

Effect of rutin on spinal cord injury through inhibition of the expression of MIP-2 and activation of MMP-9, and downregulation of Akt phosphorylation

PENG ZHANG and XUN MA

Department of Orthopaedics, The Affiliated Shanxi Da Yi Hospital of Shanxi Medical University,
Taiyuan, Shanxi 030032, P.R. China

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Abstract. Rutin has extensive pharmacological activities, including antibacterial and anti-inflammatory activities, cooling of the blood to inhibit bleeding, reducing capillary wall fragility and anti-influenza activities. However, whether rutin can ameliorate neuropathic function in spinal cord injury (SCI) in constriction-induced peripheral nerve injury remains to be elucidated. In the present study, the potential protective effects of rutin on SCI rats were investigated. Neurological function was examined using the Basso, Beattie and Bresnahan (BBB) scoring system and by measuring the water content of the spinal cord tissue in SCI rats. SCI-induced programmed cell death was measured using hematoxylin and eosin staining. In addition, the expression of macrophage inflammatory protein-2 (MIP-2) and the activation of matrix metalloproteinase-9 (MMP-9) in the SCI rats were evaluated using ELISA assay kits and zymographic analysis, respectively. The phosphorylation of protein kinase B (p-Akt) was analyzed using a western blot assay. The results demonstrated that administrating rutin began to increase BBB scores and attenuate the spinal cord water content of the SCI rats. Administrating rutin prevented SCI-induced programmed cell death. The SCI rats of in the rutin-treated group were found to exhibit lower expression levels of MIP-2 and p-Akt, reduced MMP-9 activation, compared with the SCI model rats. In conclusion, rutin was demonstrated as a potential protective agent in SCI and enhances the neurotrophic effect by inhibiting the expression of MIP-2 and activation of MMP-9, and downregulating the expression of p-Akt.

Introduction

Following spinal cord injury (SCI), ischemia-hypoxia, reperfusion injury, lipid peroxidation and inhibition of the expression of various molecules in local tissues lead to neuronal cell necrosis and axonal demyelination, and glial scar formation, which seriously impede axonal regeneration and myelination, and impact neurological recovery (1,2).

A substantial number of inflammatory cells in SCI exhibit characteristic pathological changes, which is a multi-step process with complex multifactorial involvement. Chemokines have been reported to be key in this process. Macrophage inflammatory protein-2 (MIP-2) is one member of the CXC chemokine family, which leads to specific chemotaxis of neutrophils and lymphocytes to sites of inflammation, and is important in the development of chronic bronchitis, hepatitis and other inflammatory disorders (3). Pineau *et al* indicated that astrocytes initiate inflammation through the modulation of monocyte chemoattractant protein 1 (MCP-1; CCL2), keratinocyte chemoattractant (KC; CXCL1) and MIP-2 (CXCL2) in the injured mouse spinal cord (4). Berghmans *et al* demonstrated that chlorite-oxidized oxyamylose protects significantly against hyperacute spinal cord homogenate-induced experimental autoimmune encephalomyelitis via suppression of the MIP-2/CXCL2 signal pathway (5).

Matrix metalloproteinases (MMPs) are a family of metalloprotein endonucleases dependent on Zn^{++} , which are involved in fibrosis, arthritis, tumor growth, migration, invasion and metastasis in pathological conditions (6-8). MMP, particularly MMP-9, is an important factor involved in acute spinal cord injury (9). Feng *et al* reported that ulinastatin protects against experimental autoimmune encephalomyelitis through downregulation of the expression of MMP-9 (10), and Zhang *et al* indicated that nutrient mixture attenuated SCI-induced impairment by negatively affecting the promoter activity of MMP-2 and MMP-9 in mice (11).

In previous years, the role of TNF- α in spinal cord injury has gained increasing attention, TNF- α is regarded to be an initiation factor among several cytokines, upregulating the generation of other cytokines, which is significant in the amplification of local inflammation. Protein kinase B (Akt)

Correspondence to: Dr Xun Ma, Department of Orthopaedics, The Affiliated Shanxi Da Yi Hospital of Shanxi Medical University, 99 Long Cheng Street, Taiyuan, Shanxi 030032, P.R. China
E-mail: xunmajk@163.com

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as a serine/threonine protein kinase, is a key central effector protein for multiple signal transduction pathways and, as downstream target protein for phosphoinositide 3-kinase (PI3K), it is the core of PI3K/Akt signaling pathway (12). Felix *et al* demonstrated that the inhibition of medulla Akt phosphorylation (p-Akt) signaling prevented the spontaneous respiratory recovery observed following partial cervical SCI in adult rats (13).

Rutin is a common food flavonoid belonging to the flavonols. Studies have demonstrated that flavonoid compounds have anti-inflammatory, antioxidant, antitumor, antiviral, anticardiovascular disease and immunomodulatory effects (14-16). Each phenyl ring of flavonoid compounds can be connected to a hydroxy group, sugar group or substituent group, including polysaccharides and, as the types and connection positions of the sugar are different, various flavonoid glycosides may be formed (17). However, the mechanism through which rutin regulates neurological function of SCI remains to be fully elucidated. In the present study, the predominant focus was to investigate whether rutin has a positive effect on neurological function in SCI rats, and to determine the mechanisms involved in the regulation of expression of MIP-2, activation of MMP-9 and phosphorylation of Akt in SCI rats.

Materials and methods

Drugs and chemicals. Rutin (purity >95%; Fig.1) was purchased from Nanjing Traditional Chinese Medicine Institute of Chinese Material Medica (Nanjing, China). Methylprednisolone (MPSS) was supplied by the Affiliated Shanxi Da Yi Hospital of Shanxi Medical University (Taiyuan, China). The MIP-2 ELISA assay kit was supplied by Cayman Chemicals, (Ann Arbor, MI, USA). The Bicinchoninic Acid (BCA) protein assay was supplied by Beyotime Institute of Biotechnology (Nanjing, China).

Materials. A total of 40 healthy male adult Sprague-Dawley (SD) rats, aged 2 months and weighing 250 ± 20 g, were purchased from the Chinese Academy of Medical Sciences Animal Laboratory (Beijing, China). All SD rats were allowed free access to food and water and were housed in individual cages at $23 \pm 2^\circ\text{C}$ with a humidity of $\sim 56\%$ under a 12-h light/dark cycle. The present study was approved by the ethics committee of The Affiliated Shanxi Da Yi Hospital of Shanxi Medical University. The protocols were performed in accordance with the Chinese National Natural Science Foundation animal research regulations (18) and the animal care guidelines of the National Institutes of Health (Bethesda, MA, USA).

Preparation of the rat model of SCI. The experiment rats were fixed on an operating table in a supine position and administered intraperitoneally (i.p) with pentobarbital (50 mg/kg) chloral hydrate for anesthesia. Following sterilization, the skin of the rats above the vertebral column was shaved carefully, an abdominal midline incision was made, and a 20-mm midline incision was made in the thoracic region for the purpose of exposing the vertebral column. Laminectomy was performed at vertebral level T-10, and the dura remaining intact was used to expose the dorsal cord surface.

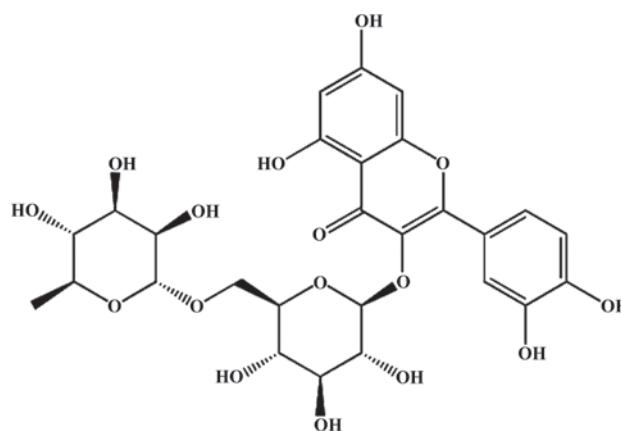


Figure 1. Chemical structure of rutin.

Drug treatment and grouping. According to a previous report (19), the dosage and dosing frequency of rutin were selected. The rats were randomly divided into five groups, each containing eight rats, as follows: (i) control group (Con), in which normal rats received physiological saline (0.1 ml/100 g, i.p.); (ii) SCI group, in which SCI model rats received physiological saline (0.1 ml/100 g, i.p.); (iii) MPSS group, in which SCI model rats received 100 mg/kg MPSS (i.p.); (iv) 1 $\mu\text{mol/kg}$ rutin group (RT group), in which SCI model rats received 1 $\mu\text{mol/kg}$ rutin; (v) 10 $\mu\text{mol/kg}$ rutin group (RT group), in which SCI model rats received 10 $\mu\text{mol/kg}$ rutin. After 6 h, the rats were sacrificed by cervical dislocation, and samples were collected for further analysis.

Evaluation of Basso, Beattie and Bresnahan (BBB) scores for evaluating neurological function. After 6 h, the locomotor recovery of the rats were evaluated using the BBB scoring system, in which locomotion was scored on a rating scale between 0 (complete paralysis) and 21 (normal locomotion) (20).

Evaluation of the water content of spinal cord tissue. The rats were administered for edema at 3 days post-SCI. The spinal cord of the rats were cut into 10 mm segments and dried for 48 h at -80°C prior to the wet weight being measured (21). The percentage of tissue water content was then calculated using the following equation: Water Content (%) = (wet weight - dry weight) / wet weight) x 100.

Evaluation of programmed cell death using hematoxylin and eosin (H&E) staining. Following treatment with rutin, the spinal cord tissues were collected and the sections of the spinal cord were washed with distilled water and stained with hematoxylin solution for 10 min (Shanghai Research Company Biological Technology Co.,Ltd., Shanghai, China). These sections were differentiated in 1% acid-alcohol for 30 sec and were then sections placed into eosin for 30 sec and dehydrated with alcohol (70, 80, 90 and 100%) for 2 min each. Subsequently, the sections were covered with xylene-based mounting medium (Invitrogen Life Technologies, Carlsbad, CA, USA) following two changes of xylene. The cells were visualized using microscopy (IX71; Olympus, Tokyo, Japan).

Evaluation of the expression of MIP-2 using ELISA assay kits. Following treatment with rutin, the spinal cord samples were collected and were homogenized in a glass homogenizer into tissue homogenates for the estimation of MIP-2. According to the manufacturer's instructions (Cayman Chemicals), the evaluation of MIP-2 was performed using commercially available ELISA assay kits (Cayman Chemicals). The protein content was determined using a BCA protein assay (Beyotime Institute of Biotechnology).

Evaluation of the activation of MMP-9 using zymographic analysis. As previously described (22), the activation of MMP-9 was measured using a gelatin zymography protease assay. Following treatment with rutin, the spinal cord tissues were collected and homogenized for the estimation of MMP-9. The samples were prepared using SDS sample buffer and then subjected to 8% SDS-PAGE (containing 0.1% gelatin; Invitrogen Life Technologies). Following electrophoresis, the gels were washed twice with 2.5% Triton X-100 (Beyotime Institute of Biotechnology) for 1 h at room temperature. The gels were then incubated at 37°C for 16-18 h in reaction buffer (Beyotime Institute of Biotechnology). Finally, the gels were stained with Coomassie Brilliant R-250 (Amresco, Inc., Framingham, MA, USA) to stain the gels.

Evaluation of the phosphorylation of Akt using western blot analysis. Following treatment with rutin, the spinal cord tissue samples were collected homogenized for the estimation of the protein expression levels of phosphorylated (p)-Akt. Briefly, 10 mg of the exposed spinal cord tissue samples were removed and incubated with 100 μ l tissue lysis buffer (pH 7.5; Beyotime Institute of Biotechnology) for 20-30 min on ice. Subsequently, the homogenates were centrifuged at 12,000 g for 20 min at 4°C. The tissue extracts were determined using a BCA protein assay (Beyotime Institute of Biotechnology). Equal quantities of protein (100 μ g) were fractionated by 10% SDS-PAGE (Invitrogen Life Technologies), followed by transferring onto polyvinylidene fluoride membranes (EMD Millipore, Bedford, MA, USA). The membranes were blocked with phosphate-buffered saline containing 0.1% Tween-20 (PBST) and 5% non-fat milk to inhibit nonspecific binding sites. Following washing with PBST, the membranes were incubated with monoclonal anti-p-Akt (cat. no. sc-293125; 1:1,500; Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) and monoclonal anti- β -actin (AC106; 1:500; Beyotime Institute of Biotechnology) overnight at 4°C. The membranes were then washed twice with PBST for 2 h at room temperature, and antibody binding was detected by incubating with a dilution of horseradish peroxidase-conjugated IgG (cat. no. sc-52336; 1:1,000; Santa Cruz Biotechnology, Inc.) for 2 h at room temperature. The western blots were developed using enhanced chemiluminescence western blotting reagents (E-CS-0050c; Wuhan Elabscience, Wuhan, China).

Statistical analysis. Statistical analyses were performed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). Results are expressed as the mean \pm standard deviation. Statistical analysis was evaluated using two-way analysis of variance. $P < 0.05$ was considered to indicate a statistically significant difference.

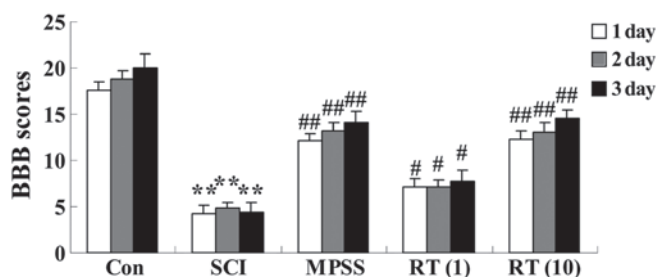


Figure 2. Effects of rutin on SCI-induced BBB scores for evaluating neurological function. Results are expressed as the mean \pm standard deviation. ** $P < 0.01$, compared with Con; # $P < 0.05$, compared with SCI; ## $P < 0.01$ compared with SCI. Con, control; SCI, spinal cord injury; MPSS, methylprednisolone-treated; RT (1), rutin (1 mg/kg) treatment; RT (10), rutin (10 mg/kg)-treated; BBB, Basso, Beattie and Bresnahan.

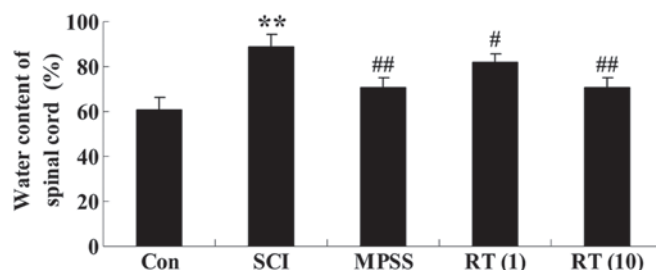


Figure 3. Effects of rutin on the water content of spinal cord tissue. Results are expressed as the mean \pm standard deviation. ** $P < 0.01$, compared with Con; # $P < 0.05$ compared with SCI; ## $P < 0.01$ compared with SCI. Con, control; SCI, spinal cord injury; MPSS, methylprednisolone-treated; RT (1), rutin (1 mg/kg)-treated; RT (10), rutin (10 mg/kg)-treated.

Results

Effects of rutin on SCI-induced BBB scores for evaluating neurological function. To measure the effect of rutin on the neurological function of the SCI rats, BBB scores were assigned to the functional abilities of the rats. Following injury, significant ($P < 0.01$) decreases in BBB scores were observed in the SCI group at 24, 48 and 72 h post-surgery, respectively, compared with those of the control group (Fig. 2). The SCI rats in the rutin-treated groups (1 and 10 μ mol/kg) exhibited increased BBB scores ($P < 0.05$ and $P < 0.01$, respectively), compared with the SCI group (Fig. 2). No significant differences were observed between the MPSS group and 10 μ mol/kg rutin-treated group ($P > 0.05$; Fig. 2).

Effects of rutin on the water content of the spinal cord tissue. At 6 h in the vehicle SCI animals, the spinal cord water content had increased significantly, compared with the control group (Fig. 3). The SCI rats in the rutin-treated (1 and 10 μ mol/kg) groups had reduced spinal cord water content ($P < 0.05$ and $P < 0.01$, respectively), compared with the SCI group (Fig. 3). Additionally, as shown in Fig. 3, the water content of the spinal cord in the 10 μ mol/kg rutin-treated group was similar to that in the MPSS group ($P > 0.05$).

Effects of rutin on SCI-induced programmed cell death. To assess effects of rutin on SCI-induced programmed cell death, cell death was detected using H&E staining. The levels

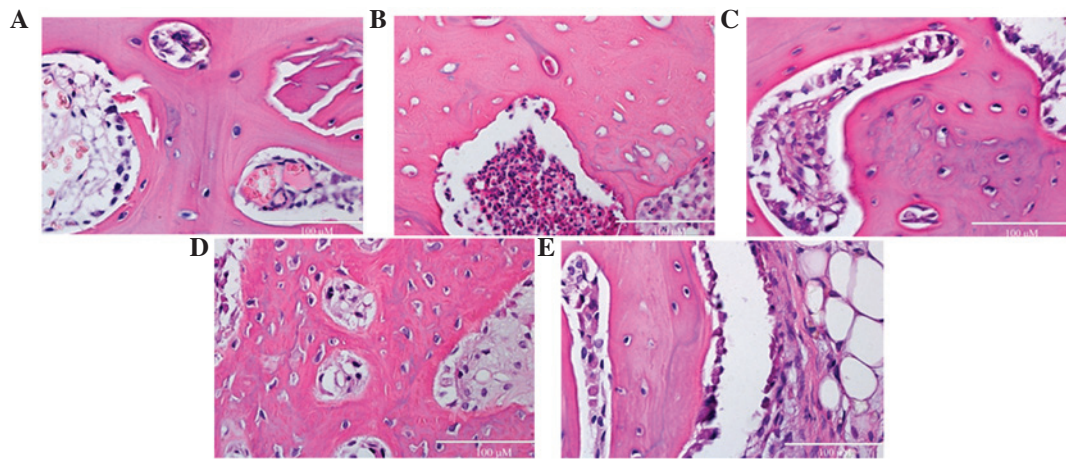


Figure 4. Effects of rutin on SCI-induced programmed cell death. Effects of rutin on SCI-induced cell death in the (A) control group, (B) SCI rat group, (C) rutin (1 mg/kg)-treated group and (E) rutin (10 mg/kg)-treated group. SCI, spinal cord injury.

of programmed cell death in the SCI group was markedly augmented, compared with the control group (Fig. 4A and B). No significant differences were observed between the MPSS group and 10 $\mu\text{mol/kg}$ rutin-treated group (Fig. 4C; $P>0.05$). However, the rutin-treated (1 and 10 $\mu\text{mol/kg}$) animals exhibited reduced programmed cell, compared with the SCI group (Fig. 4D and E).

Effects of rutin on SCI-induced MIP-2 generation. To investigate the effects of rutin on SCI-induced MIP-2 generation, the expression levels of MIP-2 in the spinal cord of the SCI rats were detected using ELISA assay kits. As shown in Fig. 5, the levels of MIP-2 in the SCI rats were augmented, compared with those of control rats. In the rutin-treated (1 and 10 $\mu\text{mol/kg}$) animals, lower levels of MIP-2 were observed, compared with the SCI group ($P<0.05$ and $P<0.01$, respectively; Fig. 5). No significant inter-group differences were observed between the MPSS group and rutin-treated (10 $\mu\text{mol/kg}$) group in the levels of MIP-2 in the SCI rats ($P>0.05$).

Effects of rutin on the SCI activation of MMP-9. To confirm the effects of rutin on the SCI activation of MMP-9, the present study detected the activation of MMP-9 using zymographic analysis. Zymography revealed that the activation of MMP-9 in the SCI rat group was significantly higher, compared with that in the control group (Fig. 6A and B). Rutin treatment (1 and 10 $\mu\text{mol/kg}$) led to a reduction in the activation of MMP-9 in the SCI rats ($P<0.05$ and $P<0.01$, respectively), compared with the SCI group (Fig. 6A and B). By contrast, no significant changes in the activation of MMP-9 were observed between the MPSS group and 10 $\mu\text{mol/kg}$ rutin-treated group ($P>0.05$).

Effects of rutin on the SCI-induced protein expression of p-Akt. To determine the protein expression levels of p-Akt in the SCI rats, the protein expression of p-Akt was determined using western blot analysis. The results demonstrated that the protein expression of p-Akt in the SCI group was significant higher than in the control group (Fig. 7A and B). These data clearly indicated that rutin (1 and 10 $\mu\text{mol/kg}$) modulated the protein expression of p-Akt in the SCI rats ($P<0.05$ and $P<0.01$, respectively), compared with the SCI group (Fig. 7A and B).

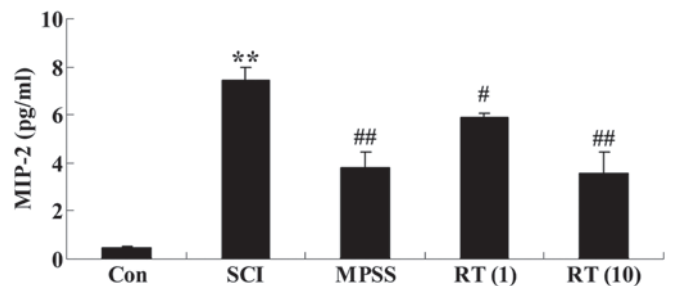


Figure 5. Effects of rutin on SCI-induced MIP-2 generation. Results are expressed as the mean \pm standard deviation. ** $P<0.01$, compared with Con; # $P<0.05$, compared with SCI; ## $P<0.01$, compared with SCI. Con, control; SCI, spinal cord injury; MPSS, methylprednisolone treatment; RT (1), rutin (1 mg/kg) treatment; RT (10), rutin (10 mg/kg)-treated. MIP2, macrophage inflammatory protein-2.

However, no significant difference was observed between the MPSS group and the 10 $\mu\text{mol/kg}$ rutin-treated group ($P>0.05$).

Discussion

The clinical morbidity rates of SCI are high, and paraplegia caused by SCI is one of the medical problems, which has remained unresolved, with no effective treatment (23). The pathological process underlying the development of SCI remains to be fully elucidated (24). Previous studies have suggested that cell necrosis is the predominant manifestation of SCI, however, with further investigation, it has been reported that the inflammatory reaction and necrosis in spinal cord nerve cells following primary mechanical tissue damage are accompanied by apoptosis, or programmed cell death (25-27). In the present study, it was demonstrated that rutin markedly augmented the BBB scores and decreased spinal cord water content in the SCI rats. In addition, rutin was observed to prevent SCI-induced programmed cell death. Xu *et al* indicated that rutin improves spatial memory and improves neurological function in Alzheimer's disease-transgenic mice (28). Rodrigues *et al* reported that rutin caused a reduction of neurodegeneration in the periphery of cortical injury (29). Aruna *et al* revealed that rutin reduces

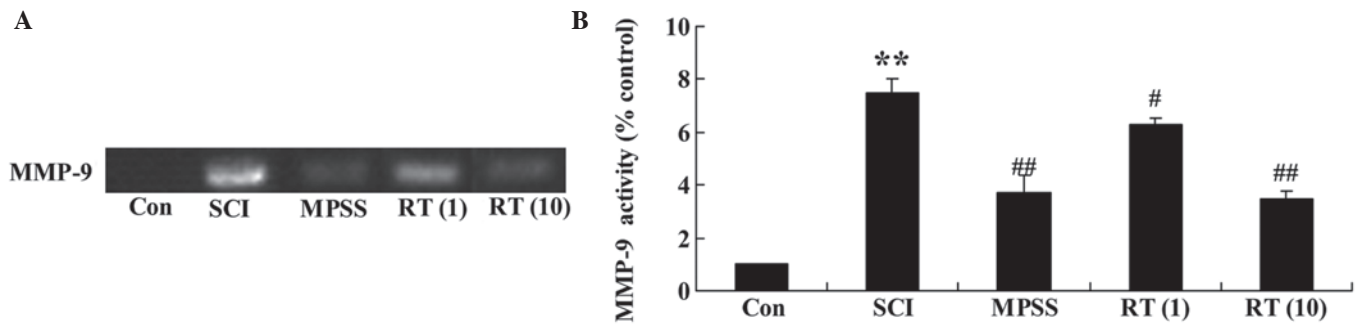


Figure 6. Effects of rutin on SCI activation of MMP-9. (A) Effects of rutin on the activation of MMP-9 were determined using zymography. (B) Statistical analysis of the activation of MMP-9 in the SCI model rats. Results are expressed as the mean \pm standard deviation. ** $P < 0.01$, compared with Con; # $P < 0.05$, compared with SCI; ## $P < 0.01$, compared with SCI. Con, control; SCI, spinal cord injury; MPSS, methylprednisolone treatment; RT (1), rutin (1 mg/kg)-treated; RT (10), rutin (10 mg/kg)-treated. MMP, matrix metalloproteinase.

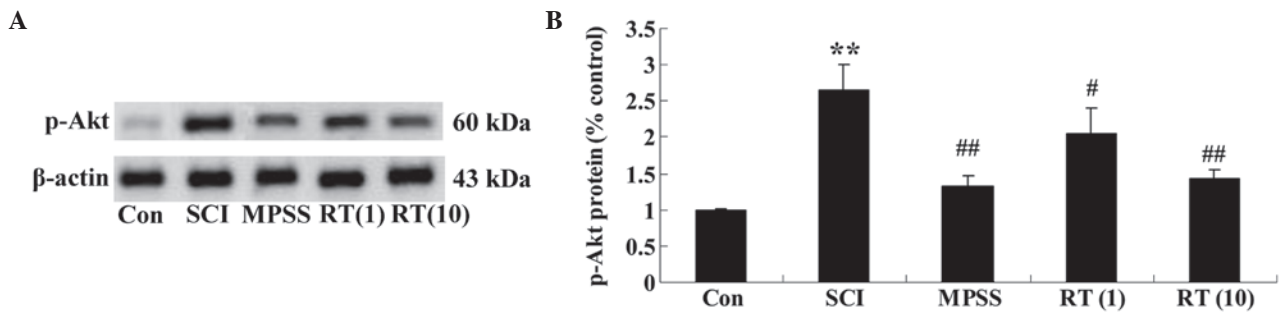


Figure 7. Effects of rutin on SCI-induced protein expression of p-Akt protein. (A) Effects of rutin on the protein expression levels of p-Akt were determined using western blot analysis. (B) Statistical analysis of the protein expression levels of p-Akt in the SCI model rats. Results are expressed as the mean \pm standard deviation. ** $P < 0.01$, compared with Con; # $P < 0.05$, compared with SCI; ## $P < 0.01$ compared with SCI. Con, control; SCI, spinal cord injury; MPSS, methylprednisolone treatment; RT (1), rutin (1 mg/kg)-treated; RT (10), rutin (10 mg/kg)-treated. p-Akt, phosphorylated protein kinase B.

programmed cell death by affecting the downregulation of apoptosis-associated speck-like protein containing-NOD-like receptor pyrin domain containing 3 (30). These curative effects of rutin (10 μ mol/kg) treatment were similar to those observed in the MPSS group.

MIP-2 is derived from a variety of cells, including macrophages, neutrophils and endothelial cells (31). The predominant biological function of MIP-2 is the chemotaxis and activation of neutrophils and lymphocytes for involvement in inflammatory reactions (32). The heparin-binding sites of MIP-2 can interact with endothelial cells, upregulate the expression of leukocyte adhesion molecules CD11b/CD18, and ultimately guide leukocytes through the vessel wall to reach the site of inflammation, thus being important in the occurrence and development of many inflammatory diseases (33). The present study observed that rutin reduced the expression levels of MIP-2 in SCI rats. Similarly, Chen *et al* reported that rutin is a potential protective agent for acute lung injury via downregulation in the expression of MIP-2 and activation MMP-9 (19).

MMPs are a class of highly conserved endogenous zinc-dependent proteases in natural evolution, and are widely distributed in plants, vertebrates and invertebrates (34). The predominant physiological role of MMPs is to degrade extracellular matrix components, including collagen, gelatin, elastin, fibronectin and proteoglycans, in which MMP-9 is the most important in degrading the endothelial basement membrane to

open the blood-brain barrier (35). Studies have reported that MMP-9 is associated with apoptosis (36,37). In the present study, a decrease in the activation of MMP-9 was observed in response to rutin, which occurred in a dose-dependent manner. Jang *et al* suggested that rutin improved functional outcome via reducing the level of MMP-9 in a photothrombotic focal ischemic model of rats (38), and Chen *et al* reported that rutin is a potential protective agent for acute lung injury via downregulation of activation of MMP-9 and inhibition of the expression of p-Akt (19).

As an important downstream signaling molecule for PI3K, Akt is a serine/threonine protein kinase, which is important in the proliferation, differentiation and apoptosis of cells (39). Previous studies have demonstrated that Akt is also important in the nociceptive information transfer process, and is involved in peripheral and central pain modulation at different levels, with inhibition of the PI3K-Akt signaling pathway resulting in a significant antinociceptive effect (40-42). Following peripheral nerve injury, p-Akt is predominantly distributed in the superficial dorsal horn of the spinal cord (43). The Akt signaling pathway is involved in the genesis and development of neuropathic pain, which is associated with its effect on the activation of nociceptive sensory neurons (44). In addition, studies have demonstrated that the Akt signaling pathway is involved in several regulatory processes of neural plasticity, and is important in the process of change in spinal cord dorsal horn neuronal plasticity caused by nerve damage (45,46). The

Akt signaling pathway is important role in the genesis and development of neuropathic pain. In the present study, rutin was observed to modulate the protein expression of p-Akt in the SCI rats. Hu *et al* suggested that rutin ameliorates activation of the renal NOD-like receptor 3 inflammasome by mediating Akt signaling (47). In addition, Jeong *et al* reported that rutin inhibits myocardial ischemia/reperfusion-induced apoptosis via extracellular signal-regulated kinase 1/2 and PI3K/Akt signals *in vitro* (48).

In conclusion, the predominant finding of the present study was that the mediated delivery of rutin successfully decreased neuropathic function behavior and associated protein expression levels. Rutin appeared to inhibit the expression of MIP-2 and activation of MMP-9, and reduce the protein expression of Akt in the SCI rats. Future investigations on the signaling pathways to rutin administration aim to provide further insights into its therapeutic action in terms of SCI-induced neuropathic function, and provide a starting point for developing novel strategies for pain control.

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