

The effects of oxyresveratrol abrogates inflammation and oxidative stress in rat model of spinal cord injury

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Abstract. Oxyresveratrol and its glycoside are important natural active materials. As an effective tyrosine kinase inhibitor, oxyresveratrol may prevent herpes virus infection, inflammation and oxidative stress, as well as protect nerves. In addition, it is known to inhibit cell apoptosis following cerebral ischemia. In recent years, oxyresveratrol and its glycoside have been widely investigated, and their useful biological activities have been explored, indicating that they may be worthy of further comprehensive research. The aim of the present study was to evaluate the photoprotective effects of oxyresveratrol and its ability to abrogate inflammation and oxidative stress in a rat model of spinal cord injury (SCI). The authors identified that oxyresveratrol significantly reversed the SCI-induced inhibition of Basso, Beattie, and Bresnahan scores, inhibited the SCI-mediated increase in spinal cord water content, significantly suppressed SCI-induced nuclear factor- κ B/p65, tumor necrosis factor- α , interleukin (IL)-1 β and IL-6 activities and reversed the malondialdehyde, superoxide dismutase, glutathione (GSH) and GSH peroxidase activities in SCI rats. SCI-induced granulocyte-macrophage colony-stimulating factor (GM-CSF), inducible nitric oxide synthase (iNOS) and cyclo-oxygenase-2 (COX-2) protein expression was significantly suppressed by oxyresveratrol, and SCI-mediated inhibition of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) protein expression was significantly increased by oxyresveratrol. In conclusion, these results suggest that the effects of oxyresveratrol restores SCI, and abrogates inflammation and oxidative stress in rat model of SCI via the GM-CSF, iNOS, COX-2 and Nrf2 signaling pathway.

Introduction

According to its pathogenesis, spinal cord injury (SCI) is divided into primary and secondary injuries. The former is the result of an initial force applied directly or indirectly to the spinal cord (1). Secondary injuries are self-destructive lesions that occur on tissues surrounding the lesions through a series of physiological and biochemical mechanisms, including oxidative stress, inflammation and the excessive release of excitatory amino acids. This leads to an increase in the degree of damage and expansion of the damaged area (2). The inflammatory response following SCI is complex, and involves the immune and nervous systems (3).

The positive expression of TGF- β in rats is observed in the endochylema of chondrocytes in the articular cartilage (4). Compared with the expression in chondrocyte in articular cartilage of normal rats, the expression on the surface layer of the *cartilage articularis* is high, and is significantly higher than that observed in the middle and deep layers, while expression in additional regions is low (5). It has been reported that during the chondrogenesis process, TGF- β 1 is highly expressed in the surface, transition and cellular layers with low maturity (6,7). In articular chondrocytes of rats with SCI, the surface, middle and deep surface layers exhibit relatively high expression levels (2). However, its expression decreases in different layers of chondrocytes. It has been demonstrated that TGF- β 1 is expressed in hematomas that develop at the site of injury following SCI, and the expression increases in the endochylema and karyon of astrocytes, capillary endothelial cells present within and outside of the marrow, as well as in motor neurons (3).

As spinal cord tissues are rich in lipids that are sensitive to lipid peroxidation, free-radical mediated oxidative stress serves an important role in secondary SCI (8). Antioxidant defense mechanisms are activated primarily through the antioxidant response element, which is a cis-acting element located upstream of antioxidant genes (9). At present, the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) signaling pathway is considered to be the most important defense mechanism in the prevention of oxidative stress (9). Nrf2 possesses extensive cytoprotective functions in preventing tumors, atherosclerosis and neurodegenerative diseases (8).

Inflammation is a self-protective reaction that occurs when organisms are confronted with damaging stimuli. Excessive inflammation results in an excessive immune response, which

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leads to the development of lesions (10). Serious inflammation may even induce tumors (10). When inflammation occurs, various cytokines induce the expression of nitric oxide synthase (NOS) (11). Inducible (i)NOS rapidly synthesizes excessive nitric oxide (NO), while peroxynitrite, a strong oxidant, is rapidly produced following a reaction between NO and a free oxygen radical (12). As a consequence, oxidative stress occurs, which leads to plasma effusion at sites of inflammation, as well as tissue damage and edema (12).

A previous study demonstrated that oxyresveratrol exhibits anti-inflammatory and detumescence functions (13). Oxyresveratrol may inhibit the expression of NOS and the accumulation of nitrous acid (13). It is therefore possible that they possess protective functions in cells that have been injured by inflammation. In addition, these compounds may increase the antioxidative capacities of cells with significant anti-inflammatory properties (14,15). As an effective tyrosine kinase inhibitor, oxyresveratrol may prevent herpes virus infection, inflammation and oxidation, as well as protect nerves (14,15). In addition, it is known to inhibit cell apoptosis following cerebral ischemia (16,17). Previous studies have suggested that oxyresveratrol is antitussive, anti-asthmatic, antioxidative, anti-inflammatory, and that they possess analgesic properties and inhibit tyrosinase activity (14,15). In addition, they may protect from free radicals (14,15). In the present study, the anti-inflammatory effects of oxyresveratrol and its associated mechanisms were investigated using a rat model of SCI.

Materials and methods

Animals and generation of the SCI model. A total of 32 adult female Sprague-Dawley rats (6-8 weeks, 160-180 g) were purchased from Experimental Animal Center of Hebei Medical University (Hebei, China) and maintained in a temperature-controlled room ($23\pm 1^\circ\text{C}$) with 12 h light/dark cycles and with access to water and food *ad libitum*. The present study was approved by the Ethics Committee of Hebei Cangzhou Central Hospital (Cangzhou, China). All rats were anesthetized with an intraperitoneal injection of 90 mg/kg ketamine (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) and 10 mg/kg xylazine (Sinopharm Chemical Reagent Co., Ltd.). Following shaving and cleansing of the skin, the paraspinal fascia, T7-10 spinous process and lamina were fully revealed and the T8-9 spinous process was removed to expose the subdural space, whilst maintaining its integrity. A 10 g object was dropped from 2.5 cm above the subject to collide with the subdural space. The wound was washed with hydrogen peroxide and incisions were sutured. Sham-operated rats were anesthetized with an intraperitoneal injection of 90 mg/kg ketamine and 10 mg/kg xylazine and their skin was shaved and cleansed, the paraspinal fascia.

Experimental groups. The animals were randomly divided into the following 4 groups (n=8): The negative control group (sham-operated); the SCI model group; the 10 mg/kg oxyresveratrol (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany)-treated SCI group; and the 20 mg/kg oxyresveratrol-treated SCI group. Rats in the 10 and 20 mg/kg oxyresveratrol-treated SCI groups were administered with

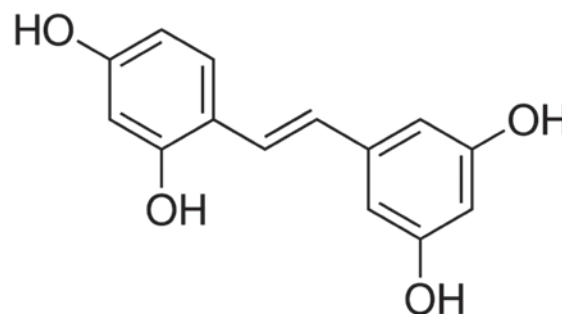


Figure 1. Chemical structure of oxyresveratrol.

intraperitoneal injections of 10 and 20 mg/kg oxyresveratrol once a day for 4 weeks, respectively. Rats from the negative control or SCI model groups were administered with intraperitoneal injections of 100 μl normal saline once a day for 4 weeks.

Motor function assessment. At 1, 2, 3 and 4 weeks following oxyresveratrol or saline treatment, rats were evaluated at 8:00 a.m. using the Basso, Beattie, and Bresnahan (BBB) locomotor rating scale from 0 to 21 (4).

Determination of spinal cord water content. After oxyresveratrol treatment for 4 weeks, spinal cord tissue samples were immediately acquired and washed with phosphate-buffered saline (PBS). Spinal cord tissue samples were weighed, and the values obtained were considered to be the wet weight. Tissue samples were then dried at 68°C for 48 h. Dried tissue samples were weighed and the values obtained were considered to be the dried weight. The percentage spinal cord water content was calculated using the following formula: (wet weight/dried weight) $\times 100$.

Biochemical analysis. After oxyresveratrol treatment for 4 weeks, rats were anesthetized with intraperitoneal injection of 90 mg/kg ketamine and 10 mg/kg xylazine. Venous blood was obtained from the eye socket of every rat and was immediately centrifuged at $2,000 \times g$ for 10 min at 4°C . Serum was collected to measure the expression of GM-CSF (cat no. H060), nuclear factor- κB (NF- κB)/p65 (cat no. H202), tumor necrosis factor (TNF)- α (cat no. H052), interleukin (IL)-1 β (cat no. H002), IL-6 (cat no. H007), malondialdehyde (MDA, cat no. A003-1), superoxide dismutase (SOD, cat no. A001-3), glutathione (GSH, cat no. A006-2) and GSH peroxidase (PX, cat no. A005) using rat ELISA kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The expression of these factors was measured using the Spectramax M2 Microplate Reader (Molecular Devices, LLC, Sunnyvale, CA, USA) at a wavelength of 450 nm.

Western blot analysis. After oxyresveratrol treatment, spinal cord tissue samples were immediately acquired and washed with PBS. The tissue samples were then lysed in radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Inc.). Protein concentrations were determined using a bicinchoninic protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). Proteins (50 μg) were separated on an 8-12% SDS-PAGE gel

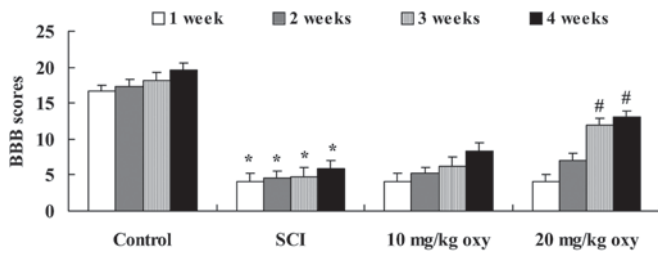


Figure 2. Oxyresveratrol treatment improves locomotor recovery in a rat model of SCI. *P<0.01 vs. control group; #P<0.05 vs. SCI model group. Control, sham-operated group; SCI, SCI model group; 10 mg/kg oxy, 10 mg/kg oxyresveratrol-treated SCI group; 20 mg/kg oxy, 20 mg/kg oxyresveratrol-treated SCI group; SCI, spinal cord injury; BBB, Basso, Beattie, and Bresnahan.

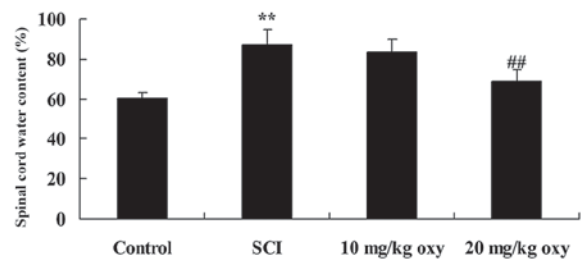


Figure 3. Oxyresveratrol reduces spinal cord water content in a rat model of SCI. The percentage spinal cord water content was calculated using the following formula: (wet weight/dried weight) x100. **P<0.01 vs. control group; ##P<0.05 vs. SCI model group. Control, sham-operated group; SCI, SCI model group; 10 mg/kg oxy, 10 mg/kg oxyresveratrol-treated SCI group; 20 mg/kg oxy, 20 mg/kg oxyresveratrol-treated SCI group. SCI, spinal cord injury.

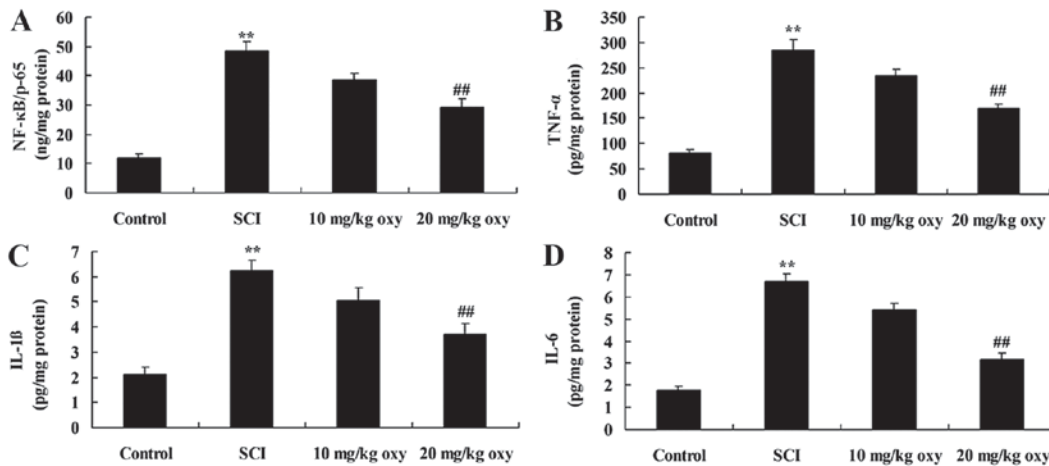


Figure 4. Oxyresveratrol treatment reduces inflammation in a rat model of SCI. The levels of (A) NF-κB/p65, (B) TNF-α, (C) IL-1β and (D) IL-6 activity in a rat model of SCI. **P<0.01 vs. control group; ##P<0.05 vs. SCI model group. Control, sham-operated group; SCI, SCI model group; 10 mg/kg oxy, 10 mg/kg oxyresveratrol-treated SCI group; 20 mg/kg oxy, 20 mg/kg oxyresveratrol-treated SCI group. SCI, spinal cord injury; NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor-α; IL, interleukin.

(with 60 V constant voltage for 3.5 h, wet-turn 14 V constant voltage for 14 h) and transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Membranes were subsequently incubated with the following primary antibodies at 4°C overnight: TGF-β1 antibody (cat. no. 3711; dilution, 1:3,000; Cell Signaling Technology, Inc., Danvers, MA, USA), iNOS antibody (cat. no. 2982; dilution, 1:2,000; Cell Signaling Technology, Inc.), cyclo-oxygenase (COX)-2 antibody (cat. no. 4842; dilution 1:5,000; Cell Signaling Technology, Inc.), Nrf2 antibody (cat. no. 12721; dilution, 1:4,000; Cell Signaling Technology, Inc.) and β-actin (cat. no. 3700; dilution, 1:5,000; Cell Signaling Technology, Inc.). This was followed by incubation with a horseradish peroxidase-conjugated antibody (cat no. A0239, dilution 1:2,000; Beyotime Institute of Biotechnology, Haimen, China) at 37°C for 1 h. Protein bands were visualized using Pierce ECL Plus™ Western Blotting Substrate (GE Healthcare Life Sciences, Chalfont, UK). Protein expression was quantified using sodium Image_Lab_3.0 software (Bio-Rad Laboratories, Inc.).

Statistical analysis. The results are expressed as the mean ± standard error using SPSS version 17.0 software (SPSS, Inc., Chicago, IL, USA). Differences among groups

were analyzed by one-way analysis of variance followed by a post hoc Tukey's test. P<0.05 was considered to indicate a statistically significant difference.

Results

The effects of oxyresveratrol improve locomotor recovery in a rat model of SCI. The chemical structure of oxyresveratrol is depicted in Fig. 1. As demonstrated in Fig. 2, the BBB scores of rats in the SCI group were significantly lower when compared with those of the sham-operated control group at 1, 2, 3 and 4 weeks following induction of the SCI model. Treatment with oxyresveratrol (20 mg/kg) significantly reversed the SCI-mediated reduction in the BBB scores of rats at 3 and 4 weeks following induction of the SCI model (Fig. 2).

Oxyresveratrol abrogates the SCI-induced increase in spinal cord water content. At 4 weeks following induction of the SCI model, a significant increase in the spinal cord water content of SCI rats was observed when compared with rats in the control group (Fig. 3). By contrast, treatment with 20 mg/kg oxyresveratrol significantly reduced the SCI-induced increase in spinal cord water content (Fig. 3).

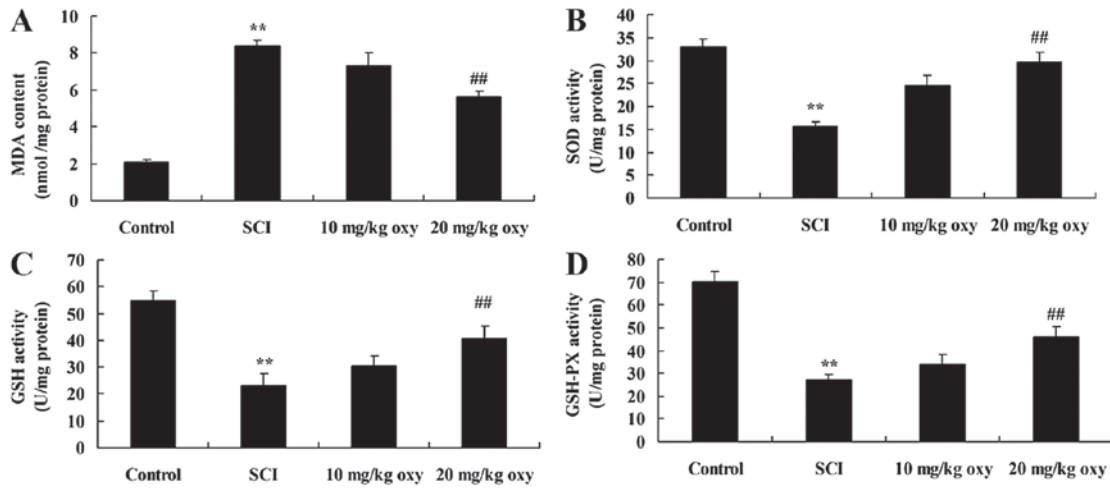


Figure 5. Oxyresveratrol reduces oxidative stress in a rat model of SCI. The levels of (A) MDA, (B) SOD, (C) GSH and (D) GSH-PX activity in a rat model of SCI. ** $P < 0.01$ vs. control group; ## $P < 0.05$ vs. SCI model group. Control, sham-operated group; SCI, SCI model group; 10 mg/kg oxy, 10 mg/kg oxyresveratrol-treated SCI group; 20 mg/kg oxy, 20 mg/kg oxyresveratrol-treated SCI group. SCI, spinal cord injury; MDA, malondialdehyde; SOD, superoxide dismutase; GSH, glutathione; PX, peroxidase.

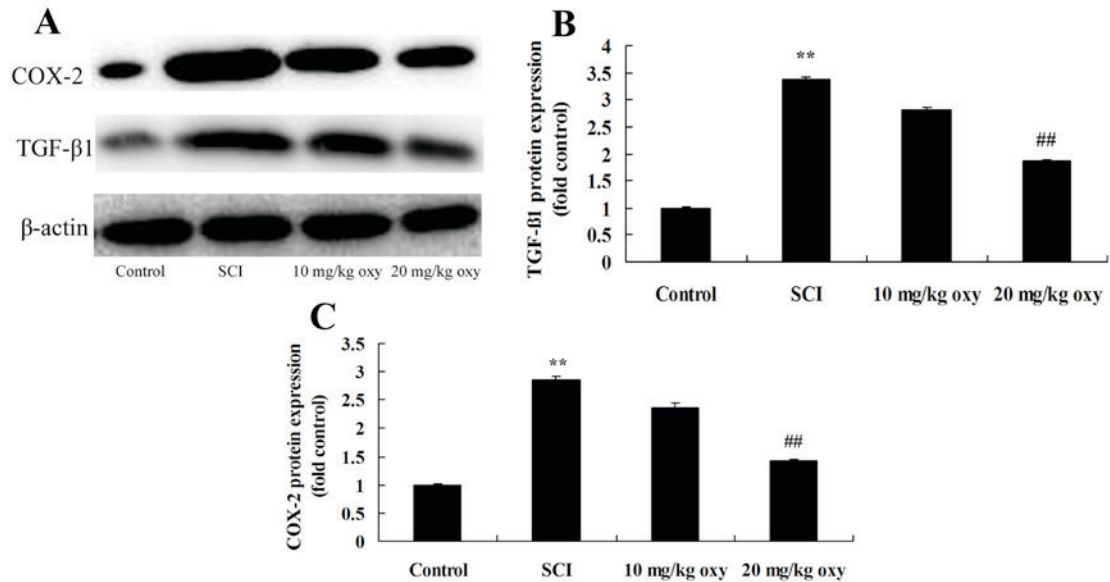


Figure 6. Oxyresveratrol abrogates SCI-induced TGF- β 1 and COX-2 expression in a rat model of SCI. (A) Western blotting analysis of TGF- β 1 and COX-2 expression in control and SCI rats treated with saline, 10 and 20 mg/kg oxy, and (B and C) quantification of band densities. ** $P < 0.01$ vs. control group; ## $P < 0.05$ vs. SCI model group. Control, sham-operated group; SCI, SCI model group; 10 mg/kg oxy, 10 mg/kg oxyresveratrol-treated SCI group; 20 mg/kg oxy, 20 mg/kg oxyresveratrol-treated SCI group. TGF- β 1, tumor necrosis factor- β 1; COX-2, cyclo-oxygenase-2; SCI, spinal cord injury.

Oxyresveratrol reduces SCI-induced inflammation. The levels of NF- κ B/p65, TNF- α , IL-1 β and IL-6 activity were used to evaluate the effects of oxyresveratrol on inflammation in SCI rats. As demonstrated in Fig. 4, the levels of NF- κ B/p65, TNF- α , IL-1 β and IL-6 were significantly increased in SCI rats when compared with the control group. By contrast, treatment with 20 mg/kg oxyresveratrol significantly suppressed SCI-induced NF- κ B/p65, TNF- α , IL-1 β and IL-6 activity in rats (Fig. 4).

Oxyresveratrol abrogates SCI-induced oxidative stress. The levels of MDA, SOD, GSH and GSH-PX activity were measured in order to investigate the anti-inflammatory effects

of oxyresveratrol in rats with SCI further. As demonstrated in Fig. 5, an increase in MDA levels and a reduction in SOD, GSH and GSH-PX levels was observed in SCI model rats when compared with the control group. However, treatment with 20 mg/kg oxyresveratrol significantly reversed MDA, SOD, GSH and GSH-PX levels when compared with the SCI model group (Fig. 5).

Oxyresveratrol abrogates SCI-induced TGF- β 1 and COX-2 expression. As demonstrated in Fig. 6, SCI significantly induced TGF- β 1 and COX-2 protein expression in SCI model rats when compared with the control group. By contrast, treatment with 20 mg/kg oxyresveratrol

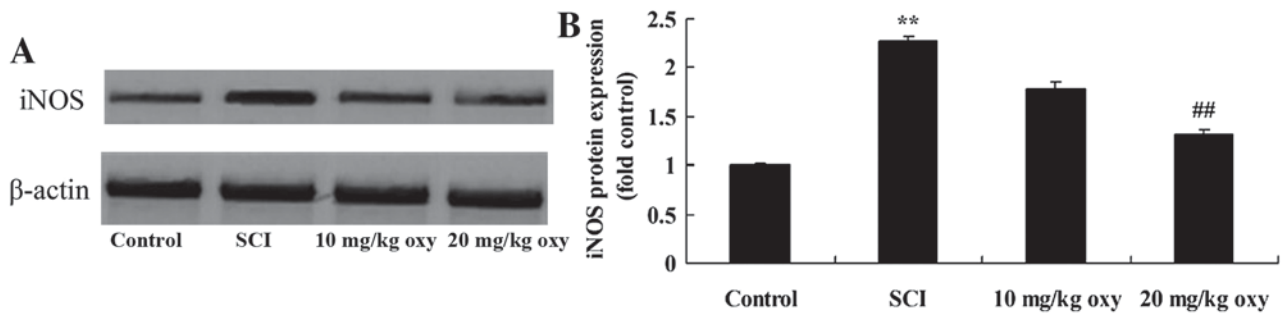


Figure 7. Oxyresveratrol abrogates SCI-induced iNOS expression in a rat model of SCI. (A) Western blotting analysis of iNOS expression in control and SCI rats treated with saline, 10 and 20 mg/kg oxy, and (B) quantification of band densities. ** $P < 0.01$ vs. control group; ## $P < 0.05$ vs. SCI model group. Control, sham-operated group; SCI, SCI model group; 10 mg/kg oxy, 10 mg/kg oxyresveratrol-treated SCI group; 20 mg/kg oxy, 20 mg/kg oxyresveratrol-treated SCI group. iNOS, inducible nitric oxide synthase; SCI, spinal cord injury.

significantly suppressed the protein expression levels of TGF- β 1 and COX-2 in SCI rats when compared with the SCI model group (Fig. 6).

Oxyresveratrol abrogates SCI-induced iNOS expression. In order to examine the effect of oxyresveratrol treatment on iNOS expression in SCI rats, iNOS protein expression was measured by western blot analysis. As demonstrated in Fig. 7, a significant increase in the protein expression levels of iNOS was observed in SCI rats when compared with the control group. Treatment with 20 mg/kg oxyresveratrol significantly reduced the SCI-induced increase in iNOS protein expression (Fig. 7).

Oxyresveratrol reverses the SCI-induced increase in GM-CSF levels. In order to determine whether oxyresveratrol affected GM-CSF expression in a rat model of SCI, the authors measured GM-CSF protein expression levels in all experimental groups by western blotting. The results demonstrated that the level of GM-CSF was significantly higher in the SCI group when compared with the control group (Fig. 8). By contrast, treatment with 20 mg/kg oxyresveratrol significantly reduced the SCI-mediated increase in GM-CSF expression levels (Fig. 8).

Oxyresveratrol reverses the SCI-mediated reduction in Nrf2 expression. In order to investigate the effects of oxyresveratrol on Nrf2 expression in SCI rats, Nrf2 protein expression levels were detected by western blot analysis. The results demonstrated that Nrf2 protein expression levels were significantly reduced in the SCI model group when compared with control group (Fig. 9). However treatment with 20 mg/kg oxyresveratrol significantly reversed the SCI-induced inhibition of Nrf2 protein expression (Fig. 9).

Discussion

Inflammatory chemokine factors and cytokines enhance the activity of and activate immune cells and neurons. They serve key roles in promoting and maintaining inflammation (18). Chemokine factors and cytokines possess a number of functions, including the transformation of cells from a proinflammatory to an anti-inflammatory state (19). If the inflammatory response at the site of injury were not inhibited

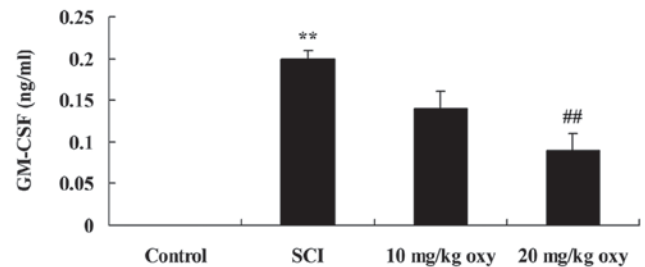


Figure 8. Oxyresveratrol abrogates SCI-induced GM-CSF expression in a rat model of SCI. ** $P < 0.01$ vs. control group; ## $P < 0.05$ vs. SCI model group. Control, sham-operated group; SCI, SCI model group; 10 mg/kg oxy, 10 mg/kg oxyresveratrol-treated SCI group; 20 mg/kg oxy, 20 mg/kg oxyresveratrol-treated SCI group. GM-CSF, granulocyte-macrophage colony-stimulating factor; SCI, spinal cord injury.

during an immune response, it may lead to a number of additional adverse effects (20). As the capabilities of injured neurons are reduced and axonal regeneration is restricted, the adverse effects of inflammation may be more obvious in the central nervous system when compared with other systems (21). The apoptosis of neurons and oligodendroglia, as well as scarring may be triggered. The excessive expression of COX-2 is associated with microvessel density and angiogenesis induced by inflammatory cytokines (22). The present study demonstrated that oxyresveratrol significantly increased the SCI-mediated activities. Andrabi *et al* (15) demonstrated that oxyresveratrol inhibits apoptotic cell death during transient cerebral ischemia, whereas Ashraf *et al* (23) indicated that the compound ameliorates inflammation in the airways following an allergic response.

As a cytokine involved in hemocytogenesis, GM-CSF promotes long-term recovery following SCI through inhibiting glial scar formation and increasing the structural integrity of axons (24). Previous studies have demonstrated that the use of TGF- β to treat primary spongocytes in an *in vitro* system, may inhibit the expression of chondroitin sulfate proteoglycans in astrocytes, which would further inhibit the formation of glial scars and provide an effective treatment for neuronal injury (25). In addition, GM-CSF may regulate the ability of macrophages to produce brain-derived neurotrophic factor (26). GM-CSF improves tactile sense and psychroesthesia in SCI rats, indicating that it may promote the recovery functional of SCI (26). In the present study, oxyresveratrol

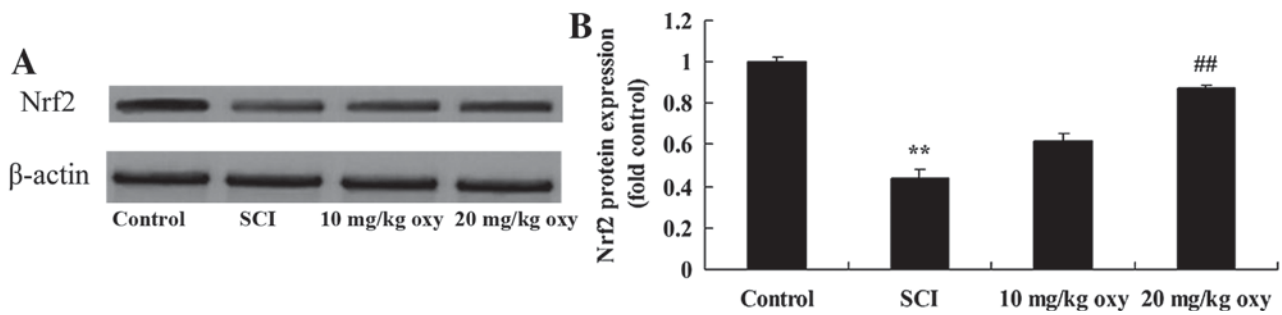


Figure 9. Oxyresveratrol reverses the SCI-mediated inhibition of Nrf2 expression in a rat model of SCI. (A) Western blotting analysis of Nrf2 expression in control and SCI rats treated with saline, 10 and 20 mg/kg oxy, and (B) quantification of band densities. ** $P < 0.01$ vs. control group; ## $P < 0.05$ vs. SCI model group. Control, sham-operated group; SCI, SCI model group; 10 mg/kg oxy, 10 mg/kg oxyresveratrol-treated SCI group; 20 mg/kg oxy, 20 mg/kg oxyresveratrol-treated SCI group. Nrf2, nuclear factor (erythroid-derived 2)-like 2; SCI, spinal cord injury.

significantly reduced the SCI-induced increase in GM-CSF levels in rats. Oxyresveratrol suppresses lipopolysaccharide-induced inflammatory responses in murine macrophages.

Following SCI, a number of factors may be involved in mediating nerve cell apoptosis (6). An increasing number of studies investigating NO have addressed its functional role in secondary SCI (6,7). NO is produced during the catalyzation of L-arginine to citrulline by NOS. Under normal physiological conditions, organisms produce NO of base quantity, and inhibit platelet aggregation and accumulation, as well as maintain conditions of vasodilatation (7). During the early stages of acute ischemic injury, small increases in NO prevent vasoconstriction induced by trauma and ischemia. With the infiltration of inflammatory cells and the production of inflammatory factors, NF- κ B is activated, which induces an increase in iNOS expression. The activity of iNOS is not calcium-dependent (22). Once synthesized, it continuously catalyzes the formation of NO (22). The results from the present study demonstrated that oxyresveratrol significantly inhibited SCI-induced iNOS protein expression in rats. Lee *et al* (14) indicated that oxyresveratrol suppresses lipopolysaccharide-induced inflammatory responses via iNOS, COX-2 and GM-CSF in murine macrophages.

It is generally thought that, under pathological conditions, including inflammation, trauma, cellular injuries and tumors, COX-2 exerts harmful functions (22). However, a study discovered that COX-2 serves a role in mediating normal physiological functions (22). For instance, COX-2 serves a functional role in glutamatergic neurons, which are constitutively expressed in the hippocampus and cortex, and demonstrates effects on long-term synaptic plasticity and the coupling of neurovascular structures during hyperemia (27). Previous studies hypothesize that the expression of COX-2 is associated with anoxia following SCI, peroxidation and neuronal death induced by excitatory amino acids (27,28). In the current study, oxyresveratrol significantly inhibited the SCI-induced increase in COX-2 protein expression in rats.

SCI is a severe traumatic disease of the central nervous system. Despite current medical strategies, functional rehabilitation is not satisfactory, even with the observed decrease in death rates (29). As a consequence, the prevention, treatment and rehabilitation of SCI have become an essential issue. In addition, secondary damage of the spinal cord following the primary damage may aggravate the degree

of injury (30). Therefore, the reduction and reversion of secondary injury has become the focus of current studies. Oxidative stress serves a fundamental role in subsequent pathological alterations in SCI (18). As a key regulator of transcription in cytophylaxis and antioxidative stress pathways, Nrf2 regulates cytoprotective genes, such as those with antioxidative and anti-inflammatory functions, as well as associated proteins in order to enhance antioxidative capacities (31). The present study demonstrated that oxyresveratrol significantly activated the SCI-mediated inhibition of Nrf2 protein expression in rats. Choi *et al* (32) confirmed that oxyresveratrol abrogates oxidative stress in the liver via activating the ERK-Nrf2 signaling pathway (32).

In conclusion, the results of the present study demonstrated that oxyresveratrol significantly increased the SCI-mediated reduction in BBB scores, inhibited the SCI-induced increase in spinal cord water content, suppressed SCI-induced NF- κ B/p65, TNF- α , IL-1 β and IL-6 activities and reversed the MDA, SOD, GSH and GSH-PX activities in SCI rats potentially via the iNOS, COX-2 and GM-CSF/Nrf2 signaling pathways. The results suggest that oxyresveratrol may present a novel therapeutic agent for the treatment of SCI.

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