Simvastatin mobilizes bone marrow stromal cells migrating to injured areas and promotes functional recovery after spinal cord injury in the rat∗

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HIGHLIGHTS

► Simvastatin treatment was given by subarachnoid injection immediately after SCI.
► Locomotive functional recovery significantly improved by simvastatin treatment.
► Bone marrow stromal cells migrated to the injured site enhanced by simvastatin.
► Simvastatin increased the expression of BDNF and VEGF in injured rats.

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ABSTRACT

This study investigated the therapeutic effects of simvastatin administered by subarachnoid injection after spinal cord injury (SCI) in rats; explored the underlying mechanism from the perspective of mobilization, migration and homing of bone marrow stromal cells (BMSCs) to the injured area induced by simvastatin. Green fluorescence protein labeled bone marrow stromal cells (GFP-BMSCs) were transplanted into rats through the tail vein for stem cell tracing. Twenty-four hours after transplantation, spinal cord injury (SCI) was produced using weight-drop method (10 g 4 cm) at the T10 level. Simvastatin (5 mg/kg) or vehicle was administered by subarachnoid injection at lumbar level 4 after SCI. Locomotor functional recovery was assessed in the 4 weeks following surgery using the open-field test and inclined-plane test. At the end of the study, MRI was used to evaluate the reparation of the injured spinal cord. Animals were then euthanized, histological evaluation was used to measure lesion cavity volumes. Immunofluorescence for GFP and cell lineage markers (NeuN and GFAP) was used to evaluate simvastatin-mediated mobilization and differentiation of transplanted BMSCs. Western blot and immunohistochemistry were used to assess the expression of vascular endothelial growth factor (VEGF) and brain-derived neurotrophic factor (BDNF). Simvastatin-treated animals showed significantly better locomotor recovery, less signal abnormality in MRI and a smaller cavity volume compared to the control group. Immunofluorescence revealed that simvastatin increased the number of GFP-positive cells in the injured spinal cord, and the number of cells double positive for GFP/NeuN or GFP/GFAP was larger in the simvastatin treated group than the control group. Western blot and immunohistochemistry showed higher expression of BDNF and VEGF in the simvastatin treated group than the control group. In conclusion, simvastatin can help to repair spinal cord injury in rat, where the underlying mechanism appears to involve the mobilization of bone marrow stromal cells to the injured area and higher expression of BDNF and VEGF.

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Spinal cord injury (SCI) is a devastating injury that can lead to irreversible neurological deficits. The current recommended treatments for SCI involve administration of high-dose methylprednisolone, which has limited efficacy and serious side effects. Recent advances in stem cell biology have heightened interest in regeneration-based treatment strategies for SCI, which can be roughly divided into exogenous or endogenous stem cell therapies. Although exogenous stem cell transplantation has made moderate progress, its application is clinically hindered by the risks of tumor...
formation, immune rejection and ethical concerns, amongst others [14]. In view of the adult contains numerous endogenous stem cells, which are normally quiescent but can be mobilized following injury or stimulation [18], therefore, a strategy that stimulates the proliferation, migration and differentiation of endogenous stem cells in vivo would be ideal for the treatment of SCI.

Bone marrow stromal cells (BMSCs) are non-hematopoietic multipotent stem cells capable of trans-differentiating into neurons, astrocytes or oligodendrocytes [22,2]. A number of studies have shown that transplantation of BMSCs significantly improves hind limb function after SCI [22,12]. Thus, BMSCs have the potential to restore injured spinal cord tissue and promote functional recovery.

Statins are cholesterol-lowering drugs that have been widely used in clinical practice for many years. Recently, statins have also been shown to have pleiotropic effects, such as anti-inflammatory, antioxidant, and neuroprotective effects. Our previous study showed that treatment with simvastatin by gavage 1 day after SCI can significantly improve locomotor recovery in rats, although the underlying mechanisms are not yet fully elucidated [10]. We also found that locally implanted simvastatin can induce endothelial progenitor cells (EPCs) homing to the bone defect and promote its repair [21]. Many studies have reported that following a stroke, traumatic brain injury (TBI), statin therapy has a synergistic effect with BMSCs, and can mobilize the engrafted BMSCs to the lesion area and promote its repair [7,25,16]. Further, Leone et al. reported that after myocardial infarction in humans, treatment with statins improved the spontaneous mobilization of endogenous BMSCs, which may have contributed to more favorable cardiac remodeling [15]. In this study, we investigated whether simvastatin administered by subarachnoid injection after SCI can mobilize BMSCs to the injured area and promote functional recovery in rats, along with the underlying mechanism.

All protocols were approved by the animal care committee of the Peking University Third Hospital. Green fluorescence protein transferred bone marrow stromal cells of rat (GFP-BMSCs) were purchased from Cyagen Bioscience Technology Co. (Guangzhou, China) and were established to be BMSCs and to steadily express green fluorescence. The cells were cultured in low glucose Dulbecco’s modified Eagle’s medium (L-DMEM) supplemented with 10% fetal bovine serum (FBS) and 4 mM l-glutamine in an incubator with 5% CO2 at 37°C. Cells between fifth and eighth passage were utilized, a small sample was selected for the morphological observation under inverted fluorescence microscope. Cultured cells were suspended at a density of 5 × 106 in 1 mL PBS, and transplanted into the rat through the tail vein.

A total of 30 female Sprague-Dawley rats (weighing 260–280 g) were used in the study. Twenty-four hours after GFP-BMSC transplantation, surgery and treatment with simvastatin were performed. Animals were randomly assigned to 3 groups (n = 10): (1) sham group, underwent laminectomy only; (2) control (vehicle) group, received SCI and vehicle treatment; and (3) sim group, received SCI and simvastatin (5 mg/kg) treatment by subarachnoid injection. SCI was induced using the modified weight-drop method, as described previously [10]. In brief, rats were anesthetized with pentobarbital (50 mg/kg intraperitoneally) and received a laminectomy at the T10 level. After the spine was immobilized stereotaxically, a moderate SCI was induced by dropping a weight of 10 g from a height of 4 cm onto an impounder (diameter, 0.2 cm) gently placed on the spinal cord. Simvastatin was dissolved in 20% dimethyl sulfoxide (DMSO) (in normal saline) to a final concentration of 500 μg/μL. For the control group, vehicle without simvastatin was prepared.

Immediately after injury, a second laminectomy was performed at lumbar lever 4. Simvastatin (5 mg/kg) was injected into the subarachnoid space using an insulin syringe with a 29-gauge needle. The insulin syringe was attached to the stereotactic device. Each injection was delivered over a 5-min period (plunge for 1 min, wait 3 min then withdraw over 1 min) to ensure optimal delivery [11]. For the control group, an equal volume of vehicle was injected into the rats in the same manner as above. After SCI, behavioral test was conducted; only rats with a Basso, Beattie and Bresnahan (BBB) locomotor rating of ≤1 (corresponding to a slight movement of 1 or 2 joints) were included in the study.

The following neurological tests were all performed before operation, and at days 1 and 3 post-operation, and then weekly until the rats were killed. Two independent examiners who were blinded to the experimental treatment observed the rats during the tests. The open-field test assessed the movement; weight support and coordination of the rats, and the results were scored using the BBB locomotor rating scale [4]. Briefly, animals were allowed to walk around freely in a circular field for 4 min while the movements of the hind limbs were closely observed. The animal’s ability to maintain postural stability was evaluated with an inclined-plane test as described previously [10]. Briefly, the rats were placed on an inclined plane, and the maximum inclination at which the rat could maintain its position for 5 s was recorded as the final angle.

Magnetic resonance imaging (MRI) experiments were conducted with a 3-T MRI with a small animal coil (Magneton Trio, Siemens Medical Solutions, Erlangen, Germany). T1- and T2-weighted images (TE = 92 ms; TR = 3620 ms; flip angle α = 120°; slice thickness: 2 mm; FOV = 80 mm) were obtained in the fourth week following the operation. Images of the thoracic spinal cord were acquired in the axial and sagittal planes. The longitudinal-sectional area was measured using Siemens Workstation software (NUMARIS/4).

Four weeks after SCI, six animals from each group were anesthetized and perfused transcardially with 4% paraformaldehyde in PBS. The spinal cord segments between T8 and T12 were removed, post-fixed in 4% paraformaldehyde overnight, stored in 30% sucrose and embedded in OCT compound. The samples were cut into serial 8-μm longitudinal sections with a cryostat and mounted onto poly-L-lysine-coated slides.

Lesion sizes were determined on longitudinal sections. Sections from control and treated rats were stained with hematoxylin and eosin (HE). On each section, intraspinal lesion cavities were outlined and measured using Image J software. The resulting areas were summed and multiplied by the spacing between sections (50 μm) to provide a mean volume (mm³).

Immunofluorescence was performed to identify the distribution and cell types of transplanted GFP-BMSCs. The primary antibodies used were as follows: mouse monoclonal anti-neuronal nuclear antigen antibody (NeuN, 1:250, Chemicon Int., Temecula, CA) for neurons, mouse monoclonal anti-glial fibrillary acidic protein antibody (GFAP, 1:500, Sigma) for astrocytes. The sections were reacted with primary antibodies overnight at 4°C. After three 10-min washes in PBS, the sections were reacted with anti-mouse IgG antibody at room temperature for 1 h. Positive signals were observed by fluorescence microscopy. For quantitation of GFP-BMSCs, and GFP/NeuN or GFP/GFAP double-positive cells, every fifth longitudinal section from six animals of each group was explored under immunofluorescence microscope at identical conditions. An area of 2 mm centered at the contusion epicenter was defined as the region of interest for quantitation. The number of GFP-positive cells and double-positive cells were summed.

Additional sections were used for immunohistochemical staining, as described previously [10]. Briefly, the sections were rinsed with PBS, blocked with bovine serum (10%) in PBS for 30 min, and then incubated overnight at 4°C with primary antibodies: rabbit anti-VEGF (1:250, Abcam), or rabbit anti-BDNF (1:250, Abcam). Immunoreactivity was visualized by staining with dianibobenzidine (DAB), and the slide was observed under a light microscope.
Four animals from each group were used for Western blot analyses. 1-cm spinal cord segment around the injured site was used for Western blot analysis. The membrane with protein samples was treated with blocking buffer for 1 h at room temperature, followed by incubation with primary antibodies for VEGF (1:100, Abcam), BDNF (1:100, Abcam) and GAPDH (1:1000, Sigma) at 4 °C overnight respectively. The membranes were then incubated with secondary antibodies at 37 °C for 1 h. Signals were detected by enhanced chemiluminescence (Amersham, USA). Densitometric analysis for the blots was performed with NIH image software.

Data were expressed as the mean ± SD and analyzed by one-way analysis of variance (ANOVA), followed by a post hoc t-test (SPSS 18.0). The difference was considered significant when p < 0.05.

As shown in Fig. 2A, under inverted fluorescence microscope, the GFP-BMSCs steadily expressed green fluorescence in the nucleus and the cytoplasm of the cell. The GFP-positive signals were stronger in the cell nuclei and cytoplasm around the nuclei.

As shown in Fig. 1A, from 1 week post-operation, the simvastatin-treated group showed significantly higher scores than vehicle-treated group (p < 0.05), and this difference was sustained until the end of the study. From 2 weeks post-operation, the simvastatin group showed significantly greater angles than vehicle group (p < 0.05), and this difference was sustained until the end of the study (Fig. 1B).

The SCI repairing induced by simvastatin was confirmed by MRI and HE staining. High signal in T2 image and low signal in T1 image indicates injury of the spinal cord. As shown in Fig. 1C, the signals were less abnormal in the simvastatin group than in the vehicle group. The mean lesion volume in the simvastatin group was smaller than that of the control group (Fig. 2D, E). These results are in line with the moderate yet significant improvements in hind limb function and less abnormal signals in MRI.

We used GFP fluorescence to trace transplanted BMSCs in the injured site. The average number of GFP-positive cells in simvastatin group was significantly higher than vehicle group (Fig. 2B, C).

**Fig. 1.** The behavioral, MRI and histological results. (A) Open-field assessment before and after SCI. (B) The inclined-plane test before and after SCI. Significant functional improvement was detected in simvastatin group compared with vehicle group. (C) MRI (T1 and T2-image) samples of the animals. (D) HE-stained samples of vehicle or simvastatin treated spinal cord. (E) Comparison of lesion volume, showing the volume of injured spinal cord in simvastatin group is significantly smaller than that of vehicle group.
Fig. 2. Immunofluorescence for GFP and cell-specific markers (NeuN, GFAP) in the injured spinal cord. (A) The morphological observation of GFP-BMSCs in vitro. Immunofluorescence for GFP in injured spinal cord of simvastatin group (B) and vehicle group (C). There was a significant difference in the number of GFP-positive cells between the simvastatin and vehicle group (F). Some GFP-positive cells were also positive for NeuN (D). There was a significant difference in the number of GFP/NeuN double-positive cells between the simvastatin and vehicle group (G). Some GFP-positive cells were also positive for GFAP (E). There was a significant difference in the number of GFP/GFAP double-positive cells between the simvastatin group and vehicle group (H).
Repair of SCI has been an important goal for neuroscience. It is now widely accepted that the adult contains plenty of endogenous stem cells, which are normally quiescent [18]. Recently, the focus of pre-clinical studies has shifted to the possibility of enhancing the response of these endogenous stem cells [14].

As the endogenous stem cells are hard to label, and the BMSCs transplanted via intravenous injection have been shown to home to the femoral bone marrow within minutes, with two-thirds of the transplanted cells drifting into the bone marrow space in the first 3 h following transplantation [19]. So, in the present study, the use of GFP-BMSCs intravenously injected 24 h before SCI not only enabled us to trace the fate of the transplanted BMSCs, but also gave us an indication of the movement and activity of endogenous stem cells.

Previous experiments have demonstrated the therapeutic efficacy of statins in acute SCI in rats [8,19]. We have also reported that treatment with simvastatin administered one day after SCI can promote functional recovery in the rat [10]. However, some controversy remains over the therapeutic efficacy of statins in treating SCI. Mann et al. failed to observe an improvement in spinal cord injury following oral treatment with statins [17]. A possible reason for this discrepancy may be that the systemic bioavailability of statins was low because of extensive first-pass metabolism in the liver, resulting in less than 5% of the oral dose reaching the systemic circulation [5], meaning the drug dose that reached the injured spinal cord may be too low to have an effect. Local administration of the drug is therefore likely to be a more effective route. Subarachnoid injection (lumbar puncture), widely used in clinical practice, is less invasive and more clinically effective [11]. In this study, we provided some important data regarding the local subarachnoid injection of simvastatin could improve the repair of SCI. It is important to note that the dose of simvastatin used in this study (5 mg/kg) was only 1.4–2.9% of the total cumulative dose used in our previous study using systemic administration [10].

BMSCs are known to trans-differentiated into neurons, astrocytes or oligodendrocytes [22,22]. Treatment with simvastatin can promote the migration of BMSCs and improve functional recovery after stroke or ischemia [7,25,16,15]. Here, we found a larger number of GFP-positive cells in simvastatin treated group than control group, where these GFP-positive cells were located mainly around the lesion site. At such a later time point (4 weeks), it might be difficult to distinguish between the enhancement of BMSCs homing and proliferation. But GFP-labeled BMSCs is usually used for tracing stem cell migration [13], and it has been proven that simvastatin inhibited proliferation of BMSCs [3], and many research reported that statins could improve the migration of BMSCs [7,25,16]. So, we postulated the improved number of GFP-positive cells might be related with the mobilization of BMSCs induced by simvastatin. Expression of NeuN in the CNS is generally considered a reliable marker and has been extensively used to characterize neurons. In this study, some GFP-BMSCs around the epicenter were also positive for NeuN in simvastatin treated group. This finding suggests that these BMSCs develop toward the neuronal line. Further, simvastatin also increased the number of cells double positive for GFP and GFAP, suggesting these BMSCs also differentiate toward the astrocytes’ line. Overall, this suggests that simvastatin induces BMSCs homing to the injured spinal cord and differentiation along the neural line. Whether these neuronal or astrocyte-like cells have a mature neural cell function needs to be further investigated.

The neurological deficits caused by SCI are not due to an intrinsic inability of CNS neurons or axons to regenerate, but rather to the formation of a CNS environment that is unfavorable to regeneration. Thus, creating a favorable neurotrophic microenvironment is essential for the regeneration of damaged neurons and axons. BDNF is a major source of neurotrophic factors and plays an important role in the survival and regeneration of neurons in SCI [23].

Next, we performed immunofluorescence for cell-specific markers to elucidate the differentiation of GFP-positive cells in the injured spinal cord. Some GFP-positive cells in lesion epicenter were also found to be positive for NeuN or GFAP (Fig. 2D, E). The number of cells double-positive for GFP/NeuN or GFP/GFAP was larger in simvastatin group than the vehicle group \( p < 0.05 \) (Fig. 2G, H).

The expression of VEGF and BDNF was detected by immunocytochemistry and Western blot assay. The expression of VEGF and BDNF was significantly increased in the simvastatin group compared with the control group \( p < 0.05 \) (Fig. 3B). In simvastatin group, strong immunohistochemical staining for BDNF or VEGF were observed in the cytoplasm of cells around the injury site. In the control group, less specific immunopositive staining was detected (Fig. 3A).

![Fig. 3](image-url)
Increased production of BDNF in the injured spinal cord has been reported to lead to functional recovery [24]. After stroke or TBI, treatment with simvastatin can improve the expression of BDNF, thereby improving neurological recovery [6]. Here, we found that simvastatin can significantly improve the expression of BDNF.

SCI leads to acute local ischemia and the severity of injury correlates with the degree of ischemia. VEGF is a potent stimulator of angiogenesis and affects blood permeability, which is important in wound healing after SCI [6]. After SCI, the VEGF therapies can improve the motor function of animals [20]. In the present study, we found that simvastatin improved the expression of VEGF in the SCI.

By boosting the expression of BDNF and VEGF, simvastatin could also modulate the environment in the injured spinal cord, resulting in better survival, proliferation and differentiation into neural lineage of BMSCs that migrate to the lesion site.

In conclusion, we report for the first time a treatment for SCI with single low dose of simvastatin administered by subarachnoid injection that can significantly promote the migration of BMSCs to the injured spinal cord, increase the expression of BDNF and VEGF, reduce the lesion cavity, and accelerate the recovery of hind limb function in rats. These results encourage the use of simvastatin to treat spinal cord injury, although further investigation is needed to develop a suitable treatment for general clinical application.

Conflict of interest

The authors declare that they have no conflict of interest.

References