Salvianolic acid B protects the myelin sheath around injured spinal cord axons
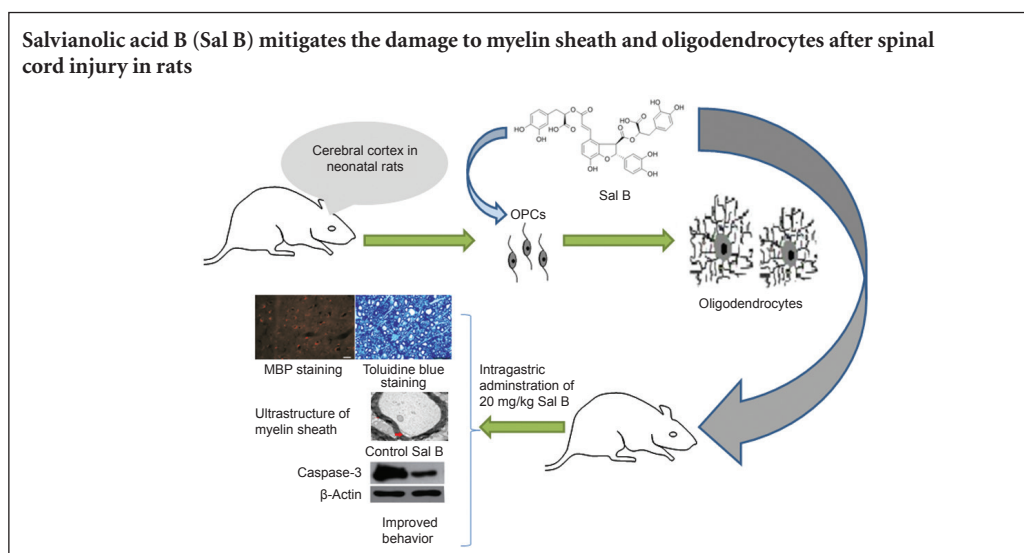
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Graphical Abstract

Salvianolic acid B (Sal B) mitigates the damage to myelin sheath and oligodendrocytes after spinal cord injury in rats

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Abstract

Salvianolic acid B, an active pharmaceutical compound present in *Salvia miltiorrhiza*, exerts a neuroprotective effect in animal models of brain and spinal cord injury. Salvianolic acid B can promote recovery of neurological function; however, its protective effect on the myelin sheath after spinal cord injury remains poorly understood. Thus, in this study, *in vitro* tests showed that salvianolic acid B contributed to oligodendrocyte precursor cell differentiation, and the most effective dose was 20 μg/mL. For *in vivo* investigation, rats with spinal cord injury were intraperitoneally injected with 20 mg/kg salvianolic acid B for 8 weeks. The amount of myelin sheath and the number of regenerating axons increased, neurological function recovered, and caspase-3 expression was decreased in the spinal cord of salvianolic acid B-treated animals compared with untreated control rats. These results indicate that salvianolic acid B can protect axons and the myelin sheath, and can promote the recovery of neurological function. Its mechanism of action is likely to be associated with inhibiting apoptosis and promoting the differentiation and maturation of oligodendrocyte precursor cells.

Key Words: nerve regeneration; spinal cord injury; salvianolic acid B; oligodendrocytes; myelin sheath; neural regeneration

Introduction

The primary cause of spinal cord injury (SCI) is an initial mechanical impact, causing compression and contusion, with damage to nerve cells, myelin, blood vessels, and supporting bone structures. Secondary inflammation and ischemia impact residual neural tissue, further aggravate neuronal death, induce demyelination, and worsen SCI (Papastefanaki and Matsas, 2015). Neuronal and axon regeneration is important after SCI. Myelin sheath and oligodendrocytes protect axons and support axon regeneration (Duncan et al., 2009; Funfschilling et al., 2012; Papastefanaki and Matsas, 2015). Necrosis and apoptosis of the myelin sheath and oligodendrocytes occur in toxic environments due to high metabolic rates, the abundance of iron-containing enzymes, and a reduced amount of reduced glutathione (Oyinbo, 2011). Saving a myelin sheath soon after injury promotes remyelination (Crowe et al., 1997; Grossman et al., 2001; Lytle and Wrathall, 2007). In contrast, slow regeneration of

the myelin sheath and oligodendrocytes is not sufficient to support axon regeneration (Franklin and Ffrench-Constant, 2008; Franklin and Gallo, 2014; Papastefanaki and Matsas, 2015), which impacts the recovery of neurological function. An extract of *Salvia miltiorrhiza* has an effect on myocardial ischemia and reperfusion (Wang et al., 2013). Animal experiments have demonstrated that salvianolic acid B (Sal B), an active ingredient of *S. miltiorrhiza*, has neuroprotective effects against cerebral ischemia and reperfusion (Tian et al., 2009) and SCI (Fu et al., 2014), and can promote the recovery of neurological function in rats. The protective effect of Sal B on injured spinal cord was associated with a reduced inflammatory reaction and an increase in blood supply to the damaged tissue (Fu et al., 2014). Nevertheless, the effects of Sal B on the myelin sheath and oligodendrocytes after SCI remain unclear.

On the basis of the above-mentioned studies, we presumed that Sal B exerted a neuroprotective effect by mitigating damage to the myelin sheath and oligodendrocytes following SCI. Thus, we sought to verify the effects of Sal B on the maturation and differentiation of oligodendrocyte precursor cells using an *in vitro* approach.

Materials and Methods

Ethics statement
All animal procedures were conducted in accordance with guidelines reviewed and approved by the Institutional Animal Care and Use Committee of Guangdong Medical University, China, and in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. Precautions were taken to minimize suffering (see anesthesia procedures below) and the number of animals used in each experiment.

Primary culture of oligodendrocyte precursor cells and pharmacological intervention
In accordance with Armstrong's method (1998), cerebral cortex was taken from 48-hour-old Sprague-Dawley rats [provided by the Experimental Animal Center of Southern Medical University of China; license No. SCXK (Yue) 2011-0015] using an operating microscope (M525F40; Leica, Wetzlar, Germany) under sterile conditions. The tissue was washed with D-Hank's solution, cut into pieces and digested with 0.25% trypsin at 37°C for 15 minutes. Cells were incubated in oligodendrocyte precursor cell medium, containing Dulbecco's modified Eagle's medium/Ham's F12 and 15% fetal bovine serum, and then placed in a polylysine-coated culture flask for 10 days. The culture flask was centrifuged at 37°C in a swing arm rotor at 200 r/min for 1.5 hours. After removal of supernatant, oligodendrocyte precursor cell medium was added and the cells incubated for 24 hours with shaking at 37°C in a swing arm rotor at 200 r/min for 1.5 hours. The cells were purified using a 74-μm sieve. Purified cells were further cultured for 24 hours. Sal B powder (Xi'an Hongsheng, Xi'an, Shaanxi Province, China) plus physiological saline were prepared into a 5 mg/mL stock solution. Oligodendrocyte precursor cells were incubated with the

medium containing 5, 10 or 20 mg/L Sal B for 3 days.

Determination of purification and differentiation of oligodendrocyte precursor cells
Oligodendrocyte precursor cells at 8 hours after purification and before drug intervention were observed with an Axioplan 2 imaging E microscope (Carl Zeiss, Oberkochen, Germany). Immunohistochemistry for A2B5 was conducted to assess the purification of oligodendrocyte precursor cells. Immunohistochemistry for myelin basic protein (MBP) and immunohistochemistry for 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase) was performed for Sal B and control (without drug treatment) groups at 3 days after drug administration. The effects of drugs on oligodendrocyte precursor cell differentiation were observed with the Axioplan 2 imaging E microscope (Carl Zeiss). Staining for oligodendrocyte markers, MBP and A2B5: cells were incubated on 7 mm × 22 mm coverslips, fixed with 2% paraformaldehyde at room temperature for 15 minutes, washed three times with phosphate buffered saline (PBS) for 5 minutes each, treated with 2% H_2O_2 at room temperature for 30 minutes, washed three times with PBS for 5 minutes each, and blocked with 5% goat serum at 4°C for 2 hours. The samples were incubated with 5% goat serum-diluted rabbit anti-rat A2B5 antibody (1:100; Millipore Corp, Billerica, MA, USA) and rabbit anti-rat MBP antibody (1:100; Epitomics, Burlingame, CA, USA) at 4°C overnight, washed three times with PBS for 5 minutes each at room temperature, incubated with biotinylated goat anti-rabbit IgG for 30 minutes, then with avidin-biotin complex reagent (Neobioscience, Shenzhen, China) at 37°C for 30 minutes, washed twice in PBS, and washed once in Tris buffer. All samples were immersed in substrate solution, placed in the dark for 20 minutes, stained with hematoxylin, dehydrated through a graded alcohol series, permeabilized with xylene, and mounted with neutral resin. Immunohistochemistry for CNPase was carried out as follows: cells were incubated on 7 mm × 22 mm coverslips, washed with PBS, blocked and permeabilized with PBS containing 0.3% Triton X-100 and 10% natural goat serum for 30 minutes, treated with rabbit anti-rat CNPase antibody (1:100; Sigma, Carlsbad, CA, USA) at 4°C overnight, washed three times with PBS, treated with rhodamine (goat anti-rabbit, 1:200; Sigma) at room temperature for 1 hour, washed twice with PBS, treated with 4′,6-diamidino-2-phenylindole (DAPI) (1:10,000; Sigma) for 10 minutes, washed with PBS, dried in the air, and mounted.

Establishing a rat model of SCI and pharmacological intervention
Twenty female Sprague-Dawley rats weighing 230–250 g were provided by the Experimental Animal Center of Southern Medical University of China [license No. SCXK (Yue) 2011-0015]. The rats were randomly assigned to treatment and control groups. Rats were intraperitoneally injected with 10% chloral hydrate 400 mg/kg (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China). A rat model of contusion was established by heavy impact (Metz et al., 2000). After

shaving, the rats were fixed in the prone position. After sterilization, T_{8-11} vertebral segments were removed, and the spinal cord was exposed. The spinal dura mater was left intact. A 10 g 2 cm-diameter metal rod was vertically dropped from a 25 mm height to impact the exposed spinal cord and to cause SCI (Ohta et al., 2004). Within the first week after SCI, the rats were assisted to urinate, and were intramuscularly injected with 50 mg/kg cefazolin, twice a day.

According to the animal experiment (Ye et al., 2011), 20 mg/L Sal B was optimal for postoperative functional recovery. The rats in the treatment group were intraperitoneally administered 20 mg/kg Sal B dissolved in 1 mL of physiological saline, once a day, for 8 consecutive weeks. The rats in the control group were given an equal volume of physiological saline.

Motor function assessment
Locomotor activity was evaluated using the Basso, Beattie, and Bresnahan locomotor rating scale (Basso et al., 1995), once a week. Paralysis is 0 points and normal locomotion is 21 points. The trunk, lower limb and tail activities were observed at 2 days after surgery. The rats were placed in a large enough space to allow free movement for 5 minutes, and their activities were video recorded. Two independent examiners, blind to the experiment, observed hindlimb movements and assessed the animals' locomotor function.

Spinal cord slice preparation
Eight weeks after surgery, three rats from each group were intraperitoneally injected with 600 mg/kg 10% chloral hydrate, and perfused with physiological saline and 4% paraformaldehyde through the heart. T_{7-12} segments were placed in 4% paraformaldehyde for 6 hours, and immersed in 30% sucrose. Frozen sections were embedded with embedding medium. The samples were sliced into 25 μm-thick longitudinal sections.

Immunohistochemistry for MBP
Twenty-five-micron-thick sections were antigen retrieved with 10 μg/mL proteinase K in a 37°C water bath for 10 minutes. One section from every four was incubated with rabbit anti-rat MBP antibody (1:400; Millipore) at 4°C overnight, washed in PBS, incubated with mouse anti-rabbit conjugated to AlexaFluor 568 secondary antibody (1:400; Invitrogen) at room temperature for 2 hours, washed with PBS, air-dried and mounted. Staining was observed under a fluorescence microscope (Carl-Zeiss Axioplan 2 imaging E).

Toluidine blue staining and electron microscopy
Four rats from each group were perfused with 0.9% physiological saline or 0.25% glutaral + 4% paraformaldehyde and the spinal cords dissected. Three animals were processed for toluidine blue staining and one was prepared for electron microscopy. The spinal cord was cut into 10 mm-long longitudinal blocks taking the injury site as the center. The samples were fixed with glutaraldehyde and osmic acid, embedded with epoxy resin, and then sliced into 1 μm-thick semi-

thin sections. These sections were stained with toluidine blue (Sigma), mounted and observed with a microscope. At least three sections of each rat were observed. Myelinated nerve fibers were quantified in ten fields of each section. The percentage of myelinated nerve fibers relative to all fibers was calculated. Fifty-nanometer-thick ultrathin sections were stained with 2% uranyl acetate (Merck Drugs & Biotechnology, New Jersey, USA) and lead citrate (SPI-Chem, West Chester, USA), and observed with a transmission electron microscope (Philips CM120, Philips, The Netherlands).

Western blot assay
Fresh spinal cord was obtained from three rats of each group 30 minutes after SCI, including 0.5 cm of tissue from the distal and proximal ends of the injury site. All samples were triturated with a pipette, lysed with radioimmunoprecipitation assay buffer, and centrifuged at 10,000 r/min. The supernatant was collected and total protein concentration was measured by the bicinchoninic acid assay. Samples were stored at –20°C. Thirty micrograms of protein were heated at 100°C for 5 minutes and then electrophoresed on a 10% sodium dodecyl sulphate-polyacrylamide gel. The proteins were then electro-transferred onto polyvinylidene difluoride membranes (Amersham, Piscataway, NJ, USA). The membranes were washed, blocked with 5% skimmed milk in Tris-buffered saline for 1 hour, incubated with rabbit anti-rat caspase-3 primary antibody (1:1,000; Sigma) at 4°C overnight, washed, incubated with horseradish peroxidase-labeled mouse anti-rabbit IgG secondary antibody (1:5,000, Sigma) for 30 minutes, and visualized by enhanced chemiluminescence. β-Actin (1:1,000, rabbit anti-rat; Sigma) served as an internal reference. The absorbance of scanned bands was determined using Image J (National Institutes of Health, Rockville, MD, USA). The results are expressed as the ratio of the target protein band intensity to that for β-actin.

Statistical analysis
The data were analyzed using GraphPad Prism 5 software (GraphPad Software, Inc., CA, USA), and are expressed as the mean ± SD. One-way analysis of variance with Bonferroni *post hoc* and Student's *t*-test were used to compare intergroup differences. A value of $P < 0.05$ was considered statistically significant.

Results
Sal B promoted the maturation and differentiation of oligodendrocyte precursor cells
Purified oligodendrocyte precursor cells were round, elliptical and uniform, and had bipolar or tripolar cell bodies (**Figure 1A**). The percentage of A2B5-positive oligodendrocyte precursor cells was more than 95% (**Figure 1B**), indicating a high purification rate of oligodendrocyte precursor cells.

Three days after intervention with different concentrations of Sal B, oligodendrocyte precursor cells underwent immunohistochemical staining for MBP and CNPase to observe cell differentiation. The percentage of cells differentiating
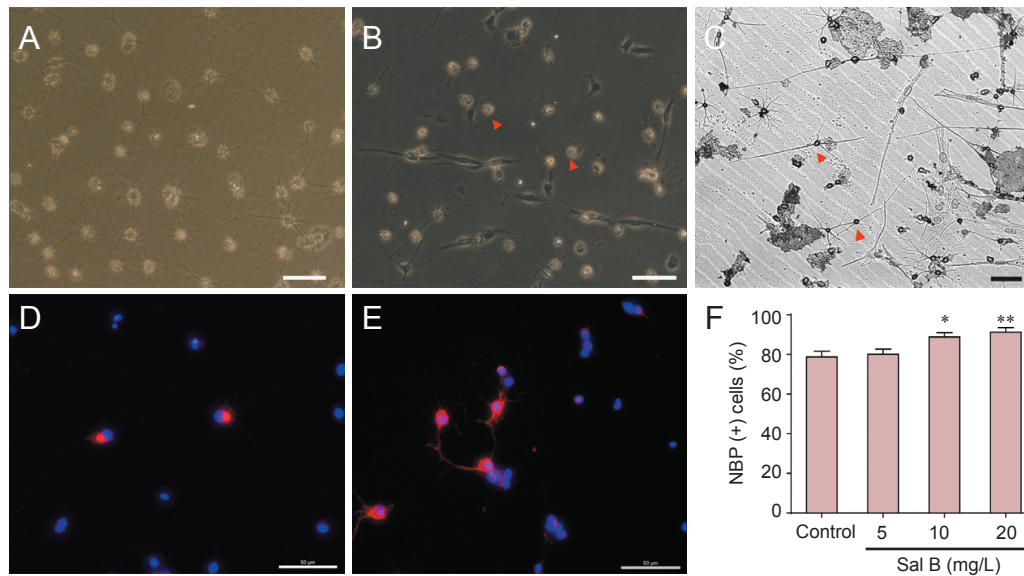
Figure 1 Effects of different concentrations of Sal B on oligodendrocyte precursor cell differentiation.
(A) Morphology of purified oligodendrocyte precursor cells. (B) A2B5-positive oligodendrocyte precursor cells (arrows). (C) Oligodendrocyte precursor cell differentiation in the 20 mg/L Sal B group, arrows point to MBP-positive cells. (D, E) Staining of oligodendrocyte precursor cells with CNPase (red) and DAPI (blue) in the control group (D) and 20 mg/L Sal B group (E). (A–E) Scale bars: 50 μm. (F) Quantitation of MBP-positive cells in the 5, 10, 20 mg/L Sal B groups and the control group. Data are presented as the mean ± SD. $*P < 0.05$, $**P < 0.01$, *vs.* control group (one-way analysis of variance with Bonferroni *post hoc* test). Sal B: Salvianolic acid B; CNPase: 2′,3′-cyclic nucleotide 3′-phosphodiesterase; MBP: myelin basic protein; DAPI: 4′,6-diamidino-2-phenylindole.

into oligodendrocytes was largest after treatment with 20 mg/L Sal B (**Figure 1F**). The number and thickness of cell processes were increased (**Figure 1C**). In the 20 mg/L Sal B group, a large number of oligodendrocyte precursor cells were positive for CNPase (**Figure 1E**). In the control group, CNPase staining was low (**Figure 1D**). Thus, 20 mg/kg was used as a therapeutic dose in the following *in vivo* experiments.

Sal B improved motor function in SCI rats
Basso, Beattie, and Bresnahan scores were significantly higher in the 20 mg/kg Sal B group compared with the control group at 3 weeks after intervention with Sal B ($P < 0.01$ or $P < 0.05$; **Figure 2A**).

Sal B enhanced remyelination in the injured spinal cord
Immunofluorescence staining for MBP demonstrated that at 8 weeks after SCI, in the control group, the quantity of myelin sheath around axons in the injured spinal cord was reduced, annuli were not intact and damaged broken myelin sheath was visible (**Figure 2B1**). In the Sal B-treated group many annuli were observed, and myelin sheath was intact (**Figure 2B2**). Also, the number of MBP-immunoreactive cells was more in the 20 mg/kg Sal B group than in the control group.

Toluidine blue staining revealed that the myelin sheath was broken and separated from axons in the control group (**Figure 2B3**). The number of myelinated nerve fibers was more in the 20 mg/kg Sal B group than in the control group. The myelin sheath was multi-layered, and the structure of the myelin sheath was intact in the 20 mg/kg Sal B group (**Figure 2B4, B7**).

In the control group, transmission electron microscopy

showed axons to be shrunk and a marked gap was observed between axons and the myelin sheath (**Figure 2B5**). In the 20 mg/kg Sal B group, the morphology of the myelin sheath and axons was noticeably improved. Axonal atrophy was mitigated, and the myelin sheath was multilayered (**Figure 2B6**).

Sal B suppressed caspase-3 expression in the injured spinal cord
Caspase-3 expression was significantly lower in the 20 mg/kg Sal B group compared with the control group 30 minutes after SCI (**Figure 2C**).

Discussion
Early intervention is very important for myelin sheath and oligodendrocyte recovery after injury. Previous studies showed that the number of oligodendrocytes was reduced by half at the injury site 1 day after SCI, so it is very important for nerve cell survival to reduce secondary injury as early as possible, and treatment within 24 hours could obtain good outcomes (Fehlings et al., 2012; Papastefanaki and Matsas, 2015). Thus, in this study, the time of intervention with Sal B was immediately after SCI.

Oligodendrocyte apoptosis can be observed at and surrounding the site of injury in humans and mice several weeks to several years after chronic injury (Kakulas, 1999; Guest et al., 2005; Totoiu and Keirstead, 2005; Lasinene et al., 2008). Demyelination and axonal degeneration impact the recovery of neurological function (Hagg and Oudega, 2006; Jiang et al., 2007; Serarslan et al., 2009). The caspase family plays a key role in apoptosis (Poter and Janicke et al., 1999; Saikumar et al., 1999; Hishikawa et al., 2000; Adjan et
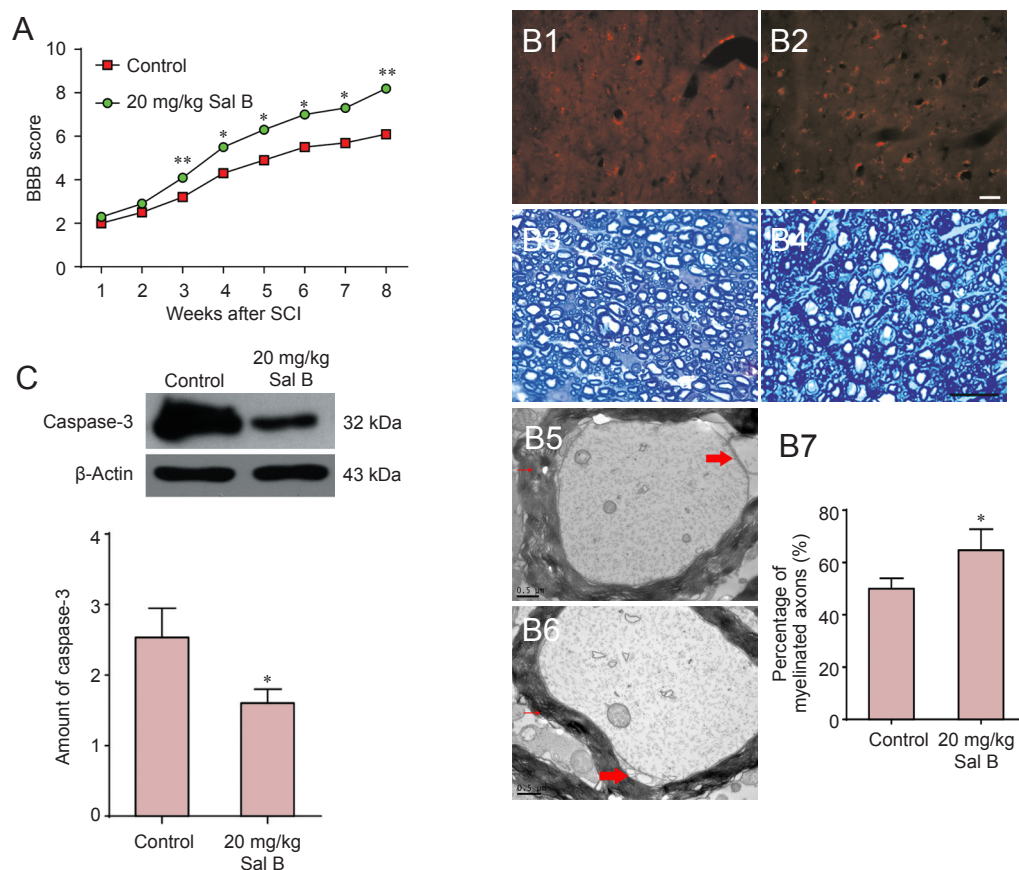
Figure 2 Effects of 20 mg/kg Sal B on remyelination, caspase-3 expression and motor function in rats with spinal cord injury.
(A) Effect of 20 mg/kg Sal B on motor function of SCI rats. BBB scores were used to assess the motor function of rats and higher scores represent better motor function. 10 animals per group were used. (B) Promoting effect of 20 mg/kg Sal B on remyelination in the injured spinal cord: 8 weeks after injury, MBP staining (red) at the site of injury in the control group (B1) and in the 20 mg/kg Sal B group (B2); toluidine blue staining at the site of injury in the control group (B3) and in the 20 mg/kg Sal B group (B4); ultrastructure of axons and myelin sheath in the control group (B5) and in the 20 mg/kg Sal B group (B6). Thin arrow points to abnormal myelin sheath; thick arrow points to the gap between axon and myelin sheath. Scale bars in B1, B2: 200 μm; scale bars in B3, B4: 50 μm; scale bars in B5, B6: 0.5 μm. (B7) Number of myelinated nerve fibers in the injured spinal cord. (C) Inhibitory effect of 20 mg/kg Sal B on caspase-3 expression in the injured spinal cord at 30 minutes after injury. The experiments were performed in triplicate. Quantitative data are presented as the mean ± SD. *$P < 0.05$, **$P < 0.01$, *vs.* control group (Student's *t*-test). Sal B: Salvianolic acid B; BBB: Basso, Beattie and Bresnahan; MBP: myelin basic protein.

al., 2007; Huey et al., 2008). Therefore, caspase-3 was used to measure the effect of Sal B on apoptosis following SCI. This study observed an anti-apoptotic effect of Sal B.

Sal B contributed to oligodendrocyte precursor cell differentiation *in vitro*, indicating that Sal B may promote remyelination after SCI. Oligodendrocyte precursor cells have been shown to produce hypoxia-inducible factor (HIF) under ischemia and hypoxia, and HIF1/2α can inhibit oligodendrocyte precursor cell maturation and myelination, which impact the recovery of neurological function (Yuen et al., 2014). Sal B has been shown to increase the blood supply to nerve tissue during neurological recovery (Fu et al., 2014). Our *in vivo* results confirmed that Sal B relieved secondary injury to the spinal cord at early stages and improved motor function recovery and promoted remyelination.

In conclusion, Sal B can promote the maturation and differentiation of oligodendrocyte precursor cells, reduce damage to the myelin sheath around axons following SCI, suppress apoptosis, and contribute to the recovery of motor function.

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