Nerve regeneration factor promotes nerve regeneration in rat*

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Abstract Nerve regeneration factor (NRF) extracted from an oral liquid of traditional Chinese medicine, as a nerve growth decoction has been reported by previous studies to exert effects of promoting nerve growth and preventing neuron apoptosis. For new insights into the function of NRF on primary cultured neurons, we investigated the neurite outgrowth in cultured rat dorsal root ganglion (DRG) explant treated with NRF by immunofluorescence and the gene and protein expressions of neurofilament-H(NF-H) and growth associated protein 43 (GAP43) in the cultured rat DRG neurons by real-time quantitative RT-PCR and Western blotting, respectively. In addition, we used a rat model of sciatic nerve crush to evaluate the effect of NRF on regeneration of injured sciatic nerve by a combination of walk track analysis, electrophysiological and histological assessments. The *in vitro* experiments indicated that NRF promoted the neurite growth of DRGs and the expression of NF-H and GAP43 at mRNA and protein levels in DRG neurons, and *in vivo* experiments showed that NRF improved peripheral nerve regeneration and functional recovery.

Keywords: nerve regeneration factor, dorsal root ganglion, sciatic nerve crush injury, neurofilament-H, growth associated protein 43.

Traditional Chinese medicine (TCM) has been using for thousands of years in China to treat various diseases and gaining increasing acceptance in western countries. The inherent advantages of TCM, such as positive efficacy, fewer side effects and abundant resources offer great promise for its application in many medical fields. Recently, the study on the working mechanisms of herbal medicine has become one of the hot topics of neuroscience. Despite considerable numbers of studies showing that TCM exerts some functions in signal transduction, regulation of gene expression and protein synthesis[1, 2], how TCM promotes nerve repair and regeneration is little known.

Nerve regeneration factor (NRF) is extracted from nerve growth decoction (NGD, a TCM oral liquid, China patent: ZL99120671.1), with the main component belonging to the chemical class of ketosteriod. NRF has been reported by previous studies to have effects of promoting nerve growth and preventing neuron apoptosis[3]. Recently, Ding et al. showed that NRF remarkably induced neuronal differentiation of PC12 cells through activating ERK1/2 in a dose-dependent and time-dependent manner[4]. But the effect of NRF on primary cultured neurons remains unclear. Here we investigated the effects of NRF on cultured dorsal root ganglion (DRG) and on the expression of NF-H and GAP43 by means of fluorescent immunocytochemistry, real-time fluorescence quantitative RT-PCR and Western blotting. Moreover, a rat model of sciatic nerve crush injury was used to investigate the promotion function of NRF on peripheral nerve regeneration *in vivo*.

1 Materials and methods

1.1 Animals and reagents

Neonatal Spragus-Dawely (SD) rats and adult SD rats (weight 220±20 g) were provided by the Experimental Animal Center of Nantong University. NRF was prepared by Key Laboratory of Neuroregeneration, Nantong University[5]. L15 medium and Trizol were purchased from GIBCO. β-NGF, rabbit anti NF-H polyclonal antibody, goat anti GAP43 polyclonal antibody, and mouse anti β-actin monoclonal antibody were from Sigma. Omniscript-TM RT Kit was from Qiagen, pGEM-T vector was from Promega. Rhodamin goat-anti-rabbit IgG, HRP goat-anti-rabbit IgG and HRP goat-anti-mouse IgG were from Santa Cruz.

1.2 DRG culture

1.2.1 Whole DRG explant culture DRGs were

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harvested from neonatal SD rats. Explanted DRGs were plated on a poly-L-lysine coated 96-well plate (Falcon) with L15 medium containing 1% FBS and maintained at 37℃, in a 5% CO_2, 95% air atmosphere. The cultured DRG explants were treated and classified into 5 groups: NRF low, middle and high dose groups with 0.1, 0.5 and 2.0 μg/mL of NRF added to the medium respectively, a NGF group treated with 0.05 μg/mL NGF added to the medium (used as positive control), and a negative control group treated without any additives to the medium.

1.2.2 DRG neurons isolation The DRG explant samples harvested from neonatal SD rats were digested with 0.125% trypsin and 0.03% collagenase for 30 min. After the enzymatic digestion, cells were gently agitated with a silicon-coated Pasteur pipette and centrifuged to remove the enzymes. The isolated cells were suspended in L15 medium and the cell density was adjusted to about 5×10^5/mL. The cells were plated on 12-well plates and kept at 37℃ in a humidified atmosphere of 95% air and 5% CO_2 for 1 h. The cells suspended in the medium were harvested and transferred on another poly-L-lysine coated 12-well plate at the density of 3×10^5/mL. The separated DRG cells were grouped for culture as the above-mentioned.

1.3 Fluorescent immunocytochemistry

After DRG explants had been cultured for 5 days immunofluorescence was performed. Briefly, DRGs were fixed for 30 min with 4% paraformaldehyde. After 3 washes in PBS, DRGs were permeabilized with 5% Triton X-100 and incubated in 0.3% goat serum (in PBS) for 30 min at 37℃. Primary antibody raised against NF-H (rabbit anti NF-H polyclonal antibody, 1∶200) was applied overnight at 4℃, and goat-anti-rabbit secondary antibody conjugated to Rhodamine (1∶200) was then added. The results were detected and photographed by a Leica fluorescent microscope.

1.4 Real-time fluorescence quantitative RT-PCR

For quantitative real-time PCR, the oligonucleotide primers and probes were designed according to the whole sequences for rat NF-H, GAP43 and GAPDH (as an internal control), respectively. The sequences are shown in Table 1.

Table 1. Oligonucleotide primers and probes of rat NF-H, GAP43 and GAPDH

Targets	Sequence (5′ to 3′)
NF-H-sense	aaggaaaccgtcattgtagaggaa
NF-H-antisense	ggagacgtagttgctgcttctt
NF-H- probe	cttctgcctccttcttcttcctcccctt
GAP43-sense	ggagcctaaacaagccgatgt
GAP43-antisense	ctacagcttctttctcctcctcag
GAP43- probe	ctgtcactgatgctgctgccaccacc
GAPDH-sense	ccttcattgacctcaactacatg
GAPDH-antisense	cttctccatggtggtggaaac
GAPDH-probe	ccatcaccatcttccaggagc

The standard plasmids of NF-H, GAP43 and GAPDH were constructed by using the above designed PCR primers[9]. They were identified by DNA sequencing, quantified, and diluted into 10^8, 10^7, 10^6, 10^5, 10^4 pg/mL, respectively.

After 12 h culture, the isolated DRG cells were homogenized in 1 mL Trizol and total RNA was extracted for reverse transcription reaction. For real-time PCR, the initial 3 min incubation at 93℃ was performed, followed by 40 cycles of denaturation (93℃ for 20 s) and annealing/extension (60℃ for 55 s). Fluorescent detection was performed after each annealing/extension step. Each assay was carried out in a separate microcentrifuge tube containing 1 μL of cDNA template and 19 μL of quantitative PCR solution and the composition of which contained 1× PCR buffer, 2.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTPs, 0.5 μmol each of NF-H, GAP43 or GAPDH upstream and down-stream primers, 0.4 μmol each of NF-H, GAP43 or GAPDH fluorescence probes, and 1U Taq-DNA polymerase. Negative control and plasmid standards were also amplified simultaneously. The mRNA quantity was automatically calculated based on the calibration curves generated by serially diluted plasmid preparations.

1.5 Western blotting

After 12 h culture, the isolated DRG cells were extracted for protein with a buffer containing 1% SDS, 100 mmol/L Tris-HCl, 1 mmol/L PMSF and 0.1 mmol/L β-mercaptoethanol. Protein concentration of each specimen was detected by the Bradford method to maintain the same loads. Protein extracts were heat denatured at 95℃ for 5 min, electrophoretically separated on a 6% (for NF-H) or

12% (for GAP43) SDS-PAGE, and transferred to PVDF membranes. The membranes were blocked with 5% non-fat dry milk in TBST buffer (50 mmol/L Tris-HCl, 100 mmol/L NaCl, and 0.1% Tween-20, pH 7.4) and incubated with a 1∶500 dilution of rabbit anti NF-H polyclonal antibody or goat anti GAP43 polyclonal antibody in 5% non-fat dry milk in TBST buffer at 4℃ overnight. The membranes were washed with TBST buffer (5 min×3), and further incubated with a 1∶200 dilution of goat anti-rabbit IgG or donkey anti-goat antibodies conjugated with horseradish peroxidase (HRP) at room temperature for 2 h. After the membrane was washed, the HRP activity was detected using an ECL kit. The image was scanned with a GS800 Densitometer Scanner (Bio-Rad), and the data were analyzed using PDQuest 7.2.0 software (Bio-Rad). β-actin (1∶4000) was used as an internal control.

1.6 Sciatic nerve crush surgery

Forty-eight SD rats (180—220 g), irrespective of gender, were randomized into 3 groups: NRF low and high dose groups, and a control group. All the SD rats were anesthetized by an injection of complex narcotics (1 mg/100 g xylazine, ketamine 9.5 mg/100 g, acepromazine 0.07 mg/100 g), and the sciatic nerve was exposed and lifted through an incision on the lateral aspect of the mid-thigh of the hind limb. The right sciatic nerve was crushed with a pair of forceps for three times (10 s/each time) with an interval of 10 s under sterile operative conditions. Crush level was marked on the muscle by a 9/0 non-absorbable silk suture, and the incision sites were then closed[7]. After operation, the animals in 2 NRF groups were intragastricly administrated every day with 0.2 or 1 mg/100 g of NRF, respectively, while the control group was given the same volume of normal saline (1 mL/100 g) instead of NRF.

1.7 Sciatic nerve regeneration evaluation

1.7.1 Walking track analysis Walking track analysis was performed to the rats of 3 groups at day 10, 15 and 20 after surgery. Three parameters including print length (PL), toe spread (TS) and intermediary toe spread (IT) were measured. Sciatic nerve function index (SFI) was calculated by Bain formula ($SFI=-38.3(EPL-NPL)/NPL+109.5(ETS-NTS)/NTS+13.3(EIT-NIT)/NIT-8.8$)[8], where the prefix E and N represent the operated side and normal unoperated side respectively. When $SFI=0$, the function is normal, and when $SFI=-100$, the nerve is totally broken down[9].

1.7.2 Electrophysiology The electrophysiological test was performed with a SUPER LAB D-95 system on day 20 post surgery. Bilateral sciatic nerves were isolated from anesthetized rats. The stimulus electrode and record electrode were put at the proximal and distal ends of the crushed nerve respectively. Complex action potential was induced by the electrical stimuli applied. Nerve conduction velocity (NCV) was calculated by latent phase and distance between two electrodes. Recovery rate was expressed as operated side/ contralateral non-operated side×100%.

1.7.3 Histological observation Following electrophysiological test, the animals were perfused with 4% paraformaldehyde. The spinal cord between L4-L6, distal end of the crushed nerve and bilateral gastrocnemius muscle were obtained, then embedded with paraffin and sectioned. Subsequently, the sections of spinal cord and muscle were deparaffinized and dehydrated gradually and examined by histology of HE staining, while the nerve sections were detected by HE staining and Masson trichome staining. Three indexes, including number of motor neurons, number of regenerated myelinate nerve fibers and cross sectional area (CSA) of muscle fibers were evaluated by a Leica Q550 IW image analysis system.

1.8 Statistical analysis

At least three repetitive assessments were performed. All data were expressed as means±SD. Student's t-test was used to compare differences between NRF or NGF group and the control group. All statistical analyses were conducted with a STATA 7.0 software package (Stata Corp), and all significance levels were set at $p<0.05$.

2 Results

2.1 Effect of NRF on neurite outgrowth of DRGs *in vitro*

In the negative control group, whole DRG explants grew very slowly and no significant neurite outgrowth could be detected until 5 days of culture. In the NRF low dose group, a little neurite formation showed on day 5. In NRF high dose group, neurite outgrowth appeared from day 2 and the neurites formed in culture were most extensive in number and radial-like in shape on day 5. In regard to the increas-

ing number of neurites and their length, NRF high dose group showed little difference with NGF group (Fig. 1). Considering that the neurite outgrowth of DRGs under such culture condition was complicated, especially the number and length of neurites being hardly measured, we used "Fractal dimension" as a parameter to describe the degree of cell aggregation[10,11]. The larger the Fractal dimension is, the rougher the cell outline is. Thus, fractal dimension is applied to describe quantitatively the irregularity of cell formation. The algorithm was completed on a computer with CPU of P4 2.0 G and memory of 256 M by the aid of Matlab 6.5 software programs, the procedure of which includes three main steps, namely transferring the color image to a 256 grey image and putting the size to 512×512; calculating the box-counting dimension and performing *t*-test analysis. The result is shown in Fig. 2.

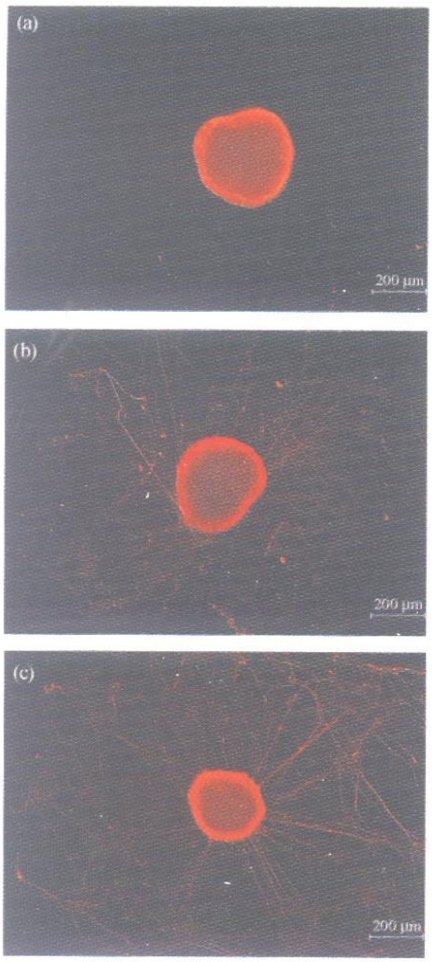

Fig. 1. The neurite outgrowth of cultured DRGs in the negative control group (a), NRF high dose group (b) and NGF group (c).

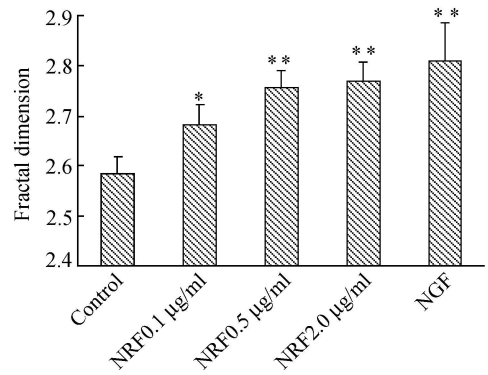

Fig. 2. Fractal dimension of cultured DRGs in 5 different groups (means±SD, $n=10$). * $p<0.05$, ** $p<0.01$, vs the negative control.

2.2 Effect of NRF on mRNA expression of NF-H, GAP43 in DRG neurons

The NF-H or GAP43 mRNA in the DRG neurons was examined by real-time PCR. GAPDH mRNA was used as an internal control for determining the relative value of NF-H and GAP43 mRNA. As shown in Fig. 3, after 12 h culture, the mRNA levels of NF-H or GAP43 for 3 NRF groups were up-regulated when compared to that of the negative control group with a significant difference between either NRF middle or high dose group and the negative control group.

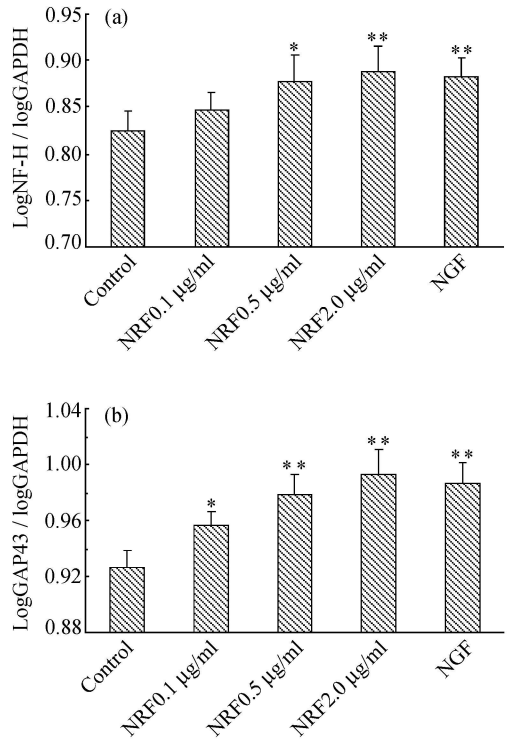

Fig. 3. The expression levels of NF-H (a) and GAP43 (b) mRNA in cultured DRG neurons in 5 different groups (means±SD, $n=10$). * $p<0.05$, ** $p<0.01$, vs control.

2.3 Effect of NRF on the protein expression of NF-H, GAP43 of DRG neurons

The results of Western blotting (Fig. 4) also showed that NRF could up-regulate the protein levels of both NF-H and GAP43 in DRG neurons. The protein expression was increased with the increase in NRF dose, with the highest appearing at the concentration of 2.0 μg/mL.

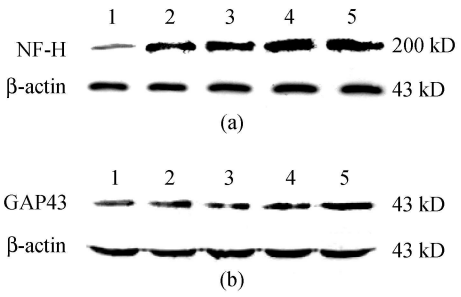

Fig. 4. The expression levels of NF-H (a) and GAP43 (b) proteins in cultured DRG neurons in 5 different groups. 1, Control; 2, NRF (0.1 μg/mL); 3, NRF (0.5 μg/mL); 4, NRF (2.0 μg/mL); 5, NGF (0.05 μg/mL).

2.4 Effect of NRF on SFI of injured sciatic nerve

The result of walking track analysis is shown in Table 2. There is no significant difference in SFI value between either of NRF groups and the control group on day 10 after surgery. On day 15, the SFI value for NRF high dose group was significantly higher than that for the control group, and on day 20 the SFI value for both NRF low and high dose groups was significantly lower than that for the control group.

Table 2. Sciatic function index for 3 different groups (means ±SD, $n=10$)

Group	day 10	day 15	day 20
NRF low dose group	−76.71±12.28	−57.22±18.93	−39.34±4.87 *
NRF high dose group	−75.49±9.59	−34.49±10.40 **	−30.99±8.03 **
The control group	−77.11±13.63	−56.54±10.22	−54.77±12.07

Note: * $p<0.05$ and ** $p<0.01$ vs the control group.

2.5 Effect of NRF on recovery rate of NCV

The results of electrophysiological tests performed on day 20 post surgery are shown in Table 3, which indicates that the NCV recovery rate for NRF high dose group was much higher than that for the control, but there was little difference between NRF low dose group and the control group.

Table 3. Effect of NRF on recovery rate of NCV (means ± SD, $n=10$)

Group	NCV recovery rate (%)
NRF low dose group	44±15
NRF high dose group	57±26 *
The control group	31±9

Note: * $p<0.05$ vs the control group

2.6 Histological study

Masson trichome staining of the sciatic nerve is shown in Fig. 5. In the control group, there appeared proliferating Schwann cells in the regenerating nerves with a few nerve fibers in disoriented arrangement. This situation was just contrary to that for NRF groups, in which the nerve fibers were aligned tightly and tidily. The number of motor neurons, regenerated myelinated nerve fibers and the CSA of muscle fibers were counted or calculated after HE staining and Masson trichome staining. The results are shown in Table 4. In high dose NRF group, the above three indexes were significantly increased compared with the control group.

Table 4. The number of motor neurons and regenerated myelinate nerve fibers and muscle fibers CSA in 3 different groups (means±SD, $n=10$)

Group	No. of motor neurons	No. of regenerated myelinate nerve fibers	Cross section muscle (μm^2)
NRF low dose group	82.2±8.7	1627±183	350.2±42.9 *
NRF high dose group	87.8±7.6 *	1879±121 **	369.0±36.8 **
The control group	77.1±10.1	1569±166	316.1±45.8

Note: * $p<0.05$ and ** $p<0.01$ vs the control group

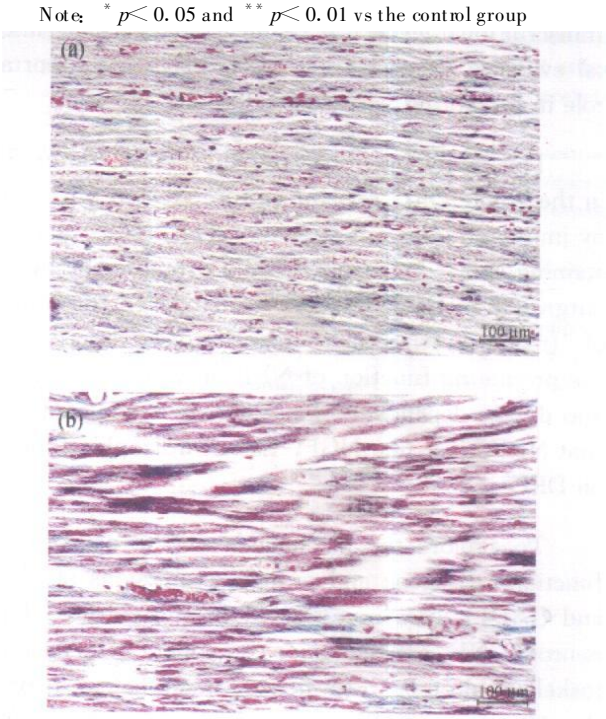

Fig. 5. Light micrographs of the regenerated sciatic nerve, obtained on day 20 post surgery. (a) NRF group; (b) the control group. Masson trichrome staining.

3 Discussion

Since the discovery of NGF in the 1950s, the exploration of neurotrophic substance has been the prime focus of neuroscience research. It has been indicated that neurotrophic factors, including NGF, CNTF, BDNF, etc., have great effects on nerve growth and repair *in vivo*. But these neurotrophic factors extracted from animals are too expensive, while those produced from genetic engineering have a controversial efficacy. Thus, seeking for alternatives to commonly used neurotrophic factors has attracted more and more interests. In this respect, TCM provides a new source for neurotrophic factors due to its unique performances. NRF examined in the present study is an effective component extracted from NGD. As indicated in previous reports, NRF significantly improved repair of injured sciatic nerve and inhibited apoptosis of rat spinal cord[3], participated in the up-regulation of proliferating gene and expression of trans-acting element in regulation of gene transcription, and was also involved in the synthesis of related structural gene by tRNA synthetase[12]. In addition, NRF could up-regulate the expression of gene correlated with cell growth, metabolism, division and proliferation in embryonic rat cortex cells, such as calmodulin binding protein, phosphorylase, ribosomal protein, mitogenic factor, microtubule protein, zinc finger protein kinase, etc., indicating that NRF could promote the growth of brain cortex cells at transcriptional level[13]. Taken together, experimental evidence suggests that NRF plays an important role in promoting nerve regeneration.

This study first observed the neurite outgrowth in the whole DRG explant culture treated with NRF by immunofluorescent staining. The results of NF-H staining showed that NRF could promote the neurite outgrowth of DRGs. Here we introduced the concept of "fractal dimension" to quantitatively describe that the promoting function of NRF on neurite outgrowth was dosage-dependent. This *in vitro* study confirmed that NRF, just like NGF, has neurotrophic function on DRGs.

To explore the mechanism of the neurotrophic function, the gene and protein expressions of NF-H and GAP43 were investigated in the cultured DRG neurons. NFs are the major component of neuron cytoskeletal proteins. In mature mammalian neurons the 10 nm neurofilaments are composed of three polypeptide subunits identified by their molecular weights as low, NF-L (68 kD), medium, NF-M (160 kD) and high, NF-H (200 kD), which are important in maintaining the unique morphological character of neurons and axoplasmic transporting necessary to normal function of nervous system[14]. Gene targeting studies revealed that loss of NFs resulted in the failure of radial growth of axons[15]. GAP43, first discovered in the 1980 s, was a fast transport membrane phosphoprotein. GAP43 plays great roles in neural development, axon regeneration and synapse reconstruction. It is expressed along the axons in developing neurons, especially in growth cones; while in mature nervous system it is distributed in nerve endings of some definite region[16]. The expression of GAP43 is elevating in the whole period of axon elongation and synapse formation, and it can regulate the response of neurons to axon targeting signal[17]. GAP43 can affect the axon-developing ability by guiding axon growing and accommodating new conjugation formation, even in the absence of other trophic factors. Thus, GAP43 is considered as the internal decision factor in neuronal developing stage and served as a marker for nerve regeneration.

In the culture of isolated DRG neurons, the method of differential adherence was applied, which could purify DRG neurons by removing non-neuronal cells. Meanwhile, no serum was used in the test to diminish the effects of various unknown factors and components in serum. This study showed that the mRNAs of NF-H and GAP43 were both up-regulated in NRF middle and high dose groups after 12 h treatment. With an increase in NRF concentration, the expression of NF-H or GAP43 was also increased. There was no difference between NRF high dose group and NGF group. These results indicated that NRF might serve the similar function as NGF to promote neurite elongation of the cultured DRGs. But the underlying mechanism remains little identified. Recent research showed that NRF could promote neuronal differentiation of PC12 cells by ERK1/2 signal transduction[4]. The phosphorylation regulation of NF was partly dependent on ERK1/2[18]. The issue of whether the promoting function of NRF on DRG is related to the ERK1/2 activity is to be studied.

In addition, this study investigated the *in vivo* effect of NRF on peripheral nerve regeneration. As we known, peripheral nerve regeneration is a complicated biochemical and cytological process involved in three aspects, namely nerve cell bodies, axons and microenvironments of nerve growth. The clinical re-

pair of injured nerve is still a difficult problem in traumatic surgery. Certain macromolecular substances such as NGF and hormones has been used in clinical practice and confirmed to be useful in promoting peripheral nerve regeneration[19, 20]. Recently research showed that some of TCM such as ginseng could promote axon elongation[21]. In this study, a rat model of sciatic nerve crush was constructed. After nerve injury and treatment with NRF, the outcome of sciatic nerve regeneration was evaluated by a combination of walk track analysis, electrophysiological and histological assessments. The results indicated that NRF could significantly increase SFI, accelerate the recovery rate of NCV, and effectively prevent apoptosis of spinal cord motor neurons, thus greatly promoting nerve regeneration.

In summary, both *in vitro* and *in vivo* experiments on the effects of NRF on neuroregeneration provided positive evidence of its promoting function. As a main component extracted from TCM preparation, NRF also has the advantages of water solubility and low side effect. Therefore, this study raises a potential possibility of NRF for clinical application to treat peripheral nerve injury.

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