ORIGINAL ARTICLE

Quercetin suppresses NLRP3 inflammasome activation and attenuates histopathology in a rat model of spinal cord injury

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Study design: Randomized experimental study.
Objectives: To investigate the molecular mechanisms of quercetin in spinal cord injury (SCI) rats.
Setting: China.
Methods: One hundred female Sprague-Dawley rats were randomly assigned into four groups: sham group, SCI group, SCI+Vehicle (Veh) group, and the SCI+Quercetin (Que) group. The influences of quercetin on proinflammatory cytokine levels, histological changes and locomotion scale were estimated.
Results: SCI significantly promoted nucleotide-binding domain-like receptor protein 3 (NLRP3) inflammasome activation and increased proinflammatory cytokine productions in the SCI group as compared with the sham group. Quercetin administration significantly decreased reactive oxygen species production, inhibited NLRP3 inflammasome activation and reduced inflammatory cytokine levels. Moreover, quercetin administration attenuated histopathology and promoted locomotion recovery.
Conclusion: Quercetin can attenuate tissue damage and improve neurological function recovery, and the mechanism may be related to the inhibition of NLRP3 inflammasome activation.

INTRODUCTION

Traumatic spinal cord injury (SCI) is a devastating condition that leads to a progressive state of degeneration with accompanying physiological, biochemical and structural changes.[1–3] The pathophysiology of SCI consists of two major mechanisms: primary injury and secondary injury, induced by diverse pathophysiological mechanisms, including inflammation and apoptosis.[4–6] Previous research has demonstrated that targeting the inflammatory response can improve nerve functional recovery in rat models of SCI.[7]

Neuroinflammatory responses include maturation and secretion of proinflammatory cytokines interleukin (IL)-1β and IL-18, which induce cell death.[8] The maturation and secretion of pro-IL-1β and pro-IL-18 require the activation of proteolytic enzyme caspase-1, which is mediated by the activation of nucleotide-binding domain-like receptor protein 3 (NLRP3) and subsequently the recruitment of apoptosis-associated speck-like protein (ASC).[9,10] The NLRP3 inflammasome, a kind of cytosolic protein signaling complex, consists of NLRP3, ASC and caspase-1 and is assembled after endogenous 'danger'.[11,12] The NLRP3 inflammasome can be activated by a variety of stimulating factors, including reactive oxygen species (ROS).[13] The NLRP3 inflammasome regulates the maturation and release of IL-1β and IL-18; and targeting of the NLRP3 inflammasome can exert neuroprotection in a rat model of SCI.[8]

Quercetin, a kind of flavonoid found in variety of vegetables, beverages, fruits and herbs, exhibits anti-inflammatory[14] and antioxidant[15] properties. Therefore, it has been used for many diseases,

such as focal cerebral ischemia[16] and intracerebral hemorrhage.[17] Previous studies have demonstrated that quercetin can reduce inflammatory lesions and exert neuroprotective effects in the hypothalamus of high-fructose-fed rats via suppressing NLRP3 inflammasome activation.[18] Recently, quercetin has been reported to exert neuroprotective effects by suppressing inflammation in SCI rats.[19] However, it is unclear whether the molecular mechanism of quercetin against SCI is related to the inhibition of NLRP3 inflammasome activation.

On the basis of the above considerations, we investigated whether quercetin could inhibit NLRP3 inflammasome activation with neuroprotection in a rat model of SCI.

MATERIALS AND METHODS

Animal selection and group allocation

Adult female Sprague-Dawley rats weighing 250–300 g were purchased from Beijing Haidian Thriving Experimental Animal Centre (Beijing, China). All procedures for these experiments complied with the guidelines of the Animal Ethics Committee of Hangzhou First People's Hospital (Hangzhou, China). Rats were housed in a standard animal room with a 12-h light/dark cycle.

One hundred rats were randomly assigned to four equal groups via a random number table: (1) sham group, where the rats only underwent laminectomy; (2) SCI group, where rats underwent SCI; (3) SCI+Vehicle (Veh) group, where rats were intraperitoneally injected with a 1-ml vehicle (1% dimethyl sulfoxide in 1 ml sterile saline) immediately after SCI; and (4) SCI+Quercetin (Que) group, where 100 mg kg^{-1} quercetin (Sigma-Aldrich, St Louis, MO, USA) in a 1-ml vehicle was intraperitoneally injected immediately after SCI. All animals in the SCI+Que and SCI+Veh groups were intraperitoneally injected with an

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equal volume of 100 mg kg^{-1} quercetin or vehicle at 12-h intervals for 3 days. The dose and timing of quercetin were based on previous researches.[15,17] Different set of animals were used to undertake the analyses.

Establishment of the SCI model
SCI was induced by a model described by Farsi et al.[20] Rats were anesthetized with an intraperitoneal injection of 3.0 ml kg^{-1} 10% chloral hydrate. Laminectomy was performed to expose the spinal cord at the vertebral T9–T11 segment without damage to the dura. The spinal cord at the vertebral T10 segment (spinal T9) underwent a 1-min compression with an aneurysm clip, horizontally. Rats were administered an intramuscular injection of penicillin (400 000 unit per animal per day) and buprenorphine to prevent infection and relieve pain postoperatively. In addition, rats underwent manual bladder emptying twice a day.

Evaluation of locomotor deficit
Locomotion deficit was evaluated using the Basso–Beattie–Bresnahan locomotion rating scale, as previously described.[8] The rating scale was from 0 to 21 (0 = complete paralysis, 21 = normal). The rats ($n = 5$ for each group) were assessed with this scale at days 1, 3, 7 and 14 after SCI by two independent investigators who were blinded to group assignment.

Histological study
Seventy-two hours after injury, rats ($n = 5$ in each group) were perfused with 0.9% saline and subsequently with 4% paraformaldehyde. For the histological analyses, some paraffin spinal cord sections were stained with hematoxylin–eosin reagent. Histological scoring was on the basis of (1) edema, (2) neutrophil infiltration and (3) hemorrhage. The score representing severity of SCI was recorded as follows: 0, none or minor; 1, limited; 2, intermediate; 3, prominent; and 4, widespread.[21]

Biochemical analysis
After the spinal cord samples at the damaged area (10 mm, $n = 5$ for each group) were removed 72 h after injury, they were immediately homogenized in phosphate-buffered saline and centrifuged at 1000 r.p.m. for 15 min at 4 °C. IL-1β, IL-18 and tumor necrosis factor (TNF)-α concentrations in the collected supernatants were determined through enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN, USA).

Protein extraction and western blotting analysis
The spinal cord samples at the damaged area (10 mm, $n = 5$ for each group) were removed 24 h after injury and stored at − 80 °C until further use. Specimens were homogenized in radio-immunoprecipitation assay buffer and then centrifuged at 12 000 r.p.m. for 30 min at 4 °C. Protein concentration in the supernatant was quantified via the bicinchoninic acid method. Total protein (20 μg) was separated with 10% sodium dodecyl sulfate polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 5% skimmed milk and subsequently incubated with specific primary antibodies overnight at 4 °C. Primary antibodies contained anti-NLRP3, anti-ASC, anti-caspase-1 and anti- β-actin (all 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing in phosphate-buffered saline with Tween 20, the membranes were incubated with a horseradish peroxidase-coupled secondary antibody (1:1000; Millipore). Detection of proteins was performed using the Enhanced Chemiluminescence Kit (Thermo Scientific, Rockford, IL, USA). Protein levels were analyzed via the imaging software (Quantity One; Bio-Rad Co. Ltd., Hercules, CA, USA).

Measurement of ROS production
The measurement of ROS production of spinal cord sample ($n = 5$ for each group) was performed through the oxidative fluorescent dye dihydroethidium (DHE) 72 h after SCI. Spinal cord cryosections (10 μm) were equilibrated with phosphate-buffered saline for 30 min at 37 °C and then incubated with DHE for 30 min at 37 °C. Oxidized DHE was determined by a fluorescence microscope (TE2000, Nikon, Tokyo, Japan).

Statistical analysis
Data in the study are expressed as the mean ± s.e.m. and were analyzed using the SPSS software version 16.0 (SPSS Inc., Chicago, IL, USA). Comparisons between different animal groups were performed by one-way analysis of variance and Dunnett post hoc test. A P-value of < 0.05 was considered to be statistically significant.

RESULTS

Quercetin administration promotes functional recovery
The Basso–Beattie–Bresnahan (BBB) score was used to assess the influence of quercetin on the progression of locomotion recovery after SCI in rats (Figure 1). We demonstrated that, after SCI, locomotion of all rats exhibited partial recovery after days. Furthermore, there was no significant difference between the SCI and the SCI+Veh groups with regard to locomotion recovery. In comparison with the SCI+Veh groups, rats in the SCI+Que group showed a significant improvement in functional recovery from day 3 onward (Figure 1).

### Quercetin administration reduces histopathological damage
Histopathological changes of the spinal cord were used to assay the protective effect of quercetin on rats with SCI (Figure 2). Compared with the sham group, congestion, edema, neutrophil infiltration and structural disruption were observed in the SCI group ($P < 0.001$). Furthermore, there was no significant difference between the SCI and the SCI+Veh groups with regard to histopathology. Nevertheless, the changes were inhibited significantly by quercetin administration in the SCI+Que group, as compared with the SCI+Veh group ($P < 0.01$). Histopathological scores were calculated and are shown in Figure 2.

### Quercetin administration reduces proinflammatory cytokine levels
SCI significantly increased proinflammatory cytokines IL-1β, IL-18 and TNF-α production 72 h after injury in the SCI group, as compared with the sham group (Figure 3). Furthermore, there was no significant difference between the SCI and the SCI+Veh groups with regard to IL-1β, IL-18 and TNF-α levels. However, quercetin administration significantly reduced inflammatory cytokine levels in the SCI+Que group as compared with the SCI+Veh group ($P < 0.01$; Figure 3).

Quercetin administration inhibits NLRP3 inflammasome activation
SCI induced significant upregulated protein expression of NLRP3, ASC and active-caspase-1 in the SCI group, as compared with the
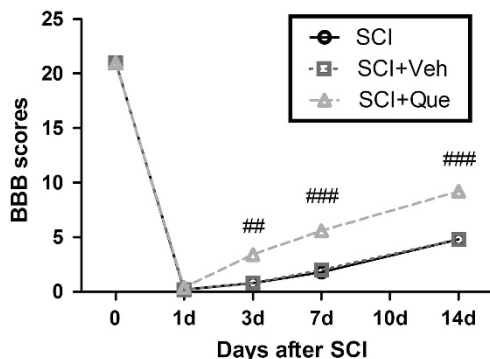
Figure 1 Time course of Basso–Beattie–Bresnahan (BBB) score after SCI, and the effect of quercetin on BBB score. ##$P < 0.01$ SCI+Que vs SCI+Veh. ###$P < 0.001$ SCI+Que vs SCI+Veh. Data represent means ± s.e.m.
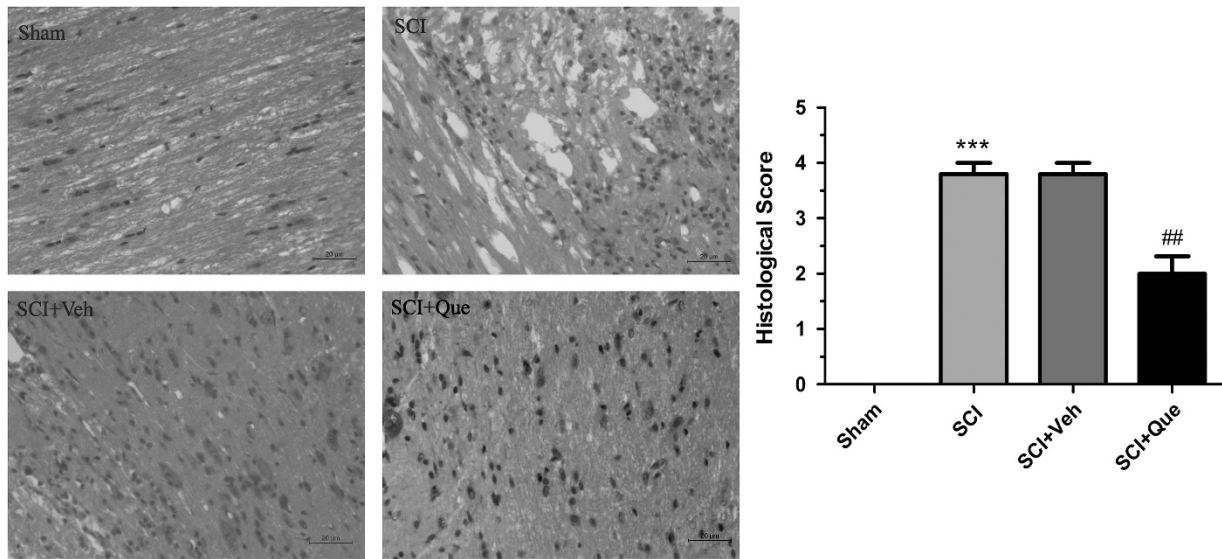
Figure 2 Effect of quercetin on histopathological change of the spinal cord tissue at 72 h after SCI. ***$P<0.001$ SCI vs Sham. ##$P<0.01$ SCI+Que vs SCI +Veh. Data represent means ± s.e.m. A full color version of this figure is available at the *Spinal Cord* journal online.
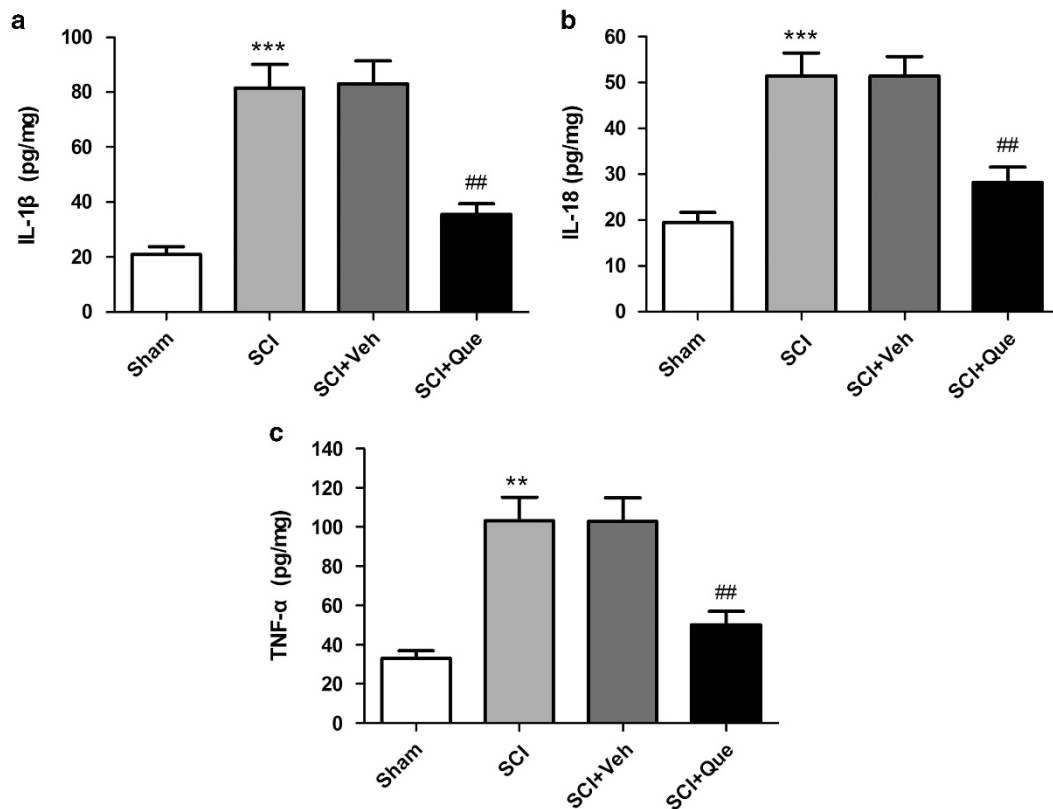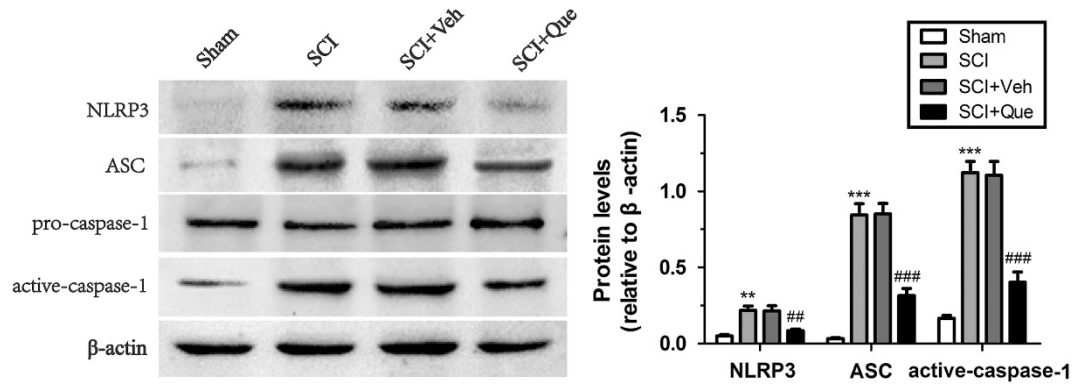

Figure 3 Effect of quercetin on proinflammatory cytokines (a) IL-1β, (b) IL-18 and (c) TNF-α protein levels in the spinal cord tissue at 72 h after SCI. **$P<0.01$ and ***$P<0.001$ SCI vs Sham and ##$P<0.01$ SCI+Que vs SCI+Veh. Data represent means ± s.e.m.

sham group (Figure 4). Furthermore, there was no significant difference between the SCI and the SCI+Veh groups in NLRP3, ASC and active-caspase-1 levels. Nevertheless, quercetin administration reduced the NLRP3, ASC and active-caspase-1 levels in the SCI+Que group as compared with the SCI+Veh group (Figure 4).

Quercetin administration reduces ROS production
ROS production was significantly increased in the SCI group compared with the sham group ($P<0.01$; Figure 5). Furthermore, there was no significant difference between the SCI group and the SCI +Veh group with regard to ROS production. ROS production was

Figure 4 Effect of quercetin on NLRP3, ASC and active-caspase-1 protein levels in the spinal cord tissue at 72 h after SCI. $**P < 0.01$ and $***P < 0.001$ SCI vs Sham, $\#\#P < 0.01$ and $\#\#\#P < 0.001$ SCI+Que vs SCI+Veh. Data represent means ± s.e.m.
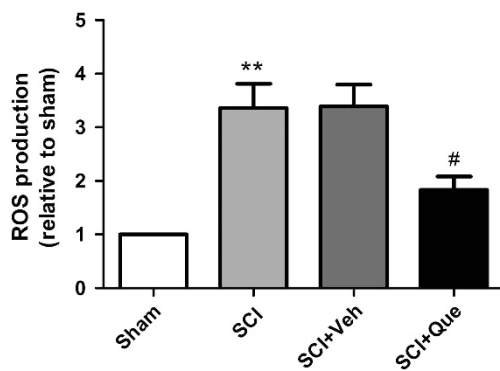
Figure 5 Effect of quercetin on ROS production in the spinal cord tissue at 72 h after SCI. $**P < 0.01$ SCI vs Sham. $\#P < 0.05$ SCI+Que vs SCI+Veh. Data represent means ± s.e.m.

markedly decreased in the SCI+Que group as compared with the SCI+Veh group ($P < 0.05$; Figure 5).

DISCUSSION

The positive effects of Chinese herbal medicine in SCI are widely recognized.[22] Quercetin, an abundant flavonoid, has been shown to promote neurological recovery in many models owing to its multiple beneficial pharmacological effects.[15,23] In the present study, we found that quercetin treatment could improve neurological recovery from day 3 onward after SCI in rats, which is in agreement with previous studies.[15] Moreover, we demonstrated that quercetin treatment reduced ROS production and inhibited NLRP3 inflammasome activation with decrease in IL-1β, IL-18 and TNF-α levels and alleviation of histopathology 72 h after SCI.

IL-1β has a key role in spinal cord damage,[24,25] which is involved in increasing other proinflammatory cytokines such as TNF-α,[26,27] enhancing vascular permeability[28] and induction of neuron apoptosis.[29] Absence of IL-1β inhibits lesion development and axonal plasticity and exerts positive effects on neurological outcome.[30] Blocking the IL-1 receptor suppresses microglial activation, promotes ventral horn neuron survival,[31] attenuates the severity of spinal cord[32] and promotes neurological recovery after SCI.[33] IL-18 is another kind of important proinflammatory cytokine after SCI.[34] Inhibition of IL-18 reduces nuclear factor-κB phosphorylation in spinal astrocytes and suppresses the induction of astroglial markers.[35] TNF-α can induce spinal cord motoneuron death[36] and exacerbate cell death after SCI.[37] Topical application of TNF-α antiserum can alleviate

edema, microvascular permeability and cell injury in rat SCI.[38] Consistent with previous studies, trauma to spinal cord causes an increase of proinflammatory cytokine levels in the spinal cord tissue.[8] Moreover, quercetin treatment markedly downregulated the protein expression of IL-1β, IL-18 and TNF-α. Furthermore, a decrease in TNF-α levels may be related to reduced IL-1β production.

Importantly, NLRP3 inflammasome is increasingly recognized as an important proinflammatory mediator regulating the maturation and release of IL-1β and IL-18.[9] Most recently, NLRP3 inflammasome is recognized to have an important role in the spinal cord tissue after SCI, and targeting the NLRP3 inflammasome can inhibit neuroinflammation, improving functional recovery in SCI rats.[8] In this study, we found that SCI caused NLRP3 inflammasome activation, which is in accordance with previous studies.[8] Moreover, quercetin administration significantly inhibited inflammasome activation, possibly via a decrease in ROS production.

ROS also has a key role in secondary oxidative stress, which contributes to spinal cord tissue damage.[15,39] A recent study has demonstrated that ROS can regulate NLRP3 inflammasome activation.[13] Inhibition of ROS production presumably suppresses inflammasome activation.[40] Another previous study showed that quercetin can increase superoxide dismutase levels, reduce malondialdehyde production and protect against SCI through regulation of secondary oxidative stress.[23] We showed that SCI induction increases ROS levels; however, quercetin obviously inhibited this process.

CONCLUSION

Quercetin can attenuate tissue damage and improve neurological function recovery, and the mechanism may be related to the inhibition of NLRP3 inflammasome activation.

DATA ARCHIVING

There were no data to deposit.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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