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ORIGINAL PAPER

# Sinomenine Attenuates Traumatic Spinal Cord Injury by Suppressing Oxidative Stress and Inflammation via Nrf2 Pathway

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## Abstract

Traumatic spinal cord injury (SCI) is a devastating condition with few efficacious drugs. Sinomenine, a bioactive alkaloid extracted from medicinal herb, has been used as a treatment of rheumatoid diseases. This present study explored the therapeutic effects of sinomenine on locomotor dysfunction and neuropathology in SCI. Our findings revealed that sinomenine mitigated neurological deficits and enhanced neuronal preservation, paralleled with a reduction of apoptosis. Also, sinomenine significantly reduced inflammatory cytokines and oxidative stress factors. We further examined erythroid-2-related factor 2 (Nrf2) nuclear translocation, which mainly controls the coordinated expression of important antioxidant and detoxification genes. An increase in Nrf2 translocation from cytoplasm to nucleus and Nrf2-mediated transactivation was observed after sinomenine administration. Knocking down Nrf2 by siRNA could counteract sinomenine-mediated anti-oxidant stress and anti-inflammation following $H_2O_2$-stimulated and LPS-stimulated PC12 cells. Together, our findings indicated that sinomenine has the potential to be an effective therapeutic agent for SCI by inhibiting inflammation and oxidative stress via Nrf2 activation.

**Keywords** Sinomenine · Spinal cord injury · Oxidative stress · Inflammation · Nrf2

## Introduction

Traumatic spinal cord injury (SCI) is a disastrous central nervous system (CNS) disorder, which inflicts severe health costs associated with permanent disabilities [1]. It is generally recognized that SCI-induced neurological deficits is not simply a consequence of the initial damage to the parenchyma, but is also attributed to a complex cascade of molecular events that contribute to early as well as delayed cell injury [2]. These secondary events occurring after the primary injury increase the complexity of the disease, including ischemia, focal hemorrhage, free radical stress and inflammatory responses, which hinder SCI recovery [3]. Neuron death resulted by the primary injury could not be reversed. Therefore, most of the attention has been paid on therapeutic interventions for the secondary injury. Although, the molecular mechanisms of the secondary damage remain poorly understood, oxidative stress and inflammation are known to play an crucial role in cell death and lesion expansion [4, 5]. Recent studies in SCI revealed that attenuating inflammation and oxidative stress could restrict the extent of tissue injury and the consequent disability [3, 6, 7].

Sinomenine (7,8-Didehydro-4-hydroxy-3, 7-dimethoxy-17-methylmorphinan-6-one) is an active alkaloid isolated from the stem and root of transitional medicinal herb *sinomenium acutum*, which has been effectively used in the clinical treatment of nephritis and rheumatoid arthritis due to its strong anti-inflammatory responses and immunomodulatory capabilities [8]. And sinomenine is found to play a protective role against various autoimmune and inflammation-associated diseases [9]. Meanwhile, the anti-oxidative stress properties of sinomenine has also been reported [10,

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11]. Recently, sinomenine was demonstrated to exert neuroprotection in several CNS disease models, including cerebral ischemia, intracerebral hemorrhage and neurodegenerative diseases [12–14]. However, few studies have conducted to address exact effects of sinomenine and underlying mechanisms on SCI.

Nuclear factor erythroid 2-related factor 2 (Nrf2), a redox-sensitive, ligand-activated transcription factor, is a pleiotropic regulator of cell survival mechanisms [15]. Nrf2 not only plays a pivotal role in modulating cellular defense against oxidative stress, it has been also found to ameliorate inflammatory responses [16]. Under normal physiological conditions, Nrf2 repressor Kelch-like ECH-associated protein 1 (Keap1) retains Nrf2 in the cytosol and facilitates its proteasomal degradation, to avoid its activation. Once cells are exposed to oxidative stress or other noxious attacks, Nrf2 dissociates from Keap1 and translocates into the nucleus [17]. Next it mediates the activations of an array of cytoprotective and antioxidant genes via binding to the antioxidant response element. Then, a battery of endogenous enzymes, such as heme oxygenase 1 (HO-1) and NAD(P)H quinone-eoxidoreductase 1 (NQO1), are produced to provide protection by regulating and maintaining intracellular redox states [18].

Based on the above observation, we investigated whether sinomenine would be beneficial in traumatic SCI and the latent mechanisms in vivo and in vitro. The influence of sinomenine on SCI-induced locomotor function deficits, tissue structure integrity, vascular permeability and cellular apoptosis were determined. Then, its properties of antioxidant stress and anti-inflammation were also determined. The potential role of the Nrf2 signaling pathway in treatment with sinomenine against SCI was further investigated. The findings of this present study could provide support for the medical use of sinomenine in treating traumatic SCI.

## Materials and Methods

### Reagents and Antibodies

Sinomenine was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were obtained from Invitrogen (Carlsbad, CA, USA). Anti-Bax (#2772), anti-cleaved Caspase 3 (#9662), anti-Histone 3 (#4499), anti-β-actin (#3700), goat anti-rabbit and anti-mouse IgGHRP were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-Bcl-2 (ab196495), anti-Nrf2 (ab137550), anti-HO-1 (ab13243) and anti-NQO1 (ab28947) were purchased from Abcam, (Cambridge, MA, USA). The chemiluminescence (ECL) kit was purchased from Bio-Rad (Hercules, CA, USA). All of the other reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless specified otherwise.

### Spinal Cord Injury and Drug Administration

Adult female Sprague–Dawley rats (220–250 g) were purchased from the Animal Center of the Chinese Academy of Sciences in Shanghai, China. The protocol for animal care and use conformed to the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health and was approved by the Animal Care and Use Committee of Wenzhou Medical University. After sodium pentobarbital (65 mg/kg intraperitoneal injection) anesthesia, animals were put on a surgery platform. A laminectomy was performed at the T9 level and the exposed dorsal surface of the spinal cord was subjected to a contusion injury as described previously [19]. For the sham-surgery controls, the rats underwent a T9 laminectomy without contusion injury. Postoperative care included manual urinary bladder emptying twice daily and the treatment of cefazolin sodium (50 mg/kg, i.p.). And all rats were housed in standard temperature conditions with a 12 h light/dark cycle and regularly fed with food and water. After operation sinomenine at 40 mg/kg was injected intraperitoneally daily until the rats were sacrificed. The final animal numbers for this research were: n = 49 (Sham), n = 49 (SCI), n = 49 (SCI/Sino).

### Cell Culture

The PC12 cells were obtained from Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Cells were seeded on sterile poly-L-lysine-coated culture plate. Cells were cultured in high glucose-containing DMEM with 10% fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin at 37 °C in a humidified atmosphere of 5% $CO_2$.

### Cell Counting Kit-8 (CCK-8) Assay

For the cell proliferation assays, PC12 cells were cultured in 96-well plates at $1 \times 10^4$ cells per well with growth culture medium with six replicate wells. After treatment, the CCK-8 reagent was diluted ten-fold with DMEM before being added (100 µl) to each well. Three hours later, the absorbance of each well at 450 nm was then measured by a microplate reader scanning.

### Nrf2 Small RNA Interference

The siRNA was used to knock down Nrf2. The siRNA oligonucleotides targeting Nrf2 was obtained from Santa Cruz Biotechnology (sc-156128). PC12 cells were transfected

with 25 nM of Nrf2 or scrambled-siRNA oligonucleotides using Lipofectamine RNAiMAX according to the manufacturer's instructions. Knockdown efficiency was examined by Western blot.

### Behavioral Tests

Behavioral analyses were conducted by two investigators who were blind as to the treatment at the indicated time points. Briefly, the Basso, Beattie, and Bresnahan (BBB) scores range from 0 points (complete paralysis) to 21 points (normal locomotion). The scale was based on the natural progression of locomotion recovery in rats after thoracic SCI. Inclined plane test was applied to evaluate the ability of rats to maintain their position for 5 s on an inclined plane. The maximum angle at which a rat could maintain its position for 5 s was recorded for each position and averaged to obtain a single score for each rat.

### Hematoxylin-Eosin (HE) Staining

One week after surgery, Spinal cord of each group containing the lesion (1 cm on each side of the lesion) was paraffin embedded. Transverse sections (5 μm thickness) of injured spinal cord (the animal number of each group was 5) were collected for histopathological examination by HE staining. Three sections at 5 mm from the lesion site were observed. The measurements were reported as the percent of preserved area in relation to the total area of each section analyzed by Image J software.

### Cell Counting of Ventral Motor Neurons

We measured the number of ventral motor neurons (VMN) by Nissl staining at 1 week after injury, as previously described [20]. Transverse sections (5 μm thick) of injured spinal cord (the animal number of each group was 5) were collected. Three sections were stained with Nissl staining reagent at each rostral, caudal 5 mm and the lesion site. Cells were counted as a VMN, which were located in the lower ventral horn and larger than half of the sampling square ($20 \times 20$ μm).

### Evan's Blue Extravasation

At day 3 after surgery, Evan's Blue dye (2 ml/kg) was intravenously administered. Two hours after injection, the injured spinal cord tissues of Evan's Blue were weighed and immersed in N,N′-dimethylformamide at 50 °C for 72 h. The optical density of the supernatant was investigated by enzyme-labelled meter. According to a standard curve, Evan's Blue was determined as μg/g of tissue. Spinal cords were fixed by 4% paraformaldehyde, and cut into 20 μm thickness using frozensection machine. The fluorescence (excitation at 493 nm and emission at 517 nm) was then measured.

### Edema Measurement

The degree of edema was assessed at the times indicated after injury by the wet weight/dry weight method. Briefly, rats were anesthetized, and the spinal cord was removed rapidly. The spinal cord was cut into 10 mm segments, and the wet weight was measured. The dry weight of spinal cord was obtained after drying at 100 °C for 48 h. The percentage of water content was calculated as: (wet weight − dry weight)/wet weight × 100.

### Real-Time Polymerase Chain Reaction

Total RNA was extracted by Trizol reagent (Invitrogen, USA) according to manufacturer's protocol. RNA was reverse-transcribed into cDNA, and real-time RT-PCR was conducted using SYBR Green Supermix (QPK-212, Tokyo, Japan) and a Light Cycler480 system (Roche, USA). RT-PCR was performed with the following specific primers: rat TNF-α: forward primer, 5′-AAATGGGCTCCCTCTATC AGTTC-3′ and reverse primer, 5′-TCTGCTTG GTGGTT TGCTACGAC-3′; rat IL-1β: forward primer, 5′-CACCTC TCAAGCAGAGCACAG-3′ and reverse primer, 5′-GGG TTCCATGGTGAAGTCAAC-3′; rat IL-6: forward primer, 5′-AA GAAAGACAAAGCCAGAGTC-3′ and reverse primer, 5′-CACAAACTGATATGCTTAGGC-3′; β-actin forward: 5′-AAGATCCTGACCGAGCGTGGC-3′, reverse: 5′-CAGCACTGTGTTGGC ATAGAGG-3′. The relative expression levels were analyzed using the $2^{-\Delta\Delta C_t}$ method.

### Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of cytokines (TNF-α, IL-1β and IL-6) in the spinal cord were assayed using cytokine ELISA kits specific for rats according to the manufacturers' instructions (TNF-α, SEA133Ra; IL-1β, SEA563Ra; IL-6, SEA079Ra; USCN Life Science, Wuhan, China). The cytokine contents are given in pg/mg protein.

### Measurement of Reactive Oxygen Species (ROS)

To detect the ROS, homogenates of spinal cord were diluted 1:10 in HEPES-Tyrode solution to obtain a concentration of 5 mg tissue/ml. 5 μl 2′,7′-dichlorodihydrofluorescein diacetate were added to 0.45 ml homogenates in 24-well plate for 30 min at 37 °C. The oxidation of the nonfluorescent compound by ROS results in the formation of the fluorescent compound 2′,7′-dichlorofluorescein which was detected by

SpectraMax M5e multi-mode microplate reader. The data were expressed as relative values of the sham group.

### Malondialdehyde (MDA) Content and Superoxide Dismutase (SOD) Activity

Samples were homogenized in phosphate-buffer saline and centrifuged at 12,000 rpm for 10 min at 4 °C to remove cell debris. For cells, after treatment, PC12 cells were lysed to get cell lysates. After centrifugation, liquid supernatant from cell lysates was collect to assess MDA content and SOD activity following the manufacturer's instructions (Beyotime, Nanjing, China). The results for MDA and SOD were expressed as the percentage of normal control.

### Western Blot

The Western blot was processed as previously described [20]. Briefly, a spinal cord segment (0.5 cm length) at the contusion epicenter was dissected and immediately stored at − 80 °C. Frozen tissues or cells were homogenized in ice-cold lysis buffer. The tissue homogenates were centrifuged at 12,000 rpm, for 20 min at 4 °C. For the measurement of Nrf2, nuclear and cytoplasmic proteins were extracted according to the instructions of the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Biotech, Nantong, China). Protein samples were loaded onto SDS–PAGE and transferred to PVDF membrane. After blocking with 5% non-fat milk, the membrane was incubated overnight at 4 °C with primary antibody solutions (Anti-Bcl-2, 1:500; anti-Bax, 1:1000; anti-cleaved Caspase 3 1:1000; anti-Histone 3 1:2000; anti-β-actin 1:3000; anti-Nrf2 1:500; anti-HO-1 1:500; anti-NQO1 1:500). Then, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature, and bands were detected using an enhanced ECL kit. Signals were visualized using the ChemiDicTM XRS + Imaging System (Bio-Rad). Densitometric quantification of the membranes was performed using Image J.

### Immunofluorescence Staining

The immunostaining was conducted as previously described [20]. The sections were probed with the primary antibodies against NeuN and cleaved Caspase 3 at 4 °C overnight (anti-cleaved Caspase 3, 1:200), followed by incubation with the corresponding fluorescent-labeled secondary antibodies. Finally, the sections were mounted with 4′,6′-diamidino-2-phenylindole (DAPI). All images were captured on a Nikon ECLIPSE Ti microscope (Nikon, Japan).

### Statistical Analysis

Data are presented as the mean ± standard error of the mean (SEM) from three independent experiments. Statistical significance between two experimental groups was analyzed using Student's t -test. When more than two groups were compared, statistical evaluation of the data was performed using one-way analysis of variance and Dunnett's post hoc test. $P < 0.05$ was considered to indicate statistical significance.

## Results

### Sinomenine Reduced Neurological Deficits After SCI

To investigate the therapeutic effect of sinomenine on locomotor recovery after SCI, behavioral testing was then evaluated for 4 weeks after injury using BBB scores and inclined plane test. At day 7 after insult, there was no significant difference between the SCI and the sinomenine administration groups (Fig. 1a). However, at day 14 after contusion, the BBB scores of rats in sinomenine group increased significantly compared to the untreated group. And such an

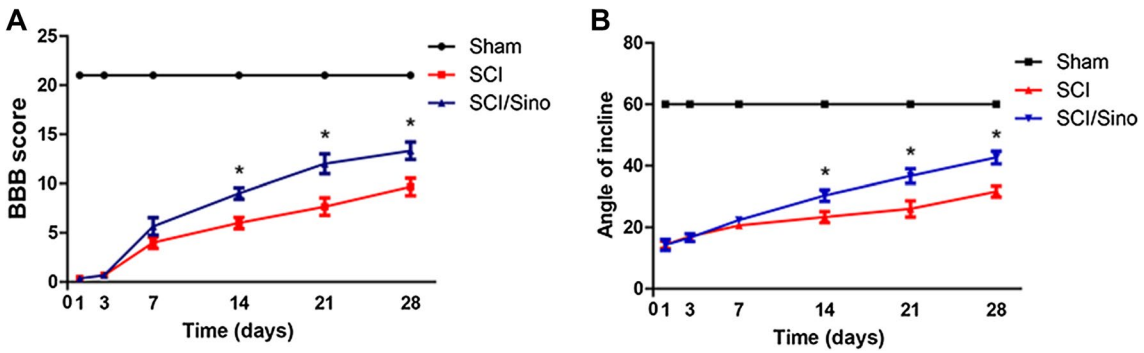


**Fig. 1** Sinomenine reduced neurological deficits after SCI. **a** The BBB scores, *$P < 0.05$ versus the SCI group, n = 9. **b** The inclined plane test scores, *$P < 0.05$ versus the SCI group, n = 9

increase lasted for up to 28 days when animals were sacrificed. Similarly, the inclined plane test scores were consistently higher in sinomenine group than that in the untreated group at day 14, 21, and 28 after injury (Fig. 1b).

## Sinomenine Decreased the Damage of Tissue Structure and Alleviated Tissue Oedema After SCI

To confirm the neuroprotective propriety of sinomenine at the histological level, HE staining and Nissl staining were analyzed at day 7 post-surgery. Results of HE staining revealed that compared with the untreated group, sinomenine decreased cavity involving the dorsal white matter and central gray matter (Fig. 2a). Quantitative results showed that sinomenine treated rats presented a higher percentage of preserved tissue in the rostral and caudal regions of the injury than untreated rats (Fig. 2b). To examine the effect of sinomenine on the loss of motor neurons in the ventral horn, Nissl staining and counting neurons were utilized. As the results shown, a massive loss of ventral motor neurons (MNV) was observed at and around the epicenter of lesion area; however sinomenine alleviated the loss of VMN around the epicenter of lesion area (Fig. 1c, d). Evan's Blue tracer is commonly used to evaluate the vascular permeability. Compared with sham group, the amount of Evan's Blue dye extravasation had a significant increase after SCI, which indicates vascular permeability disruption (Fig. 2e). While sinomenine administration reduced the amount of Evan's Blue dye extravasation at 3 days after SCI as compared to SCI group. Qualitative analysis of Evan's Blue also showed the same results (Fig. 2f). Furthermore, we also examined the magnitude of spinal cord water content after insult. The water content of spinal cord tissue significantly increased in the SCI group compared to the sham group at 12 h, 24 h, 28 h, 72 h post-injury (Fig. 2g). However, SCI-induced spinal cord edema was blunted by sinomenine administration. All of these results suggested that the damage of tissue structure and the vascular breakdown were attenuated under the treatment of sinomenine in SCI rats.

## Sinomenine Inhibited Apoptosis Caused by SCI

To explore the effects of sinomenine on modulating cellular apoptosis, the apoptosis related protein were measured by Western blot at 3 days after injury. As the results showed, after SCI the pro-apoptotic protein Bax was increased, while anti-apoptotic protein Bcl-2 was decreased, and the rate of Bax/Bcl-2 was increased. This trend was reversed by sinomenine treatment (Fig. 3a, b). Similarly, Western blot analysis of cleaved Caspase 3 indicated that the SCI-mediated increasing level of cleaved Caspase 3 was reversed by sinomenine (Fig. 3a, c). To reconfirm the level of apoptosis in neurons, cleaved Caspase 3 was detected by double staining for NeuN (red)/cleaved Caspase 3 (green). The results of fluorescence proved that following SCI, cleaved Caspase 3 was accumulated in neurons, and this trend was reversed by sinomenine treatment. (Fig. 3d).

## Sinomenine Attenuated the Production of Pro-inflammatory Cytokines in the Spinal Cord After SCI

To investigate the effects of sinomenine on inflammatory, mRNA expression and production of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α were detected by real-time PCR and ELISA. As our results showed, compared with uninjured rats, rats in SCI group had a higher mRNA expression level of IL-1β, IL-6 and TNF-α (Fig. 4a–c). While, the transcription of them was significantly attenuated by sinomenine. To reconfirmed the effect of sinomenine on the production of pro-inflammatory cytokines, we also detected them by ELISA. Similar to real-time PCR, ELISA analysis proved that sinomenine attenuated these pro-inflammatory cytokines production after SCI (Fig. 4d–f).

## Sinomenine Ameliorated Oxidative Stress Caused by SCI

We also investigated its abilities on SCI-induced oxidative stress. The effect of sinomenine on ROS generation was first tested. We found sinomenine attenuated the SCI-induced ROS production (Fig. 5a). The lipid peroxidation and antioxidant enzyme activity, including MDA and SOD, were also detected. We found the SCI led to an increase in MDA (Fig. 5b). However, sinomenine attenuated the increase in MDA. Activity of SOD, which is antioxidant enzyme and responsible for scavenging metabolites generated by free radicals, was lower after SCI than sham group, whereas this trend was reversed by sinomenine (Fig. 5c).

## Sinomenine Up-regulated Nrf2 Translocation from Cytoplasm to Nucleus and Enhanced Nrf2-Regulated Transactivation Following SCI

To explore the potential mechanisms of sinomenine, Nrf2 was detected by Western blot. Firstly, we extracted the whole spinal cord protein from each group. Our results revealed that the total cellular content of Nrf2 tended to increase by sinomenine administration, but this trend did not reach significance (Fig. 6a, b). To confirm whether sinomenine affected Nrf2 translocation, then we determined Nrf2 in the cytoplasmic and nuclear extracts separately. In sharp contrast to the lack of marked change in the whole tissue extracts, the cytoplasmic Nrf2 was decreased significantly by sinomenine treatment (Fig. 6c, d). And the nuclear Nrf2 increased after treatment with sinomenine

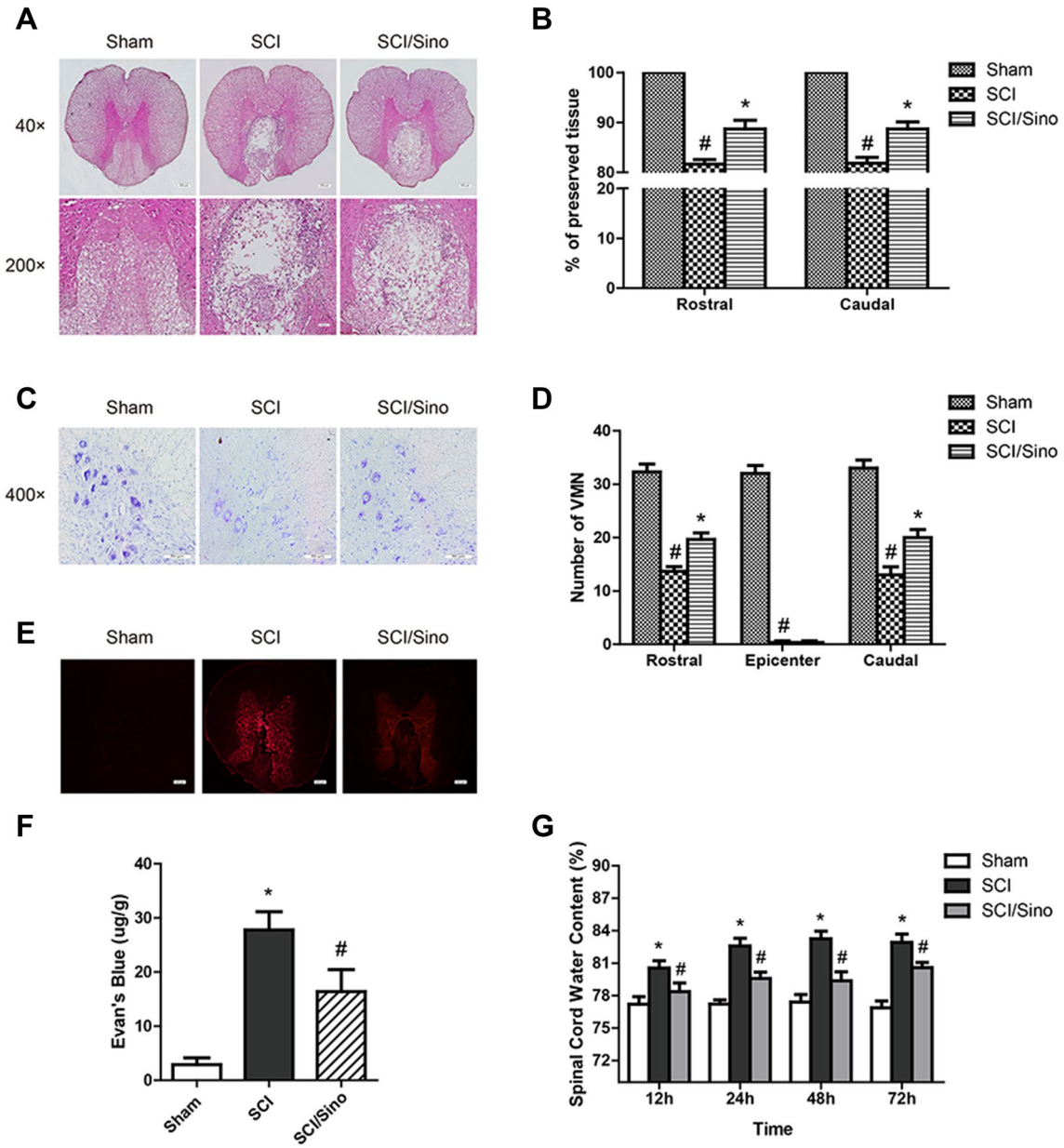


**Fig. 2** Sinomenine decreases the damage of tissue structure and alleviated tissue oedema after SCI. **a** HE staining at 7 days. Scale bars are 200 μm (×40) and 50 μm (×200). **b** Graphic presentation of the percent of preserved tissue in relation to the transverse area of the spinal cord on the seventh postoperative day. **c** Nissl staining to assess the loss of neurons at 7 days. Scale bars are 20 μm. **d** Counting analysis of VMN at rostral 5 mm, caudal 5 mm and lesion site. **e** Representative fluorescent images of Evans Blue Dye extravasation at 3 days. **f** Quantification of the amount of Evan's Blue at 3 days (μg/g). **g** Effect of sinomenine on the water content in the injured spinal cord areas at the times indicated after SCI. Columns represent mean ± SEM, *P < 0.05 versus the sham group, n = 5, #P < 0.05 versus the SCI group, n = 5

(Fig. 6c, d). All of these findings certified that sinomenine promoted the translocation of Nrf2 protein from cytoplasm to nucleus. To further explore Nrf2 activation mechanisms, we examined the effect of sinomenine on Keap1. As our results showed, Keap1 was decreased by sinomenine (Fig. 6e, f). As sinomenine increased Nrf2 translocation from cytoplasm to nucleus, then we measured the amount of HO-1 and NQO1, which are Nrf2-regulated anti-oxidation proteins. We found sinomenine enhanced the levels of NQO1 and HO-1 compared to untreated group (Fig. 6g–i). All of these results suggested that sinomenine promoted Nrf2 nuclear translocation and up-regulated the expression of Nrf2 downstream anti-oxidation proteins.

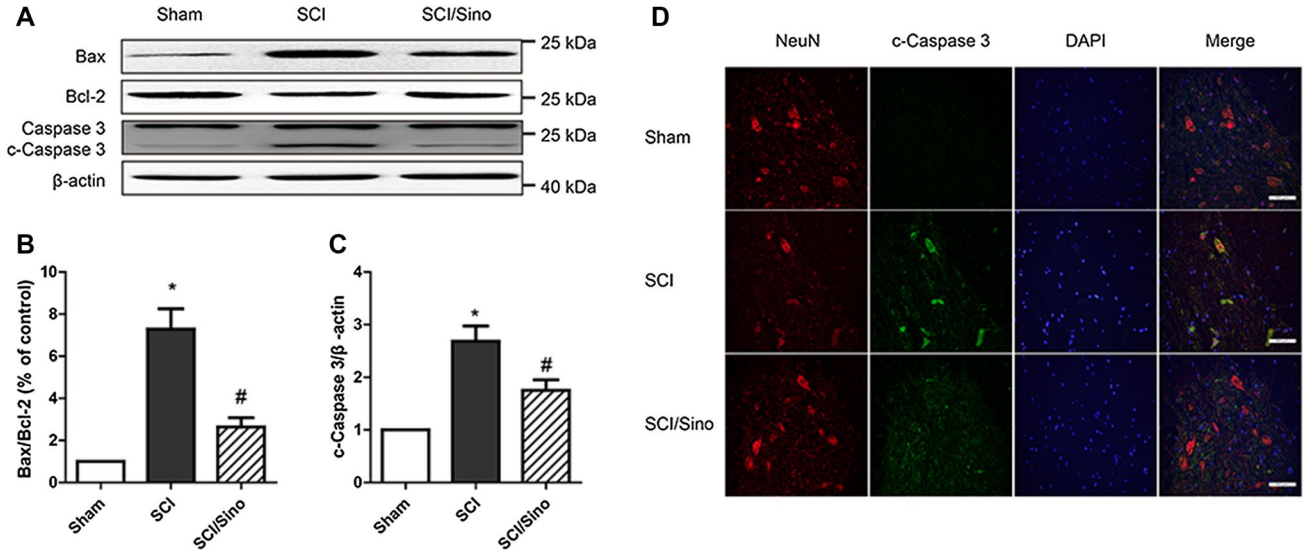


**Fig. 3** Sinomenine inhibited apoptosis caused by SCI. **a, b** Representative Western blots and quantification data of Bax, Bcl-2 and β-actin in each group. **a, c** Representative Western blots and quantification data of cleaved Caspase 3 and β-actin in each group rats. Columns represent mean ± SEM, *P < 0.05 versus the sham group, #P < 0.05 versus the SCI group, n = 5. **d** Double staining for NeuN (red)/cleaved caspase 3 (green) of sections from the spinal cord in each group rats. (Color figure online)

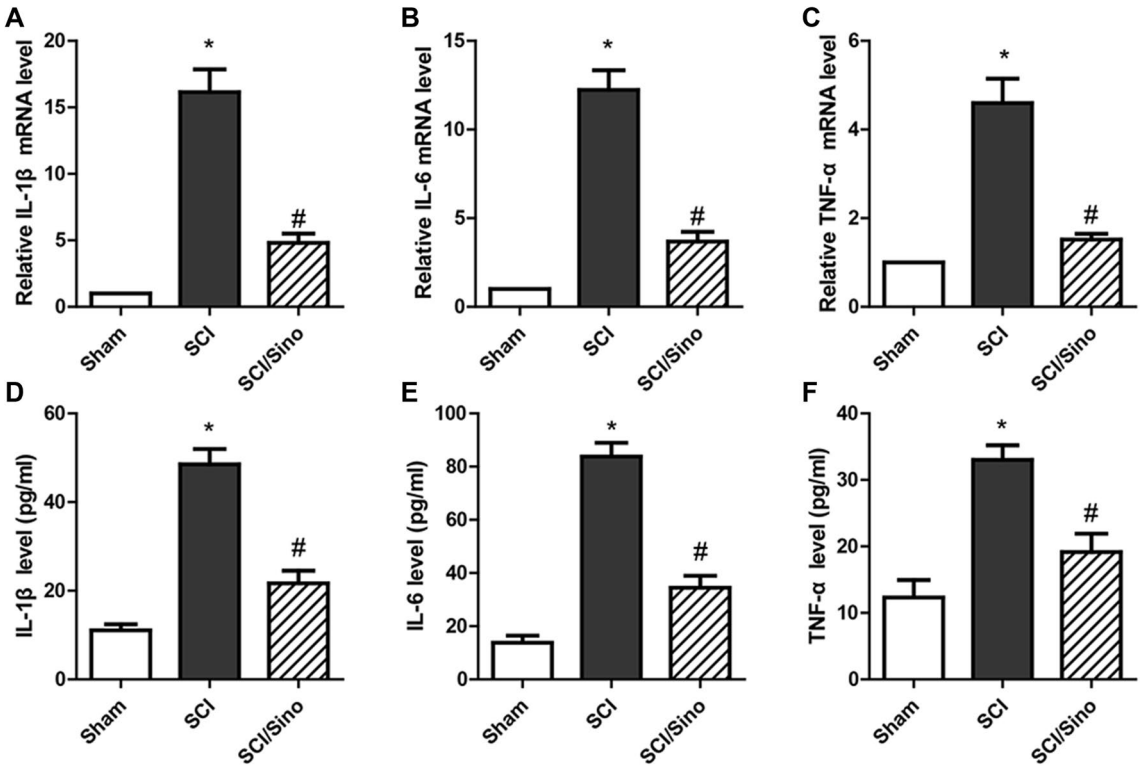


**Fig. 4** Sinomenine attenuated the production of pro-inflammatory cytokines in the spinal cord after SCI. **a, b, c** Relative mRNA levels of IL-1β, IL-6 and TNF-α. **d, e, f** The levels of IL-1β, IL-6 and TNF-α were measured by ELISA. Columns represent mean ± SEM, *P < 0.05 versus the sham group, #P < 0.05 versus the SCI group, n = 5

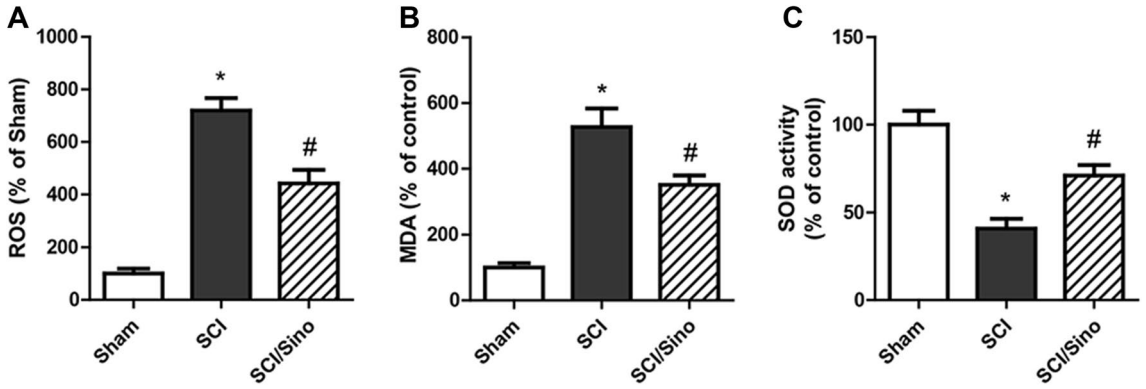


**Fig. 5** Sinomenine ameliorated oxidative stress caused by SCI. **a** The relative level of ROS in the spinal cord following SCI, columns represent mean ± SEM, *P < 0.05 versus the sham group, #P < 0.05 versus the SCI group, n = 5. **b** The relative content of MDA in the spinal cord following SCI, columns represent mean ± SEM, *P < 0.05 versus the sham group, #P < 0.05 versus the SCI group, n = 5. **c** The relative level of SOD activity in the spinal cord after SCI, columns represent mean ± SEM, *P < 0.05 versus the sham group, #P < 0.05 versus the SCI group, n = 5

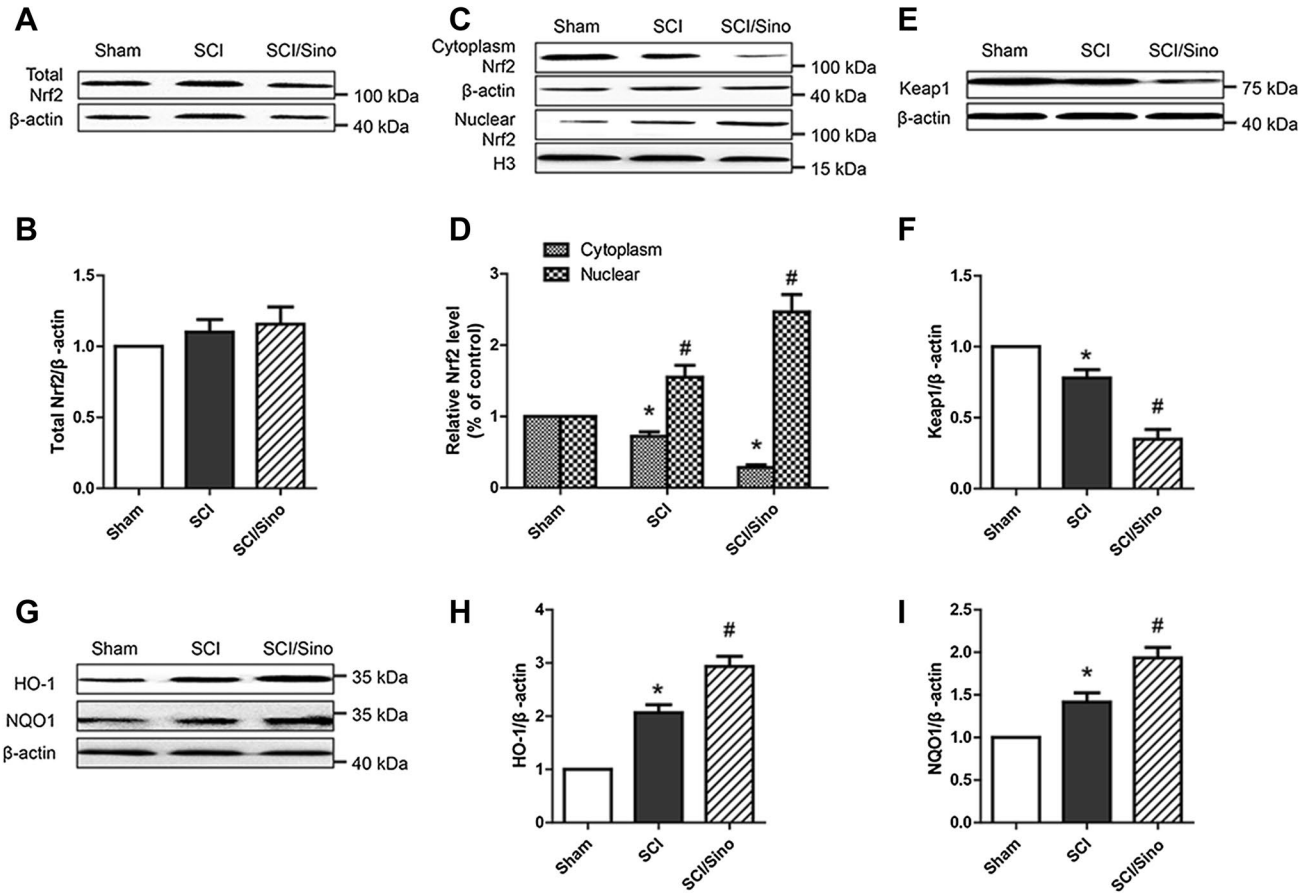


**Fig. 6** Sinomenine increased Nrf2 translocation from cytoplasm to nucleus and enhanced Nrf2-regulated transactivation following SCI. **a–d** Representative Western blots and quantification data of Total Nrf2, Cytoplasm Nrf2, Nuclear Nrf2, H3 and β-actin in each group rats. **e, f** Representative Western blots and quantification data of Keap1 and β-actin in each group rats. Columns represent mean ± SEM, *P < 0.05 versus the sham group, #P < 0.05 versus the SCI group, n = 5. **g–i** Representative Western blots and quantification data of HO-1, NQO1 and β-actin in each group rats. Columns represent mean ± SEM, *P < 0.05 versus the sham group, #P < 0.05 versus the SCI group, n = 5

## Sinomenine Attenuated $H_2O_2$-Induced Injury and Increased Accumulation of Nrf2 in the Nucleus in PC12 Cells

To evaluate the effectiveness of sinomenine in protecting PC12 cells from $H_2O_2$ injury, we first need to make sure whether sinomenine has an effect on cell viability. We found treating with different concentrations of sinomenine for 24 h could not significantly influence the viability of PC12 cells (Fig. 7a). $H_2O_2$ (200 μM) treatment for 12 h induced significant cell injury as evidenced by lower cell viability (Fig. 7b). While sinomenine greatly attenuated $H_2O_2$-induced cellular damage. And we also evaluate the effect of sinomenine on Nrf2 in PC12 cells. As our data showed, nuclear Nrf2 was increased obviously by sinomenine (10 μM) in PC12 cells (Fig. 7c, d).

## Sinomenine Protected PC12 Cells Against Cellular Damage Depending on the Nrf2

To further elucidate the role of Nrf2 in sinomenine treatment, we transfected PC12 cells with an Nrf2 siRNA. Following siRNA transfection, a decreasing level of Nrf2 was observed in PC12 cells (Fig. 7e, f). Treatment with sinomenine was still cytoprotective against $H_2O_2$-induced injury in PC12 cells transfected with the non-target siRNA but no longer induced any cytoprotective effect in cells with Nrf2 siRNA transfection (Fig. 8a), indicating that Nrf2 was critical to sinomenine-mediated cytoprotection. Furthermore, we also detected the MDA content and SOD activity. As our results showed, the $H_2O_2$-mediated increase in the MDA content and decrease in the SOD activity were attenuated by sinomenine (Fig. 8b, c). However, the effects of antioxidant

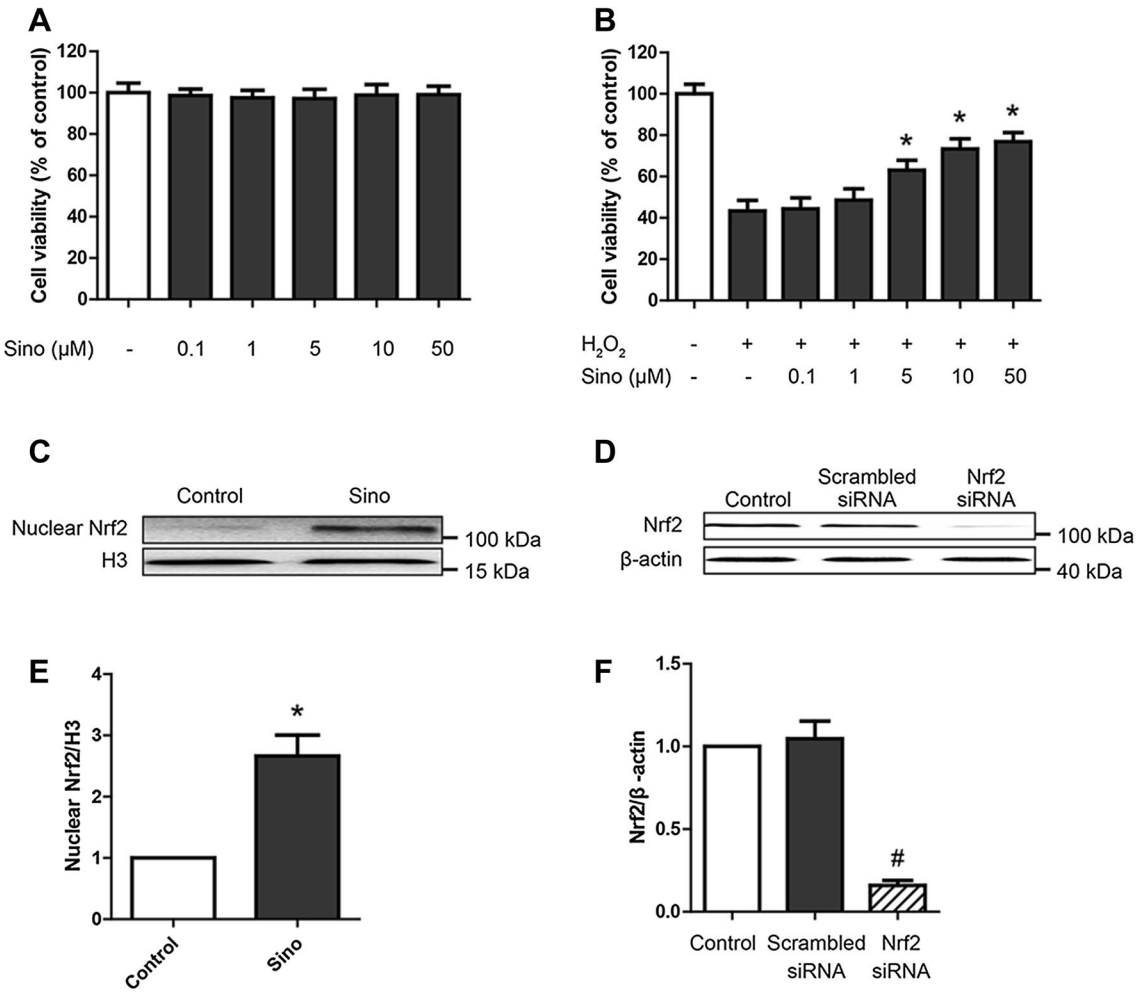


**Fig. 7** Sinomenine attenuated $H_2O_2$-induced injury and increased accumulation of Nrf2 in the nucleus in PC12 cells. **a** The effect of sinomenine on cell viability was detected by CCK-8 in each group, and there was no significant difference between each group. **b** The cell viability was measured by CCK-8 assay in each group, columns represent mean ± SEM, *P < 0.05 versus the only $H_2O_2$ treatment group. **c, d** Representative Western blots and quantification data of Nrf2 and β-actin in each group, columns represent mean ± SEM, *P < 0.05 versus the control group. **e, f** Representative Western blots and quantification data of Nrf2 and β-actin in each group, columns represent mean ± SEM, #P < 0.05 versus the scrambled siRNA group

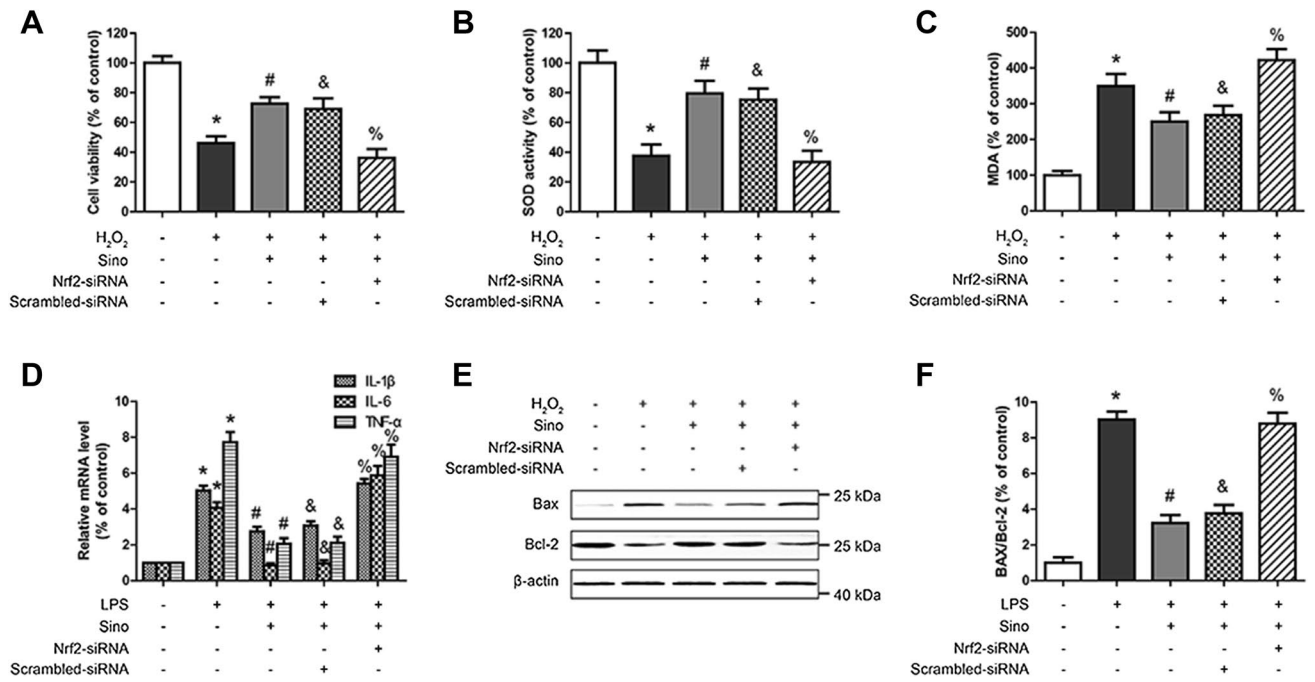


**Fig. 8** Sinomenine protected PC12 Cells against Oxidative Stress depending on the Nrf2. **a** The cell viability was measured by CCK-8 assay in each group. **b** The relative content of MDA in each group. **c** The relative level of SOD activity in each group. **d** The relative level of IL-1β, IL-6 and TNF-α were measured by ELISA in each group. **e, f** Representative Western blots and quantification data of Bax, Bcl-2 and β-actin in each group. Columns represent mean ± SEM, $^{*}P < 0.05$ versus the control group. $^{\#}P < 0.05$ versus the only $H_2O_2$ treatment group. $^{\&}P > 0.05$ versus the $H_2O_2$ and sinomenine co-treatment group. $^{\%}P < 0.05$ versus the $H_2O_2$ and sinomenine co-treatment group

stress of sinomenine were significantly blunted by Knocking down Nrf2. To investigate the anti-inflammatory mechanism, we also detected the mRNA level of pro-inflammatory cytokines in PC12 cells. Pro-inflammatory cytokines, including IL-1β, IL-6 and TNF-α, were enhance by LPS treatment, while, the increasing level of these cytokines was attenuated by sinomenine treatment via Nrf2 activation (Fig. 8d). The apoptosis related proteins, including Bax and Bcl-2, were measured to confirm the anti-apoptosis mechanism of sinomenine. We found that the $H_2O_2$-mediated apoptosis could be blunted by sinomenine, which was suppressed by knockdown of Nrf2 (Fig. 8e, f). All of above findings indicated that sinomenine protected PC12 cells against cellular damage via Nrf2 signaling pathway.

## Discussion

Traumatic SCI is a major cause of long-term paralysis in the world [21]. Currently, the only recognized pharmacologic treatment for traumatic SCI in humans is methylprednisolone, but it is still controversial, because of its adverse effects, such as metabolic complications and increased risk of wound infection [22]. Hence, improving the prognosis of SCI is still a major therapeutic challenge. It is possible to find a potentially pharmacological intervention to rehabilitate neurological deficits after SCI. Because most traumatic SCI do not involve physical transection of the spinal cord but rather compromise to the cord as a consequence of a contusive, compressive, or stretch injury [23]. These incomplete SCI could lead to the severe secondary injury lasting for a long time after the primary injury [24], which could be modulated by specific medicine treatment. Thus preservation of surviving cells during the secondary injury phase has been acknowledged as one of the main therapeutic targets of SCI [25].

As an alkaloid isolated from the stem and root of sinomenium acutum, sinomenine has been utilized successfully for centuries in traditional chinese medicine to cure various rheumatoid diseases [13]. Evidence from pharmacological research and clinical practice demonstrates that sinomenine possesses a wide spectrum of activities, including anti-inflammatory and immune-regulatory properties [8]. Previous study has demonstrated that sinomenine can be delivered across the blood–brain barrier and can become available in central nervous system tissue, therefore we think sinomenine could reach the lesion site and rescue CNS diseases [26]. For example, it has been revealed that sinomenine exerts potent protective effects against ischaemic brain injury through regulation of ion channel [12]. Sinomenine was also found to play protective effects on intracerebral hemorrhage-induced brain injury by inhibiting microglial

inflammation [13]. Based on these evidence, we conducted this study to examine the hypothesis that sinomenine could promote functional recovery after SCI. And consistent with this hypothesis, our findings displayed that sinomenine alleviated the deficiency of locomotor function and decreased the damage of tissue structure after traumatic SCI. To investigate the integrity of vascular permeability, we also examined water content and vascular permeability by Evan's blue dye extravasation. According to our results, we demonstrated that sinomenine could alleviate tissue oedema and prevent vascular breakdown, which indicated the protective effect of sinomenine on SCI.

One of traits in CNS diseases is cell death including necrosis and apoptosis. After traumatic injury, the secondary injury lead to neuronal apoptosis in spinal cord [2]. It has been well documented that the therapeutic strategies inhibiting cell apoptosis could contribute to the recovery of SCI [27]. Recently, study on ischemic cerebral injury reported that administration of sinomenine protects brain tissue from ischemia reperfusion-induced apoptosis to improve functional recovery [12]. And it has been also found that sinomenine treatment attenuates traumatic brain injury-induced neuronal apoptosis [28]. As a type of programmed cell death, apoptosis is triggered by many molecular, including cleaved Caspases 3, Bax, and Bcl-2. Among them, Bcl-2 is the protein to inhibit cell apoptosis, while Bax participates in the induction of apoptosis, and cleaved Caspase 3 is a mediator to promote programmed cell death in apoptotic signaling [29]. In the current study, we found cleaved Caspase 3 and Bax were up-regulated, and Bcl-2 was down-regulated in SCI group, which indicated that SCI could cause cell apoptosis. However, sinomenine administration attenuated the increase in cleaved Caspase 3 and Bax, and up-regulated the level of Bcl-2, which indicated that the process of apoptosis was ameliorated by sinomenine. And double staining for NeuN / cleaved Caspase 3 demonstrated sinomenine could attenuate neuronal apoptosis.

To further investigate the latent therapeutic mechanisms of sinomenine, we also detected oxidative stress level in spinal cord. It is generally recognized that ROS includes superoxide and hydroxyl radicals, formed mainly at the level of mitochondrial electron transport chain [30]. If not controlled by anti-oxidants, ROS can induce tissue injury [4]. Compared with other tissues, the CNS may be more susceptible to oxidative damage given its high rate of oxidative metabolic activity and inadequate antioxidant defense system [31]. Oxidative damage caused by ROS has been closely related to the pathological process of numerous CNS diseases [32, 33]. The pathological mechanisms of SCI have not yet been fully elucidated, but increasing evidence reveals that oxidative stress is a crucial contributor to the pathological process and mediates subsequent histopathological and neurobehavioral deficits [34]. As an endogenous antioxidant enzyme, SOD catalyzes the disproportionation of superoxide anion, and is a major anti-oxidative enzyme to scavenge oxygen free radicals [35]. The content of SOD implicates the protective effectiveness of cells from toxic injury caused by free radicals. MDA is the end product of polyunsaturated fatty acid, of which the content is an important indicator of tissue damage for lipid peroxidation [36]. Therefore, SOD activity and MDA content could be detected to evaluate oxidative stress injury secondary to SCI. In this study, our findings revealed that sinomenine attenuated the increasing content of MDA and ROS after SCI, and SOD activity was up-regulated by sinomenine to prevent cells from oxidative injury.

Nrf2 is a widely expressed transcription factor, which is known to interact with the antioxidant response element, subsequently, the downstream anti-oxidation proteins were initiated [35]. Accumulating evidence demonstrates that Nrf2 gives play to regulate the expression of a group of antioxidant and detoxification enzymes and is regarded as an important role in modulating inflammation process. Especially for CNS disorders, Nrf2 plays an pivotal role to defend against potential stress or insult [37]. Thus, the modulation of Nrf2 has been acknowledged as one of intervention methods to prevent stress-induced damage in CNS diseases. Treatment with Nrf2 activator has been reported to act neuroprotective roles in animal models of cerebral ischemia, traumatic brain injury and SCI. Mao found that Nrf2 plays a protective role against SCI-induced secondary injury, possibly by limiting the inflammatory response in the spinal cord [38]. Satoh showed that Nrf2 activation protects against cerebral ischemia in vivo [39]. Furthermore, compared with wild-type mice, Nrf2−/− mice are notably more likely to have ischemic brain injury [40].

As an Nrf2 pathway activator, sinomenine activates Nrf2 signaling pathway by the ubiquitination proteasome-mediated Keap1 degradation [8]. In a recent study, sinomenine was found to activate Nrf2 pathway and upregulate phase-II enzymes to exert renoprotective effect in fibrotic kidney [8]. It was also found that sinomenine attenuates high glucose-induced renal glomerular endothelial dysfunction by activating Nrf2 [41]. To ascertain the mechanism of sinomenine therapy, Nrf2 was detected. And our results displayed that sinomenine promoted Nrf2 translocation from the cytoplasm to the nucleus. It is widely accepted that after translocating to the nucleus, Nrf2 binds to a promoter sequence called the antioxidant response element and mediates the expression of many antioxidant enzymes, such as HO-1 and NQO1 [35]. Then, they participate in regulating oxidative stress, inflammatory reactions and apoptosis. Therefore, in the current study we investigated the level of HO-1 and NQO1. And we found sinomenine could increase HO-1 and NQO1, which may be two possible target genes to attenuate oxidative stress injury in SCI treatment.

Among the various mechanisms of injury, neuroinflammation plays another important role in the pathological process of SCI, and the inflammatory response of local damaged tissue aggravates the degree of secondary injury [42], leading to cellular necrosis and apoptosis around the epicenter of lesion area, thereby deteriorating SCI. It is well known that the pro-inflammatory cytokines at the site of injury modulates the precise cellular events after SCI [43]. In the present study, we confirmed a significant increase in the content of IL-1β, IL-6 and TNF-α following SCI. While, this tendency was decreased in tissue sections from sinomenine treated rats, compared to untreated rats. Consistent with our results, sinomenine was reported to inhibit pro-inflammatory cytokine production in retinal microglial culture [44]. And sinomenine-mediated Nrf2 activation was also found to display modulating function in inflammatory responses via NF-kB signaling in macrophages [8].

To further confirm our hypothesis in vitro, PC12 cells were treated with $H_2O_2$ to mimic oxidative damage. Consistent with our in vivo study results, sinomenine could protect PC12 cells from $H_2O_2$-induced oxidative damage, and Nrf2 pathway was activated by sinomenine. To explore whether sinomenine-mediated neuroprotection was critically dependent on the presence of Nrf2, we transfected PC12 cells with Nrf2 siRNA to knock down Nrf2. The Nrf2 protein expression in cells was significantly reduced by the appropriate siRNA. Transfected cells were then exposed to $H_2O_2$, and were treated with sinomenine. We found sinomenine could play a neuroprotective role against $H_2O_2$-induced injury in PC12 cells transfected with the non-target siRNA but no longer induced any neuroprotective effect in the cells with Nrf2 siRNA transfection. The content of MDA, SOD activity and the production of inflammatory cytokine also indicated the same results. The mechanisms of anti-apoptosis effect were also investigated. We demonstrated that knocking down Nrf2 counteracted sinomenine-casued anti-oxidant stress and anti-inflammation to abrogate the anti-apoptosis effect of sinomenine. These findings indicated that Nrf2 was critical to sinomenine-mediated cytoprotection. However, using PC12 cells to mimic neurons is one of limitations of our study, and this may warrant further investigations to reconfirm these results in primary neurons.

In conclusion, this present study investigated the effects of sinomenine on functional recovery, apoptosis, oxidative stress, and inflammation after traumatic SCI. Our findings revealed that sinomenine may provide potential therapeutic interventions for preventing oxidative stress and inflammation to attenuate SCI.

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## Compliance with Ethical Standards

**Conflict of interest** The authors declare no conflict of interest.

**Ethical Approval** All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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