Myricitrin decreases traumatic injury of the spinal cord and exhibits antioxidant and anti-inflammatory activities in a rat model via inhibition of COX-2, TGF-β1, p53 and elevation of Bcl-2/Bax signaling pathway

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Abstract. Myricitrin has multiple effects, including antagonism of platelet activating factor, regulation of blood sugar levels, oxidation resistance, protection of the liver and the relieving of ethylism. The present study evaluated how myricitrin weakens traumatic injury of the spinal cord (TISC), and exhibits antioxidant and anti-inflammatory activities in a rat model. TISC model rats were injected intraperitoneally with 5, 10 or 30 mg/kg/day of myricitrin for 5 days. Basso-Beattie-Bresnahan evaluation of locomotion and water content of spinal cord were used to analyze the effects of myricitrin on TISC. Myricitrin significantly inhibited the TISC-induced oxidative stress and inflammatory reactions. In addition, cyclooxygenase-2 (COX-2), transforming growth factor (TGF)-β1 and p53 were significantly reduced and Bcl-2/Bax rate was significantly increased by treatment with myricitrin. The results of the current study suggested that the neuroprotective effect of myricitrin exhibits significant antioxidant and anti-inflammatory activities, and a remarkable trauma protection activity in TISC rats through inhibition of COX-2, TGF-β1, p53 and elevation of Bcl-2/Bax signaling pathway.

Introduction

Characterized by high incidence rate, disability rate and cost, as well as low mortality, traumatic injury of the spinal cord (TISC) is a common trauma in spine surgery, which greatly influences quality of life of patients and increases the burdens on their family members (1). With the rise of traffic and air accidents, its morbidity is increasing year by year (1). TISC mainly occurs to young adults and does not have good therapeutic measures (2). However, it is essential for patients to take operative treatment, drug therapy and rehabilitative measures to improve their functional status.

According to its mechanisms, spinal cord injury (SCI) can be classified into primary and secondary injuries (3). Primary injury is caused by the initial force directly or indirectly acting on spinal cords (4). Secondary injury occurs on the site of primary injuries, with a series of physiochemical mechanisms including oxidative stress and excessive release of inflammatory response causing destructive lesions to complete tissues surrounding the lesions and a further deepening of the degree of injuries and the broadening of injury areas (5,6). Oxidative stress is a series of adaptive responses triggered by the loss of equilibrium between reactive oxygen species and the antioxidant system (7).

Myricitrin (Fig. 1) is the 3-O-rhamnoside of myricetin, a flavonoid. Myricitrin and tannin exist in waxberry extract (8). In addition to its confirmed pharmacological functions, myricitrin is anti-inflammatory and antineoplastic (9). Furthermore, it can prevent tooth decay, and eliminate free radicals and oxidative stress (10). Thus, the present study evaluated if myricitrin ameliorated TISC, and explored its mechanism.

Materials and methods

Animals and experimental design. All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD, USA) and were approved by the Ethics Committee for Animal Experiments, The Third Department of Orthopedics, Cangzhou Central Hospital (Cangzhou, China). A total of 60 adult female Sprague Dawley rats (7-8 weeks; 160-200 g) were kept in polypropylene cages with wood shavings as bedding, 12/12 h light/dark cycle, lights on at 8:00 a.m. at 22±2˚C and had free access to water and food. The animals were randomly divided into five groups of 12: Sham, TISC model, myricitrin (5 mg/kg/d), myricitrin (10 mg/kg/d) and myricitrin (30 mg/kg/d). TISC model rats were performed under general anesthesia, using intraperitoneal ketamine (80 mg/kg) and xylazine (10 mg/kg) injection. Rats were injured in the thoracic level 12. A laminectomy was

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also performed in the thoracic level 12, and skin and muscle overlying the spinal column were cut. A moderate-intensity weight-drop was performed using an impactor with a diameter of 2.5 mm into the thoracic level 12. In the myricitrin group, rats were injected intraperitoneally with 5, 10 or 30 mg/kg/d of myricitrin for 5 days.

Histopathology. Spinal cord tissue was collected and washed with PBS. Tissue was fixed with 4% paraformaldehyde for 24 h at room temperature, embedded in paraffin and sectioned into 4 μm sections. Then, tissue samples were dewaxed using xylene and washed with ethyl alcohol. Tissues were sectioned was stained with hematoxylin and eosin.

Basso-Beattie-Bresnahan (BBB) evaluation of locomotion and water content of spinal cord. The BBB scale evaluates the following criteria: The rating scale ranges from 0 to 21, and scores were assigned for both hind limbs by two independent observers blinded to the experiments. 0 on the scale refers to no observable hindlimb movement and 21 is normal locomotion. Following myricitrin treatment, spinal cord tissue samples were gathered and weighed as wet weight. Then, at 80˚C, spinal cord tissue samples were dried for 48 h and weighed as dry weight. The water content of the spinal cord is calculated as dry weight/wet weight.

ELISA. Spinal cord tissue homogenate was centrifuged at 8,000 x g for 10 min at 4˚C and the supernatant was harvested to measure the protein concentration using Coomassie brilliant blue G250 technique (Beyotime Institute of Biotechnology, Haimen, China). A total of 50 μl protein samples were harvested, and malondialdehyde (MDA; A003-1), superoxide dismutase (SOD; A001-3), catalase (CAT; A007-1), glutathione peroxidase (GSH-PX; A005), NF-κB p65 subunit (H202), tumor necrosis factor (TNF)-α (H052), interleukin (IL)-1β (H002) and IL-6 (H007) contents were measured using ELISA kits (Nanjing Jiancheng Biology Engineering Institute, Nanjing, China). The absorbance value at 405 nm was determined using a SpectraMax® M2e Multimode Microplate Reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from spinal cord tissue using TRIzol reagent (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). A total of 1 μg total RNA was synthesized cDNA using a one-step RT-PCR kit (Qiagen Benelux B.V., Venlo, The Netherlands). The gene expression levels of cyclooxygenase (COX)-2 and TGF-β1 were analyzed by RT-qPCR, conducted using the CFX96 Real Time PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The standard amplification program included 30 cycles, 95˚C with a 20 sec hold, annealing at 60˚C sec with a 20 sec hold, and extending at 72˚C with a 10 sec hold. The following primers were used for COX-2 forward, TTCCAATCCATGTCA AAACCGT and reverse, AGTCCGGGTACAGTCACACTT; TGF-β1 forward, 5'-AGGGCTACCATGCCAACTTC-3' and reverse, 5'-CCA CGTAGTAGACGATGGGC-3'; β-actin forward, GGCTGT ATTCCCCTCCATCG and reverse, CCAGTTGGTAAC AAT GCCATGT.

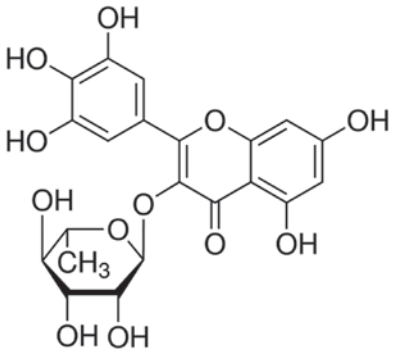

Figure 1. The chemical structure of myricitrin.

Western blotting. Spinal cord tissue homogenate was centrifuged at 8,000 x g for 10 min at 4˚C and the supernatant was harvested to measure the protein concentration using Coomassie brilliant blue G250 technique (Beyotime Institute of Biotechnology). Protein samples (50 μg) were harvested using SDS-PAGE on 10-12% gel and transferred on to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Inc.). The membrane was incubated with blocking buffer (5% skimmed milk) for 1 h at room temperature and probed overnight at 4˚C with primary antibodies against p53 (sc-1311-R; 1:200; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), Bcl-2 (sc-783; 1:400, Santa Cruz Biotechnology, Inc.), Bax (sc-6236; 1:400; Santa Cruz Biotechnology, Inc.) and β-actin (sc-7210; 1:500; Santa Cruz Biotechnology, Inc.). The membrane was incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibody (sc-2004; 1:1,000; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature.

Statistical analysis. Experimental data are expressed as means ± standard deviation and used SPSS software (version, 13.0; SPSS, Inc., Chicago, IL, USA). The Mann-Whitney U-test and Spearman's rank correlation were used for the statistical analyses. Differences with $P<0.05$ was considered to indicate a statistically significant difference.

Results

Myricitrin inhibits histological injury in SCI rats. Following treatment with myricitrin, the authors observed the histology injure of TISC model group was obviously higher than that of the sham group (Fig. 2). However, treatment with 10 and 30 mg/kg myricitrin evidently inhibited histological injury in TISC rats, compared with the TISC model group (Fig. 2).

Myricitrin decreases BBB score in SCI rats. During surgery, the effect of myricitrin on BBB score in SCI rats, the BBB locomotor rating scale, was used. Fig. 3 indicated that the mean BBB scores of the sham group were highest in all experiment groups. Following treatment by myricitrin, 10 and 30 mg/kg myricitrin significantly increased BBB score, compared with the TISC model group (Fig. 3).

Myricitrin weakens water content of spinal cord in SCI rats. To evaluate the water content of spinal cord in SCI rats, the water content was detected following treatment

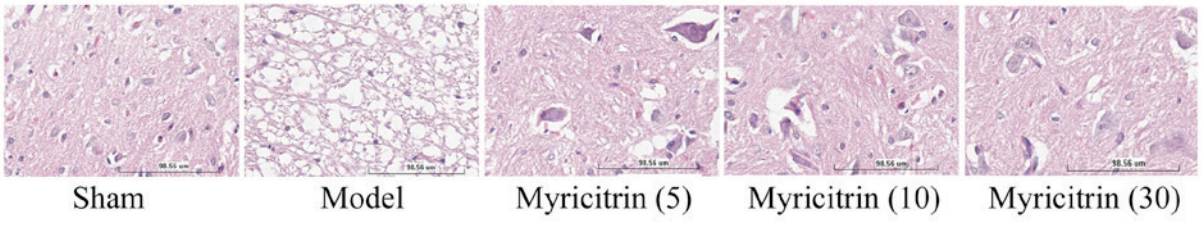

Figure 2. Myricitrin inhibited histological injury in spinal cord injury rats. Sham, sham group; Model, TISC model group; Myricitrin (5), 5 mg/kg myricitrin group; Myricitrin (10), 10 mg/kg myricitrin group; and Myricitrin (30), 30 mg/kg myricitrin group. ##P<0.01 vs. sham group; *P<0.05, **P<0.01 vs. TISC model group. TISC, traumatic injury of the spinal cord.

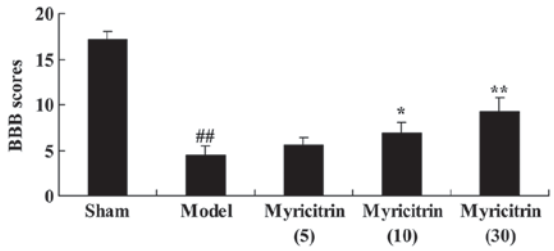

Figure 3. Myricitrin improves Basso-Beattie-Bresnahan score in spinal cord injury rats. Sham, sham group; Model, TISC model group; Myricitrin (5), 5 mg/kg myricitrin group; Myricitrin (10), 10 mg/kg myricitrin group; and Myricitrin (30), 30 mg/kg myricitrin group. ##P<0.01 vs. sham group; *P<0.05, **P<0.01 vs. TISC model group. TISC, traumatic injury of the spinal cord.

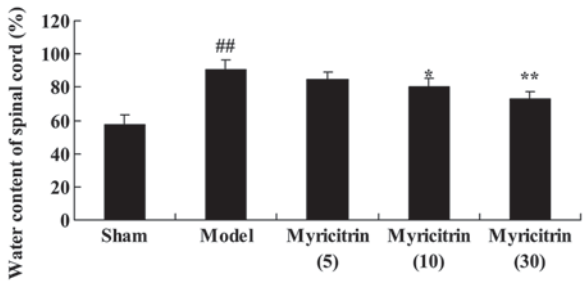

Figure 4. Myricitrin weakens water content of spinal cord in spinal cord injury rats. Sham, sham group; Model, TISC model group; Myricitrin (5), 5 mg/kg myricitrin group; Myricitrin (10), 10 mg/kg myricitrin group; and Myricitrin (30), 30 mg/kg myricitrin group. ##P<0.01 vs. sham group; *P<0.05, **P<0.01 vs. TISC model group. TISC, traumatic injury of the spinal cord.

with myricitrin. In the TISC model group, the rats presented improvements in the water content of spinal cord compared with sham group (Fig. 4). At 5 d, the water content of spinal cord was significantly suppressed by treatment with 10 and 30 mg/kg myricitrin in TISC rats, compared with the model group (Fig. 4).

Myricitrin exhibits antioxidant activity in SCI rats. Following the treatment with myricitrin, the authors researched the effect that myricitrin exhibits on antioxidant activity in SCI rats. In the TISC model group, the induction of MDA content and inhibition of SOD, CAT and GSH-PX contents was observed compared with sham group (Fig. 5). In addition, treatment with 10 and 30 mg/kg myricitrin significantly reversed the induction of MDA, SOD, CAT and GSH-PX levels in TISC rats (Fig. 5).

Myricitrin exhibits anti-inflammatory activity in SCI rats. Following SCI and administration of myricitrin, to explore the effect of myricitrin exhibits anti-inflammatory activity in SCI rats, the NF-κB p65 subunit, TNF-α, IL-1β and IL-6 contents were measured using ELISA kits. As indicated in Fig. 6, SCI-induced NF-κB p65 subunit, TNF-α, IL-1β and IL-6 contents were observed in the TISC model group, compared with the sham group. Following myricitrin administration, 10 and 30 mg/kg myricitrin significantly inhibited the SCI-induced NF-κB p65 subunit, TNF-α, IL-1β and IL-6 contents in TISC rats (Fig. 6).

Myricitrin weakens COX-2 mRNA expression in SCI rats. To further investigate the effect of myricitrin on COX-2 in SCI rats, COX-2 mRNA expression was detected by Real-time Quantitative PCR. The results revealed upregulation in COX-2 mRNA expression of TISC model group, compared with sham group (Fig. 7). Pretreatment with 10 and 30 mg/kg myricitrin significantly suppressed the COX-2 mRNA expression in TISC rats (Fig. 7).

Myricitrin weakens TGF-β1 mRNA expression in SCI rats. To examine the effect of myricitrin on TGF-β1 in SCI rats, RT-qPCR was used to detect TGF-β1 mRNA expression. There was a significant increase in TGF-β1 mRNA expression of the TISC model group, compared with the sham group (Fig. 8). Following SCI and administration of myricitrin, 10 and 30 mg/kg myricitrin significantly inhibited TGF-β1 mRNA expression in TISC rats (Fig. 8).

Myricitrin weakens p53 protein expression in SCI rats. To determine the effect of myricitrin on the p53 signaling pathway in SCI rats, p53 protein expression was analyzed using western blotting. The p53 protein expression of the TISC model group was lower than that of the sham group (Fig. 9). Administration of 10 and 30 mg/kg myricitrin significantly promoted the TISC-inducted inhibition of p53 protein expression in SCI rats (Fig. 9).

Myricitrin weakens Bcl-2/Bax rate in SCI rats. Because Bcl-2/Bax rate mediating apoptosis, Bcl-2 and Bax protein expression was measured using western blotting. As presented in Fig. 10, the Bax/Bcl-2 rate of the TISC model group was higher than that of the sham group. However, administration of 10 and 30 mg/kg myricitrin significantly inhibited Bax/Bcl-2 rate in TISC rats (Fig. 10).

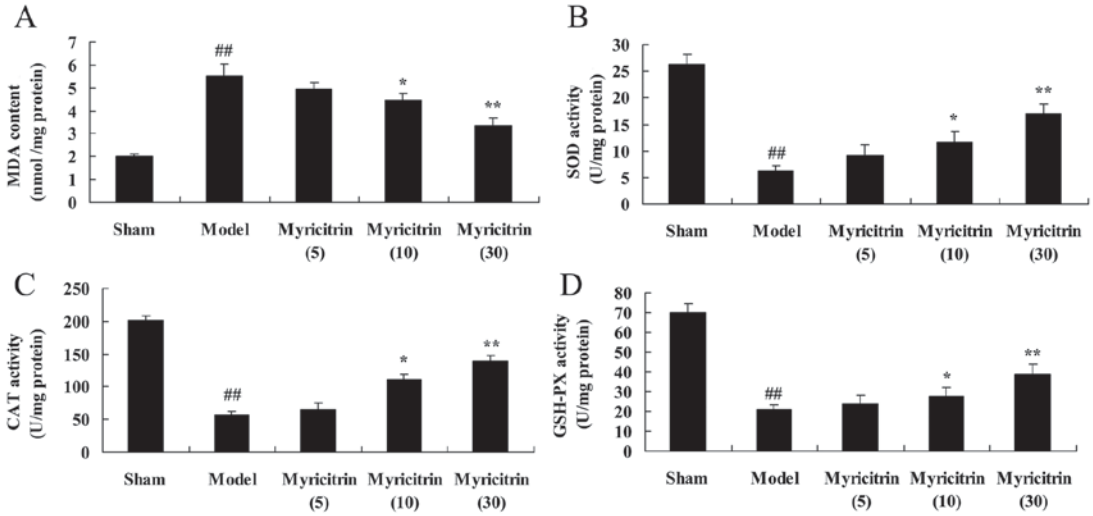

Figure 5. Myricitrin exhibits antioxidant activity in spinal cord injury rats. Myricitrin exhibits antioxidant activity as reflected by (A) MDA, (B) SOD, (C) CAT and (D) GSH-PX levels in SCI rats. Sham, sham group; Model, TISC model group; Myricitrin (5), 5 mg/kg myricitrin group; Myricitrin (10), 10 mg/kg myricitrin group; and Myricitrin (30), 30 mg/kg myricitrin group. $^{\#\#}$P<0.01 vs. sham group, *P<0.05, **P<0.01 vs. TISC model group. MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase; GSH-PX, glutathione peroxidase; TISC, traumatic injury of the spinal cord.

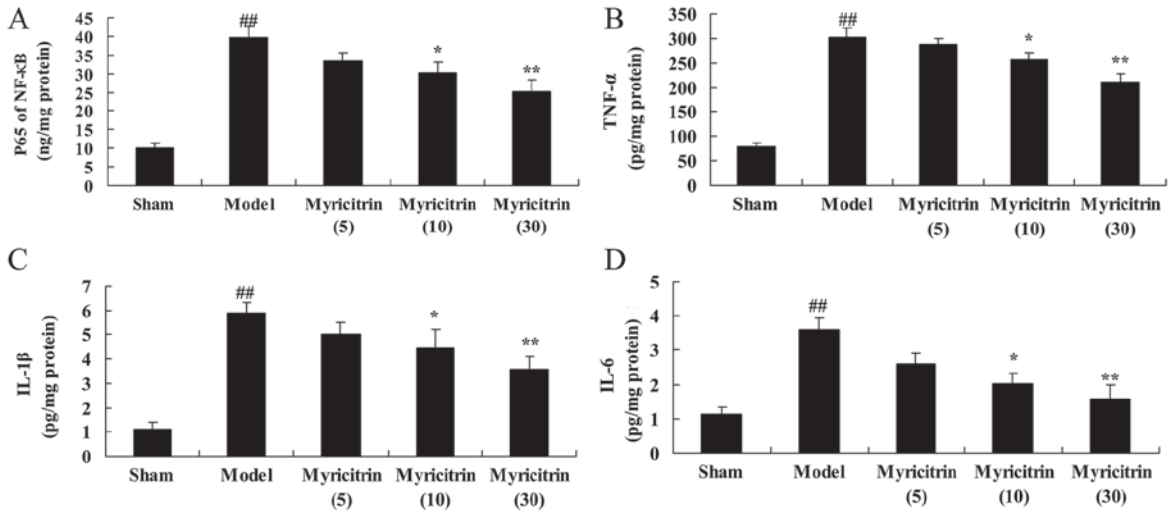

Figure 6. Myricitrin exhibits anti-inflammatory activity in SCI rats. Myricitrin exhibits anti-inflammatory activity as reflected in (A) NF-κB p65 subunit, (B) TNF-α, (C) IL-1β and (D) IL-6 levels in SCI rats. Sham, sham group; Model, TISC model group; Myricitrin (5), 5 mg/kg myricitrin group; Myricitrin (10), 10 mg/kg myricitrin group; and Myricitrin (30), 30 mg/kg myricitrin group. $^{\#\#}$P<0.01 vs. sham group, *P<0.05, **P<0.01 vs. TISC model group. SCI spinal cord injury; TISC, traumatic injury of the spinal cord; TNF-α, tumor necrosis factor-α; IL, interleukin.

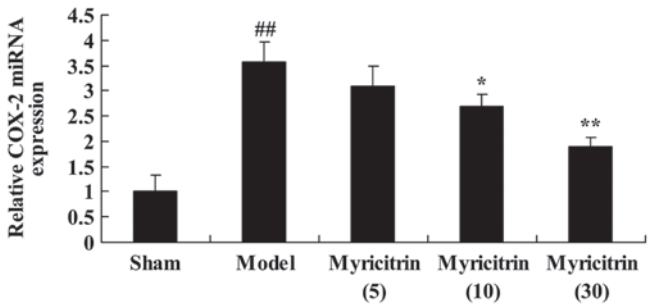

Figure 7. Myricitrin weakens COX-2 mRNA expression in spinal cord injury rats. Sham, sham group; Model, TISC model group; Myricitrin (5), 5 mg/kg myricitrin group; Myricitrin (10), 10 mg/kg myricitrin group; and Myricitrin (30), 30 mg/kg myricitrin group. $^{\#\#}$P<0.01 vs. sham group, *P<0.05, **P<0.01 vs. TISC model group. COX-2, cyclooxygenase-2; TISC, traumatic injury of the spinal cord.

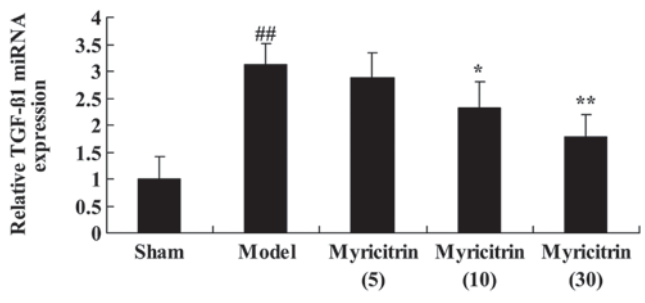

Figure 8. Myricitrin weakens TGF-β1 mRNA expression in spinal cord injury rats. Sham, sham group; Model, TISC model group; Myricitrin (5), 5 mg/kg myricitrin group; Myricitrin (10), 10 mg/kg myricitrin group; and Myricitrin (30), 30 mg/kg myricitrin group. $^{\#\#}$P<0.01 vs. sham group, *P<0.05, **P<0.01 vs. TISC model group. TGF-β1, tumor necrosis factor-β1; TISC, traumatic injury of the spinal cord.

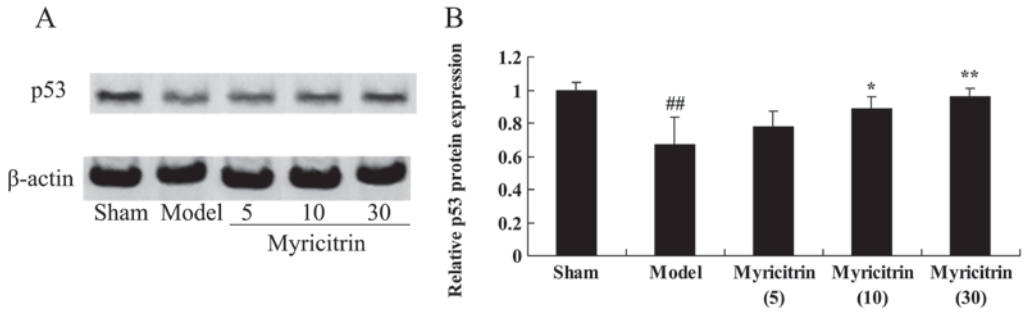

Figure 9. Myricitrin weakens p53 protein expression in SCI rats. Myricitrin weakens p53 protein expression in using (A) western blot analysis and (B) statistical analysis of p53 protein expression SCI rats. Sham, sham group; Model, TISC model group; Myricitrin (5), 5 mg/kg myricitrin group; Myricitrin (10), 10 mg/kg myricitrin group; and Myricitrin (30), 30 mg/kg myricitrin group. ##P<0.01 vs. sham group, *P<0.05, **P<0.01 vs. TISC model group. SCI, spinal cord injury; TISC, traumatic injury of the spinal cord.

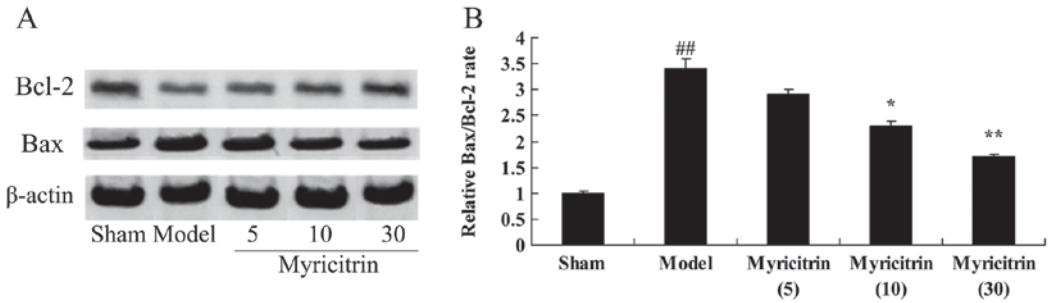

Figure 10. Myricitrin decreases Bcl-2/Bax rate in SCI rats. Myricitrin weakens Bcl-2 and Bax protein expression in using (A) western blot analysis and (B) statistical analysis of Bcl-2 and Bax protein expression SCI rats. Sham, sham group; Model, TISC model group; Myricitrin (5), 5 mg/kg myricitrin group; Myricitrin (10), 10 mg/kg myricitrin group; and Myricitrin (30), 30 mg/kg myricitrin group. ##P<0.01 compared with sham group, *P<0.05 vs. TISC model group, **P<0.01 vs. TISC model group. SCI, spinal cord injury; TISC, traumatic injury of the spinal cord.

Discussion

According to pathological changes at different phases, SCI can be divided into acute spinal cord injury and chronic spinal cord injury (11). At present, measures to treat SCI primarily include drug therapy, operative treatment and functional reconstruction following SCI (12). But the curative effects of these therapeutic measures are not definite. Thus many patients are still confronted with neurological dysfunction and permanent disability. With advances of industry, agriculture and transportation, spinal cord injury is becoming a common and frequently occurring condition (13). Therefore, intervention and treatment of SCI is urgent (14). At present, 10 and 30 mg/kg myricitrin significantly increased BBB score and suppressed the water content of spinal cord in TISC rats.

Myricitrin treatment significantly reversed the induction of MDA, SOD, CAT and GSH-PX contents and inhibited the SCI-induced NF-κB p65 subunit, TNF-α, IL-1β and IL-6 contents in TISC rats. Domitrović *et al* (10) suggested that myricitrin exhibited antioxidant and anti-inflammatory actions in carbon tetrachloride-intoxicated mice (10).

Excessive active radicals following SCI act on the postsynaptic neurons and activate adjacent astrocytes and microglial cells, resulting in ionic unbalance of nerve cells (15). Oxidative stress following SCI destabilizes the ionic homeostasis inside and outside the membrane (16). Significant amounts of Ca^{2+} enter the mitochondria and accumulate, causing destruction of the mitochondria (17) and aerobic energy metabolism disorders, inhibiting the synthesis of ATP (18). Excitatory toxicity triggered by oxidative stress serves an essential role in secondary SCI; this could extend the degeneration period of the substantia alba medullae spinalis and accelerate the apoptosis of oligodendroglia cells (14). It was previously demonstrated that free radicals may increase the release of GSH, while scavengers of free radicals have the opposite function. In addition, oxidative stress following SCI gives rise to the activation of gitter cells and astrocytes, and results in the release of inflammatory cytokines and TNF-α (19). Previous studies demonstrated that TNF-α can rapidly increase the receptor quantities of Glu in cell membrane structures, and further enhance the sensitivity of motor neurons to excitatory poisoning (18,20). Following SCI, 10 and 30 mg/kg myricitrin treatment significantly reversed the induction of MDA, SOD, CAT and GSH-PX contents and inhibited the SCI-induced NF-κB p65, TNF-α, IL-1β and IL-6 contents in TSCI rats. Furthermore, Domitrović *et al* suggested that myricitrin may exhibit antioxidant and anti-inflammatory in carbon tetrachloride-intoxicated mice (10).

A previous study suggested that cells in damage zone following SCI undergo a series of changes in form, function and metabolism (21). The causes of these changes are that cell growth loses the internal environment supported by nutrition. Injuries may induce a series of immuno-inflammatory responses; a large amount of MCP-1, TNF-α, IL-6 and IL-1β released by monocyte/macrophage increases the contents of excitatory amino acids in the damage area. In addition, the

decrease in expression of apoptosis inhibiting genes leads to cell death and secondary injury of spinal cord occurs (21-23).

Overexpression of COX-2 is associated with the increase of microvessel density (24). Moreover, COX-2 is closely related with angiogenesis, which is induced by inflammatory cytokines (25). As is well established, bone marrow mesenchymal stem cells may secrete various inflammatory cytokines and facilitate angiogenesis following injuries of the central nervous system (26). Research has discovered that obvious expression of TGF-β1 in hematoma at damage zone occurs first (27). Then, TGF-β1 expresses in the cytoplasm and the karyons of astrocytes and capillary endothelial cells (inside and outside the marrow) and motor neurons would increase (28). In the present study, it was observed that 10 and 30 mg/kg myricitrin significantly inhibited COX-2 and TGF-β1 mRNA expression in TISC rats. Domitrović *et al* (10) suggest that myricitrin exhibits antioxidant and anti-inflammatory effects in carbon tetrachloride-intoxicated mice through COX-2 and TGF-β1.

The increase in p53 expression directly causes the apoptosis or indirectly causes apoptosis by regulating other apoptosis-related genes (29). Transcriptional levels of p53 would rise and activate downstream WAF/GiP1 genes to express p21, which can inhibit the activity of cyclin dependent kinase (30). Thus, cells can stagnant between G1 and S phases. If DNA injuries cannot be repaired on time, apoptosis would happen (31). P53 genes decrease expression of endogenous Bcl-2 and inhibit its functions (32). p53 can be regarded as direct agonist of genetic transcription of Bas and increases protein expression in Bax, it also changes the proportion of Bcl-2/Bax proteins and facilitates apoptosis (33). In the present study, 10 and 30 mg/kg myricitrin administration significantly promoted the TISC-inducted the inhibition of p53 protein expression and inhibited the Bax/Bcl-2 rate in TISC rats. Sun *et al* (34) suggested that the effects of myricitrin suppressed oxidative stress damage through p53, caspase-3, Bax/Bcl-2 and the MAPK signaling pathway in ApoE-/- mice.

In conclusion, the current study has underlined that myricitrin improves BBB score and suppresses the water content of spinal cord in TISC rats through antioxidant and anti-inflammatory effects, COX-2, TGF-β1, p53 and the Bax/Bcl-2 signaling pathway. However, future studies are required to detail the mechanisms underlying how myricitrin weakens TISC and the promotion of functional recovery following SCI.

Acknowledgements

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