

Asiaticoside attenuates the effects of spinal cord injury through antioxidant and anti-inflammatory effects, and inhibition of the p38-MAPK mechanism

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Abstract. Asiaticoside has potent pharmacological activity and broader pharmacological effects, including anti-oxidant, antidepressant and hepatic protection effects, and the inhibition of tumor cell proliferation. However, the mechanism underlying the effects of asiaticoside on neurological pain in spinal cord injury (SCI) remain to be fully elucidated. Therefore, the present study investigated the specific mechanism underlying the beneficial action of asiaticoside in a SCI rat model. In the present study, Basso, Beattie and Bresnahan scores was determined to analyze the therapeutic effects of asiaticoside on the neurological function of the SCI rat model. The water content of the spinal cord was also determined to measure its effects on the SCI rats. Oxidative stress, levels of nitric oxide and inflammation were detected using commercial kits. Western blot analysis was used to measure the protein expression levels of p38-mitogen-activated protein kinase (MAPK) in the SCI rat. Asiaticoside effectively augmented the Basso, Beattie and Bresnahan scores of the SCI rats. Significant reductions in the water content of the spinal cord, the levels of inducible nitric oxide synthase (iNOS), and the activities of the nuclear factor- κ B p65 unit, tumor necrosis factor- α , interleukin(IL)-1 β and IL-6 were observed in the experimental animals. Furthermore, on examination of the oxidative stress-associated parameters, increased production of malondialdehyde and decreased levels of superoxide dismutase, glutathione and glutathione peroxidase were detected in the SCI rat model. Asiaticoside also significantly suppressed the expression of p38-MAPK, which indicated that the therapeutic effects of asiaticoside may be associated with the p38-MAPK pathway. These data confirmed that asiaticoside attenuates

SCI through antioxidant and anti-inflammatory effects, and through inhibition of the p38-MAPK mechanism.

Introduction

Spinal cord injury (SCI) is a serious complication of trauma often caused by car accidents, falls and other causes of spinal trauma, of which the symptoms include sensory and motor dysfunction below the damage plane, autonomic nerve dysfunction, difficulty in restoring nerve function following injury and high rates of morbidity (1,2). The causes of these symptoms are predominantly secondary to violent injury, however, secondary SCI includes the integrity of blood-spinal cord barrier, neutrophil inflammatory cell infiltration following primary trauma, neuronal necrosis, apoptosis and glial scar formation (3). In previous years, the incidence of the disease has exhibited a gradually increasing trend, due to a lack of effective treatment options, and surgery cannot restore lost nerve functions (4).

The pathophysiological processes of SCI include the initial primary injury and the consequent secondary injury. Secondary injury is involved in a variety of molecular mechanisms, including Ca^{2+} influx overload, excitatory amino acid toxicity and oxidative stress (5). Oxidative stress directly damages the structure and the function of nerve cells by attacking biological macromolecules in the cells, and causes death of the nerve cells, which is closely associated with neurodegenerative disease following SCI (6). Therefore, antioxidative stress is an effective strategy for SCI therapeutic intervention. Cavus *et al* reported that montelukast and methylprednisolone have a neuroprotective effect on SCI by downregulating the levels of malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH-PX) and catalase (7). Tavukçu *et al* (8) revealed that melatonin and tadalafil treatment improve erectile dysfunction following SCI through suppression of the levels of MDA, SOD and glutathione (GSH) in rats.

The induction of secondary damage following SCI can lead to tissue hemorrhage, edema and apoptosis, and immune and inflammatory grade-linking reactions are further expanded, with inflammation being important in SCI. Early relief of inflammation following SCI is involved in neural protection

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and the promotion of functional recovery. Nuclear factor (NF)- κ B, tumor necrosis factor (TNF)- α and interleukin (IL)-1 β have been reported to be significantly downregulated following plumbagin treatment in SCI rats (9). Nacar *et al* (10) revealed the beneficial effect of atorvastatin in rat SCI through anti-inflammatory effects.

The mitogen-activated protein kinase (MAPK) family is a conservative group of serine/threonine protein kinase, and comprise a major group of signaling molecules in the transduction process, which is important in the development and occurrence of disease. MAPK activation is the final step in the intracellular phosphorylation cascade (11). P38 is a phosphoric acid protein tyrosine kinase, which is purified and isolated from mammalian cells stimulated by endotoxin. P38 is the most important member of the MAPK family in the control of the inflammatory response, which may be activated due to physiological stress, lipopolysaccharides, osmotic stress or ultraviolet irradiation (11). Galan-Arriero *et al* (12) reported that oral administration of an p38 α MAPK inhibitor inhibited affective pain behavior following SCI. Qu *et al* (13) also reported that inhibition of p38-MAPK signaling reduces the microglial inflammatory response in rats following SCI.

Asiaticoside is a white needle-like crystal, which is extracted from *Centella asiatica* and has a swelling and detoxification effect, which is reported to have certain antitumor activities (14). It has been demonstrated that asiaticoside has potent pharmacological activity and broader pharmacological effects, having antioxidant, antidepressant and hepatic protective effects, and functioning in the inhibition of tumor cell proliferation. As these previous data presented only indirect evidence on the effects of asiaticoside on SCI, the present study aimed to investigate the mechanisms underlying the action of asiaticoside in neurological function using SCI model rats.

Materials and methods

Chemicals. Asiaticoside (purity >95%) was purchased from Nanjing Traditional Chinese Medicine Institute of Chinese Material Medica (Nanjing, China) and the chemical structure is indicated in Fig. 1. MPSS was purchased from the First Hospital of Jilin University (Changchun, China). MDA, SOD, GSH, GSH-PX, NF- κ B p65 unit, TNF- α , IL-1 β and IL-6 commercial immunoassay kits were purchased from Elabscience Biotechnology Co., Ltd. (Wuhan, China). The Inducible Nitric Oxide Synthase (iNOS) commercial kit was purchased from Sangon Biotech Co., Ltd. (Shanghai, China).

Animals and ethical statement. The animals used in the present study were male Sprague-Dawley rats (8-10 weeks old; 250-280 g), which were obtained from the Animal Resource Center of the First Hospital of Jilin University and approved by the ethics committee of the First Hospital of Jilin University. The rats were individually housed in Plexiglas bins with food and water continuously available, and were maintained under a controlled environment at $23 \pm 1^\circ\text{C}$ with a 12-h light/dark cycle and 60-80% humidity. All experimental procedures were approved by the guidelines of the Care and Use of Laboratory Animals of the First Hospital of Jilin University.

Experimental groups and induction of the SCI rat model. The 50 male Sprague-Dawley rats were randomly assigned into five groups. The sham group (n=10), received only physiological saline (0.1 ml/100 g, intraperitoneally). The remaining four groups underwent SCI at the T10 spinal segment impactor. The SCI group (n=10) received no treatment following SCI, the ASI (30) group (n=10) received asiaticoside at doses of 30 mg/kg once a day for 7 days following SCI (15), the ASI (60) group (n=10) received asiaticoside at a dose of 60 mg/kg once a day for 7 days following SCI, the MPSS group (n=10) received 100 mg/kg MPSS following SCI.

The rat model of SCI was induced, as previously described (16). The rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg; Sigma-Aldrich, St. Louis, MO, USA), and a laminectomy was performed at the T10 level to expose the cord beneath, without disrupting the dura. Subsequently, a rat model of spared cord injury was created by performing a laminectomy, during which the T8 and T9 vertebral peduncles were removed. The control model rats underwent the same laminectomy, but without compression.

Evaluation of neuronal function recovery. Following SCI, the evaluation of locomotor recovery was evaluated using the Basso, Beattie and Bresnahan (BBB), locomotor rating scale of 0-21, in which 0, indicated no observable hind-limb movements and 21 indicated normal locomotion (17).

Assessment of the water content of the spinal cord following SCI. At 72 h post-SCI, the spinal cord was collected, the wet weight was obtained and the spinal cord was placed in an oven at 80°C for 48 h. The dry weight of the spinal cord was then measured. The plasma supernatant was collected following centrifugation at $5,000 \times g$ for 10 min at 4°C . The nitrite concentration was spectrophotometrically determined using a CM-2600d spectrophotometer (Konica Minolta Sensing Singapore Pte Ltd., Jurong East, Singapore) and the following formula: Spinal cord water content (%) = (wet weight - dry weight) / wet weight $\times 100\%$.

Measurement of levels of MDA, SOD, GSH and GSH-PX. Following treatment with asiaticoside for seven consecutive days, the peripheral blood was collected from each group. Subsequently, the supernatant was centrifuged at $12,000 \times g$ for 20 min at 4°C . The serum levels of MDA, SOD, GSH and GSH-PX were analyzed using commercial immunoassay kits (cat. nos. E-EL-0060c, E-EL-R1424c, E-EL-R0440c and E-EL-R2491c, respectively), according to the manufacturer's instructions (Elabscience Biotechnology Co., Ltd.).

Measurement of iNOS activity. Following treatment with asiaticoside for seven consecutive days, the rats were sacrificed via cervical dislocation and spinal cord tissue was collected from each group. The activity of iNOS was determined using a commercial kit, according to the manufacturer's instructions (Sangon Biotech Co., Ltd.).

Measurement of the activities of NF- κ B p65 unit, TNF- α , IL-1 β and IL-6. Following treatment with asiaticoside for seven consecutive days, the peripheral blood was collected from

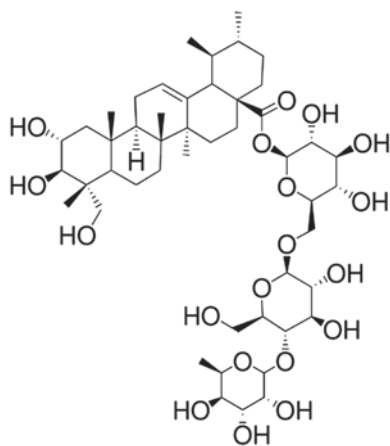


Figure 1. Chemical structure of asiaticoside.

each group. Subsequently, the supernatants were centrifuged at 12,000 x g for 20 min at 4°C. The serum activities of NF-κB p65 unit, TNF-α, IL-1β and IL-6 (cat. nos. E-EL-R0674c, E-CL-R0019c, E-EL-R0012c and E-EL-R0015c, respectively) were analyzed using the respective commercial immunoassay kits, according to the manufacturer's instructions (Elabscience Biotechnology Co., Ltd.).

Western blot analysis. Samples (~10 mg) of the exposed spinal cord tissue were removed from the rats and incubated with 100 μl ice-cold lysis buffer, containing 2 mM EDTA, 10 mM EGTA, 0.4% NaF, 20 mM Tris-HCl and protease inhibitors (pH 7.5) for 10-15 min on ice. Subsequently, the homogenates were centrifuged at 12,000 x g for 20 min at 4°C. The protein concentrations of the soluble materials were determined using a Bicinchoninic Acid protein assay (Beyotime Institute of Biotechnology, Nanjing, China). Equal quantities of protein (30 μg) were separated on 12% sodium dodecyl sulfate-polyacrylamide gels (Beyotime Institute of Biotechnology), followed by transfer onto polyvinylidene fluoride membranes (0.22 mm; EMD Millipore, Billerica, MA, USA). The membranes were blocked with phosphate-buffered saline with 5% non-fat milk to block nonspecific binding sites. Subsequently, the membranes were incubated with monoclonal mouse anti-human anti-p38-MAPK (cat. no. sc-4708 1:2,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and monoclonal anti-β-actin (cat. no. D110001; 1:500; Sangon Biotech Co., Ltd.) overnight at 4°C. The membrane was then washed three times with Tris-buffered saline with Tween 20 (Senbeijia Biotech Co., Nanjing, China) for 2 h, and then detected by incubation with anti-mouse IgG (cat. no. sc-358922; 1:1,000; Santa Cruz Biotechnology, Inc.) conjugated with horseradish peroxidase for 2 h at room temperature. The relative band intensity was determined using a gel image analysis system (Pierce Biotechnology, Inc., Rockford, IL, USA).

Statistical analysis. All the data were analyzed using SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA) and expressed as the mean ± standard deviation. Statistical analysis was performed using one-way analysis of variance, followed by Dunnett's test. P<0.05 was considered to indicate a statistically significant difference.

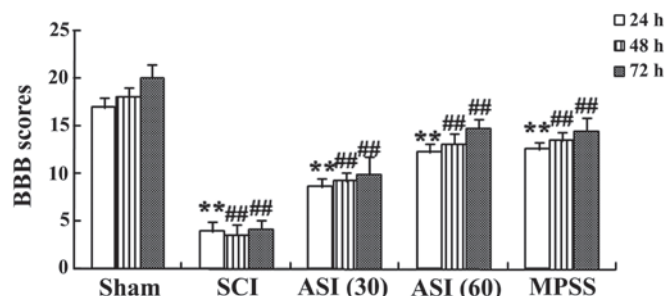


Figure 2. Effects of asiaticoside on neurological function following SCI. Data are expressed as the mean ± standard deviation. **P<0.01, compared with the control group; ##P<0.01, compared with the SCI group. Con, control; SCI, spinal cord injury; ASI (30), asiaticoside (30 mg/kg); ASI (60), asiaticoside (60 mg/kg); MPSS, methylprednisolone; BBB, Basso, Beattie and Bresnahan.

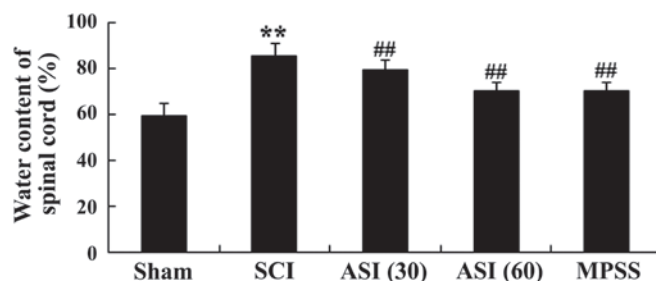


Figure 3. Effects of asiaticoside on the water content of the spinal cord following SCI. Data are expressed as the mean ± standard deviation. **P<0.01, compared with the control group; ##P<0.01, compared with the SCI group. Con, control; SCI, spinal cord injury; ASI (30), asiaticoside (30 mg/kg); ASI (60), asiaticoside (60 mg/kg); MPSS, methylprednisolone.

Results

Effects of asiaticoside on neurological function. In the present study, BBB scores were used to assess neurological function at 24 h, 48 h and 72 h following SCI, the results of which are presented in Fig. 2. The mean BBB scores of the SCI group were lower than the sham-operated group. However, it was noted that the injured rats of the asiaticoside-treated group had particularly high BBB scores. As shown in Fig. 2, the BBB scores following treatment with asiaticoside at a dose of 60 mg/kg were similar to those of the MPSS group (P>0.05).

Effects of asiaticoside on the water content of the spinal cord following SCI. The rats induced by SCI exhibited severe impairment with marked increase in water content of the spinal cord following SCI (Fig. 3). However, treatment with asiaticoside at different doses (30 and 60 mg/kg) of the injured rats significantly reduced the water content of the spinal cord, compared with the SCI model group (Fig. 3). No significant difference was observed between the MPSS group and the 60 mg/kg asiaticoside group (P>0.05).

Anti-oxidative effects of asiaticoside. It was previously reported that serum cytokine levels are relevant to SCI (18). Therefore, the serum levels of oxidant stress were determined in the present study. As shown in Fig. 4A, the MDA concentrations of the SCI group were higher than those in the sham-operated group. In the asiaticoside-treated group,

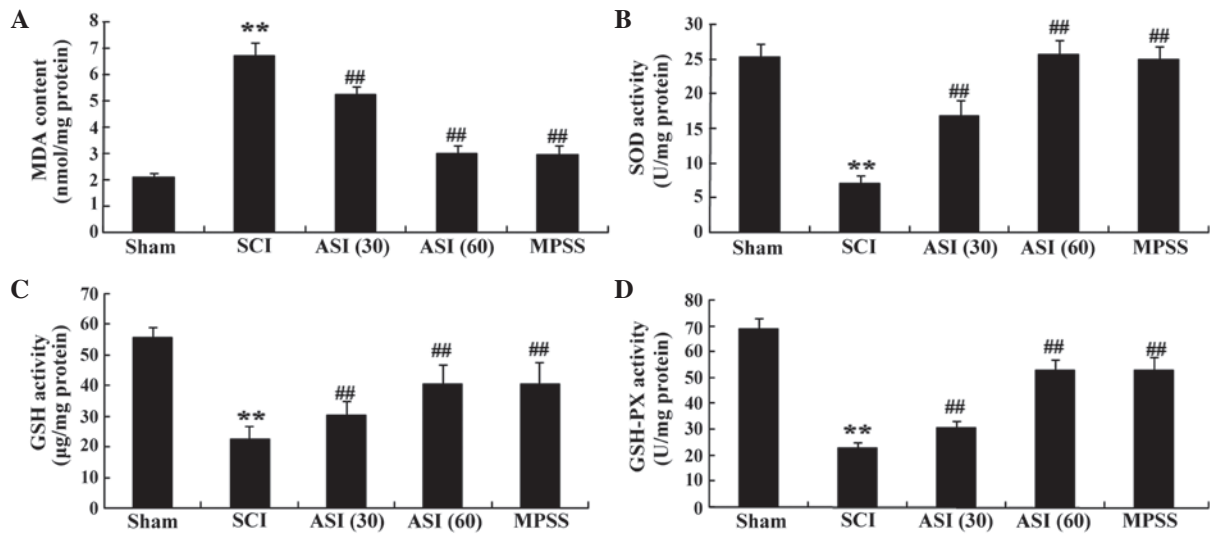


Figure 4. Anti-oxidative effects of asiaticoside following SCI. Anti-oxidative effects of asiaticoside on the concentrations of (A) MDA, (B) SOD, (C) GSH and (D) GSH-PX in the SCI model rats. Data are expressed as the mean \pm standard deviation. ** $P < 0.01$, compared with the control group; ## $P < 0.01$, compared with the SCI group. Con, control; SCI, spinal cord injury; ASI (30), asiaticoside (30 mg/kg); ASI (60), asiaticoside (60 mg/kg); MPSS, methylprednisolone; MDA, malondialdehyde; SOD, superoxide dismutase; GSH, glutathione; GSH-PX, glutathione peroxidase.

the serum levels of MDA were also lower than those in the SCI group (Fig. 4A). The levels of SOD, GSH and GSH-PX in the SCI group were lower than those of the sham-operated group (Fig. 4B-D). The expression levels of SOD, GSH and GSH-PX in the asiaticoside-treated group were gradually increased, compared with those of the SCI group. However, no significant changes in cytokine levels between the MPSS group and 60 mg/kg asiaticoside treatment group were observed (Fig. 4A-D).

Anti-oxidative effects of asiaticoside on iNOS. The present study further investigated whether asiaticoside exerted protection against SCI through the mediation of nitric oxide. As shown in Fig. 5, iNOS activity was markedly increased in the spinal cord tissues of the SCI group. However, administration with asiaticoside (30 and 60 mg/kg) generated a more pronounced reduction in iNOS activity in the SCI-induced rats (Fig. 5). In addition, the anti-oxidative effect of asiaticoside at a dose of 60 mg/kg was equipotent to that of the MPSS group (Fig. 5).

Anti-inflammatory effects of asiaticoside. The present study used ELISA commercial immunoassay kits to determine the expression levels of inflammatory factors, for assessment of the progression of the SCI. There were increases in the serum levels of the NF- κ B p65 unit, TNF- α , IL-1 β and IL-6 in the SCI group, compared with the sham group (Fig. 6A-D). However, asiaticoside treatment of the SCI-induced rats reversed these indices (Fig. 6A-D). The anti-inflammatory effect of asiaticoside (60 mg/kg) was equipotent to that in the MPSS group (Fig. 6A-D).

Astaxanthin adjusts the expression of p38-MAPK. The present study further investigated whether asiaticoside exerted protection against SCI through mediation of the expression of p38-MAPK. As shown in Fig. 7A, western blot analysis using the p38-MAPK antibody demonstrated the anticipated bands

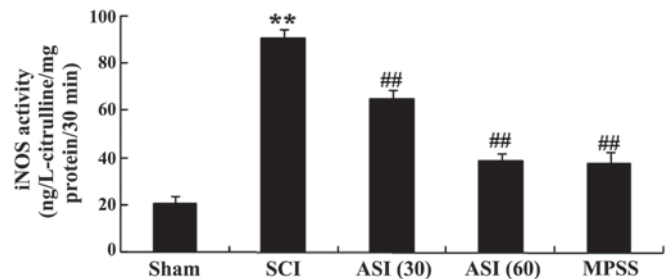


Figure 5. Anti-oxidative effects of asiaticoside on iNOS. Data are expressed as the mean \pm standard deviation. ** $P < 0.01$, compared with the control group; ## $P < 0.01$, compared with the SCI group. Con, control; SCI, spinal cord injury; ASI (30), asiaticoside (30 mg/kg); ASI (60), asiaticoside (60 mg/kg); MPSS, methylprednisolone; iNOS, inducible nitric oxide synthase.

of 43 kDa. Quantitative analysis disclosed an evident elevation of p38-MAPK protein in the SCI group, compared with the sham group (Fig. 7B). However, asiaticoside treatment (30 and 60 mg/kg) markedly decreased the protein expression of p38-MAPK, compared with the SCI group (Fig. 7A-B). No significant inter-group differences were observed between the MPSS group and asiaticoside treatment (60 mg/kg) group in the protein expression of p38-MAPK in the SCI model rat (Fig. 7A-B).

Discussion

SCI is a trauma-induced disease, the causes of which predominantly include injury from car accidents and falls from heights (18). The treatment of SCI is limited, and the majority of patients have various degrees of sensory and motor nerve dysfunction, autonomic dysfunction, a reduction or loss of self-care ability and difficulty recovering, significantly affecting quality of life and introducing a serious burden for individuals, families and society (19). In the present study, it was first demonstrated that asiaticoside increased the BBB

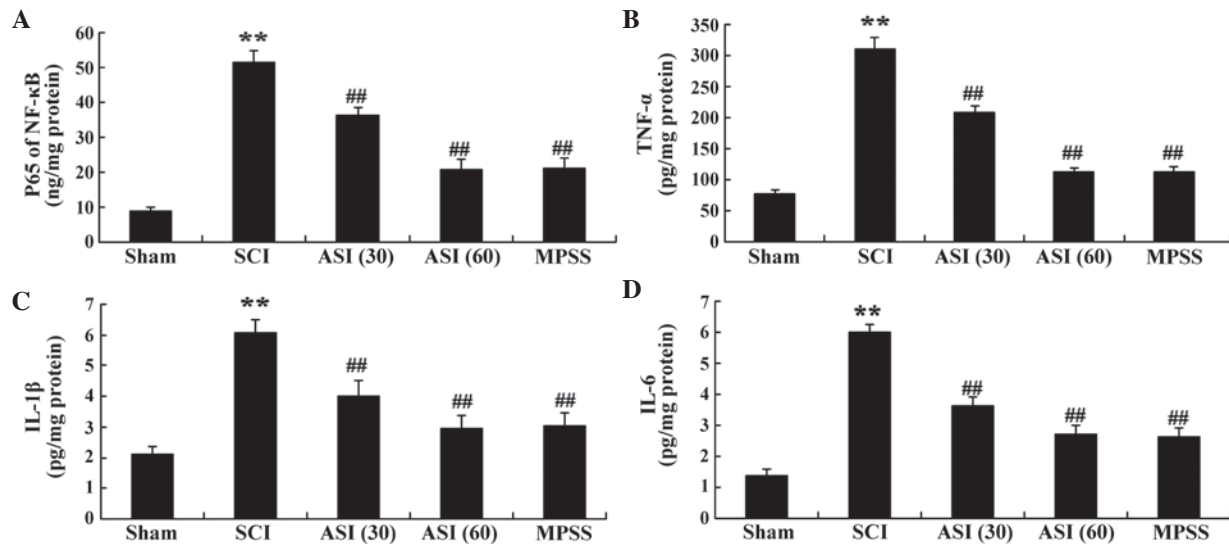


Figure 6. Anti-inflammatory effects of asiaticoside following SCI. Anti-inflammatory effects of asiaticoside on the serum activities of (A) NF-κB p65, (B) TNF-α (B), (C) IL-1β (C) and (D) IL-6 in the SCI model rats. Data are expressed as the mean ± standard deviation. **P<0.01, compared with the control group; ##P<0.01, compared with the SCI group. Con, control; SCI, spinal cord injury; ASI (30), asiaticoside (30 mg/kg); ASI (60), asiaticoside (60 mg/kg); MPSS, methylprednisolone; IL, interleukin.

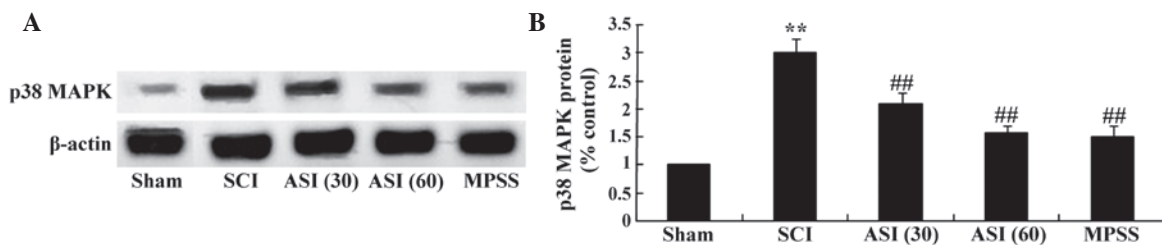


Figure 7. Astaxanthin alters the expression of the p38-MAPK pathway. (A) Effects of asiaticoside on the expression of p38-MAPK were determined using western blot analysis. (B) Statistical analysis of the protein levels of p38-MAPK in the SCI model rats. Data are expressed as the mean ± standard deviation. **P<0.01, compared with the control group; ##P<0.01, compared with the SCI group. Con, control; SCI, spinal cord injury; ASI (30), asiaticoside (30 mg/kg); ASI (60), asiaticoside (60 mg/kg); MPSS, methylprednisolone; MAPK, mitogen-activated protein kinase.

scores and reduced the water content of the spinal cord following SCI. No statistically significant differences were identified between the asiaticoside (60 mg/kg) and MPSS groups.

Currently, it is considered that a lot of oxygen free radicals (OFRs) are generated in SCI pathogenesis. OFR damage to the body may act with a trigger-like effect, with calcium overload being a final common pathway for cell damage (20). SOD is an enzyme, which catalyzes disproportionation of superoxide anions, as a major intracellular antioxidant enzyme and free radical scavenger, which can protect cells against oxygen free radicals, and the level of which indicates the strength of the effectiveness in protecting cells from toxic oxygen free radical damage (21). MDA is the end product of lipid peroxidation, and can be measured to directly reflect the level of free radicals and is an important indicator of the level of tissue injury (22). Measuring the activities of MDA and SOD can indirectly reflect the antioxidant abilities of the body (23). In the present study, it was demonstrated that asiaticoside reduced the activities of MDA and iNOS and induced the levels of SOD, GSH and GSH-PX in the SCI rats. The anti-oxidative effects of asiaticoside induce the levels of SOD, GSH and GSH-PX in healing wounds (24), and the anti-inflammatory effects of

asiaticoside dependently inhibit liver myeloperoxidase (MPO) activity and the protein expression of brain cyclooxygenase-2 (COX-2) (25). Xu *et al* (26) indicated that asiaticoside is effective in reversing 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced Parkinsonism through the levels of MDA and GSH, whereas Guo *et al* (27) indicated that the inhibitory effects of asiaticoside in gastric ulcer healing in rats via the inhibition of iNOS.

The SCI process is associated with the production and release of inflammatory mediators, microvascular endothelial function disorder, inflammatory cell infiltration and accumulation in the spinal cord tissue, and increased expression of inflammatory cytokines and adhesion molecules (16). The combined effect of these factors trigger a cascade of inflammation, thereby increasing secondary injury in the ischemic area (28). Avascular necrosis of neurons, glial cells and endothelial cells following SCI can induce the production of large quantities of inflammatory cytokines, including TNF-α, IL-1β and IL-6, which can stimulate the production of cytokines and other inflammatory mediators, affecting gene expression in glial cells (29). These cytokines can be used as a signaling molecule of endothelial cell activation, thereby stimulating the secretion of cell adhesion molecules and the adhesion between

leukocytes and endothelial cells, which induce the infiltration of leukocytes to the damage zone (30). Leukocyte infiltration damages the blood brain barrier, further aggravating SCI. Studies have demonstrated that there are several factors involved in the process of apoptosis following SCI, in which inflammatory cytokines are important. The present study demonstrated decreased activities of the NF- κ B p65 Unit, TNF- α , IL-1 β and IL-6 in SCI rats. Zhang *et al* (31) suggested that the protective effects of asiaticoside effectively protected against septic lung injury through the regulation of the iNOS, TNF- α , IL-6 and NF- κ B pathway. Bhaumik *et al* (32) reported that asiaticoside induces TNF- α to treat experimental visceral leishmaniasis via nitric oxide production.

The P38-MAPK signaling pathway is one of the three classical branches of the MAPK signaling pathway, widely involved in stress responses, including inflammation and radioactive injury. Studies have reported that activation of the p38-MAPK pathway enables the expression of downstream MAPK (MK)2 to be increased, promoting the expression of MMP-9 following SCI, leading to destruction of the blood-spinal cord barrier (33-35). The findings of the present study provide the first direct evidence, to the best of our knowledge, that asiaticoside restrained the protein expression of p38-MAPK in SCI rats. Chen *et al* (15) suggested that asiaticoside attenuates memory impairment through anti-inflammatory effects and inhibiting the overactivation of the p38-MAPK pathway. Zhang *et al* (36) reported that the protective effects of asiaticoside on acute liver injury are induced by restricting TNF- α and the p38-MAPK pathway in mice.

In conclusion, the major finding of the present study was that asiaticoside successfully decreased water content in SCI rats. Asiaticoside appeared to inhibit oxidative damage, nitric oxide activity, pro-inflammatory cytokine production and the p38-MAPK pathway. Further investigations on the signaling pathways and cross-talk consequent to asiaticoside administration may provide further insights into its therapeutic action in terms of SCI, and provide a starting point for developing novel strategies for pain control.

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