● RESEARCH ARTICLE

Propofol promotes spinal cord injury repair by bone marrow mesenchymal stem cell transplantation

Ya-jing Zhou[1], Jian-min Liu[2], Shu-ming Wei[3], Yun-hao Zhang[1], Zhen-hua Qu[1], Shu-bo Chen[4, *]

1 Department of Anesthesiology, Xingtai People's Hospital, Hebei Medical University, Xingtai, Hebei Province, China
2 Department of Orthopedic Trauma, Xingtai People's Hospital, Hebei Medical University, Xingtai, Hebei Province, China
3 Department of Anesthesiology, Third Hospital of Hebei Medical University, Shijiazhuang, Hebei Province, China
4 Department of Urinary Surgery, Xingtai People's Hospital, Hebei Medical University, Xingtai, Hebei Province, China

Abstract

Propofol is a neuroprotective anesthetic. Whether propofol can promote spinal cord injury repair by bone marrow mesenchymal stem cells remains poorly understood. We used rats to investigate spinal cord injury repair using bone marrow mesenchymal stem cell transplantation combined with propofol administration *via* the tail vein. Rat spinal cord injury was clearly alleviated; a large number of newborn non-myelinated and myelinated nerve fibers appeared in the spinal cord, the numbers of CM-Dil-labeled bone marrow mesenchymal stem cells and fluorogold-labeled nerve fibers were increased and hindlimb motor function of spinal cord-injured rats was markedly improved. These improvements were more prominent in rats subjected to bone marrow mesenchymal cell transplantation combined with propofol administration than in rats receiving monotherapy. These results indicate that propofol can enhance the therapeutic effects of bone marrow mesenchymal stem cell transplantation on spinal cord injury in rats.

Key Words: nerve regeneration; bone marrow mesenchymal stem cells; stem cell transplantation; propofol; spinal cord injury; repair; neuroprotection; anesthesia; neural regeneration

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Introduction

Effectively treating spinal cord injury, reducing morbidity and mortality, achieving optimal motor functional recovery, and improving a patient's prognosis are challenging issues for neurosurgeons (Yu et al., 2011a; Ohira et al., 2013). An increasing number of recent studies have demonstrated that under certain conditions, bone marrow mesenchymal stem cells (BMSCs) can be induced to differentiate into nerve cells for repair of injured spinal cord, providing new opportunities for treatment of spinal cord injury (Li et al., 2011). Clinical studies and basic experiments have demonstrated that propofol is a neuroprotective anesthetic (Finch et al., 1999; Haku et al., 2008) and its neuroprotective effects on the brain are likely to be associated with its anti-oxidative properties, inhibition of intracellular calcium overload, regulation of γ-aminobutyric acid receptor and inhibition of apoptosis (Feng et al., 2005). In the spinal cord, propofol has neuroprotective effects on ischemia/reperfusion injury by increasing the removal of oxygen free radicals and inhibiting lipid peroxidation (Pallini et al., 2005; Albin and Mink, 2006; Pearse et al., 2007). There is evidence that BMSC transplantation combined with propofol administration can enhance the tolerance of the spinal cord to ischemia/reperfusion injury, and the combined therapy superimposes the effect of monotherapy (Young, 2002; Ariake et al., 2012; Björklund et al., 2014). The purpose of this study was to validate neuro-protective effects of this combined therapy on rat spinal cord injury.

Materials and Methods

Animals

A single male or female 1-month-old Wistar rat and 80 healthy female adult Wistar rats weighing 200–250 g (Hebei Province Laboratory Animal Center, China; License No. SCXK (Jin) 20080004) were included in this study. The experimental protocol received approval from Animal Ethics Committee, Xingtai People's Hospital, Hebei Medical University, China. Efforts were made to minimize animal suffering.

Rat BMSC culture and identification

After sacrifice under anesthesia, the 1-month-old Wistar rat was sterilized with 75% ethanol for 10 minutes. Under aseptic conditions, rat bilateral tibia and femur were harvested. After bilateral bone stumps were resected, bone marrow cavity was flushed with 1 mL of L-DMEM complete medium (Gibco BRL, Gaithersburg, MD, USA) containing 5% fetal bovine serum (Hyclone, Logan, UT, USA). The resulting solution was made into a single cell suspension and then seeded into a 100 mL culture flask at a density of 3×10^4 cells/mL. After incubation at 37°C, 5% CO_2 with saturated humidity for 24 hours, the culture medium was

refreshed and then replaced once every 3 days. Subculture was performed at 1:2. Cell growth was observed daily under the inverted microscope (Olympus Optical Co., Ltd., Tokyo, Japan). When cells reached approximately 80% confluency, subculture was performed again at 1:3. After repeated subculture amplification, BMSCs were gradually purified. Surface antigens CD29, CD105, CD44, CD166, CD34, CD86 and CD80 were detected by a flow cytometer (Beckman Coulter, Inc., Brea, CA, USA) (Lin et al., 2012).

BMSCs labeling by CM-Dil

In the dark, 5 μL CM-Dil solution (Sigma, St. Louis, MO, USA) and 1 mL complete medium containing 5% fetal bovine serum were added to a 1.5 mL tube. After thorough stirring, the resulting solution was CM-Dil labeling solution. The adherent BMSCs that reached approximately 80% confluency washed with PBS for three times and incubated with above prepared CM-Dil dye (40 μL/cm^2) at 37°C, 5% CO_2 with saturated humidity for 20 minutes. After removal of CM-Dil dye, BMSCs were incubated with 5 mL complete medium containing 5% fetal bovine serum at 37°C for 10 minutes. After three washes with complete medium containing 5% fetal bovine serum, cells were left for 24 hours and cell morphology was observed using the fluorescence microscope (Olympus Optical Co., Ltd.). When cells reached over 80% confluency, they were sub-cultured at 1:3. Passage 4 BMSCs were taken for measuring CM-Dil labeling rate using a flow cytometer.

Spinal cord injury model

After environmental adaptation for 2 weeks, the 80 female Wistar rats were anesthetized with 10% chloral hydrate (350 mg/kg, intraperitoneal) and placed in the supine position on a surgery table. A median incision was made, with T_{8-9} spinous processes at the center, to fully expose spinous processes and vertebral plate T_{7-10}. The T_{8-9} spinous processes and part of vertebral plate were resected to expose the intact dura mater. According to the modified Allen method (Finch et al., 1999; Li et al., 2011), a 10 g weight was dropped from 2.5 cm to directly impact the exposed dura mater and spinal cord tissue. Tail spasm followed by paralysis of both lower limbs indicated success of the spinal cord injury model. The incision was rinsed using hydrogen peroxide and sutured layer by layer. Manual extrusion of the bladder was performed in spinal cord injury rats by abdominal massage twice per day (morning and afternoon).

Treatment

Six hours after surgery, spinal cord injury rats were randomly and equally allocated to four groups: (1) Model group: rats received 1 mL of L-DMEM complete medium containing 5% fetal bovine serum using 1 mL syringe (Shanghai Boguang Biotechnology Co., Ltd., Shanghai, China) *via* the tail vein. (2) BMSC group: rats received 1 mL of BMSC suspension injection (containing 3 × 10^6 CM-Dil-labeled passage 4 BMSCs) *via* the tail vein. (3) Propofol group: rats received propofol injection (2 mL/kg per hour; Xi'an Libang

Pharmacy Co., Ltd., Xi'an, Shaanxi Province, China; Approval No. H19990282) *via* the tail vein using an infusion pump within 4 hours. The BMSC transplantation + propofol infusion group (combination group): rats received 1 mL BMSC suspension (containing 3 × 10^6 CM-Dil-labeled passage 4 BMSCs) *via* the tail vein and propofol infusion (2 mL/kg per hour) within 4 hours.

Evaluation of hindlimb motor function

Before surgery, and 1, 3 days and 1, 2, 3, 4 weeks after surgery, eight rats from each group were randomly selected for evaluation of hindlimb motor function using the modified Tarlov scoring system, Basso, Beattie and Bresnahan (BBB) locomotor scale and the inclined plate test. The tests were started at 8:00 a.m. and were performed by two investigators blind to experimental grouping.

Modified Tarlov scoring system (Finch et al., 1999): Locomotion of the rats was scored as follows: 0, no movement, cannot load; 1, slight movement, cannot load; 2, frequent movement or powerful, cannot load; 3, can support body weight and able to walk one or two steps; 4, able to walk with assistance; 5, normal gait.

Inclined plane test (Shen et al., 2014): rats were placed on a smooth wooden platform. Every 5 seconds, one side of the platform was elevated by 5°. The maximum angle at which a rat was able to stay on the platform for 5 seconds was taken as the value for hindlimb motor function of that rat.

BBB locomotor scale (Shen et al., 2014): a score of 0 indicated complete paralysis and 21 represented normal function. Observation indices included range of motion, number of joints involved in motion, degree of loading, forelimb and hindlimb concordance, and movement of front and hind paws and tail.

Hematoxylin-eosin (HE) staining and immunofluorescence labeling

Four weeks after surgery, five rats from each group were randomly selected to receive an overdose of anesthesia (10% chloral hydrate, 350 mg/kg, intraperitoneal) and intubated from the left ventricle to the ascending aorta through a chest incision. The right atrium was cut open, perfused with ice-cold physiological saline and then fixed with 4% paraformaldehyde. A 1.0-cm length of spinal cord containing the injured tissue was harvested, dehydrated through a series of ethanol washes, and then longitudinally sliced into 20 μm sections. (1) HE staining: Half of the prepared rat spinal cord sections were stained with hematoxylin for 5 minutes, washed with running water, treated with HCl-ethanol solution for 10 seconds, washed with running water for 10 minutes, stained with eosin for 7 minutes, washed with running water, dehydrated with ethanol gradients, cleared with xylene, enveloped with neutral gum, and finally observed under an inverted microscope (Olympus Optical Co., Ltd.). (2) Fluorescence labeling: The remaining half of the sections were used for fluorescence microscope labeling. Ten fields per section were randomly selected. The number of CM-Dil-positive cells per field was counted under 200-fold magnification. The
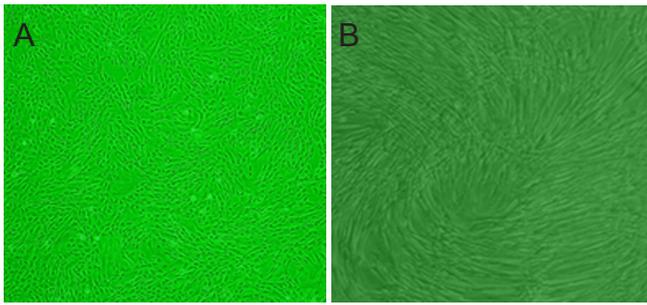
Figure 1 Morphology of bone marrow mesenchymal stem cells (BMSCs) (inverted microscope, × 200).
(A) Primary BMSCs grew in a whirlpool-like pattern; (B) passage 3 BMSCs were mainly shuttle-shaped.
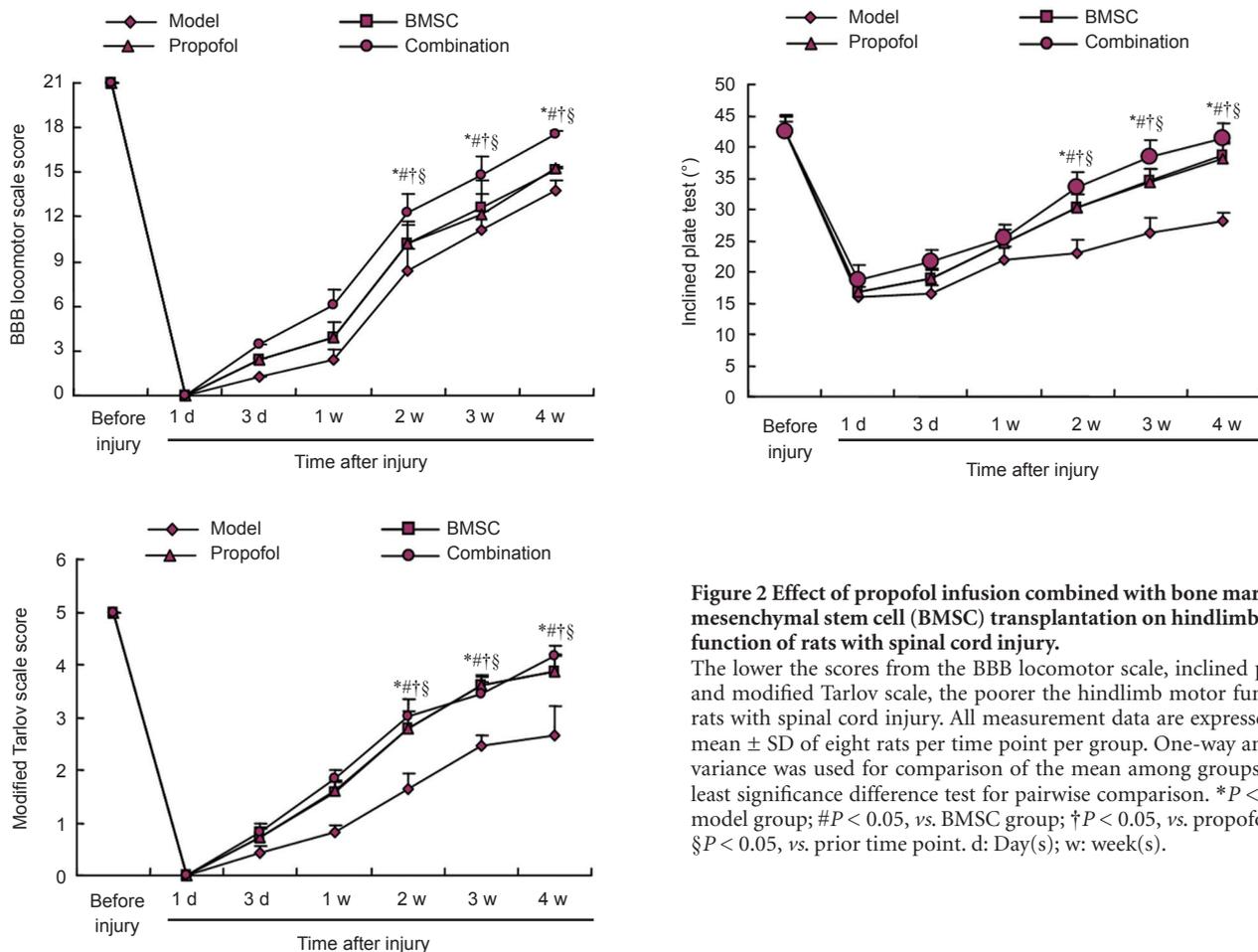
Figure 2 Effect of propofol infusion combined with bone marrow mesenchymal stem cell (BMSC) transplantation on hindlimb motor function of rats with spinal cord injury.
The lower the scores from the BBB locomotor scale, inclined plate test and modified Tarlov scale, the poorer the hindlimb motor function of rats with spinal cord injury. All measurement data are expressed as the mean ± SD of eight rats per time point per group. One-way analysis of variance was used for comparison of the mean among groups and the least significance difference test for pairwise comparison. *$P < 0.05$, *vs.* model group; #$P < 0.05$, *vs.* BMSC group; †$P < 0.05$, *vs.* propofol group; §$P < 0.05$, *vs.* prior time point. d: Day(s); w: week(s).
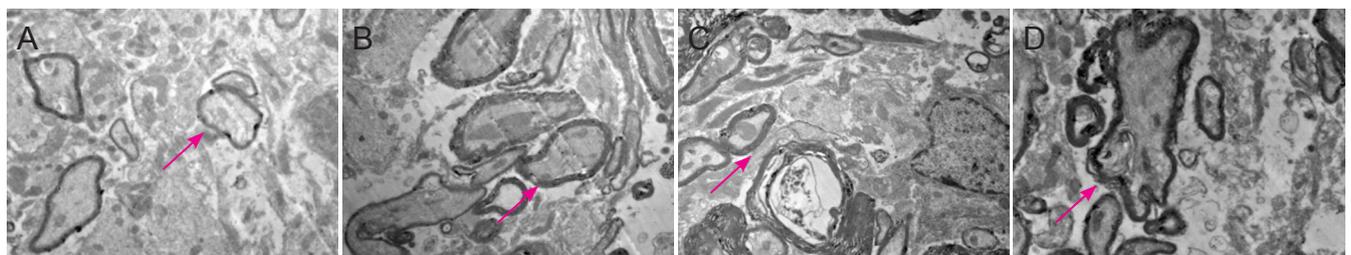
Figure 5 Effects of propofol infusion combined with bone marrow mesenchymal stem cell (BMSC) transplantation on the ultrastructure of injured rat spinal cord tissue (× 8,000).
(A) Model group: A small number of myelinated and non-myelinated nerve fibers were observed in the injured rat spinal cord. (B) BMSC group and (C) propofol group: A large number of myelinated and non-myelinated nerve fibers were observed in the injured rat spinal cord. (D) Combination group: a great number of regenerating axons with complete myelin sheath was observed. Arrows indicate nerve fibers.

mean value of CM-Dil-positive cells across 10 fields was calculated.

Retrograde labeling of the fluorescent tracer fluorogold
Four weeks after surgery, six rats from each group were randomly selected to receive an overdose of anesthesia. The sciatic nerve was fully exposed *via* the vastus lateralis muscle and the perineurium of sciatic nerve was destroyed using a haemostat to facilitate the infusion and diffusion of more fluorogold. In most circumstances, fluorogold (Invitrogen Life Technologies, Carlsbad, CA, USA) was injected into the sciatic nerve (0.4 μL 2% fluorogold per side, 0.1 μL/min) *via* the injured region using a microsyringe in the dark. After fluorogold injection, the microsyringe was left in place for 5 minutes, and the wound was washed repeatedly with saline containing 8×10^4 U penicillin and then sutured layer by layer. After surgery, rats were raised normally. One week later, tissue in the spinal cord injury region was taken, preserved in 30% sucrose at 4°C overnight, frozen at –18°C, transected, embedded and sliced into 20-μm-thick sections. At 200-fold magnification, 10 visual fields per section were randomly selected. The mean number of fluorogold-labeled nerve fibers per visual field across 10 visual fields in one section was calculated.

Transmission electron microscopy
Four weeks after spinal cord injury model, six rats from each group were randomly selected for transmission electron microscopy. The rats were perfused with 2.5% glutaraldehyde *via* the heart and fixed with 2.5% glutaraldehyde overnight. Injured spinal cord tissue was sliced into 20 μm-thick sections, fixed with osmic acid at 4°C for 2 hours, rinsed, dehydrated with acetone gradients, stained with uranyl acetate at 4°C for 4 hours, embedded with epoxy resin 6101, and finally observed under the transmission electron microscope (Beijing Weidi Kangtai Medical Instrument Co., Ltd., Beijing, China).

Statistical analysis
All measurement data are expressed as the mean ± SD. SPSS 17.0 software (SPSS, Chicago, IL, USA) was used. One-way analysis of variance was used for comparison of the mean among groups; the least significance difference test for pairwise comparison. A level of $P < 0.05$ was considered statistically significant.

Results

BMSC morphology and identification
After 5 days of culture, BMSCs and colonies in the culture flask had obviously increased in number. Passages 1, 2, 3 BMSCs proliferated actively. After subculture, the majority of cells adhered to the culture flask and exhibited a shuttle-, polygon- or triangle-shaped appearance. BMSCs developed toward mainly shuttle shape, with two or more processes and visible nuclei and nucleoli. After reaching confluence, they grew in a whirlpool-like or parallel pattern (**Figure 1**). Flow cytometry showed that these cells were positive for CD29, CD105, CD44 and CD166 but negative for CD34, CD86 and CD80, with a purity of over 95%.

Propofol infusion combined with BMSC transplantation improved hindlimb motor function of rats with spinal cord injury
Before surgery, the scores from the BBB locomotor scale, inclined plate test and modified Tarlov scale were similar between groups ($P > 0.05$). At 2–4 weeks after spinal cord injury, the scores from the BBB locomotor scale, inclined plate test and modified Tarlov scale in the propofol, BMSC and combined groups were significantly increased compared with the model group ($P < 0.05$), and the scores in the combined group were significantly increased than in the separate propofol and BMSC groups ($P < 0.05$; **Figure 2**).

Propofol infusion combined with BMSC transplantation improved pathology of rat spinal cord after spinal cord injury
Four weeks after surgery, HE staining and immunofluorescence labeling showed that (1) in the model group, spinal cord tissue in the spinal cord injury region was broken and connected by scar tissue with obvious cavity formation, but no CM-Dil-labeled cells were observed; (2) in the propofol and BMSC groups, the cavities in the rat spinal cord tissue were smaller compared to the model group and no CM-Dil-labeled cells were observed in the rat spinal cord tissue in the propofol group; (3) in the combination group, cavities in the injured spinal cord tissue almost disappeared, scattered CM-Dil-positive cells were observed in the BMSC and combination groups, and CM-Dil-positive cells in the injured rat spinal cord tissue were greater in numbers in the combination group than in the BMSC group ($P < 0.01$; **Figure 3**).

Propofol infusion combined with BMSC transplantation increased the number of fluorogold-labeled nerve fibers in the injured rat spinal cord on the cranial side
Retrograde labeling of the corticospinal tract revealed that the number of fluorogold-labeled nerve fibers in the injured rat spinal cord on the cranial side was significantly greater in the BMSC, propofol, and combination groups than in the model group ($P < 0.05$ or $P < 0.01$), and the number of fluorogold-labeled nerve fibers was significantly greater in the combination group than in the separate BMSC and propofol groups ($P < 0.05$; **Figure 4**). These results indicate that propofol infusion combined with BMSC transplantation promoted recovery of injured rat nerve fibers better than monotherapy.

Propofol infusion combined with BMSC transplantation improved the ultrastructure of injured rat spinal cord tissue
Transmission electron microscopy at 4 weeks after surgery showed that in the model group, glial scars had formed, a small amount of myelinated nerve fibers were observed in the injured rat spinal cord tissue, and macrophages had phagocytized the degenerated and necrotic myelinated nerve

fibers. In the combination group, a large number of myelinated and non-myelinated nerve fibers and regenerating axons with complete myelin sheath were observed in the injured rat spinal cord tissue. The numbers of myelinated and non-myelinated nerve fibers in the injured rat spinal cord were greater in the BMSC and propofol groups than in the model group, but they were less than in the combination group (**Figure 5**).

Discussion

The current methods of treating spinal cord injury involve rescuing dying neurons in the injured and peripheral regions, reversing spinal cord ischemia/hypoxia, and promoting recovery of neuronal function (Dittert et al., 2008; Jiang et al., 2009b; Liu et al., 2010; Xiang et al., 2013). Spinal neurons have little capacity for self-repair (Dittert et al., 2008; Jiang et al., 2009b; Liu et al., 2010; Xiang et al., 2013). Evidence exists that transplanted BMSCs can differentiate into neurons and neuroglia to repair injured neurons (Li et al., 2012). The transplanted BMSCs survive in the injured spinal cord tissue, migrate, accumulate and differentiate into neurons and glia, secrete a variety of growth factors, inhibit the expression of inflammatory factors, protect and promote neuronal regeneration, induce microvessel regeneration in the injured region, relieve secondary inflammatory reaction, and therefore treat spinal cord injury (Kouchi et al., 1998; Hsieh et al., 2007; Yao et al., 2007; Yu et al., 2011b). Other effective means are necessary for better repair of spinal cord injury, including medication, physical therapy and surgery (Yao et al., 2007).

Propofol has be shown to inhibit the production of oxygen free radicals, exhibit anti-oxidative capacity, prevent free-radical chain reaction, and inhibit lipid peroxidation (Zhao et al., 2003; Weber et al., 2005; Huang et al., 2007; Wang et al., 2009). There is strong evidence that in the early stage of spinal cord injury, propofol infusion can reduce serum level of S100β protein, decrease spinal cord calcium and water contents, and therefore protect nerves to a certain degree (Sufianova et al., 2002; Iijima et al., 2006; Kitz et al., 2006; Yu et al., 2011a). Results from this study showed that after propofol infusion combined with BMSC transplantation, a large number of myelinated and non-myelinated nerve fibers formed, the numbers of CM-Dil-labeled cells and fluorogold-labeled nerve fibers increased, and rat hindlimb motor function was markedly improved. The underlying mechanisms of rat spinal cord injury repair aim to (i) reduce the production of oxygen free radicals, increase anti-oxidase activity, remove oxygen free radicals; or (ii) increase anti-oxidase activity, improve microcirculation and tissue perfusion; or (iii) reduce the adhesion of inflammatory cells, relieve edema of injured spinal cord tissue, and protect the spinal cord against ischemia/reperfusion injury; or (iv) inhibit the expression of inflammatory factors, promote neuronal regeneration and reconstruction, induce regeneration of microvessels in the injured spinal cord, and relieve secondary inflammatory reaction (Choi et al., 2007; Jiang et al., 2009a; Shen et al., 2009).

Taken together, propofol infusion *via* the tail vein using an infusion pump can increase the survival rate of BMSCs in the injured spinal cord and optimize the repair of spinal cord injury in rats by BMSC transplantation. This method is a promising means for treatment of spinal cord injury in the clinic.

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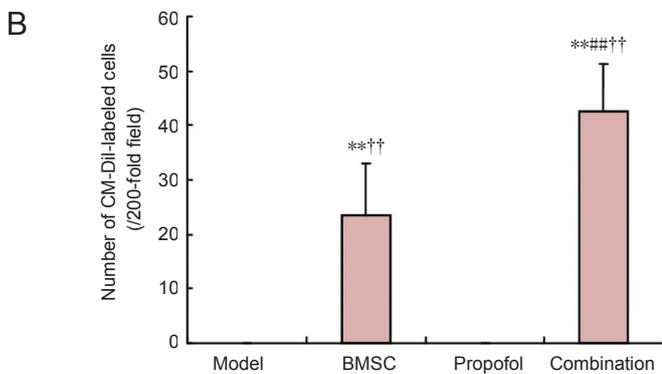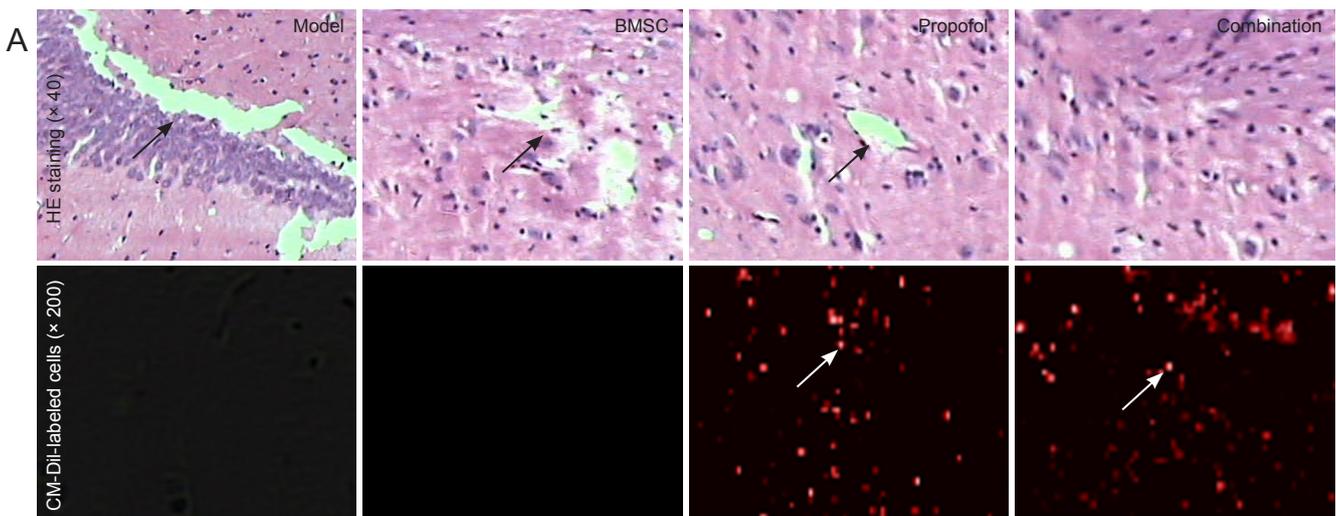
Figure 3 Effects of propofol infusion combined with bone marrow mesenchymal stem cell (BMSC) transplantation on pathological morphology of injured rat spinal cord.

(A) Pathological morphology of injured rat spinal cord at 4 weeks after injury. Black arrows indicate cavities in the injured spinal cord, and white arrows point to CM-Dil-labeled cells. (B) Quantification of CM-Dil-labeled cells in injured rat spinal cord at 4 weeks after injury. The measurement data are expressed as the mean ± SD of four rats per time point per group. One-way analysis of variance was used for comparison of the mean among groups and the least significance difference test for pairwise comparison. **$P < 0.01$, *vs.* model group; ##$P < 0.01$, *vs.* BMSC group; ††$P < 0.01$, *vs.* propofol group.
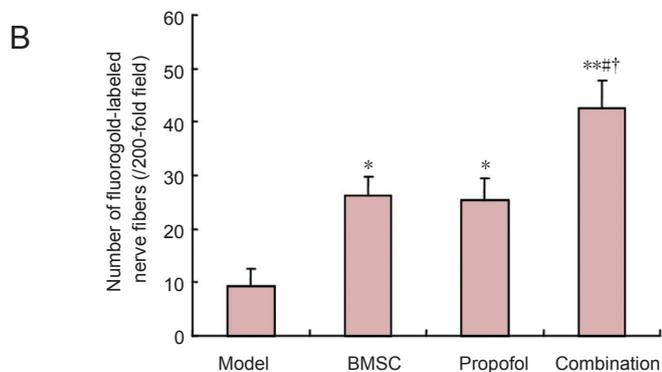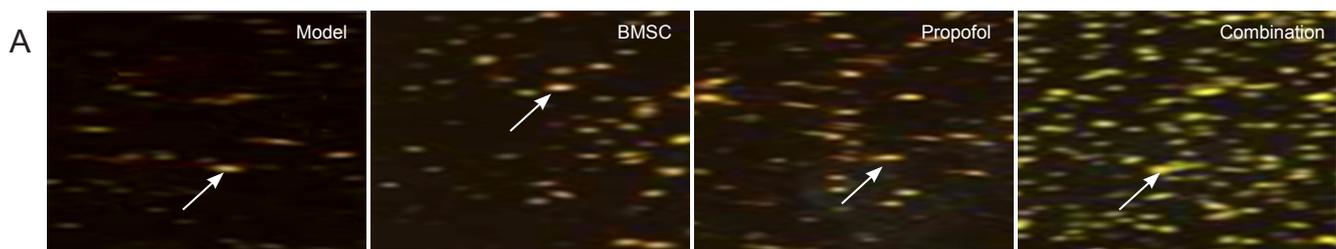
Figure 4 Effects of propofol infusion combined with bone marrow mesenchymal stem cell (BMSC) transplantation on number of fluorogold-labeled nerve fibers in the injured rat spinal cord on the cranial side.

Fluorogold-labeled nerve fibers (arrows) in the injured rat spinal cord on the cranial side at 4 weeks after spinal cord injury (A, × 200) and their quantification (B). The measurement data are expressed as the mean ± SD of six rats per time point per group. One-way analysis of variance was used for comparison of the mean among groups and the least significance difference test for pairwise comparison. *$P < 0.05$, **$P < 0.01$, *vs.* model group; #$P < 0.05$, *vs.* BMSC group; †$P < 0.05$, *vs.* propofol group.

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