Original Article
Effects of electroacupuncture on Schwann cell morphology and ciliary neurotrophic factor expression in a rabbit model of facial nerve injury

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Abstract: Objective: This study aims to investigate the biological mechanisms of acupuncture therapy in rabbit models of facial paralysis. Methods: 42 healthy adult Japanese big-ear rabbits were randomly divided into the normal control (n = 6), model (n = 18) and electroacupuncture (EA; n = 18) groups. Rabbit models of facial nerve injury were established by clamping the right facial nerve. Electron microscopy, light microscopy and immunohistochemistry were performed to observe the morphology of injured facial nerves and detect the expression of ciliary neurotrophic factor (CNTF). Results: Compared with the normal control group, the majority of myelin sheaths were damaged in the model group. There were significant differences in the number of myelinated nerve fibers, the thickness of myelin sheath, and axonal area between the model and normal control groups on days 10 and 15 after injury ($P < 0.01$ or $P < 0.05$). CNTF expression in the model group was significantly greater than that in the control group on days 5, 10 and 15 after injury ($P < 0.01$). Compared with the model group, the extent of demyelination was less severe, and there was a greater number of intact organelles in the EA group. CNTF expression in the EA group was significantly lower than that in the model group on days 5 after injury ($P < 0.05$). Subsequently, CNTF expression in the EA group increased and was significantly different from that in the model group on day 15 after injury ($P < 0.01$). In the EA group, demyelination was milder on day 5 after injury than on days 10 or 15 after injury, and was associated with the duration of low CNTF expression. Conclusions: EA rapidly promotes the repair of injured facial nerves and improves the morphology of Schwann cells during the initial stage of facial nerve injury. It may be disadvantageous to the recovery of injured facial nerve fibers and Schwann cells during the later stages of the repair process.

Keywords: Schwann cells, ciliary neurotrophic factor, electroacupuncture, facial nerve injury

Introduction

Facial paralysis,a common disease in China [1] is caused by an acute and nonsuppurative inflammation of the stylomastoid foramen. It belongs to the category of "wry mouth" (*Koupi*) or "a deviation of the eyes and mouth" (*Kouyan Waixie*), according to traditional Chinese medicine. It is primarily caused by the attack of exogenous wind to the meridian *qi*, and manifests as muscular flaccidity and weakness, with stiffness, numbness or looseness of the face, enlargement of the palpebral fissure, a deviation of the mouth to the healthy side, disappearance of wrinkles on the forehead, and flattening of the nasolabial groove.

Acupuncture has been used in China for many years to effectively treat facial paralysis [2, 3].

However, the physiological and biological mechanisms behind its effectiveness are still unknown. Schwann cells are a kind of glial cells present in peripheral nerves that form myelin sheaths around axons [4, 5]. These cells undergo Wallerian degeneration after peripheral neuroaxonal damage. Schwann cells secrete a variety of neurotrophic factors [6-8], including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF) and neurotrophin-3 (NT-3), to promote the survival of damaged neuronal cell bodies, contribute to the formation of endoneurium, and increase axonal myelination. One research team showed that acupuncture could relieve congestion, edema around nerve fibers and demyelination of the nerve root, and also help damaged Schwann cells regain their normal appearance [9].

CNTF has been shown to be much better than NGF in the promotion of facial nerve regeneration [10]. NGF mainly promotes the survival of sensory neurons [11]. BDNF has been shown to be only able to exert a protective effect during the early stage of facial nerve injury [12]. CNTF is presently a hot topic in the field of neural regeneration. It is a survival factor for various central and peripheral neurons. It is released under pathological conditions and has a particularly important role in the nervous system. In peripheral nerves, CNTF is found in Schwann cells at high concentrations. It plays a crucial role in the formation and regeneration of myelin sheaths [13] and has a unique ability to provide trophic support to both neurons and muscles. It can promote neural repair after facial nerve injury [14]. In addition, it can prevent against degeneration of facial nerve nuclei. Thus, it appears to be a promising candidate for the treatment of motor neuron diseases and may be used clinically in the near future to treat peripheral nerve injury and neurodegenerative diseases.

Electroacupuncture (EA) has been shown to enhance CNTF expression [15, 16]. Therefore, in this study, we chose a rabbit model of facial nerve injury, and focused on the factors that play a critical role in the repair of injured facial nerve-Schwann cells and CNTF. We investigated the effects of EA on the morphology of injured facial nerves, especially that of Schwann cells, and CNTF expression in Schwann cells.

Materials and methods

Animals

A total of 42 healthy Japanese big ear rabbits of either gender, aged 80-85 days, weighing 1.9-2.1 kg, were supplied by the Laboratory Animal Center of Chengdu University of Traditional Chinese Medicine, China (license NO. SCXK [chuan] 2008-14) and included in this study. Animals were kept in individual cages at room temperature and normal relative humidity. The groups were coded by one investigator and analyzed under "blind" conditions by another investigator.

Reagents and instruments

The main reagents and instruments used in this experiment are as follows: Hematoxylin-eosin (HE, Zhongshan Goldenbridge Biotech-

nology, Beijing, China), light microscope (Nikon Eclipse 80i, Nikon, Tokyo, Japan), transmission electron microscope (Hitachi 600IV, Hitachi, Tokyo, Japan), G6805-2 electric acupuncture system (Xinsheng Engineering Reagent Apparatus Factory, Qingdao, China), Leica QWin image analysis software (Huaida Instruments Co., Shanghai, China), rabbit anti-CNTF polyclonal antibody (Beijing Biosynthesis Biotechnology, China), goat anti-rabbit IgG streptavidin-biotin (SABC, Boster, Wuhan, China), and diaminobenzidine (Zhongshan Goldenbridge Biotechnology, Beijing, China).

Acupuncture instrument: Filiform needles (size 0.3 mm×25 mm, Huatuo brand) was manufactured by Suzhou Medical Appliance in Suzhou, China.

Groups and model establishment

Forty-two rabbits were divided into three groups using a table of random digits [17]: normal control (n = 6), model (n = 18) and EA (n = 18) groups. Based on previous reports [18-21] and the results of our preliminary experiments, we established rabbit models of facial nerve injury in the model and EA groups. Prior to surgery, the hair on the right side of face was removed with depilatory. Under anesthesia with 1% pentobarbital sodium (5 ml/kg) injected into the ear border vein, the facial nerve was exposed at a site on the right cheek, and a 1 cm long crush lesion was produced at the center of the cheek (about half way between the root of the ear and the right corner of the mouth, vertically 2 cm below the right pupil). A 5 minute crush of the facial nerve was performed using forceps (buckle, 5 kg of pressure). After crushing the facial nerve, the wound was sutured. Penicillin ointment was used to prevent infection of the wound. Rabbits from the model and EA groups were further assigned to one of three subgroups according to post-surgical survival period (5, 10 or 15 days). If the number of experimental animals was less than that of initially established for a particular group because of various factors, the group would be supplemented with rabbits from a standby pool by random sampling. Forty-two rabbits were included in the final analysis.

Interventions

Needling in combination with EA stimulation was performed in the EA group. No intervention

Table 1. Recovery of functional parameters in rabbits

Group	Time point	Facial muscle	Eye blink reflex	Vibrissae movement
Normal		+++	+++	+++
Model	Day 5	-	-	-
	Day 10	+	++	+
	Day 15	+	++	++
EA	Day 5	++	+++	++
	Day 10	++	+++	+++
	Day 15	+	++	+++

Notes: Functional recovery was examined in terms of facial muscle movement, eye blink reflex and vibrissae movement. These were quantified from + to +++ (- = absent).

was conducted in the normal control and model groups. For EA stimulation, rabbits were fixed using a self-made clamp. The acupoints included *Dicang* (ST4), *Jiache* (ST6), *Yifeng* (TE17) and *Hegu* (LI4) on the lesioned side. Perpendicular puncture was applied at *Yifeng*, 1 cm deep; penetrating puncture was applied at *Dicang* to *Jiache*, 1 cm deep and at *Hegu*, 0.5 cm deep. The electrodes were attached to *Yifeng* and *Hegu*, and at *Dicang* and *Jiache*, respectively, with a dense-disperse wave, at 18-20 Hz, 1.5 V [22]. 30-minute EA treatment was performed once daily. Five days constituted a treatment course and three treatment courses were used.

Sample collection

On days 5, 10 and 15 s after injury, six random rabbits from each group were sacrificed. The facial nerve was cut into 1 cm long pieces and then immediately put into 4% paraformaldehyde.

Assessment of function

Functional recovery of facial muscle movement, eye blink reflex and vibrissae movement on the affected side, compared with the normal side, were observed during the recovery process. These factors were quantified on a scale from + to +++ (- = absent) [23].

Tissues processing

The sample tissue was dehydrated, embedded and sliced using routine methods. Pathological observation with HE staining was done under the light microscope.

Morphology of injured facial nerve and Schwann cells

Samples were fixed with 3% glutaraldehyde, again with 1% osmium tetroxide, dehydrated with acetone, embedded with Epon812, cut into 5-μm-thick sections, stained with uranyl acetate and lead citrate, and finally observed under a Hitachi H-600IV transmission electron microscope.

CNTF immunohistochemistry

Immunohistochemistry was performed according to the manufacturer's instructions. Briefly, the sections were dewaxed, incubated with 3% H_2O_2 (30% H_2O_2 mixed with distilled water at 1:10) for 10 minutes at room temperature, washed with distilled water three times, treated with 0.01 mol/L citrate (pH 6.0) and heated until boiling in a microwave oven, which was repeated in 2 minutes for antigen retrieval, and finally washed with PBS. After treating with 5% bovine serum albumin blocking solution for 20 minutes at room temperature, the sections were incubated with rabbit polyclonal anti-CNTF antibody (1:200) at 37°C for 30 minutes, then overnight at 4°C, washed with PBS three times for 2 minutes each, incubated with biotinylated goat anti-rabbit IgG (1:100) at 37°C for 30 minutes, and rinsed again. They were then incubated with SABC (1:100) at room temperature for 30 minutes, washed with PBS four times for 5 minutes each and reacted with diaminobenzidine, counterstained with HE, dehydrated, cleared, mounted and observed.

Main observation indices

The morphologic changes in the facial nerve and Schwann cells were observed by electron and light microscopy. CNTF expression was evaluated by immunohistochemistry.

Statistical analyses

All statistical analyses were performed with SPSS software (version 13.0; SPSS, Chicago, IL). Values were expressed as the mean ± SD. Data were analyzed using one-way analysis of variance and post-hoc least significant differ-
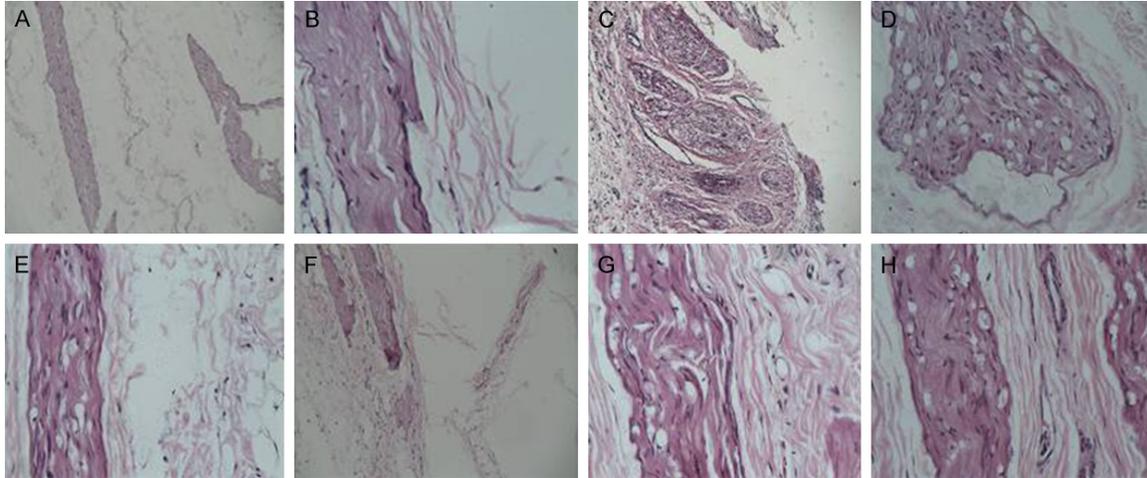
Figure 1. Hematoxylin-eosin staining. A. Normal nerve fibers, longitudinal section, ×100. B. Normal nerve fibers, longitudinal section, ×400. C. Model group on day 5 after injury, severe edema, cross section, ×100. D. Model group on day 10 after injury, moderate to severe edema, cross section, ×400. E. Model group on day 15 after injury, moderate edema, longitudinal section, ×400. F. Electroacupuncture group on day 5 after injury, mild edema, longitudinal section, ×100. G. Electroacupuncture group on day 10 after injury, moderate to severe edema, longitudinal section, ×400. H. Electroacupuncture group on day 15 after injury, moderate edema, longitudinal section, ×400.

ence test. Statistical significance was defined as $P < 0.05$.

Results

Recovery of function at various intervals after facial nerve injury

The results of functional recovery after facial nerve injury are shown in **Table 1**.

Histopathological changes in injured facial nerves

HE staining was performed to observe the pathological changes in facial nerves in each group. In the normal control group, facial nerve bundles were intact, with the smooth capsule enveloping bundles of nerve fibers. In the model group, the structural integrity of the facial nerve bundles was destroyed. Different degrees of edema were found around facial nerve fibers, and a great deal of vacuolation and inflammatory cell infiltration were observed. Compared with the model group, the histopathological changes in the EA group were much less severe at the corresponding time points (**Figure 1**).

Ultrastructure of myelin sheaths and Schwann cells in injured facial nerves

In the normal control group (**Figure 2A**, **2B**), the structure of the myelin sheath surrounding facial nerve fibers was intact, without demyelination and degeneration. Schwann cells were normal with abundant organelles, such as endoplasmic reticulum, Golgi apparati and mitochondria. In the model group (**Figure 2C-G**), the majority of myelin sheaths were disaggregated and damaged. There were many vacuoles in organelles in the cytoplasm of Schwann cells. On day 5 in the model group, the extent of demyelination was extremely severe (**Figure 2C**).

In the EA group, compared with the model group, the extent of demyelination was milder, with more abundant intact organelles, especially on day 5 after injury (**Figure 2H**, **2I**). In the EA group, demyelination was milder on day 5 than that on days 10 and 15 (**Figure 2J-M**). With continued EA treatment, the pathology worsened on day 15 in the EA group (**Figure 2L**, **2M**).

CNTF content in Schwann cells

In the normal control group, CNTF protein expression was low in Schwann cells, but in the model group, it increased significantly on days 5, 10 and 15 after injury ($P < 0.01$, $P < 0.01$ and $P < 0.05$, respectively), with a peak on day 10. On day 5 CNTF expression in the EA group was significantly lower than in the model group ($P <$
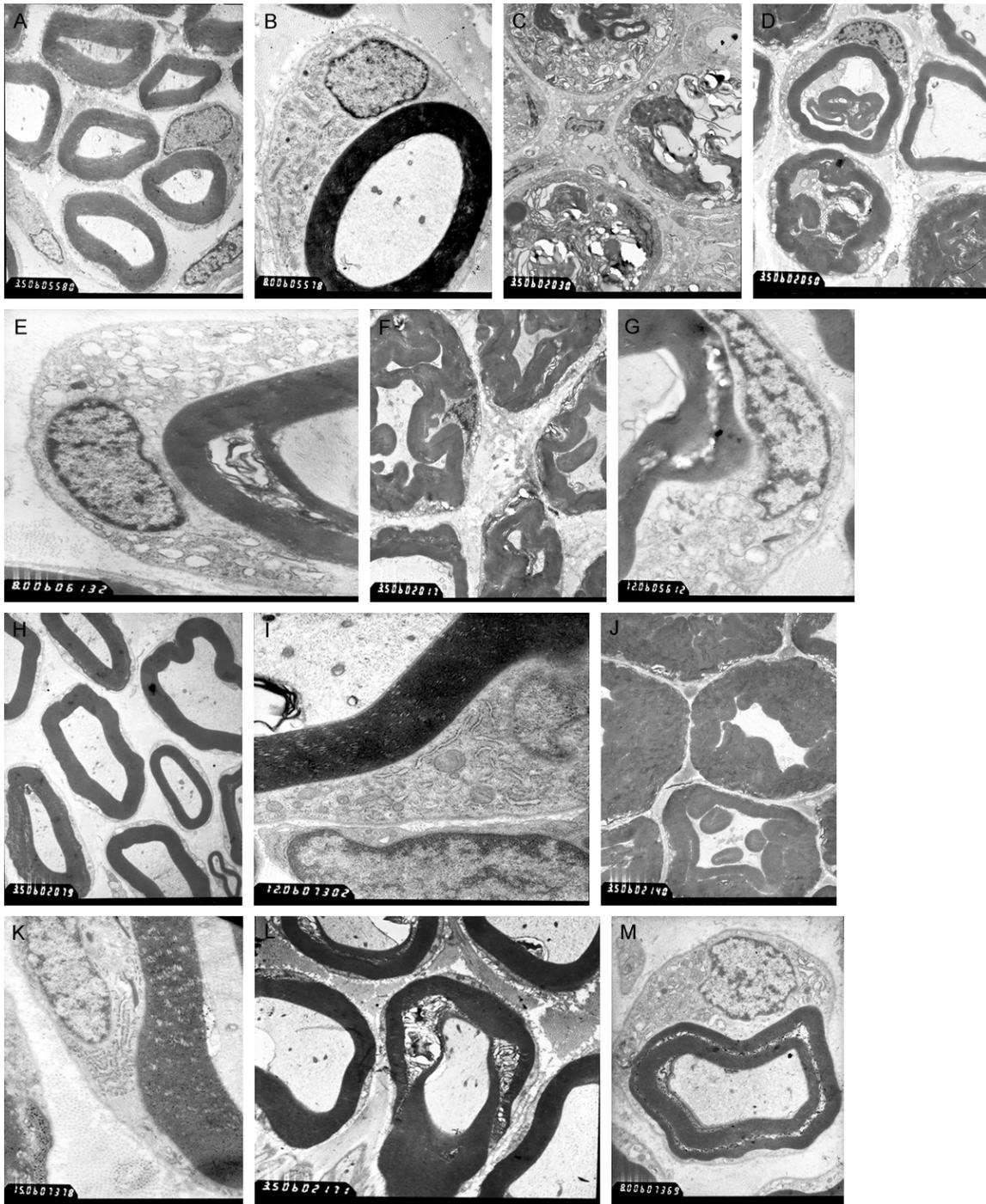
Figure 2. Ultrastructure of injured facial nerves and Schwann cells. A. Normal control group (×3500); B. Normal control group, ×8000; C. Model group (day 5 after injury), ×3500; D. Model group (day 10 after injury), ×3500; E. Model group (day 10 after injury), ×8000; F. Model group (day 15 after injury), ×3500; G. Model group (day 15 after injury), ×12000; H. Electroacupuncturegroup (day 5 after injury), ×3500; I. Electroacupuncture group (day 5 after injury), ×12000; J. Electroacupuncture group (day 10 after injury), ×3500; K. Electroacupuncture group (day 10 after injury), ×15000; L. Electroacupuncture group (day 15 after injury), ×3500; M. Electroacupuncture group (day 15 after injury), ×8000.

0.05). However, CNTF expression increased with extended duration of EA treatment, and it was higher than that in the model group at the corresponding time points. After 15 days of EA

Table 2. Concentration values for ciliary neurotrophic factor in facial nerve at different time points in each group (X ± S)

Group	n	Day 5	Day 10	Day 15
Normal control	6	93±57		
Model	6	357±222*	485±257*	327±240**
Electroacupuncture	6	126±87△	647±50	670±89△△

*$P < 0.05$, vs. normal control group; **$P < 0.01$, vs. normal control group; △$P < 0.05$, vs. model group; △△$P < 0.01$, vs. model group.

treatment, CNTF expression was significantly different from the model group on day 15 ($P < 0.01$; **Table 2** and **Figure 3**).

Discussion

Facial paralysis has been treated with EA for many years in China, about which modern physicians have accumulated abundant clinical experience. However, whether EA can be applied in the acute stage of facial paralysis is still in dispute. The main pathological features of acute facial paralysis are ischemia, edema, varying degrees of myelin sheath breakdown and axonal degeneration. EA-induced congestion and the aggravation of edema in local tissues may be disadvantageous to the recovery of facial nerve function during the acute stage of the disease. However, some studies have reported that EA can promote facial nerve repair in the acute stage of facial paralysis by improving the phagocytic function of white blood cells and increasing the number of red blood cells and exhibit an anti-inflammatory effect. EA also appears to rapidly enhance the nutritional health of injured nerves, promote absorption of inflammatory edema, and shorten the duration of compression of the facial nerve in the facial canal. All these effects combine to increase the likelihood of recovery from facial paralysis [24]. The acute stage has been shown to be an optimal period for the application of EA treatment for facial paralysis [25, 26].

There is evidence that CNTF is released when neuron is damaged, and supplementation CNTF results in attenuation of the injury [27, 28]. In our study, morphological changes in rabbit models of facial nerve injury were observed by light and electron microscopy, which showed that EA could quickly repair injured nerve fibers and Schwann cells within 5 days of injury. In addition, CNTF expression in the EA group was much lower than in the model group on day 5 after injury. The application of EA in the acute stage of facial nerve injury had positive effects on the repair of injured facial nerves and Schwann cells.

As the morphology of the injured facial nerve fibers and Schwann cells steadily returned to normal in the EA group, CNTF expression gradually decreased and reached a minimum 5 days after injury. However, continuous EA stimulation at a steady intensity may switch the effects from beneficial to harmful, subsequently leading to an increase in CNTF expression, which may be detrimental as they are positively correlated with the extent of facial nerve injury.

Some researchers have reported that the effects of EA are not positively correlated with the quantity of stimulation [29-31]. Thus, for facial paralysis, which is characterized by distinct pathological changes following injury, we should adjust the parameters of EA accordingly and select the appropriate acupuncture methods. This should be combined with careful observation of the disease, including thorough classification of various neuropathological changes and differentiation of various stages of disease development [32-34]. Moreover, the various principles mentioned in the theory of traditional Chinese medicine should be applied to current treatments [35, 36], and these should be observed in subsequent studies as well.

In conclusion, our results demonstrate that EA can promote the repair of injured facial nerves and Schwann cells in the acute stage of facial nerve injury. During later stages of facial nerve injury, continuing the EA treatment, with the same quantity of stimulation, may induce the injury and result in high CNTF expression. Consequently, the parameters of EA should be modulated during the different stages after facial nerve injury to optimize the efficacy of the treatment.
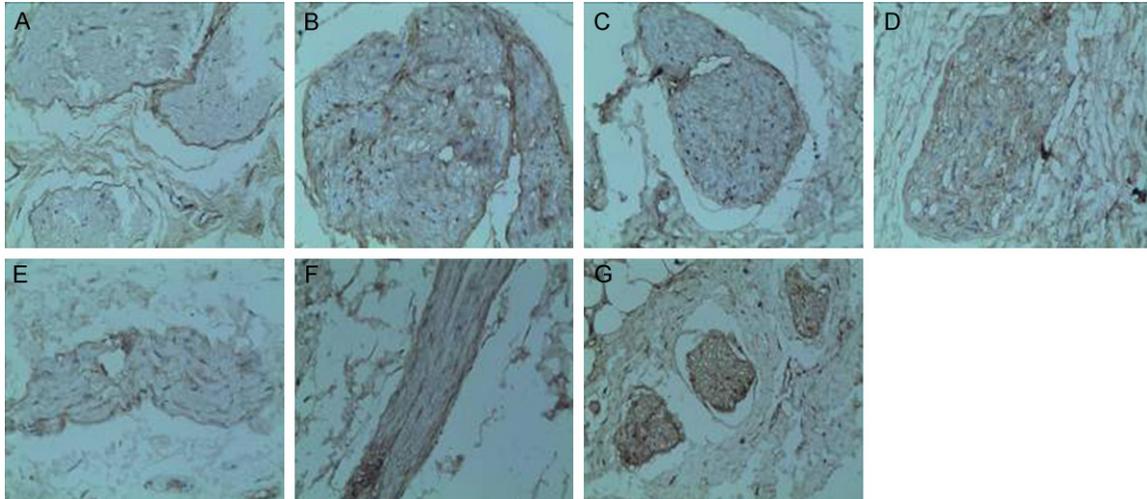
Acknowledgements

Figure 3. Ciliary neurotrophic factor immunohistochemistry (×400). A. Normal control group; B. Model group, day 5 after injury; C. Model group, day 10 after injury; D. Model group, day 15 after injury; E. Electroacupuncture group, day 5 after injury; F. Electroacupuncture group, day 10 after injury; G. Electroacupuncturegroup, day 15 after injury.

Disclosure of conflict of interest

None.

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