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Original Article

Spinal cord injury in rats treated using bone marrow mesenchymal stem-cell transplantation

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Abstract: The aim of this study was to observe the effects of bone marrow mesenchymal stem-cell transplantation (BMSCs) in repairing acute spinal cord damage in rats and to examine the potential beneficial effects. 192 Wistar rats were randomized into 8 groups. Spinal cord injury was created. Behavior and limb functions were scored. Repairing effects of BMSCs transplantation was evaluated and compared. In vitro 4',6-diamidino-2-phenylindole (DAPI)-tagged BMSCs were observed, and whether they migrated to the area of spinal cord injury after intravenous tail injection was investigated. The expression of neuron-specific protein (NSE) on BMSCs was examined. Fifteen days after transplantation, the BMSCs-treated groups scored significantly higher in limb function tests than the untreated group. Pathological sections of the bone marrow after operation showed significant recovery in treated groups in comparison to the control group. After transplantation, small amounts of fluorescent-tagged BMSCs can be found in the blood vessels in the area of spinal cord injury, and fluorescent-tagged BMSCs were diffused in extravascular tissues, whereas the DAPI-tagged BMSCs could not be detected,and BrdU/NSE double-labeled cells were found in the injured marrow. BMSCs improve behavioral responses and can repair spinal cord injuries by migrating to the injured area, where they can differentiate into neurons.

Keywords: Bone marrow-derived mesenchymal stem cells, spinal cord injury, transplantation, mechanism

Introduction

Spinal cord injury is a devastating condition, and there are no effective treatments to date; treatment by stem cell transplantation is one of the most promising methods [1-4]. Bone Mesenchymal Stem Cells (BMSCs) have received extensive attention, because of their multidirectional differentiation potential, rapid proliferation rate, they can be easily obtained, and are less likely to be rejected [5, 6]. Several experimental studies have shown that BMSCs transplantation in treating spinal cord injury has shown some therapeutic effects [7-12]. The present study aimed to analyze the repairing effects of BMSCs transplantation in spinal cord injury by assessing behavioral and histological data.

Materials and methods

Animals and grouping

One hundred ninety-two female Wistar rats, weighing 260 ± 20 g, were purchased from the Experimental Animal Center of Jilin University. Animals were randomized into eight groups as follows: (i) A1: control group, untreated; (ii) A2: operation control group, no treatment after spinal cord injury; (iii) A3: BMSCs vein transplantation group, where BMSCs were injected via tail vein at day 0; (iv) A4: BMSCs local transplantation group, BMSCs were locally injected at the area of injury at day 0; (v) B1: the control group, DAPI-tagged BMSCs were injected via tail vein; (vi) B2: BMSCs vein-transplantation group, where BMSCs were injected via tail vein after model establishment; (vii) B3: BMSCs vein-transplantation, where BrdU-tagged BMSCs were injected via tail vein after model establishment; and (viii) B4: BMSCs local transplantation group, where BrdU-tagged BMSCs were injected via tail vein after model establishment. Thirty rats were included in each of the four A groups, and 18 rats were included in each of the four B groups. Additional animals were added the moment any rat died. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use

of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Yanbian University.

Major equipment and reagents

For the execution of these experiments we used a MC01751IF type CO_2 incubator (SANYO, Japan), and a super-clean bench (Antai Company, Suzhou Jinghua Group). Immunofluorescent-tagged BMSCs and staining were visualized under an IX-71 inverted microscope (OLYMPUS Company, Japan). For in vitro experiments, we used DMEM culture medium (Gibco Company of USA), fetal bovine serum (Hyclone Company, USA), and 0.25% trypsin (Huamei Company). For immunohistochemistry, we stained the tissues using a rabbit anti-rat CD34 fluorescence-tagged antibody (BD Company, USA) and anti-BrdU. For ELISA, we used an ELISA kit (Shanghai Hufeng Chemical Co., Ltd.). Finally, BMSCs were tagged using 4,6-diamidine-2-phenylindole dihydrochloride (Shanghai Hufeng Chemical Co., Ltd.).

BMSCs extraction, isolation, and culture Five five-day old Wistar pups were selected (8.0-10.0 g) and killed by cervical dislocation, and sterilization with Iodophors was carried out three times. Bone marrow cells from femoral bones and shin bones of Wistar rats, were collected by using aseptic techniques, and BMSCs were isolated and purified by combining adherence screening and differential adhesion methods, and the cells were cultured by the adherence culture method [13]. Primary cultures were trypsinized after they reached 70-80% confluency. The cells were then subcultured to the third generation in a proportion of 1:3.

Establishment of spinal cord injury model

Rats in the test groups were used to establish the spinal cord injury model of rats by using modified Allen's impact method [14]. Spinal cord injury was induced at T12 vertebra (center of the impact area), and the size of the lesion was 3.0 mm × 4.0 mm. The impact was executed six times until both posterior limbs of the rat twitched, and their tails flicked, until complete relaxation of the limbs was observed. Subsequently, the rats were awakened and nursed in cages using conventional recovery methods. The animals received intramuscular injections of penicillin at a dose of 200 KU/kg body weight, once a day for 4 consecutive days. Urine was squeezed out of the animals twice daily, at 8 a.m. and 4 p.m.

In vitro labeling of BMSCs with DAPI

BMSCs were collected after the third passage, were rinsed with DMEM twice. We then added 10 mL DAPI application solution, and the cells were placed in an incubator at 37°C with 5% CO_2. DAPI was discarded 1 h later, the cells were rinsed with DMEM twice, followed by DMEM containing 15% fetal bovine serum, and the cells were further cultured. The cells were observed under a fluorescence microscope 24 h later and were collected successful labeling was confirmed. Cell concentration was adjusted to 1×10^6 cells/mL.

BMSCs were collected 2 days prior to transplantation after they were passaged for the third time, and had reached 70% confluency. The culture medium was discarded, and was replaced by fresh medium containing 10 μM BrdU, followed by for an additional 48-h incubation. The medium was discarded, and the cells were collected; cell concentration was adjusted to 1×10^6 cells/mL for further use [15].

BMSCs transplantation

A3, B2, and B3 groups were subjected to BMSCs transplantation on the day of spinal cord injury [8, 16-19]. The rats were anesthetized by i.p. injection of 0.3% pentobarbital sodium (30 mg/kg). A 1-mL disposable sterilized syringe of was used for the tail venipuncture, and 1-mL BMSCs, with a concentration of 1×10^6 cells/mL, was slowly injected for 2 consecutive days. BMSCs vein graft was performed simultaneously for the B1 and the B2 groups.

BMSCs cell suspension was slowly injected into the juncture (1 mm away from cord surface) of gray and white matter near the head and caudal sides of spinal cord injury, along the postcentral sulcus, by using a microinjector in an angle of about 45° (the slope of the needle was kept downward) in the A4 and B4 groups before suturing the wound after spinal cord injury. Five microliters of solution with cell concentration of 4×10^7/mL were injected at each point, thus, the total cell number that was injected for each rat was approximately 4×10^5. After injection of BMSCs, the needle was kept in place for 5 min,

Table 1. Tarlov scoring for the recovery of motor functions of Wister rats after BMSCs transplantation

Afteroperation, s		Score of 7 day	Score of 15 d	Score of 30 days
A1	5 ± 0	5 ± 0	5 ± 0	5 ± 0
A2	0 ± 0	1.25 ± 0.87	1.45 ± 0.79	1.91 ± 1.00
A3	0 ± 0	2.00 ± 0.60	2.41 ± 0.51 (1)	2.41 ± 0.90
A4	0 ± 0	1.33 ± 0.65	2.25 ± 0.45 (1)	2.75 ± 0.75 (1)

Note: (1) In comparison to the operation control group, $P < 0.01$.

and medical grade fibrin sealant was used to seal the injection holes.

Behavioral observations

Behavioral grades were assessed as follows: grade 0: no movement in posterior limbs and rats cannot be subjected to weight loading; grade 1: movement of the posterior limbs, but they cannot be subjected to weight loading; grade 3: posterior limbs can support their body weight and rats can walk 1-2 steps; grade 4: rats can walk and only suffer minor distress; grade 5: rats walk normally.

The observers were not experimenters, thus they were blinded to animal grouping, but they were very familiar with the scoring criteria. Since there is a significant difference in nyctohemeral activities in rats, the scoring was normally carried out at 9 a.m. and the rats were observed for 4 min and recorded using a camera. Their bladders were massaged to facilitate urination every 8 h, 3 days before the transplantation. All of the animals were examined to ensure the presence of an empty bladder, in order to avoid any influence of a filled bladder on their activities.

Statistical analysis

Twelve rats each were randomly selected from A1, A2, A3, and A4 groups, and behavioral scoring was performed 1, 7, 15, and 30 days after the transplantation. The measurement data are presented as mean ± standard deviation and t-test was performed using SPSS13.0 statistical software (SPSS Inc, Chicago, IL, USA). The scores of different groups at different time points were compared to determine whether any difference existed.

Histological observations

After behavioral observations on days 7, 15, and 30 post transplantation, six rats were randomly selected and killed from A1, A2, A3, and A4 groups. After the bodies became rigid, tissues from 0.5 cm above and below the area of spinal cord injury were collected for specimen preparation. The specimens were fixed in a 4% paraformaldehyde-phosphoric acid fixing solution, were then subjected to paraffin imbedding, and 5-μm thick sections were cut after dehydration. Four sections from the injured area were selected for each spinal cord specimen with an the interval of 2 mm. Hematoxylin and eosin HE staining was carried out on one section from the injured area of each rat as previously described [20-24].

Immunohistochemical staining was performed. Sections from B1 and B2 groups were subjected to air-drying for 30 min and then transferred into a wet box, where they were fixed with 70% ethanol for 10 min and then wiped dry. They were, then, digested with 1% gastric enzyme for 10 min and rinsed with 0.01 M phosphate buffer saline (PBS) twice, 10 min each time. Sections were blocked with normal goat serum for 30 min at room temperature, followed by incubation with rat anti-BrdU monoclonal antibody at 37°C for 30 min. Tissues were rinsed with 0.01 M PBS and wiped dry. Sections were then incubated with rabbit anti-human NSE at 37°C for 30 min, followed by rinses with 0.01 M PBS. Secondary antibodies (rhodamine tagged goat anti-mouse IgG and FITC tagged goat anti-rabbit IgG, 1:50) were added in the dark, and sections were kept at 37°C for 30 min, followed by PBS washes, and wiped dry. Glycerol-buffered mounting medium (pH 9.0) was used for mounting the sections in the dark.

Morphological changes in the sections from different groups at 7, 15, and 30 days after transplantation were observed, and compared under a light microscope. The distribution of DAPI-positive cells in the control groups and the treatment groups at 5, 10, and 15 days post transplantation were compared under a laser scanning confocal microscope. Similarly, BrdU/NSE double-positive fluorescent cells were examined on sections from animals sacrificed 10, 20, and 30 days after transplantation.

Results

Behavioral observations

No sign of recovery was found in A2, A3, and A4 groups 1 day after injury and the movement

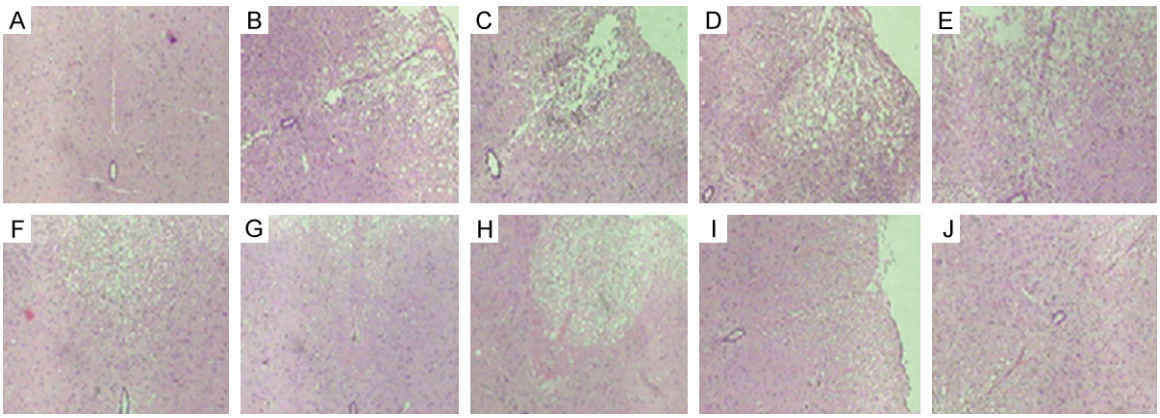

Figure 1. Histopathological sections for spine cord tissues of rats from different groups (HE, × 40).

scores were all 0, which were lower than that of A1 (5 ± 0). Motor functions in A3 and A4 groups showed recovery 7 days after the spinal cord injury, and the behavioral scores were 2.00 ± 0.60 and 1.33 ± 0.65 respectively, slightly higher than that observed for the A2 group (1.25 ± 0.87). Motor functions of A3 and A4 groups showed recovery 15 days after injury, and the behavioral scores were 1.41 ± 0.51 and 2.25 ± 0.45 respectively. These values are significantly higher in relation to A2 group (1.45 ± 0.79, $P < 0.01$). Motor functions of the rats in the A3 group further recovered 30 days after injury, achieving a behavioral score of 2.41 ± 0.90, which was slightly higher compared to the A2 group 1.91 ± 0.55, but not statistically significant. Motor functions in rats of the A3 and A4 groups further recovered reaching a behavioral score of 2.75 ± 0.75, which was significantly higher than that of the A2 group (1.91 ± 0.55, $P < 0.01$), when the two groups were compared, and the difference was statistically significant (**Table 1**).

Pathological observations

The structure of gray and white matter in the spinal cord of rats from A1 group was intact; nerve cells were evenly distributed in these areas showing normal morphology. The Nissl bodies were clear and the cell membranes were intact, nerve fibers in the white matter was arranged regularly, and the intercellular substance was uniformly distributed (**Figure 1A**). Sections from spinal cord tissues from the A2 group, showed that the injured area was mainly located in the gray matter of the spinal cord, and inflammatory cell infiltration was seen in the area of injury and surrounding areas 7 days after injury; cavity-like changes appeared in somewhat injured spine cord tissues (**Figure 1B**). A large area of softening focus appeared in the position of lesions on day 15; hemorrhagic foci can still be detected at this stage and nerve cells had disappeared (**Figure 1E**). Edema in the white matter was alleviated by day 30, in comparison to previous examination, and glial cell proliferation was evident (**Figure 1H**). Edema in tissues from A3 and A4 groups was alleviated by day 7. Inflammatory cell infiltration was reduced, vacuolar degeneration was decreased, the morphology of nerve cells had recovered and structures appeared normal (**Figure 1C**, **1D**). The morphology of nerve cells had recovered by day 15, and structures looked better sustained in comparison to day 7 (**Figure 1F**, **1G**). Nerve cells and structures appeared healthier by day 30, compared to day 15 (**Figure 1I**, **1J**).

Migration of BMSCs

Fluorescent-tagged BMSCs appeared in blood vessels 5 days after transplantation in the B2 group (**Figure 2A**). Fluorescent-tagged BMSCs were scattered in blood vessels of injured and surrounding tissues by day 10 (**Figure 2B**), and extensive diffusion of fluorescent-tagged BMSCs was found in the injured tissues by day 15 (**Figure 2C**). DAPI-tagged BMSCs were not found in the B1 group at corresponding time points.

Double labeling of neuron specific protein

Sections were observed under a laser scanning confocal microscope, and BrdU/NSE double labeled cells could be found in B3 and B4

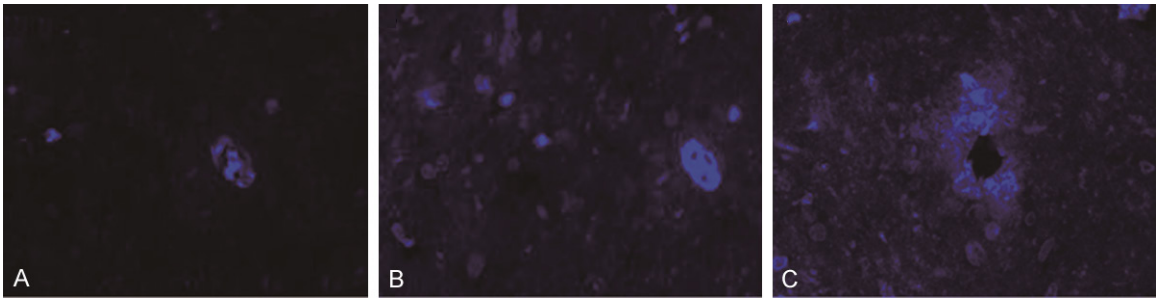

Figure 2. Migration of BMSCs in B2 group at different time points (DAPI, × 400).

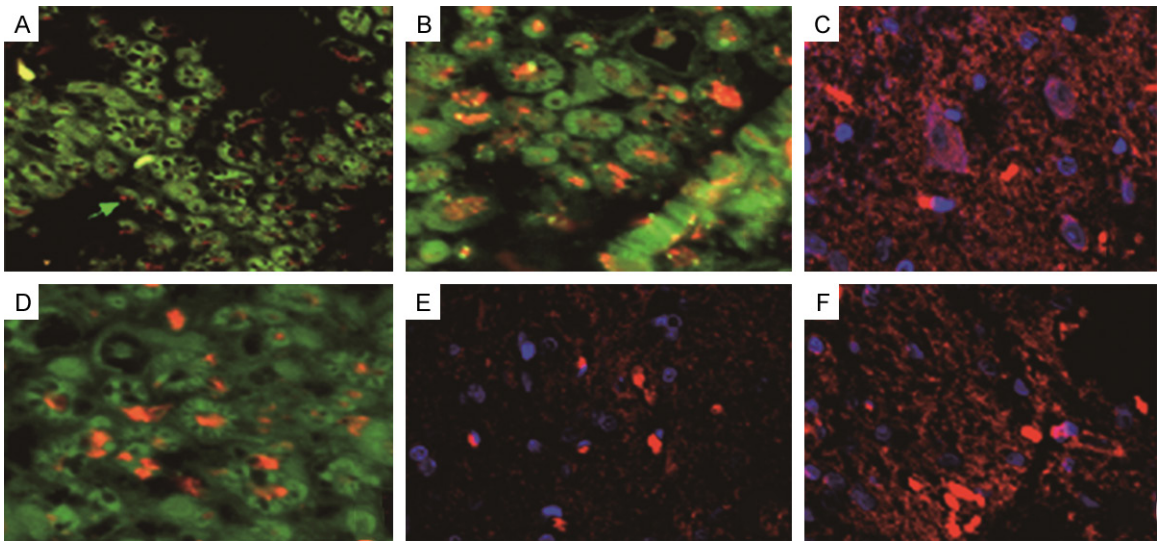

Figure 3. Observations on injured spine cord tissues under a fluorescence microscope (BrdU, DAB, ×400). A. B3 group 10 days; B. B3 group 20 days; C. B3 group 30 days; D. B4 group 10 days; E. B4 group 20 days; F. B4 group 30 days.

groups, 10, 20, and 30 days after BMSCs transplantation (**Figure 3**).

Discussion

Chopp et al. [25] were the first to transplant BMSCs into the injured spinal cord of rats and showed significant recovery in motor functions. Morphological analysis showed that neuronal and oligodendroglial protein markers are expressed in BMSCs. BMSCs have also demonstrated relatively high migratory abilities; Satake et al. [26] utilized adenovirus vector (AAV) to transfect GFP into BMSCs, and then the cells were injected into the cerebrospinal fluid of rats that suffered from spinal cord injury. The distribution of GFP-positive cells was regularly detected. The results showed that most of the transplanted cells migrated to the surrounding areas of lesions, neuronal and glial markers were expressed in some of the transplanted BMSCs, and motor functions of these rats were improved [27]. Zurita and Vaquero [27] transplanted BMSCs in a model of spinal cord contusion after a 3-month injury, and recovery of spinal cord functions, that lasted for 1 year, was observed 4 weeks after the transplantation. The transplanted BMSCs formed bridges in the cavity of the spinal cord, which provided support for regenerated axons, and expressed marker proteins of astrocytes and neurons. Hofstetter et al. [28] carried out MSC transplantation in the spinal cord of animal models that suffered spinal cord injury; it was shown that the gait of these animal significantly improved after 1 week, and penetration of newly born nerve fiber bundles could be observed between the transplantation area and the scar tissue area, 5 weeks after transplantation.

Results from the present study showed that the motor functions of animals that sustained spinal cord injury significantly improved 2 weeks after BMSCs transplantation was applied to rats. Histological observations showed that edema was reduced 7 days after the treatment, inflammatory cell infiltration decreased, vacuolar degeneration improved, the morphology of nerve cells appeared normal, and intracellular structures were evenly arranged. The recovery of nerve cells and structural arrangement was observed by day 15 as compared to tissues examined 7 days post transplantation, indicating that BMSCs transplantation into the area of spinal cord injury can promote repair and regeneration of the injured spinal cord. This experiment also demonstrated that DAPI-tagged BMSCs can penetrate the blood-spinal cord barrier and migrate to the injured spinal cord tissues after tail vein injection. The migratory BMSCs reached their target area by day 15 post transplantation; furthermore, surviving DAPI positive-tagged cells were expressing the neuronal marker, NSE, indicating that BMSCs can migrate to the injured area of the spinal cord utilizing cell-cell contact mechanisms, and promote repair by transforming into neurons. Nonetheless, the migratory mechanism of BMSCs to the area of injury needs further investigation.

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Disclosure of conflict of interest

None.

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