Original Article
Neuroprotective effects of diosgenin in rats with experimental spinal cord injury via promotion of autophagy

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Abstract: Background: Spinal cord injury (SCI) is a devastating disorder in the central nervous system with neurological deficits and loss of motor function. The aim of the present study was to evaluate the neuroprotective effects of diosgenin (DSG), a natural extract which exerts anti-inflammatory effects on many diseases, in a rat model of SCI. Methods: Forty Sprague-Dawley rats were randomized into four groups (Sham group, Vehicle group, DSG (100 mg/kg) group and DSG (200 mg/kg) group). SCI rat models were established based on the Allen's method. DSG was orally administrated (100, 200 mg/kg b.w., respectively) after induction of SCI for consecutive 21 days. Neurological function recovery was evaluated using the Basso, Beattie, Bresnahan (BBB) open-field locomotor rating scale and Rivlin's inclined plane test. Seven days after SCI, the production of proinflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6), was assessed using enzyme-linked immunosorbent assay (ELISA) kits to examine the inflammatory responses in perilesional tissues. Western blot analysis was performed to investigate the expression of apoptosis-associated proteins (Bcl-2, Bax, cleaved caspase-3) and autophagy associated proteins (Beclin 1 and light chain 3-II (LC3-II)). Spinal neurons were cultured to investigate the anti-inflammation effect of DSG *in vitro*. Results: We observed that administration of DSG significantly alleviated SCI-induced neurological deficits. Compared with Vehicle group, treatment with DSG following SCI markedly reduced the production of TNF-α, IL-1β and IL-6. Moreover, compared with Vehicle group, administration of DSG also evidently attenuated SCI-induced apoptosis, and enhanced SCI-induced autophagy. Besides, administration of DSG significantly reduced the levels of TNF-α, IL-1β and IL-6 in LPS-treated spinal cord neurons, and this reduction was suppressed by CQ treatment. Conclusion: Taken together, the results clearly showed that DSG attenuates SCI-induced secondary injury through inhibiting the inflammatory response, repressing apoptosis, and promoting autophagy in perilesional tissues.

Keywords: Spinal cord injury, diosgenin, neuroprotection, inflammation, apoptosis, autophagy

Introduction

Spinal cord injury (SCI) is a catastrophic sudden and devastating condition with an annual incidence of 12000 to 20000 cases in the United States [1], frequently caused by traumatic traffic accidents, falls, violence, sports and recreational accidents, and diseases [2]. SCI significantly affects a patient's physical and mental conditions and causes substantial economic burden on society [3]. Although great efforts have been made, SCI treatment options are still limited. The pathophysiology of SCI is featured by both primary and secondary injury mecha-

nisms. The primary damage is caused by the mechanical impact leading to direct contusion, shearing injury, or laceration of the spinal cord [4]. The subsequent secondary injury involves a complex and highly interrelated series of molecular processes, including neuroinflammation, oxidative stress, apoptosis, and necrosis [5]. Accordingly, therapies that can reduce secondary injury will effectively decrease the extent of disability resulting from SCI and improve functional recovery [6].

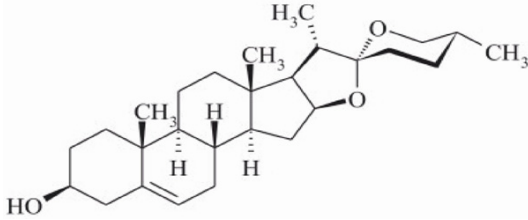Natural plant products have a potential role in exploiting innovative chemotherapy drugs for a

Figure 1. The chemical structure of diosgenin (DSG).

wide variety of human diseases. In this regard, diosgenin (DSG; **Figure 1**), the primary furostanol saponin found in several plants, including *Dioscorea* species, fenugreek, and *Costus speciosus* [7], functions as a critical starting material for the production of corticosteroids, sexual hormones, oral contraceptives and other steroidal drugs. Many studies have reported the pharmacologic effects of DSG, including anti-cancer [8, 9], anti-diabetic [10, 11], anti-arrhythmic [12] and anti-hyperlipidemic activities [13]. Meanwhile, DSG is a potent inhibitor of inflammation and reduces inflammatory response through various mechanisms. Kim *et al.* reported that phthalic anhydride-induced skin inflammation could be repressed by DSG treatment through attenuation of IL-4 and IL-6 expression [14]. Above-mentioned evidence indicates that DSG might serve a potential therapeutic role for SCI associated with inflammation. To date, however, literature survey showed little scientific evidence supporting the potential therapeutic effects of DSG in SCI.

Therefore, in the present study, we tried to determine the beneficial effects of DSG in SCI rat models via reducing neuroinflammation and neuronal apoptosis. Taken together, our results suggested that DSG might be a promising novel chemical agent for the clinical treatment of SCI.

Materials and methods

Animal uses and groups

Forty adult male healthy Sprague-Dawley (SD) rats (of SPF grade, aged 8-12 weeks), weighting 250-300 g, were obtained from Shanghai Laboratory Animal Center Co. Ltd. (Shanghai, China). All experimental protocols were approved by the Animal Use and Ethics Committee of The Affiliated 3201 Hospital of Xi'an Jiaotong University, and in accordance with the ethical guidelines of National Institutes of Health [15]. Animals were acclimatized to the environment

for three weeks prior to experimentation. The rats were housed in standard polypropylene cages at 18-24°C with a humidity of 50% and a 12-h light/dark cycle. Animals had *ad libitum* access to standard pellet chow and water. All attempts were made to minimize the number of animals and their suffering.

Spinal cord injury modeling

Rats were anesthetized with intraperitoneal 6% chloral hydrate (6 mL/kg) and placed in a supine position. After exposing the thoracic spine, a laminectomy was performed carefully at the $T_{9/10}$ level spinal cord, and then the vertebral column was completely exposed. Contusion SCI model was established as previously reported [16]. A 10-g 2-mm diameter impactor was dropped from a height of 5 cm on the $T_{9/10}$ level spinal cord to yield a moderate contusion. After contusive SCI, the bladder of rats was emptied twice a day until 14 days post-injury.

Rats (n=40) were categorized into four groups randomly, each containing 10 rats. Sham group rats (n=10) underwent only a laminectomy for 1 min; Vehicle group rats (n=10) underwent SCI only; and the remaining 20 rats underwent SCI as in Vehicle group followed by daily oral administration of DSG (100, 200 mg/kg b.w., respectively) for 21 days. DSG (purity >98%) was provided by Nanjing Spring & Autumn Biological Engineering Co., Ltd (Nanjing, China), and freshly prepared daily in dimethylsulfoxide (DMSO). According to the protocol, 20 rats (n=5 per group) were euthanized using an overdose of pentobarbital sodium on day 7, and the spinal cords were exposed from the original incisions in the back. A 1.5 cm long segment of the spinal cord containing the lesion site was dissected out. The remaining 20 rats were used to assess locomotion recovery.

Neurological function assessment

The Basso, Beattie, Bresnahan (BBB) open-field locomotor rating scale was used to detect the recovery of motor function after contusive SCI [17, 18]. The minimum score (0) was regarded as complete paralysis, whereas the maximum score (21) was considered as normal mobility. The animals' capacity to keep postural stability was detected by Rivlin's inclined plane test [19]. The maximum angle of the inclined board at which the animals could keep a con-
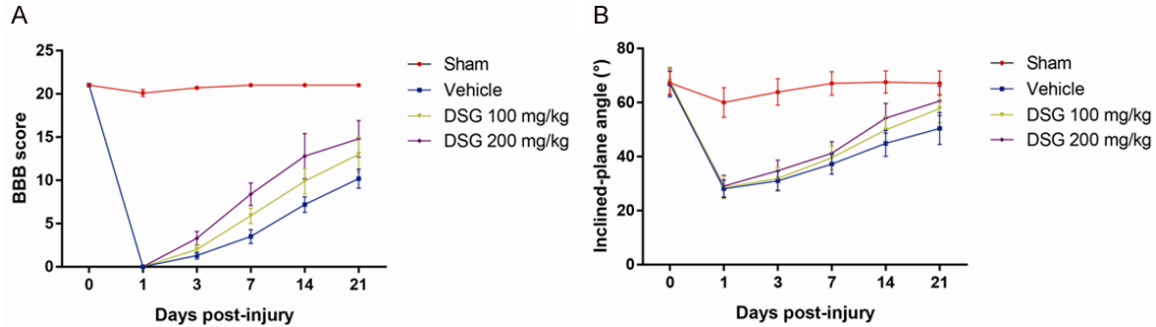
Figure 2. Neurological functional recovery was significantly improved after DSG administration. A. Hindlimb motor function was assessed by the Basso, Beattie and Bresnahan (BBB) locomotor rating scale after induction of SCI. B. The animals' ability to maintain postural stability was detected by Rivlin's inclined plane test after induction of SCI. n=10 per group. The data are shown as mean ± SD.

stant position for at least 5 s was recorded. Two independent examiners who were blind to treatment observed the rats at pre-injury and 1, 3, 7, 14, and 21 days post-injury.

Culture of primary spinal neurons

Spinal neurons were extracted from spinal cords of SD rat pups (1-2 days after birth). After euthanization, the spinal cord tissue was isolated under the microscope, freshly minced into 1 mm³ small pieces, then digested with 0.05% trypsin (Invitrogen, Carlsbad, CA, USA) and eluted with 10% BSA (bovine serum albumin, Gibco). The suspension was centrifuged at 1000 rpm for 10 min, and then the supernatant was discarded. Neuron cells were played in 6-well plates (Corning, USA) coated with poly-d-lysine and laminin (Sigma-Aldrich, St. Louis, MO, USA) at a density of 5×10^4 cells/well in serum-free neurobasal medium (Invitrogen) supplemented with 2% B27 (Invitrogen), 100 U/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), and 0.5 mM glutamine (Invitrogen). The medium was discarded every 3 days, and cultured for 10 days in a humidified incubator with 5% CO_2 at 37°C.

Neuron cell treatment

Neuron cells were allocated into five groups: Control group; LPS (lipopolysaccharide) group, where cells were treated with LPS (1 μg/mL, Sigma-Aldrich); LPS + DSG group, where cells were treated with LPS and DSG (20 μM); and LPS + DSG + CQ (chloroquine) group, where cells were treated with LPS, DSG and CQ (10 μM).

ELISA analysis

Spinal cord tissues and cultured neurons were homogenized in PBS at 4°C containing protease inhibitor cocktail. The homogenates were then centrifuged at 3000 rpm for 15 min, and aliquots of supernatant were stored at -80°C. The protein concentrations of the supernatant were investigated using BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). The concentrations of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) in spinal cord tissues and cultured neurons were detected using a colorimetric commercial kit DuoSet ELISA Development System (R&D Systems, Milan, Italy) and analyzed through microplate reader (Dynex Technology, Chantilly, USA) at an absorbance of 450 nm.

Western blot assay

Total proteins of spinal cord tissues were extracted in a RIPA lysis buffer (Beyotime, China) containing a protease inhibitor cocktail (Roche). The lysates were separated by SDS-polyacrylamide gels and then electro-transferred into PVDF membranes (Millipore, USA). The membranes were probed with primary antibodies against Bax (Santa Cruz), Bcl-2 (Santa Cruz), caspase-3 (Abcam), Beclin-1 (Cell Signaling Technology), LC3 (Cell Signaling Technology) and β-actin (Santa Cruz) overnight, followed by incubation with corresponding horseradish peroxidase-conjugated secondary antibodies. Signals were visualized with the ECL Advance reagent (Beyotime, China) and quantified using Image J 2.0 software. Target protein expression levels were determined after normalizing to β-actin.
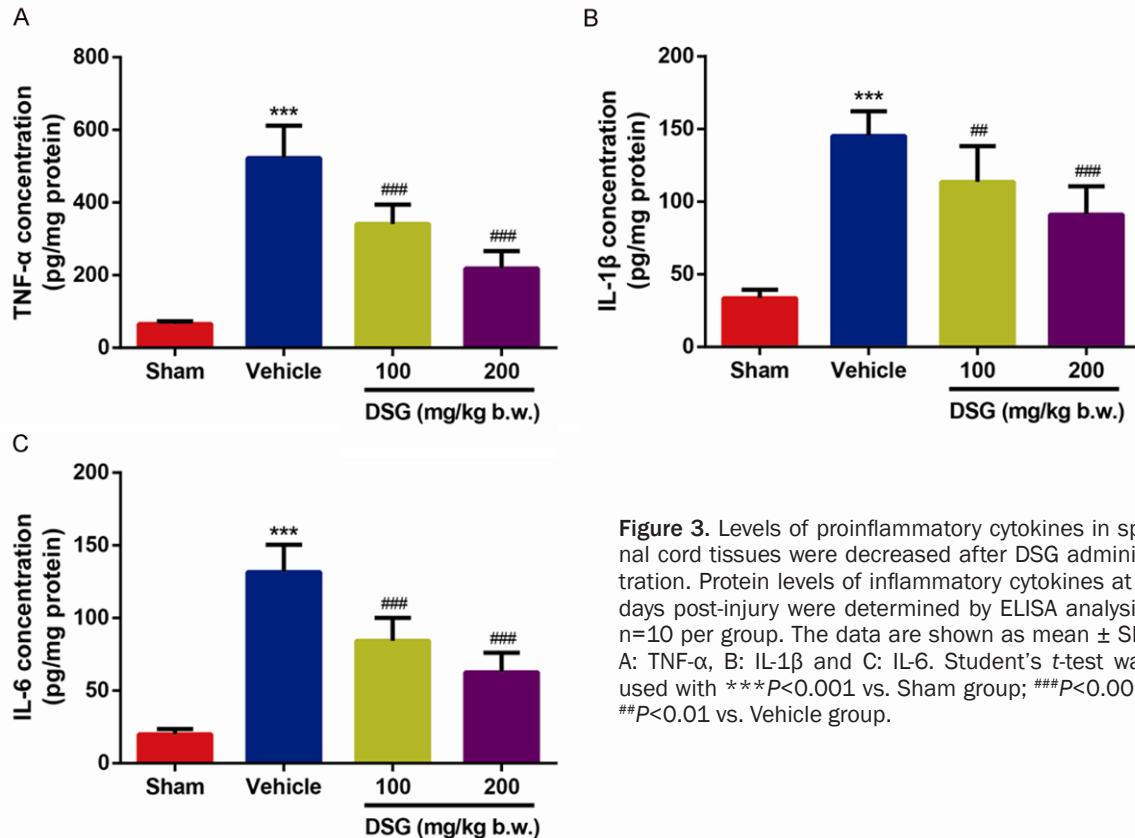
Figure 3. Levels of proinflammatory cytokines in spinal cord tissues were decreased after DSG administration. Protein levels of inflammatory cytokines at 7 days post-injury were determined by ELISA analysis. n=10 per group. The data are shown as mean ± SD. A: TNF-α, B: IL-1β and C: IL-6. Student's *t*-test was used with ***$P<0.001$ vs. Sham group; ###$P<0.001$, ##$P<0.01$ vs. Vehicle group.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). Data were addressed as mean ± standard deviation (SD) from at least three independent experiments. Comparisons among different groups were performed with unpaired Student's-*t* test. $P<0.05$ was regarded to indicate a statistically significant result in this research.

Results

Neurological functional recovery was significantly improved after DSG administration

BBB locomotor scores were measured for three weeks after surgery to evaluate the effect of DSG on locomotor functional recovery after SCI. As demonstrated in **Figure 2A**, on the 1st day post-injury, the BBB scores of rats fall in a straight line from 21 to 0, indicating severely impaired locomotor function. In the following days, a significant gradual recovery was observed. The two groups of rats which were treated

with DSG showed more significant improvements than those of rats in Vehicle group, and the effect was dose-dependent.

Rivlin's inclined plane test was performed to evaluate the effect of DSG on the rats' ability to maintain postural stability after SCI. As shown in **Figure 2B**, the inclined-plate angles of SCI rats markedly dropped compared to Sham group. Meanwhile, we found that the inclined-plate angles of rats treated with DSG increased noticeably higher than those of rats in Vehicle group in the following days, and the effect was dose-dependent. Taken together, these findings indicated DSG might accelerate the recovery of neurological function.

Levels of proinflammatory cytokines in spinal cord tissues were decreased after DSG administration

The rats were sacrificed on day 7 after SCI to investigate the anti-inflammatory effects of DSG in perilesional tissues. The results exhibited in **Figure 3** suggested that at 7 days post-injury, the concentration levels of TNF-α, IL-1β,
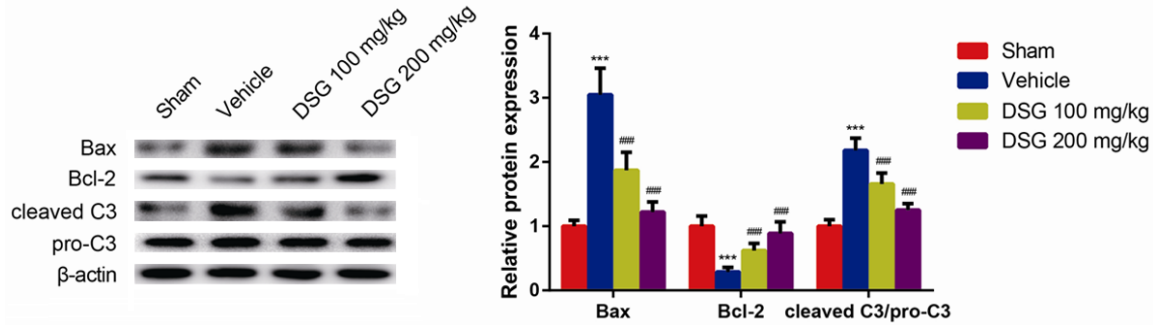
Figure 4. Apoptosis in spinal cord tissues was markedly inhibited after DSG administration. Protein levels of Bax, Bcl-2 and cleaved caspase-3 at 7 days post-injury were determined by western blot assay. n=10 per group. β-actin levels were used as an internal control. The data are shown as mean ± SD. Student's *t*-test was used with ***$P<0.001$ vs. Sham group; ###$P<0.001$ vs. Vehicle group.
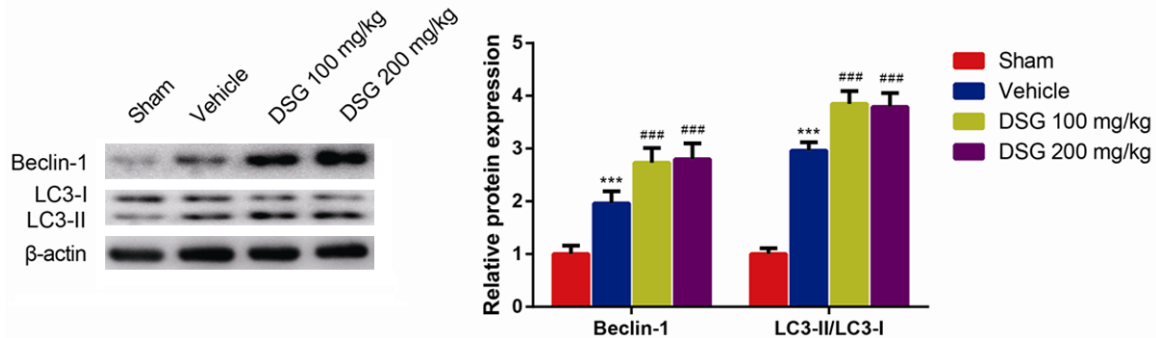

Figure 5. Autophagy in spinal cord tissues was evidently enhanced after DSG administration. Protein levels of Beclin-1 and LC3II at 7 days post-injury were determined by western blot assay. n=10 per group. β-actin levels were used as an internal control. The data are shown as mean ± SD. Student's *t*-test was used with ***$P<0.001$ vs. Sham group; ###$P<0.001$ vs. Vehicle group.

and IL-6 in the spinal cord tissues of Vehicle group rats were greatly increased, compared with those in Sham group rats. Compared to Vehicle rats, treatment with DSG for seven days led to a concentration-dependent decrease of these proinflammatory cytokines levels in perilesional tissues.

Apoptosis in spinal cord tissues was markedly inhibited after DSG administration

Apoptosis in perilesional tissues was investigated by determining the protein levels of Bcl-2 and Bax and ratio of cleaved caspase-3 to pro-caspase-3. As shown in **Figure 4**, compared to control rats, we found that spinal cord trauma elevated ratio of cleaved caspase-3 to pro-caspase-3 and protein expression of Bax and reduced Bcl-2 protein expression, indicating elevated apoptosis at injury site after trauma. However, the SCI rats receiving DSG showed a lower ratio of cleaved caspase-3 to pro-cas-

pase-3 and reduced protein expression of Bax and increased protein expression of Bcl-2 in a dose-dependent manner, possibly reflecting that SCI-induced proapoptotic Bcl-2/Bax/caspase-3 signaling was significantly attenuated by DSG administration.

Autophagy in spinal cord tissues was evidently enhanced after DSG administration

Subsequently we explored whether autophagy is induced following DSG administration as a neuroprotective mechanism. Expression levels of autophagy-related proteins Beclin-1 and LC3II in the rat spinal cord tissues at 7 d after induction of SCI were analyzed by western blot. The results demonstrated that there was nearly no Beclin-1 and LC3-II expression in Sham group. Induction of SCI promoted the expression of Beclin-1 and the conversion of LC3-I to LC3-II, whereas DSG administration remarkably enhanced SCI-induced autophagy (**Figure 5**).
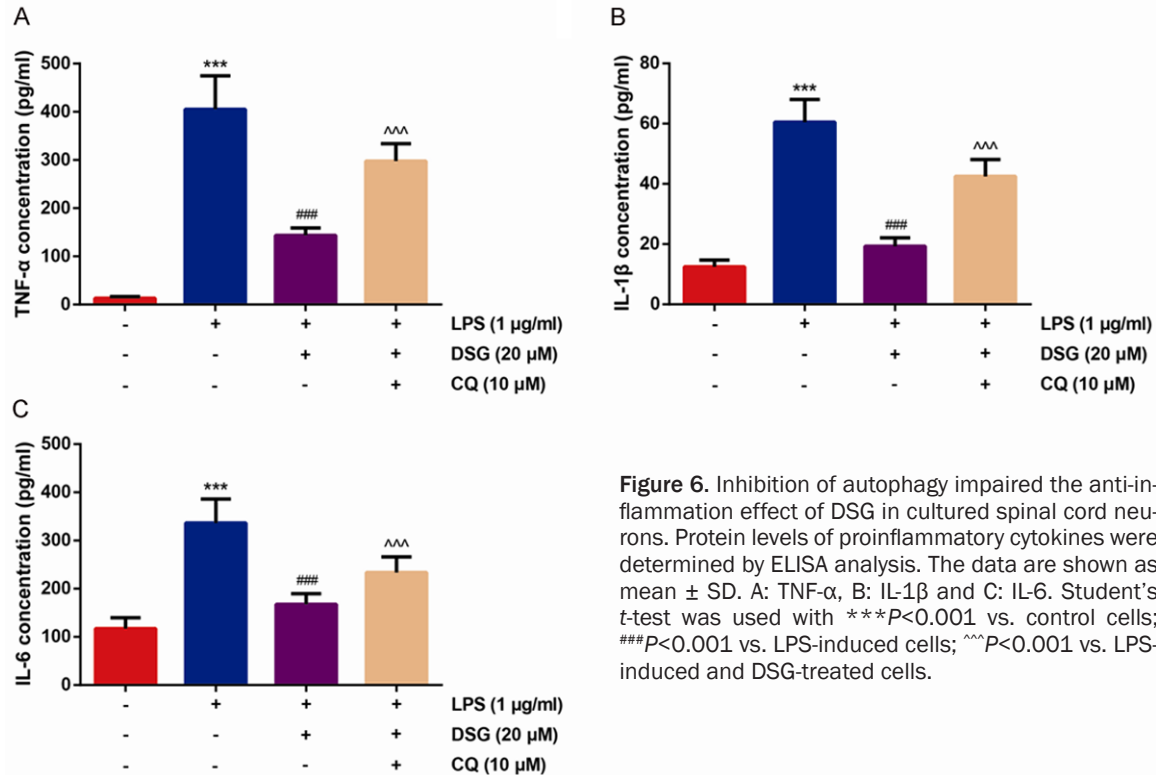
Figure 6. Inhibition of autophagy impaired the anti-inflammation effect of DSG in cultured spinal cord neurons. Protein levels of proinflammatory cytokines were determined by ELISA analysis. The data are shown as mean ± SD. A: TNF-α, B: IL-1β and C: IL-6. Student's *t*-test was used with ***$P<0.001$ vs. control cells; ###$P<0.001$ vs. LPS-induced cells; ^^^$P<0.001$ vs. LPS-induced and DSG-treated cells.

Inhibition of autophagy impaired the anti-inflammation effect of DSG in cultured spinal cord neurons

An *in vitro* inflammation model was established through exposing spinal cord neurons to LPS, and this model was used to elucidate the mechanisms involved in the anti-inflammation effect of DSG after induction of SCI. As expected, compared to control cells, LPS-induced apoptosis elevated the levels of TNF-α, IL-1β, and IL-6, which were obviously restored after DSG administration. Additionally, blocking autophagy through supplementation of CQ noticeably promoted the production of these proinflammatory cytokines (**Figure 6**), indicating that DSG can evidently suppress neuroinflammation through promotion of autophagy.

Discussion

Plants provide a vast treasure of naturally occurring compounds used as a primary source of medicine owing to their abundance in nature, inexpensiveness to produce and few side effects. The therapeutic effects of Chinese herbal extracts in SCI have been widely documented in many experimental studies up to now, including Buyang Huanwu decoction [20], ligustilide [21], and aescin [22]. DSG, a natural extract, has been demonstrated to exert its multiple beneficial pharmacologic effects in various diseases. To decipher the therapeutic effect of DSG in SCI, we established SCI rat models followed by daily oral administration of DSG. We observed that DSG administration substantially promoted hind limb motor function recovery in SCI rats. DSG also exhibited its neuroprotective function in cerebral ischemia-reperfusion-treated rats [23]. Besides, DSG can improve memory and reduce axonal degeneration in an Alzheimer's disease mouse model [24]. Then, we observed that DSG administration reduced the levels of proinflammatory cytokines (IL-1β, IL-6 and TNF-α) in SCI rats in a dose-dependent manner.

Inflammation is a common pathological process following SCI. SCI results in both acute (hours) and chronic (days to months) inflammation, which serves a pivotal role in the secondary tissue damage and cell death that follows the initial mechanical insult [25]. The cascade of inflammatory responses after SCI involves the activation of spinal cord microglia, the influx of macrophages and neutrophils, and increased

expression of free radicals and proinflammatory cytokines [26, 27]. TNF-α is released at early stage of SCI and promotes the inflammatory response by increasing the expression of chemotactic factors, which induce the recruitment of macrophages and neutrophils into the injured site [28]. IL-1β is another proinflammatory cytokine that could lead to the apoptosis of neuronal cells. Thus the detection and identification of anti-inflammatory drugs could have therapeutic values for SCI. Our present findings indicated that DSG administration significantly attenuated neuroinflammation. DSG may confer a potential anti-inflammatory effect on SCI. In addition, through *in vitro* studies, we also found that the anti-inflammatory effect of DSG in LPS-treated spinal cord neurons was dramatically attenuated by CQ, an autophagy inhibitor.

As a survival-promoting pathway ubiquitously in eukaryotes, autophagy plays a critical role in the degradation of damaged or long-lived or damaged proteins and organelles [29]. It has been reported that autophagy activation after SCI exerts a protective effect on neurons [30, 31]. Microtubule-associated protein light chain 3 (LC3) and Beclin-1 are two pacemakers in the autophagic cascade that could lead to the occurrence of autophagy. LC3 converts to LC3-I and LC3-II during autophagy, and LC3-II is a marker of autophagy [32]. Beclin-1 can mediate other autophagic proteins and reduce LC3-II accumulation [33, 34]. In the present study, DSG administration markedly enhanced SCI-induced autophagy. Moreover, DSG-suppressed inflammation was restored following inhibition of autophagy using CQ, indicating that DSG attenuates inflammatory responses after SCI, at least partially, through autophagy activation. The activation of autophagy is a double-edged sword. Autophagy can lead to the execution of cell death programs, but growing evidence suggests that autophagy is a cellular self-defense response to metabolic stresses and immunological challenges [35, 36].

It is well known that inflammatory response after SCI may also lead to apoptosis, also called programmed cell death [37]. The inhibition of TNF-α can reduce caspase family activity and interrupt neuronal apoptosis during the acute phase of SCI [38]. Neuronal apoptosis is an important hallmark of neural tissue degeneration following SCI, therefore the suppression of neural cell apoptosis is critical for functional and pathological recovery after SCI. Our results revealed that DSG administration dose-dependently elevated ratio of cleaved caspase-3 to pro-caspase-3, inhibited Bax expression, and promoted Bcl-2 expression 7 days after injury. Higher Bax/Bcl-2 is associated with increased activation of caspase-3 signaling [39], thus we can conclude that DSG suppresses apoptosis in neural cells through caspase-3 signaling via increasing the ratio of Bax/Bcl-2.

To conclude, the present study demonstrated, for the first time, that DSG treatment promotes functional recovery of SCI rats and represses the SCI-induced inflammation and apoptosis in perilesional tissues, and the neuroprotective effects of DSG might be associated with the promotion of autophagy. Therefore, our results demonstrated that administration of DSG might provide a therapeutic approach for SCI in future clinic.

Disclosure of conflict of interest

None.

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