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Ursolic acid induces neural regeneration after sciatic nerve injury

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Research Highlights

(1) Ursolic acid has anti-oxidative, anti-inflammatory, anti-apoptotic, and anti-scarring effects, and it regulates the immune system and promotes the repair of injured neurons. However, no reports have explored its role in peripheral nerve injury.
(2) This study is the first to demonstrate a role of ursolic acid in repair and regeneration following peripheral nerve injury. Ursolic acid promoted the regeneration of injured nerve myelin sheaths and reconstructed muscular functions. All experimental findings provide favorable evidence for the application of ursolic acid following peripheral nerve injury.

Abstract

In this study, we aimed to explore the role of ursolic acid in the neural regeneration of the injured sciatic nerve. BALB/c mice were used to establish models of sciatic nerve injury through unilateral sciatic nerve complete transection and microscopic anastomosis at 0.5 cm below the ischial tuberosity. The successfully generated model mice were treated with 10, 5, or 2.5 mg/kg ursolic acid *via* intraperitoneal injection. Enzyme-linked immunosorbent assay results showed that serum S100 protein expression level gradually increased at 1–4 weeks after sciatic nerve injury, and significantly decreased at 8 weeks. As such, ursolic acid has the capacity to significantly increase S100 protein expression levels. Real-time quantitative PCR showed that S100 mRNA expression in the $L_{4–6}$ segments on the injury side was increased after ursolic acid treatment. In addition, the muscular mass index in the soleus muscle was also increased in mice treated with ursolic acid. Toluidine blue staining revealed that the quantity and average diameter of myelinated nerve fibers in the injured sciatic nerve were significantly increased after treatment with ursolic acid. 10 and 5 mg/kg of ursolic acid produced stronger effects than 2.5 mg/kg of ursolic acid. Our findings indicate that ursolic acid can dose-dependently increase S100 expression and promote neural regeneration in BALB/c mice following sciatic nerve injury.

Key Words

neural regeneration; traditional Chinese medicine; ursolic acid; triterpenoid; sciatic nerve; peripheral nerve injury; S100; muscular atrophy; nerve myelin sheath; grants-supported paper; neuroregeneration

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INTRODUCTION

Neural regeneration following peripheral nerve injuries has attracted increasing attention from neurosurgical scholars. Although surgery and drug therapy are the two most commonly used treatment schemes, neither achieves precise effects. Growing evidence has confirmed that traditional Chinese medicine and its extracts can stimulate nerve growth factor expression and Schwann cell proliferation after nerve injury, thus promoting neural regeneration and functional recovery[1-3].

Ursolic acid (chemical name 3-hydroxy-12-ursen-28-oic acid) is a triterpenoid extracted from natural plant-based drugs, with a formula of $C_{30}H_{48}O_3$ and molecular weight of 456.68. Its structural formula is shown in Figure 1.

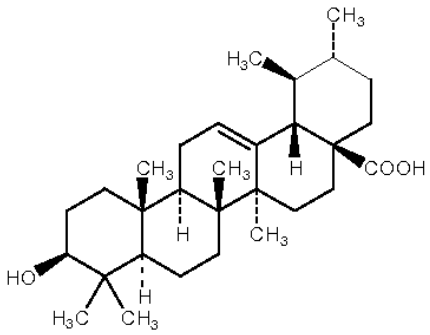

Figure 1 Structure formula of ursolic acid.

Ursolic acid has anti-cancer, anti-tumor, and anti-inflammatory effects. It also treats diabetes mellitus[4-5] and has neuroprotective effects[5-6]. In the central nervous system, Taviano *et al* [6] showed that ursolic acid antagonized Nepeta-induced injuries, protected excitability, and relieved Nepeta-induced depression. Shih *et al* [7] showed that kainic acid is excitotoxic to hippocampal neurons, and ursolic acid protects and maintains hippocampal neurons in a normal state. Pemminati *et al* [8] reported that ursolic acid can relax nerves and long-term use can alleviate haloperidol-induced catalepsy. Kunkel *et al* [9] found that ursolic acid extracted from apple peel could prevent muscular atrophy caused by muscle damage or malnutrition, indicating that ursolic acid can promote the repair of peripheral nerves in muscle tissue and provide nutrition. These evidences suggest that ursolic acid can repair damaged neurons and promote neural regeneration after peripheral nerve injury.

In this study, we observed nerve myelin sheath loss, muscular atrophy, and changes of S100 protein and mRNA expression in the peripheral blood and $L_{4–6}$ segments of the vertebral column in BALB/c mice with peripheral nerve injury following different dose ursolic acid intervention. The injury model was produced through complete transection and microscopic anastomosis of the unilateral sciatic nerve. The aim of this study was to explore the promoting effects of ursolic acid on neural regeneration following peripheral nerve injury.

RESULTS

Quantitative analysis of experimental animals

One hundred and sixty mice were included in this study. They underwent unilateral sciatic nerve transection and microscopic anastomosis at 0.5 cm below ischial tuberosity. The mice with sciatic nerve injuries were equally and randomly divided into four groups: a model group (rats were injected intraperitoneally with physiological saline) and high-, medium- and low-dose ursolic acid groups (rats were injected intraperitoneally with 10, 5, 2.5 mg/kg ursolic acid). Ten mice were selected at 1, 2, 4, and 8 weeks after injury and 160 mice were involved in the final analysis.

Ursolic acid increased serum levels of S100 in mice with sciatic nerve injury

Enzyme-linked immunosorbent assay results showed that serum S100 protein concentrations gradually increased after sciatic nerve injury and peaked at 4 weeks. They then significantly decreased at 8 weeks. Compared with the model group, serum levels of S100 significantly increased after high-, medium- and low-dose ursolic acid interventions ($P < 0.05$), and the high dose engendered the strongest effects (Table 1).

Table 1 Effect of ursolic acid on serum S100 concentrations (μg/L) in mice following sciatic nerve injury (enzyme-linked immunosorbent assay)

Group	Time after modeling (week)			
	1	2	4	8
Model	681±16	1 123±28	1 346±23	325±21
Ursolic acid				
Low-dose	714±28[a]	1 886±32[a]	2 039±33[a]	532±22[a]
Medium-dose	823±26[a]	1 945±38[ab]	2 451±68[a]	662±36[ab]
High-dose	997±53[abc]	2 282±47[ab]	3 632±73[abc]	716±28[abc]

Data are expressed as mean ± SD, and there were ten mice in each group at each time point. Mice in high-, medium-, and low-dose ursolic acid groups were intraperitoneally injected with 10, 5, 2.5 mg/kg ursolic acid. [a]$P < 0.05$, *vs*. model group; [b]$P < 0.05$, *vs*. low-dose ursolic acid group; [c]$P < 0.05$, *vs*. medium-dose ursolic acid group (pairwise comparisons using Duncan's method).

Ursolic acid promoted S100 mRNA expression in the $L_{4–6}$ spinal segments of mice with sciatic nerve injury

Real-time quantitative PCR analysis showed that in the model and low-dose ursolic acid groups S100 mRNA expression in the $L_{4–6}$ segments of the spinal cord gradually increased after sciatic nerve injury and peaked at 4 weeks, and then significantly decreased at 8 weeks. Compared with the model group, S100 mRNA expression in the high- and medium-dose ursolic acid groups was significantly higher and peaked at 1 week ($P < 0.05$), especially in the high-dose ursolic acid group (Figure 2).

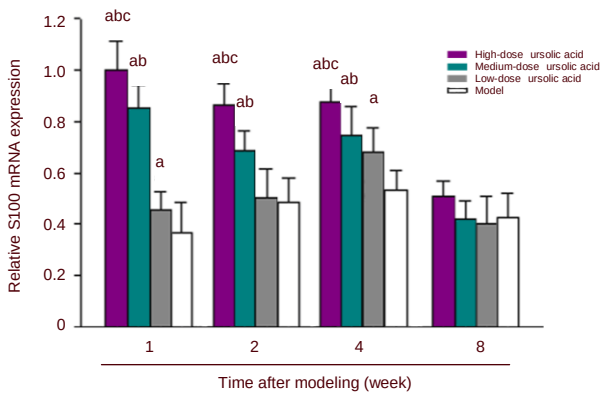

Figure 2 Effects of ursolic acid on S100 mRNA expression in $L_{4–6}$ spinal segments of mice following sciatic nerve injury (real-time quantitative PCR).

Data (fluorescent density [dRn] of S100 to GAPDH) are expressed as mean ± SD, and there were five mice in each group at each time point. Mice in high-, medium-, and low-dose ursolic acid groups were intraperitoneally injected with 10, 5, 2.5 mg/kg ursolic acid. [a]$P < 0.05$, *vs*. model group; [b]$P < 0.05$, *vs*. low-dose ursolic acid group; [c]$P < 0.05$, *vs*. medium-dose ursolic acid group (pairwise comparisons were performed using Duncan's method).

Ursolic acid promoted remyelination in mice following sciatic nerve injury

After sciatic nerve injury, toluidine blue staining showed disordered myelin sheaths, significantly proliferated fibrous connective tissue, and slow neural regeneration that did not change significantly over time. Compared with the model group, neural regeneration was more apparent in different dose ursolic acid groups at each time point, especially the high- and medium-dose groups (Figure 3).

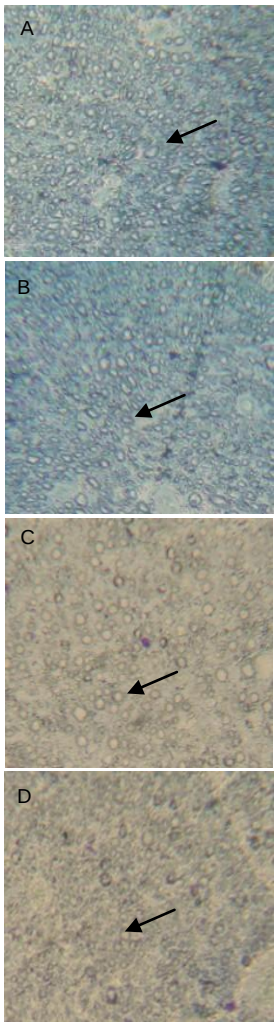

Figure 3 Effects of ursolic acid on neural regeneration in mice following sciatic nerve injury (toluidine blue staining, × 400). Arrows indicate the myelin sheath.

At 8 weeks, as seen under the light microscope, the myelin sheath was disordered and there was proliferated fibrous connective tissue in the model group (D); the high- (A; 10 mg/kg) and medium-dose (5 mg/kg) ursolic acid groups (B) showed regular arrangement of the sheath myelin, at uniform thickness and clearly visible boundary, and no fibrous tissue hyperplasia; in the low-dose (2.5 mg/kg) ursolic acid group (C), the morphology and thickness of myelin sheath were irregular, and its outline was still clear, hyperplasia of connective tissue was apparent.

Image analysis showed that the numbers and average diameters of myelinated nerve fibers in the high- and medium-dose ursolic acid groups were significantly higher than in the low-dose ursolic acid and model groups ($P < 0.05$). Moreover, the low-dose ursolic acid group showed better effects than the model group ($P < 0.05$; Table 2).

Table 2 Effects of ursolic acid on the quantity (n/mm^2) and average diameter (μm) of myelinated nerve fiber in mice at 8 weeks after sciatic nerve injury

Group	Number of myelinated nerve fibers	Average diameter of myelinated nerve fibers
Model	977±201	1.59±0.36
Ursolic acid		
Low-dose	1 032±231[a]	2.00±0.19[a]
Medium-dose	1 334±207[ab]	2.29±0.13[ab]
High-dose	1 447±286[ab]	2.56±0.27[ab]

Data are expressed as mean ± SD, and there were five mice in each group at each time point. Mice in high-, medium-, and low-dose ursolic acid groups were intraperitoneally injected with 10, 5, 2.5 mg/kg ursolic acid. [a]$P < 0.05$, *vs*. model group; [b]$P < 0.05$, *vs*. low-dose ursolic acid group (pairwise comparisons using Duncan's method).

Ursolic acid increased soleus muscle mass in mice following sciatic nerve injury

At 8 weeks, the muscular mass index in soleus muscle was significantly higher in the high-, medium-, and low-dose ursolic acid groups compared with model group ($P < 0.05$), and the high- and medium-dose ursolic acid groups showed higher indices ($P < 0.05$; Figure 4) than the low-dose ursolic acid group.

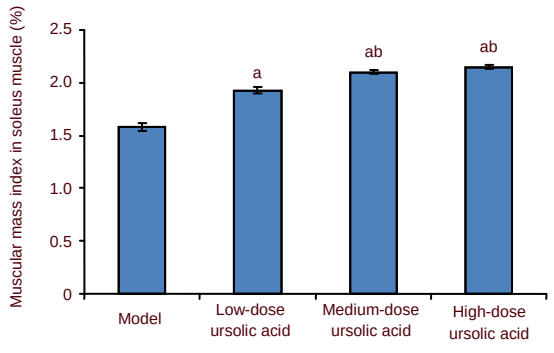

Figure 4 Effects of ursolic acid on soleus muscle atrophy in mice at 8 weeks after sciatic nerve injury.

Data are expressed as mean ± SD, and there were ten mice in each group. Mice in high-, medium-, and low-dose ursolic acid groups were injected intraperitoneally with 10, 5, 2.5 mg/kg ursolic acid.[a]$P < 0.05$, *vs*. model group; [b]$P < 0.05$, *vs*. low-dose ursolic acid group (pairwise comparisons using Duncan's method).

Muscular mass index = soleus muscle mass/body mass × 100%.

DISCUSSION

It is widely recognized that the peripheral nerve is an immune-privileged site, as because of the blood-nerve barrier, lymphocytes and antibodies cannot enter the nervous parenchyma, and no antigen-presenting cells and major histocompatibility complex II has been detected. In 1978, Saida *et al* [10] first proposed that, after peripheral nerve injury, the blood-nerve barrier in the perineurium and intima was destroyed, resulting in leakage of nerve antigens and invasion into blood circulation, where neurologic antigens induce immune responses. Subsequently, growing evidence has supported this proposal. Schwartz *et al* [11] detected anti-myelin antibodies and anti-ganglioside antibodies in peripheral blood of rats following sciatic nerve injury. Ansselin *et al* [12] found major histocompatibility complex expression and T lymphocyte aggregation following nerve transplantation. After *in vitro* cultured Schwann cells were induced with interferon-γ, it expressed the major histocompatibility complex class II antigens and activates T cells through antigen presentation, which disagrees with previous reports insisting that the peripheral nerve is an immune-privileged site. Subsequent to peripheral nerve injury, the induced immune response may inhibit nerve repair and regeneration, as the local immune response intensity is proportional to the severity of neurological impairment, with stronger immune response predicting more severe damage and poor neurological regeneration and functional recovery[13].

The peripheral nervous system, similar to other tissue, has an anatomical environment used to generate immune responses. A vascular network within nerve parenchyma contains immune response factors, such as antibodies, immune cells, and plasma proteins. Because of the existence of the blood-nerve barrier, the aforementioned immune response factors cannot enter nervous tissue.

In our study, we speculated that peripheral nerve blood-nerve barrier damage is caused by the following factors: nerve traction injury[14], nerve compression injury[15], chronic entrapment injury[16], traumatic nerve injury[17], clinical surgery or nerve graft[18], and other physical and chemical factors[19]. When the blood-brain barrier is damaged or neurological dysfunction occurs, the immune response factors in blood circulation enter nerve parenchyma and induce a series of immune responses after injury. Ansselin *et al* [12] also found marked T lymphocyte aggregation after peripheral nerve injury, indi-

cating that nerve injury may trigger cellular immune responses. The interferon-γ-activated T lymphocytes can cross through the blood-nerve barrier and further trigger allergic neuritis, and some cytokines may also produce immune reactions in peripheral nerve cells even when they remain undamaged[20].

After peripheral nerve injury, the resultant Wallerian degeneration causes macrophage accumulation, and macrophages secrete interleukin-1 after activation of phagocytosis nerve antigen. Then, interleukin-1 activates T cells and T cells proliferate to produce interleukin-2. Interleukin-2 acts on T and B lymphocytes, triggering cellular and humoral immune responses. Interleukin-1β can stimulate macrophages and Schwann cells in nerve parenchyma to secrete interleukin-1 and to exacerbate immune responses. Interleukin-4 can induce macrophage expression of major histocompatibility complex class II antigens and enhance their phagocytosis. In contrast, interleukin-10 inhibits macrophage expression of major histocompatibility complex II antigens after nerve injury[21].

The emergency of nerve immune responses allows for increasing attention on the role of immunosuppressants in the nervous system and their application in experimental research and clinical treatment of neurological diseases. In 1994, Gold *et al* [22] found that the immunosuppressant tacrolimus can improve axonal regeneration rates after sciatic nerve crush injury, and promote the recovery of nerve morphology and functions. After pre-denaturated rats were transplanted with autologous spinal cord into the tibial nerve, administration of tacrolimus promoted axonal growth and rubrospinal tract regeneration[23]. Tacrolimus also promoted *in vitro* axonal growth in cultured PC12 cells and dorsal ganglion neurons[24]. Furthermore immunosuppressants such as tacrolimus, cyclosporin A and rapamycin were involved in the repair and regeneration of injured peripheral neurons[25]. Jost *et al* [26] found that tacrolimus was superior to cyclosporine in promoting neural regeneration and repair. Tacrolimus's neurotrophic effects were associated with the dose used[27], and 5 mg/kg was the optimal dose. In the rats with sciatic nerve crush injuries, delayed (9–17 days after nerve injury) and immediate (0–8 days) administration of tacrolimus had the same effects[28], so tacrolimus treatment is insensitive to timing, thus expanding the range of clinical indications.

Short-term systemic tacrolimus intervention may promote the functional recovery of hind limbs after peripheral nerve injury[29]. After rats with brachial plexus nerve root avulsion injuries were replanted with nerve roots and treated with tacrolimus, the biceps muscle action potential, wet weight, and other indicators were significantly higher than in the blank control group[30]. However, immunosuppressive agents produce adverse reactions including nervous system disorders[31]. Animal toxicology studies have shown that different doses of tacrolimus may cause focal cerebral spinal meningitis, optic neuritis, radiculitis, tremor, and ataxia[32]. Organ transplant patients present with insomnia, tremor, headache, photophobia, clouding of consciousness, epileptic seizures, coma, or articulatory disorders after treatment with tacrolimus[33-34]. This evidence suggests that tacrolimus and other immunosuppressive agents have a two-sided effect on the nervous system, both neurotrophic and neurotoxic. The balance between these two roles may depend on drug dose, duration, and other unknown factors, thus requiring further studies.

Immunosuppressive natural drugs are characterized by few side effects and complex mechanisms, with an underlying mechanism associated with the immune system and neuroendocrine immune regulation networks[35]. A series of efficient, low-toxicity Chinese herbs have been regarded as potential immunosuppressants[36-38], such as ursolic acid. In our experiments, mice with sciatic nerve injury were treated with ursolic acid and observed for 8 weeks. Enzyme-linked immunosorbent assay and real-time quantitative PCR results showed that S100 protein in serum and spinal cord $L_{4–6}$ segments was activated and expressed in the high- and medium-dose ursolic acid groups compared with low-dose ursolic acid group and model group, and the expression was sustained for 2 weeks. S100 protein family is an acidic calcium-binding protein consisting of 16 members. It only contains amino acid sequences that are highly conserved in vertebrates[39-41], and most of them serve as a calmodulin and target protein[42]. After calcium ions bind, the S100 protein undergoes conformational changes, exposing the target protein binding site, and then acts with the corresponding target proteins to produce biological effects[43-50]. S100 protein concentrations in biological fluids can be used as a marker of functional activation after nerve injury[51-54]. Nerve injury may trigger the repair system *in vivo*, including activation of S100 protein overexpression and promotion of neural regeneration[55-58]. In our experiments, S100 protein and mRNA expression levels were significantly increased after treatment with ursolic acid at the high- and medium-dose, suggesting that ursolic acid can activate S100 protein overexpression, thus

promoting neural regeneration.

The toluidine blue staining results showed that the number, integrity, and thickness of the myelin sheaths increased during the regeneration of the damaged nerves. The number of myelinated nerve fibers and their average diameter in the high- and medium-dose ursolic acid groups were significantly higher than in the low-dose ursolic acid and model groups, and the low-dose ursolic acid group showed stronger effects than the model group. This is evidence that ursolic acid can strongly promote the growth of damaged nerve myelin. Ursolic acid at different dosages could significantly promote S100 protein expression and there were significant differences compared with the model group. Nerve injury is accompanied with partial muscle atrophy. When nerve regeneration occurs and neurological functions recover, muscle atrophy also improves. Our experimental findings showed that soleus muscle cells in the high- and medium-dose ursolic acid groups were in good physiological condition, and the recovery from muscle atrophy was significantly stronger than in the low-dose ursolic acid and model groups. These results suggest that nerve regeneration and functional recovery were improved following ursolic acid intervention. In addition, after ursolic acid given for 8 weeks, S100 mRNA expression levels showed no significant differences in the high- and medium-dose ursolic acid groups compared with low-dose ursolic acid and model groups, whereas myelinated nerve and recovery of muscular atrophy showed significant differences. These data suggested that nerve repair and regeneration have been completed at 8 weeks, and then S100 expression levels decreased, which was close to what occurring in the low-dose ursolic acid and model groups, and nerves and muscles had undergone good recovery.

Repair and regeneration after nerve injury is a complex process involving inflammation, adhesion, synergistic effects of the extracellular matrix, regulation of neurotrophic factors, neurotransmitter synthesis and release, growth cone formation and extension, and neural regeneration[59-62]. Nerve injury leads to Wallerian degeneration in nerve fibers. After neuronal cell bodies and axons appear to fracture, nerve fibers at the distal end and some axons and the myelin sheath at the proximal end will gradually rupture, disintegrate, and undergo phagocytosis. Nerve injury elicits immune responses that aggravate Wallerian degeneration, resulting in secondary nerve injury and hindering nerve repair and regeneration after injury. Meanwhile, excessive immune responses may trigger the expression of inflammatory mediators surrounding the injured tissue, and promote local scar formation and hyperplasia, which directly affects conduction recovery after nerve injury[63]. Excessive immune inflammatory response will inhibit S100 protein expression[51]. Therefore, we suggest that it is reasonable to apply immunosuppressive agents to regulate inflammatory responses, which will reduce scar formation after nerve injury and provide a favorable external environment for nerve repair and regeneration.

Ursolic acid is a pentacyclic triterpenoid that is widely found in bear berry, Ilex rotunda Thunb and other natural medicines. Ursolic acid has antioxidant and anti-inflammatory effects, promotes tumor cell apoptosis, and regulates immune functions[64-65], as well as having anti-viral and anti-scarring effects[66-67]. Our findings showed that ursolic acid promoted S100 protein expression in a dose-dependent manner after peripheral nerve injury in mice. Toluidine blue staining revealed that myelin sheath recovery after ursolic acid interventions was significantly better than in the model group. The assessments of soleus muscle mass index showed that ursolic acid reduced muscle atrophy after injury. Thus, ursolic acid may inhibit inflammatory immune response following nerve injury, thereby promoting sustained expression of S100 in spinal cord cells, reducing scar proliferation, preventing nerve demyelination, and providing favorable conditions for nerve repair and regeneration. However, we did not detect local immune responses after injury and this needs further confirmation. In addition, growth associated protein 43 expression and inflammation-related cell signaling pathways also require more attention.

MATERIALS AND METHODS

Design

A randomized, controlled animal experiment.

Time and setting

Experiments were performed in Experimental Animal Center, Norman Bethune College of Medicine, Jilin University, China, from June to August in 2011.

Materials

One hundred and sixty healthy BALB/c male mice, aged 4–6 weeks, weighing 20 ± 2 g, were provided by the Experimental Animal Center of Jilin University, China (license No. SCXK (Ji) 2007-0003). All mice were housed in a breeding room and allowed free access to

water and food. All procedures were in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[68].

Methods

Establishment of a sciatic nerve injury model in mice

Mice were intraperitoneally anesthetized with 1% thiopental (100 mg/kg) and fixed in the prone position. After the surgical site was disinfected, a 1.5 cm longitudinal incision was made on the posterior femur of a unilateral lower limb. Then, the sciatic nerve starting at the lower edge of the piriformis muscle was bluntly exposed, the surrounding tissues were stripped, and the sciatic nerve was transected at 0.5 cm below the ischial tuberosity. Five minutes later, sciatic nerve anastomosis was performed under a 100-fold stereo microscope (Nikon SM645S, Tokyo, Japan). The fascia, subcutaneous tissue, and skin wound were sutured.

Drug interventions

Ursolic acid was heated and dissolved in a 0.9% NaCl solution (purity > 90%; Aldrich U6753, Sigma, St. Louis, MO, USA). Mice in the high-, medium- and low-dose ursolic acid groups were injected intraperitoneally with 10, 5, 2.5 mg/kg ursolic acid per day[69], until specimen collection. The model group were given 25 mL/kg of 0.9% NaCl solution.

Enzyme-linked immunosorbent assay of serum S100 protein content in mice following sciatic nerve injury

At 1, 2, 4, and 8 weeks after sciatic nerve injury, ten mice in each group were selected to harvest eyeball blood samples. Blood samples were placed in sterile EP tube at room temperature for 2 hours, centrifuged at 5 000 r/min for 3 minutes, and the supernatant was collected. Serum S100 protein contents were measured using enzyme-linked immunosorbent assay in strict accordance with kit instructions (mouse (S-100) enzyme-linked immunosorbent assay kit 96T/48T, KB3773; Kaibo, Shanghai, China). Absorbance at 490 nm was measured and standard curve was plotted.

Real-time quantitative PCR assay of S100 mRNA expression in $L_{4–6}$ spinal segments of mice with sciatic nerve injury

At 1, 2, 4, and 8 weeks after sciatic nerve injury, five mice in each group were intraperitoneally anesthetized with 1% sodium thiopental (100 mg/kg), and the sciatic nerve was cut and isolated, the vertebral plate was bitten to expose the spinal segments, and the $L_{4–6}$ segments on the ipsilateral side to the injury were cut (Figure 5).

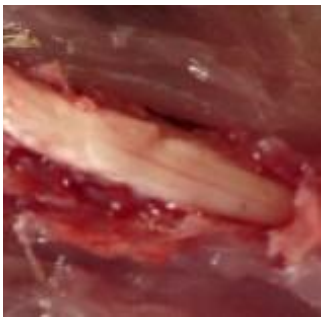

Figure 5 Spinal segments associated with the sciatic nerve.

A longitudinal incision, 1.5 cm long, was made on the posterior femur in unilateral lower limbs. Then, the sciatic nerve at the lower edge of the piriformis muscle was bluntly exposed and isolated. The vertebral plate was bitten and spinal segments associated with the sciatic nerve were exposed.

Total RNA was extracted using TRIzol reagent according to the total RNA extraction kit (Invitrogen, Carlsbad, CA, USA), and reversely transcribed into cDNA for real-time quantitative PCR amplification. PCR reaction contained 40 cycles of 95°C for 30 seconds, 58°C for 60 seconds, and 72°C for 60 seconds. 2 × SYBR real-time PCR kit was purchased from Roche (Shanghai, China). S100 primer sequences were designed with Beacon Designer™ 7 software (PREMIER Biosoft, Palo Alto, CA, USA) according to NCBI GenBank. GAPDH served as a reference (Table 3).

Table 3 S100 and GAPDH primer sequences

Primer	Sequence (5'–3')	Target fragment length (bp)
S100	Upper: ACT GAA GGA GCT TAT CAA CAA CGA Lower: AGT GTG ACT TCC AGG AGT TCA TG Probe: GTG ATG GAG ACG CTG GAC GAA GAT GG	345
GAPDH	Upper: CCA TTT GCA GTG GCA AAG Lower: CAC CCC ATT TGA TGT TAG T Probe: CAA GGC CGA GAA TGG GAA GCT TGT C	604

Toluidine blue staining of sciatic nerve myelin changes in mice

At 1, 2, 4, and 8 weeks after sciatic nerve injury, five mice in each group were anesthetized, and neural stems 0.5 cm away from sciatic nerve anastomosis was resected and fixed in formalin for 72 hours, dehydrated in ethanol and paraffin embedded, cut into 3-μm-thick sec-

tions, then dewaxed and rinsed three times with distilled water. Toluidine blue staining: specimens were soaked in 0.1% toluidine blue solution (0.1 g toluidine blue was dissolved in 100 mL of 0.1 mol/L acetate buffer) for 10 minutes, dehydrated in ethanol, put through xylene transparency, and mounted with neutral gum. Specimens were observed under a microscope (Eclipse TE-2000-U, Nikon, equipped with an attached camera, digital camera SXM1200F) and the number of myelinated nerve fibers and their average diameter were measured.

Determination of muscular mass index in the soleus muscle

At 8 weeks after sciatic nerve injury, the triceps surae were cut and weighed on an electronic analytical balance (FA214, Haikang Shanghai Scientific Instrument Co., Ltd., Shanghai, China). The muscular mass index in the soleus muscle was calculated with the Cuadros method, according to the formula: muscle mass / body mass × 100%[70].

Statistical analysis

All data are expressed as mean ± SD and were analyzed using SPSS 12.0 software (SPSS, Chicago, IL, USA). Multi-group comparisons were performed using one-way analysis of variance, and pairwise comparisons using Duncan's method. A $P < 0.05$ level was considered statistically significant.

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