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Original Article

Geniposide protects against spinal cord injury in rats through attenuating the regulation of inflammatory response and the Bcl2/Bax pathway

Su Pan[2], Chen Li[2], Ji Qu[2], Yang Qu[2], Peng Xia[2], Zhiping Qi[2], Yudan Yang[1]

[1]*Scientific Research Center, China-Japan Union Hospital of Jilin University, Changchun, China;* [2]*Department of Orthopedics, The Second Hospital of Jilin University, Changchun, China*

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Abstract: Spinal cord injury (SCI) causes loss of neurological function, depending upon the severity of injury, which may lead to paralysis. However, there is still no effective pharmacotherapy for SCI treatment so far. Geniposide (GEN), a traditional Chinese medicine, which is reported to possess a wide range of health benefits. A previous study shows that GEN displays various anti-inflammatory and anti-apoptosis properties in oxygen and glucose deprivation-induced brain microvascular injury. However, it is unclear whether GEN could protect against traumatic spinal cord injury, and the underlying molecular mechanisms associated with this process still remain unknown. In the present study, the Basso, Beattie, Bresnahan scores, and the water content of the spinal cord were used to analyze the therapeutic effects of GEN on neurological function in the SCI rats. The serum levels of nuclear factor-κB p65 unit, interleukin (IL)-4, IL-6 and IL-10 were detected using commercial kits. The expression levels of Bcl-2, Bax, Caspase-3, Caspase-6 and Caspase-7 were measured via western blot analysis. The results demonstrated that the neurological function and the water content of the spinal cord in these SCI rats began to ameliorate after GEN treatment. Meanwhile, GEN was found to have inhibitory effects on inflammatory response, compared with the SCI group. In addition, GEN significantly reduced the protein expression of Bax, Caspase-3, Caspase-6 and Caspase-7 and promoted the protein expression of Bcl-2 in the SCI rat model, which indicated that the protective effect of GEN might be associated to anti-apoptosis activation. In summary, GEN protected against spinal cord injury in rats through attenuating inflammatory response and the regulation of Bcl-2 and Bax pathway.

Keywords: Geniposide, spinal cord injury, inflammatory response, neuronal apoptosis, Bcl2/Bax

Introduction

Significant advances have been made in surgical procedures for the treatment of spinal cord injury (SCI) over the past years. Meanwhile, numerous research on SCI have been performed, however, there are very few suitable therapies due to the complex pathophysiology of SCI. Despite efforts to explore pharmacotherapy of SCI, there is no effective treatment available that will improve the locomotor function after SCI [1]. SCI can lead to local nerve tissue degeneration and necrosis, cavity formation and glial scar formation, and it can also cause atrophy of the brain and cardiovascular activities of central nuclei of neurons, degeneration and necrosis, resulting in secondary damage and cardiovascular dysfunction [2, 3]. Therefore, it is critical and necessary to develop new therapeutics that can improve locomotor and sensory function after SCI.

Local microcirculation disorders lead to edema after SCI, and the release of arachidonic acid and its derivatives, including leukotrienes, prostaglandins and thromboxane, which may cause secondary injury to local spinal cord tissues, resulting in severe inflammation, thereby causing irreversible damage to the spinal cord [4]. Research have shown that there are many factors involved in the process of apoptosis after SCI, in which inflammatory cytokines play important role. Luo et al suggested that mangiferin protects against oxidative stress and inflammation in the SCI rats [5].

The Bcl-2 family is an important regulator of apoptosis in SCI, and Bax and Bcl-2 are the

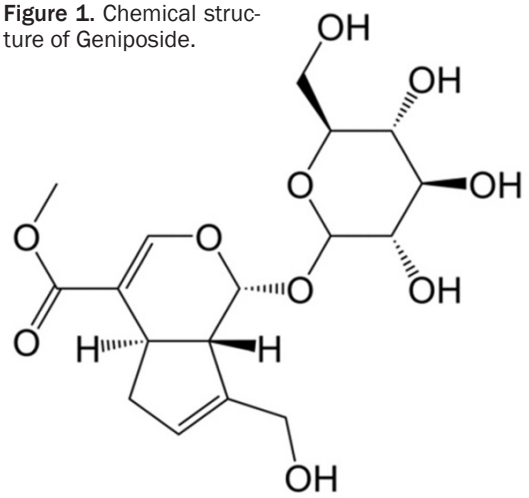

Figure 1. Chemical structure of Geniposide.

most representative genes in the Bcl-2 family, which are apoptotic and anti-apoptotic genes respectively [6, 7]. Jiang et al indicated that the administration of carvacrol inhibited neuronal apoptosis through the regulation of Bax/Bcl-2 proportion in SCI rats [8]. Meng et al reported that the injection of 3-aminobenzamide suppressed apoptosis in SCI rats through the Bax/Bcl-2 pathway [9].

Fructus gardenia, a Chinese traditional herb, was isolated from the fruit of Gardenia jasminoides Ellis, confirmed to have unique therapeutic effects in treatment of ischemic stroke [10, 11]. Geniposide, a marker component for the quality control of Gardenia, constituted the highest proportion of iridoid glycosides, which was the characteristic constituent of Gardenia. Previous research suggested that geniposide is the main ingredient in extract for anti-inflammatory and anti-thrombotic formation, compared with the plant extract [12-14]. What was more, geniposide also had effects of anti-oxidation, anti-ischemic, anti-inflammatory and anti-platelet aggregation [15], which demonstrated that the geniposide was the key bioactive ingredient related to the pharmacodynamic actions of Gardenia on nerve injury. However, to the best of our knowledge, there were no studies could be found yet on the effects of geniposide against SCI in rats up to date. Therefore, the present study designed experiments to investigate the mechanisms underlying the protective action of geniposide in the inflammation, and induction of the Bcl-2/Bax and caspase signaling pathway in SCI rats.

Materials and methods

Animals

Wistar rats weighing 220~240 g were obtained from Animal Centre of Wannan Medical College (Wuhu, China). All animals were kept in a standard environment at 21-24°C and allowed free access to water and food. Experimental procedures were performed in accordance with the guidelines of the Animal Care and Use of Committee of the Provincial Hospital Affiliated to Wannan Medical College (Wuhu, China). The study was approved by the ethics committee of the Provincial Hospital Affiliated to Wannan Medical College (Wuhu, China).

Drugs and reagents

Geniposide (Purity: >98%) was purchased from Nanjing traditional Chinese medicine Institute of Chinese Material Medica (Nanjing, China). The chemical structure is indicated in **Figure 1**. Rat nuclear factor (NF)-κB p65 unit, interleukin (IL)-4, IL-6 and IL-10 ELISA kits were acquired from R&D Institute (Minneapolis, MN, USA). Other reagents were all of analytical grade.

Establishment of an SCI rat model and drug administration

The rat model of SCI was induced according to a modified method previously [16]. Briefly, the spinal cord was performed at thoracic 10 (T10) following an established spinal cord compression model. The skin of rats above the vertebral column was incised and a laminectomy at vertebral level T10 was performed. The dorsal cord surface was exposed with the dura remaining intact. A constant weight (5 g) form a height of 10 cm was dropped onto an impounder (0.4 cm in diameter) placed on the dorsal cord. A total of 100 rats were randomly divided into five groups: Sham group only underwent laminectomy surgery and received physiological saline 1.0 ml/kg i.p.; SCI group underwent spinal cord injuries and received physiological saline 1.0 ml/kg i.p.; GEN (20) group or GEN (40) group and GEN (80) group, which received spinal cord injuries were treated by GEN at a dosage of 20, 40 and 80 mg/kg once a day for 30 consecutive days.

Evaluation of neuronal function recovery

The motor recovery of 10 rats in each group were evaluated at 24 h, 48 h and 72 h after

Table 1. BBB scores for evaluating neurological function

Groups	n	24 h	48 h	72 h
Sham	10	19.28±0.89	19.23±0.82	19.61±0.89
SCI	10	2.12±0.18**	3.46±0.47**	4.64±0.61**
GEN (20 mg/kg)	10	7.38±0.56##	8.54±0.58##	11.47±0.92##
GEN (40 mg/kg)	10	8.51±0.55##	9.87±0.62##	14.93±0.86##
GEN (80 mg/kg)	10	9.50±0.61##	13.72±0.73##	15.59±0.93##

***P*<0.01, compared with the control group; ##*P*<0.01, compared with the SCI group.BBB, Basso, Beattie and Bresnahan; Sham, sham group; SCI, spinal cord injury group; GEN (20), geniposide (20 mg/kg)-treated group; GEN (40), geniposide (40 mg/kg)-treated group and GEN (80), geniposide (80 mg/kg)-treated group. GEN, geniposide; SCI, spinal cord injury.

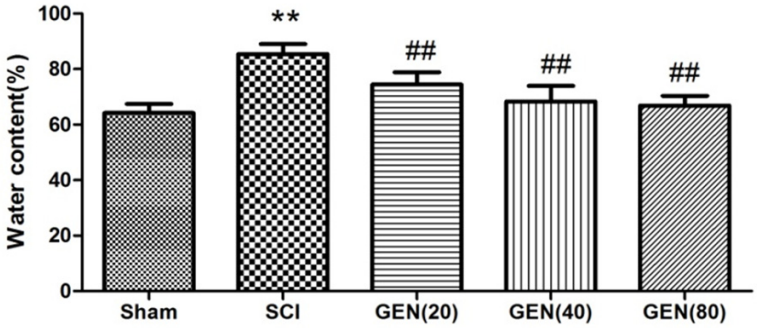

Figure 2. Effects of GEN on the water content of the spinal cord following SCI (n=10, mean ± standard deviation). ***P*<0.01, compared with the control group; ##*P*<0.01, compared with the SCI group. Sham, sham group; SCI, spinal cord injury group; GEN (20), geniposide (20 mg/kg)-treated group; GEN (40), geniposide (40 mg/kg)-treated group and GEN (80), geniposide (80 mg/kg)-treated group. GEN, geniposide.

surgery with a locomotor function scale between 0 (complete paralysis) and 21 (normal locomotion) based on the Basso, Beattie and Bresnahan (BBB) scale [17].

Assessment of water content in spinal cord tissues

The water content in the spinal cord tissues was calculated to value the spinal cord edema. After treatment with GEN for 72 h, 10 rats were sacrificed in each group by decollation, and the impaired spinal cords were dried for 48 h at 80°C conditions in order to calculate the dry weight of the impaired spinal cords. Water content of the spinal cords was calculated using the following computational method: [wet weight - dry weight]/wet weight.

Measurement of the activity of NF-κB p65, IL-4, IL-6 and IL-10

Following treatment with GEN for 72 h, 300 μl peripheral blood was collected from the animals in each group, and then the peripheral blood was centrifuged at 19,200 × g for 10 min at 4°C. Following centrifugation at 19,200 × g for 10 min at 4°C, the serum activities of NF-κB p65 unit, IL-4, IL-6 and IL-10 were measured by analyzing enzyme dynamics using commercial kits (Minneapolis, MN, USA).

Detection of the protein expression of Bcl-2, Bax, caspase-3, caspase-6 and caspase-7

Following treatment with GEN for 30 consecutive days, approximately 10 mm samples of the spinal cord tissues were homogenized in lysis buffer containing 50 mM Tris buffer, 5 mM EDTA, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 10% glycerol. Protein was collected after centrifugation at 11,800 × g for 15 min at 4°C condition, and protein quantification was calculated using BCA kit (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Equal quantities of 60 μg protein were fractioned on 10% sodium dodecyl sulfate-polyacrylamide gels (Millipore, Bedford, MA, USA), transferred onto nitrocellulose fluoride membranes (0.22 mm; Invitrogen Life Technologies, Carlsbad, CA, USA). The membranes were incubated with the following primary antibodies: anti-Bcl-2 and anti-Bax (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 1:1500 dilutions in blocking buffer, and monoclonal anti-reactivated caspase-3, 6, 7 antibody (1:1,000; Cell Signaling Technology, Danvers, MA, USA); anti-β-actin (1:2,000; ab175773, Abcam, USA) at 4°C condition. Following washing with phosphate-buffered saline (PBS), they were then incubated with Tris-buffered saline mouse anti-rabbit antibody (1:4,000; Santa Cruz Biotechnology, Inc.) for 4 h. Protein bands were quantified using enhanced chemiluminescence reagent (Pierce Biotechnology, Inc., Rockford, IL, USA) and the grey level densitometry was calculated using a

Table 2. The anti-inflammatory effects of geniposide on the serum activities of (A) NF-κB p65, (B) IL-4, (C) IL-6 and (D) IL-10 in SCI model rats (n=10, Mean ± Standard Deviation)

Groups	NF-κB p65 (ng/mg protein)	IL-4 (pg/mg protein)	IL-6 (pg/mg protein)	IL-10 (pg/mg protein)
Sham	13.74±1.22	8.68±1.37	2.23±0.46	5.06±1.16
SCI	58.79±1.66**	20.52±1.45**	12.40±0.79**	37.14±2.17**
GEN (20 mg/kg)	42.51±1.48##	16.89±1.67##	8.52±0.51##	27.82±2.32##
GEN (40 mg/kg)	38.82±1.54##	12.81±1.86##	8.43±0.47##	25.54±3.27##
GEN (80 mg/kg)	35.62±1.49##	10.19±1.05##	6.67±0.66##	22.51±3.03##

**P<0.01, compared with the control group; ##P<0.01, compared with the SCI group. Sham, sham group; SCI, spinal cord injury group; GEN (20), geniposide (20 mg/kg)-treated group; GEN (40), geniposide (40 mg/kg)-treated group and GEN (80), geniposide (80 mg/kg)-treated group. GEN, geniposide; NF-κB, nuclear factor κB; IL, interleukin.

gel image analysis system (Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis

The data were presented as the mean ± standard deviation and were analyzed using SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). The statistical comparisons of BBB scores, water content, the serum activities of inflammatory factors and protein expression of apoptosis were performed using one-way analysis of variance (ANOVA) followed by Dunnett's test. P<0.05 was considered to be statistically significant.

Results

Evaluation of neural function

The chemical structure of GEN is shown in **Figure 1**. It was noted that BBB scores in the sham group were 19.28±0.89, 19.23±0.82 and 19.61±0.89 at 24, 48 and 72 h post surgery, respectively, as summarized in **Table 1**. By contrast, the SCI group rats demonstrated severe neurological impairment with marked reductions in BBB scores (2.12±0.18, 3.46± 0.47 and 4.64±0.61) at the selected time points. However, GEN at doses of 20, 40 and 80 mg/kg significantly improved neurological function (P<0.01) in injured animals, compared with the SCI model group, particularly at 72 h post-surgery (**Table 1**). Thus, this time point was selected for subsequent investigations.

Assessment of GEN on water content in spinal cord tissues following SCI

As shown in **Figure 2**, there was a marked elevation in water content of the spinal cord (P<0.01) in the SCI group compared with the sham group. Following treating SCI-induced rats with GEN, the water content in spinal cord tissues was significantly decreased in a dose dependent manner (P<0.01) compared with that in the control group.

Measurement of GEN on inflammatory response following SCI

To determine the anti-inflammatory effect of mangiferin on SCI, the serum activities of NF-κB p65 unit, IL-4, IL-6 and IL-10 were analyzed in the present study. The results revealed that SCI induced the inflammatory reaction and increased the serum activities of NF-κB p65 unit, IL-4, IL-6 and IL-10 in the SCI model rat group, compared with those of the control group. However, these inflammatory factors were reduced in the GEN-treated (20, 40 and 80 mg/kg) groups, compared with those in the SCI model group (**Table 2**).

Detection of GEN on cellular apoptosis following SCI

In order to determine the effect of GEN on cellular apoptosis following SCI, the protein expression of apoptosis regulated proteins, including Bcl-2, Bax, Caspase-3, Caspase-6 and Caspase-7, which were detected by western blot analysis. As shown in **Figure 3**. Bcl-2 and Bax exhibited specific bands of 26 and 23 kDa, respectively. Following one way ANOVA analysis, there was evident decreases in Bcl-2 expression and increases in Bax expression following SCI, versus the control (P<0.01). GEN treatment to the SCI-induced rats increased the expression of Bcl-2 and decreased Bax at the protein level in a dose-dependent manner (**Figure 3**). Meanwhile, Caspase-3, Caspase-6 and Caspase-7 exhibited specific bands of 35, 18 and 30 kDa, respectively. Following one way

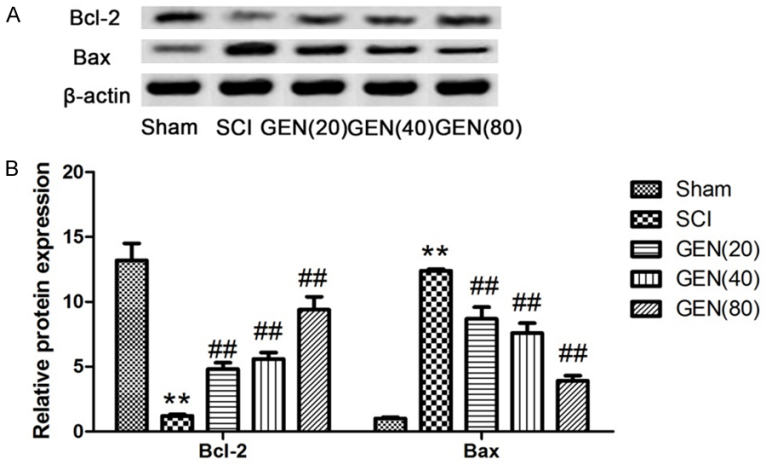

Figure 3. Geniposide alters the expression levels of Bcl-2 and Bax. A. The effects of mangiferin on the expression levels of Bcl-2 and Bax were determined using western blot analysis. B. Statistical analysis for quantification of the protein levels of Bcl-2 and Bax in SCI model rats. Data are presented as the mean ± standard deviation. ***P*<0.01, compared with the control group; ##*P*<0.01, compared with the SCI group. Sham, sham group; SCI, spinal cord injury group; GEN (20), geniposide (20 mg/kg)-treated group; GEN (40), geniposide (40 mg/kg)-treated group and GEN (80), geniposide (80 mg/kg)-treated group. GEN, geniposide.

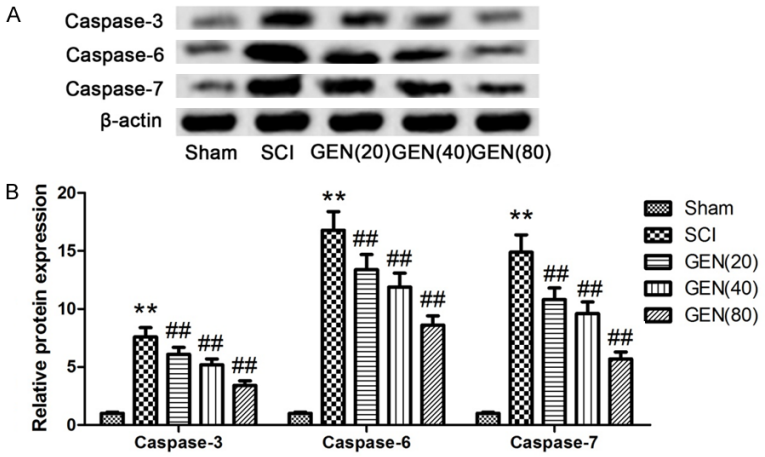

Figure 4. Geniposide alters the expression levels of Caspase-3, Caspase-6 and Caspase-7. A. The effects of mangiferin on the expression levels of Caspase-3, Caspase-6 and Caspase-7 were determined using western blot analysis. B. Statistical analysis for quantification of the protein levels of Caspase-3, Caspase-6 and Caspase-7 in SCI model rats. Data are presented as the mean ± standard deviation. ***P*<0.01, compared with the control group; ##*P*<0.01, compared with the SCI group. Sham, sham group; SCI, spinal cord injury group; GEN (20), geniposide (20 mg/kg)-treated group; GEN (40), geniposide (40 mg/kg)-treated group and GEN (80), geniposide (80 mg/kg)-treated group. GEN, geniposide.

ANOVA analysis, there was evident increases in Caspase-3, Caspase-6 and Caspase-7 expression following SCI (*P*<0.01), versus the control. GEN treatment to the SCI decreased Bax at the protein level in a dose-dependent manner (**Figure 4**). Besides, HE staining and Bax immunohistochemical staining were observed in the Supplementary Figure 1.

Discussion

Spinal cord injury (SCI) is characterized by high morbidity rates with serious complications and difficult to treat, causing rigorous of significant economic and social burdens for individuals, families and the communities [18]. In the present study, GEN significantly improved BBB scores and reduced the water content of the spinal cord in the SCI rats, and the neuro-protective mechanism might be associated with the regulation of suppressing inflammatory response and the Bcl2/Bax pathway.

Inflammation is a common pathologic process in neurodegeneration in the central nervous system diseases and injuries. According to the type of trauma and the pathophysiological changes, SCI could be divided into the primary spinal cord injury and the secondary spinal cord injury. Inflammation plays an important role in the consequent secondary spinal cord injury [19]. NF-κB in glial cells, neural cells and vascular endothelial cells can be activated in acute SCI generally, and the early activation of NF-κB regulates the expression levels of a series of immune and inflammatory-associated genes at the transcriptional level, sensitizing a variety of inflammatory factors [20]. Therefore, inhibiting the expression of NF-κB activity is the key point to the suppression of inflammatory response, which may reduce the nuero-damage from the secondary spinal cord injury [21]. Interleukin-

(IL-)4, an inflammatory cytokine with a great variety of biological activities, played an important role in the inflammatory response and immune regulation. IL-4 might be up-regulated in acute SCI according to the research report [22]. IL-6 and IL-10 were also typical inflammatory cytokines in the pathologic process of SCI, further promoted the inflammatory response and increased the promotion of nerve damage [23, 24]. In addition, results from the current study demonstrated that GEN effectively decreased the activity of NF-κB p65, IL-4, IL-6 and IL-10, which implies that the protective role of GEN against SCI may be associated with the inhibition of inflammatory response.

The secondary lesion induced by SCI is harmful to the spinal neurons, which may also trigger the apoptotic cascades in vivo. Therefore, the pharmacological inhibition of apoptosis may be considered as a potential therapeutic strategy in treatment of SCI. Report has been demonstrated that targeted retrograde gene delivery of neurotrophic cytokine can suppress the cellular apoptosis and restore neurological function following spinal cord injury [25]. Bcl-2 family proteins have been proved to play a vital function in cellular apoptosis process. Under normal physiological circumstances, Bcl-2 itself serves as an anti-apoptotic protein, whereas another member of the family, Bax, acts as an apoptosis-inducing protein molecule [26]. These results of the present study demonstrated that the evident reduction of Bcl-2 and the increase of Bax protein expression were analyzed in the spinal cord tissues via western blot method. However, treatment with GEN dose dependently caused elevated levels of Bcl-2 and reduced Bax protein in SCI rats. In addition, the activities of caspase-3, caspase-6 and caspase-7, important executioner molecules played a key part in the apoptotic signaling pathway, were firstly found to be markedly elevated in rats following SCI and GEN could significantly inhibited this index. In summary, these findings indicated that the neuro-protective effects of GEN may involve in the suppression of inflammatory response and the regulation of Bcl-2/ Bax and caspase-3, 6, 7 pathways in the spinal cord following SCI.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yudan Yang, Department of Central Laboratory, China-Japan Union Hospital of Jilin University, Changchun 130033, China. Tel: 86-431-84995382; Fax: 86-431-84995382; E-mail: yangyudan@jlu.edu.cn

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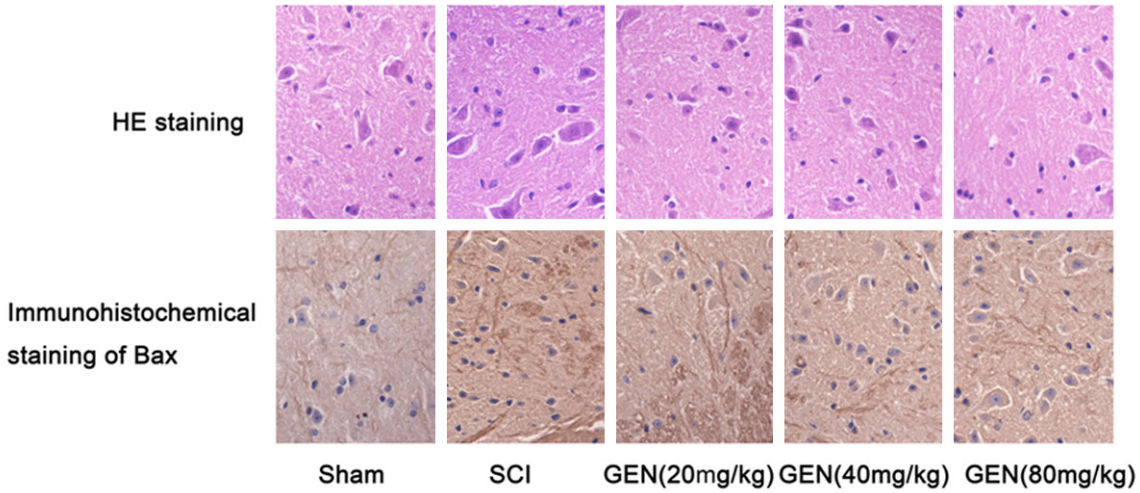

Supplementary Figure 1. HE staining and Bax immunohistochemical staining in the spinal cord injury at 30 days following SCI (Inverted phase contrast microscope × 40).