Transforming growth factor-β3 promotes facial nerve injury repair in rabbits

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Abstract. The present study investigated the effects of transforming growth factor (TGF)-β3 on the regeneration of facial nerves in rabbits. A total of 20 adult rabbits were randomly divided into three equal groups: Normal control (n=10), surgical control (n=10) and TGF-β3 treatment (n=10). The total number and diameter of the regenerated nerve fibers was significantly increased in the TGF-β3 treatment group, as compared with in the surgical control group (P<0.01). Furthermore, in the TGF-β3 treatment group, the epineurial repair of the facial nerves was intact and the nerve fibers, which were arranged in neat rows, were morphologically intact with visible myelin swelling. However, in the surgical control group, the epineurial repair was incomplete, as demonstrated by: Atrophic nerve fibers, partially disappeared axons and myelin of uneven thickness with fuzzy borders. Electron microscopy demonstrated that the regenerated fibers in the TGF-β3 treatment group were predominantly myelinated, with clear-layered myelin sheath structures and axoplasms rich in organelles. Although typical layered myelin sheath structures were observed in the surgical control group, the myelin sheaths of the myelinated nerve fibers were poorly developed and few organelles were detected in the axoplasms. Neuro-electrophysiological examination demonstrated that, as compared with the surgical control group, the latency period of the action potentials in the TGF-β3 treatment group were shorter, whereas the stimulus amplitudes of the action potentials were significantly increased (P<0.01). The results of the present study suggest that TGF-β3 may improve the regeneration of facial nerves following trauma or injury.

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Introduction

The facial nerve is the longest peripheral nerve in the human body. Its anatomical structure is unique and its position, which is superficial and closely associated with the parotid gland, leaves it vulnerable to damage during an operation or as a result of trauma (1,2). Damage to the facial nerve can lead to paralysis of the muscles that control facial expressions (3), which may seriously affect the patients' quality of life and result in psychological stress for patients and their families. Therefore it is unsurprising that facial nerve defect repair is widely studied (4,5).

Current treatments for damaged facial nerves following facial nerve injury include surgical repair, catheter facial nerve repair and molecular repair (6). Most neurotrophic factors are capable of inducing axonal growth and current research is predominantly focused on three groups of neurotrophic factors which are involved in the regulation of endogenous repair processes: Neurotrophic factors, including insulin-like growth factor (IGF)-1, IGF-2 and BDNF, transforming growth factor (TGF)-β, glial cell-derived neurotrophic factor and IL-6 cytokines, including leukemia inhibitory factor (7,8). Treatment with exogenous IGF-1 and IGF-2 improves the rate of axonal regeneration, whereas the application of corresponding antibodies can block this effect (9). The underlying mechanism may involve IGF-mediated activation of RAS/MAPK and PI3K/Akt signaling pathways, thereby inhibiting the apoptosis of neurons and Schwann cells and promoting the growth of axon.

TGF-β superfamily consists of pleiotropic polypeptide growth factors with extensive biological activities, of which TGF-β3 is a subtype. With the development of tissue engineering, an increasing number of studies have investigated the role of neural regulatory factors in the regeneration of injured facial nerves (10). However, the effects of TGF-β3 on the repair of injured facial nerves, and the underlying mechanisms, are yet to be elucidated (11-16).

In the present study, a rabbit model was established with trauma sustained to the bilateral superior buccal branches of the facial nerve. In order to facilitate the interior administration of TGF-β3 to investigate nerve regeneration, a nerve growth chamber was constructed using a silicone tube. Dynamic, continuous and comparative observations of the morphological changes of the repaired nerve were performed at various time

points, utilizing various methods. Through a combination of neural electrical physiological detection and image analysis, the effects and possible mechanisms of TGF-β3 on facial nerve injury repair were investigated.

Materials and methods

Reagents. Paraformaldehyde was obtained from Beijing Chemical Reagent Company (Beijing, China). The JEOL 1230 transmission electron microscope was purchased from JEOL, Ltd. (Tokyo, Japan). The operating microscope and BX26 optical microscope were purchased from Olympus Corporation (Tokyo, Japan). A Canon digital camera was obtained from Canon Inc. (D70; Tokyo, Japan) and the EM UC6 ultramicrotome was purchased from Leica Microsystems (Wetzlar, Germany).

Experimental animals and animal modeling. A total of 20 adult rabbits, weighing 2.5-3.0 kg, were provided by the Department of Experimental Animals of Xinjiang Medical University (Urumchi, China). The rabbits were maintained under standard conditions with *ad libitum* access to food and water. The present study was approved by the Experimental Animal Ethics Committee at Xinjiang Medical University.

Facial nerve trauma and repair models were established in 10 rabbits. Rabbits were anesthetized with 30 mg/kg pentobarbital via the ear vein and a transverse incision was subsequently made in the bilateral cheek to expose ~2 cm of the buccal branches of the facial nerve prior to the transection of ~5 mm of the superior buccal branches of facial nerve. A silicone chamber, to be used as a nerve conduit, was created using a surgical microscope (ASOM-4C; Chengdu Corder Optics & Electronics Co., Ltd., Chengdu, China). In order to form a nerve regeneration chamber (Fig. 1A), the broken ends of the facial nerve were embedded 1.5 mm into a sterile silicon tube (outer diameter, 3 mm; inner diameter, 2 mm; length, 10 mm). Suture fixation of both ends of the epineurium to the silica gel tube was performed using 10-0 thread. A collagen sponge was used for support and as a vehicle for TGF-β3 implantation. Following this, the right silicone chambers were loaded with 10 µl TGF-β3 (50ng/µl) and were used as the TGF-β3 treatment group (n=10); whereas the left chambers were filled with phosphate-buffered saline and used as the surgical control group (n=10). The wounds were subsequently sutured using 4-0 thread. A total of 10 rabbits were randomly assigned to the normal control group, and did not receive any treatment.

Examination of gross morphology. Preoperative and postoperative changes in the vibrissal movements of the rabbits were observed, including trembling symptoms. Gross morphology, anastomosis of neural connections, stump neuroma and scars on the buccal branches of the facial nerve were examined 12 weeks post-operation.

Hematoxylin and eosin (HE) staining. Rabbits were anaesthetized using 3% pentobarbital sodium and sacrificed via strangulation. The rabbits were perfused with 4% paraformaldehyde, via the heart, for fixation and proximal and distal allograft neural tissue samples (length, ~1 mm) were

subsequently collected from each group. Briefly, the buccal branch of the facial nerve was exposed. Subsequently, the facial nerve was dissected 5 mm from the distal end of the silicone tube and 5 mm from the proximal end of the silicone tube. Following removal of the silicone tube, the facial nerve specimens were harvested. Specimens were fixed with 4% paraformaldehyde, embedded in paraffin, sliced into sections and stained with HE. Subsequently, >20 fields in each specimen were randomly selected for observation of the regenerated nerve fibers. The number and diameter of regenerating nerve fibers in the cross-section of the regenerated nerve and the longitudinal section of the proximal anastomosis were observed using light microscopy (SZM-45B1; Zhong Tu Ao Si Micro-Optical Equipment Company, Shenzhen, China, Shenzhen, China).

Light microscopy and transmission electron microscopy examination. The neural tissue samples were fixed with 2.5% glutaraldehyde at 4˚C for 6 h prior to immobilization with 1% osmic acid and subsequent gradient acetone dehydration. Following this, the tissues were washed with 0.1 M phosphate buffer solution, embedded overnight with anhydrous Epoxy resin 618 (Wuxi Resin Factory, Wuxi, China) and incubated in the dryer for pure embedding at 37˚C for 2 h and 60˚C for 48 h, until the resin had totally polymerized. Part of the embedded tissue was sliced into semi-thin sections and stained with toluidine blue (Shanghai Mai Ke Lin Biochemical Company, Shanghai, China). Ten sections of each specimen were used for examination of the regenerated nerve using light microscopy. Part of the embedded tissue was sliced into ultrathin sections, saturated with uranyl acetate for 30 min and stained with citric acid for 15 min. Subsequently >20 fields in each specimen were randomly selected for observation of regenerated nerve fibers. The ultrathin sections of the regenerated nerves were observed under a transmission electron microscope.

Electrophysiological examination. The compound muscle action potentials of the facial nerve were detected using an electromyogram evoked potential instrument (A3101-b; Shanghai Hai Shen Medical Electronic Instrument Co., Ltd., Shanghai, China), 12 weeks post-operation. The latency period and amplitude values of the facial nerve action potentials were recorded and analyzed using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA).

Statistical analysis. All data were analyzed by SPSS 16.0 software. Paired t-tests were performed to analyze the difference between the treatment and control groups. Data are presented as the mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Changes in gross morphology. In order to determine whether TGF-β3 had an effect on nerve regeneration, the general condition and gross morphology of the rabbits in the various groups were observed. The general condition of the rabbits was observed immediately after surgery, various facial paralysis symptoms were detected in all of the rabbits with facial

nerve trauma, including: Varying degrees of photophobia, a pendulous ear on the operative side, 1-2 cm eyelid distance, a drooping upper lip, and a failure to completely close eyelids, even following stimulation (Fig. 1B).

A total of 12 weeks after the operation, the original incision was reopened under general anesthesia, in order to assess the repair of the nerve injury. The silicone tube was encapsulated by fibrous connective tissue in both the TGF-β3 treatment and surgical control groups (Fig. 2A and B), as observed with the naked eye. Furthermore, when the connective tissue was dissected from the translucent silicone tubes, both ends of the nerve had successfully crossed the defect area and connected; however, the wall of the silica gel tube had no adhesion with the regenerated nerve and it was easy to separate them. In the TGF-β3 treatment group, the diameters of the regenerated nerves were similar to those of the nerve stems in the distal and proximal ends. Furthermore, no neuroma was detected and the newly formed epineurium comprised of a complex network of blood vessels gray in color, and was firm and tough, as demonstrated by the visible tension caused by pulling with tweezers without snapping (Fig. 2C). In the surgical control group, the end of the regenerated nerve was slightly thicker and the nerve appeared delicate, with poor elasticity; therefore, it was easy to break (Fig. 2D). The results suggest that the general condition and gross morphology of the damaged facial nerves was improved following treatment with TGF-β3.

Observation of the regenerated facial nerve using a light microscope. In order to analyze the effects of TGF-β3 on the regeneration of facial nerves, the number and diameter of the regenerated nerve fibers were measured at 12 weeks post-operation. As outlined in Table I, the total number and diameter of the regenerated nerve fibers was significantly increased (P<0.01) in the TGF-β3 treatment group (1052.00±144.34 root and 6.16±0.45 cm), as compared with those in the surgical control group (555.30±86.74 root and 3.59±0.61 cm).

In the TGF-β3 treatment group, the epineurium of the facial nerve was intact and the nerve fibers were morphologically intact and arranged in neat rows with visible myelin swelling. However, in the surgical control group, the epineurium was incomplete, atrophy was detected in the nerve fiber, the axons had partially disappeared, and the myelin was of uneven thickness with fuzzy borders.

In order to observe any pathological changes in the newly regenerated nerve fibers, the facial nerves were subsequently stained with HE and examined. As demonstrated in Fig. 3A, numerous regenerated nerve fibers were observed in the cross sections from the TGF-β3 treatment group, and the majority of these fibers formed into bundles containing blood vessels. Furthermore, hypertrophy and hyperplasia of the Schwann cells was visible, the size of the regenerated fibers was not uniform, the nuclei were thick, and in the longitudinal sections, the regenerated nerve fibers twisted in parallel across the distal anastomosis. In the cross sections obtained from the surgical control group (Fig.3B), minimal newborn nerve fibers and few myelin fibers were detected, in addition to fat cell infiltration and considerable scar tissue. In the longitudinal sections, the nerve fibers were arranged

Table I. Comparison of the total number and diameter of the regenerated nerve fibers at 12 weeks post-operation (n=10).

Group	Total number (root)	Diameter (μm)
TGF-β3 treatment	1052.00±144.34	6.16±0.45
Surgical control	555.30±86.74	3.59±0.61
T-value	9.327	10.689
P-value	<0.001	<0.001

Data are presented as the mean ± standard deviation. TGF, transforming growth factor.
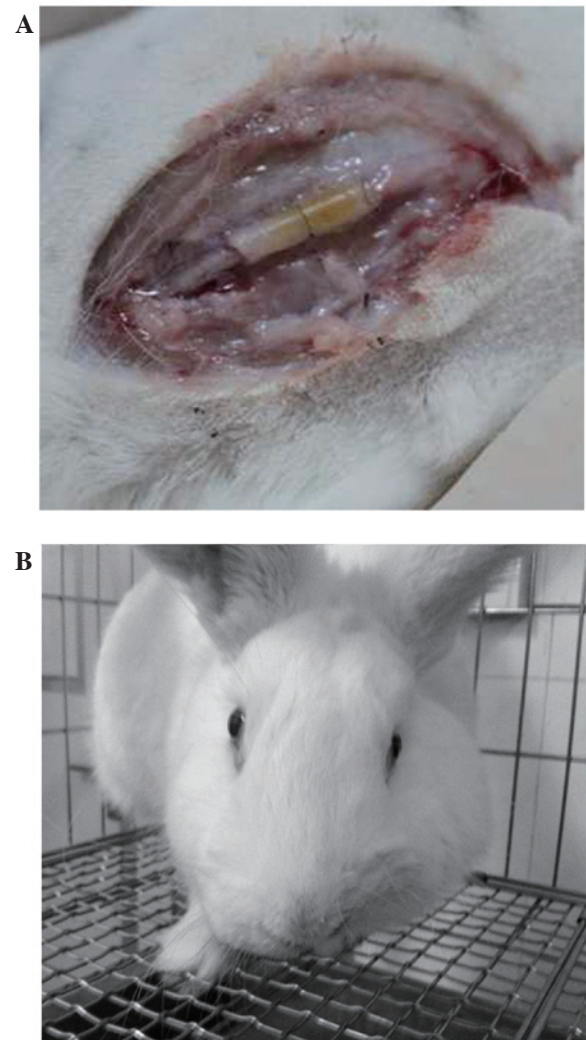

Figure 1. Representative nerve regeneration chamber and facial symptoms in a rabbit model for the regeneration of facial nerves. (A) To form the nerve regeneration chamber, both broken ends of the transected superior buccal branches of facial nerve were embedded 1.5 mm into a sterile silicon tube (outer diameter, 3 mm; inner diameter, 2 mm; length 10 mm). (B) Representative facial symptoms of a rabbit with facial nerve injury. Facial paralysis symptoms were observed.

in a disordered and discontinuous manner and some were partially dissolved. The regenerated nerve fibers with obvious distortions presented as spirals with substantial scar tissue. In addition, the axons were unevenly distributed and their
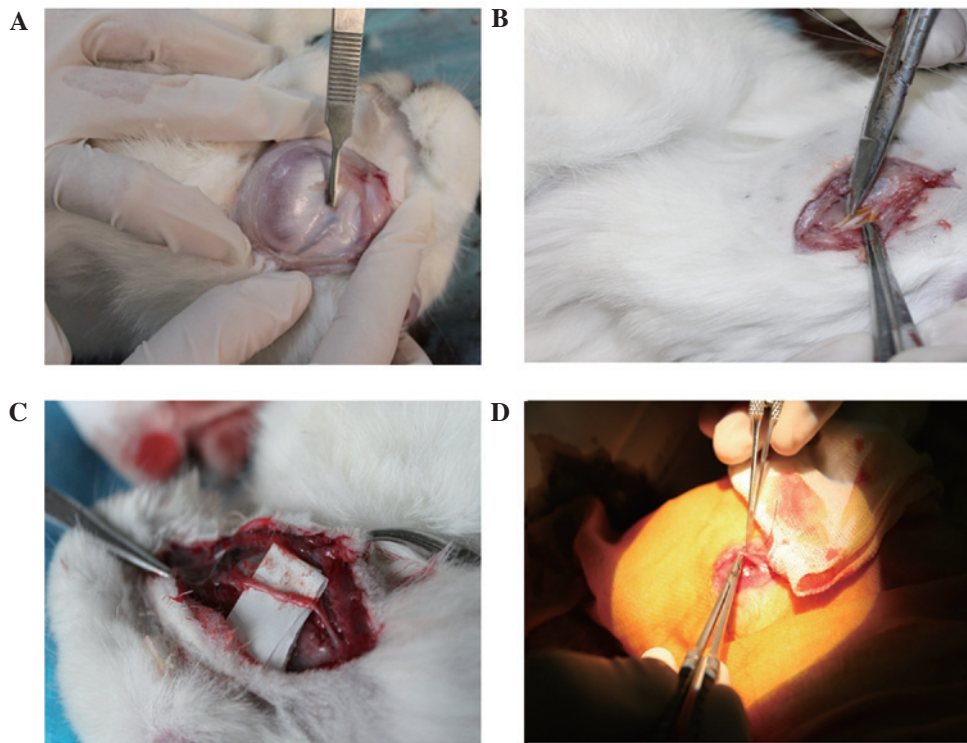
Figure 2. Gross morphological observations of the (A and B) nerve regeneration chamber and (C and D) regenerated facial nerve, as observed at 12 weeks post-operation. (A and C) Representative images of the transforming growth factor (TGF)-β treatment and (B and D) the surgical control groups.
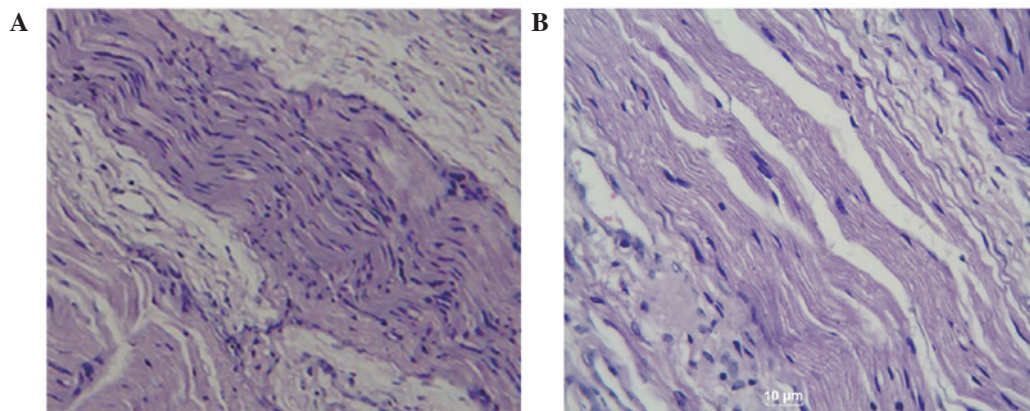

Figure 3. Histological observation of the regenerated facial nerves. Hematoxylin and eosin (HE) staining was performed in order to observe the regenerated facial nerves at 12 weeks post-operation. Representative HE staining images are shown (magnification, x40). (A) The transforming growth factor (TGF)-β3 treatment and (B) the surgical control groups.

numbers were significantly reduced, with no obvious myelin sheath. Collectively, these results suggest that TGF-β3 may effectively promote the repair of transected facial nerves.

Observation of the facial nerve under a transmission electron microscope. In order to analyze the structure of the regenerated nerve fiber, the regenerated facial nerve was observed under a transmission electron microscope. As outlined in Fig. 4A and B, the regenerated facial nerves in the TGF-β3 treatment group were predominantly myelinated nerves with regular form. Furthermore, the layered structure of the myelin sheath was clear with distinct dark and light banding and there were abundant organelles in the axoplasm.

Notably, most of the regenerated fibers formed into bundles with rich blood vessels; whereas in the surgical control group, nerve regeneration and repair only occurred in five injured facial nerves. The remaining facial nerve injuries demonstrated scar tissue and when the ultrathin sections were observed under a transmission electron microscope the regenerated nerve fibers were irregular in shape. In addition, the axoplasmic organelles were unclear, the lamellar structures were disordered, and myelin dysplasia and degeneration of the nerve fiber was observed (Fig. 4C and D). These results suggest that 12 weeks post-operation, the regenerated facial nerve fibers of rabbits in the experimental group were predominantly myelinated nerve fibers with a clear lamellar
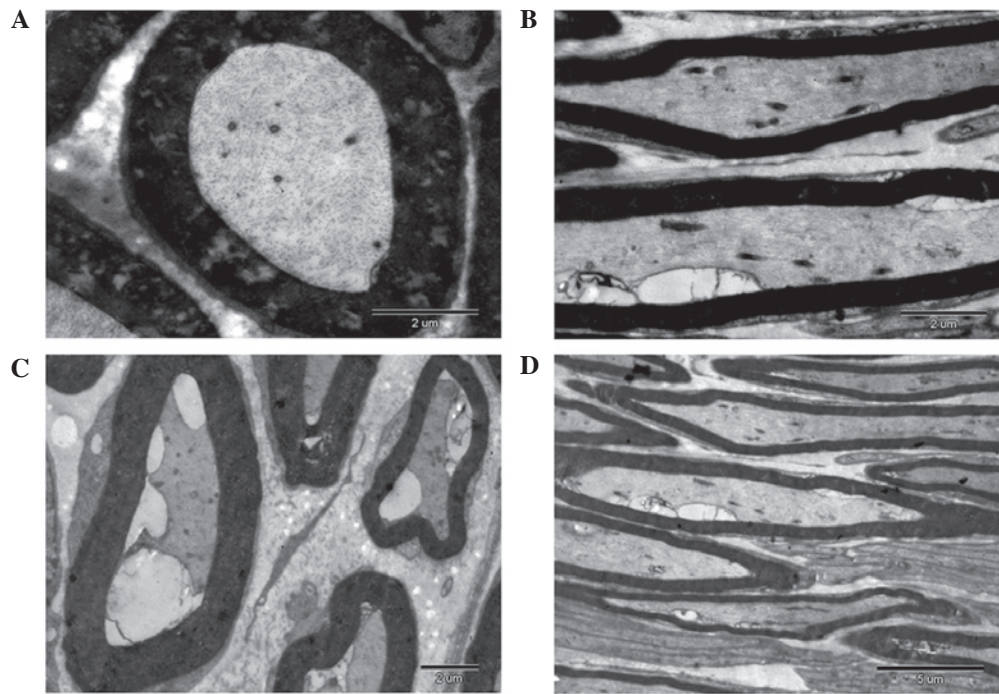
Figure 4. Observation of regenerated facial nerves under transmission electron microscope. At 12 weeks post-operation, semi-thin and ultra-thin sections of regenerated facial nerve tissues were observed under a transmission electron microscope. Representative images are shown (magnification, x6,000). (A) Cross section of the facial nerve in the transforming growth factor-β3 treatment group. (B) Longitudinal section of facial nerve in the TGF-β3 treatment group. (C) Cross section of the facial nerve in the surgical control group. (D) Longitudinal section of facial nerve in the surgical control group.

Table II. Comparison of the latency period and amplitude of action potentials at 12 weeks post-operation (n=10).

Group	Latency period (ms)	Amplitude (mV)
TGF-β3 treatment	2.35±0.41[a,b]	8.60±1.87[a,b]
Surgical control	3.42±0.56[a]	4.62±0.77[a]
Normal control	1.47±0.42	11.32±5.36
T-value	8.596	23.731

Data are presented as the mean ± standard deviation. [a]P<0.01 vs. the normal control group; [b]P<0.01 vs. the surgical control group. TGF, transforming growth factor.

structure and rich axoplasmic organelles, as compared with the control group.

Electrophysiological examination. To evaluate the functional recovery of the facial nerves, an electrophysiological examination was performed 12 weeks post-operation. In order to detect the compound muscle action potentials of the facial nerve, latency period and amplitude values were recorded and analyzed using an electromyogram evoked potential instrument. As shown in Table II, the action potential latency periods were increased in the surgical control (3.42±0.56 msec) and TGF-β3 treatment groups (2.35±0.41 msec), as compared with the normal control group (1.47±0.42 msec). The latency periods of the action potentials in the TGF-β3 treatment group were significantly decreased (P<0.01), as compared with the surgical control group. As compared with the normal

control group (11.32±5.36 mV), the amplitudes of the action potentials in the surgical control group (4.62±0.77 mV) were significantly decreased (P<0.01); whereas a significant increase (P<0.01) was detected in the TGF-β3 treatment group (8.60±1.87 mV). These results suggest that administration of TGF-β3 into the regeneration chamber following the transection of the superior buccal branches of the facial nerve may significantly promote the conduction velocity of the facial nerve.

Discussion

Regeneration of an injured nerve is a complex process and it is a comprehensive reflection of the reconstruction of neural pathways, recovery of metabolism and functional repair (17,18). Commonly used methods for the repair of nerves include: Autologous nerve transplantation, nerve allograft transplantation, end-to-end anastomosis, facial nerve transplantation, vascularized nerve graft and non-nervous tissue transplantation (19-21). End-to-end anastomosis cannot be performed when there is a large defect in the facial nerve, therefore the most effective method for the repair of facial nerves with larger defects is an autologous nerve transplantation (22,23). However, for peripheral nerve defects involving thick and long segments this method is not appropriate, as the autologous nerve graft source is difficult to acquire and the following problems may occur: Neuroma formation, scarring in the donor area, sensorimotor disorder and errors in the neural network (24,25). It has been suggested that nerve conduit technology may be an effective method to solve these problems; Lundborg *et al* (26) demonstrated that silicone tubes could be

used for nerve defect repair and this promoted the development of nerve catheter technology. Previous studies (27,28) have used nerve substitutes and various materials in the repair of facial nerve injury and have demonstrated that nerve catheter orientation and the nutrition of the regenerative microenvironment have a vital role in regeneration of the damaged nerve. Therefore, the use of nerve regeneration chambers to alter the regenerative microenvironment and the addition of nerve regeneration factors into the chamber is now widely used in neural regeneration (29,30).

In the present study, a silicone tube was used as a nerve conduit to simulate the epineurium, and a collagen sponge with a 3D network structure was used for support and as a vehicle for TGF-β3 implantation. The nerve conduit provided a combination of nutrition, support and contact guidance as the chamber prevented the formation of scar tissue and the invasion of connective tissue, in addition to maintaining the stability of the microenvironment for nerve regeneration.

The results of the present study demonstrated that administration of TGF-β3 into the regeneration chamber may effectively promote the growth and maturity of regenerating facial nerves. The repair effects demonstrated by the TGF-β3 treatment group were significantly improved, as compared with the surgical control group. Therefore, the local application of TGF-β3 may significantly increase the diameter of axons and nerve conduction velocity, and accelerate facial nerve fiber regeneration and myelination, thus promoting the overall repair of facial nerves.

In conclusion, TGF-β3 may promote the regeneration of facial nerves following trauma; however, the process of facial nerve repair is rather complicated. Therefore, whether TGF-β3 can be used clinically remains unknown and requires further investigation.

Acknowledgements

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