RESEARCH ARTICLE

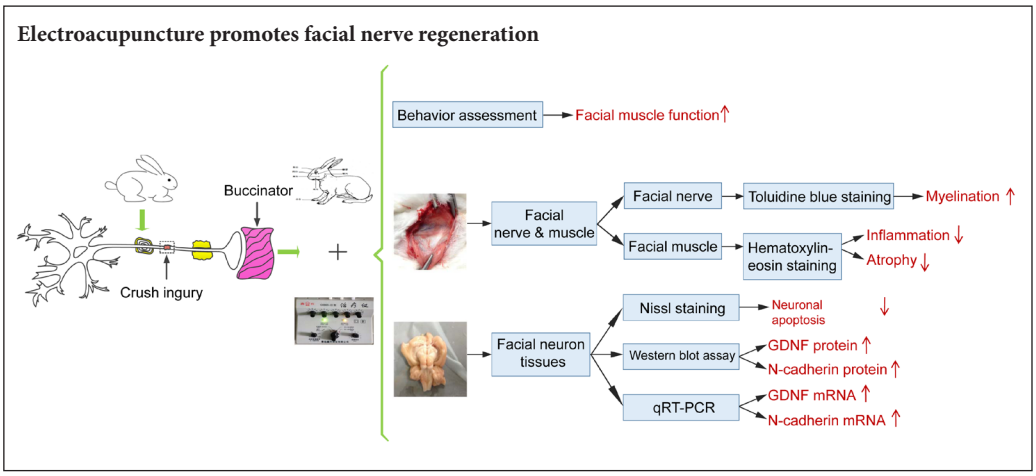
Electroacupuncture promotes peripheral nerve regeneration after facial nerve crush injury and upregulates the expression of glial cell-derived neurotrophic factor

Jing Fei[1], **Lin Gao**[1], **Huan-Huan Li**[2], **Qiong-Lan Yuan**[2], **Lei-Ji Li**[1,*]

1 Department of Otorhinolaryngology, Head and Neck Surgery, Affiliated Hospital of Southwest Medical University, Luzhou, Sichuan Province, China

2 Department of Anatomy and Neurobiology, Tongji University School of Medicine, Shanghai, China

Graphical Abstract



Correspondence to:
Lei-Ji Li, MD,
83193604@qq.com.

orcid:
0000-0002-9531-7400
(Lei-Ji Li)

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Abstract

The efficacy of electroacupuncture in the treatment of peripheral facial paralysis is known, but the specific mechanism has not been clarified. Glial cell-derived neurotrophic factor (GDNF) has been shown to protect neurons by binding to N-cadherin. Our previous results have shown that electroacupuncture could increase the expression of N-cadherin mRNA in facial neurons and promote facial nerve regeneration. In this study, the potential mechanisms by which electroacupuncture promotes nerve regeneration were elucidated through assessing the effects of electroacupuncture on GDNF and N-cadherin expression in facial motoneurons of rabbits with peripheral facial nerve crush injury. New Zealand rabbits were randomly divided into a normal group (normal control, n = 21), injury group (n = 45) and electroacupuncture group (n = 45). Model rabbits underwent facial nerve crush injury only. Rabbits in the electroacupuncture group received facial nerve injury, and then underwent electroacupuncture at *Yifeng* (TE17), *Jiache* (ST6), *Sibai* (ST2), *Dicang* (ST4), *Yangbai* (GB14), *Quanliao* (SI18), and *Hegu* (LI4; only acupuncture, no electrical stimulation). The results showed that in behavioral assessments, the total scores of blink reflex, vibrissae movement, and position of apex nasi, were markedly lower in the EA group than those in the injury group. Hematoxylin-eosin staining of the right buccinator muscle of each group showed that the cross-sectional area of buccinator was larger in the electroacupuncture group than in the injury group on days 1, 14 and 21 post-surgery. Toluidine blue staining of the right facial nerve tissue of each group revealed that on day 14 post-surgery, there was less axonal demyelination and fewer inflammatory cells in the electroacupuncture group compared with the injury group. Quantitative real time-polymerase chain reaction showed that compared with the injury group, N-cadherin mRNA levels on days 4, 7, 14 and 21 and GDNF mRNA levels on days 4, 7 and 14 were significantly higher in the electroacupuncture group. Western blot assay displayed that compared with the injury group, the expression of GDNF protein levels on days 7, 14 and 21 were significantly upregulated in the electroacupuncture group. The histology with hematoxylin-eosin staining and Nissl staining of brainstem tissues containing facial neurons in the middle and lower part of the pons exhibited that on day 7 post-surgery, there were significantly fewer apoptotic neurons in the electroacupuncture group than in the injury group. By day 21, there was no significantly difference in the number of neurons between the electroacupuncture and normal groups. Taken together, these results have confirmed that electroacupuncture promotes regeneration of peripheral facial nerve injury in rabbits, inhibits neuronal apoptosis, and reduces peripheral inflammatory response, resulting in the recovery of facial muscle function. This is achieved by up-regulating the expression of GDNF and N-cadherin in central facial neurons.

Key Words: *nerve regeneration; facial paralysis; electroacupuncture; glial cell-derived neurotrophic factor; N-cadherin; crush injury; neuronal apoptosis; facial neuron; nerve demyelination; neural regeneration*

Chinese Library Classification No. *R493; R364*

Introduction

Peripheral facial nerve paralysis can result in drooping brows, incomplete eyelid closure, dry eyes, hyperacusis, impaired taste and problems with mouth closure, which may lead to severe impairments to function and appearance (Wang et al., 2016; Ma et al., 2018). The annual incidence of facial nerve paralysis is estimated at 20–25 cases per 100,000 people worldwide, but reaches 258/100,000 people in China (Li et al., 2010; Hong et al., 2013). At present, cortical hormone treatment, anti-virus treatment, angiectasis therapy, vitamin treatment, massage, functional training and facial nerve decompression, facial nerve anastomosis and face-lift operations, are often used. However, there is no common measure of therapeutic effectiveness that is applicable to all the various treatments (Jin et al., 2016; Huang et al., 2017).

Acupuncture, an important part of traditional Chinese medicine, can markedly aid recovery from functional impairments after facial nerve injury with epineurium integrity in both clinical practice and animal experiments (Sun et al., 2011; Zhou et al., 2013). One of the most popular types of acupuncture, electroacupuncture (EA) has been shown to affect neuron proliferation and differentiation in animal models of intervertebral disc protrusion (Jiang et al., 2015), demyelinating diseases, hypoxic-ischemic encephalopathy and stroke (Hong et al., 2013; Xu et al., 2014; Liu et al., 2015). The mechanisms underlying the effects of EA for related acupoints in facial nerve regeneration have been widely studied, but remain unclear (Li et al., 2018).

The success of peripheral nerve regeneration is mainly determined by the survival of central neurons (Kim et al., 2016). Glial cell-derived neurotrophic factor (GDNF) is a distantly related member of the transforming growth factor-β superfamily, and belongs to the neurotrophin polypeptide family of proteins (Liu et al., 2012; Xiao et al., 2016). Previous research showed that GDNF was the most potent survival factor described for motoneurons *in vitro* (Höke et al., 2002) and more recent studies have confirmed its role in neuronal protection and axonal regeneration *in vivo* (Allen et al., 2013; Pascual et al., 2015). Not only does GDNF keep all axotomized facial motoneurons alive *in vivo*, but it is also the only factor known to prevent axotomy-induced motoneuron atrophy (Henderson et al., 1994).

GDNF exerts a protective role in neurons through its receptors: (1) the ligand binding receptor GDNF family receptor α1, and (2) the known transmembrane signal transduction receptors Ret, NCAM140, and integrin β1 (Zuo et al., 2013). N-cadherin, a calcium-dependent neuronal cell surface protein with a structure and function similar to those of NCAM and integrin β1, also mediates adhesion and signal transduction (Neugebauer et al., 1988). The extracellular portion of Ret that binds to the GDNF/GDNF family receptor α1 also has a cadherin-like domain (Oppenheim et al., 1995; Kjaer et al., 2003). Our previous study demonstrated that EA upregulates N-cadherin mRNA in neurons and promotes facial nerve regeneration (Li et al., 2015). Studies concerning the correlation between GDNF and N-cadherin are very limited, especially those focusing on the mechanism of EA. The present study aimed to assess EA effect on peripheral facial nerve crush injury and to explore the underlying mechanism of GDNF and N-cadherin involvement in facial motoneuron protection.

Materials and Methods

Animals

One hundred and eleven 6–8 months male and female New Zealand white rabbits weighing 3.00 ± 0.25 kg were obtained from the Experimental Animal Center of Southwest Medical University of China (license No. SYXK (Chuan) 2013-065). The rabbits were individually housed in a controlled environment at 23 ± 2°C and humidity of 50% in 12-hour light-dark cycle with free access to food and water. All experimental protocols and animal handling procedures were approved and conducted in accordance with the Guide for the Animal Care and Use Committee of Southwest Medical University (approval No. 20170120001), and were consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Following acclimatization, all rabbits were randomly assigned to the following three groups: a normal group (normal control; $n = 21$) that received no treatment; an injury group ($n = 45$) that underwent facial nerve crush injury; an EA group ($n = 45$) that received EA treatment after facial nerve crush injury. The injury and EA groups were divided into five subgroups according to 1, 4, 7, 14, and 21 days after surgery.

Model establishment

The buccal branches of the facial nerve of rabbits were subjected to crush injury to set the pathological model in accordance with a previously described method (Zhang et al., 2012). In brief, rabbits were intraperitoneally anesthetized with 10% chloral hydrate (Shan Pu Chemical, Shanghai, China) (3 mL/kg), followed by shaving of the face. After an appropriate curvilinear infra-auricular incision of the skin had been made, the fascia was dissociated to expose the facial nerve, located on the line between the mouth and the pretragal, and approximately 2 cm below the pupil (**Figure 1A**). Using hemostatic forceps (Jin Zhong Hemostat, Shanghai, China), 2 cm length of facial nerve was crushed for 5 minutes using a 5 kg force. The hemostat was locked in three notched teeth as described elsewhere (Zhang et al., 2012). Finally, the incision was sutured with non-absorbable surgical suture materials. Clindamycin was injected intramuscularly daily for 5 days to prevent infection. Crush injury was performed on the right side of the face. The surgical procedures were performed by the same surgeon to maintain consistency. We performed animal behavioral assessment 2 hours after surgery. When a total score was 5 points, the modeling was considered successful.

EA intervention

After routine disinfection of the acupuncture sites with 75% alcohol, perpendicular or oblique needling at an angle of 45 degrees was done with stainless steel disposable acupuncture needles (0.25 mm in diameter, 25 mm in length; Xinxinglin brand, Tianyuheng Technology Company, Beijing, China)

that were inserted at a depth of approximately 15–20 mm at each point in each rabbit. Mechanical stimulation was performed at *Yifeng* (TE17: posterior to the ear lobe, between the mastoid process and mandible angle), *Jiache* (ST6: one finger-breadth anterosuperior to the mandibular angle where the masseter is easy to see when the teeth are clenched), *Sibai* (ST2: directly below the pupil, and in the depression of the infraorbital foramen), *Dicang* (ST4: directly below the pupil, and 0.4 cun (1.3 cm) lateral to the corner of the mouth), *Yangbai* (GB14: directly above the pupil, 1 cm above the midpoint of the eyebrow), *Quanliao* (SI18: at the bottom edge of the zygomatic bone), and *Hegu* (LI4: between the 1st and 2nd metacarpal bones, and in the midpoint of the radial side of the 2nd metacarpal bone. L14 received acupuncture only, without electrical stimulation) at the injury side as described previously (Ya et al., 1999, 2000a, b, 2001) (**Figure 1B**).

After hand manipulation to achieve the desired sensation, the positive and negative electrodes of three groups of output wires from the electrostimulator (G6805-1A; Xin Sheng Industrial Co., Ltd., Qingdao, China) were connected to the needle handles at the above-mentioned acupuncture points, pairing as follows: ST4-ST6, TE17-SI18, and ST2-GB14. A sparse-dense wave with a frequency of approximately 18–20 Hz and a voltage intensity of 1.5–2.0 V was selected; the magnitude of the stimulation was adjusted to the extent that the needle could slightly vibrate and keep the rabbits quiet. The EA treatment was first performed for 30 minutes, 2 hours post-surgery and repeated once daily.

Tissue sample preparation

At each time point, three rabbits in each group were used for each of histological analysis, quantitative real time-polymerase chain reaction (qRT-PCR), and western blot assay (For western blot assay, three normal rabbits were used to compare with the injury and EA groups at each time point). For qRT-PCR and western blot assay, the rabbits were sacrificed with intravenous injection of air. The brainstem containing facial motoneurons was extracted and placed on ice, washed with PBS, then rapidly dissected into coronal blocks of an approximate thickness of 3 mm, and stored in a freezer at −80°C (Hu et al., 2017). For histological analysis, the rabbits were perfused with saline solution and 4% paraformaldehyde; the extracted brainstem was immersed in 10% formalin, conventionally dehydrated through a graded series of alcohol (75%, 85%, 95%, and 100%), and paraffin embedded (Gao et al., 2016).

Animal behavior assessment

The facial muscle function was evaluated using the animal behaviors scores for blink reflex, vibrissae movement and the position of apex nasi at every time point. An 18-gauge needle with a 5 mL syringe was used to blow air onto the eye twice to evoke the blink reflex. The degree of blink reflex was graded on a 0 to 2 scale (0, no difference between the two sides; 1, the blink reflex was delayed compared with the unaffected side; 2, the blink reflex disappeared completely). Vibrissae movement was observed for 30 seconds and scored on a 0 to 2 scale (0, no difference between both sides; 1, the vibrissae movement was weaker than that on the healthy side; 2, the vibrissae movement disappeared completely). The position of the apex nasi was scored on a 0 to 1 scale (0, the position was in the middle; 1: the position was towards the unaffected side). The total score was defined as the sum of all scores. The higher the score was, the more obvious the facial paralysis symptoms were. There were no facial nerve paralysis symptoms in the normal group when the total score was 0. When a total score was more than 3 points, we believed that facial paralysis symptoms existed (Takahashi et al., 2001).

Histological observation

For hematoxylin-eosin staining, a series of 5 μm-thick sections were obtained from paraffin embedded specimens. The specimens were deparaffinized, stained with hematoxylin-eosin reagents (Solarbio, Shanghai, China), cleared with xylene, and mounted with neutral resin. For toluidine blue staining, tissue sections were deparaffinized, soaked in a toluidine blue dye (Solarbio) at room temperature for 20 minutes, and rinsed in 95% ethanol solution for 10 seconds. Finally, the slides were washed with distilled water, directly dehydrated with 100% alcohol, cleared, and mounted. For Nissl staining, the sections were deparaffinized, treated with Nissl dye (Solarbio) in oven at 56°C for 1.5 hours, washed, differentiated in 95% alcohol, dehydrated, cleared, and mounted. The morphological changes were observed and photographed under the microscope (Olympus, Tokyo, Japan).

Facial motoneurons in the brainstem were identified after hematoxylin-eosin staining. After Nissl staining, five randomly sections from each rabbit at each time point were selected, and the facial neurons that containing Nissl bodies were counted. Then the ratios of facial neurons numbers in the EA group or the injury group to that in the normal group was used to evaluate neuronal loss and function (Ribeiro et al., 2015).

In hematoxylin-eosin staining of cross section of the buccinator, five sections from each rabbit at every time point were randomly selected, and five fields of view on each slide were randomly selected for observation with the microscope. The cross-sectional areas of buccinator were detected with the ImageJ software (ImageJ program 1.42; National Institutes of Health, Bethesda, MD, USA). The ratio of the EA group, or the injury group, to the normal group was used to observe buccinator atrophy and inflammatory responses.

Toluidine blue staining of sections of facial nerves was performed to assess remyelination and inflammatory reactions.

qRT-PCR

To identify whether GDNF (NCBI Accession Number: XM_008262127.2) and N-cadherin (NCBI Accession Number: XM_017344097.1) were involved in the neuroprotective effects of EA, their mRNA levels were evaluated by qRT-PCR. All rabbits were sacrificed after their last behavior assessment. Facial neurons in the brainstem tissues were collected. Total RNA was extracted from tissues specimens

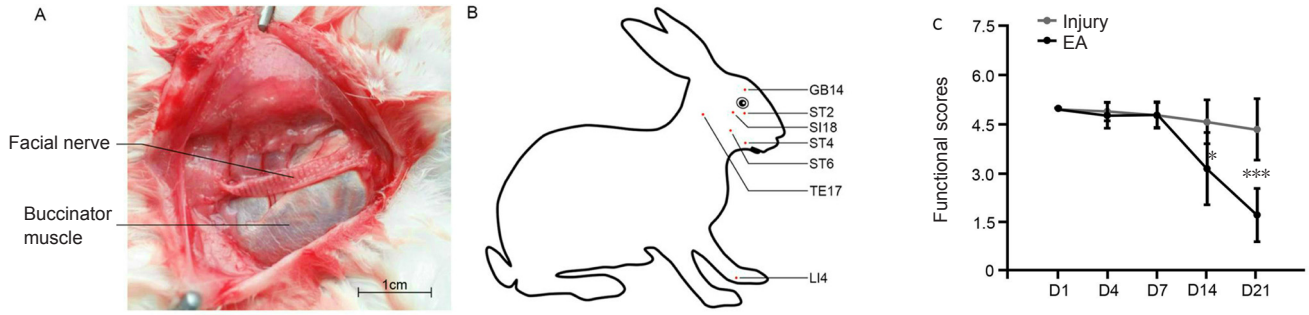

Figure 1 Animal model establishment, EA intervention and behavior assessment.
(A) The facial nerve injury model was established by clamping 2 cm of the right facial nerve for 5 minutes using a 5 kg force. (B) EA was applied to *Yifeng* (TE17), *Jiache* (ST6), *Sibai* (ST2), *Dicang* (ST4), *Yangbai* (GB14), *Quanliao* (SI18), and *Hegu* (LI4) on the affected side for 30 minutes, respectively, once daily. (C) Scores in evaluation of facial muscle function in rabbits during treatment. The total number at each time point is the sum of blink reflex, vibrissae movement, and position of apex nasi. The higher the score is, the more severe the facial paralysis symptoms are. Facial paralysis is diagnosed when a total score is greater than 3 points. The total scores of the EA group were significantly lower than those in the injury group from 14 days post-surgery. There was no significant recovery in the symptoms of rabbits in the injury group during treatment. Data are presented as the mean ± SD (n = 3; two-way analysis of variance followed by least significant difference *post hoc* test). $*P < 0.05$, $***P < 0.001$, *vs.* injury group (underwent facial nerve crush injury). Scale bar: 1 cm. D: Day; EA: electroacupuncture.

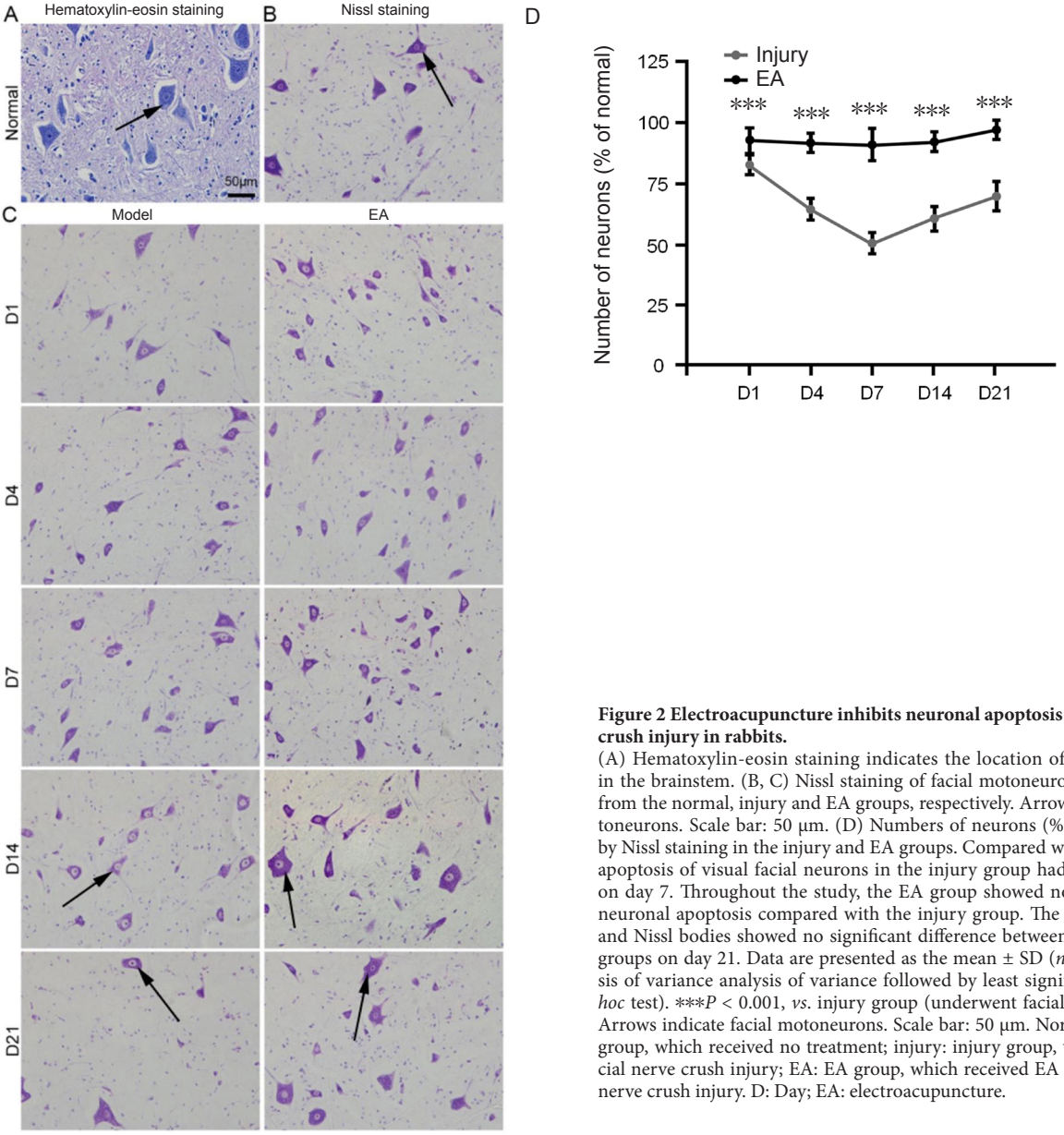

Figure 2 Electroacupuncture inhibits neuronal apoptosis after facial nerve crush injury in rabbits.
(A) Hematoxylin-eosin staining indicates the location of facial motoneurons in the brainstem. (B, C) Nissl staining of facial motoneurons in the brainstems from the normal, injury and EA groups, respectively. Arrows indicate facial motoneurons. Scale bar: 50 μm. (D) Numbers of neurons (% of normal) detected by Nissl staining in the injury and EA groups. Compared with the EA group, the apoptosis of visual facial neurons in the injury group had markedly increased on day 7. Throughout the study, the EA group showed no significant signs of neuronal apoptosis compared with the injury group. The numbers of neurons and Nissl bodies showed no significant difference between the EA and normal groups on day 21. Data are presented as the mean ± SD (n = 3; two-way analysis of variance analysis of variance followed by least significant difference *post hoc* test). $***P < 0.001$, *vs.* injury group (underwent facial nerve crush injury). Arrows indicate facial motoneurons. Scale bar: 50 μm. Normal: Normal control group, which received no treatment; injury: injury group, which underwent facial nerve crush injury; EA: EA group, which received EA treatment after facial nerve crush injury. D: Day; EA: electroacupuncture.

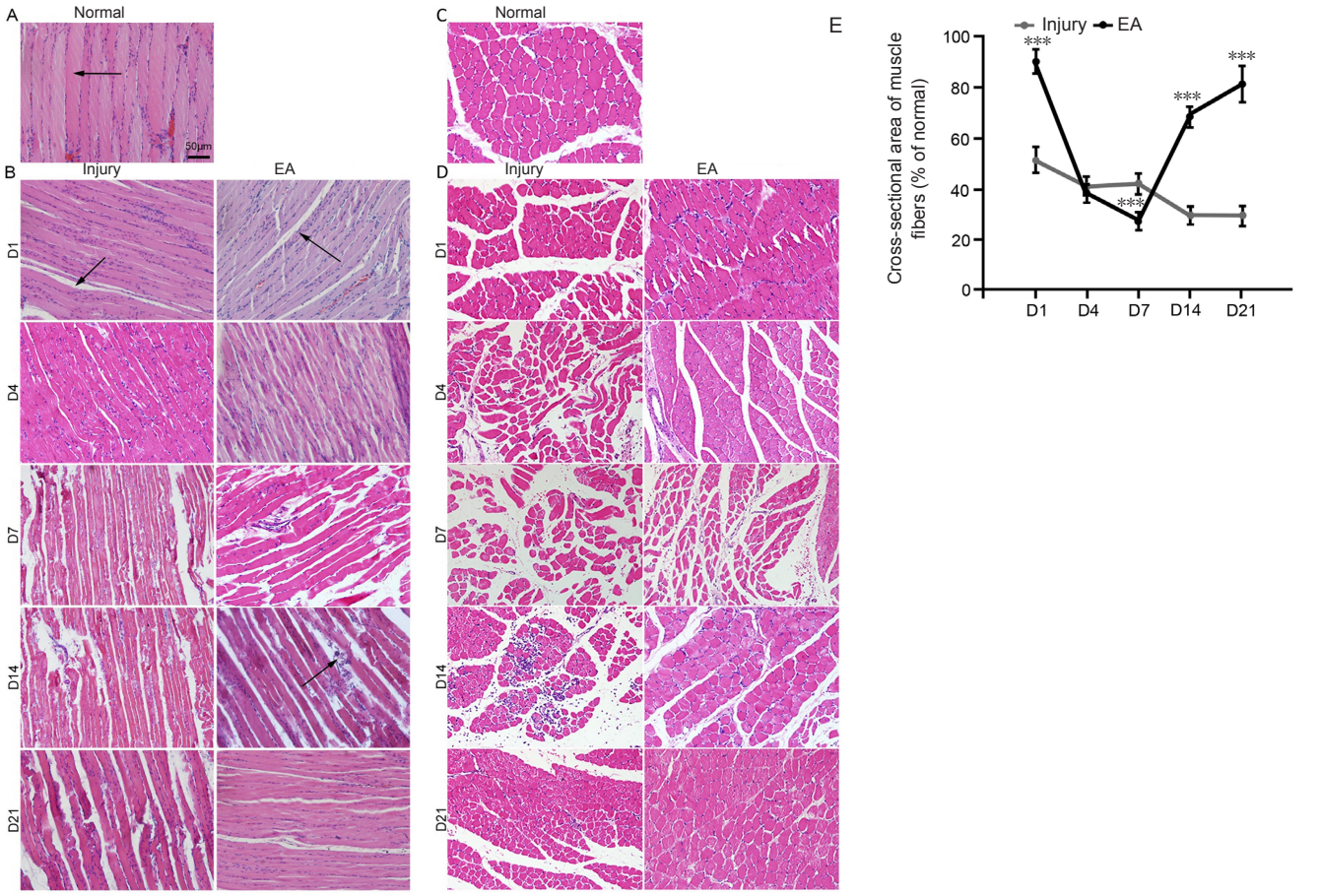

Figure 3 EA relieves buccinator and muscle atrophy.
(A, B) Hematoxylin-eosin staining of longitudinal sections of buccinator muscle on the right side in each group. After facial nerve crush injury, gradual degeneration was observed with thin diameter muscle fibers. Inflammatory cells clustered and small vessels increased in the muscle gap. Treatment with EA markedly improved buccinator morphology and architecture, with reduced inflammatory cell infiltration, and the nucleus location shifted significantly from the edge to the center. (C, D) Hematoxylin-eosin staining of cross-sections of the buccinator on the right side in the normal, injury and EA groups. (E) Analysis of cross-sectional areas of the buccinator in the normal, injury and EA groups detected by hematoxylin-eosin staining on days 1, 4, 7, 14, 21 post-surgery. There was obvious muscle atrophy in the injury and EA groups compared with the normal group after facial nerve crush. However, EA treatment delayed the buccinator atrophy on day 1 and hastened its recovery on days 14, and 21 compared with the injury group. Data are presented as the mean ± SD (n = 3; two-way analysis of variance analysis of variance followed by least significant difference *post hoc* test). ***P < 0.001, *vs.* injury group (underwent facial nerve crush injury). Arrows indicate muscle fibers (A), myocutaneous space (B-injury-D1), muscle nucleus (B-EA-D1) and inflammatory cells (B-EA-D14). Scale bar: 50 μm. Normal: Normal control group, which received no treatment; injury: injury group, which underwent facial nerve crush injury; EA: EA group, which received EA treatment after facial nerve crush injury. D: Day; EA: electroacupuncture.

with TRIzol (Ambion Inc., Carlsbad, CA, USA) following the manufacturer's instructions. Total RNA concentration and quality were determined on a Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA); cDNA was reverse transcribed with a reverse transcription kit (TAKARA, Japan). All primers used are shown in **Table 1**. Each reaction consisted of 10 μL SYBR Green Mixture, 1 μL cDNA, 0.5 μL (10 μmol/μL) of each primer and 8 μL PCR-grade water, to yield a final volume of 20 μL. PCR was performed on Real-time 7500 PCR detection system (Bio-Rad, USA) under the following conditions: 95°C, 3 minutes; 95°C, 5 seconds; 56°C, 10 seconds; 72°C, 25 seconds, 39 cycles; 65°C, 5 seconds; 95°C, 50 seconds. β-Actin was used as a reference gene. Relative mRNA levels were determined by the $2^{-\Delta\Delta Ct}$ method (Livak et al., 2001).

Table 1 Quantitative real time polymerase chain reaction primers

Gene	Sequence (5′–3′)	Product size (bp)
GDNF	Forward: CTG ACT TGG GTT TGG GCT AC	124
	Reverse: AGC CTC CCT GGT CCC TCC CC	
N-cadherin	Forward: CGG CCT TAA CGG AGG ATT CA	151
	Reverse: GGT CCT CGG GAG TTT TCT GG	
β-Actin	Forward: CCA CTC CTG CCA AGG AGA AG	106
	Reverse: CAC CAC CAT GTA CCC CGG CA	

GDNF: Glial cell-derived neurotrophic factor.

Western blot assay

To assess EA effects on the GDNF and N-cadherin protein expression levels, western blot assay was performed. All rabbits were sacrificed after their last behavior assessment. The brainstem tissues containing facial neurons were collected. Total protein was extracted from tissue samples with a Protein Extraction kit (Bioswamp, Wuhan, China) according to the manufacturer's instructions. Protein concentration was determined with a bicinchoninic acid Protein Assay Kit

(Bioswamp). Proteins (20 μg per well) were separated by 12% sodium dodecyl sulfate-polyacrylamide electrophoresis under a constant voltage of 80 V for 30 minutes followed by a constant voltage of 120 V until the bromophenol blue reached the bottom of the separation gel. Proteins were then electrophoretically transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA) at 90 V for 50 minutes. The membranes were blocked with 5% skim milk at room temperature for 2 hours. The primary antibodies, rabbit anti-GDNF (Bioss, Beijing, China) (diluted 1:200) and mouse anti-N-cadherin (Abcam, MA, USA) (diluted 1:1000), were added for overnight incubation at 4°C. After three subsequent washes with phosphate-buffered saline with 0.1% Tween 20 (5 min/wash), the membranes were incubated with goat anti-rabbit IgG (Bioswamp) (diluted 1:1000) and goat anti-mouse IgG (Bioswamp) (diluted 1:1000) antibodies conjugated to horseradish peroxidase at room temperature for 1 hour and washed three times with phosphate-buffered saline in 0.1% Tween 20 (10 minutes/wash) with gentle shaking. Finally, the membranes were detected by chemiluminescence with BeyoECL Star Kit (Beyotime, Shanghai, China). Band densities were quantified with the ImageJ software. The results were expressed as the relative expression of grayscale values of the interested protein to β-actin amounts in the same sample.

Statistical analysis

Data, expressed as the mean ± SD, were analyzed with SPSS 22.0 software (IBM, Armonk, NY, USA). Results were analyzed by two-way analysis of variance. For multiple group comparison at the same time point, the least significant difference *post hoc* test was performed. A value of $P < 0.05$ was considered statistically significant.

Results

EA promotes the recovery of facial muscle function

Rabbits in the normal group showed no symptoms of facial nerve paralysis, and the score for each assessment was 0. No differences were observed between the EA and injury groups before day 14 post-surgery. Subsequently, the total scores of the injury and EA groups decreased, but the scores in the EA group decreased more rapidly. However, up until 14 days post-surgery, the total scores in the injury and EA groups were greater than 3. The total scores for the blink reflex, vibrissae movement, and the position of apex nasi in the EA group were significantly lower than those in the injury group ($P < 0.05$ on day 14, $P < 0.001$ on day 21). The above symptoms exhibited by the rabbits in the injury only group showed no sign of recovery over the 21 days. The differences of facial nerve paralysis symptoms in all groups are shown in **Figure 1C**.

EA inhibits neuronal apoptosis after peripheral facial nerve crush injury

As shown in **Figure 2A**, polygonal facial motoneurons were clustered in the ventral part of the pons and near the abdominal midline. The deep blue tigroid structures in neuron cytoplasm represented Nissl bodies, which are characteristics of neuron survival. Nissl bodies in facial neurons from the normal group were many and discrete and were evenly distributed in the cytoplasm (**Figure 2B**). The size of Nissl bodies decreased after the crush injury that caused pyknosis and loss of neurons in the injury group. The apoptosis of visual facial neurons was significantly increased on day 7 in the injury group compared with the EA group ($P < 0.001$), as indicated by incomplete membrane and extracellular leakage. In the EA group, Nissl bodies were fuzzy, reduced or absent, and pyknotic neurons were obvious on day 4. After 14 days of EA treatment, Nissl bodies had recovered to their preoperative state. Throughout the study, the EA group showed no significant signs of neuronal apoptosis compared with the injury group ($P < 0.001$) (**Figure 2C** and **D**). The numbers of neurons and Nissl bodies showed no significant difference between the EA and normal groups on day 21.

EA alleviates the buccinator inflammation and atrophy

The morphology and inflammation of the buccinator muscle were investigated as it is innervated by the facial nerve. Hematoxylin-eosin staining showed normal architecture of the buccinator in the normal group (**Figure 3A**). In the injury group, degeneration and inflammation were observed. Muscle fibers were weakly stained, with fuzzy outlines and had thinner diameters. There were clusters of inflammatory cells and small vessels increased in the muscle gap (**Figure 3B**). Subsequently, muscle fibers recovered slowly, and the location of their nuclei moved from the edge to the center. There was no sign of recovery in the injury group. In contrast, treatment with EA markedly improved the buccinator morphology and architecture, with reduced inflammatory cell infiltration (**Figure 3B**). Statistical analysis of the cross-sectional area of the buccinator in the three groups indicated that there was obvious muscle atrophy in the injury and EA groups compared with the normal group after facial nerve crush (**Figure 3C–E**). However, EA treatment significantly alleviated the buccinator atrophy on days 1, 14, and 21 compared with the injury group ($P < 0.001$).

EA promotes myelination of the injured peripheral facial nerve

Toluidine blue staining of the normal facial nerve shows uncolored axons and uniformly blue stained myelin. As shown in **Figure 4**, the postoperative structure of axons was severely damaged, and dark blue stained inflammatory cells had also infiltrated the nerve bundles. In addition, myelin was extensively disrupted in the injury group compared with the normal group. Signs of demyelination and inflammation were still evident on day 21 in the injury group. However, on days 14 and 21 more myelinated axons and fewer inflammatory cells were found in the EA group.

EA upregulates GDNF and N-cadherin mRNA in neurons

GDNF and N-cadherin mRNA expressions in brainstem samples, containing the facial motoneurons, were observed using qRT-PCR. N-cadherin mRNA levels in the EA group were significantly higher on days 4, 7, 14, and 21 compared with the injury group ($P < 0.001$). Similarly, GDNF mRNA

levels in the EA group were significantly higher on days 4, 7, and 14 ($P < 0.001$; **Figure 5A**, **B**).

EA treatment upregulates GDNF and N-cadherin protein levels

The protein expressions of GDNF and N-cadherin in brainstem samples were investigated using western blot assay. GDNF and N-cadherin protein levels had remarkably decreased in the EA and injury groups compared with the normal group on days 1 and 4. Notably, EA treatment induced a slow increase of N-cadherin expression from day 4. There was continued increase in the expression of N-cadherin on days 4, 7, and 14 post-injury in the EA group, greater than in the injury group. Similarly, a significant decrease of GDNF protein levels in the EA and injury groups was observed compared with the normal group on days 1 and 4. GDNF protein levels in the EA group were significantly upregulated on days 7, 14, and 21 compared with the injury group ($P < 0.001$; **Figure 5D–E**).

Discussion

Repair, regeneration, and neuroprotection are the main challenges in rescuing neurological function from injury (Ribeiro et al., 2015; Szepanowski and Kieseier, 2016; Tallon and Farah, 2017). Potential for regeneration following nerve fiber injury is reflected by the *de novo* expression or upregulation of a wide variety of molecules in the distal nerve fiber tracts (Gordon et al., 2017). Neuronal response is also associated with the expression of neurotrophic factors, transcription factors, growth-stimulating substances, cell adhesion molecules (*e.g.*, L1, NCAM and N-cadherin), extracellular matrix components, intracellular signaling enzymes, and proteins regulating cell-surface cytoskeletal interactions (Raivich et al., 2007). This study aimed to explore the mechanism of EA treatment in nerve regeneration in a rabbit model of facial nerve crush.

To our knowledge, this is the first time that EA effects on the expression of GDNF and N-cadherin during functional facial nerve regeneration in a rabbit model. Histological evidence of regenerative facial nerve provides a structural basis for functional recovery. In this study, toluidine blue staining revealed more axons and reduced inflammatory cells in the EA group compared with the injury group, which may be helpful in promoting the recovery of retrograde transport function. After facial nerve injury in rabbits, denervation of the targeted facial muscles will induce facial paralysis with ptosis, decreased whisker movement, leakage of drinking water, and buccinator atrophy (Tang et al., 2014). When re-innervation is complete, atrophy of the targeted muscle is discontinued, followed by a gradual functional recovery (Bodine et al., 2001), as shown in the EA group. Hematoxylin-eosin staining indicated that recovery of the buccinator was improved after EA treatment compared with the injury group. The blink reflex score, vibrissae movement score, position of apex nasi score, and total score were clearly lower in the EA group than in the injury group after day 7, indicating that facial nerve function recovered more quickly in the EA group. Similar results (Liu et al., 2017) have been described following facial nerve crush in rabbits and, as in the present study, showed the appearance of recovery of the peripheral facial nerve and its targeted facial muscle after facial nerve crush.

Denervation of a targeted muscle occurs as a consequence of motor nerve injury, followed by alterations in a series of molecular events (Gan et al., 2016). Nissl bodies, also known as Nissl substance, shoals and granules, or the tigroid substance, in neuronal cytoplasm, are used as a sign of cellular and molecular changes in injured neurons or retrograde reaction following axonal injury (Raivich et al., 2007). In this study, the EA group showed earlier recovery than the injury group morphologically and the neuronal apoptosis was not obvious. According to their Nissl function, EA treatment may increase cellular metabolism and the synthesis and supply of protein in facial motoneurons after facial nerve crush.

N-cadherin is a transmembrane cell adhesive molecule to which GDNF binds (Cao et al., 2010; Patil et al., 2011) to exert its neuroprotective effect in dopaminergic neurons (Zuo et al., 2013). GDNF binding to N-cadherin activates the intracellular PI3K/Akt signaling pathway to protect neurons (Wang et al., 2014). It had been demonstrated that GDNF mRNA in adult rat brain is located mainly in neurons (Pochon et al., 1997), and its synthesis is not restricted to dopaminergic areas. Suzuki et al. (1998) found that GDNF mRNA is mainly expressed in the cytoplasm membrane, especially in the neuromuscular junctions and less in the axons and Schwann cells. Synthesis of the GDNF protein in the brainstem is rarely reported. Retrograde transport of GDNF from the muscle may be the main way to promote the survival of the facial motoneurons.

The acupoints applied in our study are located on facial nerve branches (Xi et al., 2005). Earlier reports (Watson et al., 1982; Hinrichsen et al., 1984; Satoda et al., 1988; Populin et al., 1995) showed that horseradish peroxidase applied to the superior labial branch of the facial nerve labeled neurons within the dorsal and lateral divisions of the facial nucleus. Therefore, retrograde transport of nutrients, such as GDNF and N-cadherin, is blocked after peripheral nerve injury as Waller degeneration and axon discontinuity occurred at the proximal tip of the lesioned axons. Facial motoneurons would therefore lose their supply of the targeted-derived neurotrophic factor GDNF. In the present study, GDNF protein levels in the EA group decreased on day 1 and 4, but increased on day 7, 14, and 21 to above normal. The levels in the injured group remained below normal. However, in the EA group, GDNF mRNA increased on day 4, 7 and 14 and no difference was found on day 1 and 21 compared with the injury group. In contrast, N-cadherin protein levels fell until day 4 then increased but had not reached normal levels by day 21, although N-cadherin mRNA levels increased except on day 1. This discrepancy in mRNA and protein may be due to the following factors: first, mRNA indicates transcript changes; protein translation can be affected by multiple factors, such as mRNA level, processing of polypeptide chain in protein translation and post-translation, siRNA and miR-

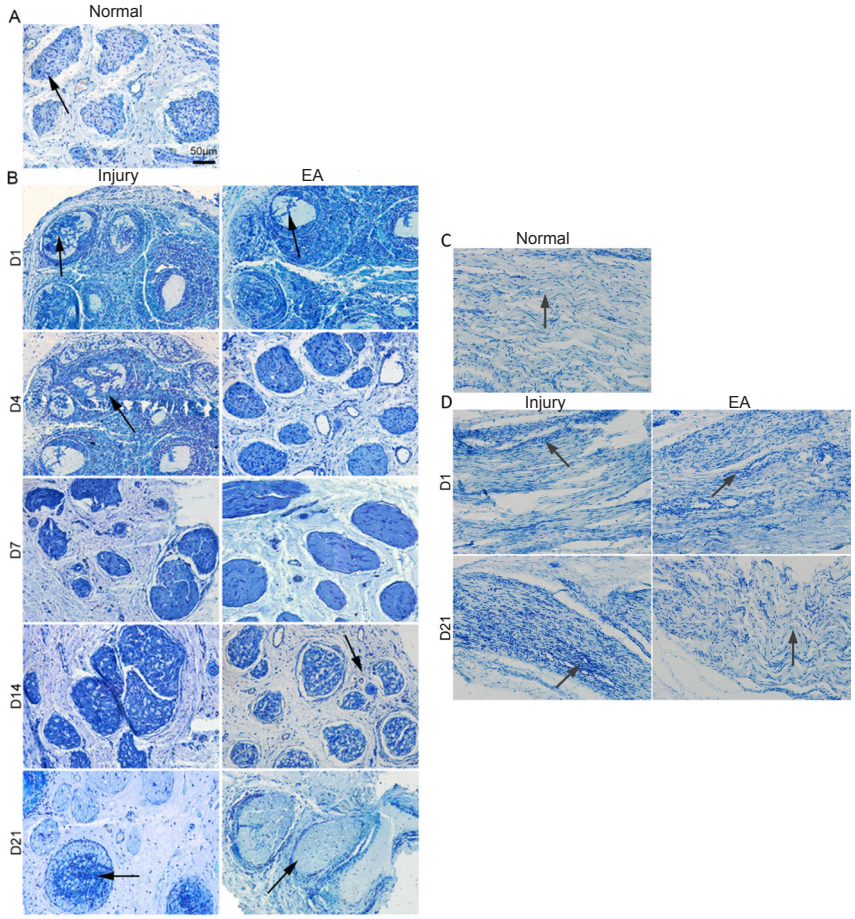

Figure 4 EA promotes myelination after facial nerve injury.
(A, B) Toluidine blue staining of cross sections of facial nerves in the normal, injury and EA groups, respectively. (C, D) Toluidine blue staining of longitudinal sections of facial nerves in the normal, injury and EA groups. The axonal structure was severely damaged postoperatively, and dark blue stained inflammatory cells also infiltrated the nerve bundles. Myelin was extensively disrupted in the injury group compared with the normal group. Signs of demyelination and inflammation were still evident on day 21 in the injury group. In the EA group, several axons were myelinated and only a few inflammatory cells were found on day 14. Arrows indicate normal axon (A, B-EA-D21), degenerated myelin (B-injury-D4), inflammatory cells (B-D1, B-injury-D21), regenerative myelin (B-EA-D14), normal nerve fiber (C, D-EA-D21), and inflammatory cells (D-D1, D21). Scale bar: 50 μm. Normal: Normal control group, which received no treatment; injury: injury group, which underwent facial nerve crush injury; EA: EA group, which received EA treatment after facial nerve crush injury. D: Day; EA: electroacupuncture.

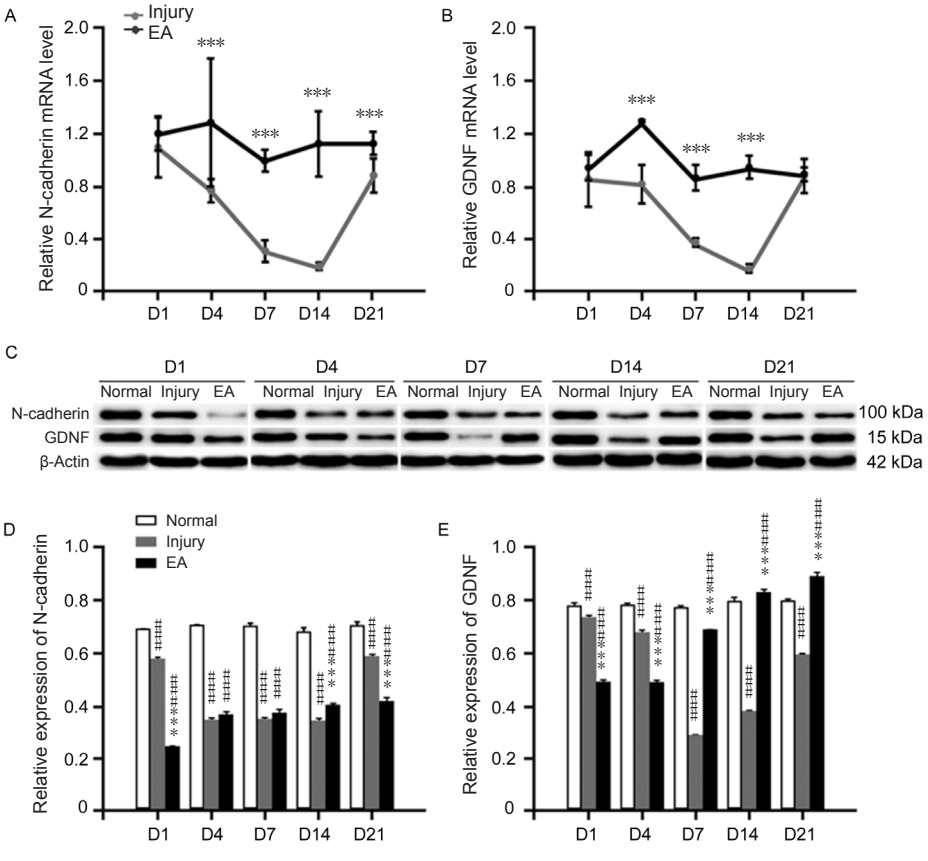

Figure 5 qRT-PCR and western blot assay of N-cadherin and GDNF.
(A, B) mRNA levels of N-cadherin and GDNF of the brainstem containing facial motoneurons in the injury and EA groups relative to the normal group detected by qRT-PCR on days 1, 4, 7, 14, and 21 post-surgery. N-cadherin mRNA levels in the EA group were significantly higher on days 4, 7, 14, and 21 compared with both injury and normal groups. Similarly, GDNF mRNA levels in the EA group were significantly higher on days 4, 7, and 14. (C) Protein expressions of N-cadherin and GDNF of the brainstem containing facial motoneurons were detected by western blot assay using β-actin as an internal control. (D, E) Statistical results showed protein expressions of N-cadherin and GDNF. The expression of N-cadherin in the EA group showed an increased trend from day 4, and EA significantly increased the expression of N-cadherin on day 14 post-injury compared with the injury group. GDNF protein levels in the EA group were significantly upregulated on day 7, 14, and 21 compared with the injury group. Data are presented as the mean ± SD (n = 3; two-way analysis of variance followed by least significant difference *post hoc* tests). ***P < 0.001, *vs.* injury group; ###P < 0.001, *vs.* normal group. Normal: Normal control group, which received no treatment; injury: injury group, which underwent facial nerve crush injury; EA: EA group, which received EA treatment after facial nerve crush injury. D: Day; EA: electroacupuncture; GDNF: Glial cell-derived neurotrophic factor; qRT-PCR: quantitative real time-polymerase chain reaction.

NA; Second, PCR reflected mRNA levels only from neurons and glial cells in brainstem. In contrast, western blot assay reflected protein levels from neurons and glial cells, as well as GDNF retrograde transport from muscle. In addition, the sensitivity of PCR is generally higher than that of western blot assay. Most importantly, our results showed the significant increase of GDNF and N-cadherin proteins in the brainstems from the EA group from day 4 to day 21. These might suggest strong neuronal intrinsic growth progress in the EA group, which probably promotes the survival of facial motoneurons and then leads to a better microenvironment for peripheral nerve regeneration. EA treatment induced regeneration of lost enteric neurons in diabetic rats probably by increasing GDNF expression (Du et al., 2015).

In addition, western blot assay showed that the intensity of the GDNF increase in the EA group was higher than that of the N-cadherin increase in the EA group, which might indicate that EA has more effective on inducing GDNF expression than that of N-cadherin. On one hand, N-cadherin may promote the binding of GDNF to other receptors. On the other hand, it is possible that GDNF could prevent intracellular calcium overload through a combination of calcium dependent adhesion molecules with a prolonged action time in neurons. Thus maintaining the cell activity and avoiding injury. We also found that the relative expression levels of GDNF were always higher than those of N-cadherin in the normal group. Considering the connection between neurons and other cells within the nervous system, GDNF may have other off-target effects, such as paracrine and autocrine activities (Wang et al., 2014). New Zealand rabbits have some self-healing ability to recovery from external injuries on the premise of nerve integrity. We have shown that the injury group showed the start of a recovery trend, morphologically, on day 21 post-operation but not functionally, within the observation period.

In our model of facial nerve crush injury, EA treatment could relieve the inflammatory reactions and peripheral nerve retrograde degeneration. Indeed, EA promoted neuronal survival, to ensure successful peripheral facial nerve regeneration. We deduced the possible mechanism underlying the protective effects observed may be that EA upregulated the expression of GDNF and N-cadherin in neurons, and the enhanced signal transduction between the two molecules. However, further investigation is required for clarification.

EA promotes functional and histological recovery in the rabbit model of facial nerve crush defect, indicating that EA could be used to meet the needs of clinical application. EA upregulates GDNF and N-cadherin in facial motoneurons, which may be the mechanism underlying its effects. However, the specific signaling pathway in which EA plays a role remains to be elucidated.

Limitations

This study has some limitations. Electrophysiological tests, such as electromyography, can be used to objectively determine the function of the whole motor neuron; that is, the peripheral nerve, the neuromuscular junction and the muscle itself. Future studies, such as the relationship between EA and GDNF and N-cadherin in facial nerve paralysis *in vitro* and the signal pathway could directly elucidate the mechanism that EA promotes facial nerve regeneration.

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