Oxysophoridine rescues spinal cord injury via anti-inflammatory, anti-oxidative stress and anti-apoptosis effects

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Abstract. Oxysophoridine (OSR) is an alkaloid extracted from *Sophora alopecuroides* L and has various pharmacological activities. The present study aimed to investigate the protective effects and underlying mechanisms of OSR on spinal cord injury (SCI), a clinically common serious trauma, in a rat model. The results of the present study demonstrated that the anti-inflammatory effect of OSR improved Basso, Beatie and Bresnahan Locomotor Rating Scale scores and reduced spinal cord tissue water contents in an SCI rat model. Inflammatory activation was measured by ELISA, and Prostaglandin E2 (PGE2), intercellular adhesion molecule-1 (ICAM-1), cyclooxygenase-2 (COX-2), nuclear factor-κB (NF-κB) and B-cell lymphoma 2 (Bcl-2)/Bcl-2-associated X (Bax) protein expression levels using western blotting. The results revealed that treatment with OSR reduced tumor necrosis factor-α, interleukin (IL)-1β, IL-6, IL-8 and malondialdehyde, and increased superoxide dismutase and glutathione peroxidase levels in the serum of an SCI rat model. OSR significantly reduced the protein expression of inflammation-associated proteins PGE2, ICAM-1, COX-2, NF-κB and Bcl-2/Bax ratio in the spinal cord tissue of an SCI rat model. Furthermore, the results of the current study demonstrate that OSR ameliorates SCI via anti-inflammatory, anti-oxidative stress and anti-apoptosis effects.

Introduction

Spinal cord injury (SCI) is a clinically common serious trauma. The occurrence rate has the tendency to rise with increases in annual traffic accidents (1,2). Furthermore, SCI has a characteristically high disability rate and low mortality, which burden society and individuals (2). Pathophysiological changes of SCI are divided into primary mechanical damage and secondary damage that occurs subsequent to primary damage (3). When primary mechanical damage occurs in SCI, the degree of damage is directly associated with the degree of damaged spinal cord tissue. Clinical intervention in SCI is currently difficult (4). At present, SCI treatment primarily aims to cure secondary damage, prevent further intensification of secondary damage and recover spinal cord functions (5).

Secondary SCI is further damage that is caused by various factors following primary damage of spinal cord. The mechanism is extremely complicated (6). A series of neurobiochemical, microcirculatory and inflammatory responses continue to occur within the damage zone of SCI, which results in local vasospasm, coagulation and thrombogenesis (7). A series of pathological reactions damage persistent nerve cells and also cause damage to spinal cord tissue (8). Currently, it is considered that the mechanisms implicated in secondary SCI primarily include blood vessels, oxidative stress, inflammatory responses and apoptosis (3,9).

The membrane structure of spinal cord tissues contains abundant lipids. Following damage, the generation and release of oxygen radicals is increased. Oxygen radicals act on the polyunsaturated fatty acids of the cytomembrane to generate lipid peroxidase, change membrane permeability, disintegrate lysosomes and cause necrocytosis, which results in secondary damage of the spinal cord. Free radical scavenging in cells primarily involves two antioxidant systems, including enzymatic defense reactions superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) (10).

SCI is the continuous destruction of the spinal canal caused by external force, fracture or instant damage of the spinal cord caused by dislocation upon trauma and is the opposite to physical trauma (11). Secondary damage is further injury to the spinal cord, which is caused by a series of biochemical reaction events, including bleeding, edema, local ischemia reperfusion, inflammatory response, Ca^{2+} overflow and reactive oxide species after the primary damage has occurred (11). The degree of damage in secondary SCI far exceeds primary damage and is the principal factor for resulting neuronal death and neurofunction deficit (12). SCI is associated with local microcirculation disturbance, damage induced by neuroinflammation and reactive oxygen species, and toxic effects

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induced by excitatory amino acids, electrolyte imbalance and nerve cell apoptosis (13). Spinal nerve cell apoptosis caused by a series of immune-inflammatory responses is considered to be a major factor involved in the aggravation of secondary damage of the spinal cord. Therefore, the development of methods to preventing secondary damage in the spinal cord after primary damage has occurred is required (13).

The pharmacological action of oxysophoridine (OSR), whose chemical structure is presented in Fig. 1 (14). OSR belongs to the quinolizidine alkaloid group as do other alkaloids of *Sophora alopecuroides* (14). A previous study demonstrated that OSR has various pharmacological actions, including antiarrhythmic, protection of cardiac muscle, antiviral, antineoplastic effects (15). Furthermore, OSR has antiviral pharmacological actions, which is similar to sophoridine. OSR exhibits anti-inflammatory action and inhibits the biosynthesis of leukotriene B4 (16). The present study hypothesizes that the anti-inflammatory effect of OSR rescues SCI via anti-inflammatory, anti-oxidative stress and anti-apoptosis effects.

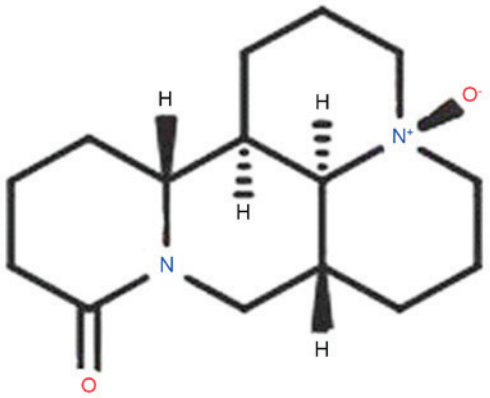

Figure 1. Chemical structure of oxysophoridine.

Materials and methods

Animals. The experimental protocol was approved by Shandong Provincial Hospital Affiliated to Shandong University Animal Care and Ethics Committee. Female adult Sprague-Dawley rats (weight, 200-230 g, n=50) were purchased from the Experimental Animal Center of Shandong University, and maintained in standard cages (22-24˚C and 55-60% humidity) with water and food *ad libitum* and a 12-h light/dark cycle. All rats were randomly assigned into five groups; sham-operation group, SCI model group, 60 mg/kg OSR group, 120 mg/kg OSR group and 180 mg/kg OSR group. Anesthetized Sprague-Dawley rats received a midline 150 kdyne contusion injury in spinal level T10 using an Infinite Horizon impactor device, which was considered to be the SCI model. The establishment of the SCI model was confirmed by analysis of the Basso, Beatie and Bresnahan (BBB) Locomotor Rating Scale (17) and spinal cord tissue water content. In the 60, 120 and 180 mg/kg OSR groups, SCI rats were administered intragastrically with 60, 120 and 180 mg/kg OSR once per day for 10 days. OSR was purchased from Jinghua Pharmaceutical Group Co., Ltd. (Yanchi, China). In sham-operation group and SCI model group, rats were administered normal saline intragastrically.

Behavioral assessments. Functional recovery was assessed following treatment with OSR using the BBB Locomotor Rating Scale to ensure consistency of the lesion (17). Following 10 days treatment with OSR, the rats were narcotized with 35 mg/kg of pentobarbital and then sacrificed using decollation. Subsequently, abdomen of rats was cut open, spinal level T10 was peeled and spinal cord tissues were collected and washed with PBS. Spinal cord tissues were weighed as wet weight and heated at 80˚C for 48 h, and subsequently weighed as dry weight. Spinal cord tissue water content was calculated by (wet weight/dry weight) x100.

Inflammatory activation as measured by ELISA. Whole blood (500 μl) was centrifuged at 2,000 x g for 10 min at 4˚C and serum was collected in every rat to determine the levels of tumor necrosis factor-α (TNF-α; H052), interleukin (IL)-1β (H002), IL-6 (H007), IL-8 (H008), malondialdehyde (MDA; A003-1), SOD (A001-1) and GSH-Px (A005) using commercial ELISA kits from Nanjing Jiancheng Biology Engineering Institute (Nanjing, China) according to the manufacturer's protocol.

Western blotting. Spinal cord tissues were isolated from every rat and homogenized in RIPA assay (Beyotime Institute of Biotechnology, Haimen, China). Protein concentrations were measured using a BCA protein assay kit (Beyotime Institute of Biotechnology, Haimen, China). Proteins (50-80 μg) were fractionated by 12% SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were blocked in 5% skim milk in TBS-Tween-20 (TBS-T; 0.05%) at room temperature for 1 h on a shaker and incubated with the following primary antibodies: Prostaglandin E2 (PGE2; sc-20771; 1:500), intercellular adhesion molecule-1 (ICAM-1; sc-7891; 1:500), cyclooxygenase-2 (COX-2; sc-7951; 1:500), nuclear factor-κB (NF-κB; sc-109; 1:500), Bcl-2-associated X (Bax; sc-6236; 1:500), Bcl-2 (sc-783; 1:500) and GAPDH (sc-367714; 1:500; all from Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4˚C overnight. The membrane was washed with TBS-T and treated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:3,000), visualized with a BeyoECL Star (Beyotime Institute of Biotechnology) and quantified using Bio-Rad Laboratories Quantity One software version 3.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Data are presented as the mean ± standard deviation using SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). Statistical differences were determined using one-way ANOVA followed by a Tukey test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

OSR rescues BBB scores and reduces spinal cord tissue water content in SCI rats. The BBB scores in the SCI model group were significantly reduced, compared with the sham group (Fig. 2A). Furthermore, treatment with OSR (120 or 180 mg/kg) significantly increased the BBB scores in SCI rats, compared with the SCI model group (Fig. 2A). Inversely, the spinal cord tissue water content of the SCI model group was

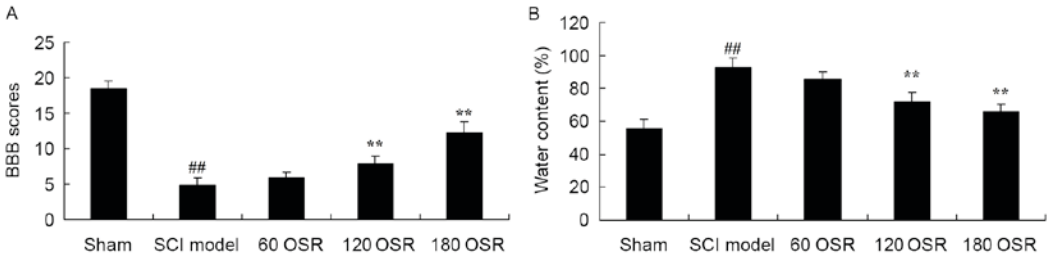

Figure 2. OSR rescues BBB scores and spinal cord water content in SCI model rats. OSR reduces the effects of SCI on (A) BBB scores and (B) spinal cord water content in SCI model rats. ##P<0.01 vs. sham group and **P<0.01 vs. SCI model group. OSR, oxysophoridine; BBB, Basso, Beatie and Bresnahan; SCI, spinal cord injury; 60 OSR, 60 mg/kg OSR; 120 OSR, 120 mg/kg OSR; 180 OSR, 180 mg/kg OSR.

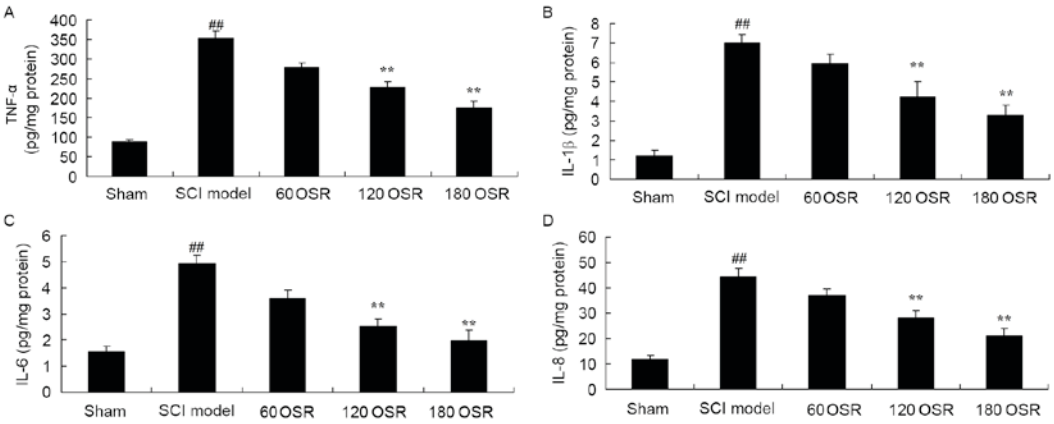

Figure 3. OSR suppresses inflammation in SCI model rats. OSR suppresses serum levels of (A) TNF-α, (B) IL-1β, (C) IL-6 and (D) IL-8 in SCI model rats. ##P<0.01 vs. sham group and **P<0.01 vs. SCI model group. OSR, oxysophoridine; SCI, spinal cord injury; TNF, tumor necrosis factor; IL, interleukin; 60 OSR, 60 mg/kg OSR; 120 OSR, 120 mg/kg OSR; 180 OSR, 180 mg/kg OSR.

significantly higher compared with the sham group (Fig. 2B), and 120 or 180 mg/kg of OSR significantly inhibited the increased water content of SCI model rats, compared with the SCI model group (Fig. 2B).

OSR suppresses inflammation in SCI rats. The present study confirmed the anti-inflammatory effect of OSR on inflammation in SCI rats by measuring the levels of TNF-α, IL-1β, IL-6 and IL-8 in the serum using ELISA kits. The results indicate that TNF-α, IL-1β, IL-6 and IL-8 levels in SCI model rats were significantly higher compared with levels in the sham group (Fig. 3). After 10 days of treatment with 120 or 180 mg/kg OSR, serum TNF-α, IL-1β, IL-6 and IL-8 levels in SCI rats were significantly reduced compared with levels in the SCI model group (Fig. 3).

OSR suppresses oxidative stress in SCI rats. To confirm the anti-oxidative stress effect of OSR in SCI rats, the levels of MDA, SOD and GSH-Px in the serum were measured using ELISA kits. Compared with the sham group, MDA levels were significantly increased, and SOD and GSH-Px activities were significantly reduced in SCI model rats (Fig. 4). Treatment with 120 or 180 mg/kg OSR significantly reduced MDA levels, and increased SOD and GSH-Px levels in SCI rats compared with the SCI model group (Fig. 4).

Anti-inflammatory effect of OSR suppresses PGE2 protein expression in SCI rats. PGE2 protein expression in the

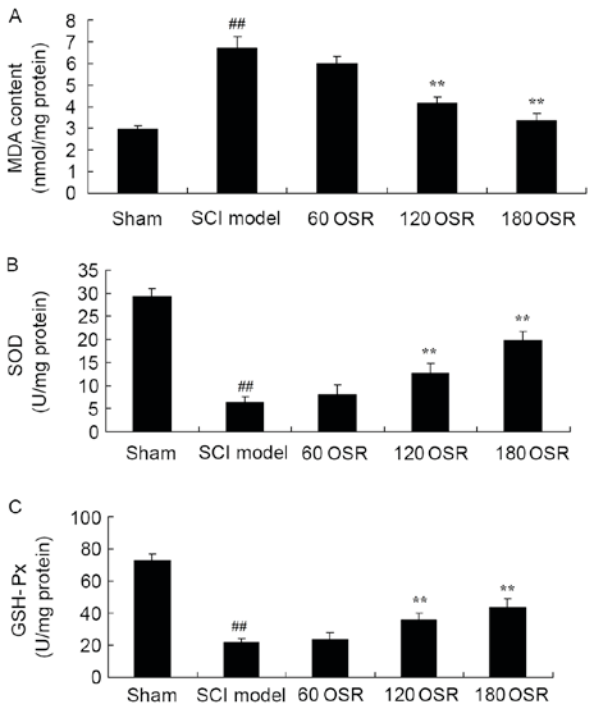

Figure 4. OSR suppresses oxidative stress in SCI model rats. OSR suppresses levels of (A) MDA, and increases levels of (B) SOD and (C) GSH-Px in SCI model rats. ##P<0.01 vs. sham group and **P<0.01 vs. SCI model group. OSR, oxysophoridine; SCI, spinal cord injury; MDA, malondialdehyde; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; 60 OSR, 60 mg/kg OSR; 120 OSR, 120 mg/kg OSR; 180 OSR, 180 mg/kg OSR.

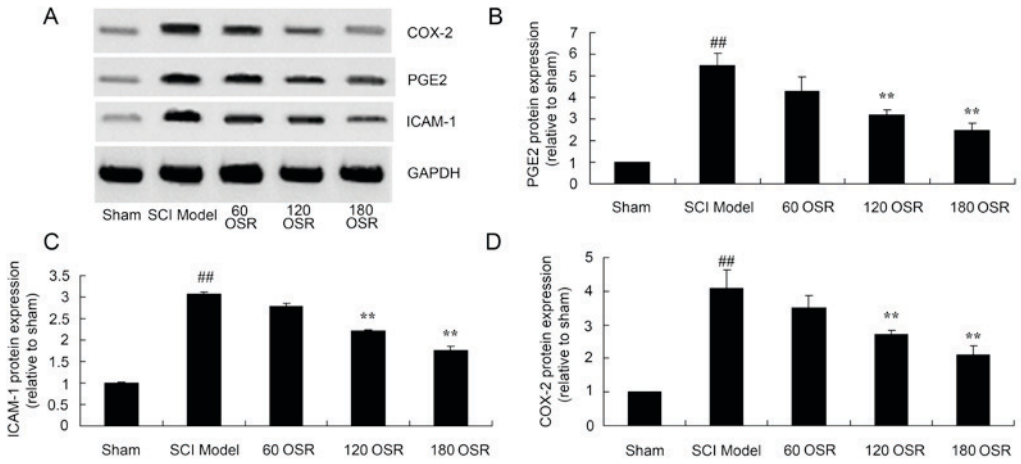

Figure 5. OSR suppresses the protein expression of PGE2, ICAM-1 and COX-2 in SCI model rats. (A) Representative image of western blot analysis of PGE2, ICAM-1 and COX-2 protein expression. Densitometric analysis indicated that OSR suppressed the protein expression of (B) PGE2, (C) ICAM-1 and (D) COX-2 in SCI model rats. $^{\#\#}$P<0.01 vs. sham group and **P<0.01 vs. SCI model group. OSR, oxysophoridine; PGE2, prostaglandin E2; ICAM-1, intercellular adhesion molecule-1; COX-2, cyclooxygenase-2; SCI, spinal cord injury; 60 OSR, 60 mg/kg OSR; 120 OSR, 120 mg/kg OSR; 180 OSR, 180 mg/kg OSR.

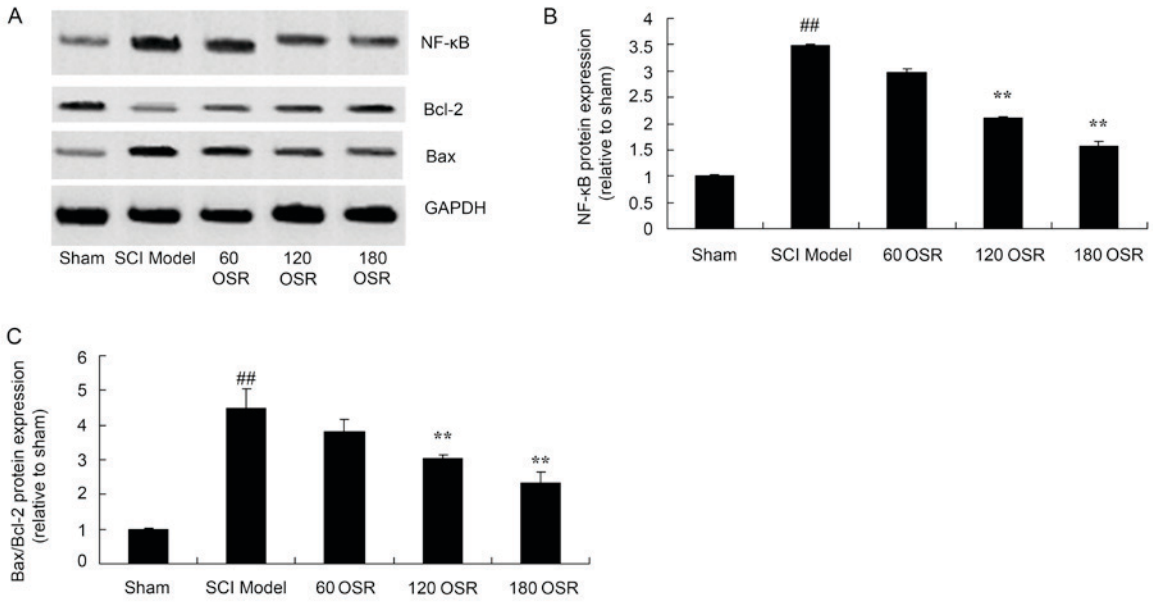

Figure 6. OSR suppresses NF-κB protein expression and the ratio of Bax/Bcl-2 in SCI model rats. (A) Representative image of western blot analysis of NF-κB, Bcl-2 and Bax protein expression. Densitometric analysis indicated that OSR reduced (B) NF-κB protein expression and (C) Bax/Bcl-2 ratio in SCI model rats. $^{\#\#}$P<0.01 vs. sham group and **P<0.01 vs. SCI model group. OSR, oxysophoridine; NF-κB, nuclear factor-κB; Bax, Bcl-2-associated X; SCI, spinal cord injury; 60 OSR, 60 mg/kg OSR; 120 OSR, 120 mg/kg OSR; 180 OSR, 180 mg/kg OSR.

spinal cord tissue of SCI model rats was significantly higher compared with the sham group (Fig. 5A and B). Treatment with 120 or 180 mg/kg OSR significantly suppressed PGE2 protein expression in SCI rats compared with the SCI model group (Fig. 5A and B).

Anti-inflammatory effect of OSR suppresses ICAM-1 protein expression in SCI rats. The ICAM-1 protein expression in the spinal cord tissue of SCI model rats was significantly increased compared with the sham group (Fig. 5A and C). Administration of 120 or 180 mg/kg OSR significantly reduced the induction of ICAM-1 protein expression in SCI rats compared with the SCI model group (Fig. 5A and C).

Anti-inflammatory effect of OSR suppresses COX-2 protein expression in SCI rats. As demonstrated in Fig. 5A and D, the COX-2 protein expression in the spinal cord tissue of SCI model rats was significantly increased compared with the sham group. Furthermore, treatment with 120 or 180 mg/kg OSR significantly suppressed COX-2 protein expression compared with the SCI model group (Fig. 5A and D).

Anti-inflammatory effect of OSR suppresses NF-κB protein expression in SCI rats. To investigate the involvement of the NF-κB pathway in the anti-inflammatory effect of OSR in SCI rats, NF-κB protein expression in the spinal cord tissue was measured using western blotting. SCI significantly induced

NF-κB protein expression compared with the sham group (Fig. 6A and B). Treatment with 120 or 180 mg/kg OSR significantly inhibited the NF-κB protein expression induced by SCI, compared with the SCI model group (Fig. 6A and B).

OSR suppresses the Bax/Bcl-2 protein expression ratio in SCI rats. To investigate the effect of OSR on apoptosis in the SCI rat model, the Bax/Bcl-2 protein expression ratio in the spinal cord tissue of rats was measured by western blotting. The ratio of Bax/Bcl-2 protein expression in SCI model rats was significantly higher compared with the sham group (Fig. 6A and C). However, treatment with 120 or 180 mg/kg OSR significantly reduced the ratio of Bax/Bcl-2 protein expression in SCI model rats, compared with the SCI model group (Fig. 6A and C).

Discussion

Pathophysiological changes in SCI are divided into primary mechanical damage and secondary damage that occurs subsequent to primary damage (1). When primary damage occurs in SCI, it is impossible to intervene clinically, and, currently, treatment of SCI only aims to cure secondary damage (18). Secondary damage of the spinal cord is further injury to the spinal cord that is caused by various factors following primary damage of the spinal cord (19). Spinal damage that results from secondary damage may greatly exceed primary damage. A recent study has investigated the mechanism of SCI, and the mechanisms that are thought to be involved include oxidative stress, inflammatory responses and apoptosis (8). The results of the present study demonstrated that OSR increased BBB scores and reduced spinal cord water content in SCI model rats. These results are similar to the Yang *et al* (16) which demonstrated that OSR presents effective antinociception in the spinal cord of mice.

A previous study demonstrated that inflammatory responses have an important role on the pathogenesis of secondary damage (20). Another previous study indicated that there is a high number of various types of inflammation-associated proteins in the tissue following spinal cord damage, including TNF-α, IL-1β, IL-6, IL-8, ICAM-1 and vascular cell adhesion molecule 1 (VCAM-1), suggesting that they may have an important role in the pathophysiological mechanism of secondary damage following SCI (10). The results of the current study demonstrate that treatment with OSR suppressed TNF-α, IL-1β, IL-6 and IL-8 levels in SCI model rats. Previously, Meng *et al* (15) demonstrated that OSR attenuated acute myocardial infarction through anti-oxidative, anti-inflammatory and anti-apoptotic pathways.

SOD has an important role in the oxidation and antioxidant balance, and scavenges free radicals and protect cells from damage (21). MDA is the lipolysis product of lipid peroxidase. The body can generate oxygen radicals through the enzyme system (12), which can attach polyunsaturated fatty acids to the biological membrane, causing lipid peroxidation, forming MDA from lipid peroxide, and causing cell damage. Measurement of MDA is often coordinated with measurement of SOD (21). The level of activity of SOD indicates the ability of the body to scavenge free radicals directly. GSH-Px is an important peroxidase enzyme that is expressed throughout the body (12). GSH-Px eliminates harmful peroxide metabolites in cells and intercepts lipid peroxidation chain reactions, which protects cytomembrane structure (21). The results of the present study indicate that OSR significantly reduced MDA levels, and increased SOD and GSH-Px levels in SCI model rats. This anti-oxidative stress effect of OSR was also reported in a study by Meng *et al* (15), which demonstrated that OSR attenuated acute myocardial infarction through anti-oxidative, anti-inflammatory and anti-apoptotic pathways.

NF-κB is a transcription factor that has an important regulatory role in inflammatory responses, immune responses, cell growth, differentiation and apoptosis (22). NF-κB consists of p65 and p50 subunits, which combine to form a heterodimer. Activated NF-κB participates in multiple inflammatory cell factors individually or cooperates with other transcription factors, including TNF-α, IL-1β, IL-6, IL-8, ICAM-1 and VACM-1 (13). The gene products induced by NF-κB action further participate in inflammatory and immune responses, and have important functions in physiological and pathological conditions of the body (23). The current study demonstrated that OSR significantly suppressed PGE2, ICAM-1 and COX-2 protein expression in the spinal cord tissue of SCI model rats. Wang *et al* (24) also reported that OSR protected against ischemia-induced injury via PGE2, COX-2 and ICAM-1 expression, and the NF-κB pathway.

The inflammatory response is one of primary mechanisms of aggravating secondary SCI. The process is primarily induced by the toll-like receptor (TLR)-NF-κB signaling pathways (25). Knowledge of the signal transduction process associated with TLRs-NF-κB signaling is largely based on the investigation of TLR2 and TLR4 (26). Activated TLRs not only induce inflammatory responses, but also promote the differentiation and maturity of antigen-specificity acquired immune responses (26). The present study demonstrated that OSR significantly inhibited NF-κB protein expression in SCI model rats. Wang *et al* (24) also reported that effects on the NF-κB pathway were associated with the anti-inflammatory effects of OSR, which protected against ischemia-induced injury.

Bcl-2 is a cytoplasmic protein that has a higher level of expression during development of the central nervous system (27). Once development of the nervous system is complete, Bcl-2 expression is maintained at a low level. Bcl-2 is an anti-apoptotic gene and can prevent various apoptosis pathways following spinal damage (21). Previous research has demonstrated that overexpression of Bcl-2 in experimental animals alleviated nerve injury, improved the anti-injury function of the tissue, promoted outward growth of axons, repaired damaged central nervous system tissue and prevented nerve cell apoptosis (28). Bax is located in the cytoplasm. After receiving an apoptosis signal, it is activated to alter the membrane permeability of the mitochondrial membrane and promote the apoptosis of cells (28). The current study demonstrated that OSR significantly reduced the Bax/Bcl-2 protein expression ratio in SCI model rats. Wang *et al* (29) previously reported that OSR also protects against focal cerebral ischemic injury via Bax/Bcl-2 expression.

In summary, OSR rescues SCI as treatment with OSR increased BBB scores, reduced spinal cord tissue water content, reduced the production of pro-inflammatory cytokines, increased the levels of anti-oxidant enzymes and reduced the ratio of Bax/Bcl-2 protein expression in SCI model rats,

which revealed anti-inflammatory, anti-oxidative stress and anti-apoptosis effects of OSR that may be mediated via NF-κB and the Bax/Bcl-2 pathway. In conclusion, OSR may exert a protective effect on SCI by anti-inflammatory, anti-oxidative stress and anti-apoptosis effects, which indicates that OSR may be a potential therapeutic agent for SCI.

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