

Polyaniline promotes peripheral nerve regeneration by enhancement of the brain-derived neurotrophic factor and ciliary neurotrophic factor expression and activation of the ERK1/2/MAPK signaling pathway

LIN FAN¹, YAN XIONG¹, ZHEN FU², DINGFENG XU³, LEI WANG¹, YONG CHEN⁴, HAOYANG XIA¹, NA PENG¹, SHAOJUN YE¹, YANFENG WANG¹, LINA ZHANG³ and QIFA YE^{1,2}

¹Zhongnan Hospital of Wuhan University, Institute of Hepatobiliary Diseases of Wuhan University, Transplant Center of Wuhan University, Hubei Key Laboratory of Medical Technology on Transplantation, Wuhan, Hubei 430071; ²Research Center of National Health Ministry on Transplantation Medicine Engineering and Technology, The 3rd Xiangya Hospital of Central South University, Changsha, Hunan 410013; ³College of Chemistry & Molecule Sciences, Wuhan University, Wuhan, Hubei 430081; ⁴Department of Abdominal Laparoscopic Surgery, Qinghai University Affiliated Hospital, Xining, Qinghai 810001, P.R. China

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Abstract. A previous study has demonstrated a progression in the nerve regeneration by polyaniline/cellulose (PANI/RC), although the underlying mechanism was not elucidated. In the present study, regenerated nerves were investigated, using histological techniques, functional assays and western blot analysis. The *triceps surae* muscle weight ratio percentages of the sham, regenerated cellulose (RC) and the PANI/RC groups were 38.88 ± 4.76 and $76.32 \pm 7.11\%$, respectively. The thickness of the myelin sheath for the aforementioned groups were as follows: 1.2 ± 0.27 ; 0.49 ± 0.21 and $0.93 \pm 0.28 \mu\text{l}$. Western blot analysis demonstrated that the ciliary neurotrophic factor (CNTF) and brain-derived neurotrophic factor (BDNF) were highly expressed in the regenerated nerve in the presence of polyaniline. Phosphorylated extracellular kinase (p-ERK)1/2 expression in the PANI/RC group was significantly elevated compared with the RC group (1.83-fold) and the sham group (4.92-fold). The expression of the axon sprout-associated

proteins, such as Tau, α -tubulin and growth associated protein-43, were increased (1.64, 1.59 and 1.24-fold, respectively) compared with the RC group. The results demonstrated that PANI enhances the expression and secretion of BDNF and CNTF, activates the ERK1/2 signaling pathway and increases the expression levels of the GAP-43, Tau and α -tubulin, suggesting an insight into nerve regeneration and possible clinical interventions in nerve injury.

Introduction

The incidence of new onset peripheral nerve injury is increasing worldwide. More than one million cases with new onset peripheral nerve injury is presented annually worldwide (1). The peripheral nerve injuries can result from trauma, cancer or congenital defects. Regarding the clinical and basic research, the sciatic nerve injury and regeneration process has been a focus of major investigation in medicine. The nerve fiber growth velocity is very slow and is estimated to 1-2 mm/day in humans (2,3). The autologous nerve graft is considered the best treatment option, although significant drawbacks are encountered, such as the limited donor source, the donor site morbidity, the multiple surgical sites and the possible size mismatch (4). Consequently, novel methods to replace the autologous nerve graft for nerve gap reconstruction are continuously evaluated.

In the previous study conducted by our group, the RSC96 cell line was used to evaluate the biological applications of the polyaniline/regenerated cellulose (PANI/RC) that enhanced RSC96 cell adhesion and proliferation (5). Scanning electron microscopy demonstrated the adhesion of RSC96 cells via pseudopodium extension on the PANI sub-micrometer dendritic particles (5). It is well established that Schwann cells serve a critical role in the peripheral nerve regeneration (6). In the present study, it was hypothesized that PANI is involved

Correspondence to: Dr Qifa Ye, Zhongnan Hospital of Wuhan University, Institute of Hepatobiliary Diseases of Wuhan University, Transplant Center of Wuhan University, Hubei Key Laboratory of Medical Technology on Transplantation, Wuhan University, 169 East Lake Road, Wuchang, Wuhan, Hubei 430071, P.R. China
E-mail: yqf_china@163.com

Professor Lina Zhang, College of Chemistry & Molecule Sciences, Wuhan University, 299 Bayi Road, Wuhan, Hubei 430081, P.R. China
E-mail: zhangln@whu.edu.cn

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in the latter process via the regulation of Schwann cells. The specific function of PANI in nerve regeneration was detected at the molecular level. During the aforementioned process, the involvement of a complex interlinked cell signaling pathway was identified. The recruitment of cytokines and neurotrophins at the site of the lesion resulted in the activation of a series of molecular and cellular signaling pathways involved in neuronal and non-neuronal cell communication (7,8). Such mechanisms may include the generation of new neurons, glia, axons, myelin or synapses that in turn promote nerve regeneration (8).

Neurotrophins are proteins that help stimulate and control neurogenesis. Brain derived neurotrophic factor (BDNF) is one of the most active neurotrophins (9) that supports the survival of existing neurons and promotes the growth and differentiation of new neurons and synapses (10,11). In addition, the application of exogenous BDNF to the nerve topical lesion has been reported to potentiate axon regrowth and maturation during peripheral nerve regeneration (12).

Cytokine ciliary neurotrophic factor (CNTF) is an additional neurotrophin that is required for nerve regeneration (13). CNTF is secreted by Schwann cells and astrocytes, and the application of exogenous CNTF to the nerve lesion has also been reported to promote axon regrowth and maturation during peripheral nerve regeneration (14-17). However, it remains unclear whether the role of endogenous CNTF in the activation of cell growth and differentiation is associated with PANI, following peripheral nerve injury.

In the present study, the ability of PANI/RC to promote rat sciatic nerve regeneration was demonstrated by monitoring the morphological and molecular changes of the regenerated nerve fibers using histological, immunohistochemical and western blotting techniques.

Materials and methods

Preparation of PANI/RC conduit and cellulose conduit. The PANI/RC hydrogels were prepared through the interfacial polymerization via a U tube, of which a cellulose hydrogel was sandwiched in the middle as previous described (5). The PANI/RC and RC hydrogels were rolled to fabricate PANI/RC and RC conduits. The final length of conduits was 12 mm, with an inner diameter of 1.5 mm and a tube wall thickness of 1 mm.

Animals. Adult male Sprague-Dawley rats, (age, 8-10 weeks, weight, 210±10 g, n=45) were obtained from the Hubei Provincial Center for Disease Control and Prevention in Wuhan, China. The experimental procedures involving animals were carried out in accordance with National Institutes of Health (Bethesda, MD, USA) guide for the care and use of laboratory animals and The Code of Ethics of the World Medical Association. The study received ethics approval from the Ethics Committee of Zhongnan Hospital of Wuhan University (Wuhan, China).

Surgical procedure. The animals were divided into three groups (15 rats for each group): Sham (the sciatic nerve was exposed in the absence of nerve tissue disposal), RC (the defected sciatic nerve was connected with the RC conduit) and

PANI/RC (the defected sciatic nerve was connected with the PANI/RC conduit). The 5 mm defects in the sciatic nerve were produced by removing the nerve tissue which was repaired with the nerve conduits as reported previously (18-20).

Triceps weight analysis. The *triceps surae* muscles from the experimental and the contra lateral sides (non-operated) were weighed in order to estimate the relative weight ratio 3 months following surgery, according to the following formula:

$$\text{triceps weight \%} = \frac{\text{triceps weight of the operated leg}}{\text{triceps weight of the unoperated leg}} \times 100\%$$

Histological assessment. The harvested nerve grafts were fixed in a cold buffered 4% paraformaldehyde solution or cold buffered 3% glutaraldehyde solution. Following fixation, the former part of tissues in each group were embedded in olefin, cut in 4 mm slices and stained with hematoxylin (10 min at room temperature) and eosin (2 min at room temperature; H&E) or toluidine blue (3 min at room temperature). The latter part of the nerve sections were fixed in a cold buffered 3% glutaraldehyde solution for transmission electron microscopy TEM examination (JEM-1200 EX, JEOL Ltd., Tokyo, Japan). A total of 200 nerve fibers in 10 random areas were selected by two pathologists for the fiber size analysis. All nerve sections were observed under a light microscope (TE2000-U, Nikon Corporation, Tokyo, Japan). An image analysis system (Image-Pro Plus Version 6.0, Media Cybernetics Inc., Rockville, MD, USA) was used to determine the number and areas of the individual myelinated axons.

Immunohistochemical evaluation. The expression of the extracellular signal-regulated kinase (ERK) 1/2/ mitogen-associated protein kinase (MAPK) was evaluated in nerve paraffin sections of the regenerated nerve tissues via immunohistochemical analysis. After being blocked (10 min at room temperature) using 5% sham goat serum (cat. no. AR1009; Wuhan Boster Biological Technology Ltd., Wuhan, China), the sections were incubated with a rabbit anti-rat ERK1/2 antibody (cat. no. 16443-1-AP; 1:50; ProteinTech Group, Inc., Wuhan, China) or MAPK (cat. no. 9212; 1:100; Cell Signaling Technology Ltd., Danvers, MA, USA) at 4°C overnight and then with biotinylated secondary antibodies (cat. no. AB501-01A; 1:500; SinoBiological Inc., Shanghai, China) for 20 min at 37°C. The sections were reacted with a Strept Avidin Biotin Complex (100 µl; cat. no. PV-9000; Zhongshan Gold Bridge Biological Technology Ltd., Beijing, China) for 20 min at room temperature and finally stained with 3,3-Diaminobenzidine (cat. no. AR1022; Wuhan Boster Biological Technology Ltd., Wuhan, China). The positive ratios (200 cells in 10 random areas counted) of ERK1/2(MAPK) were observed by microscope (TE2000-U, Nikon Corporation, Tokyo, Japan) and analyzed by an image analysis system (Image-Pro Plus version 6.0; Media Cybernetics Inc., Rockville, MD, USA).

Western blot analysis. The total protein content was extracted from regenerated or sham nerve in a lysis buffer (cat. no. P0013B; Beyotime Biotechnology Ltd., Shanghai, China)

containing 50 mM Tris HCl, 1% NP-40, 150 mM NaCl, 0.1% SDS, 1 mM PMSF and 1 mM Na_3VO_4 . The protein concentration of the samples was determined by the Bicinchoninic Acid assay kit. Bovine serum albumin (5%; cat. no. 164210-100; Procell Life Science & Technology Co., Ltd., Wuhan, China) was used as a standard. An equal amount (30 μg) of protein was loaded on polyacrylamide gels containing 10-12% SDS and transferred to polyvinylidene fluoride membranes. The membranes were probed with specific antibodies against nerve growth factor (cat. no. BA0611-2; 1:200; Wuhan Boster Biotechnology Co., Ltd); tau (cat. no. ab32057; 1:5,000; Abcam, Cambridge, MA, USA); S100 (cat. no. ab52642; 1:5,000; Abcam); growth-associated protein-43 (GAP-43; cat. no. ab75810; 1:5,000; Abcam); α -tubulin (cat. no. ab15246; 1:500; Abcam), BDNF (cat. no. SC-546; 1:300; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), CNTF (cat. no. ab46172; 1:3,000; Abcam); ERK (cat. no. 16443-1-AP; 1:1,000; ProteinTech Group, Inc.); phosphorylated (p)-ERK1/2 (cat. no. 4370; 1:2,000; Cell Signaling Technology Inc.); β -actin (cat. no. BM0627; 1:200; Wuhan Boster Biotechnology Co., Ltd.) and GAPDH (cat. no. AB-P-R 001; 1:1,000; Hangzhou Goodhere Biotechnology Co., Ltd., Hangzhou, China). GAPDH and β -actin were used as internal controls. The protein detection was carried out using the enhanced chemiluminescence detection system (cat. no. NCI5079; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and the membranes were exposed on Kodak X-Omat films. The protein levels were semi quantified by densitometric analysis of the integrated area (pixels) of the protein bands using the BandScan software version 4.3 (<http://download1.bio1000.com/soft/biology/BandScan5.0.rar>). The numerical values for the protein band intensities were produced following normalization of the target protein expression with the expression value corresponding to the GAPDH or β -actin band.

Statistical analysis. SPSS 21.0 statistical software was used for the statistical analysis of the relevant data (IBM Corp., Armonk, NY, USA). The data are expressed as the mean \pm standard deviation. The differences between the two groups were compared using the student t-test. The differences among several groups were analyzed using one-way analysis of variance followed by least significance difference multiple-comparison tests. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

General post-operative observations. The conduits for nerve reconstruction are indicated in the nerve condition intra-operatively (Fig. 1A and B) and 3 months post-operation (Fig. 1C and D). Fig. 1 indicates the sciatic nerve reconstruction that was conducted using the following materials: PANI/RC (Fig. 1A and C) and RC (Fig. 1B and D). The PANI/RC conduit indicated favorable biocompatibility performance (Fig. 1C), while the RC conduit was wrapped with thick fibrous connective tissue (Fig. 1D).

Triceps weight analysis. Nerve recovery is indirectly reflected by muscle recovery. The *triceps surae* muscle weight ratio is estimated by the formula: Operation side/contralateral side

$\times 100\%$. Sham group: 99.70 ± 2.29 ; RC group: 38.88 ± 4.76 ; PANI/RC group: 76.32 ± 7.11 , $P < 0.01$ (Fig. 1E). In terms of *triceps surae* muscle weight ratio, the PANI/RC group demonstrated better motor function recovery compared with the RC group.

Axon regeneration, myelination and activation of Schwann cells. The images of H&E sections indicated the regenerated nerve fibers in the three groups (Fig. 2A). The number of Schwann cells (blue arrow) detected in the PANI/RC group was greater than that in the RC and sham groups. The axons (black arrow) regenerated more efficiently in the PANI/RC compared with the RC group (Fig. 2A). The blood vessels that were necessary for nutrient supply and neurite growth were detectable in the PANI/RC group. A vast number of fibrous connective tissues (white arrow) were crawled into the nerve that prevented nerve regeneration (Fig. 2A). The protein S100 (marker of Schwann cells) revealed a 3-fold increase in the PANI/RC compared with the sham group (Fig. 2B). The aforementioned protein demonstrated a 1.6-fold increase in the PANI/RC compared with the RC group ($P < 0.01$, Fig. 2B). The images of toluidine blue sections (Fig. 2B) indicated that the regenerated nerve fibers in the PANI/RC group were smaller and less uniform compared with those in the sham group. However, the nerve fibers in the RC group were the smallest in size and most irregular in shape with numerous fibrous connective tissues. The fiber diameter analysis (Fig. 2D) indicated that the size range in the PANI/RC group was between 3 and 5 μm , whereas the percentage was estimated to 37.6 ± 5.8 . The latter was similar to the percentage of fiber diameter noted in the sham group (34.2 ± 6.4) and significantly greater compared with that in the RC group (19.2 ± 6 ; $P < 0.01$).

TEM analysis of the regenerated nerve tissues revealed that the formation of regenerated myelinated fibers occurred at similar levels in both the PANI/RC and sham groups (Fig. 2E). Statistical analysis was carried out on the average axon diameter (Fig. 2D), the thickness of the regenerated myelin sheath (Fig. 2F). A significant difference between the PANI/RC and the RC groups was noted for all of the parameters measured ($P < 0.05$). A thicker myelin sheath was observed in the PANI/RC group ($0.93 \pm 0.28 \mu\text{m}$) compared with the RC group ($0.49 \pm 0.21 \mu\text{m}$, $P < 0.05$), yet still smaller than that in sham group ($1.2 \pm 0.27 \mu\text{m}$; $P > 0.05$).

Activation of the ERK1/2/MAPK signaling pathway. Immunohistochemical analysis was employed to analyze the positive expression levels of ERK1/2, 3 months post-implantation, in all groups in order to determine whether the signaling pathways of MAPK/ERK1/2 were activated following the increase in expression of CNTF and BDNF proteins. The positive ratio of MAPK/ERK1/2 proteins in the PANI/RC group was significantly greater than that of the RC group ($P < 0.01$, Fig. 3A-C). The results further demonstrated that ERK1/2 is activated in the PANI/RC and RC group compared with the sham group (Fig. 3C), as indicated by the increased phosphorylation levels of ERK1/2 (Fig. 3D). The increase in the p-ERK1/2 levels was highly significant between the PANI/RC and the RC and the sham groups ($P < 0.01$), while the levels of ERK1/2 were unchanged (Fig. 3D; $P > 0.05$).

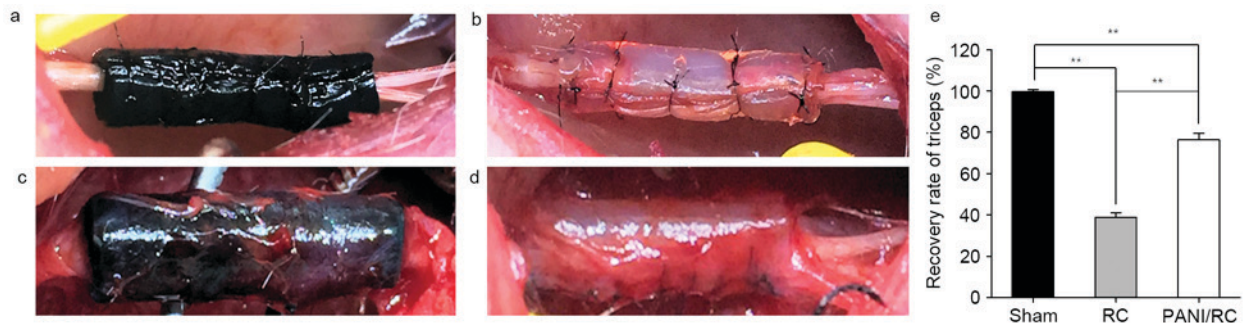


Figure 1. Pre-operative images in the (A) PANI/RC and (B) RC groups and 3-month postoperative images in the (C) PANI/RC and (D) RC groups. (E) Repaired surae comparison between contra lateral operation side with different conduits sham, RC and PANI/RC groups. Data are expressed as the mean \pm standard deviation. $n=5$, ** $P<0.01$. PANI/RC, polyaniline/regenerated cellulose; RC, regenerated cellulose.

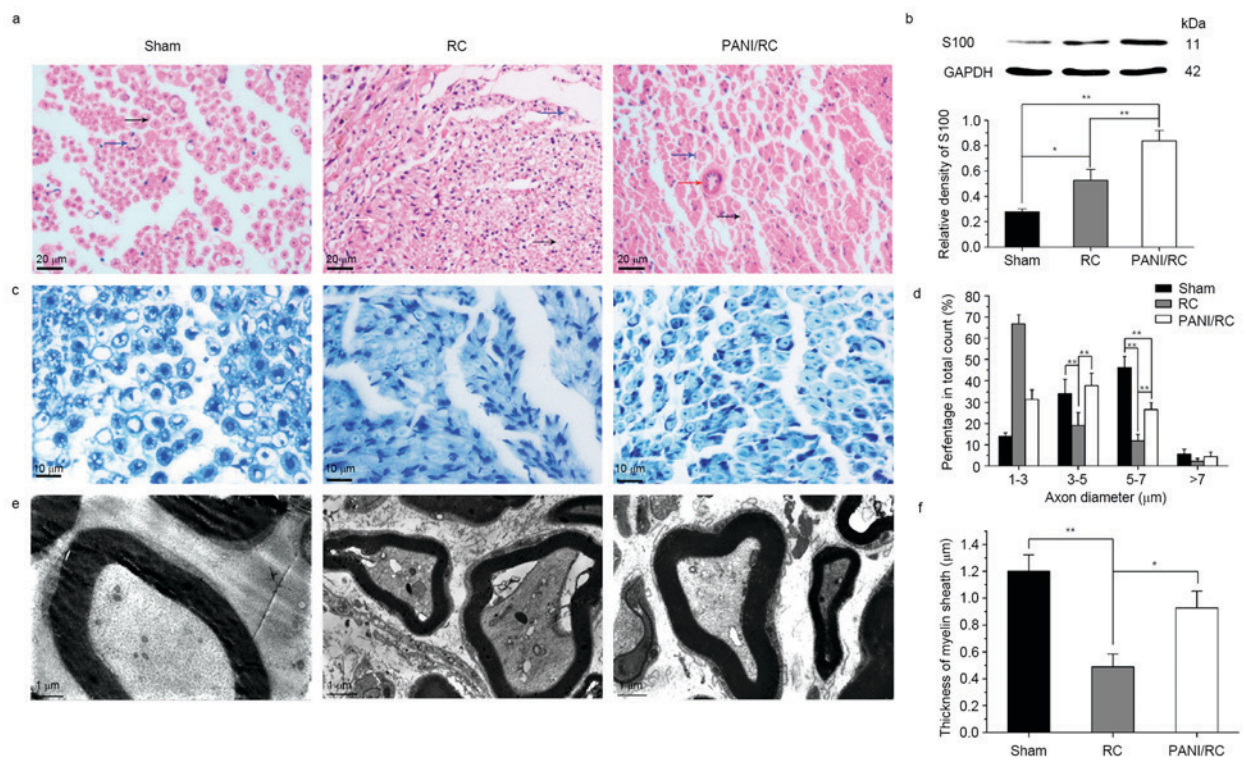


Figure 2. Histology images from cross sections of regenerated nerves extracted from specific types of nerve conduits implanted in rats following 3 months of implantation. Tissues were stained with (A) hematoxylin and eosin, and (B) S100 protein expression levels were measured. (C) Toluidine blue staining. (D) Axon diameter of regenerated and non-operated sciatic nerves. (E) Transmission electron microscopy images. (F) Analysis of the thickness of the myelin sheath. Black arrows indicate the myelinated axons. Blue arrows indicate the Schwann cells. Red arrows indicate the red blood cells/vessels. White arrows indicate the fibrous connective tissue. Data are expressed as the mean \pm standard deviation. $n=5$, * $P<0.05$, ** $P<0.01$. PANI/RC, polyaniline/cellulose; RC, regenerated cellulose.

Recruitment of growth factors, cytokines and neurotrophins. The expression of the proteins CNTF, BDNF, Tau, GAP-43, NGF and α -tubulin following implantation of the conduits was examined using western blot analysis in order to determine the activation of the Schwann cells following nerve injury. The expression levels of the proteins corresponding to the PANI/RC group were significantly higher compared with the expression levels of the proteins corresponding to the sham and RC groups (Fig. 4). Specifically, the expression levels of the proteins BDNF, CNTF, NGF, GAP-43, Tau and α -tubulin were significantly increased in the RC and the PANI/RC groups compared with the sham group ($P<0.01$, Fig. 4). The proteins

BDNF, CNTF, NGF, GAP-43, Tau and α -tubulin revealed a 3.07-, 2.17-, 3.05-, 3.01-, 3.48- and 2.44-fold increase in the PANI/RC group compared with the sham group. The aforementioned proteins demonstrated a 1.27-, 1.18-, 1.43-, 1.59-, 1.64- and 1.24-fold increase in the PANI/RC group compared with the RC group (Fig. 4).

Discussion

In the present study, the parameters of axons, axon diameter and thickness of myelin sheath were greater in the PANI/RC group compared with the corresponding parameters in the

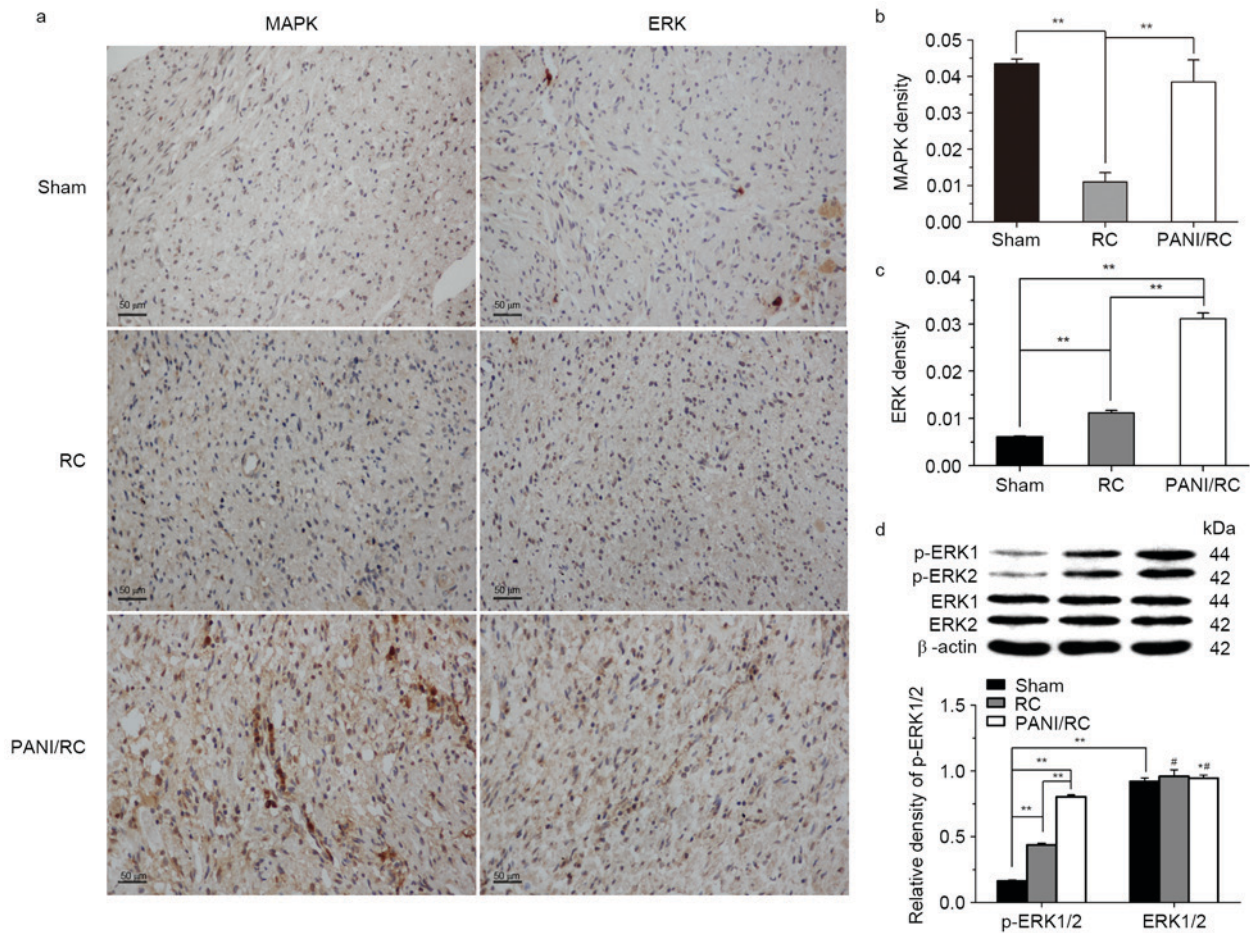


Figure 3. Immunohistochemical images of (A) regenerated nerves and quantification of (B) MAPK and (C) ERK1/2 positive cells. (D) Western blot analysis of p-ERK1/2 in the Sham, RC and PANI/RC groups. Brown staining indicates positive expression. Data are expressed as the mean \pm standard deviation. (n=5, **P<0.01, #P<0.01 vs. p-ERK1/2 in the RC group, *P<0.01 vs. p-ERK1/2 in the PANI/RC group). PANI/RC, polyaniline/cellulose; ERK, extracellular signal-regulated kinase; MAPK, mitogen-associated protein kinase; p, phosphorylated; RC, regenerated cellulose.

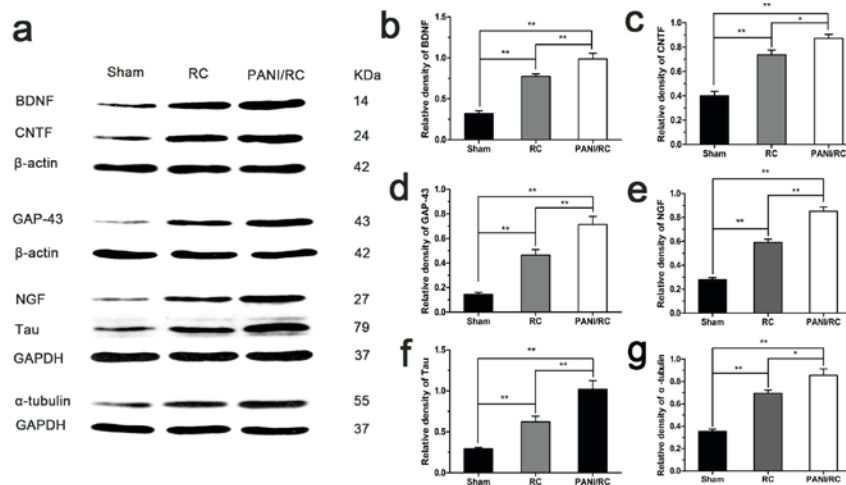


Figure 4. Protein expression levels of BDNF, CNTF, NGF, GAP-43, Tau and α -tubulin. (A) Representative western blot images. Protein expression levels of (B) BDNF, (C) CNTF, (D) NGF, (E) GAP-43, (F) Tau and (G) α -tubulin, in the three groups. Data are expressed as the mean \pm standard deviation. n=5, *P<0.05, **P<0.01. PANI/RC, polyaniline/cellulose; BDNF, brain-derived neurotrophic factor; CNTF, ciliary neurotrophic factor; NGF, nerve growth factor; GAP, growth-associated protein; RC, regenerated cellulose.

RC group and lower than those noted in the sham group. The histological data were similar with the findings reported in previous studies (17-19).

In the present study, the PANI/RC conduit exhibited two main characteristics: The hierarchical micro-nanostructure and the conductivity (5). The aforementioned factors may

promote RSC96 cellular proliferation via a synergistic mode of action (5).

Regarding conductivity, some authors have previously reported that the conductive material promotes nerve regeneration even without electrical stimulation (ES) (21-23). The conductive scaffold in the absence of ES can in turn be used to mediate a bioelectricity signal to the distal end for nerve regeneration (21,22). Initial studies in the field of nerve regeneration demonstrated the beneficial effects of a conducting polymer with ES on neurite outgrowth (23). However, an implanted bioelectronic conduit can establish a stable and long-term electrical communication with living tissues, thereby allowing regeneration of the tissues or an exchange of physiological signals in order to recover damaged biological functions (24). The therapeutic actions of ES are mediated by the enhanced BDNF-stimulated myelination that in turn promotes the promyelination effect on Schwann cells at the onset of myelination (25). The results regarding the expression of NGF and BDNF are in agreement with the aforementioned findings. Taken together, the data suggested that the presence of PANI promotes the electrical conductivity of the PANI/RC conduits on axon regeneration and myelination, compared with the RC conduits.

In addition, BDNF activates the MAPK/ERK1/2 cascade, which may lead to transcriptional regulation and protein synthesis in the postsynaptic neuron (26). In the present study, the expression level of BDNF in the PANI/RC group was significantly increased compared with the RC and sham groups. The ERK1/2 (MAPK) signaling pathway of the has demonstrated to be involved in axon regeneration (27). Furthermore, Park *et al* (28) revealed that CNTF promoted retinal ganglion cell survival and axonal regeneration via the ERK1/2/MAPK signaling pathway, which is similar with the present results.

The aforementioned evidence demonstrated that the release of CNTF and the activation of ERK1/2 were essentially involved in axon growth modulation following nerve injury. Furthermore, the ERK signaling pathway was reported to be involved in the modulation of cytoskeletal protein expression, such as GAP-43 and α -tubulin during axon regeneration (29,30). GAP-43 is synthesized at high levels during axonal outgrowth and transported to the growth cone (31). α -tubulin participates directly in the regulation of the microtubule polymerization during neuronal development and axonal regeneration (32). Tau is a highly soluble microtubule-associated protein that modulates the stability of axonal microtubules (33). The proteins GAP-43, α -tubulin and Tau serve a crucial role in the local cytoskeletal rearrangement at the axonal terminal, causing the growth cone to reform and sprout (34). The level of protein synthesis of GAP-43, Tau and α -tubulin during the axon regrowth suggested that these proteins may directly participate in the processes of growth cone formation or axon elongation (30,32,34). In the present study, western blot analysis was carried out to determine whether BDNF, CNTF, GAP-43, Tau and/or α -tubulin proteins were activated during axon regeneration, so as to establish a link between cytokine signaling and axon regeneration. The present study indicated that the expression of the GAP-43, α -tubulin and Tau proteins in the PANI/RC group was elevated compared with the expression of the corresponding proteins in the sham and RC groups.

These findings are in agreement with previous studies that investigated the expression levels of GAP-43 and α -tubulin (~10-fold and ~3-5-fold increase, respectively) (35).

In the present study, the synthetic PANI/RC conduit exhibited favorable efficacy for nerve tissue regeneration. The aforementioned PANI conduit enhanced the sciatic nerve elongation and provided a favorable environment for axon regeneration by promoting the secretion of numerous cytokines, growth factors and neurotrophins, as opposed to the RC conduit. Taken together, the increasing levels of the CNTF and BDNF proteins induced by PANI regulated the activation of the ERK1/2 (MAPK) signaling pathway that in turn promoted the expression of the GAP-43, tau and α -tubulin cytoskeletal proteins.

In conclusion, the findings of the present study suggested that in the presence of PANI, the PANI/RC conduit serves a pivotal role in the stimulation of Schwann cells, in the activation of the ERK1/2 (MAPK) signaling pathway and in the release of significant growth factors that are required for the enhancement of axon regeneration and myelination following static nerve injury. Despite the promising findings, additional research is required to elucidate the exact mechanism(s) of the conduit action and which characteristic is more important for nerve regeneration: The hierarchical micro-nanostructure or the conductivity.

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