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The mechanism of astragaloside IV promoting sciatic nerve regeneration

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Research Highlights

(1) Astragaloside IV, the main component of the traditional Chinese medicine astragalus membranaceus, has been shown to exert neuroprotective effects, but studies regarding this compound are limited.
(2) This study assumed that astragaloside IV promoted the repair of sciatic nerve injury, and observed whether its mechanism of action was influenced by growth-associated protein-43 expression.
(3) Astragaloside IV accelerated nerve myelin sheath growth in mice with sciatic nerve injury, increased the diameter and number of myelinated nerve fibers, and elevated and accelerated pheral nerve regeneration and functional reconstruction by upregulating growth-associated tein-43 expression.

Abstract

3-O-beta-D-xylopyranosyl-6-O-beta-D-glucopyranosyl-cycloastragenol (astragaloside IV), the main active component of the traditional Chinese medicine astragalus membranaceus, has been shown to be neuroprotective. This study investigated whether astragaloside IV could promote the repair of injured sciatic nerve. Denervated sciatic nerve of mice was subjected to anastomosis. The mice were intraperitoneally injected with 10, 5, 2.5 mg/kg astragaloside IV per day for 8 consecutive days. Western blot assay and real-time PCR results demonstrated that growth-associated protein-43 expression was upregulated in mouse spinal cord segments L_{4-6} after intervention with 10, 5, 2.5 mg/kg astragaloside IV per day in a dose-dependent manner. Luxol fast blue staining and electrophysiological detection suggested that astragaloside IV elevated the number and diameter of myelinated nerve fibers, and simultaneously increased motor nerve conduction velocity and action potential amplitude in the sciatic nerve of mice. These results indicated that astragaloside IV contributed to sciatic nerve regeneration and functional recovery in mice. The mechanism underlying this effect may be associated with the upregulation of growth-associated protein-43 expression.

Key Words

neural regeneration; traditional Chinese medicine; peripheral nerve injury; astragaloside IV; growth-associated protein-43; sciatic nerve; nerve myelin sheath; myelinated nerve; axons; neuroregeneration

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Conflicts of interest: None declared.

Ethical approval: This study was approved by the Animal Ethics Committee of Jilin University in China.

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INTRODUCTION

The outcomes of conventional surgical and conservative medical treatment for peripheral nerve injury have been disappointing[1-2]. Recent studies confirmed that natural medicine can stimulate nerve growth factor expression after nerve injury, and promote peripheral nerve regeneration and functional recovery[2-9]. These results indicated that natural medicine could be a new method for promoting the repair of peripheral nerve injury.

3-O-beta-D-xylopyranosyl-6-O-beta-D-glucopyranosyl-cycloastragenol, astragaloside IV, is a major active component of the traditional Chinese medicine *astragalus membranaceus*, and its structure is shown in Figure 1.

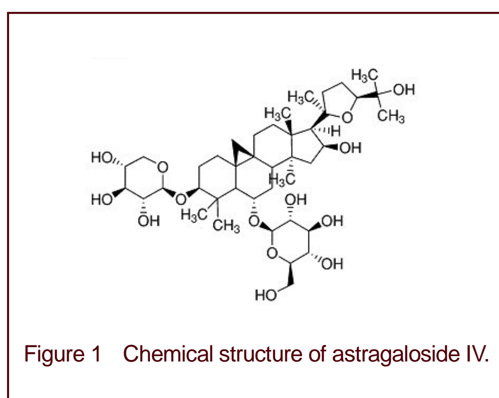

Figure 1 Chemical structure of astragaloside IV.

Astragaloside IV can inhibit inflammation, oxidation, and apoptosis, and exerts immunoregulatory effects. This compound also protects the heart, intestines and stomach[10-11]. In the central nervous system, astragaloside IV has contributed to nerve repair after cerebral ischemia/reperfusion injury[8], accelerated axon growth in mouse hippocampi, prevented neuronal atrophy and memory loss[12], and suppressed spontaneous synaptic conduction and Ca^{2+} oscillation in hippocampal neurons[10]. Astragaloside IV also protected primary cultured nigral cells against 6-hydro- xydopamine-induced injury[13]. Additionally, astragaloside IV combined with ginsenoside prevented oxidative damage of adult PC 12 cells and protected mitochondrial membrane potential[14]. In the peripheral nervous system, astragaloside IV also can protect and promote peripheral nerve regeneration[1-2].

Neural growth-associated protein 43 guides neuronal growth and branching during neural development and regeneration, possibly inducing changes in the presynaptic membrane and neurotransmitter release, thus promoting long-term potentiation, spatial memory and learning[15-17].

Previous studies suggested that growth- associated protein 43 expression gradually increased in the corpus striatum and ipsilateral ischemic cortex after cerebral ischemia/ reperfusion. This increase in the ischemic hemisphere occurred to different extents[16-27].

This study supposed that astragaloside IV contributed to the repair of sciatic nerve injury, and its mechanisms were associated with the upregulation of growth-associated protein 43 that is involved in axonal regeneration. This study observed the effects of astragaloside IV on peripheral nerve regeneration and functional recovery in a mouse model of sciatic nerve injury, and also determined the expression of growth-associated protein 43 during peripheral nerve regeneration. The mechanism of action of astragaloside IV on peripheral nerve regeneration and functional recovery was also investigated.

RESULTS

Quantitative analysis of experimental animals

A total of 169 BALB/c mice were used in the study, with nine dying during anesthesia. The remaining 160 mice were used to establish a model of sciatic nerve injury by denervating the left sciatic nerve. After model induction, mice were equally and randomly assigned to model, high-, moderate-, or low-dose astragaloside IV groups, and were intraperitoneally injected with saline 1 mL, or 10, 5, or 2.5 mg/kg astragaloside IV per day, respectively, for 1, 2, 4, and 8 consecutive weeks. Ten mice from each group were used at each time point. One hundred and sixty mice were included in the final analysis.

Astragaloside IV accelerated the recovery of sciatic nerve function in mice

Electrophysiology results suggested that at 1, 2, 4, and 8 weeks after sciatic nerve injury, sciatic nerve conduction amplitude and velocity were gradually increased in each group. The recovery of sciatic nerve was significantly better in the high- and moderate-dose astragaloside IV groups than that in the low-dose astragaloside IV and model groups (P < 0.05; Tables 1, 2).

Table 1 Effects of astragaloside IV on sciatic nerve conduction amplitude (mV) in mice with sciatic nerve injury

Group	Post-injury (week)			
	1	2	4	8
Astragaloside IV				
High-dose	2.08±0.21[ab]	4.42±0.15[ab]	24.98±0.15[ab]	26.11±0.91[ab]
Moderate-dose	2.07±0.18[ab]	4.29±0.17[ab]	21.28±0.23[ab]	22.14±0.29[ab]
Low-dose	1.32±0.13	2.51±0.05	16.66±0.30	18.71±0.30
Model	1.19±0.06	2.17±0.14	12.19±0.06	16.78±0.71

[a]P < 0.05, *vs.* model group; [b]P < 0.05, *vs.* low-dose astragaloside IV group. Data are expressed as mean ± SD, n = 10 mice at each time point (one-way analysis of variance and least significant difference t-test). High-, moderate- and low-dose astragaloside IV groups were intraperitoneally injected with 10, 5 and 2.5 mg/kg per day, respectively.

Table 2 Effects of astragaloside IV on motor nerve conduction velocity (m/s) in mice with sciatic nerve injury

Group	Post-injury (week)			
	1	2	4	8
Astragaloside IV				
High-dose	19.96±0.32[ab]	41.43±0.57[ab]	65.31±1.58[ab]	64.11±1.42[ab]
Moderate-dose	18.73±0.31[ab]	34.22±0.72[a]	54.39±1.13[ab]	59.29±0.63[ab]
Low-dose	12.88±0.19	31.98±0.89[a]	51.07±0.63	52.86±0.29[a]
Model	10.41±0.18	26.71±0.56	48.22±0.29	44.18±2.44

[a]P < 0.05, *vs.* model group; [b]P < 0.05, *vs.* low-dose astragaloside IV group. Data are expressed as mean ± SD, n = 10 mice at each time point (one-way analysis of variance and least significant difference t-test). High-, moderate- and low-dose astragaloside IV groups were intraperitoneally injected with 10, 5 and 2.5 mg/kg per day, respectively.

Astragaloside IV upregulated growth-associated protein 43 mRNA expression in spinal cord segments L_{4-6} of mice with sciatic nerve injury

Real-time PCR revealed that growth-associated protein 43 mRNA contents gradually increased in corresponding spinal cord segments in the low-dose astragaloside IV and model groups. This mRNA expression peaked at 4 weeks after sciatic nerve injury, and decreased at 8 weeks. Compared with the low-dose astragaloside IV and model groups, growth-associated protein 43 mRNA expression significantly increased in the high-dose and moderate-dose astragaloside IV groups (P < 0.05). Growth- associated protein 43 expression peaked at 1 week following sciatic nerve injury, and decreased at 8 weeks. The upregulation of growth-associated protein 43 mRNA expression was most significant in the high-dose astragaloside IV group (Figure 2).

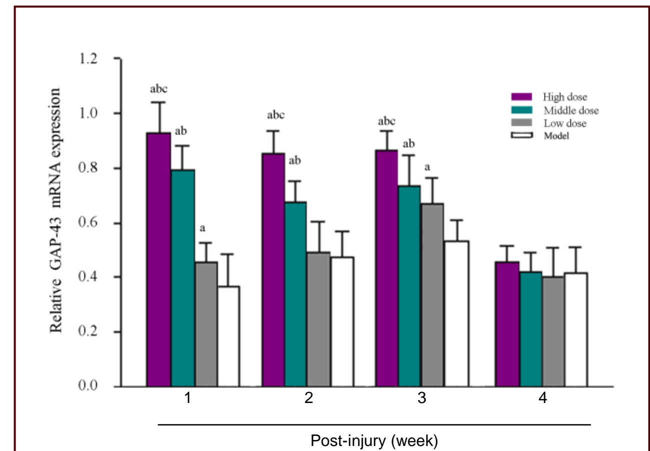

Figure 2 Effects of astragaloside IV on growth-associated protein 43 (GAP-43) mRNA expression in spinal cord segments L_{4-6} in mice with sciatic nerve injury.

High-, moderate- and low-dose astragaloside IV groups were intraperitoneally injected with 10, 5 and 2.5 mg/kg per day, respectively. The measurement data were expressed as the Ct ratio of growth-associated protein 43/ glyceraldehyde-phosphate dehydrogenase (GAPDH). Data are expressed as mean ± SD. Ten mice from each group were used at each time point. [a]P < 0.05, *vs.* model group; [b]P < 0.05, *vs.* low-dose astragaloside IV group; [c]P < 0.05, *vs.* moderate-dose astragaloside IV group. One-way analysis of variance and least significant difference t-test were used.

Astragaloside IV upregulated growth-associated protein 43 protein expression in spinal cord segments L_{4-6} of mice with sciatic nerve injury

Western blot assay results revealed that growth-associated protein 43 protein expression reached a peak in each group at 1 week after sciatic nerve injury, and then gradually decreased. Compared with the model group, growth-associated protein 43 protein expression was significantly higher in the high-dose and moderate-dose astragaloside IV groups at various time points (P < 0.05). Moreover, growth-associated protein 43 protein expression was also significantly upregulated in the low-dose astragaloside IV group at 1, 2 and 4 weeks after injury (P < 0.05). No significant difference in growth-associated protein 43 protein expression was detectable between the low-dose astragaloside IV group and model group at 8 weeks. Growth-associated protein 43 protein expression was significantly greater in the high-dose and moderate-dose astragaloside IV groups than that in the low-dose astragaloside IV group at various time points after injury (P < 0.05), and the upregula-

tion was most significant in the high-dose astragaloside IV group (Figure 3, Table 3).

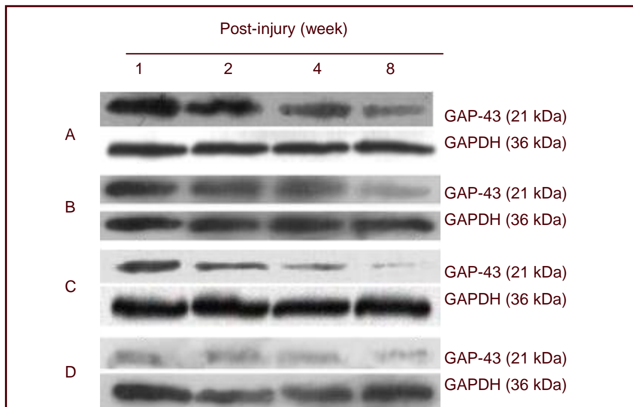

Figure 3 Effects of astragaloside IV on growth-associated protein 43 protein expression in spinal cord segments $L_{4–6}$ in mice with sciatic nerve injury.

10, 5 and 2.5 mg/kg per day astragaloside IV were used. Compared with the model group (injected with saline, D), high, moderate and low-dose astragaloside IV (A, B, C) can upregulate growth-associated protein 43 protein expression in spinal cord segments $L_{4–6}$ in mice with sciatic nerve injury.

Table 3 Effects of astragaloside IV on growth-associated protein 43 protein expressions in spinal cord segments $L_{4–6}$ in mice at various time points after sciatic nerve injury

Group	Post-injury (week)			
	1	2	4	8
Astragaloside IV				
High-dose	0.59±0.02^{ab}	0.46±0.03^{ab}	0.27±0.03^{a}	0.21±0.04^{ab}
Moderate-dose	0.51±0.02^{ab}	0.43±0.02^{ab}	0.37±0.01^{ab}	0.21±0.02^{ab}
Low-dose	0.41±0.03^{a}	0.34±0.04^{a}	0.32±0.03^{a}	0.12±0.02
Model	0.27±0.03	0.20±0.02	0.19±0.02	0.12±0.02

Gray value ratio of growth-associated protein 43/reduced glyceraldehyde-phosphate dehydrogenase (GAPDH) was expressed as mean ± SD. High grayscale ratio indicates a high level of growth-associated protein 43 protein expression. Ten mice were designated for each time point in each group. $^{a}P < 0.05$, *vs.* model group; $^{b}P < 0.05$, *vs.* low-dose astragaloside IV group (n = 10). One-way analysis of variance and least significant difference *t*-test were used. High-, moderate- and low-dose astragaloside IV groups were intraperitoneally injected with 10, 5 and 2.5 mg/kg per day, respectively.

Astragaloside IV promoted the remyelination of sciatic nerve in model mice

Luxol fast blue staining revealed the structure of the myelin sheath around mouse sciatic nerves. Function of the sciatic nerve was mostly recovered at 8 weeks after sciatic nerve injury; therefore, sciatic nerve samples were stained with Luxol fast blue at 8 weeks after injury to observe the effects of astragaloside IV on the sciatic nerve myelin sheath. The sciatic nerve myelin sheath was found to be regular and uniform, with an obvious outline in the high-dose and moderate-dose astragaloside IV groups (Figure 4A, B). By contrast, the sciatic nerve myelin sheath was irregular, with a clear outline in the low-dose astragaloside IV group. Furthermore, fibrous connective tissue hyperplasia was visible among nerve bundles (Figure 4C). The sciatic nerve myelin sheath was irregular, and fibrous connective tissue hyperplasia was also apparent in the model group (Figure 4D). Compared with the model group, sciatic nerve myelin sheath regeneration was clearly promoted in the high-dose and moderate-dose astragaloside IV groups, especially in the high-dose astragaloside IV group.

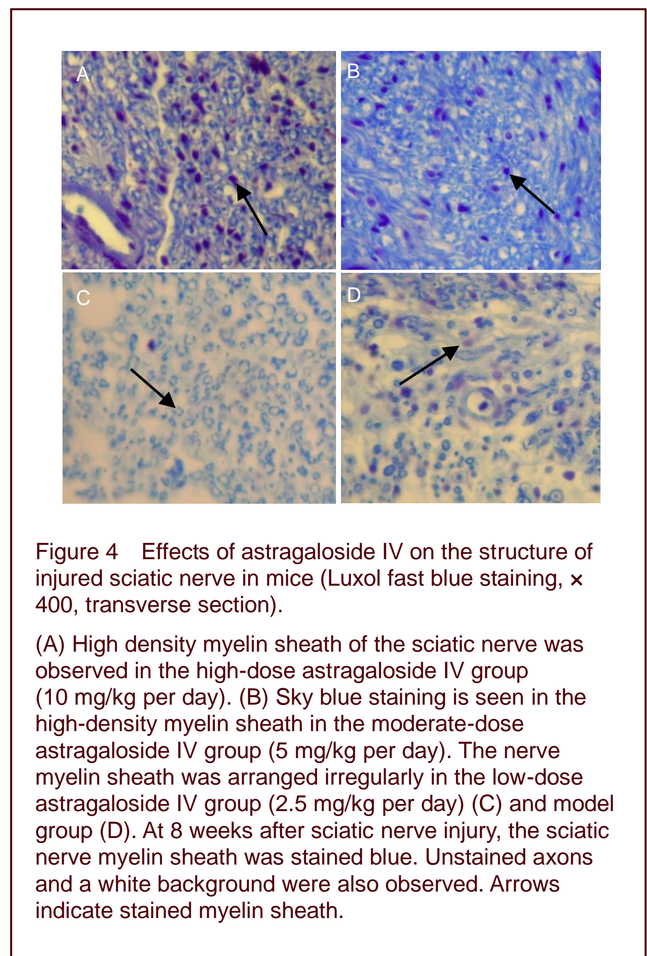

Figure 4 Effects of astragaloside IV on the structure of injured sciatic nerve in mice (Luxol fast blue staining, × 400, transverse section).

(A) High density myelin sheath of the sciatic nerve was observed in the high-dose astragaloside IV group (10 mg/kg per day). (B) Sky blue staining is seen in the high-density myelin sheath in the moderate-dose astragaloside IV group (5 mg/kg per day). The nerve myelin sheath was arranged irregularly in the low-dose astragaloside IV group (2.5 mg/kg per day) (C) and model group (D). At 8 weeks after sciatic nerve injury, the sciatic nerve myelin sheath was stained blue. Unstained axons and a white background were also observed. Arrows indicate stained myelin sheath.

The number and diameter of myelinated nerve fibers were significantly higher in the high-, moderate- and low-dose astragaloside IV groups when compared with the model group ($P < 0.05$). The number and diameter of myelinated nerve fibers were significantly greater in the high-dose and moderate-dose astragaloside IV groups compared with the low-dose astragaloside IV group ($P < 0.05$). No significant difference in the number and diameter of myelinated nerve fibers was detected between the high-dose and moderate-dose astragaloside IV groups (Table 4).

Table 4 Effects of astragaloside IV on the number and diameter of myelinated nerve fibers in mice at 8 weeks after sciatic nerve injury

Group	The number of myelinated nerve fibers (n/mm^2)	Diameter of myelinated nerve fiber (μm)
Astragaloside IV		
High-dose	75±3^{ab}	2.47±0.31^{ab}
Moderate-dose	71±1^{ab}	2.31±0.16^{ab}
Low-dose	56±4^{a}	1.99±0.32^{a}
Model	50±2	1.57±0.26

Data are expressed as mean ± SD (n = 10). ^aP < 0.05, *vs.* model group; ^bP < 0.05, *vs.* low-dose astragaloside IV group. One-way analysis of variance and least significant difference t-test were used. High-, moderate- and low-dose astragaloside IV groups were intraperitoneally injected with 10, 5 and 2.5 mg/kg per day, respectively.

DISCUSSION

Astragaloside IV promotes neural regeneration or prevents neural injury through various mechanisms[1, 9-14]. Growth-associated protein 43 is highly expressed in individual neurons during development and adult cerebral hippocampal neurons[28-31]. Growth-associated protein 43 is a sensitive specific molecular marker present during nerve injury and repair, and plays an important role in nerve growth, development and regeneration[32-35]. During axon regeneration in the central nervous system, growth-associated protein 43 expression is significantly upregulated, which clearly promotes neural regeneration and repair[36-41]. Thus, this study established a mouse model of sciatic nerve injury, and observed the effects of astragaloside IV on peripheral nerve regeneration, functional recovery, and growth-associated protein 43 expression during peripheral nerve regeneration.

In this study, Luxol fast blue staining was used to determine the changes in sciatic nerve myelin sheath structure, and results revealed that high-dose and moderate-dose astragaloside IV accelerated the growth of myelin sheath surrounding injured sciatic nerve, increased the number and diameter of myelinated nerve fibers, contributed to neural regeneration and structural reconstruction, and elevated the quality of regenerating nerve. Electrophysiological detection suggested that regenerating sciatic nerve had good structure at 8 weeks, showing good motor nerve conduction velocity and action potential amplitude. Results of electrophysiological detection completely coincided with the results of Luxol fast blue staining.

Growth-associated protein 43 expression was lower in spinal cord segments L_{4-6} from normal Balb/c mice[42-47], active in growing neuronal axons, and strongly associated with neuronal axon growth[34, 48-50]. In the present study, western blot assay and real-time PCR results demonstrated that growth-associated protein 43 expression was activated and highly expressed in corresponding spinal cord segments of mice with sciatic nerve injury. Growth-associated protein 43 expression was significantly greater in the high-dose and moderate-dose astragaloside IV groups than in the low-dose astragaloside IV group and model group within 4 weeks. These results suggested that high-dose and moderate-dose astragaloside IV apparently upregulated growth-associated protein 43 expression in regenerating nerve tissues. Moreover, this promoting effect could last at least 4 weeks. At 8 weeks, growth-associated protein 43 expression was identical between high- and moderate-dose astragaloside IV groups and the model group. However, astragaloside IV promoted the upregulation of growth- associated protein 43 expression for up to 4 weeks, so the structure and function of the sciatic nerve in the astragaloside IV groups were better than those in the model group. Gerin *et al* [37] confirmed that endogenous repair genes including growth-associated protein 43 are persistently expressed for 12 weeks following spinal cord injury. Therefore, this study presumed that growth-associated protein 43 was probably persistently expressed in the 4 weeks following insult in each group[46], which contributed to the regeneration and repair of sciatic nerve, but was further stimulated by the treatment with astragaloside IV.

Appropriate expression of growth-associated protein 43 promotes neural recovery and regeneration, but excessive expression can contribute to inflammatory reaction and scar formation, and suppress myelin sheath growth[2, 51-57]. In this study, upregulation of growth-associated protein 43 expression promoted peripheral nerve regeneration, indicating that high-dose and moderate-dose astragaloside IV was in an appropriate range, all of which contributed to growth-associated protein 43 expression and exerted an accelerated regulatory effect on neural regeneration. In the high-dose and moderate-dose astragaloside IV groups, growth-associated protein 43 mRNA maintained a stable high expression at 1, 2 and 4 weeks, and showed a decreased expression at 8 weeks. Growth-associated protein 43 protein expression peaked at 1 week, and then gradually diminished. The expression of mRNA and protein showed a certain asynchrony, indicating that there was endogenous translational control or

post-translational control. High levels of growth-associated protein 43 protein may have been upregulated to avoid neural injury. The promoting effect of astragaloside IV on neural regeneration possibly has multiple molecular mechanisms[1, 8-10, 13, 14]. This study also presumed that astragaloside IV had a persistent promoting effect (at least for 4 weeks) on activation of growth-associated protein 43 in the spinal cord segments $L_{4–6}$ after sciatic nerve injury, resulting in a positive regulatory effect on peripheral nerve regeneration and functional recovery.

In summary, astragaloside IV elevated and accelerated peripheral nerve regeneration and functional reconstruction, which is associated with its regulatory effect on growth-associated protein 43 expression.

MATERIALS AND METHODS

Design
A randomized, controlled animal study.

Time and setting
Experiments were performed at the National Key Laboratory, Central Laboratory, China-Japan Union Hospital, Jilin University, China from November 2010 to May 2011.

Materials
Animals
Healthy clean male BALB/c mice, aged 8 weeks old and weighing 20 ± 2 g, were provided by the Experimental Animal Center, School of Basic Medicine, Jilin University, China (license No. SCXK (Ji) 2007-0001). The protocols were conducted in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by Ministry of Science and Technology of China[58].

Drugs
Astragaloside IV was purchased from the National Institute for Food and Drug Control in China. Astragaloside IV was dissolved using dimethyl sulfoxide to create a stock solution of 10 g/L, and diluted with saline to working concentrations[59].

Methods
Preparation of a mouse model of sciatic nerve injury
BALB/c mice were intraperitoneally anesthetized with 1% sodium thiopental, and fixed in the prone position. Under aseptic conditions, a 2-cm longitudinal incision was made at the posterior thigh of the left hindlimb. The sciatic nerve was exposed at the inferior border of the piriform muscle. The trunk and surrounding tissues of the sciatic nerve were bluntly separated using a glass needle. The sciatic nerve was completely denervated at 0.5 cm below the ischial tuberosity. Ten minutes later, the sciatic nerve was anastomosed using 11/0 sutures under a 12-fold microscope (Surgery Microinstrument Factory, Zhenjiang, Jiangsu Province, China), and muscle and skin were separately sutured closed (Figure 5)[60].

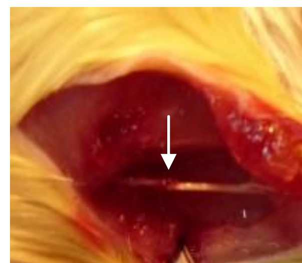

Figure 5 Injured sciatic nerve of a mouse.
Arrow shows sciatic nerve and the denervated site.

Drug intervention
The dose of astragaloside IV was consistent with the amount of original astragaloside IV administered clinically, and was found to be equivalent to the intraperitoneal dose of 5 mg/kg per day. This amount was considered a moderate dose. 10 mg/kg per day and 2.5 mg/kg per day were considered high and low doses, respectively. Administration was performed immediately after model induction. Mice in the high-, moderate- and low-dose astragaloside IV groups were intraperitoneally injected with 10, 5, 2.5 mg/kg astragaloside IV per day[59] (dissolved in dimethyl sulfoxide, diluted in saline), respectively. Mice in the model group were given 1 mL/d saline. Drug administration was consecutively performed until experiments were complete or samples were collected.

Neuroeletrophysiology for the recovery of sciatic nerve function in mice after astragaloside IV intervention
Bilateral sciatic nerves of mice were investigated using a Medtronic Keypoint electromyograph machine (Medtronic Corporation, Minneapolis, MN, USA) at 1, 2, 4 and 8 weeks after model establishment. Room temperature was maintained at 24°C. Animals were intraperitoneally anesthetized with 1% sodium triopental. After disinfection, the sciatic nerve was exposed in a prone position. Concentric needle electrodes were placed in the mouse soleus muscle as a recording electrode (M point). A ground electrode was placed in the mouse tail. Super-strong stimuli (current 10 mA) were given at the level of the

ischial tuberosity proximal to the anastomotic stoma (P point) and the branch of the distal sciatic nerve (D point) using double head stimulating electrodes (the distance between the two electrodes was 2 mm). The distance between the electrodes P-D was measured using a vernier caliper to calculate the difference of action potential latency, which represents the time for the nerve impulse to traverse the two points. Motor nerve conduction velocity of sciatic nerve was equal to the distance of P-D/difference of action potential latency (m/s). Simultaneously, motor nerve action potential amplitude of the sciatic nerve (mV) was measured using the vernier caliper.

Sample collection

A total of 10 mice from each group were obtained at 1, 2, 4 and 8 weeks after injury. They were intraperitoneally anesthetized with 1% sodium thiopental. A median incision was made at the posterior spine, and the vertebral canal was opened with rongeur forceps. Spinal cord segments $L_{4–6}$, connected to the sciatic nerve, were intact and exposed (Figure 6). Spinal cord segments $L_{4–6}$ from the injured side were obtained, marked and rapidly immersed in liquid nitrogen for western blot assay and real-time PCR. In addition, sciatic nerve trunk from anastomotic stoma (0.5 cm, including the anastomotic stoma) to the distal end was obtained and fixed in 10% neutral formalin over 72 hours, dehydrated through a graded alcohol series, embedded in paraffin, and sliced into 2-μm-thick sections.

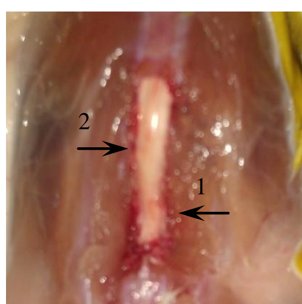

Figure 6 Morphology of mouse spinal cord segments $L_{4–6}$ after high-dose astragaloside IV intervention at 8 weeks following sciatic nerve injury.

Arrows show anastomotic stoma (1) and distal nerve trunk (2).

Real-time PCR for growth-associated protein 43 mRNA expression in spinal cord segments $L_{4–6}$ of mice

Growth-associated protein 43 and reduced glyceraldehyde-phosphate dehydrogenase (GAPDH) primers were designed using Beacon designer 7 software (PREMIER Biosoft, Palo Alto, CA, USA) in accordance with NCBI Genbank (http://www.ncbi.nlm.nih.gov/genbank/). Primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd., Shanghai, China. Primer sequences are listed in Table 5.

Table 5 Primer sequences of GAP-43 and GAPDH

Primer name		Sequence	Product size (bp)
GAP-43	Upstream	5′-GCC TAA ACA AGC CGA TGT GC-3′	276
	Downstream	5′-TTC GTC TAC AGC GTC TTT CTC C-3′	
	Probe	5′-TGC TGC TGT CAC TGA TGC TGC TGC-3′	
GAPDH	Upstream	5′-AAT GTG TCC GTC GTG GAT CTG-3′	462
	Downstream	5′-CAA CCT GGT CCT CAG TGT AGC-3′	
	Probe	5′-CGT GCC GCC TGG AGA AAC CTG CC-3′	

GAP-43: Growth-associated protein-43; GAPDH: reduced glyceraldehyde-phosphate dehydrogenase.

Spinal cord segments $L_{4–6}$ were obtained at various time points. Total RNA was extracted using an extraction kit [Sangon Biotech (Shanghai) Co., Ltd.] using the TRIzol reagent method. First-strand cDNA was synthesized with a reverse transcription kit (M-MLV RTase Kit, Promega, Beijing, China), and cDNA was synthesized further. cDNA served as a template. Real-time PCR was used to amplify growth-associated protein 43 in the spinal cord segments $L_{4–6}$. A 2 × SYBR real-time quantitative PCR kit was purchased from Roche (Beijing, China). Reaction conditions were as follows: 95°C for 30 seconds, 58°C for 60 seconds, and 72°C for 60 seconds, 40 cycles in total. GAPDH served as an internal reference. Stratagene Mx3000P Real-Time QPCR System (Agilent Technologies, Beijing, China) was used to detect the Ct values of growth-associated protein 43 and internal reference gene (Stratagene MxPro QPCR Software, Agilent Technologies, Beijing, China). Relative mRNA expression of growth-associated protein 43/GAPDH was calculated.

Western blot assay for growth-associated protein 43 protein expression in the spinal cord segments $L_{4–6}$ of mice

Spinal cord segments $L_{4–6}$ were obtained at various time points and triturated in a mortar with RIPA lysis buffer. Total protein was extracted with an extraction kit (Beyotime Institute of Biotechnology, Nanjing, Jiangsu Province, China). After adding loading buffer, samples were boiled in water for 15 minutes. After centrifugation, the supernatant was obtained and electrophoresed on 12% sodium dodecyl sulfate-polyacrylamide gel, and electrotransferred onto polyvinylidene fluoride membrane using

the wet method[9]. The membrane was immersed in rabbit anti-mouse growth-associated protein 43 or GAPDH antibody (1:1 000; Roche, Shanghai, China) at 4°C overnight. After four washes with 0.01 mol/L PBS (each for 5 minutes), the membrane was incubated in goat anti-rabbit IgG (1:10 000; Roche) at room temperature for 1 hour. After four washes with 0.01 mol/L PBS (each for 5 minutes), the membrane was visualized in accordance with the instructions from the enhanced chemiluminescence kit (Roche). X-Ray film was exposed and analyzed using a gel image processing system (UVP EC3 600 Imaging System, UVP LLC, Upland, CA, USA). Gray values of target blots were analyzed with an image processing system using VisionWorksLS software, and relative gray values (growth-associated protein-43/GAPDH) were calculated.

Luxol fast blue staining for the diameter and number of myelinated nerve fibers in mouse sciatic nerve

Sciatic nerve trunk from the anastomotic stoma (0.5 cm, including the anastomotic stoma) to the distal end was obtained and fixed in 10% neutral formalin for > 72 hours, dehydrated through a graded alcohol series, embedded in paraffin, and sliced into sections of 3.0 μm. After deparaffinization, the samples were immersed in Luxol fast blue staining solution at 60°C for 12 hours, then subsequently immersed in 95% alcohol for 5 minutes, and treated with 0.05% lithium carbonate for 15 seconds. After a wash with 70% alcohol, the samples were washed with distilled water, dehydrated, permeabilized, mounted, observed and photographed with a light microscope (Eclipse TE-2000-U, Nikon, equipped with an attached digital camera SXM1200F, Tokyo, Japan). The diameter and number of myelinated nerve fibers were determined using an image scanner (Olympus, Tokyo, Japan).

Statistical analysis

Measurement data were expressed as mean ± SD and analyzed using SPSS 17.0 software (SPSS, Chicago, IL, USA). Mean difference among multiple groups was compared using one-way analysis of variance. Paired comparison of intergroup mean difference was performed using least significant difference *t*-test. A value of $P < 0.05$ was considered statistically significant.

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