

Original Article

Effects of NP-1 on proliferation, migration, and apoptosis of Schwann cell line RSC96 through the NF- κ B signaling pathway

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Abstract: Peripheral nerve injury is a common refractory disease in the clinic that often leads to dysfunction of movement and sensation. Different from other tissue injuries, peripheral nerve injury needs a longer time for regeneration. Therefore, effective drug therapy is needed to promote nerve regeneration in the treatment of peripheral nerve injury. Our preliminary studies have shown that continuous intramuscular injection of NP-1 promotes the regeneration of injured sciatic nerve in rats, but the mechanisms were still unknown. Schwann cells are very important cells in the formation of myelin sheath of peripheral nerves and participate in the repair and regeneration of peripheral nerve injury. To further investigate the effect of NP-1 on rat Schwann cells and the underlying mechanism, different concentrations of NP-1 were used to treat rat Schwann cell line RSC96. Light microscopy, CCK-8 assay, cell scratch assay, and special cell staining were performed to investigate RSC96 cell aging and apoptosis. mRNA and protein expression of NF- κ B signaling pathway-related factors were determined using qPCR and immunohistochemistry respectively. Light microscopy, CCK-8 assay, cell scratch assay, and special cell staining showed NP-1 could improve the ability of proliferation, immigration of Schwann cells. QPCR and immunohistochemistry showed NP-1 influenced the expression of multiple factors associated with nerve regeneration which NF- κ B signaling pathway played a key role. The results show that NP-1 promoted the proliferation and migration of RSC96 cells and inhibited cell aging and apoptosis possibly through the NF- κ B signaling pathway. These findings provide a potential target for clinical treatment of peripheral neuropathy and experimental data support.

Keywords: Schwann cells, NP-1, NF- κ B signaling pathway, proliferation, migration

Introduction

Peripheral nerve injury is a common complication of trauma, and can cause serious dysfunction, even limb disability [1]. Traction injury [2], crush injury [3], and infant shoulder dystocia [4] can cause peripheral nerve injuries. Nerve transplantation [5] and sutures [6] are often used to restore the continuity of the damaged nerve. This clinical repair process, depending on the location of the nerve injury, takes several months, usually, more than 10 months. However, in most patients, the clinical repair is unsatisfactory and nerve function is not com-

pletely restored. Therefore, the study of peripheral nerve injury has become an important area of research [7, 8].

Schwann cells are special glial cells of the peripheral nervous system that surround the axons of neurons to form the myelin sheath [9]. They play important roles in the growth, development, maturation, function, and maintenance of peripheral nerves [10, 11]. The number of Schwann cells present at the site of injury as well as their regeneration and proliferation abilities are the key factors responsible for the repair of a peripheral nerve injury [12]. Proteins

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[13], microRNAs [14], and long non-coding RNAs [15] affect the repair process of peripheral nerve injury by regulating the proliferation of Schwann cells. Moreover, the neurotrophic and nerve growth factors secreted by Schwann cells are involved in the repair of peripheral nerve injury [16, 17]. Thus, the functional status of Schwann cells is very important for repair of injured peripheral nerves. The Schwann cell line, RSC96, was established through spontaneous transformation of primary cultures of rat Schwann cells that had been cultured for long durations. RSC96 cells have retained the characteristics of primary Schwann cells and can be sub-cultured several times, and therefore, are widely used in research involving peripheral nerves [18, 19].

Neutrophil peptide 1 (NP-1), also known as α defensin 1, is a member of the α defensin family that is mainly secreted by neutrophils [20]. NP-1 is closely related to the repair of peripheral nerve injuries. Studies have shown that continuous injection of NP-1 is beneficial to the repair and regeneration of injured peripheral nerves in rats; moreover, it has a positive effect on the rate of regeneration after nerve injury and excitation and conduction of nerve impulses [21, 22]. NP-1 can regulate synaptic transmission [23], affect neuronal function [24], and thereby exhibit a great potential for peripheral nerve repair. NF- κ B signaling pathway is central to the responses of cells to inflammation, stress, and injury [25, 26], and plays an important role in the repair of injured peripheral nerves [27, 28]. It has been reported that NF- κ B signaling pathway can regulate inflammation [29], proliferation [30], migration [31], aging, and apoptosis [32] of Schwann cells. However, few reports have described the effects of NP-1 on Schwann cell function. Moreover, whether the effects of NP-1 are mediated by the NF- κ B signaling pathway has not been investigated in detail.

Herein, we investigated the effects of NP-1 on the repair of injured peripheral nerves and on Schwann cell proliferation, migration, aging, and apoptosis, as well as the underlying mechanism. Moreover, we investigated the underlying mechanism at the cellular level to provide additional experimental evidence for the use of NP-1 in the clinical treatment of peripheral nerve injury.

Methods and materials

Detection of RSC96 cell activity using CCK-8 assay

RSC96 cells (the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China) (3000-4000 per well) were seeded in a 96-well plate. Cells were cultured for 12-24 h with culture medium supplemented with 2% fetal bovine serum (FBS) (Gibco, Life Technologies, Carlsbad, USA). Cells were then treated with different concentrations of NP-1 (0, 4, 8, 12, and 16 μ g/mL) (Shanghai Qiangyao Biological Technology Co., Ltd., Shanghai, China) prepared in culture medium supplemented with 2% FBS. Cells in the negative control group were treated with 100 μ L of culture medium supplemented with 2% FBS, but without NP-1. After treatment for 12, 24, 36, 48, and 60 h, cells in each well were treated with 10 μ L of CCK-8 solution (Dongren Chemical Technology, Shanghai, China) at 37°C for 2 h. Optical density at 450 nm was measured using an ELISA reader (Bio-Rad, USA). Cell survival and inhibition rates were calculated according to the following formulas: cell survival rate = $(As-Ab)/(Ac-Ab)$; cell inhibition rate = $(Ac-As)/(Ac-Ab)$, where As represents the absorbance of experimental well (cell-containing culture medium + CCK-8 + different concentrations of NP-1), Ac represents the absorbance of control well (cell-containing culture medium + CCK-8), and Ab represents the absorbance of blank control well (CCK-8 + culture medium without cells + NP-1).

Cell cycle analysis by flow cytometry

RSC96 cells were seeded in a 12-well plate at a density of 3×10^5 /well. Cells were cultured for 12-24 h with culture medium supplemented with 2% FBS. Cells were then treated for 36 h with different concentrations of NP-1 (0, 4, and 8 μ g/mL) prepared in culture medium supplemented with 2% FBS. The following experimental steps were carried out according to the instructions of the biological cell cycle test kit: Cells from each well of the 12-well plate were transferred into a separate 1.5 mL Eppendorf tube (Eppendorf, Hamburg, Germany) and centrifuged at 2930 rcf for 5 min at 23°C-27°C. Supernatant was discarded, and cells were washed with phosphate-buffered saline (PBS)

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(Gibco, Life Technologies, Carlsbad, USA) and then centrifuged again at 2930 rcf for 5 min at 23°C-27°C. After discarding the supernatant, 1 mL DNA staining solution and 10 µL permeabilization solution were added to each tube. Subsequently, cells were suspended in working solution, shaken for 5-10 min, incubated for 30 min in the dark at room temperature, and then assessed by flow cytometry (Beckman Coulter, Brea, CA, USA).

Cell scratch assays

RSC96 cells were seeded in a 48-well plate with 2×10^5 per well and then incubated for 36 h in culture medium supplemented with 2% FBS. Cell monolayers were then scraped in horizontal direction to with create a “scratch” using a 200 µL pipet tip and washed with PBS. Subsequently, cells were treated with different concentrations of NP-1 (0, 4, and 8 µg/mL) prepared in culture medium supplemented with 2% FBS for 36 h. After treatment, cells were photographed using an optical microscope (Shanghai BIEM Optical Instrument Manufacturing Co., Ltd, Shanghai, China).

Detection of cell aging using β -galactosidase staining

RSC96 cells (3000-4000 per well) were seeded in a 96-well plate. Cells were cultured for 36 h with culture medium supplemented with 2% FBS for 12-24 h. Cells were then treated with different concentrations of NP-1 (0, 4, and 8 µg/mL) prepared in culture medium supplemented with 2% FBS. Subsequently, cells were stained with a β -galactosidase staining kit (Beyotime Biotechnology, Shanghai, China) and then photographed using an optical microscope (Olympus, Japan).

Detection of changes in cell nuclei using 4',6-diamidino-2-phenylindole (DAPI) staining

RSC96 cells were cultured in an incubator with 1-3% CO₂ at 37°C and seeded in a 96-well plate at approximately 3000-4000 cells/well. Cells were cultured for 12-24 h with culture medium supplemented with 2% FBS and then treated for 36 h with different concentrations of NP-1 (0, 4, and 8 µg/mL) prepared in culture medium supplemented with 2% FBS. Subsequently, cells were washed three times with PBS for 5 min each, fixed with 4% paraformaldehyde

overnight, washed three times with PBS for 5 min each, stained with DAPI (Solarbio, Beijing, China) at room temperature for 10-15 min, washed three times with distilled water for 5 min each, and photographed using an optical microscope.

Detection of cell apoptosis using acridine orange (AO)/propidium iodide (PI) staining

RSC96 cells were seeded in a 48-well plate at approximately 2×10^4 cells/well. Cells were cultured for 12-24 h with culture medium supplemented with 2% FBS and then treated for 36 h with different concentrations of NP-1 (0, 4, and 8 µg/mL) prepared in culture medium supplemented with 2% FBS. Subsequent steps were performed according to the instructions provided by the AO/PI staining kits (BestBio, Shanghai, China). Finally, cell images were acquired at 488 nm laser excitation using fluorescence microscopy.

Detection of cell apoptosis using trypan blue staining

RSC96 cells were seeded in a 48-well plate at approximately 2×10^4 cells/well. Cells were cultured in medium supplemented with 2% FBS for 12-24 h and then treated for 36 h with different concentrations of NP-1 (0, 4, and 8 µg/mL) prepared in culture medium supplemented with 2% FBS. After washing with PBS, cells were stained with 0.4% trypan blue (Solarbio, Beijing, China), 100 µL/well, for 5 min at room temperature. Then, cells were washed with PBS and photographed using an optical microscope.

Quantitative fluorescence PCR

RSC96 cells were seeded in a 6-well plate at approximately 1.5×10^6 cells/well. Cells were cultured for 12-24 h with culture medium supplemented with 2% FBS and treated for 36 h with different concentrations of NP-1 (0, 4, and 8 µg/mL) prepared in culture medium supplemented with 2% FBS. Gene primers were designed and synthesized by BGI Tech (Shenzhen, Guangdong Province, China) and are shown in **Table 1**. Total RNA was extracted using the Trizol method and cDNA was synthesized using a kit (Abm Inc., Canada). Forty cycles of gene amplification were completed according to the annealing temperature of each target gene.

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Table 1. Sequences of primers used in PCR

Gene	Sequences (5'-3')
ICAM-1	Forward: GATCATACGGGTTGGGCTTCTCC Reverse: GCCACTGCTCGTCCACATAGTATT
IL-6	Forward: AGTCACAGAAGGAGTGGCTAAGGA Reverse: GCACACTAGGTTGCCGAGTAGAC
IL-1 α	Forward: TCGTCTAAGTCACTCGCATGG Reverse: GGGCTGGTTCCACTAGGCTTTG
p65	Forward: AGGATTACATCAAGCACCCAC Reverse: TACGCCATCTTCTCCTGTG
iNOS	Forward: CACCTTTATGTTTGTGGCGATGT Reverse: GAAGTAATCCTCAACCTGCTCCTC
COX-2	Forward: GTGGGTGCTTGGCTTGTGACTT Reverse: TTTTGAAGAAGCCCACTGATACC
AKT	Forward: CGCTTCTTTGCCAACATCG Reverse: TCATCAAAATACCTGGTGTCCG
JUN	Forward: GAACTCGGACCTTCTCACG Reverse: GCAGCGTATTCTGGCTATG
mTOR	Forward: TTTGGACGGTGTAGAACTTGGGA Reverse: CTTGTTTAAAGCTTCTGGTTTCAC
GAPDH	Forward: ATCTGACATGCCGCTGGAGAA Reverse: ACAACCTGGTCTCAGTGTAGCC

Table 2. RSC96 cell survival rates

Treatment duration (h)	NP-1 concentration ($\mu\text{g}/\text{mL}$)				
	0	4	8	12	16
12	1.00	0.95	1.10	1.04	1.12
24	1.00	1.28*	1.12	1.30*	1.21*
36	1.00	1.35**	1.31*	1.26	1.28
48	1.00	1.04	1.14	1.09	1.18
60	1.00	1.01	0.99	1.13	1.07

*Absorbance value of other concentration vs. 0 $\mu\text{g}/\text{mL}$ NP-1 at the same time point. SPSS v20.0 software was used for statistical analysis. Comparisons between two groups were made with independent-sample t-tests or nonparametric tests. *P < 0.05, **P < 0.05.

Table 3. RSC96 cell inhibition rates

Treatment duration (h)	NP-1 concentration ($\mu\text{g}/\text{mL}$)				
	0	4	8	12	16
12	0.00	0.05	-0.10	-0.04	-0.12
24	0.00	-0.28*	-0.12	-0.30*	-0.21*
36	0.00	-0.35**	-0.31*	-0.26	-0.28
48	0.00	-0.04	-0.14	-0.09	-0.18
60	0.00	-0.01	0.01	-0.13	-0.07

Absorbance value of other concentration vs. 0 $\mu\text{g}/\text{mL}$ NP-1 at the same time point. SPSS v20.0 software was used for statistical analysis. Comparisons between two groups were made with independent-sample t-tests or nonparametric tests. *P < 0.05, **P < 0.05.

The specificity of the amplification products was determined using the fusion curve, and

data analysis was carried out using the $2^{-\Delta\Delta\text{Ct}}$ method.

Detection of the expression of related proteins using immunohistochemistry

RSC96 cells were seeded in a 96-well plate at a density of 1×10^4 cells/well. Cells were cultured for 12-24 h with culture medium supplemented with 2% FBS and then treated for 36 h with different concentrations of NP-1 (0, 4, and 8 $\mu\text{g}/\text{mL}$) prepared in culture medium supplemented with 2% FBS. Cells were then washed with PBS, and treated with H_2O_2 (Beijing Chemical Plant, Beijing, China) for 10-15 min in the dark. Then, cells were washed with PBS for 5 min, treated with 50 μL primary antibody working solution including NGF (1:100; Abcam, USA), iNOS (1:100; Abcam, USA), p65 (1:400; CST, USA), IL-1 β (1:400; Bioss, Beijing, China), and NP-1 (1:100; ABclonal, Wuhan, Hubei Province, China) at 4°C overnight. After washing with PBS for 5 min, cells were treated with secondary antibody (GTVisionTM III anti mouse/rabbit immunohistochemistry test kit, Gene Tech, China) at 23°C-27°C for 30 min, washed again with PBS for 5 min, and then incubated with DAB working solution [solution B (DAB developer) and solution C (color buffer) was mixed at a volume ratio of 20 μL solution B to 1 mL solution C] for 3-10 min at room temperature. Subsequently, cells were washed with PBS for 5 min, stained with hematoxylin for 1 min, and washed with water. Finally, cells were photographed using an optical microscope. Under 400 \times magnification, screenshots of the same size were randomly selected for each well. Total positive area was determined using Image Pro Plus v6.0 software (Media Cybernetics, Rockville, USA).

Statistical analysis

SPSS v20.0 software (SPSS, Chicago, IL, USA) was used for statistical analysis and Image-pro plus v6.0 software was used for picture analysis. All measurement data are expressed as the mean \pm SD.

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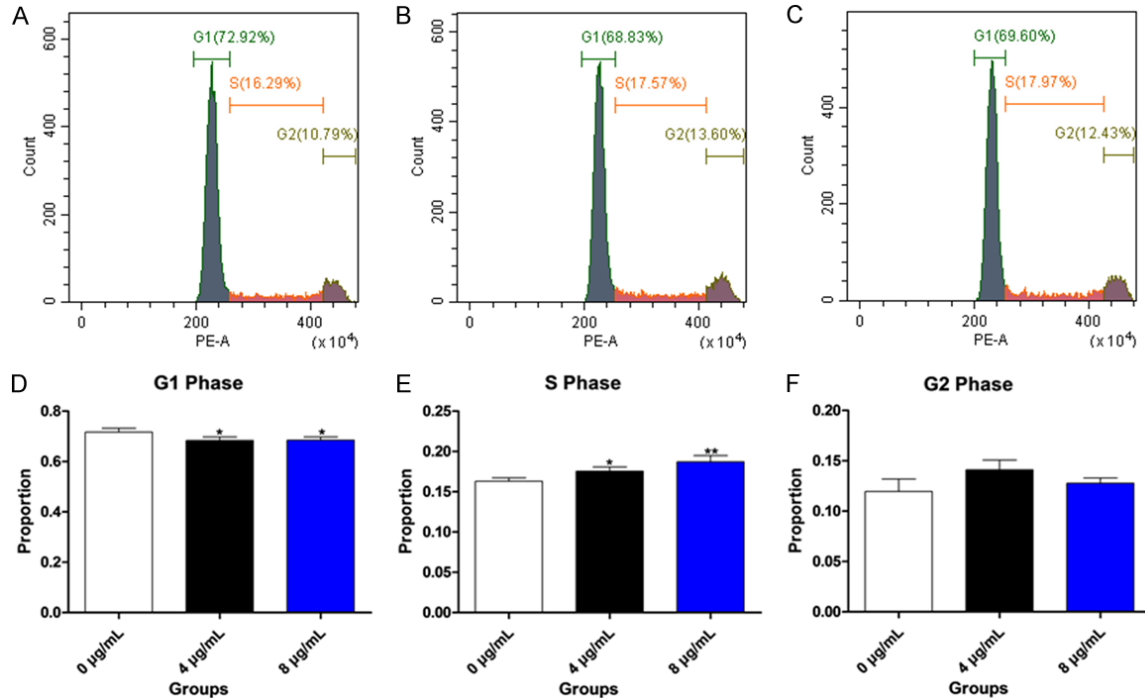


Figure 1. RSC96 cell cycle analysis using flow cytometry. Treatment with 0 µg/mL (A), 4 µg/mL (B), and 8 µg/mL (C) NP-1 for 36 h. Ratio of cells at phases G1 (D), S (E), and G2 (F). SPSS v20.0 software was used for statistical analysis. Comparisons between two groups were made with independent-sample t-tests or nonparametric tests. * $P < 0.05$, ** $P < 0.01$.

Comparisons between two groups were made with independent-sample t-tests and nonparametric tests. A level of $P < 0.05$ was considered statistically significant.

Results

Effects of NP-1 on RSC96 cell activity

RSC96 cells were treated with 0, 4, and 8 µg/mL NP-1 for 36 h and their viability was assessed by the CCK-8 assay. RSC96 cells treated with 4 µg/mL NP-1 showed the highest survival rate (i.e. the lowest cell inhibition rate) and significant cell activity followed by cells treated with 8 µg/mL NP-1 (Tables 2, 3).

Effects of NP-1 on RSC96 cell cycle

We evaluated the effects of NP-1 on cell cycle of RSC96 cells. After treatment with 0, 4, and 8 µg/mL NP-1 for 36 h, there were $72 \pm 2\%$, $68 \pm 1\%$, and $69 \pm 1\%$ cells in G1 phase, respectively. The proportion of cells in phase G1 was significantly different between cells treated with 4 and 8 µg/mL NP-1 as compared with those in

the control group (0 µg/mL NP-1; $P = 0.049$ and $P = 0.048$, respectively). After treatment with 0, 4, and 8 µg/mL NP-1 for 36 h, there were $16\% \pm 0.00$, $18 \pm 1\%$, and $19 \pm 1\%$ cells in S phase, respectively. The proportion of cells in phase S was significantly different between cells treated with 4 and 8 µg/mL NP-1 as compared with those in the control group (0 µg/mL NP-1; $P = 0.030$ and $P = 0.009$, respectively). After treatment with 0, 4, and 8 µg/mL NP-1 for 36 h, there were $12 \pm 1\%$, $14 \pm 1\%$, and $13 \pm 1\%$ cells in phase G2, respectively. However, there was no significant difference in the proportion of cells in phase G2 between cells treated with 4 and 8 µg/mL NP-1 as compared with those in the control group (0 µg/mL NP-1) (Figure 1).

Effects of NP-1 on RSC96 cell migration

To determine the effects of NP-1 on the migration of RSC96 cells, the cells were scratched and treated with 0, 4, and 8 µg/mL NP-1 for 36 h. At the end of treatment, the remaining area of scratch wound was 72751.67 ± 2468.89 , 60589.67 ± 6728.84 , and $68462.00 \pm$

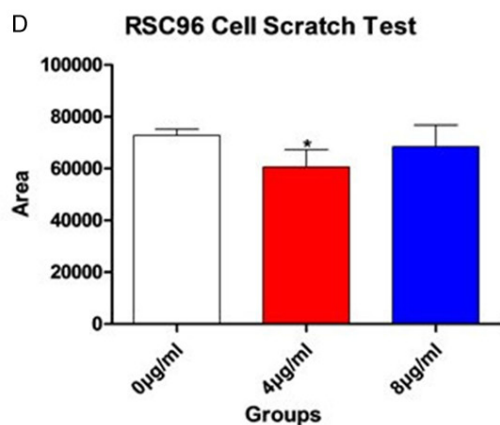
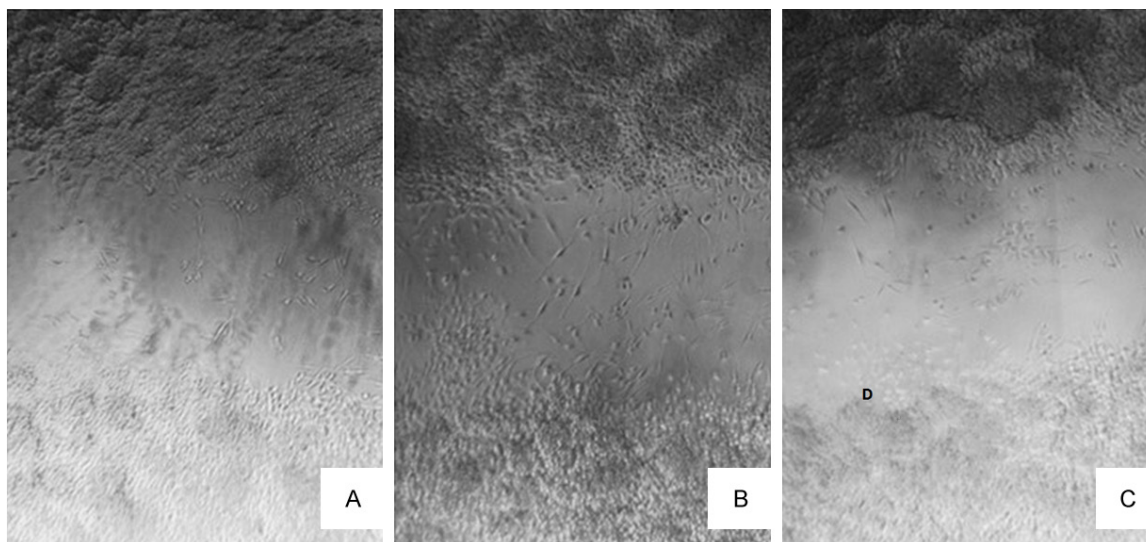


Figure 2. Detection of RSC96 cell migration using cell scratch assays. Treatment with 0 µg/mL (A), 4 µg/mL (B), and 8 µg/mL (C) NP-1 for 36 h. (D) Remaining cell scratch area in each group. SPSS v20.0 software was used for statistical analysis. Image-pro plus v6.0 software was used for picture analysis. Comparisons between two groups were made with independent-sample t-tests or nonparametric tests. * $P < 0.05$.

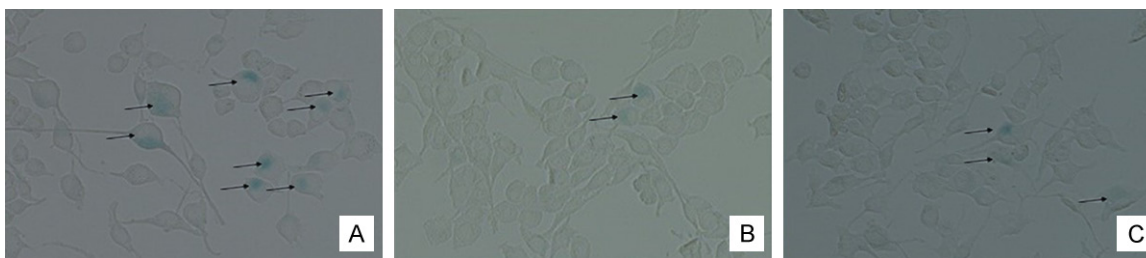


Figure 3. Detection of cell aging using β -galactosidase staining (200 \times). Treatment with 0 µg/mL (A), 4 µg/mL (B), and 8 µg/mL (C) NP-1 for 36 h. Arrowheads indicate blue-stained aging cells.

8289.36, respectively. The cell scratch area remaining in the 4 µg/mL NP-1-treated group was significantly smaller than that in the control group (0 µg/mL NP-1) ($P = 0.042$). However, there was no significant difference in the cell scratch area remaining between 8 µg/mL NP-1-treated and control group (0 µg/mL NP-1) (Figure 2).

Effects of NP-1 on RSC96 cell aging and apoptosis

The number of aging cells was the highest in the control group (0 µg/mL NP-1), which decreased after treatment with 4 and 8 µg/mL NP-1 for 36 h (Figure 3). Moreover, the number of broken nuclei, cells with red fluorescence,

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Figure 4. 4',6-Diamidino-2-phenylindole (DAPI) staining of nuclei (200 ×). Treatment with 0 µg/mL (A), 4 µg/mL (B), and 8 µg/mL (C) NP-1 for 36 h. Arrowheads indicate fragmented nuclei.

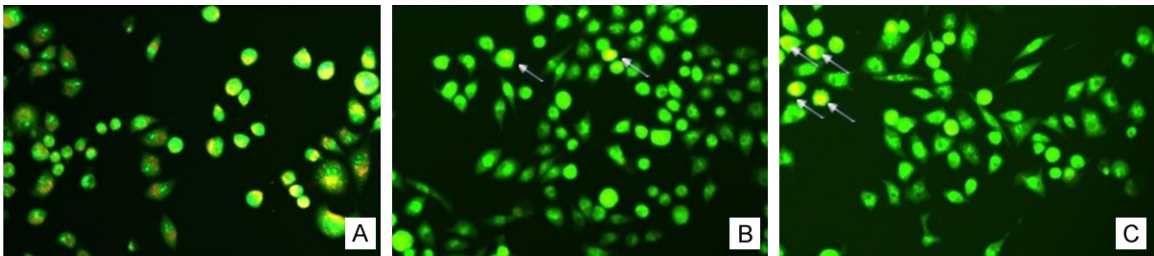


Figure 5. Acridine orange (AO)/propidium iodide (PI) double staining (200 ×) of cells. Treatment with 0 µg/mL (A) 4 µg/mL (B), and 8 µg/mL (C) NP-1 for 36 h. The nuclei of normal cells exhibited yellow green fluorescence, and the nuclei of apoptotic cells exhibited red fluorescence. Arrowheads indicate cells with red fluorescence. There are too many fluorescent cells in A, and thus they are not marked with arrowheads.

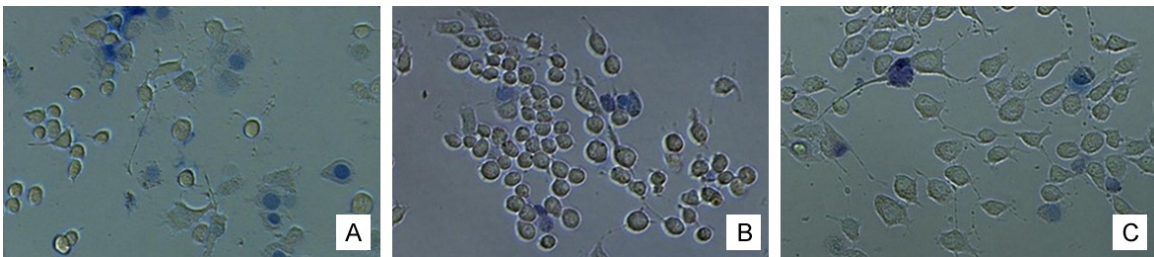


Figure 6. Trypan blue staining (200 ×) of cells. Treatment with 0 µg/mL (A), 4 µg/mL (B), and 8 µg/mL (C) NP-1 for 36 h. Blue cells are apoptotic cells.

and cells stained with trypan blue were the highest in the control group cells (treated with 0 µg/mL NP-1), but these were decreased in cells treated with 4 and 8 µg/mL NP-1 (**Figures 4-6**).

Effect of NP-1 on mRNA expression of regulatory factors associated with NF-κB signaling pathway

The mRNA expression of nine factors involved in NF-κB signaling pathway (ICAM-1, IL-1α, IL-6, p65, iNOS, COX2, AKT, JUN, and mTOR) was determined by qPCR. The factors whose expression showed 15% change as compared

to that in the control group are shown in **Figure 7** and **Table 4**.

Effect of NP-1 on protein expression of factors related to the NF-κB signaling pathway

The effects of NP-1 on protein expression of four factors (p65, iNOS, IL-1β, and NGF) related to the NF-κB signaling pathway were evaluated. The protein expression of p65, iNOS, IL-1β, and NGF in cells treated with 4 and 8 µg/mL NP-1 for 36 h was significantly higher than that in the control group cells (0 µg/mL NP-1) ($P < 0.05$). There was no significant difference in protein expression between cells treated

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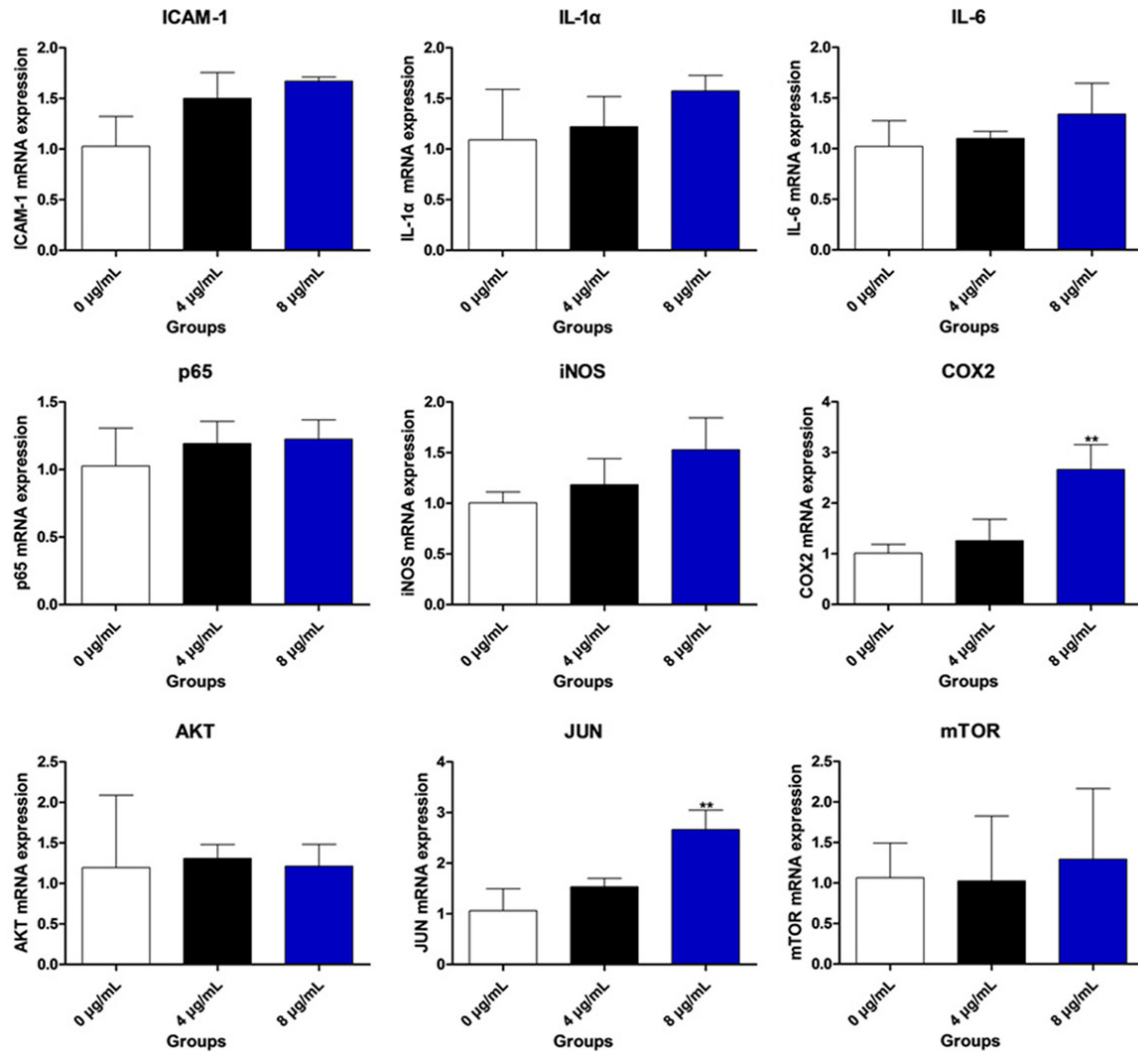


Figure 7. mRNA expression of factors related to the NF- κ B signaling pathway. Graphs correspond to 4 and 8 μ g/mL NP-1-treated cells vs. 0 μ g/mL NP-1-treated cells. * $P < 0.05$, ** $P < 0.01$. SPSS v20.0 software was used for statistical analysis. Comparisons between two groups were made with independent-sample t-tests or nonparametric tests.

with NP-1 and those in the control group (0 μ g/mL NP-1) (Figures 8, 9; Table 5).

Discussion

Schwann cells are essential for the formation of myelin sheaths of peripheral nerves [33-36]. NP-1 is a small polypeptide involved in the repair of peripheral nerve injury. Our previous studies revealed that continuous injections of NP-1 into the gluteus maximus muscle promoted the repair and regeneration of injured rat sciatic nerve [29]. NP-1 has been reported to regulate the synaptic transmission of frog vestibular hair cells [23] and the function of primary neurons in the small intestine of guinea pigs

[24], suggesting that NP-1 plays an important role in regulation of nerve cells. However, only a few studies have reported the effects of NP-1 on Schwann cells. Therefore, we aimed to investigate the effects of NP-1 on Schwann cells.

We first observed the specific morphology of Schwann cells (RSC96) on different days of culture. RSC96 cells showed fusiform, triangular, or polygonal morphology before reaching 100% confluence, after which, they clustered together exhibiting a morphology similar to “paving stones” with round or quasi round nuclei, fusiform, triangular, polygonal, or round cytoplasm.

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Table 4. mRNA expression of genes related to the NF- κ B signaling pathway

Gene	NP-1 concentration		
	0 μ g/mL	4 μ g/mL	8 μ g/mL
ICAM-1	1.03 \pm 0.30	1.50 \pm 0.25#	1.67 \pm 0.04#
IL-1 α	1.09 \pm 0.50	1.22 \pm 0.30	1.57 \pm 0.15#
IL-6	1.02 \pm 0.25	1.10 \pm 0.07	1.34 \pm 0.30#
p65	1.03 \pm 0.28	1.19 \pm 0.16#	1.23 \pm 0.14#
iNOS	1.00 \pm 0.11	1.18 \pm 0.26#	1.53 \pm 0.31#
COX2	1.01 \pm 0.17	1.26 \pm 0.42#	2.66 \pm 0.49**,#
AKT	1.20 \pm 0.89	1.31 \pm 0.17	1.21 \pm 0.27
JUN	1.06 \pm 0.43	1.54 \pm 0.17#	2.66 \pm 0.38**,#
mTOR	1.07 \pm 0.43	1.03 \pm 0.80	1.29 \pm 0.87#

*Treatment with 4 or 8 μ g/mL NP-1 for 36 h vs. intervention with 0 μ g/mL NP-1 for 36 h. SPSS v20.0 software was used for statistical analysis. Comparisons between two groups were made with independent-sample t-tests or nonparametric tests. **P < 0.01. #indicates the factor that changed over 15% in the 4 μ g/mL NP-1-treated and 8 μ g/mL NP-1-treated groups compared with the 0 μ g/mL NP-1-treated group.

Treatment with NP-1 (4 and 8 μ g/mL) promoted the growth and migration of RSC96 cells, with the most obvious growth stimulation observed after 36 h. Therefore, the optimal concentration of NP-1 and duration of treatment for obtaining these effects were determined to be 4 and 8 μ g/mL and 36 h, respectively. It has been reported that low concentrations of NP-1 promotes cell proliferation, whereas high concentrations of NP-1 exerts cytotoxic effects [37]. NP-1 stimulates cell proliferation, and p42/44 MAP kinase and Akt activation in a dose-dependent manner [38] and promotes cell migration through two-way activation of ERK1/2 [39]. Kang et al. [40] reported that folic acid treatment-mediated improvement in proliferation and migration of Schwann cells was helpful in the repair of peripheral nerve injury. The proliferation and migration of Schwann cells are also affected by small RNAs and nerve growth factors [41, 42]. The results of the present study showed that low concentrations of NP-1 increased the proliferation and migration of Schwann cells, which affects the repair of injured peripheral nerves.

β -galactosidase, DAPI, AO/PI, and trypan blue staining results showed that treatment with 4 and 8 μ g/mL NP-1 for 36 h inhibited RSC96 cell aging and apoptosis. A previous study reported that high concentrations of NP-1-3 (50 μ g/mL) caused cell apoptosis through activa-

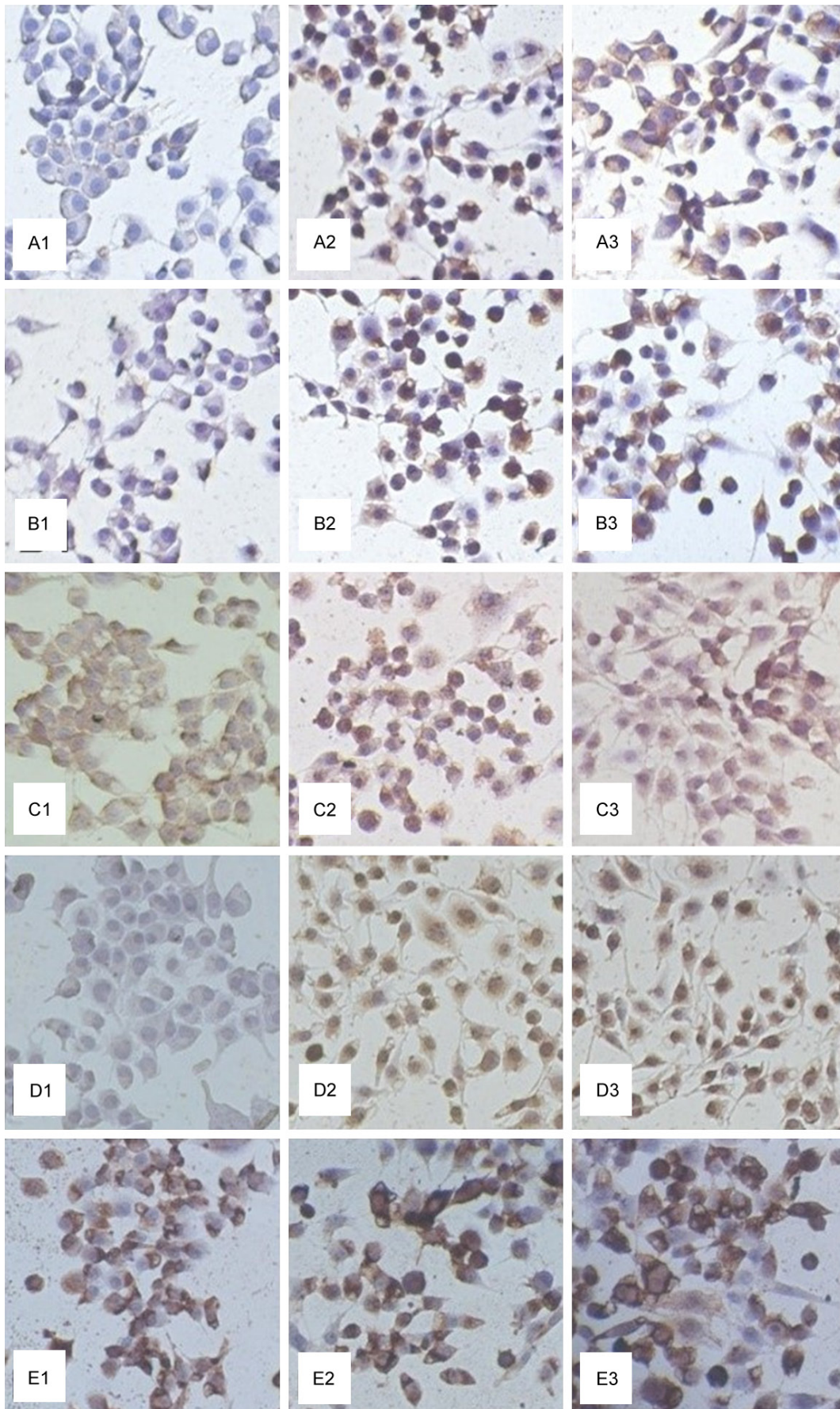
tion of caspase 3/7 [43]. However, aging and apoptosis of Schwann cells are not conducive to the repair of peripheral nerve injury [44, 45]. In this study, we found that low concentrations of NP-1 (4 and 8 μ g/mL) slowed down the aging and apoptosis of RSC96 cells. Therefore, we believe that low concentrations of NP-1 can slow down the aging and apoptosis of Schwann cells at the site of peripheral nerve injury, providing protection to sciatic nerve function. Moreover, as NP-1 promotes the proliferation and migration of Schwann cells, it can accelerate the repair of injured peripheral nerves as well as provide conducive environment for the recovery of nerves.

NF- κ B is a family of transcription factors, including p65, RelB, c-Rel, p50, and p52. Several chemical mediators involved in various stages of inflammation and early stages of the immune response can be regulated by the NF- κ B signaling pathway [46, 47]. P65 has a transactivation domain that can activate target genes. The increased expression of p65 protein can initiate the activation of NF- κ B signaling pathway. NF- κ B and iNOS levels are increased in inflammatory diseases [48]. However, inhibition of the NF- κ B signaling pathway results in downregulation of iNOS, COX-2, and IL-1 β , indicating its anti-inflammatory role [49].

IL-1 α , an upstream factor regulating NF- κ B activity, is involved in cell aging [50] and inflammation [51]. Mei et al. [52] found that siRNA could inhibit cell proliferation and migration and decrease the expression of p65 and CCND1; moreover, the decrease in CCND1 expression was related to decrease in cell proliferation and increase in apoptosis [53]. Sahin et al. [54] found that tomato powder improves age-related inflammatory responses by inhibiting the expression of NF- κ B and mTOR.

In this study, we determined the mRNA expression of mediators directly related to inflammation (ICAM-1, IL-1 α , IL-6, p65, iNOS, and COX2) and cell function (AKT, JUN, and mTOR), all of which take part in NF- κ B signaling pathway. We also determined the protein expression of five regulatory molecules: NGF, inflammation-related proteins (p65, iNOS, and IL-1 β), and NP-1. We found that treatment with 4 and 8 μ g/mL NP-1 for 36 h increased the protein expression of p65, iNOS, IL-1 β , and NGF, as compared to that in the control group, whereas

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Figure 8. Images of immunohistochemistry analysis of cells (200 ×). (A) p65 protein, (B) iNOS protein, (C) IL-1 β protein, (D) NGF protein, and (E) NP-1 protein. 1: 0 μ g/mL NP-1-treated group; 2: 4 μ g/mL NP-1-treated group; and 3: 8 μ g/mL NP-1-treated group. Image-pro plus v6.0 software was used for picture analysis.

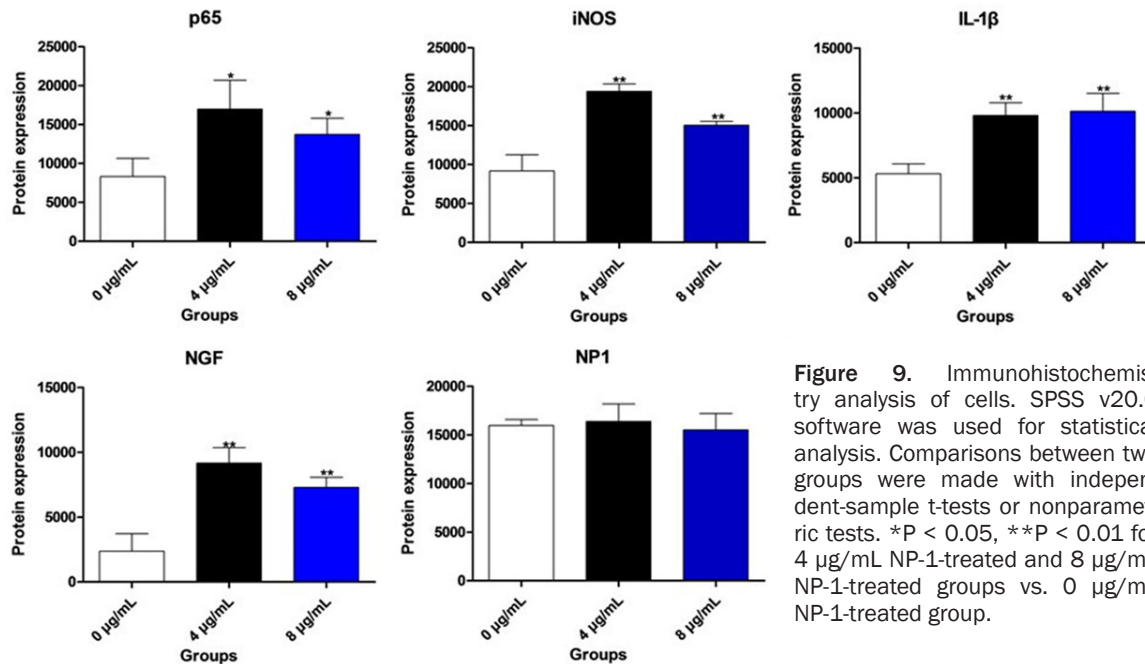


Figure 9. Immunohistochemistry analysis of cells. SPSS v20.0 software was used for statistical analysis. Comparisons between two groups were made with independent-sample t-tests or nonparametric tests. *P < 0.05, **P < 0.01 for 4 μ g/mL NP-1-treated and 8 μ g/mL NP-1-treated groups vs. 0 μ g/mL NP-1-treated group.

Table 5. Expression of proteins related to the NF- κ B signaling pathway

Protein	NP-1 concentration		
	0 μ g/mL	4 μ g/mL	8 μ g/mL
P65	8319.00 \pm 2330.62	16995.00 \pm 3702.14*	13736.33 \pm 2074.40*
iNOS	9170.00 \pm 2087.41	19413.33 \pm 946.34**	15047.33 \pm 507.01**
IL-1 β	5308.33 \pm 754.91	9800.00 \pm 981.08**	10136.67 \pm 1374.06**
NGF	2372.33 \pm 1349.73	9171.67 \pm 1178.91**	7288.00 \pm 784.90**
NP-1	15952.00 \pm 626.71	16381.33 \pm 1788.95	15504.67 \pm 1694.44

*P < 0.05, **P < 0.01 for 4 μ g/mL NP-1-treated and 8 μ g/mL NP-1-treated groups vs. control (0 μ g/mL NP-1) group.

no obvious effects were observed on NP-1 protein expression. Moreover, 4 or 8 μ g/mL NP-1 upregulated the mRNA expression of ICAM-1, IL-1 α , IL-6, p65, iNOS, COX2, JUN, and mTOR, as compared to that in the control group. Low concentrations of NP-1 increased the expression of iNOS, IL-1 β , COX2, and JUN by activating NF- κ B signaling pathway; this also altered the secretion of inflammatory mediators and chemokines in RSC96 cells. We suggest that activation of the NF- κ B signaling pathway resulted in the upregulation of iNOS, IL-1 β , COX2, ICAM-1, IL-1 α , and IL-6, which stimulated inflammation and promoted NGF secretion. NF- κ B has been associated with the secretion of b-NGF

in metabolic syndrome [55]. Moreover, NP-1 promoted cell proliferation and migration, and inhibited cell aging and apoptosis, via IL-1 α , JUN, and mTOR modulation. Li et al. [56] reported that p65 knockout decreased the phosphorylation levels of c-jun and increased squamous apoptosis. Furthermore, mTOR activation was reported to promote cell proliferation [57], whereas mTOR inhibition was found to reduce cell migration ability [58]. Overall, these findings indicated that activation of NF- κ B signaling pathway promoted the repair of injured peripheral nerves. Interestingly, NP-1 treatment did not produce significant changes in NP-1 protein expression in RSC96 cells, suggesting that exogenous NP-1 may not affect the levels of endogenous NP-1 in cells.

A limitation of the present study is that we only investigated the effect of NP-1 on the NF- κ B signaling pathway, but we did not investigate

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the effect of this pathway on NP-1, which will be studied in the future.

In conclusion, we elucidated the possible mechanism underlying NP-1-induced changes in the function of RSC96 Schwann cells at the cellular level. Based on the results, we suggest that NP-1 promotes the proliferation and migration of RSC96 cells and inhibits their aging and apoptosis. These findings also indicate a possible mechanism of repair and regeneration of injured peripheral nerves and suggest that NP-1 can be a potential target for clinical treatment of peripheral nerve injury.

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Disclosure of conflict of interest

None.

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