

Original Article

Low-level laser therapy (LLLT) promotes facial nerve regeneration after crush-injury in rats

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Abstract: The aim of this study was to evaluate whether the effects of low level laser therapy (LLLT) can enhance nerve regeneration on crush facial nerve. A 4-mm crush injury in a buccal branch of the facial nerve was made as the control group; low level laser treatment (wavelength, 980 nm; optical power output of potency, 70 mW; energy density, 16.71 J/cm²) on the crush injury after operation for 5 weeks was performed in the experiment group. A sham group of uninjured animals represented normal controls. Vibrissa movement, morphological analyses of regenerated nerves (immunohistochemistry, light microscopy and transmission electron microscopy), and real-time PCR for quantifying the mRNA expression levels of nerve growth factor (NGF), low-affinity NGF receptor (p75NTR) and its tyrosine receptor kinase A (trkA) were performed to assess nerve regeneration. NGF, p75NTR, and trkA mRNA expression was significantly increased in laser + crush group. The recovery of Vibrissa movement in the laser + crush group was markedly higher than that of the crush group, and similar to sham group values. Furthermore, larger regenerated axons and thicker myelin sheaths were observed in laser + crush treated animals compared with the crush group. LLLT promoted effective regeneration of crush-injured facial nerve.

Keywords: Crush injury, facial nerve regeneration, low-level laser therapy

Introduction

Peripheral facial injury is a common clinical condition, which can result in functional and psychological disorders to the patient. Due to the complex anatomical structure of the extra-temporal facial nerve, facial paralysis can be caused by the injury of some or all branches of the facial nerve [1-7]. Complications of long-standing facial nerve injury include flaccid paralysis, incomplete recovery of facial function, and aberrant motor axon regeneration causing involuntary muscle contraction, also termed synkinesis. In addition to esthetic and functional issues, facial paralysis can impair communication, hinder expression of emotion, and cause disabling psychological complications [1, 8, 9].

Low-level laser therapy (LLLT) is an effective tool for the treatment of peripheral nerve regeneration. LLLT was introduced in the 1980s, and has recently attracted considerable attention, for showing immediate protective effects, bac-

teria-inhibiting, anti-inflammation, reducing scar tissue formation on the wound, and significantly increasing axonal outgrowth and myelination [10-12]. Moreover, LLLT can reduce mononuclear cell migration, leading to decreased edema area, analgesia, and anti-inflammatory reactions, promoting a more rapid regeneration [8, 13]. Laser treatment has bio-stimulatory effects in peripheral nerve repair, and helps improve blood supply, cell proliferation, and collagen synthesis. This therapy provides analgesic, anti-inflammatory, and anti-edema effects [9]. Improved peripheral nerve function leading to significant functional recovery following LLLT was also reported by Rochkind et al. [14].

The aim of this study was to evaluate the effects of low-level laser therapy (LLLT) at 980-nm on the facial nerve following crush injury. Assessment of an experimental model with functional recovery could provide relevant data and a basis for future clinical applications in the treatment of nerve injuries.

Table 1. Primer sequences

Gene	Sequence (5'-3')	mRNA position (start)	mRNA position (end)	Product size (bp)
NGF	Forward: AAG ACC ACA GCC ACG GAC AT Reverse: CGC CTT GAC AAA GGT GTG AG	722	916	195
p75 ^{NTR}	Forward: TGG CGG ATA TGG TGA CCA CT Reverse: GCA GCT GTT CCA CCT CTT GA	770	921	152
TrkA	Forward: AGC CGT GGA ACA GCA TCA CT Reverse: CGC ATG GTC TCA TTG GTC AG	943	1095	153
GAPDH	Forward: GCA TCC TGC ACC AAC T Reverse: GCA GTG ATG GCA TGG ACT GT	802	902	101

the facial nerve, on the skin surface, at a wavelength of 980 nm, an optical power output of potency of 70 mW, an energy density of 16.71 J/cm², a beam area of 0.13 cm², a power density of 557.32 mW/cm², and an exposure time of 30 s (per point). The laser source had been previously tested to confirm the dose. Laser therapy applications were performed

Materials and methods

Animals and crush injury

Thirty-six male Sprague-Dawley rats (6 weeks, 200-250 g) were purchased from an animal supplier (KeYu Co., China) (SCXK (Jing) 2016-0002). The experiments were performed 1 week after adaptation in the animal facility.

Sprague-Dawley rats were housed in rooms with constant temperature (22°C) and humidity (50%). All animal experiments followed a protocol approved by the Committee for Animal Experimentation of the 309 Hospital of PLA, China (CNU IACUC-H-2013-16). Rats were anesthetized via intraperitoneal injection of Zolletil® (a 1:1 combination of tiletamine and zolazepam, Virbac, Carros, France) and xylazine hydrochloride. A post-auricular incision was made at the left side. The main trunk of the facial nerve was exposed, at its exits from the stylomastoid foramen before branching of the main trunk. The main trunk was identified by electrical stimulation for the entire hemifacial movement. Then, it was crushed with hemostatic forceps for 30 seconds from its origin, generating a facial nerve crush defect [14, 15].

Rats were equally and randomly divided into three groups: sham, crush (crush injury of facial nerve), and crush + laser (laser treatment after crush injury of facial nerve).

Low-level laser therapy (LLLT)

Low-level laser therapy with continuous pulse using a diode laser (Daheng New Epoch technology, China) was applied. The protocol was applied transcutaneously to three points along

med 1 day after surgery and in the postoperative period (three times weekly for a total of 5 weeks) [8, 9].

Vibrissa movement evaluation

Vibrissa movement was graded to assess the whisking behavior of the animals, based on a five different scores: 0, no whisker movement; 1, slight whisker movement; 2, slow movement; 3, rapid movement, but distinguishable from the contralateral normal side; 4, symmetric movement compared to the normal side. Vibrissa movement evaluation was performed at 4, 8, and 12 weeks after operation. To avoid subjective bias, the assay was performed in a blinded manner [16].

Quantification of NGF, p75^{NTR} and trkA mRNA expression by real-time PCR

Five weeks after surgery, 3 animals per group were anesthetized, and the facial nucleus (FNs) of left side was harvested. Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA), and reverse transcribed into cDNA using a first-strand synthesis kit (Invitrogen); cDNA amounts were quantified by real-time PCR. The following primers were used to amplify specific cDNA segments: NGF (GenBank Reference Sequence no: XM_227523.3), p75^{NTR} (GenBank Reference Sequence no. X05137.1), trkA (GenBank Reference Sequence no. M85214.1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (GenBank Reference Sequence no: NM_017008.3) (**Table 1**). GAPDH was used as an internal control for normalization. The relative mRNA levels were derived by the cycle threshold (Δ Ct) method as described previously

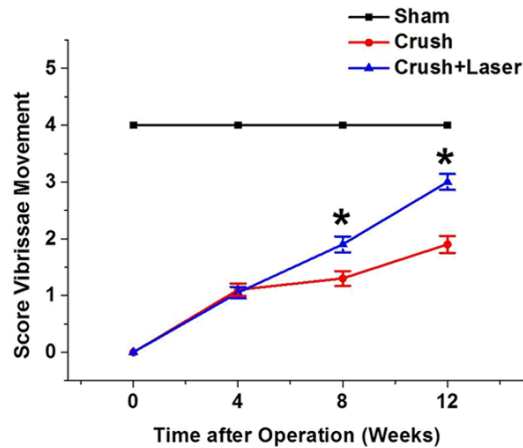


Figure 1. Vibrissa movement grade. The vibrissa movement was assessed at the 4th, 8th, and 12th weeks after surgery. The recovery rate of vibrissae movement in the crush + laser groups was faster than in the crush groups at 8th and 12th points ($P < 0.05$).

(Applied Biosystems Manual, Foster City, CA); PCR reactions were assessed at least twice [2].

Immunofluorescent histopathology

The left side buccal branch of the facial nerve was removed after twelve week of operation, fixed in 4% paraformaldehyde for 12 h, and placed in 30% sucrose phosphate buffer for 24 h until the specimens sunk to the bottom of the container. 20-30 μ m thick cross sections were cut by a freezing microtome. Mouse anti-S100 (1:500, ABCAM, USA) and anti-neurofilament 200 (1:80; Sigma, USA) primary antibodies were applied for overnight incubation at 4°C. Goat anti-rabbit IgG (FITC) and anti-mouse IgG (TRITC) secondary antibodies (Dako Japan) were applied for 1 h at room temperature. Images were acquired under a laser confocal microscope (FV10i-oil, push around, Tokyo, Japan).

Histomorphometric evaluation

At the end of the 12-week follow-up period, 6 rats per group were anesthetized. The facial nerve was exposed again, and the nerve segment, including the crush-injury site, was harvested. The samples were immediately immersed into a fixation solution containing 2.5% glutaraldehyde in PBS (pH 7.4) at 4°C for 24 hours. Only the distal portion (5 mm distal to the injury) was used for histomorphometric evaluation. The nerve segment was then post-

fixed with 2% osmium tetroxide for 2 h, washed with PBS (pH 7.4), and routinely processed and epoxy resin embedded. Serial transverse sections of 1 μ m thickness were cut with a microtome (Leica, Ultracut, UCT, Vienna, Austria) and stained with 1% toluidine blue for light microscopy examination (Olympus, BX41, TF, Japan). Images were acquired using the specialized system SPOT RT-KE color mosaic (Diagnostic Instruments, Inc., MI) and digitized with the SPOT software Ver. 4.6 (Diagnostic Instruments, Inc.). To simplify myelinated axon counting, the total cross-sectional area of the nerve was measured at 40 \times , and 3 high power fields were randomly selected at 200 \times as previously reported [2]. Mean fiber density was calculated by dividing the total number of myelinated nerve fibers within the sampling field by the corresponding area (N/mm^2). Total fiber number (N) was then estimated by multiplying mean fiber density by the total cross-sectional area of the whole nerve, assuming a uniform distribution of nerve fibers across the entire section.

For assessing myelin thickness and G-ratio, ultrathin sections were obtained using the same ultra-microtome (Leica, Ultracut), and double-stained with uranyl acetate and lead citrate. The sections were analyzed on a transmission electron microscope (TEM) (JSM 1200 IIE, JEOL, Tokyo, Japan); after imaging, OP-TIMAS Ver. 6.5 (Media Cybernetics, Bethesda, MD) was used to measure myelin thickness. G-ratio was defined as the ratio of axon diameter to that of the myelinated fiber.

Statistical analysis

All measurements and histologic observations were carried out blindly by the person who was not involved in the animal surgery. The data were expressed as means \pm standard error of the mean (SEM). Statistical analysis was performed with one-way ANOVA and Scheffe's post-hoc test. A P -value of 0.05 or less was considered statistically significant.

Results

Functional recovery of vibrissa movement

All animals displayed normal vibrissa movement before the surgery. **Figure 1** shows the time course of vibrissae movement recovery. The rats in sham group performed normal vibrissae movement in the whole experimental

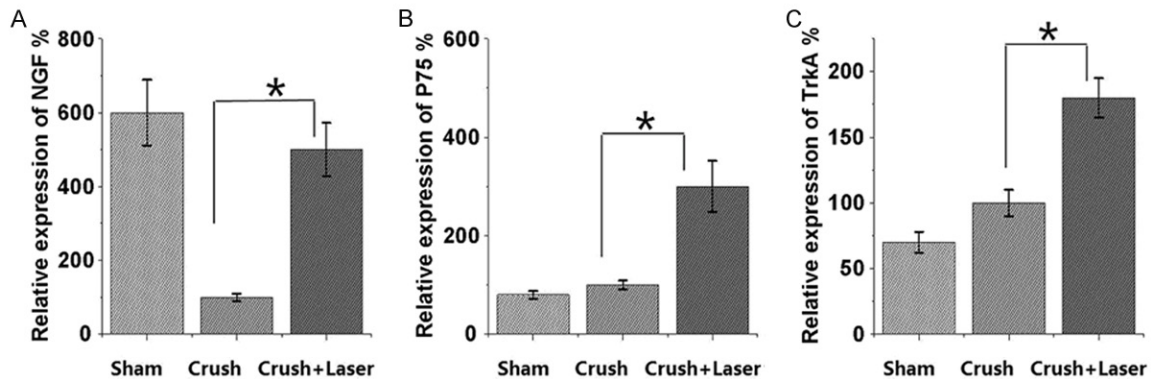


Figure 2. Quantification (relative expression, %) of NGF, p75NTR, and TrkA mRNA expression levels in the sciatic nerve 4 weeks after operation by quantitative real time-polymerase chain reaction.

process. Both crush group and crush + laser group, vibrissae movement was eliminated after the surgery, and it gradually recovered. However, at eight and twelve weeks after surgery, the average vibrissae movement grade of rats in crush + laser group (2.66 ± 0.50) was higher than crush group.

Evaluation of NGF, p75NTR and trkA expression levels by quantitative real-time PCR

Quantitative RT-PCR revealed that NGF, p75NTR, and trkA mRNA levels were significantly higher in the crush + laser group than the crush group. The mRNA expression levels of TrkA and P75 were even significantly higher in the crush + laser group compared with the sham group (* $P < 0.05$, **Figure 2**).

Immunofluorescent histopathology of NF-200 and S100

Twelve weeks after surgery, cross sections of the distal nerve stump were evaluated after staining with NF-200 (Red) and S100 (green). Interestingly, S100 positive (green) myelin sheaths surrounded NF200 positive (red) axons in all cross sections, but myelin sheaths were thicker in the crush + laser group than in the crush group. In addition, there was more vigorous axonal regeneration and myelin sheath coverage in crush + laser treated animals than in the crush group (**Figure 3**). The fluorescence signal intensity of NF-200 (Red) and S100 (green) were higher in crush + laser group compared with compared with crush group.

Histomorphometric evaluation of axon

Twelve weeks after surgery, semi-thin cross sections of the facial nerve regenerated in the

crushed side were analyzed by toluidine blue. Regenerated nerve fibers in the crush + laser group were well-regulated in micro-fascicles, similar to normal nerve fibers, and somewhat larger than those in the crush group. Meanwhile, the formation of blood vessels was also observed, as shown in **Figure 4**. Reduced vessel formation and less connective tissues were formed in the crush + laser group compared with the crush group (**Figure 4**). We used transmission electron microscopy (TEM) to evaluate degree of denervation. Those results showed deficits in myelinated fiber population in crush group compared to crush + laser group (**Figure 2C**). An increase of myelinated fibers was noted in crush + laser group than the crush group (**Figure 2B and 2C**). It is widely recognized that the g-ratio were calculated for assessing axonal myelination. The g-ratio was higher in crush + laser group than in the laser group.

Discussion

LLLT has been widely applied in clinical practice to facilitate nerve regeneration [17]. Studies have shown that Schwann cells, the principal glial cells of the peripheral nervous system, secrete neurotrophic factors that promote peripheral nerve regeneration [18]. Phototherapy can stimulate Schwann cell proliferation *in vitro* [19]. *In vivo* studies assessing functional recovery, histological and micro-morphological changes, and electrophysiological improvement, demonstrated that LLLT has beneficial effects on the regeneration of rat sciatic nerve injury [14]. A review of the literature on phototherapy for peripheral nerve repair found that the use of laser is based on several wavelengths (632-904 nm), lesion types (crushing,

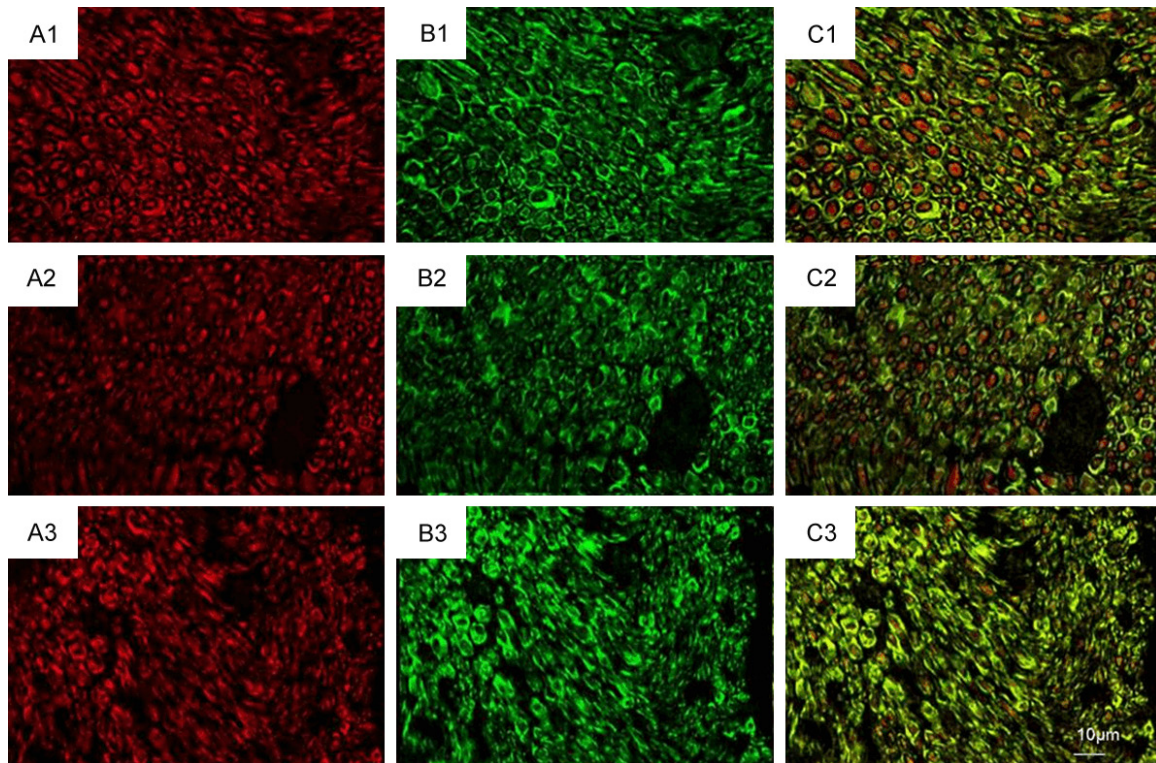


Figure 3. Immunohistochemistry micrographs after S-100 (red) and NF-200 (green) staining of the transverse-sectioned distal portion of the regenerated segment. (A1, B1, C1) Sham group; (A2, B2, C2) Crush group; (A3, B3, C3) Crush + laser group. (C1, C2, C3) are merged S-100 and NF-200. Scale bars indicate 10 μ m.

neurorrhaphy, and tubulation), sample types, duration and manner of emission, and measurement types (such as electrophysiological, morphometric, and functional) [11]. Multiple reports concluded that this treatment promotes the regeneration of traumatic injury [8, 9, 20]. However, other authors did not observe any beneficial effects [21]. The laser affects biological tissues differently according to usage parameters, such as dose, wavelength, continuous or pulsed mode, treatment duration, and application site [17, 22]. Based on this study, effective results were observed in nerve regeneration and the functional recovery of vibrissa movement, when comparing the crush + laser and crush groups.

In the morphological analysis of experimental groups in general, myelin fibers occurred with predominance, with irregular placement and diameters in the crush group. However, myelin fibers showed a better fascicle organization in the crush + laser group in relation to the early 5-week period, which is characteristic of the nerve regeneration process. In transmission electron microscopy, the myelinated fibers in

the crush + laser group showed better nerve fascicle organization compared with the crush group. Microscopy also showed oblique and longitudinal collagen fibers, indicating a random orientation of fibers in the regenerating tissue.

The effects of LLLT on nerve regeneration are well known. Irradiation ranging from 632 to 901 nm with LLLT light dose-dependently modulates the proliferation of Schwann cells *in vitro*, enhances nervous cell differentiation, increases axonal growth and myelination, and improves morphological recovery in experimental sciatic nervous lesions [19]. The current findings regarding the functional recovery of vibrissa movement also indicated that the crush and crush + laser groups both returned gradually to normal levels, with the crush + laser group closer to the sham group at 8 and 12 weeks.

This study had some limitations. Alcantara et al. recommended LLLT to be started as soon as possible after peripheral nerve injury [20]. Wang et al. indicated that application of 808-

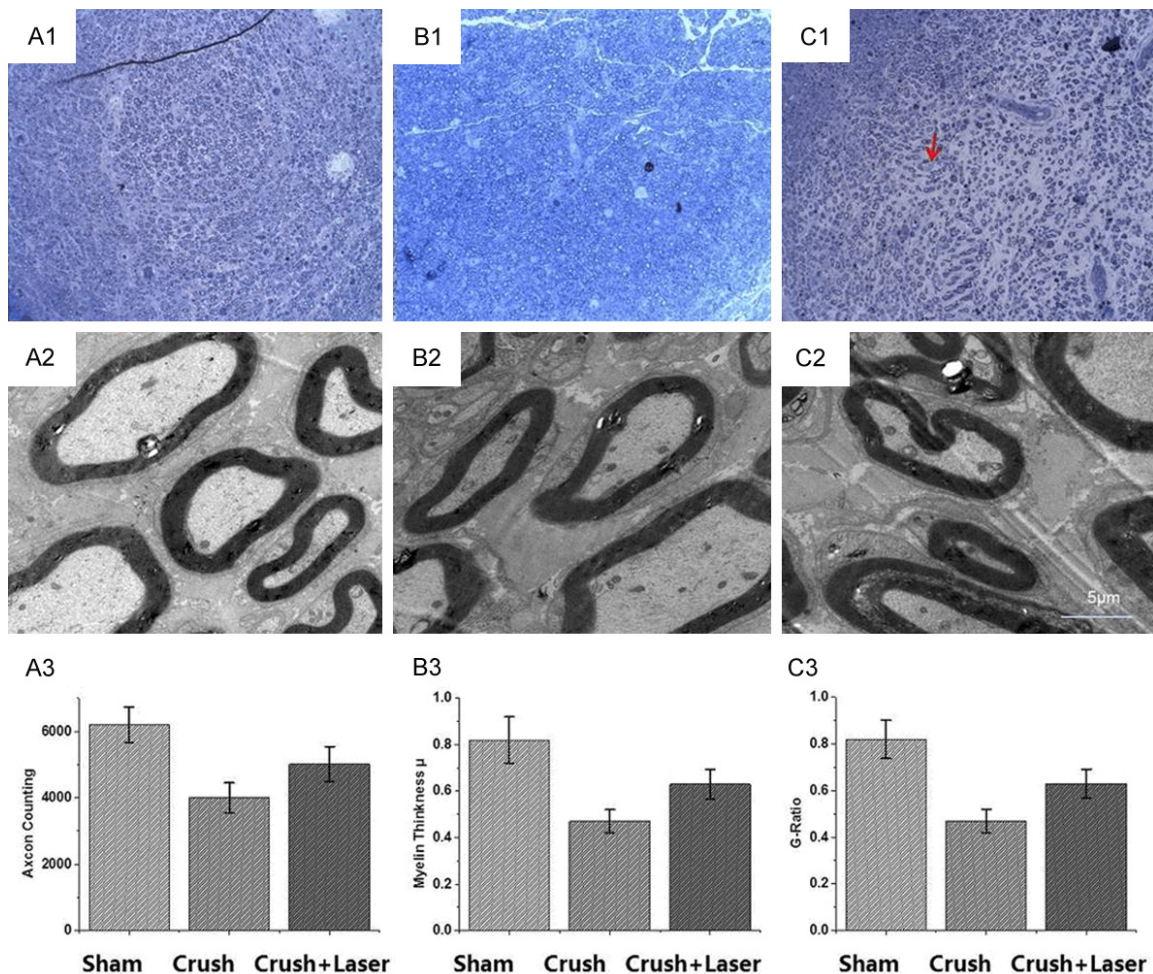


Figure 4. Structure of regenerated facial nerves. The first row shows the morphological properties of regenerated nerves by light microscopy; the second row depicts the ultrastructure of regenerated nerves by transmission electron microscopy; the third row displays the quantitation of axon count, myelin sheath thickness, and G-ratio ($n = 6$). A1, B1, C1. Sham group; A2, B2, C2. Crush group; A3, B3, C3. Crush + laser group; * $P < 0.05$ compared with the Normal group. The scale bar is 5 μm in semi-section micrographs, and 20 nm in ultra-thin micrographs.

nm LLLT at 3 and 8 J/cm^2 , respectively, for 20 days is effective in promoting sciatic nerve regeneration [14]. We only check the energy density 16.71 J/cm^2 on facial nerve crush. Except for laser treatment time, we only focused on effective results on crush nerve, and did not address whether it has negative effects on the normal natural nerve. Wang et al. assessed 8 J/cm^2 laser on natural nerves, and found no deleterious effects.

In conclusion, these findings indicated that low-level laser therapy is effective in the treatment of peripheral facial nerve injury, therefore representing a useful technique for peripheral nerve crush injury.

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Disclosure of conflict of interest

None.

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