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Dihydratanshinone I Alleviates Spinal Cord Injury via Suppressing Inflammatory Response, Oxidative Stress and Apoptosis in Rats

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Data Interpretation D
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Background: Spinal cord injury (SCI) is a serious nervous system injury, causing extremely low quality of life and immeasurable economic losses. However, there is few therapies that can effectively cure the injury. The goal of the present study was to explore the potential therapeutic effects of dihydratanshinone I (DI) for SCI and the involving mechanism.





Material/Methods: A SCI rat model was structured to investigate the effects of DI on recovery of SCI. Tarlov's scale was employed to assess the neuronal function and histopathological examination was carried out by hematoxylin and eosin staining. In addition, tumor necrosis factor (TNF)- α , interleukin (IL)-6, IL-1 β , inducible nitric oxide synthase (iNOS), total oxidant status (TOS) and total antioxidant status (TAS) levels were detected. Tunel assay and western blot analysis were performed to evaluate cell apoptosis. Furthermore, western blot assay was used to measure the protein expressions.

Results: The results demonstrated that the treatment of DI alleviated the pathological damage induced by SCI and promoted the neuronal functional recovery. DI suppressed TNF- α , IL-1 β , IL-6, iNOS, and TOS levels while improved the TAS level. Moreover, increased cell apoptosis in SCI rats was inhibited by administration of DI. Most importantly, DI reserved the soaring of TLR4, MyD88, HMGB1, and NOX4 level after induction of SCI. Thus, the observation revealed that the HMGB1/TLR4/NOX4 pathway may be involved in the protective effects of DI on SCI.

Conclusions: In conclusion, the findings suggest that DI alleviates SCI by restraining secretion of inflammatory factors, and occurrence of oxidative stress and apoptosis *in vivo*. DI may be developed into an effective alternative therapy for SCI in clinic.

MeSH Keywords: **Inflammation • Oxidative Stress • Rats • Spinal Cord Injuries**

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Background

Spinal cord injury (SCI) is a serious complication of spine injury, putting patients in debilitating pathological conditions physically and psychologically, and may cause huge economic challenges to society [1–3]. SCI leads to physical damages, including infarction of the spinal cord, dyskinesia, and sensory deficits in the area below the injury, muscle wasting, chronic pain, and paralysis [4,5], resulting in acceleration of tissue edema, neural cell apoptosis, and the inflammatory cytokine release [6]. Nowadays, supportive measures are still the primary treatment because of the complex pathophysiology of SCI [7]. Pharmacological therapies, such as high-dose corticosteroids, GM-1 ganglioside, and mecobalamin, have been widely performed while the therapeutic effects for SCI of these treatments are uncertain and unexpected side effects are reported [8]. Therefore, to develop more effective and stable therapies with fewer side effects is urgent need.

Traditional Chinese medicine (TCM) has been proven to play multiple therapeutic roles in various diseases. *Salvia miltiorrhiza* (Danshen), a well-known TCM, has been used in treatment of cardiovascular diseases, amenorrhea, hemorrhage, and hepatitis for several hundred years [9]. The active components of *Salvia miltiorrhiza* can be divided into 2 components: hydrophilic and lipophilic fractions [10]. The major active constituents in hydrophilic fractions are salvianolic acid derivatives and diterpenoids, while those in lipophilic fractions are the diversified tanshinones [11]. Dihydrotanshinone I (DI) is one of the tanshinones extracted from *Salvia miltiorrhiza* and has been widely researched for its extensive biological activities. DI has exhibited anti-inflammatory effects *in vitro* and *in vivo* [12], reverses the multidrug resistance [13], promotes vasorelaxation of coronary arteries in rats [14], and even acts as a tumor suppressor by inducing cytotoxicity and inhibiting angiogenesis [15]. However, little study has investigated the ability and the underlying mechanism of DI to protect from spinal cord injury.

In the current study, we established the spinal cord injury rat model and explained the protection of DI against spinal cord injury by inhibition of inflammatory cytokine response, decreases of oxidative stress and cell apoptosis.

Material and Methods

DI preparation

DI powder (HPLC $\geq 95.0\%$) was obtained from Sigma–Aldrich Corporation (St. Louis, MO, USA) and purity of the red powder was greater than 95.0% according to the detection of HPLC–UV. It was dissolved with absolute alcohol and stored at -20°C .

Animals

Sprague-Dawley (SD) rats weighing 250–270 g were purchased from Comparative Medicine Centre of Yangzhou University. The rats were raised under a controllable condition of $22\pm 3^{\circ}\text{C}$, relative humidity $50\pm 10\%$, and 12-hour light and 12-hour darkness, supplied with standard laboratory feed. All experiments were approved by the Animal Care and Use Committee of Dajiangdong Hospital.

SCI model and group

The SCI rat model was constructed as previously described [16,17]. Briefly, the SD rats were injected intraperitoneally with 100 mg/kg ketamine and 1 mg/kg chlorpromazine for anesthesia. Then the T8 and T9 vertebral peduncles of the rats were removed by a laminectomy. Following this, all rats were individually housed in a room with the aforementioned conditions. After inducing the SCI model for 24 hours, rats were orally administered DI treatment. The 40 SD rats were divided into 5 groups ($n=8$). In the sham group, rats were subjected to surgical procedures of laminectomy without the removal of T8 and T9 vertebral peduncles, and given normal saline orally. In the SCI group, rats underwent the laminectomy and were treated with normal saline. In the SCI+DI group, SCI rats were administered orally with 2 mg/kg and 4 mg/kg of DI. All groups were administrated once a day for 28 days and were sacrificed at 24 hours after the last dose.

Histopathological examination

Spinal cord samples from rats of the 5 groups were fixed with 10% neutral formaldehyde buffer overnight, dehydrated in different concentrations of ethanol, embedded in paraffin, and cut into 4 μm sections. The sections were then stained with Hematoxylin and eosin (H and E) staining. The pathological changes of the samples after SCI or treatment of DI were observed by a light microscope

Assessment of neuronal function

Neurological function of SCI rats was evaluated after treatment of DI for 28 day after surgery in accordance to the Tarlov scale: 0 point, no lower extremity movement and could not bear gravity; 1 point, lower extremity motion and could not bear gravity; 2 point, lower extremity motion against gravity; 3 point, lower extremity could support the weight and walk for 1 to 2 steps; 4 point, walk with mild obstacles; 5 point, walk normally.

Measurement of TNF- α , IL-6, and IL-1 β levels

To measure effects of DI on pro-inflammatory cytokine in rats with SCI, blood was harvested and curdled. After centrifugation

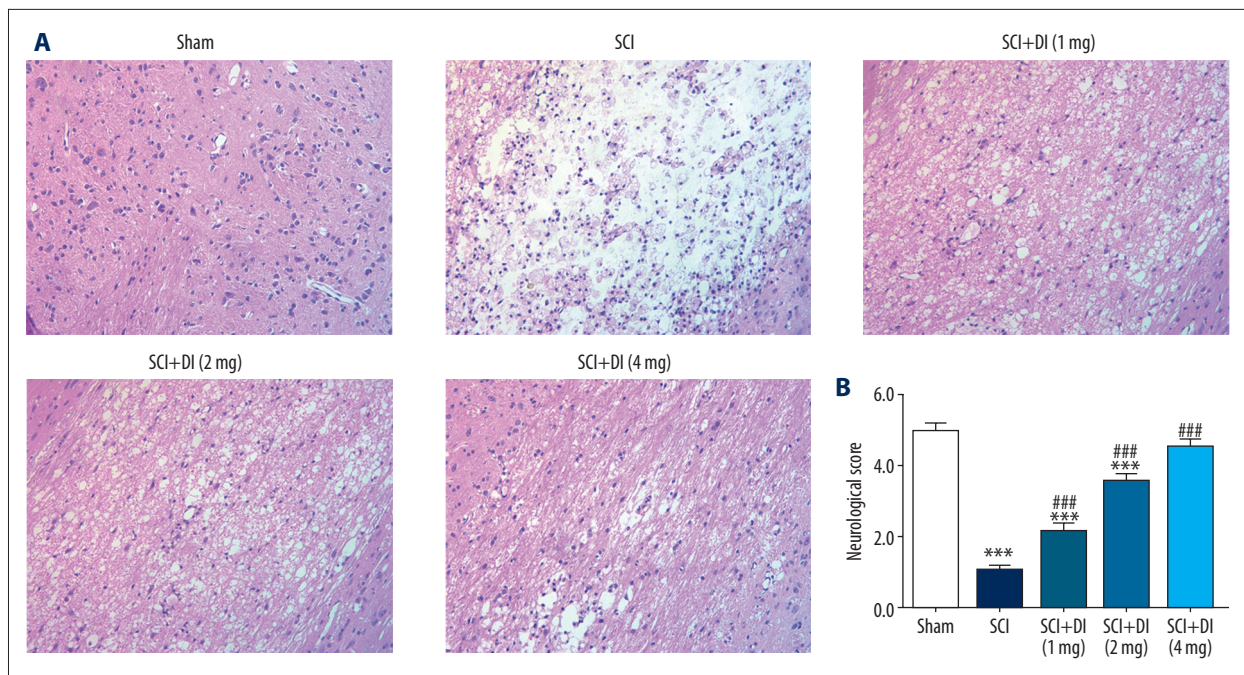


Figure 1. DI reduced histopathological changes in spinal cord tissues and recovered motor function after SCI. **(A)** H and E staining of spinal cord section was performed with or without DI. Original magnification: 200×. **(B)** Neurological function of the SCI rats in each group was evaluated by Tarlov scores. Data are expressed as mean±SD. *** $P<0.001$ versus sham group; ### $P<0.001$ versus SCI group. DI – dihydrotanshinone I; SCI – spinal cord injury; H and E – hematoxylin and eosin; SD – standard deviation.

at 1000 g for 20 minutes, the supernatant was collected and the levels of serum TNF- α , IL-6, and IL-1 β were examined by enzyme-linked immunosorbent assay (ELISA). All assays were replicated for 3 times and 6 replicates were established in each group.

Western blot assay

Spinal cord tissues from rats were collected and homogenized in a cold RIPA lysis buffer. The concentration of extracted total proteins was measured with a BCA protein assay kit (Thermo, USA). Sample proteins were then loaded onto 12% SDS-PAGE and electrotransferred to nitrocellulose membranes. The membranes were used to blocked with 5% non-fat milk at 37°C for 1 hour, and incubated with primary antibodies (IL-6, 1: 1000, ab6672; IL-1 β , 1: 1000, ab2105; TNF- α , 1: 1000, ab6671; iNOS, 1: 1000, ab15323; Bcl-2, 1: 1000, ab32124; Bax, 1: 1000, ab32503; cleaved caspase 3, 1 μ g/ml, ab2302; TLR4, 1: 500, ab13556; MyD88, 1: 1000, ab133739; HMGB1, 1: 1000, ab79823; and NOX4, 1: 2000, ab133303. All were purchased from Abcam) at 4°C overnight. Following that, the membranes were washed 3 times and incubated with secondary goat anti-rabbit (1: 2000, ab6721) or anti-mouse IgG (1: 1000, ab7068) antibodies for 2 hours at 37°C. The bands were visualized with enhanced chemiluminescent (ECL) detection kit.

Measurement of oxidative stress

The levels of serum total oxidant status (TOS), serum total antioxidant status (TAS) and nitric oxide (NO) were detected by a commercially available kit (Jiancheng Bio., Nanjing, China) following the manufacturer's protocol, respectively. Each group was replicated for 5 times and all assays were independently repeated 3 times.

Tunel assay

TUNEL assay was carried out to evaluate the cell apoptosis induced by SCI. Spinal cord cells were washed 3 times with DPBS and fixed with 4% paraformaldehyde for 30 minutes without light. The cells were then incubated with 15 μ g/mL proteinase K for 15 minutes and 3% H₂O₂ for 15 minutes at 37°C. After washing several times in PBS, cells were stained with 3,3'-diaminobenzidine (DAB). Positive stained cells were counted with a fluorescence microscopy.

Statistical analysis

All data were analyzed by SPSS 20.0 software and are presented as mean \pm standard deviation (SD). The comparison of the 2 groups was conducted by Student's *t*-test and the significance of multiple group differences were analyzed using

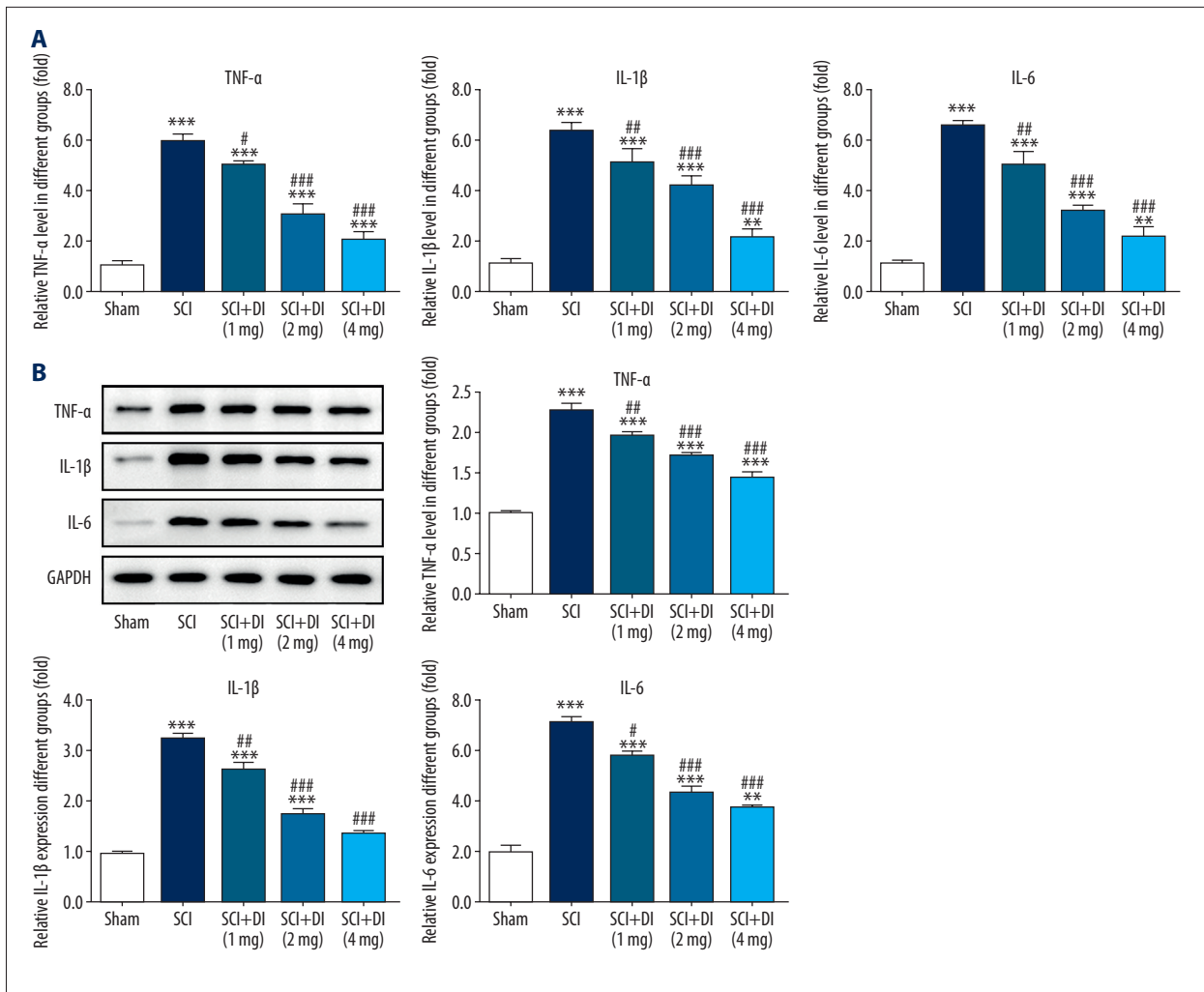


Figure 2. Effects of DI on inflammatory cytokines in rat spinal cord. **(A)** Levels of IL-6, IL-1 β and TNF- α in serum were assessed by ELISA assay. **(B)** Western blot assay was employed to determine the generation of TNF- α , IL-6, and IL-1 β in spinal cord tissues of rats after sham surgery, and SCI and DI treatment. Data are expressed as mean \pm SD. ** $P < 0.01$, *** $P < 0.001$ versus sham group; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ versus SCI group. DI – dihydrotanshinone I; IL – interleukin; TNF – tumor necrosis factor; ELISA – enzyme-linked immunosorbent assay; SCI – spinal cord injury; SD – standard deviation.

one-way analysis of variance (ANOVA). A P value < 0.05 was considered as the significant difference.

Results

DI alleviated pathological changes of spinal cord in rats with SCI

SCI results in multiple alteration of pathology and morphology in spinal cord tissues [18]. To investigate the influence of DI on histopathology of spinal cord segments in SCI rats, SCI rat model was established. As presented in Figure 1A, the SCI rats showed the significant alteration of neuron's morphology, degeneration of gray matter, infiltration of leukocytes and

increased cavitation areas in spinal cord tissue compared with sham-operated rats. However, with administration of different dose of DI, pathological lesions induced by SCI were rescued in a dose-dependent manner. In addition to, the scope of cavitation areas was shrunken as well. These data indicated that DI may reduce alteration of spinal cord morphology in SCI rats.

DI promoted neurological functional recovery

To explore the role of DI in functional recovery after SCI, neurological examination was performed with a Tarlov scale. As shown in Figure 1B, the average neurological functional scale in the SCI group was significantly lower than that in sham group. The score of neurological function was remarkably increased after the treatment of DI compared with the group with

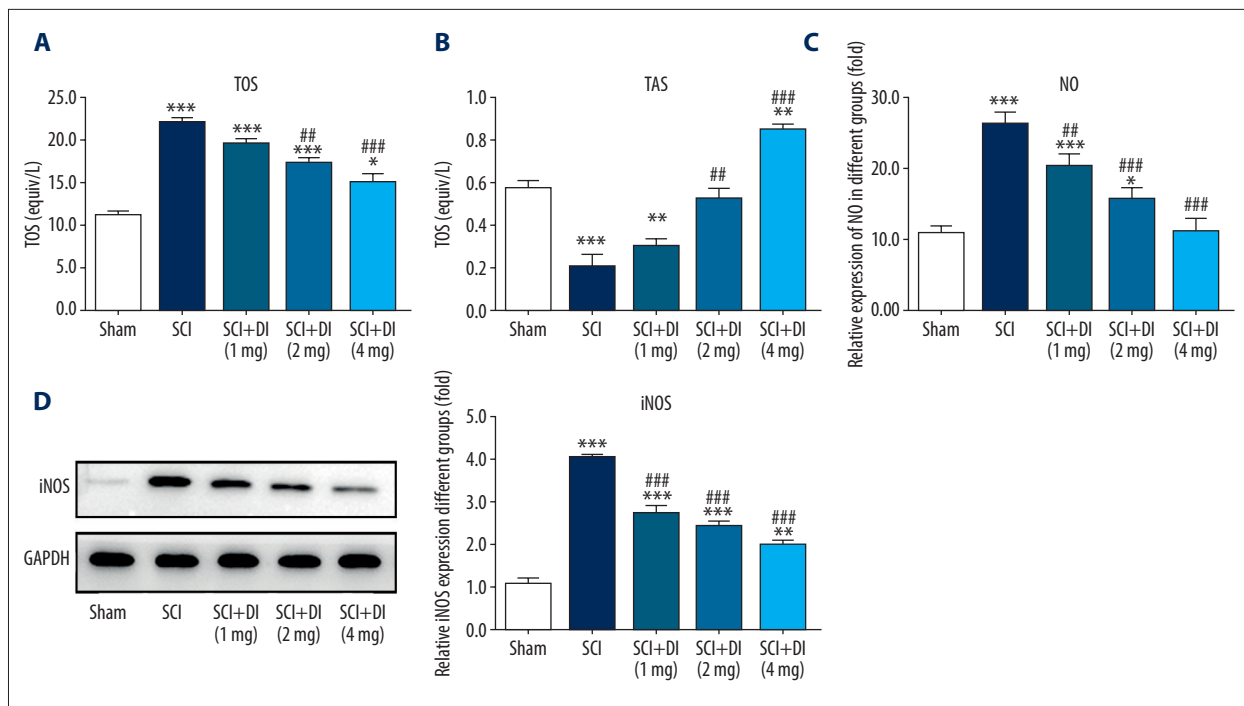


Figure 3. Effects of DI on oxidative stress in rat spinal cord. TOS (A), TAS (B), and NO level (C) in serum of SCI-treated rats were detected in the absence or presence of DI. (D) iNOS protein level was assessed by western blot analysis. Data are expressed as mean±SD. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ versus sham group; # $P<0.01$, ### $P<0.001$ versus SCI group. DI – dihydroanthinone I; TOS – total oxidant status (TOS); TAS – total antioxidant status; iNOS – inducible nitric oxide synthase; SD – standard deviation.

induction of SCI. Besides, we found that there was no significant difference between the scores of the DI-treated SCI rats and the sham-operated rats, suggesting that enough dose of DI had obvious effects on accelerating neurological and motor functional recovery in rats subjected to SCI.

DI reduced inflammatory cytokines in serum and spinal cord tissues

To identify the effects of DI on SCI-induced inflammatory response, inflammatory cytokines in both serum and spinal cord tissues were evaluated. The results from ELISA assay revealed that SCI dramatically increased the secretions of IL-6, IL-1 β , and TNF- α in serum in comparison with the sham group. However, diminishing levels of IL-6, IL-1 β , and TNF- α were observed in DI-treated SCI group (Figure 2A). In addition, western blot analysis result demonstrated that the production of IL-6, IL-1 β , and TNF- α were induced after SCI while DI treatment reversed the increases in spinal cord tissues (Figure 2B). These results indicated the suppressive effects of DI on the generation of pro-inflammatory factors in SCI-induced serum and spinal cord tissues.

DI suppressed SCI induced oxidative stress and iNOS generation

In addition to inflammatory response, serum oxidative stress level after SCI was measured. As shown in Figure 3A and 3B, SCI markedly enhanced TOS while reduced TAS in comparison with the sham group. Meanwhile, DI treatment prevented the aberrant alteration and elevated the capacity of antioxidant after SCI. In addition, NO level was found to be increased in SCI-stimulated rats and the excessive expression of NO was suppressed by treatment of DI (Figure 3C). Western blot assay showed that increased protein level of iNOS after SCI was significantly reduced by DI treatment (Figure 3D). The results suggested that DI exerted strong anti-oxidative activity in SCI rat model.

DI exerted anti-apoptosis activity in SCI rats

Neural cell apoptosis stimulated by SCI affects the physiological activities of central nervous system (CNS) and represses the recovery of white matter and neurological functions [19]. To investigate the role of DI in SCI-induced cell apoptosis, TUNEL assay and western blot analysis were performed. As shown in Figure 4A, SCI induced an obviously increased apoptosis rate whereas DI treatment greatly reduced cell apoptosis compared with the cells treated with SCI. Additionally, western

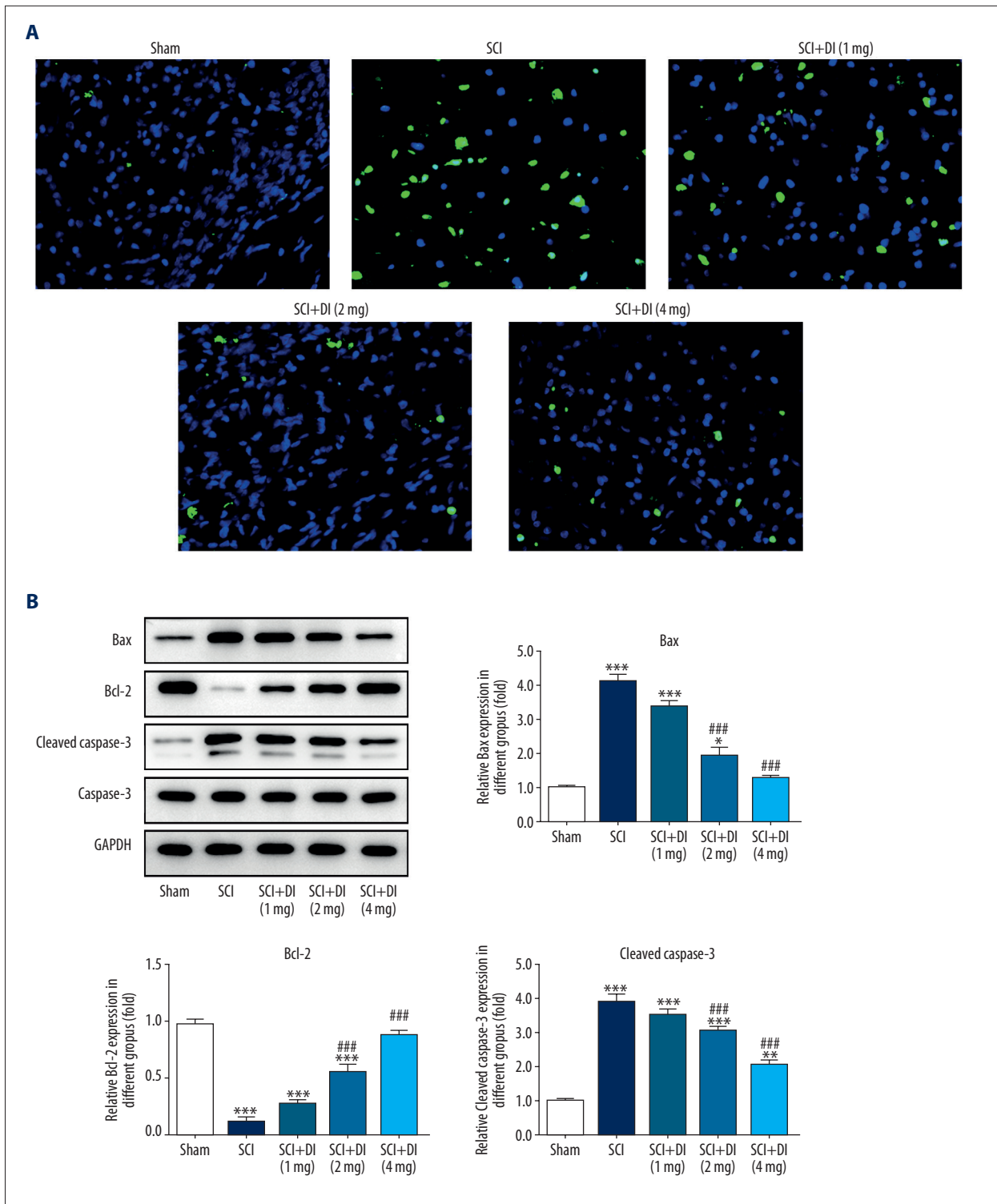


Figure 4. DI suppressed SCI-induced cell apoptosis in rats. **(A)** TUNEL assay was carried out to assess the apoptosis after SCI in the absence or presence of DI. Original magnification: 400×. **(B)** Western blot assay was implemented to measure the levels of proteins involved in cell apoptosis. Data are expressed as mean±SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus sham group; ## $P < 0.01$, ### $P < 0.001$ versus SCI group. DI – dihydratanshinone I; SCI – spinal cord injury; SD – standard deviation.

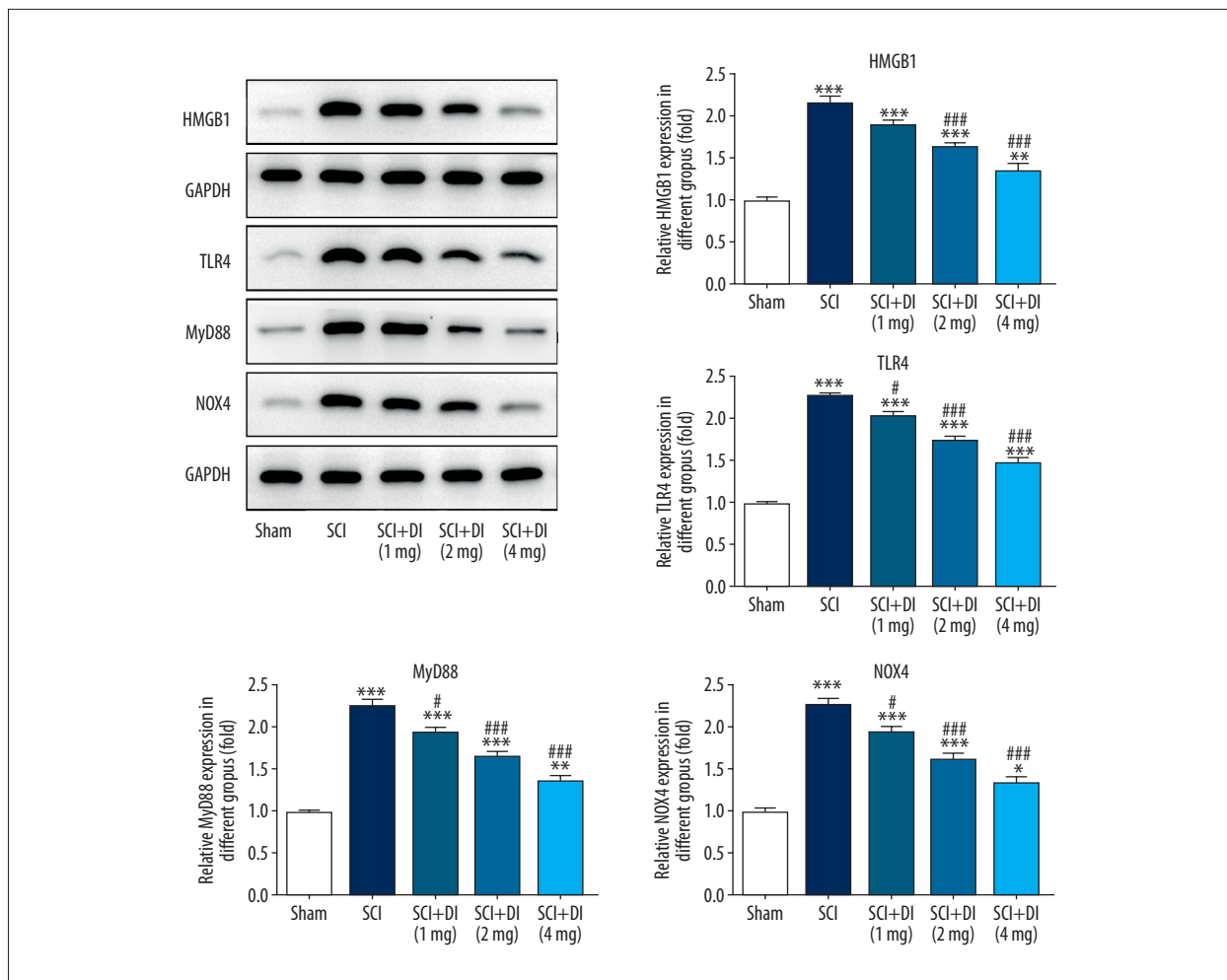


Figure 5. Effect of DI on HMGB1/TLR4/NOX4 activation in rat spinal cord. Levels of MGB1, TLR4, MyD88 and NOX4 were decreased in SCI rats after administration of DI. Data are expressed as mean±SD. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ versus sham group; # $P<0.05$, ### $P<0.001$ versus SCI group. DI – dihydrotanshinone I; SCI – spinal cord injury; SD – standard deviation.

blot results showed that protein levels of Bax and cleaved caspase-3 were significantly increased and Bcl-2 level was decreased in SCI rats. DI treatment reduced the expression levels of Bax and cleaved caspase-3, and enhanced Bcl-2 level after SCI (Figure 4B). The data indicated DI evidently suppressed cell apoptosis after SCI lesions.

DI inhibited the HMGB1/TLR4/NOX4 signaling pathway

Further, we examined the underlying mechanism by which DI protects rats against SCI. As shown in Figure 5, when compared with the sham group, the contents of HMGB1, TLR4, MyD88, and NOX4 in SCI-treated spinal cord tissues were extremely increased. However, SCI-induced increase levels of 4 proteins were notably inhibited by DI treatment in a concentration-dependent manner, which indicate that DI may exert its protective effects on SCI rats via inactivating the HMGB1/TLR4/NOX4 signaling pathway.

Discussion

In the present study, we observed that DI ameliorated pathological damages by reversing histological changes in spinal cord and recovered neurological and motor functions with an improved Tarlov scale. Moreover, we also revealed that DI alleviated inflammatory response by reduction of TNF- α , IL-6 and IL-1 β in serum and spinal cord, inhibited oxidative stress by a decreased TOS and an increased TAS, and suppressed cell apoptosis following the SCI. Furthermore, HMGB1/TLR4/NOX4 signaling pathway was found to be associated with the protective effects of DI on SCI-treated rats.

Dihydrotanshinone I (DI), as a potent active component of *Salvia miltiorrhiza* (Danshen), have been extensively used for gynecological and cardiovascular diseases [20–22]. It is well established that DI shows strong anti-oxidative and anti-inflammatory properties in many inflammation and other diseases.

Yuan et al. found that DI showed anti-inflammatory effects by inhibited the secretions of inflammatory cytokines and activation of NF- κ B pathway *in vitro* and alleviating LPS-induced acute kidney injury *in vitro* [12]. Wang et al. revealed that DI remarkably suppressed the expression of NF- κ B reporter gene and phosphorylation of I κ B α and p65 induced by TNF- α , and also repressed the expressions of NF- κ B target genes TNF- α , IL-6, and MCP1 [23]. In addition, previous study demonstrated that lipophilic fractions of *Salvia miltiorrhiza* inhibited the oxidative stress level in diabetic nephropathy by abolishing high glucose-induced reactive oxygen species and enhancing the Nrf2 level [24]. Dai et al. reported that Tanshinone I exerted neuroprotection through ameliorating motor and cognitive functions damaged by hypoxia-ischemia via inhibiting oxidative stress in the neonatal rats [25]. In the current study, our results showed that DI not only blocked the production of IL-6, IL-1 β , TNF- α , and iNOS improved anti-oxidative level and suppressed the NO level after SCI, but reduced tissue lesions and rescued neurological function, which is consistent with previous reports. These data indicate the suppressive effects of DI on SCI-stimulated inflammation and oxidative stress in spinal cord tissues.

It is widely accepted that inflammatory reaction under abnormal conditions may cause cell apoptosis [26,27]. Neutrophils release reactive oxygen free radicals, damage the vascular endothelium cells, which accelerate tissue edema, necrosis and apoptosis after SCI [28,29]. Previous study has showed that DI exerted anti-tumor activity in colon cancer by promoting caspase dependent apoptosis and autophagy *in vitro*, meanwhile increasing the induction of apoptosis and caspase-3 activation in xenograft rats [30]. Cao et al. elucidated that DI increased the activation of caspase-3/9 and the release of cytochrome c induces apoptosis and inhibits proliferation so as to induce apoptosis and inhibit proliferation in glioma cells [31]. However, Wei et al. revealed that DI reduced myocardial apoptosis by inhibiting the expressions of Bax, cleaved caspase-3 and cleaved caspase-9 while promoting the Bcl-2 expression after myocardial ischemia-reperfusion injury (MIRI) [32]. In our study, results from Tunel assay demonstrated that DI evidently diminished SCI-induced apoptosis, with a decrease level of Bax and cleaved caspase-3 and an increased level of Bcl-2. Based on the aforementioned result, we speculate that

DI plays different roles in the apoptosis associated with various diseases and the underlying molecular mechanism remains to be further illustrated.

Studies have shown that acute SCI resulted in an increase of HMGB1 level which is related to neuronal apoptosis [33]. For example, glycyrrhizin protected rats against ischemic spinal cord injury by inhibiting the secretions of inflammatory cytokines and HMGB1 [34]. Besides, TLR4 is intimately linked with the autoimmune and non-infectious immune response. Kang et al. reported that the intervention of hyperbaric oxygen alleviated secondary spinal cord injury by reduced the levels of HMGB1, NF- κ B and TLR4 *in vivo* [35]. Another study revealed that Shikonin alleviated tissue injury and recovered motor function induced by spinal cord injury through regulating the inflammation response via the inactivation of HMGB1/TLR4/NF- κ B pathway [36]. NOX4 has been also regarded as a strong pro-inflammatory factor for its role of the downstream of TLR4 under certain circumstances and the interaction with TLR4 in both endogenous and exogenous TLR4 ligand-induced ROS generation [37-39]. In the present study, the expressions of HMGB1/TLR4/NOX4 pathway in SCI rats were detected following SCI in the presence and absence of DI. We found that stimulation of SCI induced the HMGB1/TLR4/NOX4 signaling while DI suppressed the high expressions of this signaling after SCI. These results indicate HMGB1/TLR4/NOX4 pathway is involved in the protection of DI against SCI *in vivo*.

Conclusions

Our study demonstrated that DI ameliorated spinal cord injury through suppressing the progression of inflammatory response and oxidative stress, as well as apoptosis in rats. The beneficial effects of DI may be associate with blockages of HMGB1/TLR4/NOX4 signaling. Collectively, the data indicate that DI has the potential to serve as a therapeutic candidate for patients with SCI.

Conflict of interest

None.

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