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## The Feasibility of Delayed Repair of Facial Nerve Trauma-electrophysiological Studies and Research of Neurons and Specificity of Regeneration

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**Abstract:** To study aimed to evaluate the feasibility of delayed repair of facial nerve trauma in case immediate repair was not possible. Guinea pigs were randomly assigned to one of eight groups; normal group, group of immediate Facial-facial Anastomosis (FFA), group of delayed FFA for 7 days, group of delayed FFA for 14 days, group of delayed FFA for 21 days, group of delayed FFA for 30 days, group of delayed FFA for 60 days and group of delayed FFA for 90 days, by turns. The survival time was 2 months after nerve suture. The group of immediate FFA and the group of delayed FFA for 7 days had shorter latency than other groups of delayed FFA ( $p < 0.05$ ). The amplitude of the group of delayed FFA for 7 days was larger than that of immediately sutured and other delayed sutured ones. The group of delayed FFA for 60 and 90 days had longer latency and smaller amplitude than the group of immediate FFA and other group of delayed FFA. The facial neurons labeled by HRP in immediate suture group were more than that in any other delayed suture group ( $p < 0.05$ ). There was no significant difference among groups of delayed FFA for 7, 14, 21 and 30 days ( $p = 0.326$ ). Immediate repair of facial nerve trauma showed best results; however delayed nerve repair was also an option. The best curative effects were with repairs within 60 days.

**Key words:** Collateral branches, feasibility, delayed facial-facial anastomosis, electrophysiological, HRP-labeled neurons, myelinated axon per motoneuron ratio

### INTRODUCTION

The benefits of immediate or delayed sutures for better nerve regeneration, following facial nerve trauma have been well established. Since World War II, several studies on nerve repair have been carried out. Delayed nerve repair is not a favorable option in some of these studies, but is carried out in the absence of immediate treatment. Many researchers have reported comparable functional recovery between delayed and immediate nerve sutures (Guntinas-Lichius *et al.*, 1997, 2000a, b).

The morphological changes occurring in denervated human skeletal muscles (Brooke and Kaiser, 1974) have been widely investigated. Facial nerve is very complex in its function and anatomy and is easily injured by various mechanisms. In routine clinical setting, treatment of nerve lesions are often performed by delayed nerve suture. With the exception of iatrogenic lesions it is usually not possible to carry out a nerve suture immediately after injury to a peripheral nerve. A good understanding of the function and anatomical process helps in identifying and repairing nerve lesions. Facial nerve monitors are useful

to identify and map injury and prognosis. These monitors use mechanical or electrical stimulation to find the exact location of nerves. A number of experimental animal studies on the optimal timing of delayed nerve suture have been published, but the results are varied (Barrs, 1991; Bignotti *et al.*, 1986; Bolesta *et al.*, 1988). The histochemical profile of a muscle is dependent on its state of innervation. Any experimentally induced changes in innervation which can be measured are reflected in the histochemical profile of the muscle fibers (Brooke and Kaiser, 1974). The experimental study explored the feasibility of delayed repair after facial nerve trauma. Functional recovery of facial muscles and the specificity of regenerated fibers in terms of nerve extension and re-innervation after trauma were used as observational indices.

### MATERIALS AND METHODS

**Experimental animals:** Guinea pigs were bought from Suzhou Agriculture Institute. There were 8 animals per group; all were males and weighed from 300 to 350 g. In

the study, a survival time of 2 months for nerve regeneration for all groups of animals was set.

**Medicines and reagents:** Horseradish peroxidase (HRP) in powder, tetramethylbenzidine (TMB), sodium nitroprusside, ammonium molybdate, sodium acetate, dimethyl sulfoxide (DMSO) were all procured from SIGMA®.

**Main equipments:** Operation microscope (West Germany), freezing microtome, computer image processing system (KS400 image analysis system, Carl Zeiss Far East Co., Ltd in Germany, ZEISS; UMSP micro spectrophotometer; ACE camera, differently enhanced camera with 930-line resolution; image analysis software package, KS400 Ver. 2.0).

**Grouping of animals:** Male guinea pigs (n = 64), with short hair, weighing around 300 g were included and variegated. They were randomly assigned to one of the eight groups: normal group, group of immediate FFA, group of delayed FFA for 7 days, group of delayed FFA for 14 days, group of delayed FFA for 21 days, group of delayed FFA for 30 days, group of delayed FFA for 60 days and group of delayed FFA for 90 days by turns. Eight guinea pigs were assigned to each group. The HRP-labeled neurons and myelinated axon per motoneuron ratio were observed (direct observation for normal group) two months after the operation. In guinea pigs it usually takes about 21-30 days for facial nerve to recover for axonal regeneration after trauma. In addition, delayed reconstruction may need more time.

#### **Experimental animal models:**

- **Cutting guinea pigs' facial nerve:** Guinea pigs were anesthetized with pentobarbital sodium (with intraperitoneal injection, 20 mg kg<sup>-1</sup>) and ketamine hydrochloride (with intramuscular injection, 80 mg kg<sup>-1</sup>) and the right facial nerve trunk was exposed and separated. It was cut 2 mm apart from stylomastoid foramen. For the groups of delayed FFA, double transfixion were given to the two traumatic ends with 11 No. 0 sutures respectively and they were folded back and fixed on trapezius muscle and the incision was closed
- **Immediate nerve suture:** For the group of immediate FFA, the two ends of the cut facial nerve were sutured with two stitches in 11 No.0 sutures and the incision was closed
- **Delayed nerve suture:** For the groups of delayed FFA, the incision was reopened on the 7th, 14th,

21st, 30th, 60th or 90th days. The two traumatic nerve ends fixed on the trapezius muscle were located, carefully peeled off from the scar tissue and cut from the surrounding tissues. They were sutured with two stitches in 11 No.0 sutures and the incision was closed

After two months of facial nerve anastomosis, each group of eight guinea pigs was anesthetized with pentobarbital sodium mixed with chloride ketone, which induced action potential latency of orbicularis oris muscle. The EMG activity and amplitude changes were observed as the indicators. After the injured facial nerve was exposed, bipolar needle silver electrode was placed in the proximal anastomotic as stimulating electrodes, with the DC stimulation of 0.75 mA pulsed square wave, pulse width 0.1 ms, stimulation frequency of 1 Hz, low-pass filter 100 Hz, high-pass filter 2 KHz, the scanning time 2 msec and the sensitivity of 2 mV. Subsequently, the needle recording electrode was placed in the ipsilateral orbicularis oris muscle and contralateral auricle ground.

**Retrograde tracing of facial neurons with HRP:** The complete process has been described elsewhere in detail (Jiongjiong *et al.*, 2004). Two months after FFA, subcutaneous injection of 2 mg HRP dissolved in 0.2 mL distilled water containing 2% DMSO was administered to the guinea pigs' right vibrissa pad. About 48 hours later, these guinea pigs were anesthetized and after cordis perfusion with warm normal saline, they were perfused by 500 mL mixture of 1.25% paraformaldehyde, 2.5% glutaraldehyde and 0.1 M phosphate buffer solution (pH 7.4). The whole brain and brainstem were taken out to mark the operation side and 30 continual frozen sections (50 µm) on the level of facial nucleus were made. After washing with double-distilled water, they were set in an incubation buffer containing 0.005% TMB, 0.1% sodium nitroprusside, 0.03% H<sub>2</sub>O<sub>2</sub> and 5% 0.1 M sodium acetate buffer (Ph 3.7) to pre-react for 20 min in dark. About 50 µL of 30% H<sub>2</sub>O<sub>2</sub> was added into the incubation buffer and the incubation was continued for 20 min in the dark. They were washed in 0.1 M sodium acetate buffer (5×6 min) and re-stained in 0.1 M sodium acetate buffer containing 5% ammonium molybdate for 15 min. After washing in 0.1 M sodium acetate buffer (5×6 min), the sections were dehydrated, vitrified and sealed.

**Staining of myelinated fibers:** Following two months after nerve suture, six guinea pigs from each group were anesthetized. Before this process cordis perfusion was done with warm normal saline, which removed blood making it convenient to take the facial nerve sample. The

incision below the guinea pigs' right ear was reopened under operation microscope. The right facial nerve trunk was taken (2 mm from the stoma toward the proximal stump and distal stump, respectively) and fixed in formalin-calcium acetate solution. Myelin sheaths were stained with Ponceau 2R-brilliant green stain method described by Yuwang *et al.* (2003). Trans-sections from the two ends of the stoma were taken. The sections were deparaffinized and rehydrated. The sections were stained in Ponceau 2R staining solution for 5 min (1 g Ponceau 2R, 1 mL glacial acetic acid, 99 mL distilled water). After washing with distilled water, they were put in 2.5% phosphotungstic acid solution for 1 min (2.5 g phosphotungstic acid, 100 mL distilled water). They were re-stained in brilliant green staining solution for 4 min (1.0 g brilliant green, 1 mL glacial acetic acid, 99 mL distilled water). They were put in 1% acetonitrile acetic acid solution for differentiation; washed by tap water and dehydrated with graded ethanol. After which they were vitrified by dimethylbenzene and sealed in neutral resin. After staining, the myelin sheath was pink; axon, lamellar sheath and endoneurium were green; the degenerated myelin sheath was unstained due to absence of phospholipid.

**Counting:** The counting of HRP-labeled neurons and myelinated fibers was done with a computer image processing system by Biophysics TRG at the Shanghai Second Medical University. The HRP-labeled neurons, were counted every three sections for one brainstem and the sum was multiplied by three. For counting of myelinated fibers, three non-overlapping views were enlarged 1000 times for each section. Myelinated fibers were counted in each view and the mean number was calculated. The view was enlarged 63 times and the total area where nerve tissue was distributed was counted. The number of myelinated fibers of the whole nerve was calculated as that total area divided by view area and then multiplied by the mean number. The number of myelinated fibers on the distal stump/the number of myelinated fibers on the proximal stump was the passing rate of myelinated fibers.

Myelinated axon per motoneuron ratio was the number of myelinated fibers divided by the number of HRP labeled neurons in one guinea pig.

**Statistical methodology:** All data are presented as Mean±SD. A one-way Analysis of Variance (ANOVA, S-N-K) was applied to detect any differences between the different types of delayed nerve suture and immediate nerve suture. The p value of less than 0.05 was considered statistically significant.

## RESULTS

The evoked EMG incubation period of the orbicularis oris was 2.7500±0.2881 ms in guinea pigs in the normal group. In the facial nerve immediate anastomosis group and delayed anastomosis group, the evoked EMG incubation period of the orbicularis oris was significantly longer than that of normal group ( $p < 0.05$ ). The incubation period for immediate suture group (3.5667±0.2733 msec) and for the group of delayed suture for seven days (3.4167±0.2927 msec) was lower than any other group of delayed sutures. However, there was no significant difference between the two groups ( $p = 0.478$ ). The incubation periods of delayed FFA for 60 days group (5.3167±0.1722 msec) and for delayed FFA for 90 days group (5.4667±0.1366 msec) were longer than other groups of delayed suture. The incubation period of the group of delayed FFA for 7 days was significantly lower than that of delayed FFA for 14 days (4.2000±0.4817 msec) ( $p < 0.05$ ). There was no significant difference ( $p = 0.307$ ) between the delayed FFA for 14 days and delayed FFA for 21 days groups (4.4167±0.6585 msec). Further, there was also no significant difference ( $p = 0.119$ ) between the delayed FFA for 21 days and delayed for 30 days groups (4.7500±0.3082 msec) (Fig. 1).

The EMG amplitude of normal group (3.2083±0.5589 mv) was more than that of immediate suture group and groups of delayed suture ( $p < 0.05$ ). However, the amplitude of group with delayed FFA for 60 days (0.8000±0.1897 mv) and the group with delayed FFA for 90 days (0.7167±0.1941 mv) was lower than other groups of delayed suture, while there was no statistically significant difference between these two groups ( $p = 0.673$ ). The amplitude of delayed FFA for 7 days group (2.000±0.3098 mv) was higher than that of immediate suture group (1.3167±0.2137mv). There was no significant difference between immediate suture group

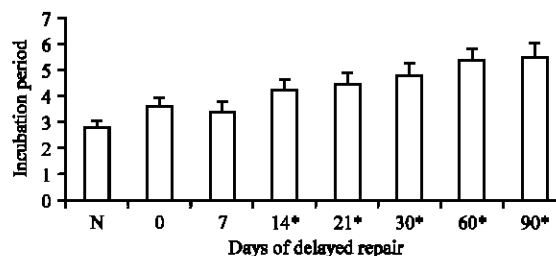


Fig. 1: Comparison among induced EMG incubation period of the orbicularis oris N is normal group and \*represent the comparison with immediate repair group ( $p < 0.05$ )

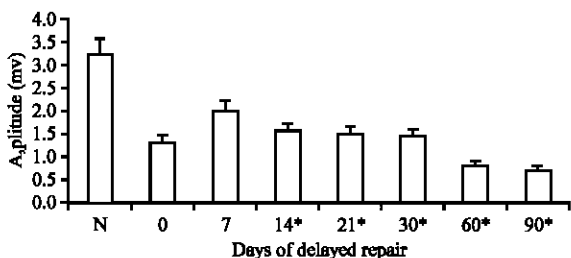


Fig. 2: Comparison among induced EMG amplitude of the orbicularis oris N is normal group and \*represent the comparison with immediate repair group ( $p < 0.05$ )

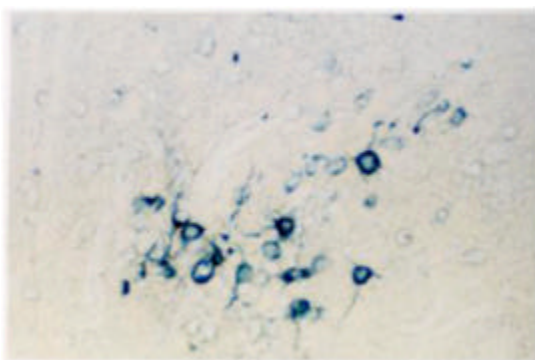


Fig. 3: HRP labeled facial neurons of normal guinea pig (100 times enlarged)

and delayed suture for 14 days ( $1.5500 \pm 0.2588$  mv), delayed FFA for 21 days ( $1.4833 \pm 0.4119$  mv) and delayed FFA for 30 days ( $1.4333 \pm 0.3983$  mv) groups (Fig. 2).

**HRP-labeled neurons:** After staining, the HRP-labeled neurons were blue-black and the axon and dendrites were clearly identified. The background was white or light yellow. Generally, the labeled neurons were lateral to the ventral brain stem.

HRP-labeled neurons were concentrated into a mass in normal guinea pigs (Fig. 3); while 2 months after FFA, HRP-labeled neurons were scattered, less than those in normal guinea pigs (Fig. 4-6). According to the results of the experiment, HRP-labeled facial neurons in normal male guinea were counted  $928.5000 \pm 147.1255$ , more than sutured groups ( $p < 0.05$ ). HRP facial neurons in group of immediate FFA ( $550.5000 \pm 38.8265$ ) were more than groups of delayed FFA ( $p < 0.05$ ). However, there was no significant difference among the groups of delayed FFA for 7 days ( $477.5000 \pm 44.6352$ ), for 14 days ( $436.0000 \pm 28.5657$ ), for 21 days ( $416.5000 \pm 30.9112$ ) and for 30 days ( $424.5000 \pm 41.7313$ ) ( $p = 0.326$ ) for HRP facial

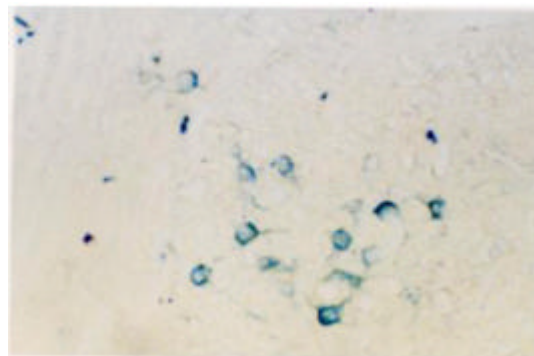


Fig. 4: HRP labeled facial neurons of guinea pigs in group of immediate FFA (100 times enlarged)

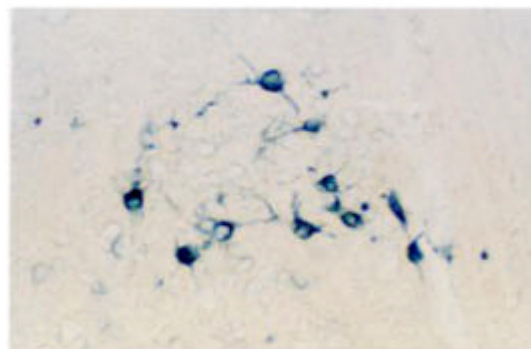


Fig. 5: HRP labeled facial neurons of guinea pig in group of delayed FFA for 7 days (100 times enlarged)

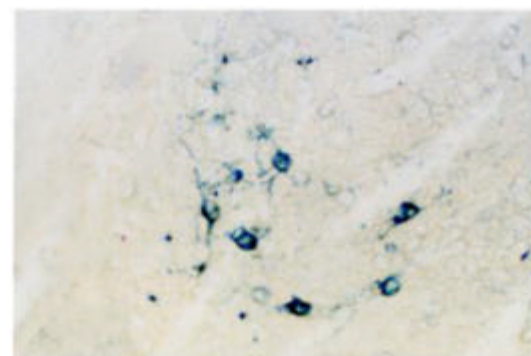


Fig. 6: HRP labeled facial neurons of guinea pig in group of delayed FFA for 90 days (100 times enlarged)

neurons. Similarly, there was no significant difference among the groups of delayed FFA for 14 days, for 21 days, for 30 days, for 60 days

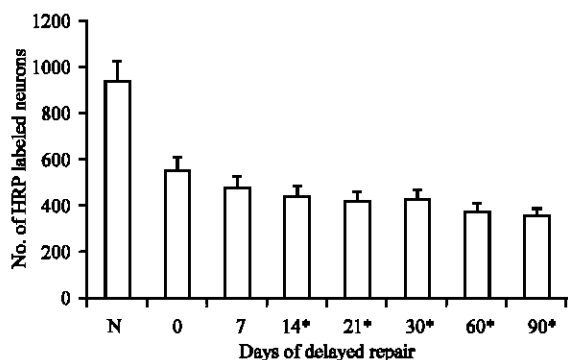


Fig. 7: The comparison of the numbers of HRP labeled neurons among groups N is normal group \*Compared to group of immediate FFA ( $p < 0.05$ )

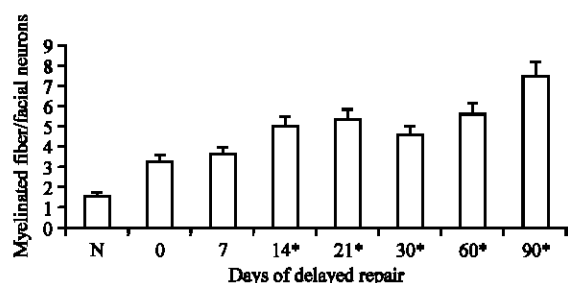


Fig. 8: The comparison of buccal branches' myelinated axon per motoneuron ratios among groups N is normal group \* Compared to group of immediate FFA ( $p < 0.05$ )

( $372.0000 \pm 23.3923$ ) and for 90 days ( $350.0000 \pm 30.2192$ ) ( $p = 0.129$ ) (Fig. 7) for HRP facial neurons.

**Myelinated axon per motoneuron ratio:** The myelinated axon per motoneuron ratio of normal group was  $1.6100 \pm 0.1778$ , which was lower than those of sutured groups ( $p < 0.05$ ); those of groups of immediate FFA ( $3.2869 \pm 0.4258$ ) and delayed FFA for 7 days ( $3.6105 \pm 0.2200$ ) were lower than other groups of delayed FFA ( $p < 0.05$ ), but there was no statistically significant difference between them ( $p = 0.260$ ). The myelinated axon per motoneuron ratio of group of delayed FFA for 90 days ( $7.1408 \pm 0.7306$ ) was higher than other sutured groups ( $p < 0.05$ ). There was no significant difference between those of groups of delayed FFA for 14 days ( $4.9247 \pm 0.4533$ ) and for 30 days ( $4.5637 \pm 0.6245$ ) ( $p = 0.209$ ). There was no significant difference between those of groups of delayed FFA for 14 days, for 21 days ( $5.2951 \pm 0.6051$ ) and for 60 days ( $5.5441 \pm 0.4062$ ) ( $p = 0.085$ ) (Fig. 8).

## DISCUSSION

Nerve regeneration following trauma is a complicated process and involves various biological, physical and chemical factors. When a nerve injury occurs, the connection between neuron pericaryon and axon is broken. The number of regenerated axons entering the distal nerves determines the effect of nerve regeneration. It has been reported that considerable numbers of perikaryons die after nerve trauma. The closer the neuron pericaryon was to the trauma site, the severe was the injury to it. This death was reported to be due to apoptosis and confirmed by the change in shape of the pericaryon, which is a typical characteristic of apoptosis. The process further included DNA degradation, while the survived neurons changed shape at biological and molecular levels. This was called the alternation of neuron from "transmission" to "growth" (Fu and Gordon, 1997). After trauma, the axon extends during nerve regeneration, with the regeneration of axonal terminals to form synaptic connections with the target tissue, which results in the recovery of form and function. Neuron pericaryon is the nutrition center of a cell and is responsible for regeneration of axon (Burnett and Zager, 2004; Fenrich and Gordon, 2004).

Some researchers believe that nerve suture delayed for some time may reduce the axons of the nerve sheath growing into the target muscle, accompanied by the target muscle atrophy, suggesting that delay in muscle contraction can occur after nerve regeneration (Fu and Gordon, 1995). This can further change the nature of muscle fibers irreversibly (Constantinidis *et al.*, 2003; Jergovic *et al.*, 2001). According to the results of facial nerve EMG, the therapeutic effect of suture delayed for 7 days was equal to or better than that of immediate suture group. The therapeutic effect of suture delayed for 60 days and 90 days was worse than that of immediate suture group and other group of delayed suture and the therapeutic effect of suture delayed for 14, 21 and 30 days was almost equal.

In the experiment, 2 mg HRP was injected on one side of normal male guinea pigs' vibrissa pad. The HRP-labeled neurons in nerve sutured groups were less than that of normal group. It was observed that longer delay in administration of suture was inversely related to the number of HRP neurons (Fig. 7). Two months after nerve suture, HRP-labeled neurons of immediate FFA group were more than those of groups of delayed FFA. Among all the groups, HRP-labeled neurons of groups of delayed FFA for 60 days and 90 days were the least.

Several researches in China and internationally have confirmed that the death of sensory nerves and motor nerves can be totally or partially prevented, if external NTFs are supplied to the traumatic nerves (Spector *et al.*, 1993; Baumgartner and Shine, 1997, 1998). The faster the nerve grows, the sooner the connection between the neuron and the target organ recovers. This in turn prevents the death of the neuron. Orlando's research using hypoglossal nerve -facial nerve anastomosis (HFA) indicated that nerve regeneration can be accelerated if facial nerve repair is delayed for appropriate time (14 or 56-112 days) (Guntinas-Lichius *et al.*, 2000a).

It was observed that, if sutures were delayed for too long, for the period when the connection between neurons and target organ was blocked, there was an increase in neuronal deaths. However, it was seen that the survival rate of neurons of properly delayed suture could be as high as that of immediate suture. After trauma, Schwann cells in distal facial nerves produced some substance to promote nerve regeneration. Maria Adele Rueger discovered that the production of 43 kDa (one of neuronal growth associated proteins, molecular mass=43 KDa, PI 4.3, also called GAP-43), the nerve-growth promoting protein, erbB2 and erbB4, the neuromedin receptors, by Schwann cells could reach peak levels several weeks after nerve trauma. The amount and activity of Schwann cells do not decrease until 32 weeks after the trauma (Rueger *et al.*, 2008; Mirsky and Jessen, 1999). The expressions of various NTFs, such as BDNF and GDNF and their receptors inside the neurons and distal Schwann cells will multiply after nerve trauma (Hoke *et al.*, 2006). Hoke *et al.* (2006) found that 48 hours after nerve trauma, distal Schwann cells started to express the mRNA of glial cell line-derived neurotrophic factor (GDNF). A high density was maintained during six months after nerve trauma (Hoke *et al.*, 2002). If the traumatic facial nerve was repaired at a favorable time when the factors inhibiting nerve regeneration, such as degradation product of myelin sheath and GIF (Yuguchi *et al.*, 1995) were at low levels and the factors promoting nerve regeneration, such as neurotrophic factor, were at high levels, a satisfying regeneration was obtained. In case, the microenvironment for nerve regeneration was the best, nerve regeneration after trauma accelerated and time frame for the arrival of the regenerated axons at the target tissue was greatly shortened (Guntinas-Lichius *et al.*, 2000b). The total time of properly delayed suture was not longer than that of immediate suture when the connection between the neurons and the target organ was blocked. Without innervation for long, the distal Schwann cells will atrophy and apoptise and their ability of promoting nerve

regeneration will decrease considerably (Sulaiman and Gordon, 2009; Saito and Kanje, 2009) and the GDNF synthesized by Schwann cells will reduce greatly (Hoke *et al.*, 2002). This causes slow nerve growth or failed regeneration and lowered survival rate of neurons. The regenerated axon usually appears at the last Ranvier's node of the proximal stump of the trauma, so it is called "nodal" or "collateral" branch. In general, the formation of a branch is finished within 3 h after trauma (Torigoe *et al.*, 1996), in which there are many variably sized growth vesicles. For the specificity of nerve regeneration in extending and re-innervation, there were mainly two mechanisms. The first being neural tropism in which degenerated distal nerves and the target organ can synthesize and secrete some soluble, diffusible proteins, which attract or repel the regenerated axons to grow toward the target tissue (Gundersen and Barrett, 1979; Kuffler, 1989; Baier and Bonhoeffer, 1992). The second mechanism is filtering mechanism for regenerated axons, where proximal nerves regenerate, there are some "recognition molecules" for guiding the correctly regenerated axons towards growth and stopping the growth of incorrectly regenerated axons (Schachner, 1992; Martini *et al.*, 1992).

In fact, nerve regeneration cannot restore the complete functionality of the nerve as it was before trauma. In terms of the linkage and group motions of regenerated facial nerves in clinics and experiments, there are quite nonspecific re-innervations and wrong re-innervations in facial nerve regeneration. It can be said that even if the neurons wrongly re-innervate new regions, it does not mean that they will give up the original ones. Due of the collateral branches, a neuron can innervate various target organs in different regions at the same time (Monserrat and Benito, 1988; Thomas, 1988). The amount of the collateral nerve branches not eliminated is directly related with the degree of neuronal non-specificity and wrong re-innervation (Thomander, 1984). Only retrograde tracing of facial neurons cannot reflect the number of the collateral branches. No matter how many of them exist, a neuron can be stained only once. The myelinated fibers in the regenerated buccal branches of facial nerve were counted and it was observed that myelinated axon per motoneuron ratio can objectively reflect the number of collateral branches and indirectly, can affect the degree of non-specificity and incorrect re-innervation in the facial nerve regeneration.

In the experiment, the myelinated axon per motoneuron ratio of normal guinea pig was the lowest, which thoroughly proved that the innervation of normal guinea pigs' facial nerve was the most specific. The

ratios of group of immediate FFA and group of delayed FFA for 7 days were lower than other groups of delayed FFA. The ratio of group of delayed FFA for 90 days was the highest.

For now, it is still an open question whether signals induce branch shoot or cause the degeneration and disappearance of the collateral branches after nerve trauma. Some experts think that most of the dendrites entering the neuron pericaryon inhibit the neuron and that the reduction of dendrites after trauma makes the surviving neurons over-excited, leading to over regeneration of the fibers and lowered specificity of regeneration (Graeber *et al.*, 1993).

The result of this experiment corresponds to some previous theories, which report that the less time the regenerated nerve takes to reach the target organ, the lesser the collateral branches grow (Guntinas-Lichius *et al.*, 2000a). When the nerve successfully arrives at the target organ, it may produce some factors or absorb some factors from the target organ to inhibit a further growth of the collateral branches (Diamond *et al.*, 1976). Further, Nitric Oxide (NO) may also play an important role in the process (Wang *et al.*, 1995).

### CONCLUSION

This study showed that when immediate repair of facial nerve trauma was not permissible, delayed repair was also practicable. If the facial nerve is repaired within 60 days, reasonably good curative effect can be expected.

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