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Propofol's effect on the sciatic nerve

Harmful or protective?[☆]

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Research Highlights

(1) Little data is available on the neuroprotective effects of propofol after peripheral nerve injury. Therefore, this study aimed to explore the effect of local propofol injection on sciatic nerve injury in BALB/c mice.
(2) We also examined the spatiotemporal expression of nuclear factor kappa B, which plays a crucial role in inflammatory and immune responses, after sciatic nerve injury.
(3) Propofol can inhibit nuclear factor kappa B expression in the $L_{4–6}$ segments of the spinal cord, suppress apoptosis, and alleviate focal inflammation on the side of the injured sciatic nerve, thereby promoting recovery following nerve injury.

Abstract

Propofol can inhibit the inflammatory response and reduce the secretion and harmful effects of astrocyte-derived proinflammatory cytokines. In this study, after propofol was injected into the injured sciatic nerve of mice, nuclear factor kappa B expression in the $L_{4–6}$ segments of the spinal cord in the injured side was reduced, apoptosis was decreased, nerve myelin defects were alleviated, and the nerve conduction block was lessened. The experimental findings indicate that propofol inhibits the inflammatory and immune responses, decreases the expression of nuclear factor kappa B, and reduces apoptosis. These effects of propofol promote regeneration following sciatic nerve injury.

Key Words

neural regeneration; peripheral nerve injury; propofol; nuclear factor kappa B; sciatic nerve injury; inflammatory response; nerve conduction; immunosuppression; myelin sheath; grant-supported paper; neuroregeneration

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Conflicts of interest: None declared.

INTRODUCTION

Peripheral nerve injury results in Wallerian degeneration at the distal end and retrograde degeneration at the proximal end, in which the inflammatory response is the most critical event. The inflammation may affect peripheral nerve degeneration and regeneration, and cause the accumulation of macrophages. The macrophages can be activated by phagocytosing antigen, which then secrete interleukin-1[1] and induce neuropathic pain[2-9]. Gold *et al* [10] were the first to demonstrate that the immunosuppressant FK506 increases the rate of axonal regeneration in rats after sciatic nerve injury[11-17]. In addition, short-term systemic administration of FK506 accelerates limb functional recovery in rats after peripheral nerve injury[18-22]. FK506 was also shown to significantly improve upper limb muscle action potentials and increase muscle wet weight after nerve root replantation into the spinal cord of rats after brachial plexus nerve avulsion injury[23].

Ethical approval: The experimental procedures were approved by the Animal Ethics Committee of Chifeng City Hospital in China.

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Therefore, FK506 has been widely used in the treatment of nervous system diseases. However, FK506 and other immunosuppressive agents exert a variety of side effects, such as nervous system disorders[24-25]. An immunosuppressive agent with high efficacy and low toxicity is urgently needed.

Propofol is a rapid, but short-acting, intravenous drug that is preferentially used for the induction and maintenance of anesthesia, as well as sedation of critically ill patients. Propofol can inhibit inflammation and suppress the secretion of the proinflammatory cytokines interleukin-1, interleukin-6 and tumor necrosis factor alpha from astrocytes, and enhance the synthesis and release of the anti-inflammatory cytokine interleukin-10.

Consequently, propofol can inhibit damage caused by proinflammatory cytokines[26] and exert protective effects on the central nervous system[27]. However, the role of propofol on peripheral nerve injury has not been studied adequately.

Nuclear factor kappa B, discovered by Sen and Baltimore[28] in 1986, is a nuclear transcription factor that specifically binds kappa B sequences and enhances the transcription of numerous genes, including various immunoglobulins. Nuclear factor kappa B is widely expressed in the nervous system, being inactive in the cytoplasm of quiescent cells. When cells are stimulated, nuclear factor kappa B is activated and translocates from the cytoplasm to the nucleus, where it binds specific gene sequences. The binding may trigger the transcription of target genes, such as bcl-2, inducible nitric oxide synthase and various adhesion molecules[29-30]. The nuclear factor kappa B target genes regulate the host immune and inflammatory responses[31-40], as well as neuronal survival, plasticity, neural degeneration and neuropathic pain[41-50].

In this study, we aimed to observe the effect of propofol on the inflammatory and immune responses, as well as its effect on neurological functional recovery in BALB/c mice with sciatic nerve injury.

RESULTS

Quantitative analysis of experimental animals

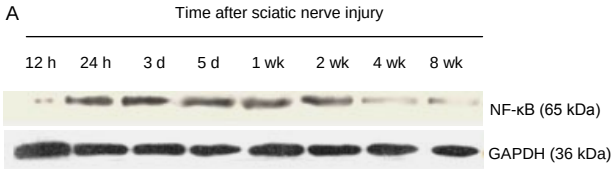
A total of 250 BALB/c mice were included in this study; 80 mice were randomly selected for the control group, while 170 mice were used to model sciatic nerve injury by unilateral sciatic nerve transection. Ten mice were excluded because of accidental death during surgery. The remaining 240 mice were randomly divided into model and propofol groups, receiving saline and propofol injection, respectively, at the site of the injured sciatic nerve for 1 week. All 240 mice were involved in the final analyses.

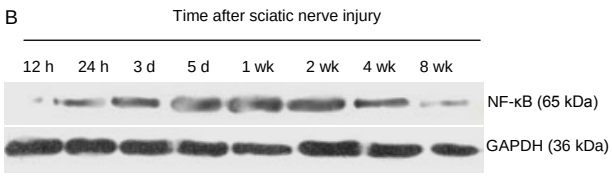
Propofol decreased nuclear factor kappa B expression in spinal cord $L_{4–6}$ segments in mice with sciatic nerve injury

Western blot analysis showed that nuclear factor kappa B expression in spinal cord $L_{4–6}$ segments began to increase 12 hours after sciatic nerve injury, reached a peak at 1 week, and then gradually decreased ($P < 0.05$). Propofol administration decreased nuclear factor kappa B expression in spinal cord $L_{4–6}$ segments after sciatic nerve injury ($P < 0.05$; Figure 1, Table 1).

Propofol decreased nuclear factor kappa B mRNA expression in spinal cord $L_{4–6}$ segments in mice with sciatic nerve injury

Real-time PCR analysis revealed that nuclear factor kappa B mRNA was minimally expressed in spinal cord $L_{4–6}$ segments in normal mice. After sciatic nerve injury, nuclear factor kappa B mRNA levels rapidly increased, indicating the presence of local inflammation. Expression gradually increased between 12 hours and 2 weeks after injury, and these levels were significantly higher than in the control group ($P < 0.05$). Propofol administration significantly reduced nuclear factor kappa B mRNA expression in spinal cord $L_{4–6}$ segments in mice with sciatic nerve injury ($P < 0.05$), but expression levels were still higher than in the control group ($P < 0.05$; Figure 2).





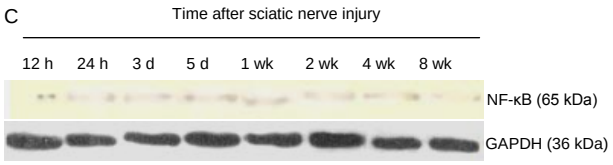


Figure 1 Effect of propofol on nuclear factor kappa B (NF-κB) expression in spinal cord $L_{4–6}$ segments in mice with sciatic nerve injury.

Western blot analysis showed that, compared with the model group (A), NF-κB protein expression in spinal cord $L_{4–6}$ segments was decreased in the propofol group (B). There was no significant difference in NF-κB expression between the propofol group and the control group (C) at any time point. h: Hour; d: day; wk: week.

Table 1 Effect of propofol on nuclear factor kappa B expression in spinal cord $L_{4–6}$ segments in mice with sciatic nerve injury (western blot analysis)

Group	Time after sciatic nerve injury							
	12 h	24 h	3 d	5 d	1 wk	2 wk	4 wk	8 wk
Propofol	0.005±0.009[a]	0.273±0.002[a]	0.676±0.028[a]	1.099±0.022[a]	0.624±0.039[a]	0.669±0.041[a]	0.288±0.03[a]	0.200±0.029[a]
Model	0.026±0.033[ab]	0.487±0.002[abc]	0.787±0.033[abc]	1.332±0.022[abc]	1.886±0.024[abc]	1.021±0.023[abc]	0.560±0.005[abc]	0.300±0.027[abc]
Control	0.008±0.022	0.092±0.012	0.012±0.019	0.039±.0.023	0.034±0.032	0.039±0.026	0.032±0.022	0.030±0.023

Nuclear factor kappa B expression is represented as the absorbance ratio of nuclear factor kappa B to GAPDH. A higher absorbance ratio indicates stronger nuclear factor kappa B expression. Data are expressed as mean ± SD. Multiple group comparisons were performed using least significant difference *t*-test. [a]*P* < 0.05, *vs*. control group; [b]*P* < 0.05, *vs*. propofol group; [c]*P* < 0.05, *vs*. the same group at the previous time point. h: Hour; d: day; wk: week.

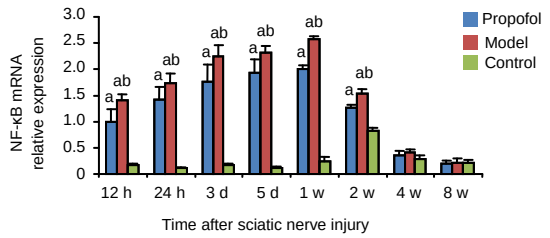


Figure 2 Effect of propofol on nuclear factor kappa B (NF-κB) mRNA expression in spinal cord $L_{4–6}$ segments in mice with sciatic nerve injury.

NF-κB mRNA relative expression is represented as NF-κB mRNA Ct value as detected by real-time PCR. Data are expressed as mean ± SD. Group comparisons were performed using group *t*-test (independent samples *t*-test). [a]*P* < 0.05, *vs*. control group; [b]*P* < 0.05, *vs*. propofol group. h: Hour; d: day; w: week.

Effects of propofol on nerve conduction velocity and electromyographic amplitude in mice with sciatic nerve injury

The electromyography results showed that, at 1–4 weeks after sciatic nerve injury, the electromyographic amplitude and motor nerve conduction velocity were decreased in the propofol group, while the latency was prolonged, compared with the control group (*P* < 0.05). These changes were time-dependent (*P* < 0.05). Propofol injection into the injured sciatic nerve significantly alleviated the nerve and muscle deficits in a time-dependent manner (*P* < 0.05). There was no apparent change in electromyographic amplitude, motor nerve conduction velocity, or latency in the control group (Tables 2–4).

Table 2 Effect of propofol on electromyographic amplitude (mV) in mice with sciatic nerve injury

Group	Time after sciatic nerve injury (week)			
	1	2	4	8
Propofol	2.32±0.18[b]	4.32±0.17[bc]	18.67±0.27[bc]	25.07±0.30[bc]
Model	1.76±0.11[ab]	2.36±0.11[abc]	16.43±0.32[abc]	18.70±0.32[abc]
Control	26.43±0.06	27.01±0.15	26.54±0.08	26.90±0.71

Data are expressed as mean ± SD; *n* = 5 in each group per time point. Group comparisons were performed using paired *t*-test. [a]*P* < 0.05, *vs*. propofol group; [b]*P* < 0.05, *vs*. control group; [c]*P* < 0.05, *vs*. the same group at the previous time point.

Propofol improved the morphology of myelin sheath in spinal cord $L_{4–6}$ segments in mice with sciatic nerve injury

Luxol fast blue staining showed that the myelin sheaths in spinal cord $L_{4–6}$ segments in control rats had a normal

morphology, an even thickness and clear contours, with no hyperplasia of the adjacent connective tissue. At 8 weeks after sciatic nerve injury, the myelin sheath in spinal cord $L_{4–6}$ segments displayed an irregular morphology with apparent hyperplasia in the fibrous connective tissue. After 1 week of propofol treatment, the myelin sheath recovered its normal morphology, and had an appearance similar to that in the control group (Figure 3).

Table 3 Effect of propofol on motor nerve conduction velocity (m/s) in mice with sciatic nerve injury

Group	Time after sciatic nerve injury (week)			
	1	2	4	8
Propofol	19.9±0.26[b]	39.7±0.81[bc]	58.9±1.34[bc]	60.1±0.67[bc]
Model	13.1±0.16[ab]	32.1±0.68[abc]	44.1±0.80[abc]	50.3±0.21[abc]
Control	69.3±0.17	68.7±0.57	69.2±0.46	67.5±3.37

Motor nerve conduction velocity = distance between the two stimulating electrodes/difference in action potential latency. Data are expressed as mean ± SD; n = 5 in each group per time point. Group comparisons were performed using least significant difference t-test. [a]P < 0.05, *vs*. propofol group; [b]P < 0.05, *vs*. control group; [c]P < 0.05, *vs*. the same group at the previous time point.

Table 4 Effect of propofol on electromyography latency (ms) of sciatic nerve injury mice

Group	Time after sciatic nerve injury (week)			
	1	2	4	8
Propofol	1.88±0.82[b]	1.53±0.32[bc]	1.42±0.32[bc]	1.19±0.22[bc]
Model	1.82±0.91[ab]	1.76±0.54[abc]	1.61±0.52[abc]	1.32±0.21[abc]
Control	1.08±0.18	1.11±0.17	1.06±0.10	1.09±0.14

Data are expressed as mean ± SD; n = 5 in each group per time point. Group comparisons were performed using least significant difference t-test. [a]P < 0.05, *vs*. propofol group; [b]P < 0.05, *vs*. control group; [c]P < 0.05, *vs*. the same group at the previous time point.

Visual fields were randomly selected for image analysis, and the number of myelinated nerve fibers and their average diameter were calculated, and the results were statistically analyzed. The number and the average diameter of myelinated nerve fibers in the spinal cord $L_{4–6}$ segments were significantly decreased in the model group compared with the control group (P < 0.05). After propofol administration, the number and the average diameter of myelinated nerve fibers in the spinal cord $L_{4–6}$ segments were significantly increased compared with the model group (P < 0.05). These results indicate that propofol can significantly improve neural regeneration after sciatic nerve injury in mice (Table 5).

Propofol inhibited apoptosis in spinal cord $L_{4–6}$ segments in mice with sciatic nerve injury

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was performed to detect apoptotic cells. The number of TUNEL-positive (apoptotic) cells in the spinal cord $L_{4–6}$ segments on the injured side was increased after sciatic nerve injury (P < 0.05). The number of TUNEL-positive cells gradually decreased with increasing time after injury (P < 0.05). Propofol administration significantly reduced the number of apoptotic cells in spinal cord $L_{4–6}$ segments on the injured side (P < 0.05). No apoptotic cells were found in the control group at any time point (Figure 4, Table 6).

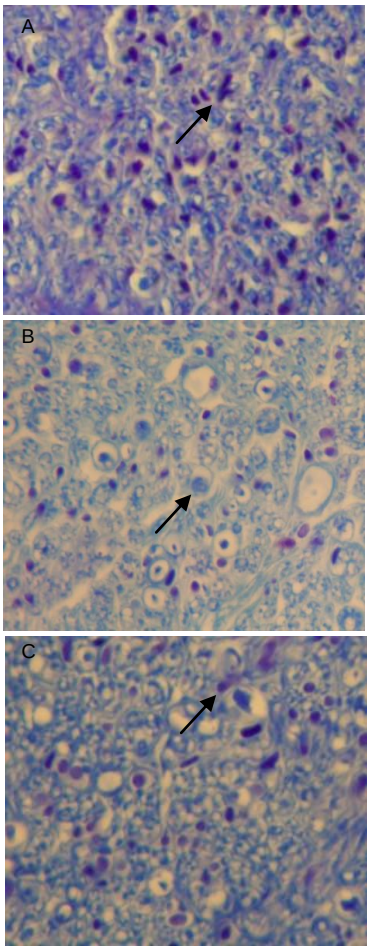


Figure 3 Effect of propofol on the morphology of myelin sheath in spinal cord $L_{4–6}$ segments of mice 8 weeks after sciatic nerve injury (luxol fast blue staining, × 40).

The myelin sheath is stained blue (arrows), while axons are not stained. The background is white.

(A) In the propofol group, the morphology of the myelin sheath in spinal cord $L_{4–6}$ segments on the injury side is similar to that in the control group.

(B) In the model group, irregular morphology of the myelin sheath and apparent hyperplasia in fibrous connective tissue is visible.

(C) In the control group, the morphology of the myelin sheath in spinal cord $L_{4–6}$ segments is normal.

Table 5 Effect of propofol on the number (n/mm^2) and the average diameter (μm) of myelinated nerve fibers in spinal cord $L_{4–6}$ segments in mice 8 weeks after sciatic nerve injury

Group	Number of myelinated nerve fibers	Average diameter of myelinated nerve fibers
Propofol	72±2[b]	2.38±0.30[b]
Model	51±1[ab]	1.61±0.17[ab]
Control	73±3	2.47±0.31

Data are expressed as mean ± SD; n = 5 in each group per time point. Group comparisons were performed using paired t-test. [a]P < 0.05, vs. propofol group; [b]P < 0.05, vs. control group.

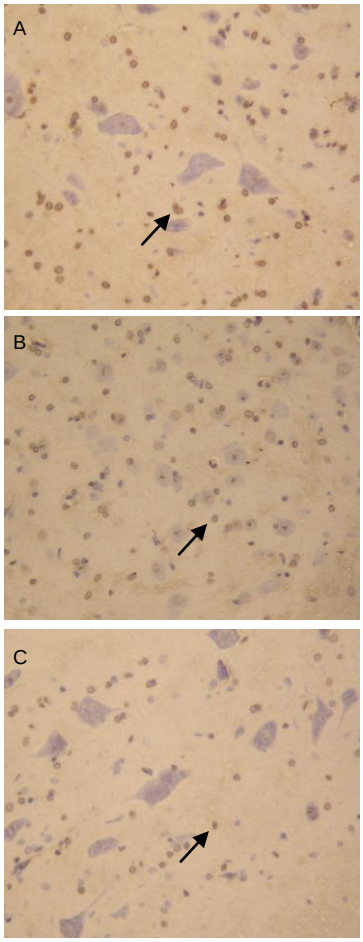


Figure 4 Effect of 1-week propofol treatment on apoptosis in spinal cord $L_{4–6}$ segments in mice with sciatic nerve injury (TUNEL staining, × 200).

TUNEL labeling, visible as brownish yellow staining, was mainly present in the cytoplasm of anterior horn cells (arrows).

(A) In the propofol group, the number of apoptotic cells was significantly reduced.

(B) In the model group, the number of apoptotic cells was significantly increased.

(C) In the control group, no apoptotic cells were found.

TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling.

Table 6 Effect of propofol on the number of apoptotic cells (n/field at 400 × magnification) in spinal cord $L_{4–6}$ segments in mice with sciatic nerve injury

Group	Time after sciatic nerve injury (week)			
	1	2	4	8
Propofol	6.71±0.31[b]	3.28±0.22[bc]	2.76±0.20[bc]	2.08±0.18[bc]
Model	9.82±0.43[ab]	4.18±0.32[abc]	3.91±0.30[abc]	3.02±0.20[abc]
Control	0.11±0.03	0.10±0.02	0.12±0.01	0.11±0.02

Data are expressed as mean ± SD; n = 5 in each group per time point. Group comparisons were performed using one-way analysis of variance. [a]P < 0.05, vs. propofol group; [b]P < 0.05, vs. control group; [c]P < 0.05, vs. the same group at the previous time point.

DISCUSSION

Repair and neuronal regeneration following peripheral nerve injury is a long and complex physiological process. Saida *et al* [51] were the first to show that when peripheral nerve injury occurs, the blood-nerve barrier is compromised, resulting in the leakage of antigens and a localized immune response, and immune cells, such as macrophages, infiltrate the perineurium and endoneurium. Schwartz *et al* [52] detected anti-myelin sheath antibodies and anti-ganglioside antibodies in the peripheral blood of rats with sciatic nerve injury. Ansselin *et al* [53] found that the major histocompatibility complex proteins accumulate at the nerve transplantation site, where T lymphocytes also gather. Growing evidence indicates that after peripheral nerve injury, the immune and inflammatory responses may inhibit nerve repair and regeneration. Furthermore, the intensity of the immune response is proportional to the severity of nerve injury. The greater the severity of the nerve injury, the stronger the immune response, and the more limited the degree of neuronal regeneration and functional restoration[54].

Many factors can compromise the integrity of the blood-nerve barrier, including nerve traction injury[55], nerve compressive injury[56], nerve chronic constriction injury[57], traumatic nerve injury, nerve repair surgery and nerve transplantation. Blood-nerve barrier damage or dysfunction allows immune factors in the blood to enter the nerve parenchyma, triggering a series of post-injury immune responses, potentially inducing allergic neuritis, and leading to immune and inflammatory damage to the uninjured nerve tissue[53].

Propofol pretreatment can reduce aquaporin 4 overexpression, protect rats with cerebral ischemia/reperfusion injury, and alleviate cerebral ischemia/reperfusion-indu-

ced brain edema and other complications[27]. Zvara *et al* [58] demonstrated the protective effect of transient ischemia/reperfusion preconditioning on nerve injury, and a number of studies support the concept that propofol preconditioning attenuates the severity of spinal cord injury. Lei *et al* [59] showed that propofol significantly suppresses the rise in serum concentrations of cytosolic enzymes during spinal cord ischemia/reperfusion injury, and has a protective effect on aortic occlusion-induced spinal cord injury. Propofol administration immediately following spinal cord ischemia can lower levels of malondialdehyde and excitatory amino acids, enhance the activity of superoxide dismutase, and effectively alleviate pathomorphological changes to nerve cells due to ischemia/reperfusion injury[60]. During thoracic and abdominal artery surgery, propofol administration *via* the abdominal aorta can attenuate side effects caused by spinal cord injury[61]. In addition, propofol has been shown to inhibit the expression of inflammatory cytokines, such as interleukin 6 and tumor necrosis factor alpha[62]. Ren *et al* [63] found that propofol treatment can significantly reduce nerve cell swelling, necrosis and apoptosis in rats after focal cerebral ischemia/reperfusion, and decrease the expression of hypoxia-inducible factor 1 alpha and heat shock protein 70. Ma *et al* [64] found that propofol improved neurobehavioral scores to varying degrees, decreased brain water content, lowered serum lactate dehydrogenase and creatine kinase levels, and alleviated pathomorphological changes to brain tissue in rats with cerebral hemorrhage. Although the effects of propofol on the central nervous system have been intensively investigated, little is known of its role in repair after peripheral nerve injury.

In this study, mice with sciatic nerve injury were followed for 8 weeks after propofol treatment. Western blot analysis and real-time PCR results showed that nuclear factor kappa B protein and mRNA levels rose in the spinal cord segments after peripheral nerve injury, and that expression levels in the propofol group were significantly lower than in the model group, but significantly higher than in the control group. No significant differences in expression were found among the groups at 2 weeks. Activation of the nuclear factor kappa B signaling pathway during peripheral nerve development and after injury may be mediated by nerve growth factor *via* the neurotrophin receptor $p75^{NTR}$. In turn, nuclear factor kappa B can induce the expression of various inflammatory cytokines[65-68].

After mice with sciatic nerve injury were treated with propofol, nuclear factor kappa B protein and mRNA levels significantly diminished compared with the model group, suggesting that propofol could inhibit nuclear factor kappa B protein expression after peripheral nerve injury. We conclude that propofol may further suppress the excessive inflammatory response, decrease local scar hyperplasia, and provide favorable conditions for neural regeneration.

Luxol fast blue staining showed that there was an increase in the number of myelinated fibers and in the integrity and thickness of the myelin sheath during the neural regeneration process. After propofol treatment, the number and average diameter of myelinated nerve fibers in $L_{4–6}$ segments of the spinal cord increased significantly, indicating that propofol protects against damage and promotes repair and regeneration following nerve injury.

The number of apoptotic cells was determined using TUNEL staining. The number of apoptotic cells in $L_{4–6}$ segments of the spinal cord apparently decreased after propofol treatment, indicating that propofol inhibits apoptosis of motor neurons following nerve injury.

Nerve injury is accompanied by local muscle atrophy. Neural regeneration and recovery may help restore nerve conduction velocity[69]. Nerve electrophysiology showed that the nerve in mice with sciatic nerve injury underwent substantial physiological recovery after propofol treatment.

The nerve repair and regeneration process after injury is very complex, involving inflammation, adhesion, effects of various extracellular matrix components, neurotrophic factors, neurotransmitter synthesis and release, growth cone formation and extension, and neuronal regeneration[70-73]. Nuclear factor kappa B can regulate host immune and inflammatory responses, and plays a critical role in neuronal survival, plasticity, neural degeneration and neuropathic pain after injury[41]. Excessive expression of nuclear factor kappa B is not conducive to recovery after nerve injury[42-50]. Therefore, the use of immunosuppressive agents to control inflammation following nerve injury is of benefit, reducing scar formation and apoptosis, and providing a favorable environment for nerve repair and regeneration.

In summary, in this study, we examined nuclear factor kappa B protein and mRNA expression, myelin injury, number of apoptotic cells, and nerve electromyographic changes in the $L_{4–6}$ segments of the spinal cord. Propofol may inhibit the inflammatory response after nerve injury, decrease nuclear factor kappa B expression in spinal

cord cells, reduce apoptosis and local scar formation, and limit demyelination during the neural regeneration process. All of these effects of nuclear factor kappa B favor nerve repair and regeneration after injury.

MATERIALS AND METHODS

Design

A randomized, controlled animal experiment.

Time and setting

Experiments were performed from July 2011 to June 2012 at the Center Laboratory of China-Japan Union Hospital of Jilin University (National Key Laboratory, biosafety level-3), China.

Materials

Animals

Healthy, clean, adult, male BALB/c mice, aged 8 weeks, weighing 20 ± 2 g, were provided by the Experimental Animal Center of Basic Medical College of Jilin University, China, with license No. SCXK (Ji) 2007-0001. The use of experimental animals was in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China[74].

Drug

Propofol was purchased from Xi'an Libang Pharmaceutical Co., Ltd., Xi'an, Shaanxi Province, China (Approval Document No. National Medicine Permit No. H20010368, batch No. 11003141). Chemical name: 2,6-di-isopropylphenol; specification: 10 mg/mL.

Methods

Establishment of peripheral nerve injury model in BALB/c mice

Mice were anesthetized with 1% sodium pentothal (100 mg/kg; Langde Chemical & Pharmaceutical Co., Ltd., Yancheng, Jiangsu Province, China) *via* intraperitoneal injection, and were fixed in the prone position. After the skin was disinfected, a 1.5-cm longitudinal incision was made at the unilateral (right) leg, and the sciatic nerve trunk at the lower margin of the piriformis muscle was separated from the surrounding tissue. The sciatic nerve was dissected 0.5 cm below the ischial tuberosity, and anastomosed under 12 × magnification using a microscope (Zhenjiang Operation Microscope Instrument Factory, Zhenjiang, Jiangsu Province, China). Then, the fascia, subcutaneous tissue and skin were sutured. Postoperatively, mice were placed under a heating lamp to promote recovery from anesthesia[69].

Propofol injection into the site of sciatic nerve injury

Mice in the propofol and model groups were injected with propofol (3 mg/kg per day; local administration dose equivalent to the clinical dose[75]) or saline (1 mL per day), respectively, into the site of the injured sciatic nerve, for 1 week.

Specimens

At 12, 24 hours, 3, 5, 7 days, 2, 4 and 8 weeks, five mice selected from each group were anesthetized with 1% sodium pentothal (100 mg/kg) *via* intraperitoneal injection. A median incision was made on the posterior surface of the spinal cord, and the subcutaneous tissue and muscle were separated, exposing the spinous processes and lamina. The spinal canal was exposed using microscopic forceps, and the portion of the spinal cord containing the $L_{4–6}$ segments was cut. Specimens were preserved in freezing tubes and transferred to liquid nitrogen.

Five mice were randomly selected from each group for each time point and were intraperitoneally anesthetized. Then, the mice were subjected to thoracotomy, exposing the heart. Left ventricular catheterization was performed, and the animals were perfused with heparin saline and then with 4% paraformaldehyde buffer. Perfusion was terminated when clear perfusate flowed out of the right atrial appendage. Subsequently, the nerve trunk, 0.5–0.7 cm from the distal end of the sciatic nerve anastomotic stoma, was cut, and a portion of the ipsilateral spinal cord containing the $L_{4–6}$ segments was also cut. Tissues were fixed with 10% neutral formaldehyde for 72 hours, followed by ethanol dehydration and paraffin embedding.

Western blot analysis for the detection of nuclear factor kappa B expression in $L_{4–6}$ segments of the spinal cord on the side of injury

Specimens were taken out of liquid nitrogen, ground, boiled with loading buffer for 15 minutes, and centrifuged at 2 500 r/min for 5 minutes. The supernatant was discarded and RIPA lysate was added to separate and extract proteins. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed. Proteins were transferred to a polyvinylidene difluoride membrane and incubated with rabbit anti-mouse nuclear factor kappa B monoclonal antibody (1:1 000; Roche Molecular Biochemicals, Indianapolis, IN, USA) at 4°C overnight, rinsed with 0.01 mol/L PBS four times for 5 minutes each, incubated with goat anti-rabbit IgG (1:1 000; Roche) at room temperature for 1 hour, and

rinsed with 0.01 mol/L PBS four times for 5 minutes each. Immunoreactive bands were detected with 3,3'-diaminobenzidine according to the kit instructions (Roche) and the membrane was exposed to X-ray film. Then, scanning and analysis of the film was performed, and the absorbance value of the target band was analyzed using a gel image processing system (Alpha-Innotech Company, San Leandro, CA, USA). Values were expressed as the ratio of the intensity of the target band to that of GAPDH.

Real-time PCR for the detection of nuclear factor kappa B mRNA expression in $L_{4–6}$ segments of the spinal cord on the side of injury

Specimens were taken out of liquid nitrogen, total RNA was extracted with Trizol, RNA concentration was determined, and cDNA was prepared through reverse transcription. Nuclear factor kappa B primers were designed with Beacon Designer 8 software (Premier Biosoft, Palo Alto, CA, USA), and GAPDH served as the reference. The specificity of the primers was verified using Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Primer sequences are as follows:

Primer	Sequence (5′–3′)
Nuclear factor kappa B	S: GCA AAG GAA ACG CCA GAA GC
	A: CAC TAC CGA ACA TGC CTC CAC
	Probe: CGC TCC ACT GCC GCC ACC GAA G
GAPDH	S: AAT GTG TCC GTC GTG GAT CTG
	A: CAA CCT GGT CCT CAG TGT AGC
	Probe: CGT GCC GCC TGG AGA AAC CTG CC

S: Sense strand; A: antisense strand.

Reaction conditions: 40 cycles of 95°C for 30 seconds, 58°C for 60 seconds, and 72°C for 60 seconds. The C_t values of the target and reference genes in each group were determined and entered into a standard curve (supplementary Figure 1 online). Quantitative analysis of the target gene was performed, and nuclear factor kappa B mRNA expression was calculated using Sigma Plot 8.0 (Bio-Rad, Hercules, CA, USA).

Luxol fast blue staining for assessing morphological changes in the myelin sheath in spinal cord $L_{4–6}$ segments

Eight weeks after surgery, the paraffin embedded neural tissues were dewaxed and stained with hematoxylin-eosin. The basic structure of the adjacent nerve tissue and the presence of pathological changes were observed. Specimens were dewaxed again and immersed in luxol fast blue solution (Roche) at 60°C for 12 hours, rinsed with 95% ethanol for 5 minutes, treated with 0.05% lithium carbonate for 15 seconds, rinsed with 70% ethanol, dehydrated, cleared, and mounted. Specimens were observed under an inverted microscope (Olympus, Tokyo, Japan).

Cellular apoptosis in spinal cord $L_{4–6}$ segments detected using the TUNEL assay

At 1, 2, 4 and 8 weeks after surgery, the $L_{4–6}$ segments of the spinal cord were dewaxed and fixed in 4% paraformaldehyde and PBS for 15 minutes, and rinsed with PBS twice for 5 minutes each. Specimens were incubated with 100 μL of 20 μg/mL protease K solution for 8–10 minutes at room temperature, and rinsed with PBS for 5 minutes. Then, specimens were immersed in 4% paraformaldehyde and PBS for 5 minutes, and rinsed with PBS twice for 5 minutes each. Specimens were equilibrated in 100 μL buffer for 5–10 minutes at room temperature, and immersed in 100 μL TUNEL reaction solution (Roche) in the dark at 37°C for 1 hour. Then, specimens were immersed in 2 × SSC for 15 minutes, rinsed with PBS three times for 5 minutes each, and incubated in 0.3% H_2O_2 for 15 minutes. After specimens were stained with 100 μL 3,3'-diaminobenzidine for 10 minutes, a light brown background was visible under the microscope. Specimens were rinsed with deionized water five times, dehydrated in a graded ethanol series, cleared with xylene, and mounted with neutral gum. Five visual fields for each slice were randomly selected under an optical microscope (Olympus), and the number of apoptotic cells was counted and the average value obtained.

Electrophysiological examination of the sciatic nerve

At 1, 2, 4 and 8 weeks after surgery, five mice in each group were examined with an electromyography/evoked potential tester (Medtronic Keypoint, Medtronic Company, Minneapolis, MN, USA) at 24°C. Mice were intraperitoneally anesthetized, and the skin was disinfected at the level of the sciatic nerve, which was exposed. A recording electrode was inserted into the soleus muscle belly (M point), and the ground electrode was placed at the tail. Two parallel stimulating electrodes (2-mm apart) in the distal sciatic nerve bifurcation (D point) and at the proximal ischial tuberosity level (P point) were applied to produce supramaximal stimulation (10 mA current). The distance between the two stimulation electrodes was measured using a vernier caliper (Edward Measurement Equipment Co., Ltd., Xi'an, Shaanxi Province, China). Motor nerve conduction velocity was calculated according to the formula: motor nerve conduction velocity = distance between the two stimulating electrodes / differ-

ence in action potential latency.

Statistical analysis

Data were expressed as mean ± SD and were statistically analyzed using SPSS 19.0 software (SPSS, Chicago, IL, USA). Difference in mean values among groups was compared using one-way analysis of variance. The expression of nuclear factor kappa B mRNA was compared with group *t*-test (independent sample *t*-test), while electromyographic amplitude and the number and average diameter of myelinated nerve fibers were compared with paired *t*-test. Differences in nuclear factor kappa B expression, motor nerve conduction velocity and latency between groups were compared with least significant difference *t*-test. A *P* value less than 0.05 was regarded to indicate a significant difference.

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