Administration of chlorogenic acid alleviates spinal cord injury via TLR4/NF-κB and p38 signaling pathway anti-inflammatory activity

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Abstract. Chlorogenic acid, as a secondary metabolite of plants, exhibits a variety of effects including free radical scavenging, antiseptic, anti-inflammatory and anti-viral, in addition to its ability to reduce blood glucose, protect the liver and act as an anti-hyperlipidemic agent and cholagogue. The present study demonstrated that administration of chlorogenic acid alleviated spinal cord injury (SCI) via anti-inflammatory activity mediated by nuclear factor (NF)-κB and p38 signaling pathways. Wistar rats were used to structure a SCI model rat to explore the effects of administration of chlorogenic acid on SCI. The Basso, Beattie and Bresnahan test was executed for assessment of neuronal functional recovery and then spinal cord tissue wet/dry weight ratio was recorded. The present study demonstrated that chlorogenic acid increased SCI-inhibition of BBB scores and decreased SCI-induction of spinal cord wet/dry weight ratio in rats. In addition, chlorogenic acid suppressed SCI-induced inflammatory activity, inducible nitric oxide synthase activity and cyclooxygenase-2 protein expression in the SCI rat. Furthermore, chlorogenic acid suppressed Toll like receptor (TLR)-4/myeloid differentiation primary response 88 (MyD88)/NF-κB/IκB signaling pathways and downregulated p38 mitogen activated protein kinase protein expression in SCI rats. The findings suggest that administration of chlorogenic acid alleviates SCI via anti-inflammatory activity mediated by TLR4/MyD88/NF-κB and p38 signaling pathways.

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Introduction

Spinal cord injury (SCI) frequently occurs as a result of traffic, falling, industrial or athletic accidents (1,2). The subsequent symptoms resulting from these injuries are associated with a high disability rate, high cost and low mortality rate. There is a high incidence rate of SCI in China. As a serious nervous system injury, the majority of SCI cases result in paralysis, pain and burden to patients and their families (3). Therefore, therapeutic intervention to aid treatment of these symptoms is of primary concern (4).

There are two primary mechanisms involved in the development of SCI, including primary mechanical injury and sequential injury. The latter was proposed in 1911 and has been accepted and acts as a foundation to current research investigations (3). The self-destruction fracture degree of sequential organization involves multiple factors and exceeds even that of primary injury mechanism (5). Prevention of sequential injury may reserve the residual functions of the surviving nerve tissue (6). In addition, it may correct microcirculation dysfunction, which is an important part of secondary injury. The mechanisms currently known to participate in sequential injury include immune inflammatory reaction, vascular mechanism, lipid peroxidation and free radical theory, theory of amino acid, calcium mediated mechanism and electrolyte imbalance and inflammatory mechanisms (2). Of these, the immune inflammatory reaction results from early microcirculation dysfunction and is important in the development of sequential injury (7).

SCI, as a severe central nervous system injury, lacks an effective therapeutic method and development of treatment for patients is of primary concern (8). Considerable research has previously been conducted regarding injury mechanism, treatment and other aspects of SCI. The injury mechanism has been elucidated however, no breakthrough progress regarding specific treatment has been obtained and therefore SCI remains a worldwide issue.

The inflammatory reaction is the primary mediator of the sequential injury in SCI (9), exhibiting a key role in the pathophysiological mechanism of the injury. SCI may trigger a series of molecular events, leading to activation of inflammatory cells in myeloid tissue resulting from circulatory

system infiltration, release a large amount of proinflammatory medium and neurotoxins, and generate oxygen free radicals and nitroso compounds which may lead to cell injury (10,11).

Chlorogenic acid is a phenylpropanoid substance synthesized during aerobic respiration in plants. The molecular formula is $C_{16}H_{18}O_9$ and the molecular weight is 345.30 g/mol (12). It is one of the primary active components of numerous Chinese herbal medicines, including *lonicera japonica*, *eucommia ulmoides* and oriental wormwood (13). It is additionally an important active ingredient of various fruits and vegetables. Chlorogenic acid has a variety of effects including free radical scavenging, antisepsis and anti-inflammation, anti-virus, reducing blood glucose, anti-hyperlipidemia, and acts to protect the liver and as a cholagogue (13). Chlorogenic acid reduces tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 production by suppressing the toll like receptor 4-mediated nuclear factor (NF)-κB signaling pathway (14,15). It has previously been demonstrated that chlorogenic acid additionally exhibits anti-cancer and anti-AIDS effects and may therefore be used as a foundation medicine to design and develop anti-cancer and anti-AIDS drugs. Furthermore, due to its actions as an anti-oxidant, chlorogenic acid may be used in the pharmaceutical industry and its actions applied in daily chemical, food and further fields (14). The present study investigated the effects of administration of chlorogenic acid on SCI and the potential underlying mechanism.

Materials and methods

Animals and experimental protocol. Female Wistar rats (n=50, weight, 250-300 g; age, 8-10 weeks) were purchased from the Center for Experimental Animals of Central South University (Changsha, China), and then maintained at 22-24˚C, under a 12 h dark/light cycle, relative humidity 40-60%, with free access to standard laboratory diet and water *ad libitum*. The use of Wistar rats was approved by the Animal Care and Use Committee of Central South University.

Models and grouping. All rats were randomly assigned into five groups (10 rats/group): Sham; SCI; Chlorogenic acid (10 mg/kg); Chlorogenic acid (30 mg/kg); Chlorogenic acid (100 mg/kg). The Wistar rats were intraperitoneally injected with ketamine (80 mg/kg) and xylazine (10 mg/kg) for anesthesia. The SCI model was established as previously described (16). Following this, the T8 and T9 vertebral peduncles of narcotized rats were removed. The same laminectomy without compression was carried out for control group. The following treatments were administered to the rats after inducing the SCI model for 24 h: Sham group, rats were gavaged with normal saline; SCI group, SCI rats were gavaged with normal saline; Chlorogenic acid groups, SCI rats were gavaged with 10, 30 or 100 mg/kg Chlorogenic acid once every three days for 3 weeks.

Assessment of neuronal functional recovery. Following administration of chlorogenic acid (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), the assessment of neuronal functional recovery was executed using the Basso, Beattie and Bresnahan (BBB) test. The BBB test indicates a locomotor rating scale of 0 (no observable hind-limb movements) to 21 (normal locomotion) (17).

Wet/dry weight ratio. Following administration of chlorogenic acid, spinal cord tissue was acquired and weighed immediately as wet weight, and then dried weight to constant weight at 80˚C for 72 h, and weighed again. Wet/dry weight ratio was calculated by dividing the wet weight by the dry weight.

Assessment of TNF-α, IL-1β and IL-6 levels and inducible nitric oxide synthase (iNOS) activity. Following administration of chlorogenic acid, spinal cord tissue was acquired and homogenized in cool phosphate-buffered saline. Then, the protein concentration was measured using a bicinchoninic acid protein assay kit (BCA; Beyotime Institute of Biotechnology, Nanjing, China). TNF-α (PT516), IL-1β (PI303) and IL-6 (IL-6) levels and iNOS activity (S0025) were measured with corresponding ELISA kits according to the manufacturer's protocol (Beyotime institute of Biotechnology) and analyzed using an ELISA reader.

Western blot analysis. Following administration of chlorogenic acid, spinal cord tissue was acquired and homogenized in cool radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology). Proteins were extracted using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology) and the concentration was measured using a BCA protein assay kit (Beyotime Institute of Biotechnology). Proteins (50 μg) were separated by 10% SDS-PAGE and electrotransferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat milk for 1 h at 37˚C and incubated overnight with primary antibodies against NF-κB (dilution, 1:1,000; cat. no. sc-7178), IκB (dilution, 1:1,000; cat. no. sc-371), phosphorylated IκB (p-IκB; dilution, 1:1,000; cat. no. sc-101713), phosphorylated p38 (p-p38; dilution, 1:1,000; cat. no. sc-101759) and β-actin (dilution, 1:800; cat. no. sc-7210), all obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), at 4˚C. Membranes were then incubated with the secondary goat anti-mouse or anti-rabbit IgG antibodies (A0208 or A0216; dilution, 1:5,000; Beyotime Institute of Biotechnology) for 1 h at 37˚C and detected by an enhanced chemiluminescent detection system (GE Healthcare Life Sciences, Little Chalfont, UK) according to the manufacturer's protocol.

Statistical analysis. All data are expressed as the mean ± standard deviation and analyzed using SPSS software, version 17.0 (SPSS Inc., Chicago, IL, USA). Data were compared between groups using a Student's t-test. One-way analysis of variance followed by Bonferroni's post hoc test were utilized to determine the significant difference among multiple groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Administration of chlorogenic acid increases BBB scores in SCI rats. The chemical structure of chlorogenic acid is presented in Fig. 1. The present study investigated the effects of administration of chlorogenic acid on neuronal function

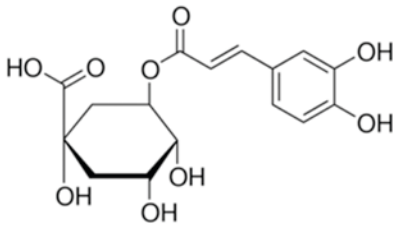

Figure 1. Chemical structure of chlorogenic acid.

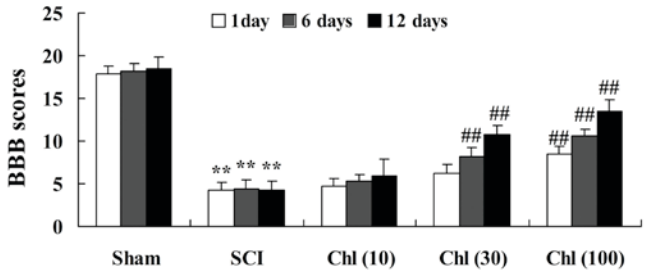

Figure 2. Administration of chlorogenic acid increases BBB scores in SCI rats. SCI rats were treated with differing doses of chlorogenic acid and neuronal functional recovery was then assessed using the BBB test. **P<0.01 vs. sham group; ##P<0.01 vs. SCI group. BBB, Basso, Beattie and Bresnahan; SCI, spinal cord injury; Chl, chlorogenic acid.

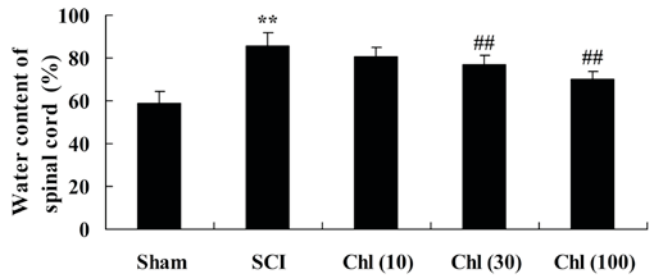

Figure 3. Administration of chlorogenic acid decreases spinal cord wet/dry weight ratio in SCI rat. SCI rats were treated with chlorogenic acid and wet/dry weight ratio of the spinal cord was then measured. **P<0.01 vs. sham group; ##P<0.01 vs. SCI group. SCI, spinal cord injury; Chl, chlorogenic acid.

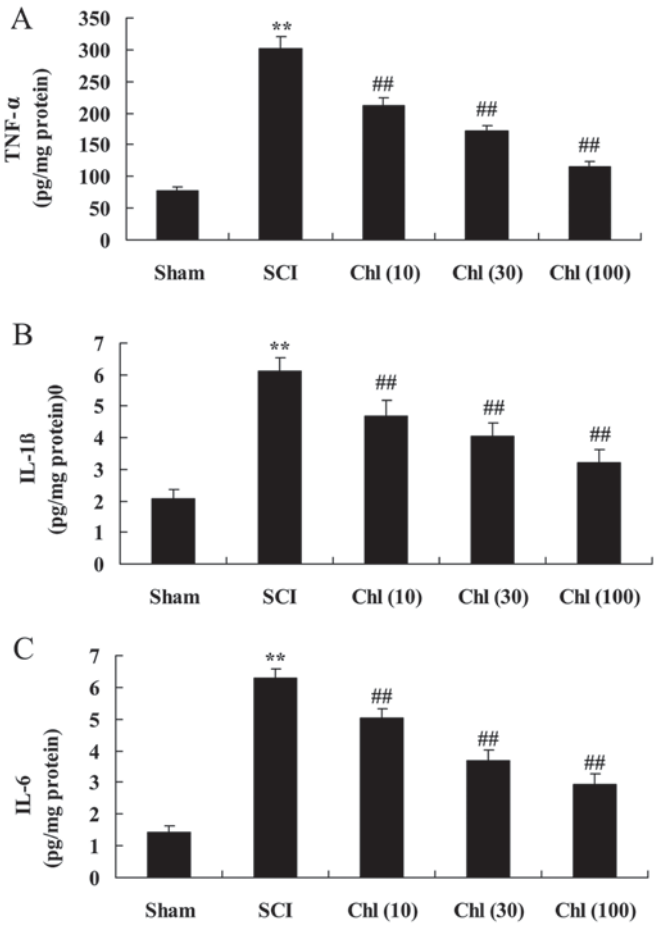

Figure. 4. Administration of chlorogenic acid suppresses inflammatory activity in SCI rat. Administration of chlorogenic acid suppressed (A) TNF-α, (B) IL-1β and (C) IL-6 levels in SCI rats. **P<0.01 vs. sham group; ##P<0.01 vs. SCI group. SCI, spinal cord injury; Chl, chlorogenic acid; IL, interleukin; TNF-α, tumor necrosis factor.

recovery, assessed using the BBB test. When compared with sham-operated group, BBB scores were significantly reduced in SCI rats (Fig. 2). However, treatment with chlorogenic acid (30 and 100 mg/kg) significantly elevated the SCI-induced inhibition of BBB scores in the rats (Fig. 2).

Administration of chlorogenic acid decreases spinal cord wet/dry weight ratio in SCI rats. It was then investigated if chlorogenic acid decreased spinal cord wet/dry weight ratio in the SCI rat. Following a single day of treatment with chlorogenic acid, SCI rats demonstrated an increased spinal cord wet/dry weight ratio compared with the sham-operated group (Fig. 3). Treatment with chlorogenic acid significantly reduced the SCI-induced increase in wet/dry weight ratio compared with the SCI group rats (Fig. 3).

Administration of chlorogenic acid suppresses inflammatory activity in SCI rat. In order to detect alteration in inflammatory activity in rats with SCI following chlorogenic acid treatment, assessment of TNF-α, IL-1β and IL-6 levels was performed. As presented in Fig. 4, TNF-α, IL-1β and IL-6 levels in SCI tissue were significantly increased compared with the sham-operated group. Furthermore, TNF-α, IL-1β and IL-6 levels of SCI rats exposed to chlorogenic acid were decreased compared with the SCI rats (Fig. 4).

Administration of chlorogenic acid suppresses iNOS activity in SCI rat. To investigate iNOS activity in SCI rats following chlorogenic acid treatment, ELISA kits were used. SCI induced increased iNOS activity in the rats compared with the sham-operated group (Fig. 5). Following treatment with chlorogenic acid, iNOS activity was significantly reduced compared with SCI rats (Fig. 5).

Administration of chlorogenic acid suppresses COX-2 protein expression in SCI rat. The present study investigated if administration of chlorogenic acid suppressed COX-2 protein expression in SCI rats. As presented in Fig. 6, COX-2 protein expression levels in the SCI model group were increased compared with control group. Treatment with chlorogenic acid significantly suppressed COX-2 protein expression in SCI rats, compared with the SCI rat model group (Fig. 6).

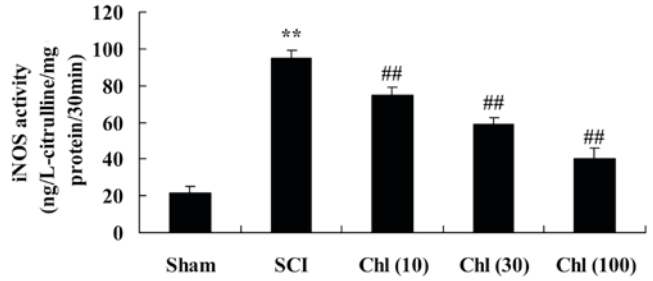

Figure. 5. Administration of chlorogenic acid suppresses iNOS activity in SCI rat. SCI rats were treated with chlorogenic acid and iNOS activity was detected. **P<0.01 vs. sham group; ##P<0.01 vs. SCI group. SCI, spinal cord injury; Chl, chlorogenic acid; iNOS, inducible nitric oxide synthase.

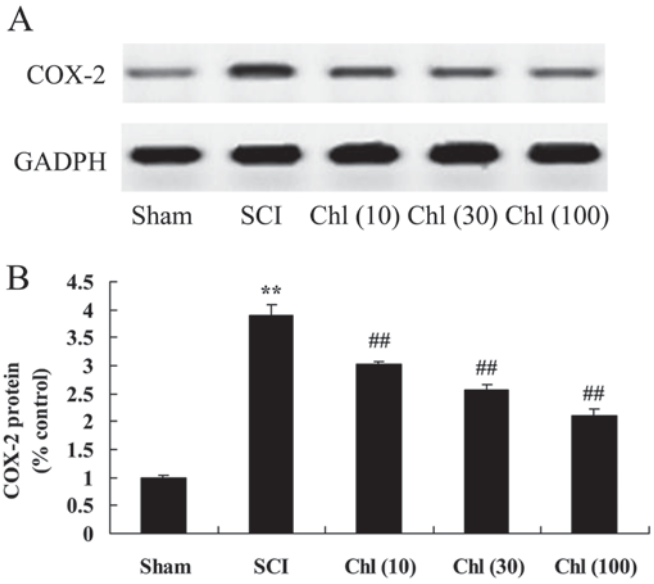

Figure 6. Administration of chlorogenic acid suppresses COX-2 protein expression in SCI rat. (A) Representative image and (B) quantitative representation of COX-2 protein expression following administration of chlorogenic acid in SCI rats, detected via western blot analysis. **P<0.01 vs. sham group; ##P<0.01 vs. SCI group. SCI, spinal cord injury; Chl, chlorogenic acid; COX-2, cyclo-oxygenase 2.

Administration of chlorogenic acid suppresses toll like receptor (TLR) 4 protein expression in SCI rat. TLR4 protein expression in the SCI rat treated with chlorogenic acid was assessed using western blot analysis. TLR4 protein expression levels in the SCI rat were significantly increased, compared with control group (Fig. 7). The increase in TLR4 protein expression was then significantly suppressed by administration of chlorogenic acid in SCI rats, compared with SCI rat model group (Fig. 7).

Administration of chlorogenic acid suppresses myeloid differentiation primary response (MyD)88 protein expression in SCI rats. The present study additionally analyzed whether chlorogenic acid suppressed MyD88 protein expression in SCI rats. There was a significant increase in MyD88 protein expression in SCI rats, compared with control group (Fig. 8). Chlorogenic acid then significantly suppressed MyD88 protein expression in SCI rats, compared with SCI rat model group (Fig. 8).

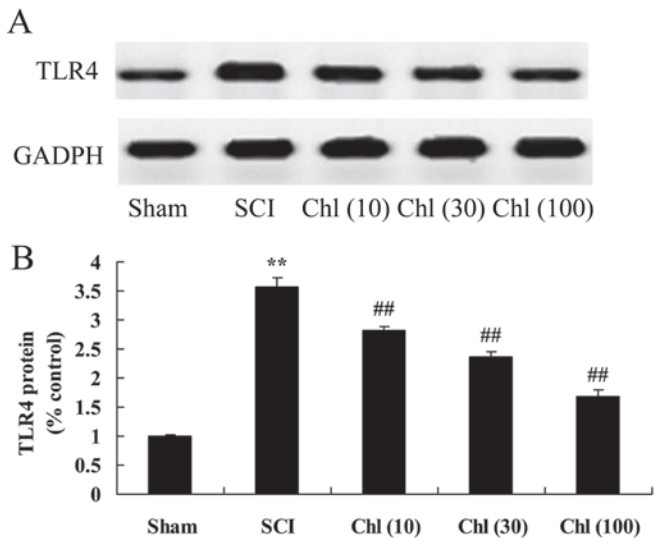

Figure 7. Administration of chlorogenic acid suppresses TLR4 protein expression in SCI rat. (A) Representative image and (B) quantitative representation of TLR4 protein expression following administration of chlorogenic acid in SCI rats, detected via western blot analysis. **P<0.01 vs. sham group; ##P<0.01 vs. SCI group. SCI, spinal cord injury; Chl, chlorogenic acid; TLR4, Toll like receptor 4.

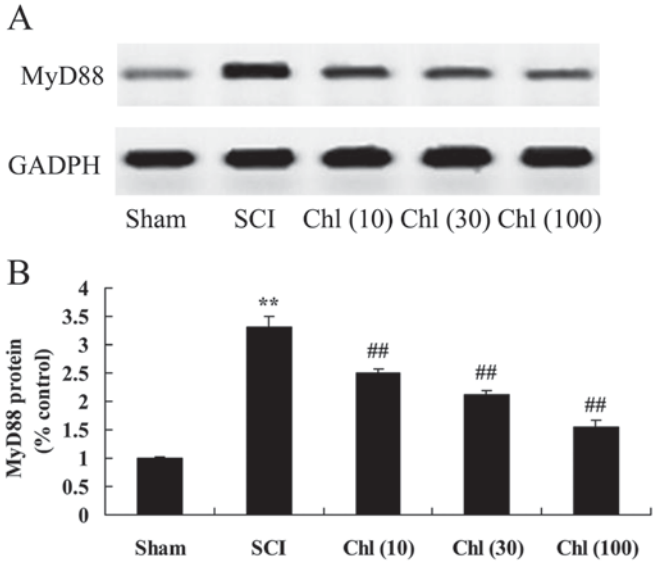

Figure 8. Administration of chlorogenic acid suppresses MyD88 protein expression in SCI rat. (A) Representative image and (B) quantitative representation of MyD88 protein expression following administration of chlorogenic acid in SCI rats, detected via western blot analysis. **P<0.01 vs. sham group; ##P<0.01 vs. SCI group. MyD88, myeloid differentiation primary response; SCI, spinal cord injury; Chl, chlorogenic acid.

Administration of chlorogenic acid suppresses nuclear factor (NF)-κB expression in SCI rat. The effect of chlorogenic acid on NF-κB protein expression in SCI rats was analyzed using western blot analysis. As presented in Fig. 9, NF-κB protein expression was increased compared with sham-operated group. Pretreatment with chlorogenic acid significantly reduced NF-κB protein expression in the SCI rat (Fig. 9).

Administration of chlorogenic acid has differing effects on IκB and p-IκB expression in SCI rat. The present study

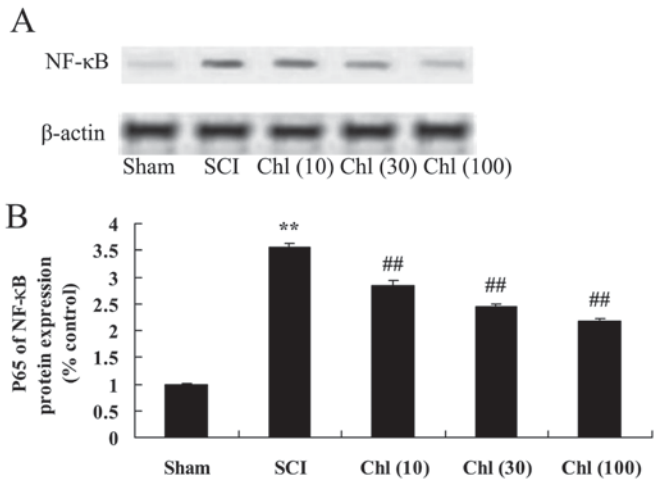

Figure 9. Administration of chlorogenic acid suppresses NF-κB expression in SCI rat. (A) Representative image of NF-κB and (B) quantitative representation of p65 of NF-κB, following administration of chlorogenic acid in SCI rats, detected via western blot analysis. **P<0.01 vs. sham group; ##P<0.01 vs. SCI group. NF-κB, nuclear factor-κB; SCI, spinal cord injury; Chl, chlorogenic acid.

investigated the *in vivo* effects of chlorogenic acid on IκB and p-IκB expression, and it was demonstrated that IκB protein expression levels in the SCI rat were decreased compared with the sham group, whereas p-IκB expression levels were increased. Administration of chlorogenic acid significantly increased activated IκB protein expression and suppressed p-IκB expression in SCI rats (Fig. 10).

Administration of chlorogenic acid suppresses p-p38 mitogen activated protein kinase (MAPK) expression in SCI rat. The present study finally investigated the effects of chlorogenic acid on p-p38 MAPK expression in SCI rat. As presented in Fig. 11, p-p38 MAPK protein expression was enhanced by SCI compared with sham-operated group. However, administration of chlorogenic acid significantly suppressed p-p38 MAPK protein expression in SCI rats (Fig. 11).

Discussion

Due to the complicated pathophysiological basis of SCI, identification of an effective therapeutic method has become one of the most difficult challenges in the medical field (18). Numerous tests and experiments have been conducted on neurofunctional deficit following SCI, which have significantly enriched understanding of the SCI pathological mechanism (19). However, there is almost no current research focused at the clinical application stage. At present, SCI treatment research primarily focuses on two aspects: To limit or reduce the sequential injury to protect nerve function, and to take measures to promote nerve regeneration (20). The data from the present study revealed that administration of chlorogenic acid increased BBB scores and decreased spinal cord wet/dry weight ratio in SCI rats.

Previously, with an increase in research regarding nitric oxide, the effect of secondary spinal cord injury has become of primary concern and research interest (21). Under the physiological status, nitric oxide maintains and organizes

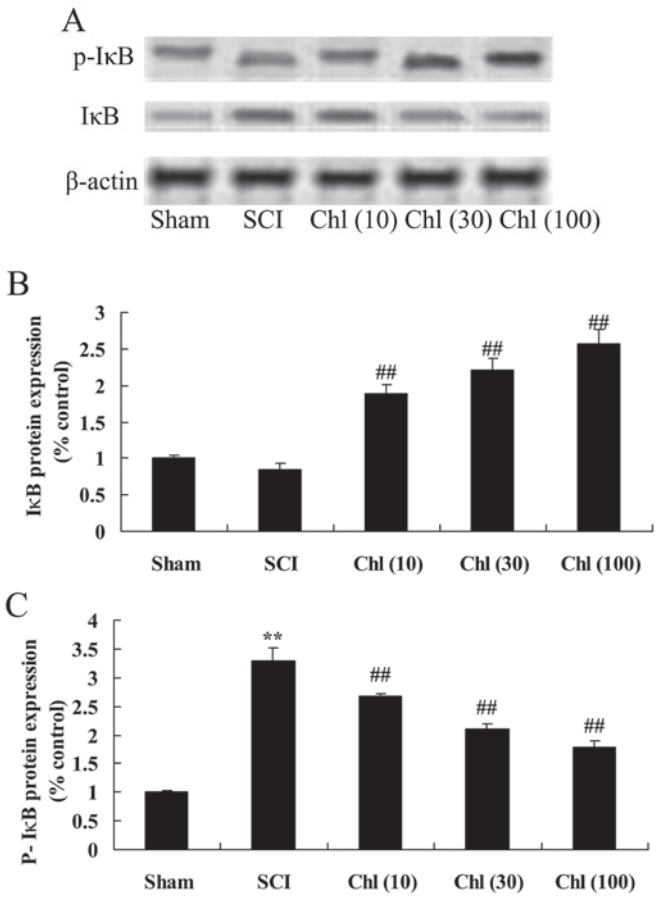

Figure 10. Effects of chlorogenic acid on p-IκB and IκB expression in SCI rat. (A) Representative image and quantitative representation of (B) IκB and (C) p-IκB expression levels following administration of chlorogenic acid in SCI rats, detected via western blot analysis. **P<0.01 vs. sham group; ##P<0.01 vs. SCI group. p, phosphorylated, SCI, spinal cord injury; Chl, chlorogenic acid.

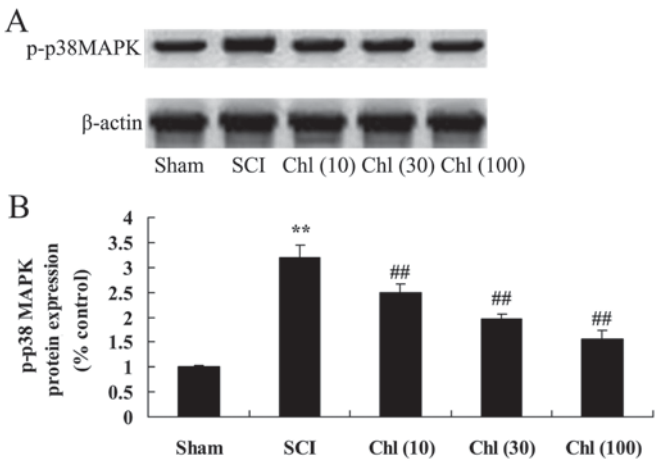

Figure 11. Administration of chlorogenic acid suppresses p-p38 MAPK expression in SCI rat. (A) Representative image and (B) quantitative representation of p-p38 MAPK expression levels following administration of chlorogenic acid in SCI rats, detected via western blot analysis. **P<0.01 vs. sham group; ##P<0.01 vs. SCI group. p, phosphorylated; MAPK, mitogen activated protein kinase; SCI, spinal cord injury; Chl, chlorogenic acid.

the blood circulation (21). Under abnormal conditions, nitric oxide may participate in the inflammatory reaction and cell

apoptosis, and neuronal apoptosis has been demonstrated to be important in the secondary injury of the spinal cord (22,23). It has previously been demonstrated that COX-2 exerts various pathological roles in the body, including a role in the inflammatory reaction, sensation of pain, cellular damage and cancer (24). However, another study suggested that COX-2 additionally affects the normal physiological functioning of the human body (24). In the central nervous system, COX-2 is expressed in glutamic-acid nerve cells in seahorses and in the human cortex, and is important in synaptic activity, long-term synaptic plasticity and nerve and blood vessel interactions during functional congestive periods. The present study demonstrated that chlorogenic acid significantly inhibited iNOS activity and COX-2 protein expression in SCI rats.

The presence of inflammatory cytokines may further induce the degree of sequential SCI, due to the fact that following injury, inflammatory cells, particularly neutrophile granulocytes, gather at the injured area (25). Neutrophils release elastase and reactive oxygen free radicals which destroy the integrity of the vascular endothelium, increase degree of tissue edema and necrosis and deteriorate neural functions. They additionally result in neuronal and oligodendrocyte apoptosis via caspase-3; stimulate astrocyte proliferation, lead to local glial scar formation, restrain the axon regeneration, upregulate expression of associated inflammatory genes and subsequently induce the inflammatory response following the SCI (26,27). Therefore, the presence of inflammatory cytokines is one of the important events at the early stages following SCI. In the present study, chlorogenic acid significantly reduced TNF-α, IL-1β and IL-6 levels of SCI rats through the TLR4/MyD88/NF-κB signaling pathway. Furthermore, Shi *et al* (28) indicates that chlorogenic acid reduces liver inflammation through the inhibition of TLR4/MyD88/NF-κB signaling pathway. Ruifeng *et al* (15) suggested that chlorogenic acid reduces TNF-α, IL-1β and IL-6 production by suppressing TLR4-mediated NF-κB signaling pathway.

The NF-κB family is the primary regulatory factor of inflammatory gene expression. It regulates the expression of numerous cytokines and regulates the inflammatory reaction in central nervous system injury (29). In central nervous system trauma, excitatory damage, ischemic damage and neurodegenerative diseases, abnormal activated NF-κB may induce neuronal apoptosis. Abnormal activation of NF-κB, co-localization staining of activated NF-κB and its target gene product iNOS have been revealed in nerve cells of SCI. A large number of genes with expressions levels regulated by NF-κB were detected in SCI, including the pro inflammatory cytokines, TNF-α, IL-1β, IL-6, iNOS and matrix metalloproteinases (MMPs) (30). It was demonstrated that chlorogenic acid significantly inhibited NF-κB protein expression in SCI rats. Chen and Wu (13) indicated that chlorogenic acid suppresses IL-1β-induced inflammation via iNOS and COX-2 in human chondrocytes.

During the activation process of NF-κB, which is regulated by IKKβ, IKKβ phosphorylates I-κBα protein, leading to ubiquitination and degradation of I-κBα protein (31). Following SCI, the protein expression of phosphorylated I-κBα in myeloid tissue increases significantly. BMS-345541 intervention may reduce >50% of the protein expression of phosphorylated I-κBα (32). These results further demonstrate that the IKKβ kinase activity in the tissue following SCI significantly increases, and that this increase may be inhibited by BMS-345541 intervention (13). In the present study, chlorogenic acid significantly suppressed p-IκB and p38 MAPK protein expression and activated IκB protein expression in SCI rats. Chen and Wu (13) indicate that chlorogenic acid suppresses IL-1β-induced rabbit chondrocytes through IκB. These results suggest that NF-κB and p38 signaling pathways are involved in the effects of chlorogenic acid on SCI.

The p38 MAPK signaling pathway is one of the classic three branches of the MAPK signal pathway, which widely participates in the inflammatory reaction, radioactive injury and stress reactions. It has previously been demonstrated that the p38 MAPK signal pathway promotes MMP-9 expression following SCI and enters the blood-spinal cord barrier. The results of the present study demonstrated that chlorogenic acid significantly suppressed p38 MAPK protein expression in SCI rats.

In conclusion, the findings demonstrated that administration of chlorogenic acid alleviated spinal cord injury via anti-inflammatory activities *in vivo*. The therapeutic effect of chlorogenic acid is associated with suppression of TLR4/MyD88/NF-κB/IκB, and down-regulation of p38 MAPK expression. Therefore, chlorogenic acid may act as a potential therapeutic candidate for the treatment of SCI through its anti-inflammatory properties mediated by the TLR4/MyD88/NF-κB and p38 MAPK signaling pathways.

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