antibody (1:200; cat. no. ZB2307; ZSGB-BIO, Beijing, China). Stable 3,3'-diaminobenzidine (ZSGB-BIO) was then used as a chromogen for light microscopy (Eclipse 80i). Counterstaining of sections was performed with hematoxylin and eosin (Shanghai BetterBioChem Co., Ltd., Shanghai, China). The primary antibodies used in the study were as follows: Rabbit anti-Caspase-3 polyclonal antibody (1:100; PA5-16335; LabVision/Neomarkers; Thermo Fisher Scientific, Inc.), rabbit anti-Bcl-2 antibody (1:80; sc-492; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), rabbit anti-Nogo-A antibody (1:30; PA1060; Wuhan Boster Biological Technology, Ltd., Wuhan, China), rabbit anti-OMgp antibody (1:80; BS-1200R; BIOSS, Beijing, China) and rabbit anti-MAG antibody (1:100; BS-0257R; BIOSS). Photomicrographs were acquired at x200 magnification under an inverted microscope (Eclipse 80i). The distribution of Nogo-A, OMgp, MAG, Caspase-3, in addition to Bcl-2 expression levels, were assessed by counting the number of positively stained cells in 10 distinct regions of ischemic penumbra in each section.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from the rat brain tissue was extracted using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Brain tissue was homogenized in ~1 ml TRIzol reagent. After homogenization, insoluble material from the homogenate was removed by centrifuging at 10,387 x g for 10 min at 4°C. Following isopropyl alcohol precipitation, total RNA was treated with DNase (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol, in order to remove contaminating DNA. cDNA was synthesized from 1 µg total RNA using the SuperScript III Reverse-Transcriptase kit (Invitrogen; Thermo Fisher Scientific, Inc.) in a final volume of 20 µl. The reactions were incubated in 96-well optical plates at 95°C for 10 min, followed by 40 cycles of 2 min at 95°C, 45 sec at 54°C, 45 sec at 68°C, and extension at 68°C for 8 min. qPCR was performed using the SYBR Prime Script kit (Invitrogen; Thermo Fisher Scientific, Inc.) and the Applied Biosystems 7500 Fluorescent Quantitative PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primer sequences used were as follows: Caspase-3 forward, 5'-CATGGCCCTGAAATACGAAGTC-3' and reverse, 5'-CCG TCCCTTGAATTTCTCCAG-3'; Bcl-2 forward, 5'-GGGAGA TCGTGATGAAGTACATCC-3' and reverse, 5'-AGTTCC ACAAAGGCATCCCAG-3'; β-actin forward, 5'-AAGAGA GGCATCCTGACCCTG-3' and reverse, 5'-TCCAGACGC AGGATGGCATG-3' (GenePharma Co., Ltd., Shanghai, China). Relative quantification was performed using the 2^{-ΔΔC_q} method (24), with normalization to the β-actin gene. As a negative control, RT-qPCR was performed using all reagents except for the template, and for an RT-minus control, RT-qPCR was conducted in the absence of reverse transcriptase.

Table I. Nogo-A-positively stained cells at different time points (n=10).

Group	Day 3	Day 7	Day 14
PBS	31.02±3.85	25.07±3.00	52.28±4.55
MSCs	24.97±4.20 ^a	21.93±2.68	40.77±6.68 ^a

^aP<0.05 vs. PBS group. PBS, phosphate-buffered saline; MSCs, mesenchymal stem cells.

Table II. Oligodendrocyte myelin glycoprotein-positively stained cells at different time points (n=10).

Group	Day 3	Day 7	Day 14
PBS	32.60±5.14	40.87±3.12	46.02±3.35
MSCs	30.42±3.58	31.22±3.22 ^a	32.55±5.94 ^a

^aP<0.05 vs. PBS group. PBS, phosphate-buffered saline; MSCs, mesenchymal stem cells.

Statistical analysis. Statistical analyses were performed using the SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). Statistical significance of mNSS was analyzed using one-way analysis of variance. All data are presented as the mean ± standard deviation and analyzed for statistical significance by Pearson's χ^2 test. P<0.05 was considered to indicate a statistically significant difference.

Results

Morphology and MSC migration. MSCs were collected from the bone marrow of femurs and tibias of SD rats and were evaluated morphologically using a phase contrast microscope. Subsequent to 14 days of culture, primary bone marrow derived MSCs reached 70% confluence and exhibited a fibroblast-like morphology (Fig. 1A). The third passage of MSCs also exhibited a fibroblastic morphology and reached 80-90% confluency after 7 days of culture (Fig. 1B). A model of MCAO was then established in rats to detect the effect of transplantation of MSCs by intravenous injection to cerebral ischemia. Green fluorescent CFSE-labeled MSCs were observed 3 days following the establishment of MCAO using a fluorescence microscope. CFSE-labeled MSCs migrated to the brains of rats and survived in the ischemic brain (Fig. 1C).

Evaluation of neurological deficit. The cerebral ischemia-induced neurological deficits were determined by mNSS assessments at 3, 7 and 14 days. As displayed in Fig. 2, no significant differences in mNSS scores were observed between the MSCs group and the PBS group on day 3. However, on days 7 and 14, the mNSS scores of the MSCs group were significantly lower compared with those of the PBS group (P<0.05), indicating that the transplantation of MSCs promoted the functional recovery of the CNS following cerebral ischemia.

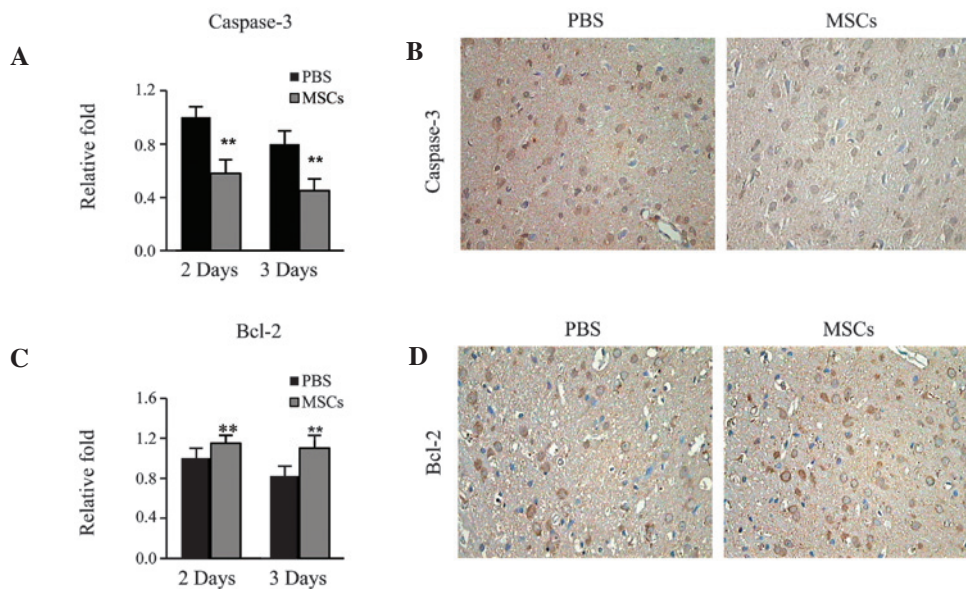


Figure 4. Evaluation of Caspase-3 and Bcl-2 expression levels in ischemic rat brains. (A) Relative mRNA expression levels of Caspase 3 on days 2 and 3 after the establishment of middle cerebral artery occlusion (MCAO) in the PBS and MSCs groups. Data are presented as the mean \pm standard deviation (SD). * $P < 0.05$ vs. PBS group. (B) Immunohistochemical staining of Caspase-3 on day 3 following the establishment of MCAO in PBS and MSCs groups (magnification, $\times 200$). (C) Relative mRNA expression levels of Bcl-2 on days 2 and 3 following the establishment of MCAO in PBS and MSCs group. Data are presented as the mean \pm standard deviation. ** $P < 0.01$ vs. PBS group. (D) Immunohistochemical staining of Bcl-2 on day 3 after MCAO in PBS and MSCs group (magnification, $\times 200$). $n = 10$ in each group at each time point. PBS, phosphate-buffered saline; MSCs, mesenchymal stem cells; Bcl-2, B-cell lymphoma 2.

Expression of Nogo-A, MAG and OMgp. As Nogo-A, MAG and OMgp are involved in the prevention of CNS regeneration following brain injury, we subsequently investigated whether transplantation of MSCs was able to affect their cellular expression. The MSCs group displayed a significantly reduced number of Nogo-A positive cells compared with the PBS group on days 3 and 14 ($P < 0.05$); however, the difference was not significant on day 7 (Fig. 3 and Table I). With regard to OMgp, the PBS and the MCAO group displayed a similar number of OMgp-positive cells on day 3. However, the MSCs group displayed a significantly lower number of OMgp-positive cells compared with the MCAO group on days 7 and 14 ($P < 0.05$; Table II). Furthermore, the MSCs group displayed a significantly lower number of MAG-positive cells compared with the MCAO group between days 3 and 14 ($P < 0.05$; Table III). The aforementioned results suggest that the transplantation of MSCs reduced the cellular expression levels of Nogo-A, OMgp and MAG and thus contributed to the functional recovery of the CNS following cerebral ischemia in rats.

Expression of Caspase-3 and Bcl-2. Finally, it was determined whether the transplantation of MSCs was able to affect cell apoptosis, which is crucial factor in the functional recovery of CNS after cerebral ischemia. MSCs group had significantly lower Caspase-3 mRNA expression levels compared with the MCAO group on days 2 and 3 ($P < 0.01$; Fig. 4A and B). Conversely, the MSCs group displayed significantly higher Bcl-2 expression levels compared with the MCAO group on days 2 and 3 ($P < 0.01$; Fig. 4C and D). Thus, the present results indicate that the transplantation of MSCs is able to reduce apoptosis, which was likely to have accounted for the functional recovery of the CNS following cerebral ischemia.

Table III. Myelin-associated glycoprotein-positively stained cells at different time points ($n = 10$).

Group	Day 3	Day 7	Day 14
MCAO	68.48 \pm 6.35	66.70 \pm 5.01	67.32 \pm 6.69
MSCs	51.68 \pm 7.21 ^a	55.28 \pm 9.91 ^a	53.52 \pm 7.70 ^a

^a $P < 0.05$ vs. the MCAO group. MCAO, middle cerebral artery occlusion; MSCs, mesenchymal stem cells.

Discussion

In the present study, the effect of transplantation of bone marrow derived MSCs on functional recovery of CNS after brain ischemia in a rat MCAO model was assessed, and the possible underlying mechanisms investigated.

Initially, it was confirmed that transplantation of MSCs provided therapeutic benefit to cerebral ischemia through the establishment of a rat model of MCAO. MSCs were isolated from rats and injected intravenously into rats with MCAO. The MSCs were able to survive and migrate to the damaged brain, and the transplantation of MSCs significantly improved the functional recovery of the CNS.

The possible mechanisms by which transplantation of MSCs promoted the functional recovery of the CNS following cerebral ischemia were then examined. The present study determined that transplantation of MSCs reduced the expression of axon regeneration inhibitors, including Nogo-A, MAG and OMgp. Furthermore, transplantation of MSCs inhibited neuronal apoptosis, decreasing Caspase-3 and increasing Bcl-2 protein expression levels. The two mechanisms function

synergistically to promote the functional recovery of the CNS following cerebral ischemia.

The underlying mechanisms by which transplantation of MSCs reduces the expression of axon regeneration inhibitors and inhibits neuronal apoptosis have yet to be elucidated. It has previously been confirmed that MSCs constitutively secrete several growth factors, including nerve growth factors (NGF), brain derived neurotrophic factors (BDNF) and glioma derived neurotrophic factors (GDNF) (25). Exogenous NGF has an important role in neuronal plasticity and regenerative potential, in addition to the inhibition of neural apoptosis (26). BDNF is able to support the survival of injured CNS neurons *in vitro* and *in vivo* and inducing neurite outgrowth (27). GDNF is able to protect against neuronal death after brain injury (28,29). Therefore, it is plausible that MSCs secrete these growth factors, which in turn reduce the expression of axon regeneration inhibitors and inhibit neuronal apoptosis.

In conclusion, the present study revealed that transplantation of bone marrow derived MSCs promotes the functional recovery of the CNS subsequent to cerebral ischemia through decreasing the expression of myelin-associated inhibitors (MAIs) and inhibiting neuronal apoptosis. We propose that inhibitors targeting the aforementioned MAIs and the apoptotic pathway may have potential for the treatment of cerebral ischemia.

Acknowledgements

This work was supported by grants from the Science and Technology Project of the Education Department of Heilongjiang Province (grant nos. 12531311 and 12521336) and the Science Project of the Health Department of Heilongjiang Province (grant no. 2010-095).

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