Original Article
Neuroprotective effect of 7,8-dihydroxyflavone in spinal cord injury

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Abstract: Objective: To investigate the protective effect of the specific tyrosine receptor kinase B (TrkB) agonist 7,8-dihydroxyflavone in spinal cord injury and the involved mechanisms. Methods: A total of 130 male ICR mice were randomly divided into five groups, including a sham-operated group, spinal cord injury group, spinal cord injury + solvent group, spinal cord injury + 7,8-dihydroxyflavone (3 mg/kg) group, and spinal cord injury + 7,8-dihydroxyflavone (5 mg/kg) group. The spinal cord injury model was established via the cross-clamp method (the spinal cord at the level of T7-T11 was exposed). 7,8-dihydroxyflavone or a solvent was injected intraperitoneally 30 minutes after the induction of injury. Protein expression of p-TrkB, p-Akt, caspase-3, and Bcl-2 was examined using immunoblotting 24 hours after the spinal cord injury was induced. HE staining and in situ TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling) assays were used to detect neuronal damage. Another batch of mice were maintained for 14 days after the spinal cord injury was induced, administered 7,8-dihydroxyflavone daily at the same time point, and assessed for neurological function. Results: 7,8-dihydroxyflavone significantly upregulated the protein expression of p-TrkB and p-Akt, suppressed the expression of the caspase-3 protein, increased Bcl-2 protein expression, and reduced neuronal apoptosis. Furthermore, 7,8-dihydroxyflavone remarkably improved the neurological function scores after the occurrence of spinal cord injury. Conclusions: A significant neuroprotective effect was induced by 7,8-dihydroxyflavone in mice with spinal cord injury, and 7,8-dihydroxyflavone may be used for the clinical treatment of patients with spinal cord injury.

Keywords: Spinal cord injury, 7,8-dihydroxyflavone

Introduction

Spinal cord injury is associated with high morbidity and mortality and seriously affects the quality of life of the patients. The pathogenic mechanisms of spinal cord injury mainly include primary spinal cord injury and secondary spinal cord injury. Primary spinal cord injuries are the inevitable immediate injuries that result from a variety of traumas. Secondary spinal cord injuries are the injuries that occur several hours or days after the trauma and are the hot spots and focus of current research on drug therapy [1]. Although the molecular mechanisms of spinal cord injury have been elucidated to a certain degree with the progress of related research, drugs for effective clinical treatment of spinal cord injury are still lacking. Therefore,

it is imperative to determine effective drugs for the treatment of these injuries. Previous studies have shown that brain-derived neurotrophic factor (BDNF) exerts its neuroprotective effect through activation of the TrkB signaling pathway in a spinal cord injury model [2, 3], suggesting that drugs the target TrkB have important implications for the treatment of spinal cord injury. This study intended to provide a new direction for the clinical treatment of spinal cord injury through the examination of the protective effect of the specific TrkB agonist 7,8-dihydroxyflavone against spinal cord injury.

Materials and methods

The ICR mice used in this study were purchased from the Laboratory Animal Medical Center of

Nanjing Medical University. Nissl staining solution and total protein extraction solution were purchased from Beyotime Biotechnology Co., and antibodies against p-TrkB, p-Akt, p-CREB, cleaved caspase 3, and Bcl-2 were obtained from Cell Signaling Co. The TUNEL assay kit was purchased from Roche Co. (US). The cross-clamp mouse model was prepared using a vascular clamp manufactured by Kent Scientific Co. (US). The tested compound, 7,8-dihydroxyflavone, was purchased from Sigma Co. (US).

Animal groups and preparation of the spinal cord injury mouse model

The ICR mice were randomly divided into five groups, including the sham-operated group, spinal cord injury group, spinal cord injury + solvent group, spinal cord injury + 7,8-dihydroxyflavone (3 mg/kg) group, and spinal cord injury + 7,8-dihydroxyflavone (5 mg/kg) group, with 26 animals in each group. The spinal cord injury mouse model was prepared according to the description in the literature [4]. Briefly, after the mice were anesthetized with chloral hydrate (4 mg/kg), a 3-cm incision was made in the middle of the back. The T5-8 vertebrae were exposed under a surgical microscope. The vertebral endplate at the level of T9 was removed using a vascular clamp to fully expose the spinal cord. The spinal cord was pressed with a mouse vascular clamp for one minute, with a force of 10 g. The back muscle and skin were closed by layers with sutures and adequate hemostasis. The animals in the sham-operated control group underwent removal of the vertebral endplate, exposure of the spinal cord, and closure of the incision with sutures, but without clamping of the spinal cord with a vascular clamp. The mice were placed on a heating pad after the surgery and then were returned to their cages and received a normal diet when they were fully awake. Twelve mice were euthanized one day after the injury was induced for molecular mechanism and histology assays. The remaining 14 mice were maintained for an additional 20 days for neurological function assessment before they were euthanized via cervical dislocation.

Immunoblotting

The mice were anesthetized 24 hours after spinal cord injury, and the thoracic cavity was opened. Perfusion was performed with 80 mL of saline via the left heart apex. An incision was made in the back to expose the T5-8 vertebrae. The vertebral endplate was removed, and a 1.5-cm fragment of spinal cord tissue, with the clamping site in the middle, was collected and stored in liquid nitrogen. Then, tissue lysis buffer was added at a ratio of 1:1000 to 50 mg of spinal cord tissue from each mouse, followed by homogenization. The homogenate was centrifuged at 12,000 rpm for 10 min. The supernatant was collected, and 5 × loading buffer was added at a 4:1 ratio followed by incubation in boiling water for 10 min. Electrophoresis was performed with 35 mg of protein in each well. The membrane with the transferred protein was blocked at room temperature for one hour using 5% milk and then incubated with diluted primary antibody at 4°C overnight. The membrane was washed with TBST and then incubated with diluted secondary antibody at room temperature for one hour. Following the TBST wash, the developing buffer was added to the membrane for visualization. Image J software was used for quantitative analysis.

TUNEL and HE staining

Perfusion was performed with 100 mL of saline via the left ventricle at 24 hours after spinal cord injury. Spinal cord tissue specimens were fixed with 4% paraformaldehyde and embedded with paraffin, and 6-mm consecutive sections were prepared. A TUNEL assay was performed to detect apoptotic neurons by strictly following the user's manual. Ten visual fields were randomly selected under the microscope (400 ×) for positive cell counting. The mean number of positive cells was recorded for each slide. Five mice were used from each group for HE staining following the procedure in the user's manual.

Motor function assessment

The mice with spinal cord injury were assessed for motor function using the Basso mouse scale for locomotion [5]. The first assessment was performed at 24 hours after the injury, and the assessment was repeated at the same time 13 days after the injury.

Statistical analysis

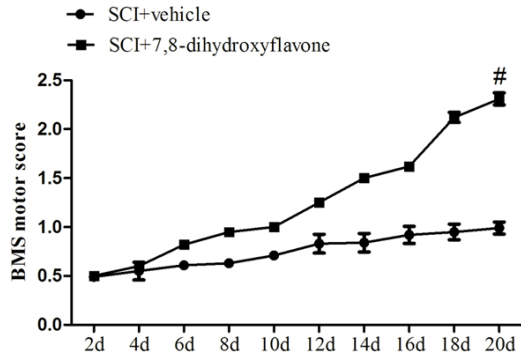The experimental data were processed with SPSS17.0 and expressed as means ± standard

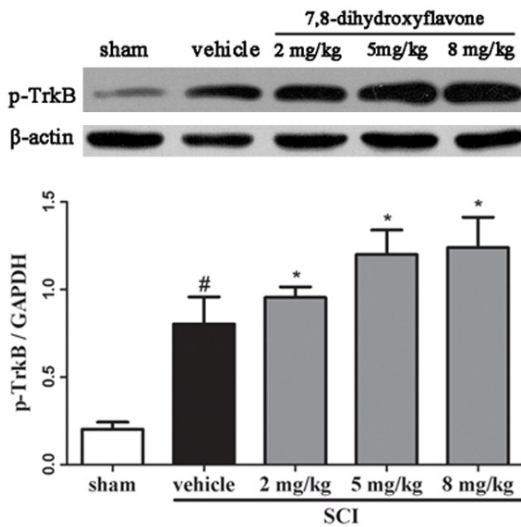**Figure 1.** Comparison of BMS motor scores in two groups.

Figure 2. Different expression of p-TrkB in different groups.

Figure 3. Expression comparison of p-TrkB in different groups.

deviations ($\overline{x}$ ± s). A Mann-Whitney test was used to analyze the neurological function scores. Intergroup comparisons were performed with one-way analysis of variance. A value of $P < 0.05$ was considered a statistically significant difference.

Results

7,8-dihydroxyflavone significantly improved neurological function after spinal cord injury in a dose-dependent manner

The neurological function scores of the sham-operated group remained at a value of 2.5 for 20 days. Compared to the sham-operated group, the motor function scores of the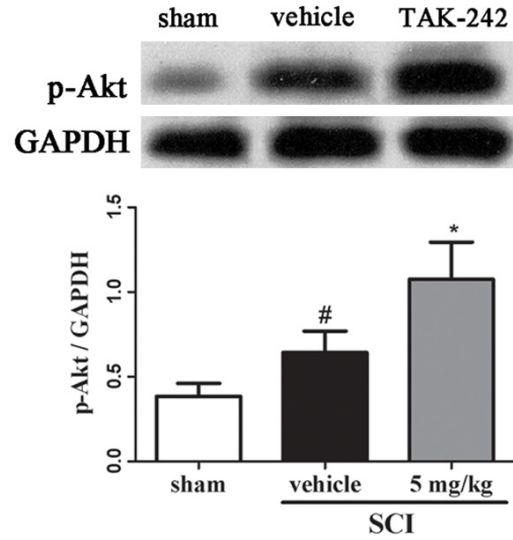 spinal cord injury group were significantly lower (**Figure 1**, $P < 0.05$) and were significantly improved by 7,8-dihydroxyflavone treatment (**Figure 1**, $P < 0.05$).

Effect of 7,8-dihydroxyflavone on the expression of p-TrkB, p-Akt and p-CERB

The results of this study showed that 7,8-dihydroxyflavone significantly upregulated the phosphorylation level of p-TrkB in a dose-dependent manner, and the effect of the 5 mg/kg dose showed a significant improvement over the 3 mg/kg dose (**Figure 2**, $P < 0.05$). The results of p-Akt measurements indicated that 7,8-dihydroxyflavone remarkably upregulated the level of p-Akt in a dose-dependent manner (**Figure 3**, $P < 0.05$). Moreover, the solvent had no effect on the expression of p-TrkB and p-Akt. In addition, 7,8-dihydroxyflavone significantly increased the expression of p-CREB (**Figure 4**, $P < 0.05$).

7,8-dihydroxyflavone significantly inhibited the expression of the apoptosis-related protein caspase-3, while it enhanced the expression of the anti-apoptotic protein Bcl-2

Based on the findings described above, immunohistochemical staining was performed on mice that received the most effective dose of 7,8-dihydroxyflavone (5 mg/kg). Caspase-3 expression was significantly increased in the spinal cord injury group (**Figure 5**, $P < 0.05$), while the Bcl-2 expression level was significant-
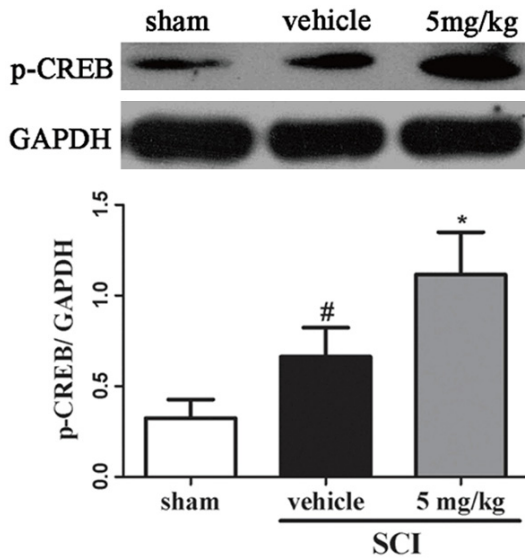
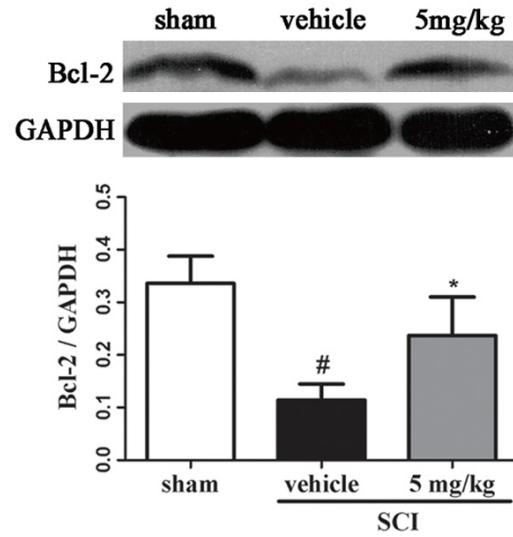Figure 4. Expression comparison of p-CREB in different groups.

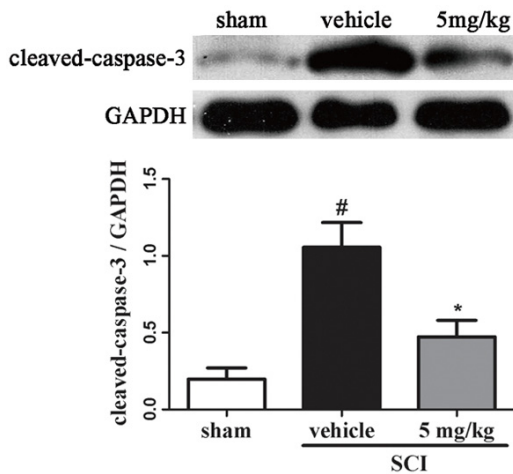Figure 6. Expression comparison of Bcl-2 in different groups.

Figure 5. Expression comparison of cleaved-caspase-3 in different groups.

ly decreased (**Figure 6**, *P* < 0.05), suggesting that 7,8-dihydroxyflavone can significantly inhibit the expression of caspase-3 (**Figure 5**, *P* < 0.05) and enhance the expression of the anti-apoptotic protein Bcl-2 (**Figure 6**, *P* < 0.05).

Histological changes

Apparent hemorrhage loci were found in the gray matter of the injured spinal cord 24 hours after injury induction via light microscopy (**Figure 7A**). Under 400× magnification, the neurons of the sham-operated group showed a regular structure and morphology (**Figure 7B**)

with dense white matter, while a large number of the neurons in the spinal cord injury group appeared shrunken and had undergone necrosis (**Figure 7C**). The number of damaged neurons was significantly reduced in the 7,8-dihydroxyflavone treatment group (**Figure 7D**). In the TUNEL staining assay, the nuclei of TUNEL-positive cells were brown, and TUNEL-negative cells were colorless. Only a small number of cells were TUNEL-positive in the sham-operated group (**Figure 8A**, *P* < 0.05). The number of apoptotic neurons in the spinal cord tissue 24 hours post spinal cord injury was significantly increased (**Figure 8B**, *P* < 0.05), while intraperitoneal injection of 7,8-dihydroxyflavone significantly reduced neuronal apoptosis (**Figure 8C**, *P* < 0.05). These results suggest that 7,8-dihydroxyflavone plays an anti-apoptotic role in spinal cord injuries.

Discussion

BDNF is a member of the endogenous neurotrophic factor family and exerts its neuroprotective function through binding to its specific receptor TrkB [6]. BDNF promotes the recovery of cellular functions by maintaining a stable intracellular calcium ion level. In addition, BDNF can also repair cell damage by protecting cells from damage caused by free radicals and increasing the activity of intracellular antioxidative enzymes. BDNF has been shown to promote the growth of striatal and sensory neu-
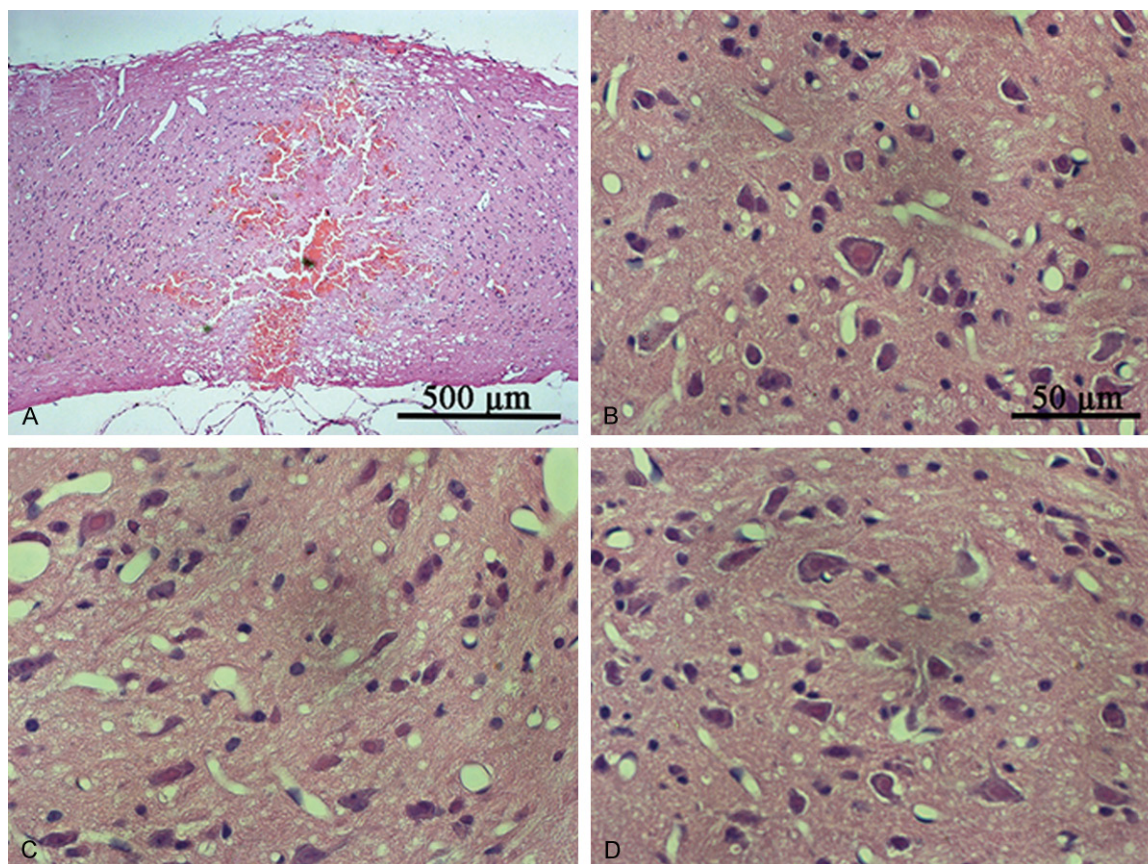
Figure 7. Staining assay of injured spinal cord in different groups.

rons, protect motor neurons from cell death induced by surgical resection, inhibit apoptosis of hippocampal neurons induced by nitric oxide, and protect cerebellar granule neurons from damage caused by metabolism and excitotoxicity. BDNF shows elevated expression after spinal cord injuries, which prevents the atrophy of damaged neurons and promotes the regeneration of axons from rubrospinal neurons and corticospinal neurons. BDNF activates Akt after binding to its receptor, TrkB, and the Akt-mediated signaling pathway plays an important role in the survival and apoptosis of neurons. Activated Akt suppresses the mitochondria-dependent apoptosis program. Previous studies have found that suppression of Akt activity aggravates the severity of spinal cord injuries [4, 7], while activation of Akt significantly improves the prognosis of spinal cord injury, suggesting that targeting the TrkB/Akt signaling pathway can exert a neuroprotective effect in spinal cord injuries. As a newly designed specific TrkB activator [8], 7,8-dihydroxyflavone showed neuroprotective activity, which is also

exhibited by BDNF. It prevented neuronal apoptosis, resisted kainic acid-induced cytotoxicity, and reduced the damaged area in cerebral stroke. It also showed a neuroprotective effect in an animal model of Parkinson's disease [9]. The results from this study showed that 7,8-dihydroxyflavone significantly improved neurological function scores in mice with spinal cord injury, which is likely related to the activation of the TrkB/Akt signaling pathway.

Akt upregulates CREB activity after its activation, and activated CREB induces the expression of the anti-apoptotic protein Bcl-2. Bcl-2 is an important anti-apoptotic protein and exerts its anti-apoptotic function by suppressing Ca^{2+} release from the endoplasmic reticulum and signal transduction of tissue apoptosis pathways and inhibiting free radicals. Inducing Bcl-2 protein expression exhibits marked anti-apoptotic effects in spinal cord injury animal models. Previous studies have shown that Akt activation following spinal cord injury upregulates CREB activity, which exerts its neuroprotective
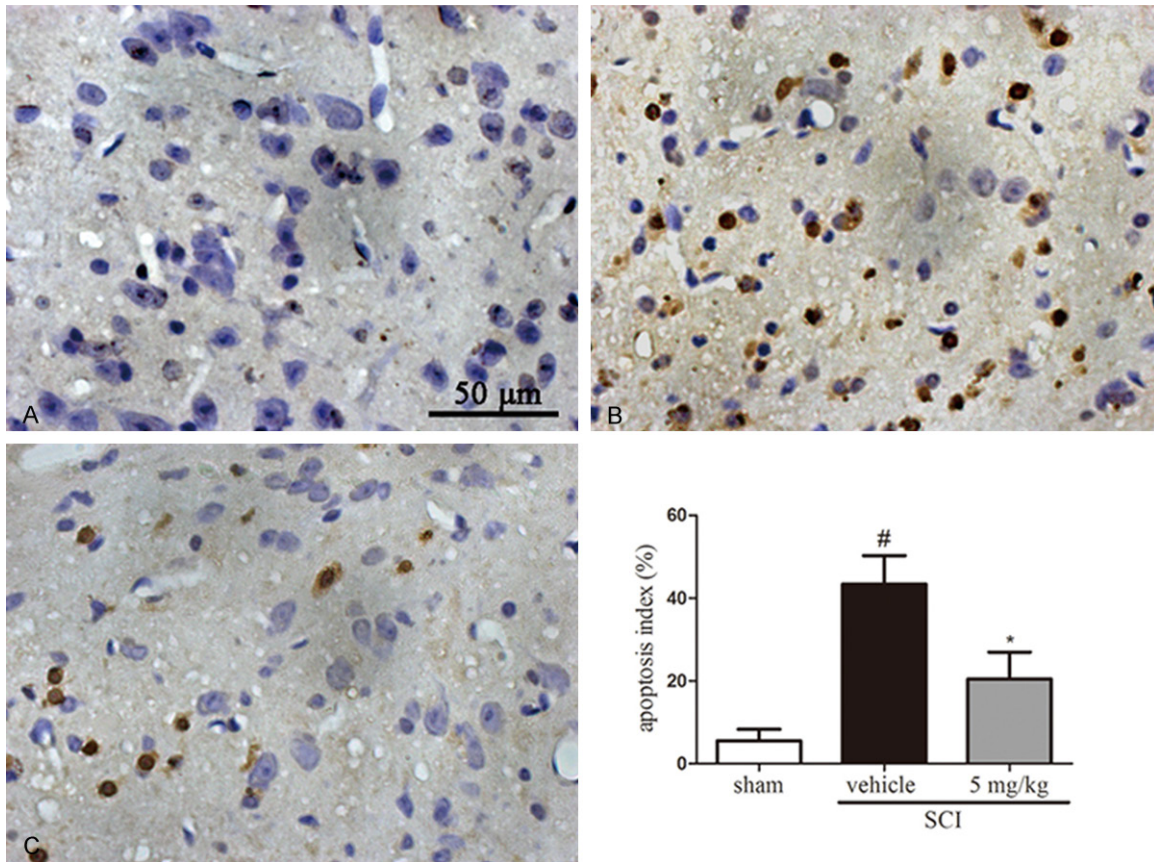
Figure 8. Comparison of apoptosis ratio in different groups.

effect through regulation of Bcl-2 expression [10]. Furthermore, activated Akt suppresses the expression of caspase-3, the most important executor of apoptosis. The main substrate of caspase-3 is poly (ADP-ribose) polymerase PARP. When the cell apoptosis program is initiated, PARP is cleaved into two fragments by caspase-3, leading to the separation of the two zinc finger structures that bind DNA in the PARP catalytic domain at the carboxyl terminus, which in turn results in increased Ca^{2+}/Mg^{2+}-dependent endonuclease activity, DNA cleavage between nucleosomes, and thus cell apoptosis. The expression of caspase-3 is significantly upregulated in animal models of spinal cord injury [11, 12]. Caspase-3 expression has been mainly detected in motor neurons, and suppression of caspase-3 expression significantly reduces neuronal apoptosis. The results from this study demonstrated that 7,8-dihydroxyflavone remarkably inhibited the expression of the apoptotic protein caspase-3 and increased the expression of the anti-apoptotic protein Bcl-2, which is likely one of the impor-

tant mechanisms underlying the neuroprotective effect of 7,8-dihydroxyflavone.

In summary, as a TrkB specific activator, 7,8-dihydroxyflavone plays an important role in neuroprotection, and its mechanism may be related to the activation of Akt/CREB. Moreover, 7,8-dihydroxyflavone has good lipid solubility, which ensure its easy penetration through the blood-brain barrier and quick enrichment in the nervous tissue. Intraperitoneal and intravenous administration can be employed for delivering 7,8-dihydroxyflavone, which permits easy clinical application of this drug. No significant side effects of 7,8-dihydroxyflavone were found in this study. Its mechanism and possible side effects require further verification in future studies to produce a solid theoretical foundation for its use in the clinical treatment of spinal cord injury.

Disclosure of conflict of interest

None.

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