Electrophysiological functional recovery in a rat model of spinal cord hemisection injury following bone marrow-derived mesenchymal stem cell transplantation under hypothermia*

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Abstract
Following successful establishment of a rat model of spinal cord hemisection injury by resecting right spinal cord tissues, bone marrow stem cells were transplanted into the spinal cord lesions via the caudal vein while maintaining rectal temperature at 34 ± 0.5°C for 6 hours (mild hypothermia). Hematoxylin-eosin staining showed that astrocytes gathered around the injury site and formed scars at 4 weeks post-transplantation. Compared with rats transplanted with bone marrow stem cells under normal temperature, rats transplanted with bone marrow stem cells under hypothermia showed increased numbers of proliferating cells (bromodeoxyuridine-positive cells), better recovery of somatosensory-evoked and motor-evoked potentials, greater Basso, Beattie, and Bresnahan locomotor rating scores, and an increased degree of angle in the incline plate test. These findings suggested that hypothermia combined with bone marrow mesenchymal stem cells transplantation effectively promoted electrical conduction and nerve functional repair in a rat model of spinal cord hemisection injury.

Key Words: bone marrow mesenchymal stem cells; electrophysiological function; hypothermia; spinal cord hemisection injury; transplantation

Abbreviations: BMSCs, bone marrow-derived mesenchymal stem cells; SCI, spinal cord injury; SEP, somatosensory-evoked potential; MEP, motor-evoked potential

INTRODUCTION

Bone marrow-derived mesenchymal stem cells (BMSCs) can differentiate into a variety of nervous system cells, thereby providing possible therapeutic strategies for nerve injury repair[1-6]. However, BMSC transplantation alone is not sufficient to repair spinal cord injury (SCI), because the majority of BMSCs transplanted into the spinal cord differentiate into glial cells and rarely survive[7]. The microenvironment of the injured spinal cord plays a crucial role in inducing differentiation and survival of transplanted BMSCs[8-11]. In recent years, hypothermia (33-35°C) has been widely studied as a beneficial approach due to its neuroprotective effects against secondary injury, providing hope for the treatment of brain injury and SCI[12]. The present study hypothesized that hypothermia influences differentiation and survival of transplanted BMSCs, as well as improves the microenvironment of the injured spinal cord. To test this hypothesis, the microenvironment was modified through hypothermia. The aim of the present study was to analyze the effects of BMSC transplantation in combination with hypothermia for the recovery of SCI in a rat model.

Electrophysiological tests, such as somatosensory-evoked potential (SEP) and motor-evoked potential (MEP), provide objective methods to monitor descending, ascending, and segmental pathways in the spinal cord[13-15]. These tests have supplemented the behavioral evaluation of neurological functions in humans and animals[16]. The technique of combined MEP and SEP recordings could potentially allow for the assessment of rats with or at risk for SCI, as well as assessment of treatments for experimental cord injury[17]. In the present study, SEP and MEP were utilized to continuously monitor spinal cord hemisection injury and subsequent treatment.

RESULTS

Quantitative analysis of experimental animals
Sixty-eight 3-month-old, Sprague-Dawley...
rats were included in the study for model establishment through resection of the right hemisphere of the spinal cord. However, eight rats died during SCI modeling. The remaining 60 rats were equally assigned to three groups ($n = 20$) for analysis: a model group, a BMSC + normal temperature group and a BMSC + mild hypothermia group. In the BMSC + normal temperature group and the model group, rectal temperature was maintained at $37 \pm 0.5^\circ$C. In the BMSC + mild hypothermia group, rectal temperature was maintained at $34 \pm 0.5^\circ$C, and BMSCs were transplanted via tail vein injection 6 hours later.

**Morphology of transplanted BMSCs**

The numbers of BMSCs and colonies significantly increased after 5 days in culture. After 1–3 passages, the cells actively proliferated, the majority of cells adhered to a monolayer with various forms such as spindle-shaped, oval-shaped, flat-shaped, triangular and irregular, and cells exhibited a strong refraction and extended more than two processes, some of which connected to each other. The nucleus and nucleolus remained intact and the cells grew in a confluent layer (Figure 1).

**Mild hypothermia and BMSC transplantation significantly improved histology at the injury site**

At 4 weeks after injury, spinal cord tissue breakage, scar connections, and structural disorder were visible at the affected site in the model group, as well as a clear cavity formation (Figure 2A). In the normal temperature + BMSC transplantation group, astrocytes aggregated at the edge of the affected site and formed scars at the junction of the intact spinal cord and damaged spinal cord; the tissue cavity was less than in the model group, but larger than in the mild hypothermia + BMSC transplantation group (Figure 2B). In the mild hypothermia + BMSC transplantation group, astrocytes produced reactive hypertrophy, aggregated, and formed scars at the edge of the affected site; some cells were spindle-shaped, with a dense network between processes, and the size of the cavity was reduced (Figure 2C). Immunohistochemical staining showed an increase in the number of bromodeoxyuridine (BrdU)-positive cells at the affected site in the mild hypothermia + BMSC transplantation group and normal temperature + BMSC transplantation group compared with the model group ($P < 0.05$, $P < 0.01$; Figure 3) at 4 weeks post-injury.

![Figure 1](image1.jpg)

**Figure 1** Morphology of bone marrow-derived mesenchymal stem cells (scale bars: 50 μm).

(A) After 2 days in culture, adherent cells are extended and appear spindle-shaped.

(B) After 7 days in culture, the cells grew around a clone.

(C) Cells from the third passage are fused together and arranged in a bunched or radiated shape.

![Figure 2](image2.jpg)

**Figure 2** Histological analysis of rats with spinal cord hemisection injury (hematoxylin-eosin staining; scale bars: 100 μm).

(BMCS: Bone marrow-derived mesenchymal stem cells.

(A) At 4 weeks after injury in the model group, the damaged spinal cord at the affected site exhibits clear cavity formation (arrow).

(B) In the normal temperature + BMSC transplantation group, astrocytes are aggregated at the edge of the affected site and scars formed at the junction between the intact and damaged spinal cord. The size of the tissue cavity (arrow) is less than in the model group but larger than in the mild hypothermia + BMSC transplantation group.

(C) In the mild hypothermia + BMSC transplantation group, astrocytes produce a reactive hypertrophy, aggregate and form scars at the edge of the affected site. In addition, the cavity is absent (arrow).
Mild hypothermia + BMSC transplantation improved electrophysiological function in rats

SEP and MEP testing results showed that both waveforms disappeared after the establishment of spinal cord hemisection injury. At 8 weeks post-injury, SEP and MEP measurements were slightly restored in the normal temperature + BMSC transplantation group. In addition, SEP and MEP levels were completely restored in the mild hypothermia + BMSC transplantation group, as well as increased volatility. SEP and MEP latency and amplitude are shown in Table 1.

Mild hypothermia + BMSC transplantation improved behaviors

Basso, Beattie, and Bresnahan (BBB) scores

Following injury, the rats exhibited full paraplegia, with no activity in the hind limb or tail, as well as urination dysfunctions without defecation dysfunction. Retraction to the puncture began to emerge at 1 week post-injury, hind limb movement occurred at 2 weeks post-injury and became clearer at 4 weeks. Hind limbs exhibited coordinated activities at 6 weeks, and urinary function was partially restored, although residual urine remained in the bladder. The three groups exhibited similar changes after injury. BBB scores in the normal temperature + BMSC transplantation and mild hypothermia + BMSC transplantation groups were greater than in the model group at 4 weeks post-injury ($P < 0.01$). BBB scores in the mild hypothermia + BMSC transplantation group were greater than in the normal atmospheric temperature + BMSC transplantation group ($P < 0.05$; Table 2).

Incline plate test

At 4-8 weeks post-injury, the tilt angle in the incline plate test was greater in the mild hypothermia + BMSC transplantation group than in the model group or normal temperature + BMSC transplantation group ($P < 0.05$, $P < 0.01$, respectively; Table 3).

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**Table 1** Somatosensory-evoked potential (SEP) and motor-evoked potential (MEP) in each group at 8 weeks post-transplantation

| Group                              | SEP          | MEP          |
|------------------------------------|--------------|--------------|
|                                   | Latency (ms) | Amplitude (μV) | Latency (ms) | Amplitude (μV) |
| Model                             | 35.31±1.421  | 1.33±0.132   | 15.73±0.421  | 1.72±0.154    |
| Normal temperature + BMSC         | 26.46±1.32±a | 1.75±0.18±a  | 11.85±0.347  | 2.45±0.282±a  |
| transplantation                   | 14.71±0.90±b | 2.12±0.23±b  | 7.82±0.19±h  | 4.15±0.27±h   |

Data are expressed as mean ± SD from eight rats in each group by analysis of variance and Dunnett’s t-test comparison. $^aP < 0.05$, $^bP < 0.01$, vs. model group; $^hP < 0.05$, vs. normal temperature + BMSC transplantation group.
DISCUSSION

In recent years, mild hypothermia (33-35°C) has received increased attention for the treatment of central nervous system injury. Clinical studies have shown that mild hypothermia effectively reduces secondary nerve injury and protects the brain and spinal cord against severe injury. The protective effects depend on the following mechanisms: reduced release of excitatory amino acids, inhibition of calcium influx, regulation of calmodulin kinase II and protein kinase C activity, inhibition of inflammatory response following cerebral ischemia, suppression of edema formation, decreased oxygen metabolic rate, decreased production of free radicals, and inhibition of necrosis and mitochondrial release of cytochrome C-induced neuronal apoptosis.[16-20]

The present study analyzed the effects of mild hypothermia combined with BMSC transplantation in a rat model of SCI. Histological and hematoxylin-eosin staining results exhibited typical neuronal-like morphological changes with a reduced cavity in the mild hypothermia + BMSC transplantation group at 4 weeks post-injury. Tissue repair was better than in the model group or normal temperature + BMSC transplantation group. Immunohistochemical staining was also utilized to determine the number of BrdU-positive neurons at the affected site in rats: mild hypothermia + BMSC transplantation group > normal temperature + BMSC transplantation group > model group. BBB scores and inclined plane test results were statistically significant at 4 weeks post-injury: mild hypothermia + BMSC transplantation group > normal temperature + BMSC transplantation group > model group.

| Group                                      | Time after modeling (week) | 1   | 2   | 4   | 6   | 8   |
|--------------------------------------------|---------------------------|-----|-----|-----|-----|-----|
| Model                                      | 0.54±0.15                 | 1.49±0.28 | 4.97±0.68 | 6.39±0.59 | 7.02±0.61 |
| Normal temperature+BMSC transplantation    | 0.53±0.12                 | 1.62±0.23 | 5.42±0.83° | 8.68±0.52° | 9.04±0.62° |
| Mild hypothermia+BMSC transplantation      | 0.54±0.11                 | 1.73±0.14 | 6.39±0.67° | 9.98±0.64° | 12.62±0.73° |

A higher score indicates better locomotion behavior. Data are expressed as mean ± SD from eight rats in each group by analysis of variance and Dunnett’s t-test comparison. °P < 0.01, vs. model group; °°P < 0.05, vs. normal temperature + BMSC transplantation group. BMSC: Bone marrow-derived mesenchymal stem cell.

MATERIALS AND METHODS

Design
A randomized, controlled, animal study.

Time and setting
The present study was performed between March 2009 and May 2011 at the Laboratory Animal Center of Tianjin Medical University Hospital in China.
Materials

One 1-month-old, Sprague-Dawley rat was used for BMSC extraction, and 68 healthy, female, 3-month-old, Sprague-Dawley rats (weighing 200–250 g) were purchased from the Animal Laboratory, Chinese Academy of Medical Sciences, China (license No. SCXK (Jin) 20070001). Rats were housed at 18–26°C in 40–70% relative humidity. The temperature within the cage was 1–2°C greater than the general environment, and humidity was 5–10% greater. Ventilation of the cages was performed 8–12 times per hour. The experimental disposal of animals complied with the Guidance Suggestions for the Care and Use of Laboratory Animals, issued by the Ministry of Science and Technology of China[25].

Methods

Rat BMSC cultivation

The 1-month-old Sprague-Dawley rat was anesthetized with an intraperitoneal injection of 2.5% ketamine 20 mg/kg and then sacrificed. The rats were then disinfected in 75% alcohol for approximately 10 minutes. Bilateral tibia and femur were removed under sterile conditions; the bone ends were resected, and 1 mL of Dulbecco’s modified Eagle’s medium (DMEM) complete medium (Gibco BRL Life Technologies, Gaithersburg, ML, USA) was collected using a No. 10 syringe. The cells were washed and prepared into a single-cell suspension at the density of 3 × 10^4/mL. In brief, the flask was fully washed and 15 mL of DMEM containing 0.05% bovine serum albumin was added; 24 hours later, the medium was collected and cells were centrifuged using 3-kDa centrifugal ultrafiltration tubes at 3 800 r/min for 30 minutes. The cells were then inoculated into 100-mL culture flasks and incubated at 37°C in 5% CO₂ saturated humidity. Culture medium was replenished 24 hours later and then replaced every 3 days. Non-adherent cells were removed and adherent cells were expanded until the cultures were subconfluent. The cells were then processed through sequential passages. The majority of contaminating hematopoietic stem cells was progressively lost, and after the second passage, cultures contained a morphologically homogenous cell population that was designated BMSCs. This cell population was confirmed by fluorescence-activated cell-sorting analysis, showing a lack of typical hematopoietic cell surface marker expression, including CD45, CD34, and CD14, but positive expression of CD71, CD105, and CD44[26]. Cells between passage 3 and passage 6 were used for the experiments. Proliferating cells were labeled by BrdU diluted in culture medium (15 μM BrdU) (Takara Biotechnology (Dalian), Dalian, China)[27-28].

Establishment of SCI models

A total of 68 female, Sprague-Dawley rats were maintained in the laboratory for 2 weeks. At the beginning of the experiment, the rats were anesthetized with an intraperitoneal injection of 2.5% ketamine (20 mg/kg). The rats were fixed in a prone position on the surgery table to prepare the skin specimens, which were then disinfected. T₉ spinous processes were selected, and a 2–3 cm length of skin and subcutaneous tissue were incised along the posterior median line. The paraspinal muscles were stripped, exposing T₉ spinous processes and lamina. Using forceps, the T₉ spinous processes and lamina were resected, exposing dura mater. The right hemisphere of the spinal cord was then resected. The wound was rinsed with a penicillin-saline solution and sutured. The rat bladders were squeezed twice daily, morning and evening, to express urine, until resumption of micturition reflex. Paralysis of the injured right hind limb was considered successful model establishment[29-30].

Mild hypothermia and normal temperature BMSC transplantation

A HP-V26 temperature meter (Beijing Shun Jie Xinlong Technology, Beijing, China) was used for continuous monitoring of rectal temperature. Following SCI, rats in normal temperature + BMSC transplantation group were placed on the surgical table at room temperature and rectal temperature was maintained at 37 ± 0.5°C. After 6 hours, a 1-mL BMSC suspension (1 × 10^5/L) suspension was injected through the tail vein using a 1-mL syringe. In the mild hypothermia + BMSC transplantation group, the rats were placed on an ice blanket, maintaining rectal temperature at 34 ± 0.5°C; after 6 hours, a 1-mL BMSC suspension (1 × 10^5/L) suspension was injected into the tail vein using a 1-mL syringe. Then animals were then housed in individual cages.

Evaluation of functional recovery

Eight rats from each group were selected to assess functional recovery. Each behavioral test was observed by two independent investigators.

BBB score: The open-field locomotion scale was used to assess movement, weight support, and coordination. Scores were achieved using a standardized BBB locomotor scoring system. The BBB scores range from 0 (flaccid paralysis) to 21 (normal gait). Rats were acclimated to the testing environment (90 cm diameter plastic wading pool; 4 cm height) prior to testing[31-32], and testing was performed at 1, 2, 4, 6, and 8 weeks post-surgery. BBB scores were averaged for each group. Inclined plate test: An inclined plate surface was covered with a 6-mm-thick rubber pad, and the rats were placed on an axis perpendicular to the longitudinal axis of the inclined plate. The incline angle gradually increased, and the rats were forced to remain on the inclined plate for at least 5 seconds to record maximum angle. The inclined plate angle was measured three times in each rat to obtain an average value. Three groups were measured at 1, 2, 4, 6, and 8 weeks post-surgery[33-35].

Histological analysis after spinal cord hemisection

At 4 weeks post-surgery, two rats were randomly selected from each group and were anesthetized intraperitoneally with chloral hydrate (35 mg/100 g). The dissected spinal cord tissues (T₂) were fixed for 3 hours in 4% paraformaldehyde, soaked overnight in 10% glutaraldehyde, washed in 0.1 M phosphate buffer, and then post-fixed in 1% osmium tetroxide for 1 hour. Following dehydration in a graded ethanol series, the specimens were embedded in Spurr’s resin and ultrathin sections were stained with uranyl acetate and lead citrate, and observed using a transmission electron microscope (Hitachi H7650, Tokyo, Japan). The number of pia mater was counted on the photomicrographs. The number of pia mater was averaged for each group.
parafomaldehyde, followed by 30% sucrose, and cut into 15-mm thick sagittal and parasagittal sections using a cryostat (Oxford Instruments, Oxford, UK). Hematoxylin-eosin staining and 1% cresyl violet staining were performed for general histological examination under microscope observation (Olympus, Tokyo, Japan). **Immunohistochemical analysis of BrdU-positive cells at the affected site**

At 4 weeks post-surgery, two rats were randomly selected from each group for BrdU detection. Immunohistochemistry for BrdU detection required 2 N HCl-pretreatment of spinal cord tissue sections (T₃) to open histones and make DNA accessible. All stainings were performed on free-floating, 40-μm thick sections. The sections were incubated in monoclonal mouse anti-rat BrdU antibody (10 μM; Boehringer Mannheim, Santa Cruz, CA, USA) at 4°C overnight in a humidified chamber, followed by biotinylated horse anti-mouse IgG antibody (1:167; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C for 45 minutes. A total of 10 visual fields were randomly selected from each section under a high-power microscope (Olympus) to quantify BrdU-positive cells from each field (200 ×) of vision. The mean served as the number of BrdU-positive cells per group.

**SEP and MEP measurements in rats following spinal cord hemisection**

At 8 weeks post-model establishment, SEPs and MEPs were measured in eight rats per group using Keypoint myoelectricity-evoked potential equipment (Medtronic, Minneapolis, MN, USA). Rats were anesthetized intraperitoneally with 10% chloral hydrate, and a stimulus electrode was subsequently fixed to the hind legs. Recording electrodes were placed on the hind-limb cortical sensory area between the coronal suture and sagittal suture lines using a brain localization testing instrument (Wuhan Vaidya Medical Technology, Wuhan, China). Reference electrodes were placed 0.5 cm posterior to the recording electrodes, eliciting direct-current square wave electrical stimulation with an intensity of 5–15 mA, pulse width of 0.2 ms, and frequency of 3 Hz, which was superimposed for 50–60 times. SEP latency and amplitude were recorded, and nerve electrophysiological recovery was observed. Stimulating electrodes were placed in the cerebral cortex motor area, with a stimulus intensity of 40 mA, a pulse width of 0.1 ms, and a frequency of 1 Hz, which was superimposed 300–500 times. The scanning speed was 5 ms/D and sensitivity was 5 μV/D. MEP latency and amplitude changes were recorded.

**Statistical analysis**

Data are expressed as mean ± SD, and a completely randomized design analysis of variance was performed using SPSS 15.0 statistical software. Two-sample comparison was performed with the Dunnett’s t-test, and a level of *P* < 0.05 was considered significant. All analyses were performed using SPSS statistical software (Version 15.0; SPSS, Chicago, IL, USA).

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**Author contributions**: Dong Wang had full access to study design, as well as implemented data analysis and wrote the manuscript. Jianjun Zhang revised the manuscript using a blind method and was responsible for statistical analysis.

**Conflicts of interest**: None declared.

**Ethical approval**: This study was approved by the Scientific Review Committee and the Institutional Review Board of Tianjin Medical University in China.

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