

# 7,8-dihydroxycoumarin may promote sciatic nerve regeneration by suppressing NF- $\kappa$ B expression in mice

JIAN-SHI DU, QING ZHAO, YING-LI ZHANG, YU WANG and MING MA

Department of Neurology, China-Japan Union Hospital, Jilin University, Changchun, Jilin 130033, P.R. China

Received March 17, 2013; Accepted September 4, 2013

DOI: 10.3892/mmr.2013.1682

**Abstract.** Nuclear factor (NF)- $\kappa$ B expression occurs during sciatic injury. In addition, 7,8-dihydroxycoumarin exhibits a neurotrophic effect on peripheral nerve regeneration. To investigate the effects of 7,8-dihydroxycoumarin on the expression levels of NF- $\kappa$ B in L4-6 spinal cord segments of the injured sciatic nerve in mice and on the functional recovery and regeneration following nerve injury, a total of 160 healthy adult male BALB/c mice underwent unilateral sciatic nerve interruption and anastomosis. The mice were separated into groups and subsequently treated with physiological saline (control) or high, medium or low doses of 7,8-dihydroxycoumarin. NF- $\kappa$ B levels were detected by western blot analysis and quantitative polymerase chain reaction (qPCR), and the sciatic functional index (SFI) was measured. Neuronal apoptosis was detected by terminal-deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) staining. The results revealed that NF- $\kappa$ B was activated in the L4-6 spinal cord connected to the injured sciatic nerve. qPCR and western-blot analysis results showed that the expression levels of NF- $\kappa$ B in the high- and medium-dose groups were significantly lower compared with the low-dose and control groups at 12 h, one day, three days, five days and one week ( $P < 0.05$  for each). SFI and TUNEL results demonstrated that the high- and medium-dose groups exhibited improved functional nerve regeneration and reduced apoptosis compared with the low-dose and control groups. In conclusion, 7,8-dihydroxycoumarin is capable of suppressing the immune activation of NF- $\kappa$ B in the neurons of the L4-6 spinal cord connected with the injured sciatic nerve, thereby reducing the focal infiltration of inflammatory cells, producing the optimum environment for nerve regeneration.

## Introduction

Nerve repair and regeneration of the peripheral nerve following injury, is a long and complex biological process, and is not able to be surgically treated. The immune response induced by peripheral nerve injury inhibits nerve repair and regeneration (1). The strength of the local immune response is proportional to the severity of the nerve injury. The more severe the nerve injury the stronger the immune response, which in turn, results in a decreased ability of nerve regeneration and functional recovery (2,3). Immunosuppressants, such as FK506, have been widely used for axon regeneration (4-9). However, FK506 is associated with the side effect of nervous system disorder. Varying dosages of immunosuppressants may result in multifocal cerebrospinal meningitis, optic neuritis, inflammation of the spinal nerve root, tremors and discoordination (10,11). Patients who have received an organ transplant also exhibit other mild side effects such as insomnia, tremors, headaches and photophobia, or more apparent side effects, such as clouding of consciousness, epileptic seizures, comas and dysarthroses (5,12).

Studies have shown that natural immunosuppressive drugs have fewer side effects and more complex underlying mechanisms (13-15). Their mechanism of action is related to the immune system and the regulatory neuroendocrine-immune network (16). Previous, studies concerning traditional Chinese medicines with immunosuppressive potential for the purpose of a high benefit-risk ratio have located novel candidates (13,14,17,18), one of which is 7,8-dihydroxycoumarin. 7,8-Dihydroxycoumarin is an effective monomer isolated from *Thymelaeaceae Daphne* plants, with a molecular formula of  $C_9H_6O_4$  (18). 7,8-Dihydroxycoumarin is involved in cleaning necrosis-facilitating substances, balancing water electrolytes, neurotransmitter and energy metabolism, and the secretion of neurotrophic factor, thus it is involved in the functional recovery of neurons (18,19). 7,8-Dihydroxycoumarin exhibited certain protective and inhibitive effects on the cardiovascular system, and is capable of passing through the blood brain barrier (18-20). Following injury to the peripheral nerve, Wallerian degeneration occurs at the distal end of the injured nerve, and retrograde degeneration is observed at the proximate end (21,22). The key to controlling the degenerative process is to suppress the immune inflammatory response (1) as contaminant inflammation in nerve injury not only affects nerve repair and regeneration, but also leads to pathological neuralgia.

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*Correspondence to:* Dr Qing Zhao, Department of Neurology, China-Japan Union Hospital, Jilin University, 126 Xiantai Street, Changchun, Jilin 130033, P.R. China  
E-mail: zhaoqingjlu@sina.cn

**Key words:** 7,8-dihydroxycoumarin, nuclear factor- $\kappa$ B, peripheral nerve injury, nerve regeneration

Nuclear factor (NF)- $\kappa$ B, a nucleoprotein factor is capable of binding to a specific  $\kappa$ B enhancer sequence of the immunoglobulin  $\kappa$  light chain gene, and has a wide range of expression in the nervous system (23,24). NF- $\kappa$ B is able to regulate immune and inflammatory responses, as well as the activity, plasticity and neuropathic pain of neurons following injury (25). Brambilla *et al* (26) demonstrated that the immunosuppression of NF- $\kappa$ B in astrocytes may alleviate the inflammatory response following spinal cord injury and promote the functional recovery of the spinal cord. However, to the best of our knowledge, there have been no studies regarding the effect of 7,8-dihydroxycoumarin on the expression of NF- $\kappa$ B in peripheral nerve regeneration following injury.

In this study, the sciatic nerve injury BALB/c mouse model was treated with 7,8-dihydroxycoumarin, in order to observe the expression of NF- $\kappa$ B in spinal motor neurons and the effects of 7,8-dihydroxycoumarin on peripheral nerve regeneration following injury.

## Materials and methods

**Materials and animals.** 7,8-Dihydroxycoumarin powder (purity, 95.5%; Fig. 1) was purchased from Xidian Pharmaceutical Co., Ltd. (Changchun, China). The powder was dissolved in 0.9% saline solution, sterilized through a membrane filter ( $\Phi$  0.2  $\mu$ m) and stored at -20°C for later use. In total 160 healthy adult male BALB/c mice (weight, 20 $\pm$ 2 g) were donated by the Laboratory Animal Center of the Fundamental Medical College of Jilin University (Changchun, China) and were raised at room temperature with a routine diet and access to water *ad libitum*. The mice were randomized into control (treated with physiological saline), high-dose, medium-dose and low-dose groups, respectively, with 40 mice per group. The animal experimental protocols were approved by the Jilin University Laboratory Animal Ethics Committee (27).

**Animal modeling.** All BALB/c mice were anesthetized by intraperitoneal injection of 1% sodium thiopental with a dosage of 100 mg/kg body weight. Mice were fixed in the prone position and 1.5 cm-longitudinal incisions were aseptically prepared on the unilateral rear thigh. The subcutaneous tissues were separated and the infrapiriformis muscle underwent blunt dissection to expose the cord of sciatic nerves. The sciatic nerve cords were carefully separated from the surrounding tissues using blunt glass needles. Subsequently, the sciatic nerve cords were interrupted at 0.3 cm below the ischial tuberosity and were microsurgically anastomosed by 11-0 microsutures (SharpPoint™ MicroSutures; Angiotech Pharmaceuticals Inc., Vancouver, BC, Canada), with the aid of a microscope (magnification, x12; OLYMPUS SZX16; Olympus Corp. OCN, Beijing, China), to close the muscle and skin.

**Drug intervention.** 7,8-Dihydroxycoumarin was dissolved in physiological saline and administered to the mice intragastrically (ig). The ig dose was determined by equivalent conversion on the basis of the clinical dose of 7,8-dihydroxycoumarin (28) and this dose value served as the medium dose. The high and low doses were determined by geometric progression. The final high-, medium- and low-doses were 16, 8 and 4 g/kg/day,

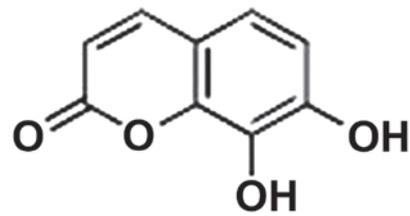


Figure 1. Structure of 7,8-dihydroxycoumarin.

respectively. Physiological saline of an equivalent volume served as the control. The injections were administered daily for seven continuous days.

**Sciatic functional index (SFI) test.** At four and eight weeks following injury, the walking gait of each group was observed and analyzed and the SFI was calculated. The specification of the box was 5cm x 5cm x 8 cm. A cage with a unilateral door was placed at the remote end of the box and a piece of white paper, the same length and width as the box, was placed beneath. The mice were placed into the near end of the box after their pelma had been dyed with ink, and they were encouraged to move towards the remote end by tapping the box. Approximately 6-7 mouse footprints were marked on the paper. The footprints of the injured foot (E) and normal foot (N) were recorded and the following three indicators were measured: Podogram length (PL), the longest distance of footprints from the heel to toe; the width between the first and fifth toes (TW), the ligature distance from the first toe to the fifth toe, i.e. the ligature distance was the same as the width; and the inter-toe distance (IT)—the ligature distance from the second toe to the fourth toe. The data group with the largest numerical value was selected for use. The three variables were placed into the following formula and SFI was calculated:  $SFI = -38.3(EPL - NPL) / NPL + 109.5(ETW - NTW) / NTW + 13.3(EIT - NIT) / NIT - 8.8$ . SFI=0 refers to normal and SFI=-100 refers to a complete nerve injury.

**Spinal cord sampling.** Five mice from each group were anesthetized by intraperitoneal injection of 1% sodium thiopental with a dosage of 100 mg/kg body weight at predetermined times (12 h, 24 h, three days, five days, seven days, two weeks, four weeks and eight weeks following surgery). The *canalis vertebralis* was exposed via a midline incision to the posterior vertebral column. The L4-6 spinal cord segment connected to the injured sciatic nerves was dissected intact, dissociated and removed. Subsequently the tissue samples were stored in liquid nitrogen for use in quantitative-polymerase chain reaction (qPCR) and western blot analysis.

Five mice from each group were selected and individually fixed in the supine position. A perfusion needle was placed to the aortic root by the left ventricular cardiac apex. Rapid perfusion of 50-100 ml physiological saline was performed to wash the blood. As the outflow from the *auricula dextra* became limpid, perfusion was performed with 300-400 ml fresh 4% paraformaldehyde in phosphate-buffered saline (PBS). The perfusion lasted 30 min for tissue fixation. Following the completion of the perfusion, the

*canalis vertebralis* was exposed. L4-6 spinal cord segments connected with the injured sciatic nerves were dissected, dissociated and removed. These tissues were stored until the terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling (TUNEL) assay was performed.

**Western blot analysis detects NF- $\kappa$ B protein.** Tissue samples stored in liquid nitrogen were removed quickly and ground with a pestle and mortar. Cells were lysed in an ice-cold radioimmunoprecipitation assay lysis buffer (Beyotime, Nanjing, China) for 15 min. The proteins were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and electroblotted onto a polyvinylidene fluoride (PDVF) film. The PDVF film was incubated in rabbit anti-mouse NF- $\kappa$ B antibodies (diluted 1,000 times with PBS containing 1% bovine serum albumin; Beyotime) overnight at 4°C and rinsed four times with 0.01 mol/l PBS, for five min per rinse. The color was developed using a western blotting 3,3'-diaminobenzidine tetrahydrochloride (DAB) testing kit (Beyotime). X-ray film exposure was performed and the samples were scanned and analyzed.

**qPCR analysis.** NF- $\kappa$ B and reduced glyceraldehyde-phosphate dehydrogenase (GAPDH) primers were designed by Beacon Designer 7 software (Premier Biosoft Int., Palo Alto, CA, USA) and synthesized by Sangon Biotech (Shanghai, China). Primer sequences are shown in Table I. Total RNA was extracted from tissue samples using TRIzol. cDNA was cloned by reverse transcription using total RNA templates. The NF- $\kappa$ B gene was detected by qPCR using cDNA templates. GAPDH served as an inner control for each reaction system. The reaction conditions were as follows: 95°C for 30 sec; 58°C for 60 sec and 72°C for 60 sec, for a total of 40 cycles. Subsequently, the relative mRNA content of NF- $\kappa$ B/GAPDH was determined.

**Apoptosis of neurons (TUNEL method).** The tissues of the L4-6 spinal cord segment were routinely prepared into 3- $\mu$ m thick, paraffin-embedded slices (20). The specimen slices on the glass slides were immersed in a 4% paraformaldehyde/PBS solution for 15 min, incubated in 20  $\mu$ g/ml proteinase K solution for 10 min at room temperature, immersed in 4% paraformaldehyde/PBS solution for a further 5 min and then incubated in the balancing saline solution for 10 min. The TUNEL reaction solution (29) was added dropwise onto the specimens, a cover slip was added to cover the specimens, followed by the reaction in a wet box in the dark at 37°C for 1 h. Specimens were immersed in 2X saline sodium citrate solution for 15 min. Specimens were incubated in 0.3% H<sub>2</sub>O<sub>2</sub> for 15 min. A DAB mixture was added for 10 min for color development. Subsequently, specimens were rinsed with deionized water, dehydrated with gradient ethanol (1 min at 50, 70, 85, 95 and 100% respectively), hyalinized with xylene twice, for 1 min each time, and sealed with neutral resin. Five fields were randomly selected for each slide and the apoptotic cells were observed.

**Statistical analysis.** SPSS 10.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis and Student's t-test was conducted for comparison. Data are presented as the mean  $\pm$  SD. P<0.05 was considered to indicate a statistically significant difference.

Table I. NF- $\kappa$ B and GAPDH primer sequences.

| Primer name          | Sequence (5'-3')        |
|----------------------|-------------------------|
| NF- $\kappa$ B-S     | GCAAAGGAAACGCCAGAAGC    |
| NF- $\kappa$ B-A     | CACTACCGAACATGCCTCCAC   |
| NF- $\kappa$ B-probe | CGCTCCACTGCCGCCACCGAAG  |
| GAPDH-S              | AATGTGTCCGTCGTGGATCTG   |
| GAPDH-A              | CAACCTGGTCCTCAGTGTAGC   |
| GAPDH-probe          | CGTGCCGCCTGGAGAAACCTGCC |

NF, nuclear factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; -S, sense; -A, antisense.

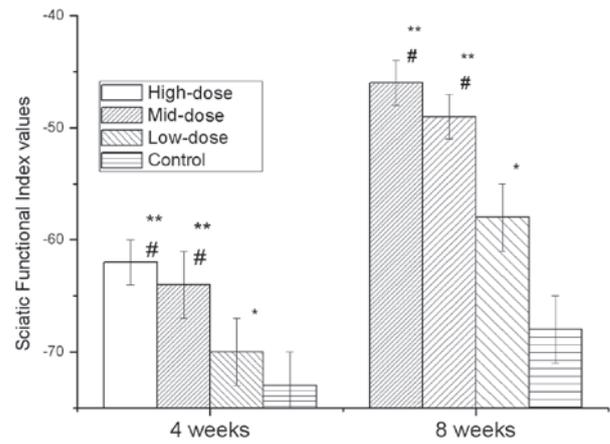


Figure 2. Effects of 7,8-dihydroxycoumarin on the sciatic functional index in mice following sciatic nerve injury. \*\*P<0.01 and \*P<0.05 compared with the control group; #P<0.05 compared with the low-dose group (Student's t-test).

## Results

**SFI test.** Fig. 2 shows the SFI of each group at two time points, four and eight weeks, after modeling. The high-, mid- and low-dose groups exhibited significant differences compared with the control (P<0.05). The high- and mid-dose groups exhibited significant differences compared with the low-dose group (P<0.05), but the high-dose group was not significantly different compared with the mid-dose group. These results revealed that the application of 7,8-dihydroxycoumarin improved the SFI following injury to the sciatic nerve.

**Western blot analysis.** Fig. 3 shows the results of the western blot analysis. Grayscale analysis (Table II) demonstrated that the level of NF- $\kappa$ B protein in each group increased between 12 and 24 h following injury to the sciatic nerve, then the expression levels of NF- $\kappa$ B in the high-dose group decreased to a normal level in one week and expression levels of NF- $\kappa$ B in the medium- and low-dose groups decreased to normal levels in two and four weeks, respectively. However, the NF- $\kappa$ B levels in the control group remained elevated after eight weeks. The expression levels of NF- $\kappa$ B between the post-surgical times of 12 h, 24 h, three days, five days and one week in each experimental group exhibited statistically significant differences (P<0.05). NF- $\kappa$ B expression levels in the high- and

Table II. Relative grayscale of NF- $\kappa$ B/GAPDH blots in different groups (n=5).

| Group         | Time                   |                        |                        |                        |                        |                        |                        |                        |
|---------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|
|               | 12 h                   | 1 day                  | 3 days                 | 5 days                 | 1 week                 | 2 weeks                | 4 weeks                | 8 weeks                |
| High-dose     | 0.01±0.03 <sup>a</sup> | 0.27±0.00 <sup>a</sup> | 0.68±0.03 <sup>a</sup> | 1.21±0.02 <sup>b</sup> | 0.53±0.02 <sup>a</sup> | 0.18±0.03 <sup>a</sup> | 0.09±0.03 <sup>a</sup> | 0.03±0.01 <sup>b</sup> |
| Mid-dose      | 0.01±0.01 <sup>a</sup> | 0.39±0.01 <sup>a</sup> | 0.79±0.03 <sup>a</sup> | 1.33±0.02              | 0.62±0.04 <sup>a</sup> | 0.66±0.03 <sup>b</sup> | 0.16±0.01 <sup>b</sup> | 0.03±0.01 <sup>b</sup> |
| Low-dose      | 0.01±0.02 <sup>a</sup> | 0.49±0.01 <sup>a</sup> | 1.11±0.02 <sup>b</sup> | 1.60±0.02              | 1.89±0.02 <sup>b</sup> | 0.67±0.04 <sup>b</sup> | 0.29±0.03              | 0.03±0.01 <sup>b</sup> |
| Model control | 1.25±0.01              | 2.21±0.01              | 2.01±0.02              | 1.74±0.24              | 3.30±0.03              | 1.02±0.03 <sup>b</sup> | 0.23±0.02              | 0.07±0.02              |

<sup>a</sup>P<0.01 and <sup>b</sup>P<0.05 compared with the control group (Student's t-test). NF, nuclear factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Values are provided as the mean ± SD.

Table III. Apoptosis cell count of neurons (n=5).

| Group         | Time (weeks)           |                        |                        |                        |
|---------------|------------------------|------------------------|------------------------|------------------------|
|               | 1                      | 2                      | 4                      | 8                      |
| High-dose     | 6.61±0.32 <sup>a</sup> | 3.33±0.21 <sup>a</sup> | 2.76±0.20 <sup>a</sup> | 2.08±0.18 <sup>a</sup> |
| Mid-dose      | 6.82±0.43 <sup>a</sup> | 4.18±0.32 <sup>a</sup> | 3.91±0.30 <sup>a</sup> | 3.02±0.20 <sup>a</sup> |
| Low-dose      | 9.72±0.31              | 8.18±0.32              | 6.18±0.32              | 5.18±0.32              |
| Model control | 10.11±0.03             | 8.86±0.02              | 7.12±0.01              | 6.31±0.02              |

<sup>a</sup>P<0.05 compared with the control group. Values are provided as the mean ± SD.

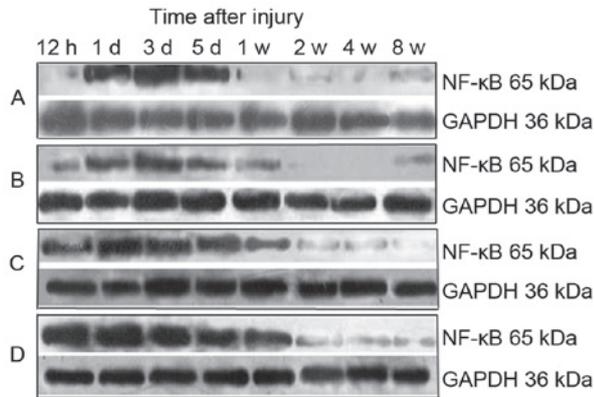


Figure 3. NF- $\kappa$ B protein blots in the L4-6 spinal cord segments of mice with sciatic injury. (A) High-dose group, (B) medium-dose group and (C) low-dose group. 7,8-dihydroxycoumarin reduced NF- $\kappa$ B protein expression in the L4-6 segments compared with the (D) control group. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NF, nuclear factor.

medium-dose groups were significantly inhibited compared with the control group (P<0.01).

**qPCR analysis.** qPCR analysis of relative NF- $\kappa$ B mRNA in the spinal cord are shown in Fig. 4. Between 12 to 24 h following injury to the sciatic nerve, NF- $\kappa$ B mRNA contents in the spinal cord segments connected with the injured sciatic nerve were increased in the short term and the increments in the high- and medium-dose groups were significantly less than those in the low-dose and control groups (P<0.05). After one week, the NF- $\kappa$ B mRNA content was not significantly

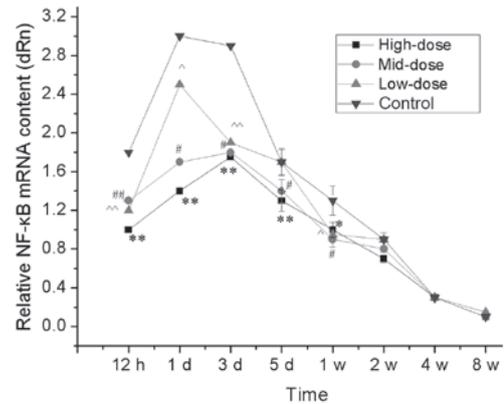


Figure 4. Relative quantity of NF- $\kappa$ B mRNA expression between 12 h to 8 weeks following sciatic nerve injury in mice treated with 7,8-dihydroxycoumarin. <sup>\*\*\*</sup>^P<0.01, <sup>\*,#</sup>^P<0.05 compared with the control group. The results are expressed as the mean ± SD of the five mice in each group. Statistical differences were compared using Student's t-test. NF, nuclear factor.

different between the groups. These results demonstrated that the high- and medium-dose of 7,8-dihydroxycoumarin suppressed the expression of NF- $\kappa$ B.

**TUNEL detects the apoptosis of neurons.** The results of neuron apoptosis following TUNEL staining are shown in Table III. The cell count of neuron apoptosis peaked at one week following injury and decreased at two, four and eight weeks. The apoptotic cell count of the high- and mid-dose groups at each time point was significantly lower than that of the low-dose and control groups (P<0.05).

## Discussion

In this study, 7,8-dihydroxycoumarin was injected into the BACB/c mice following sciatic nerve injury, and the mice were tracked and tested for 8 weeks. The western-blot analysis and qPCR analysis results respectively revealed that the expression levels of NF- $\kappa$ B protein in the L4-6 spinal cord segment of the high- and mid-dose groups were lower than that of the low-dose and control groups. The SFI test results demonstrated that the nerve function index increased during the regeneration of injured nerves; the SFIs of the high- and mid-dose groups were significantly higher than that of the low-dose and control groups, and the SFI of the low-dose group was significantly higher than that of the control group. This indicates that 7,8-dihydroxycoumarin promotes the functional recovery of injured nerves (18,19). The low-dose group was incapable of significantly inhibiting NF- $\kappa$ B protein expression (it was not significantly different when compared with the control group), but significantly promoted the functional recovery of injured nerves. These results suggested that the application of 7,8-dihydroxycoumarin following nerve injury promotes nerve regeneration and functional recovery (18,19).

Following the continuous injection of 7,8-dihydroxycoumarin for eight weeks, the expression levels of NF- $\kappa$ B in the high- and mid-dose groups were not significantly different compared with the low-dose and control groups, yet the SFI, which refers to the degree of recovery following nerve injury, exhibited significant differences. These results suggest that the nerve repair and regeneration process of high- and mid-dose groups are completed after eight weeks, thus the NF- $\kappa$ B expression levels were gradually reduced, to approximately that of the low-dose and control groups. Thus, at eight weeks the neural function appeared to have been well recovered. The neuron apoptosis count of results following TUNEL staining reveal that the neuron apoptosis count of each group peaks one week after injury and declines at two, four and eight weeks. The apoptotic cell count of the high-dose group at each point was significantly lower than that of the low-dose and control groups.

NF- $\kappa$ B is a key nuclear transcription factor that exists in eukaryotes and is present in the cytoplasm as inactive homologous or heterologous dimers, which form complexes with its inhibitory proteins, I $\kappa$ Bs (25). Following different types of internal and external stimuli (certain cytokines, growth factors, immunity receptors, ischemia, anoxia and injury), NF- $\kappa$ B dissociates from I $\kappa$ Bs and is rapidly transferred into the nucleus where it binds to the target gene promoter or enhancer  $\kappa$ B motif to modulate the synthesis of the mRNA of target genes. This results in the participation of NF- $\kappa$ B in various biological processes, such as immune response, inflammation, apoptosis and cell proliferation (27). NF- $\kappa$ B has multiple regulatory actions on gene transcription, such as binding to specific sequences of the immunoglobulin gene  $\kappa$ -light chain enhancer  $\kappa$ B, it participates in inflammatory nerve lesions, pathologic nerve pain and functional change that inhibits axonal regeneration and promotes neuronal apoptosis following nerve injury (25). Protecting neuronal soma and avoiding irreversible degeneration are prerequisites for successful regeneration following nerve injury to aid nerve regeneration by promoting injured neuron survival and inhibiting neuron degeneration (25,26). In the present study, the potential nerve repair accelerator, 7,8-dihydroxycoumarin,

was used. The application of 7,8-dihydroxycoumarin following injury to the peripheral nerve in mice was capable of restraining the expression of NF- $\kappa$ B protein in a dose-dependent manner. The results of the SFI test demonstrated that the nerve function recovery with 7,8-dihydroxycoumarin was markedly improved compared with the control group. Therefore, it is hypothesized that 7,8-dihydroxycoumarin reduces neuron cell apoptosis in the course of repair and regeneration by inhibiting NF- $\kappa$ B expression in L4-6 spinal cord neurons following nerve injury, this provides favorable conditions for nerve repair and regeneration and is protective in nerve regeneration following injury.

The present study has certain shortcomings. The local immune response following nerve injury has not yet been tested. Furthermore, further studies are required regarding the cell signaling pathways related to the local inflammatory reaction following nerve injury. In conclusion, 7,8-dihydroxycoumarin is a potential drug for use in peripheral nerve repair and regeneration.

## Acknowledgements

The study was funded by the Jilin Provincial Science & Technology Development (Project no. 20100751).

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