Original Article
Berberine improves motor function recovery by inhibiting endoplasmic reticulum stress-induced neuronal apoptosis via AMPK activation in rats with spinal cord injury

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Abstract: Evidences have supported the neuroprotective potential of berberine (BBR) in brain ischemia and injury. This study aimed to investigating whether BBR has neuroprotective effects on SCI and its mechanisms. Adult male Sprague-Dawley rats (220-250 g) were randomly divided into Sham, SCI, SCI + BBR (40 mg/kg/day), and SCI + BBR (40 mg/kg/day) + Compound C groups. The BBB score showed BBR improved functional recovery obviously 7 days later after SCI. Spinal cord tissue samples were harvested three days after SCI. Apoptotic neurons were assessed by TUNEL assay, and the expression levels of p-AMPK, AMPK, CHOP, cleaved caspase-12, Bcl-2, Bax, and cleaved caspase-3 were determined via Western blot. Furthermore, the influence of BBR on p-AMPK, caspase-12 and CHOP expression was determined via immunofluorescence. Neuronal cell apoptosis after SCI was significantly attenuated by BBR (P<0.01). Moreover, Western blot demonstrated that the expressions of cleaved caspase-12, CHOP, Bax, and caspase-3, which were linked to endoplasmic reticulum stress (ERS) associated apoptosis pathways, were significantly increased after SCI and were inhibited by BBR (P<0.01). However, the expressions of p-AMPK and Bcl-2 were significantly decreased after SCI and ameliorated by BBR (P<0.01). Immunofluorescence analysis indicated that BBR increased p-AMPK positive neurons number, reduced caspase-12 and CHOP positive neurons number following SCI (P<0.01). However, the protective effect of BBR on ERS-related protein expression was abolished by Compound C (P>0.05). Thus, BBR attenuates neuronal apoptosis and improves functional recovery in rats with SCI, and these neuroprotective effects may be associated with ERS inhibition and AMPK activation.

Keywords: Spinal cord injury, apoptosis, berberine, endoplasmic reticulum stress, AMPK, neuron

Introduction
SCI is a serious clinical problem linked to high morbidity worldwide, which often leads to devastating and catastrophic dysfunction [1]. Permanent neurological deficits result from massive cell death through immediate cell death by necrosis and prolonged apoptotic cell death, which results from secondary injury after SCI [2]. Secondary injury includes disturbances in ionic homeostasis, local edema, focal hemorrhage, excitotoxicity, oxidative stress, endoplasmic reticulum stress (ERS), and inflammation [3]. Neuronal apoptosis is a critical mechanism of secondary injury after SCI [4] and is triggered by various complex mechanisms. Recent concerns have been raised regarding the role of ERS, neuronal apoptosis, and their interactions in pathological deficits following SCI [5, 6]. Previous studies have demonstrated that prolonged ERS may play an important role in cell apoptosis regulation during SCI [6, 7]. Furthermore, ERS-mediated cell death has been associated with two central pathways: the transcription activation of the C/EBP homologous transcription factor (CHOP) [8, 9] and the activation of ER-associated caspase-12 [10, 11]. Therefore, blocking CHOP and caspase-12 mediated ERS cell death pathways may represent a potential therapeutic target to terminate the apoptotic course after injury.
Berberine (BBR) is an alkaloid extract in traditional medicine herbs. It is an AMP-activated protein kinase (AMPK) activator that is widely used in oriental medicine to treat diarrhea [12, 13]. BBR also exerts neuroprotective effects in brain ischemia [14-16], traumatic brain injury [17], and diabetic neuropathy in rats [18, 19]. Furthermore, previous studies have demonstrated that BBR attenuates ERS-induced cell apoptosis in several cell types [20, 21]. In addition, AMPK activation has been demonstrated to inhibit ERS-mediated neuroblastoma cell apoptosis [22]. However, whether BBR has a neuroprotective effect on SCI and the neuroprotective molecular mechanisms of BBR in the nervous system remain unknown. This study aimed to determine the effect of BBR on neuronal apoptosis and ERS in a rat model of SCI using behavioral, biochemical, and immunofluorescent assessments.

**Materials and methods**

**Animals and experimental design**

All animal experiments were conducted in accordance with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals. Adult male Sprague-Dawley rats (220-250 g) were purchased from the Experimental Animals Center of Liaoning Medical University. The animals were randomly divided into four groups: the sham group, SCI group, SCI + BBR group, and SCI + BBR + Compound C group. Berberine hydrochloride (BBR) (Nanjing Chunqiu Biology Engineering Company, 141433-60-5) was administered via intraperitoneal injection at a dose of 20 mg/kg every 12 hours per day following SCI. To investigate whether AMPK activation inhibits ERS-mediated neuronal apoptosis after SCI in rats, a specific inhibitor of AMPK, Compound C (Sigma-Aldrich, St. Louis, MO, USA), was administered via intraperitoneal injection at a dose of 20 mg/kg every 12 hours per day following SCI. To investigate whether AMPK activation inhibits ERS-mediated neuronal apoptosis after SCI in rats, a specific inhibitor of AMPK, Compound C (Sigma-Aldrich, St. Louis, MO, USA), was administered. Compound C was dissolved in 10% DMSO (Dimethyl sulfoxide; Sigma-Aldrich, St. Louis, MO, USA), and aliquots were stored at -20°C until use. Aliquots of Compound C at a concentration of 5 μg/μl were prepared in normal saline for single intravenous (5 μl) injection 30 minutes prior to BBR treatment (the final concentration of DMSO <0.1%).

**Induction of SCI in rats**

All rats were injured at the thoracic level 10 (T10) following a modified weight-drop model, which was performed to induce SCI [23]. Briefly, the rats were anaesthetized (10% chloral hydrate 0.03 ml/kg, i.p.), and the model was induced under sterile conditions. The skin and muscle were incised, and a laminectomy was performed at T10, leaving the dura intact. The impact of a 10-g weight at a height of 20 mm was subsequently applied to the dorsal surface of the spinal cord. The force was implemented by a steel rod (2-mm diameter tip), which was vertically dropped through a ruler tube perpendicular to the spinal cord. Following removal of the device, the muscle and skin were sutured. Treatments were subsequently administered as indicated for each group.

**Behavioral tests**

To investigate the functional recovery of the rats’ hind limbs after SCI, the Basso Beattie Bresnahan (BBB) behavioral test in an open-field was performed at four time points after SCI [24]. The scale used to measure hind limb function after SCI ranged from zero, which indicates no spontaneous movement, to a maximum score of 21, which indicates normal motor function. The inclined plane test was performed by a rubber mat secured to a flat board. The maximum angle of the flat board position was increased from 0° horizontally in 5° increments until the rat was unable to maintain its position on the board for five seconds without falling. Each rat was scored by two observers blinded to the experimental groups.

**Collection of spinal cord tissues**

Seventy-two hours following sham surgery or injury, a subgroup of animals in each group were sacrificed by cervical dislocation. For western blot assays, tissues were collected as 2-cm sections on ice, which were centered at the T10 lesion site. The tissues were rapidly excised and stored at -80°C until further analysis. Lesion site ± 3 mm of cord tissues either side of the lesion epicenter for rats were removed and prepared for western blot assays. For Nissl staining, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL), and immunostaining analysis, rats in each group were anaesthetized as previously described and intracardially perfused with cold saline (4°C), followed by 4% paraformaldehyde in phosphate buffered saline (PBS). The injured spinal cords within the 10-mm rostral, epicen-
Berberine improves motor function recovery by inhibiting endoplasmic reticulums

Figure 1. BBR improved locomotor function after spinal cord injury. Locomotor function was assessed by the BBB scale. Rats treated with BBR demonstrated a significant improvement in locomotor function from days 7 to 14 post-injury compared with the SCI group (**P <0.01). **P <0.01 vs. the SCI group. Data are presented as means ± standard deviations (SDs), n = 6.

after, and caudal regions were harvested and stored in 4% paraformaldehyde for subsequent analysis. The formalin-fixed spinal cord tissue samples were subsequently embedded in OCT media and coronally sectioned at 5-µm thickness at two spinal cord levels (2-mm rostral and caudal to the epicenter of the injury) with a freezing microtome (Leica CM3050S, Heidelberg, Germany).

TUNEL assay

To determine the ratio of cell apoptosis in the two tissue sections (2-mm rostral and caudal to the epicenter of the injury) of each spinal cord tissue sample, an in situ cell death detection kit (Roche, Mannheim, Germany, 10711900) was applied according to the manufacturer’s instructions. Images were captured by a confocal microscope (Olympus IX71, Tokyo, Japan). Positive apoptotic neurons were counted in six randomly selected fields per sections in the spinal anterior horn by Image J 1.42q analyzer software (Liaoning Medical University, Jinzhou, Liaoning, China). The average number of TUNEL-positive neurons (red dots neurons) and all neurons for the two sections were counted respectively as the number in a sample, at 400 × magnification (Scale bars = 20 μm) [25]. The percentage of apoptotic neurons in a sample was defined as follows: the percentage of apoptotic cells (%) = 100 × (total apoptotic neurons/total neurons).

Immunofluorescence staining

The two frozen sections of each spinal cord tissue sample from the 2-mm rostral and caudal to the epicenter of the injury were rewarmed at room temperature for 20 minutes and then rinsed three times in PBS for 15 minutes. The sections were subsequently blocked with 5% normal donkey sera (Jackson Immuno Research, West Grove, PA, USA; 118727), 0.05% Triton X-100 for one hour. The sections were then incubated with a suitable dilution of primary IgG antibody, such as p-AMPK(1:50; Santa Cruz, sc-101630), Mouse mAb CHOP (1:3,200; CST, USA; L63F7, #2895S) or Rabbit mAb Caspase-12 (1:1000; Abcam, ab18766) mixture, overnight at 4°C and then rinsed three times in PBS for 15 minutes. The sections were subsequently incubated with IFKine Red AffiniPure Donkey Anti-Rabbit IgG (1:1,000; Abbkine, Redlands, CA, USA; 142401B, 100 μl/500 μl) or IFKine Green AffiniPure Donkey Anti-Mouse IgG (1:1,000; Abbkine, Redlands, CA, USA; 1337-02A, 100 μl/500 μl) for one hour at room temperature. After washing in PBS for 15 minutes, the nuclei were marked with 4′6-diamidino-2-phenylindole (DAPI) according to the manufacturer’s instructions (Sigma-Aldrich, St Louis, MO, USA; #033M4064V, 1 mg). Images were captured by a confocal microscope (Olympus IX71, Tokyo, Japan) at a high magnification (Scale bars = 20 μm). The positive cell numbers of CHOP and caspase-12 were counted in six randomly selected fields per sections in the spinal anterior horn by Image J 1.42q analyzer software (Liaoning Medical University, Jinzhou, Liaoning, China). The average positive cell number for the two sections (2-mm rostral and caudal to the epicenter of the injury) was then calculated as the number of positive cells in a sample.

Western blot

The prepared spinal cord tissues were minced using eye scissors on ice, homogenized in lysis buffer that contained 1 mmol/L of PMSF, 1 μg/ml of aprotinin, 1 μg/ml pepstatin A, 50 mM of NaF and centrifuged at 12,000 revolutions per minute (rpm) at 4°C for 20 minutes to collect the supernatant. BCA protein assay was used to determine the protein concentrations. Proteins were separated via SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a PVDF membrane. The membrane was blocked with 0.1% BSA in Tris-buffered saline (TBS) for two h at room temperature and subsequently incubated overnight at 4°C with antibodies for p-AMPK (1:1000; Santa Cruz, sc-10-
Berberine improves motor function recovery by inhibiting endoplasmic reticulums

1630), AMPK (1:1,000; Santa Cruz, sc-74461), CHOP (1:1,000; Cell Signaling, USA, L63F7, #2895S), Bcl-2 (1:1000; Abcam, ab183656), Bax (1:1000; Abcam, ab199613), caspase-12 (1:500; Abcam, ab18766), cleaved-caspase-3 (1:500; Abcam, Cambridge; ab32042), and β-actin (1:500; Santa Cruz Biotechnology USA, #D1713). The membrane was then incubated with HRP-conjugated secondary antibody (1:2,000; Santa Cruz, #L3902, 200 μg/0.5 ml) in TBS-T for one hour at room temperature and visualized using a chemiluminescence system (ECL kit; Beyotime Institute of Biotechnology, Nanjing, Jiangsu, China). Optical density (OD) was determined using Image J 1.42q analyzer software (Liaoning Medical University, Jinzhou, Liaoing, China).

Statistical analysis

Data were analyzed using SPSS version 16.0 statistical software package (SPSS Inc., Chicago, IL, USA). All data are expressed as means ± standard deviations (SDs). Comparisons between groups were performed via one-way analysis of variance (ANOVA) and Dunnett’s post-hoc test for the statistical analysis. P<0.05 was considered as statistically significance.

Results

Effects of BBR on neural function recovery after SCI

To identify the effects of BBR on neural function recovery during the two weeks after SCI,
Berberine improves motor function recovery by inhibiting endoplasmic reticulums

the evaluation of BBB scoring at four time points [23] post-surgery was used to quantify locomotor activity. In the sham group, the BBB scores were 20.17 ± 0.983, 20.33 ± 0.816, 20.5 ± 0.837, and 20.83 ± 0.408 at the four time points post-surgery, respectively. The rats in the two experimental groups exhibited gradual function improvement during the subsequent seven days. In contrast, the rats in the BBR group exhibited significantly increased scores at days 7 ($P<0.01$) and 14 ($P<0.01$) compared with the SCI group. Two weeks after SCI, the final BBB scores in the SCI (n = 6) and BBR (n = 6) groups were 6.17 ± 0.753 and 9.67 ± 0.816, respectively ($P<0.01$, Figure 1).

**BBR attenuates neuronal apoptosis in spinal cord tissues after SCI**

Neuron survival was examined via an immunofluorescence technique using a specific antibody for neuron (NeuN) and TUNEL analysis (Figure 2). The number of TUNEL positive neurons in each group was counted with a color image analyzer (Image-ProPlus, Liaoning Medical University, Jinzhou, Liaoning, China). The percentages of apoptotic spinal cord neurons in the sham, SCI, and SCI + BBR groups were 3.56 ± 1.53%, 44.58 ± 4.52%, and 30.26 ± 3.22%, respectively. These findings indicate that BBR reduced spinal cord neuronal death after SCI.

**Effects of BBR on Bcl-2, Bax, and cleaved caspase-3 expression in spinal cord tissues**

The SCI group exhibited decreased Bcl-2 expression and increased Bax and cleaved caspase-3 expression compared with the sham group. In contrast, the BBR group exhibited a significantly increased Bcl-2 expression and significantly decreased Bax and cleaved caspase-3 expression compared with the SCI group ($P<0.01$; Figure 3A, 3B).

**Effects of BBR on the expression of p-AMPK, AMPK, CHOP, and cleaved caspase-12 in spinal cord tissues**

Western blotting was used to determine the effects of BBR on the expressions of p-AMPK, AMPK, and cleaved caspase-12 in each group. The SCI group exhibited significantly decreased p-AMPK expression and significantly increased CHOP and cleaved caspase-12 expressions compared with the sham group ($P<0.05$). In contrast, the BBR group exhibited a significantly increased p-AMPK expression and significantly decreased CHOP and cleaved caspase-12 expressions compared with the SCI group ($P<0.05$, Figure 4).

**Immunofluorescence staining of p-AMPK, caspase-12 and CHOP in spinal cord neurons**

Immunofluorescence staining was used to identify the effects of BBR administration on the expressions of p-AMPK, caspase-12 and CHOP in spinal cord tissues three days after SCI in each group (Figure 5A-F). The number of p-AMPK, caspase-12 and CHOP positive neurons in each group was counted with a color image analyzer (Image-ProPlus, Liaoning Medical University, Jinzhou, Liaoning, China). The
average numbers of p-AMPK, caspase-12 and CHOP positive neurons per section in each group were as follows (Figure 5B, 5D, 5F): sham group 25.67 ± 2.73, 8.33 ± 1.63 and 3.32 ± 1.42/mm², respectively; SCI group 6 ± 1.78, 31.17 ± 3.19 and 24.51 ± 2.73/mm², respectively; and BBR group 17.5 ± 2.59, 20.51 ± 2.88 and 15.83 ± 1.72/mm², respectively. Immunofluorescence staining indicated increased CHOP and caspase-12 but decreased p-AMPK positive neuron numbers in the spinal cord tissues in the SCI group compared with the sham group (P<0.01). However, the spinal cord tissues in the BBR group exhibited significantly higher p-AMPK but lower CHOP and caspase-12 positive neuron numbers compared with the SCI group (P<0.01).

**Effects of compound C on the expressions of p-AMPK, CHOP, and cleaved caspase-12 in SCI with BBR administration**

To investigate the molecular mechanism of the influence of BBR on ERS-induced neuronal apoptosis in the SCI model, the AMPK inhibitor Compound C was administered. The activations of AMPK, CHOP, and cleaved caspase-12 were determined by Western blot analysis. The expression levels of CHOP and caspase-12 were significantly reduced, but p-AMPK was significantly increased in the SCI + BBR group compared with the SCI group (P<0.01). However, the expression levels of CHOP and cleaved caspase-12 were significantly increased, but p-AMPK was significantly decreased in the SCI + BBR + Compound C group compared with the SCI + BBR group (P<0.01). The expressions of CHOP and cleaved caspase-12 in the SCI + BBR + Compound C group was not significantly different from the SCI group (P>0.05). Together, these findings indicate that the protective role of BBR in ERS-induced apoptosis in SCI is related to AMPK activation (Figure 6A, 6B).

**Discussion**

Following SCI, the pathological changes that ensue are a result of direct damage at the injured location and include disturbances in ionic homeostasis, local edema, focal hemorrhage, excitotoxicity, oxidative stress, inflammation, and ERS. These changes have been referred to as secondary damages [3]. Although the damage initiated by an original injury is irreversible, secondary injury is an active process that occurs at molecular and cellular levels and is thus reversible and modifiable [26]. This suggests that therapeutic treatments to limit the pathological process and improve neural function recovery are possible. Although the precise mechanisms of secondary spinal cord damage remain elusive, neuronal and glial cell apoptosis plays a role in SCI, and the inhibition of neuronal and oligodendroglial apoptosis may be critical for improving neural function recovery [4, 27].

BBR is an alkaloid extracted from plants of the berberidaceae family and has a long history in Chinese medicines [28]. BBR can penetrate the blood-brain barrier via intraperitoneal administration [29]. However, to our knowledge, it has not been previously reported whether BBR has
Berberine improves motor function recovery by inhibiting endoplasmic reticulums.
Berberine improves motor function recovery by inhibiting endoplasmic reticulums

Figure 5. Immunofluorescence staining of the proteins: p-AMPK (A), caspase-12 (C) and CHOP (E) in rat spinal cord tissues at 72 h after injury. There were lots of p-AMPK positive neurons but few caspase-12 and CHOP positive neurons in the sham group. After SCI the number of p-AMPK positive neurons decreased significantly. What more, Injury resulted in a substantial number of caspase-12 and CHOP positive neurons in the SCI group. This increase was significantly attenuated by BBR treatment. *P<0.05 vs. the sham group, **P<0.05 vs. the SCI group. (B, D, F) The quantitative analysis of P-AMPK, caspase-12 and CHOP positive neurons per longitudinal section in both groups between 5 mm rostral and caudal to the injury epicenter (six views per section) is shown. Values are presented as means ± SDs, n = 6.

neuroprotective effects in SCI. To assess the effect of BBR on neurological function in SCI rats, an extensively used BBB scoring method was implemented in the current study. The findings indicated that BBR administration after SCI was associated with significant improvement in locomotion at one week post-treatment (Figure 1). This finding suggests that BBR ameliorates deficits in locomotor function in rats with SCI. Furthermore, BBR not only significantly decreased the percentage of TUNEL positive neurons but also decreased the Bax/Bcl-2 ratio and suppressed cleaved caspase-3 expression (Figures 2 and 3). Together, these findings indicate that BBR has neuroprotective effects in SCI.

Early research demonstrated that BBR, an AMPK activator, suppresses neuroinflammatory responses via AMPK activation in BV-2 microglia [30]. Other research has also demonstrated that AMPK activation plays a protective role in brain ischemia in rats [31, 32]. AMPK is a protein kinase that maintains the energy balance in an organism [33, 34]. Disturbances in energy metabolism after SCI have been demonstrated in previous research [35]. The central nervous system has a high metabolic rate and a poor capacity for nutrient storage. However, the changes in p-AMPK protein expression in rats after SCI remain unknown. Therefore, it is important to investigate the influence of BBR on AMPK and the role of AMPK in neuronal survival and death following SCI. Our research demonstrates that the p-AMPK expression after SCI is lower than in normal spinal cord tissues; however, BBR administration after SCI promotes AMPK activation (Figures 4 and 5A, 5B). These findings indicate that the p-AMPK expression decreased following SCI,
Berberine improves motor function recovery by inhibiting endoplasmic reticulums

which suggests that the energy metabolism in SCI is turbulent. Nevertheless, BBR administration activated AMPK in rats after SCI. Together, the current findings suggest that AMPK activation may be associated with the effect of BBR inhibition of neuronal apoptosis following SCI.

Nevertheless, the exact mechanism of the interaction between apoptosis and AMPK activation remains unclear. Several studies have demonstrated that the protein expression of CHOP and caspase-12 progressively increased to a maximum at three days post-injury [5, 7]. The protein expression levels of CHOP and caspase-12 at three days after SCI were determined to investigate the molecular mechanism of BBR in SCI in this study. The results demonstrated that ERS was involved in SCI, and the expressions of CHOP and caspase-12 significantly increased, which is similar to a previous study [5]. Furthermore, the current study demonstrated that ERS was inhibited by BBR administration three days after SCI in rats (Figures 4 and 5C-F), which suggests that the neuroprotective effect of BBR in SCI may be relevant to ERS inhibition. It has been reported that AMPK activation has neuroprotective effects [36] and that this is an important defensive response to stress [37]. AMPK activation protects cardiomyocytes against hypoxic injury via the attenuation of ERS [38]. Moreover, AMPK activation may function against ERS-mediated neurotoxicity [39]. To investigate whether AMPK activation inhibits ERS-mediated neuronal apoptosis following SCI in rats, a specific inhibitor of AMPK, Compound C, was used. The protective effect of BBR on the expression of ERS-related proteins was abolished by Compound C (Figure 6), which indicates that the protective role of BBR in SCI may be associated with ERS-mediated cell apoptosis attenuated by AMPK activation.

Despite these promising findings, several study limitations must be considered in the interpretation of these results. First, our research suggests that the administration of BBR (40 mg/kg/day) has the effect to inhibit ER stress-induced apoptosis in rats after SCI, while a previous study indicated that a relatively high concentration of berberine induces apoptosis via the mitochondrial-dependent pathway in human glioblastoma T98G cells [40]. It is necessary to clarify whether the different concentrations of berberine is associated with its different drug effects in cell apoptosis and investigate the optimal therapeutic dose for SCI. Several studies have demonstrated that BBR has the potential to promote axonal regeneration in injured nerves of the peripheral nervous system [41] and promote Nrf2-related neurite outgrowth [18]. In addition, BBR also attenuates axonal transport impairment and axonopathy in neuroblastoma-2a cells [42]. Nevertheless, whether the positive effect of BBR on functional recovery after SCI is related to its potential to pro-

Figure 6. Blocking AMPK activation facilitated ERS-induced apoptosis. A. Expression of p-AMPK, CHOP, and cleaved caspase-12 proteins in the sham, SCI, SCI + BBR, and SCI + BBR + compound C groups. B. The optical density analysis of p-AMPK, CHOP, and cleaved caspase-12 in each group are shown as histograms. β-actin was used as the loading control and for band density normalization. *P<0.01 vs. the sham group; **P<0.01 vs. the SCI group; ##P>0.05 vs. the SCI group. Data are presented as means ± SDs, n = 6.
Berberine improves motor function recovery by inhibiting endoplasmic reticulums

In summary, the main findings in this study indicate that BBR treatment inhibits the activation of the ERS-induced apoptotic pathway in a rat SCI model, and this protective effect may occur via AMPK activation.

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Disclosure of conflict of interest

None.

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