

EXPERIMENTAL STUDY

Experimental research on end-to-side anastomosis of peripheral nerves and effect of FK506 on end-to-side anastomosis

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Abstract: *Objective:* To study the effects of end-to-side anastomosis of initially-denatured nerves at different times. *Method:* 60 male Wistar albino rats were used to fabricate animal models for the experiment on end-to-side anastomosis of peripheral nerves and 50 female Wistar albino rats were used to fabricate animal models for the experiment on the effect of FK506 on end-to-side anastomosis. Bilateral common peroneal nerve, tibialis anterior muscle electrophysiological and histological examinations, tibialis anterior muscle wet muscle weight determination, and motor end plate examination were performed 3 months after operation. *Results:* All recovery rates of action potential, single muscle contraction force and tetanic contraction force of the FK506 experimental group are significantly higher than those of the control group and the sectional area of muscle fiber is also higher than that of the control group of normal saline. *Conclusion:* The best time for end-to-side anastomosis of nerves should be controlled within 2 weeks and the effect of end-to-side anastomosis of nerves will gradually become unsatisfactory. FK506 plays a role in promoting functional rehabilitation following nerve end-to-side anastomosis (Tab. 7, Fig. 4, Ref. 31). Text in PDF www.elis.sk. Key words: peripheral nerves, end-to-side anastomosis, FK506, motor end plate, electrophysiological.

Clinically we often encounter cases with distal end of the nerve intact and proximal end avulsed or severely injured which are not eligible for nerve reconstruction by the method of regular end-to-end anastomosis. Another nerve will be injured and the functions of its innervation area sacrificed although the neural transplantation or neural transposition can reconstruct the injured nerve (1, 2). As a method for neural restoration, the end-to-side anastomosis of peripheral nerves repairs nerves through lateral branch sprouting with the normal nerve as a donor without influencing the functions of the donor nerve. In recent years, extensive and in-depth researches have been conducted worldwide and some clinical applications have been performed (3). The dominating muscles lose innervation, atrophy, degenerate and fibrillate after peripheral nerves are injured. The motor end plate degenerates and disappears. Whether the motor end plate can regenerate after its disappearance and nerve regeneration is still controversial (4). Many scholars have found that nerves can regenerate and obtain partial physiological functions through end-to-side anastomosis but many issues remain to be solved on applying nerve end-to-side anastomosis to clinic treatment of nerve injury and making it a regular surgical method (5).

Nerves cannot grow without appropriate microenvironment. As a new method for dealing with nerve defect and nerve injury with missing proximal end, end-to-side anastomosis has been increasingly drawing people's attention to how to provide an appropriate microenvironment for lateral branch sprouting and promote lateral branch growth in addition to sophisticated microsurgical techniques and methods (6). In recent years, a series of researches have shown that FK506 also plays a vital role in promoting nerve growth in addition to its immunosuppressive action and its eutrophic effect and neuroprotective effect have been increasingly drawing people's attention (7).

Material and methods

Model making and grouping

Making and grouping of animal models for the experiment on end-to-side anastomosis of peripheral nerves.

Sixty female Wistar albino rats (supplied by SHANGHAI SLAC LABORATORY ANIMAL CO. LTD.) weighing 200–220 g were divided into 6 groups with 10 for each group. Sterilization and surgery were performed on the posterior-lateral of the right thigh to expose the ischiadic nerve and common peroneal nerve after anesthesia through intraperitoneal injection with 1 % thiopental sodium. It was cut 5 mm below the branch of the common peroneal nerve. The proximal end was ligated, reversed and sutured to the adjacent muscles. End-to-side anastomosis was performed for one of the groups. A small window with diameter of approxi-

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mately 1mm was opened on the epineurium of the tibial nerve. The distal end of the common peroneal nerve was anastomosed end-to-side to the window of the tibial nerve with microsurgical suture lines. The incision was sutured layer by layer and end-to-side anastomosis was performed for the five remaining groups 1, 2, 4, 8 and 16 week(s) thereafter, respectively (8).

Making and grouping of animal models for the experiment on effect of FK506 end-to-side anastomosis of peripheral nerves

Fifty female Wistar albino rats (supplied by SHANGHAI SLAC LABORATORY ANIMAL CO. LTD.) weighing 200–220 g were divided into 6 groups with 10 for each group. Sterilization and surgery were performed on the posterior-lateral of the right thigh to expose the ischiadic nerve and common peroneal nerve after anesthesia through intraperitoneal injection with 1 % thiopental sodium. It was cut 5 mm below the branch of the common peroneal nerve. The proximal end was ligated, reversed and sutured to the adjacent muscles. End-to-side anastomosis was performed for one of the groups. A small window with diameter of approximately 1mm was opened on the epineurium of the tibial nerve. The distal end of the common peroneal nerve was anastomosed end-to-side to the window of the tibial nerve with microsurgical suture lines. The incision was sutured layer by layer. The left side served as normal control. The animals were divided into 5 groups with 10 for each group. The lateral muscles of the right crura of the animals in the five groups were injected with normal saline and FK506 diluted with normal saline respectively, at a dose of 1 mg/kg/d, 2 mg/kg/d, 4 mg/kg/d, and 8 mg/kg/d respectively, for two consecutive weeks, after the day of operation (including the same day), and raised in cages for 3 months regularly (9).

General observation

Bilateral ischiadic nerves were dissected out and also the common peroneal nerve along the original incision 3 months after operation. Separation was performed until anterolateral muscle group of the crus innervated along with the common peroneal nerve to observe the appearance of the nerves and muscles.

Electrophysiological detection

The branches were sheared off and tibialis anterior muscle was dissected out after the bilateral ischiadic nerve and common peroneal nerve were dissected out. A needle recording electrode was inserted into tibialis anterior muscle in a direction consistent with the muscle fiber. A 0.5" acupuncture needle was inserted into the rat tail and grounded. The stimulating electrode was hooked with the ischiadic nerve and stimulation was performed for several times after the stimulus intensity was adjusted. The diagram was frozen after the diagram for induced composite action potentials was stable and the starting points of artifacts and action potentials were clear. The amplitudes of various composite action potentials

were read. Finally, distal tendon of tibialis anterior muscle was cut and sutured with silk thread for fixation. The other end of the silk thread was connected to the recorder. Electrical stimulation was performed on the proximal end of the anastomotic stoma at a stimulation voltage of 2 V, time interval of 0.5 s and tetanic stimulation frequency of 30 times/s. Single muscle contraction force and tetanic contraction force were recorded.

Histological examination

Histological examination for the common peroneal nerve: segments of the common peroneal nerve were cut at 3mm from the anastomotic stoma, placed into the glutaraldehyde fixing solution buffered with phosphoric acid, fixed for 24 hours at 4 °C, embedded directionally with Epon812, cut into semi-thin transverse sections, stained with toluidine blue and observed with an optical microscope.

Determination of wet muscle weight: tibialis anterior muscles on both sides were taken completely and weighed on an analytical scale (0.0001 g).

Sectional area of muscle fibre: a muscle strip with length of approximately 10 mm and diameter of 4 mm was cut from the middle and lower section of the posterior tibialis anterior muscle and weighed, placed into 4 % paraformaldehyde, fixed at 4 °C for 24 hours, embedded with paraffin, cut into 5 µm-thick sections, stained with HE, dehydrated, clarified and sealed. It was observed under an optical microscope.

Motor end plate: the muscle belly was cut from the middle and upper part of the posterior tibialis anterior muscle and weighed, cut into longitudinal sections, stained with ACHE and observed for morphology and staining of motor end plate under an optical microscope.

Statistical treatment

The number of common peroneal nerve fibres, cross sectional area of the tibialis anterior muscle fibres, area and staining of motor end plate were determined with the HPIAS-1000 high-definition colour pathological graphic analysis system. All experimental data obtained used the ratio between the value measured on the right side and that on the left side (i.e. recovery rate) and were expressed with mean and standard deviation (\pm s). A variance analysis and statistical treatment were performed for the result.

Results

General observation of rats for the experiment on end-to-side anastomosis of peripheral nerves

The common peroneal nerves of all groups on the experimental side recover to a certain extent. The common peroneal nerves of the groups of 0, 1 and 2 week(s) post initial denaturation are mellow and plump with excellent glossiness and anterolateral

Tab. 1. Comparison of recovery rates of wet muscle weight among various experimental.

Initial Denaturation Time (group)	0 Week	1 Week	2 Week	4 Week	8 Week	16 Week
Numb of Samples	10	10	10	10	10	10
Wet Muscle Weight	0.767 \pm 0.104	0.773 \pm 0.112	0.779 \pm 0.110	0.527 \pm 0.098	0.322 \pm 0.082	0.134 \pm 0.061

Tab. 2. Comparison of recovery rates of nerve fibers among various experimental groups.

Initial Denaturation Time (group)	0 Week	1 Week	2 Week	4 Week	8 Week	16 Week
Num of Samples	10	10	10	10	10	10
Number of Nerve Fibers	0.825±0.126	0.836±0.127	0.799±0.118	0.621±0.107	0.436±0.097	0.265±0.088

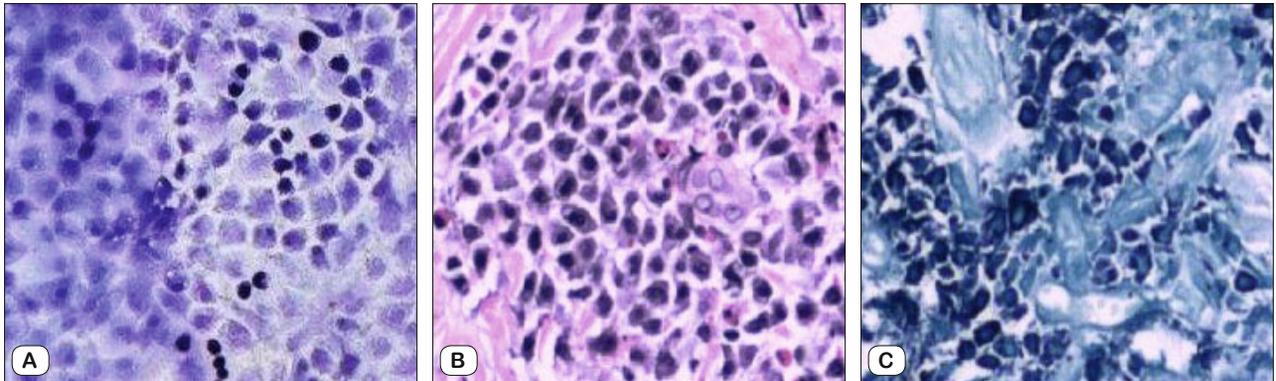


Fig. 1. Histological Examination of Cross Sections of Lateral Common Peroneal Nerves (×200, stained with toluidine blue). A – cross sections of lateral common peroneal nerves treated with end-to-side anastomosis 1 week post initial denaturation; B – cross sections of lateral common peroneal nerves treated with end-to-side anastomosis 4 weeks post initial denaturation; C – cross sections of lateral common peroneal nerves treated with end-to-side anastomosis 16 weeks post initial denaturation.

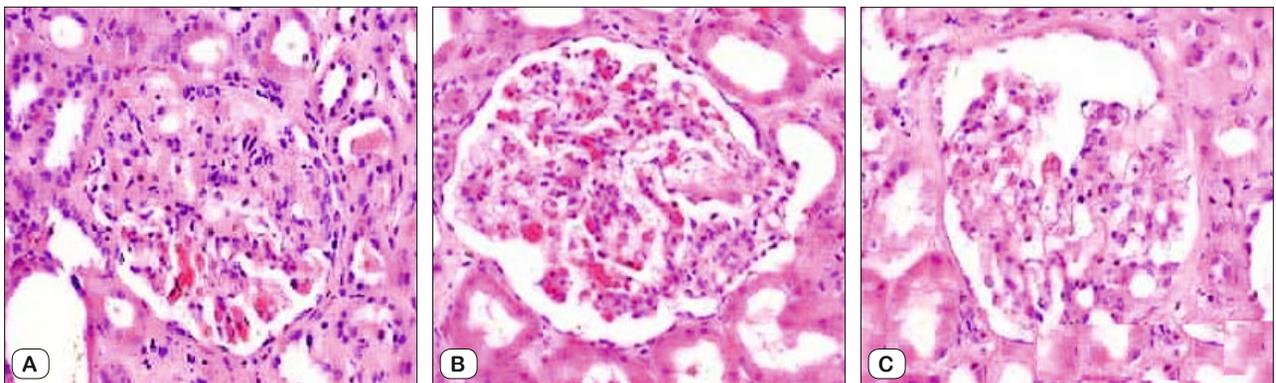


Fig. 2. Histological Examination of Cross Section of Tibialis Anterior Muscle. A – cross sections of lateral tibialis anterior muscle treated with end-to-side anastomosis 1 week post initial denaturation; B – cross sections of lateral tibialis anterior muscle treated with end-to-side anastomosis 4 weeks post initial denaturation; C – cross sections of lateral tibialis anterior muscle treated with end-to-side anastomosis 16 weeks post initial denaturation.

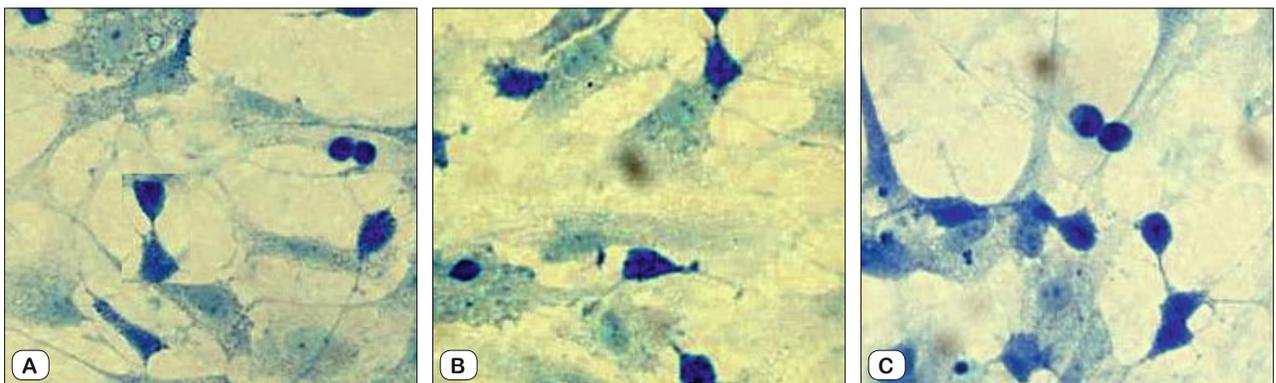


Fig. 3. Histological Examination of Motor End Plates. A – cross sections of motor end plate of lateral tibialis anterior muscle treated with end-to-side anastomosis 1 week post initial denaturation; B – cross sections of motor end plate of lateral tibialis anterior muscle treated with end-to-side anastomosis 4 weeks post initial denaturation; C – cross sections of motor end plate of lateral tibialis anterior muscle treated with end-to-side anastomosis 16 weeks post initial denaturation.

Tab. 3. Comparison of recovery rates of sectional area of muscle fiber among various experimental groups.

Initial Denaturation Time (group)	0 Week	1 Week	2 Week	4 Week	8 Week	16 Week
Numb of Samples	10	10	10	10	10	10
Cross Sectional Area Of Muscle Fiber	0.778±0.127	0.762±0.124	0.748±0.118	0.616±0.106	0.483±0.089	0.305±0.087

Tab. 4. Comparison of recovery rates of area and staining of motor end plates of various experimental groups.

Initial Denaturation Time (group)	0 Week	1 Week	2 Week	4 Week	8 Week	16 Week
Numb of Samples	10	10	10	10	10	10
Area of Motor End Plate	0.712±0.117	0.697±0.104	0.724±0.108	0.553±0.099	0.398±0.095	0.227±0.083
Staining of Motor End Plate	0.792±0.128	0.811±0.130	0.784±0.115	0.606±0.11	0.472±0.097	0.362±0.086

Tab. 5. Comparison of electrophysiological indexes.

Initial Denaturation Time (group)	0 Week	1 Week	2 Week	4 Week	8 Week	16 Week
Numb of Samples	10	10	10	10	10	10
Recovery Rate of Action Potential	0.713±0.112	0.726±0.118	0.717±0.109	0.554±0.095	0.332±0.087	0.184±0.082
Recovery Rate of Single Contraction Force	0.685±0.096	0.704±0.103	0.692±0.094	0.467±0.091	0.318±0.082	0.164±0.081
Recovery Rate of Tetanic Contraction Force	0.672±0.098	0.690±0.097	0.661±0.088	0.478±0.082	0.341±0.081	0.172±0.079

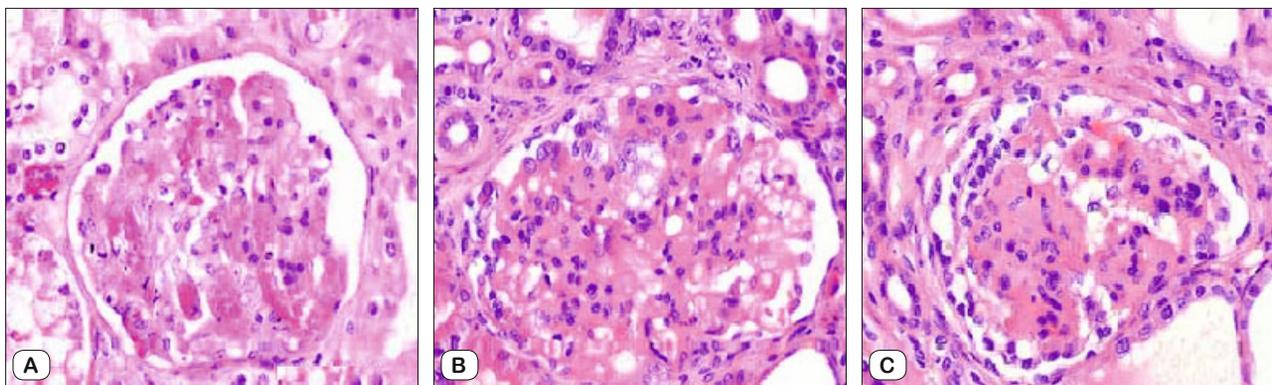


Fig. 4. Histological Examination of Cross Section of Tibialis Anterior Muscle. A – cross section of tibialis anterior muscle of the normal saline group; B – cross section of tibialis anterior muscle of the 2 mg/kg/d group.

muscle groups of the crus connected are plump and elastic. They approximate the normal side. The common peroneal nerves of the groups of 4, 8 and 16 weeks post initial denaturation are relatively thin and small and muscle development is relatively poor. Such conditions are particularly significant in the group of 16 weeks post initial denaturation.

Wet weight of the tibialis anterior of rats for the experiment on end-to-side anastomosis of peripheral nerves

The recovery rates of wet weight of the tibialis anterior of the groups of 0, 1 and 2 week(s) post initial denaturation are higher than those of the groups of 4, 8 and 16 weeks post initial denaturation. The statistical result shows no significant difference among the groups of 0, 1 and 2 week(s) post initial denaturation ($p > 0.05$). The recovery rate of wet muscle weight of the tibialis anterior muscle is decreasing with prolonging time of initial denaturation among the groups of 2, 4, 8 and 16 weeks post initial denaturation.

A significant difference was showed between the 2-week group and 4-week group, 4-week group and 8-week group, and 8-week group and 16-week group ($p < 0.05$) (Tab. 1).

Neurohistological examination of rats for the experiment on end-to-side anastomosis of peripheral nerves

Compared with various groups with longer initial denaturation time, the cross sectional area of common peroneal nerve is large, the number of regenerated nerve fibres is big, arranged in sequence, the nerve fibres are thick, myelin sheath is thick and the number of inter-nerve tract connective tissues is small on the experimental side of the groups of 0, 1 and 2 week(s) post initial denaturation. The statistical results show no significant difference among the groups of 0, 1 and 2 week(s) post initial denaturation ($p > 0.05$). The recovery rate of the number of regenerated nerve fibres is decreasing with prolonging time of initial denaturation among the groups of 2, 4, 8 and 16 weeks post initial denaturation.

Tab. 6. Comparison of recovery rates of sectional area of muscle fiber among various experimental groups.

Initial Denaturation Time (group)	Normal Saline Group	1mg/kg/d	2mg/kg/d	4mg/kg/d	8mg/kg/d
Numb of Samples	10	10	10	10	10
Cross Sectional Area Of Muscle Fiber	0.365±0.092	0.551±0.104	0.742±0.113	0.782±0.118	0.711±0.121

Tab. 7. Comparison of electrophysiological indexes.

Initial Denaturation Time (group)	Normal Saline Group	1mg/kg/d	2mg/kg/d	4mg/kg/d	8mg/kg/d
Numb of Samples	10	10	10	10	10
Recovery Rate of Action Potential	0.381±0.091	0.512±0.096	0.693±0.104	0.722±0.110	0.681±0.106
Recovery Rate of Single Contraction Force	0.376±0.095	0.524±0.101	0.698±0.099	0.735±0.109	0.713±0.110
Recovery Rate of Tetanic Contraction Force	0.394±0.086	0.589±0.092	0.726±0.108	0.763±0.117	0.751±0.113

A significant difference was shown between the 2-week group and 4-week group, 4-week group and 8-week group, and 8-week group and 16-week group ($p < 0.05$) (Tab. 2 and Fig. 1).

Histological examination of muscle and determination of cross sectional area of muscle fibres of rats end-to-side anastomosis of peripheral nerves

Compared to various groups with longer initial denaturation time, the structure of the cross section of tibialis anterior muscle on the experimental side is clear and muscle fibre is thick and close to the morphological structure of normal muscles in the groups of 0, 1 and 2 week(s) post initial denaturation. The statistical result shows no significant difference among the groups of 0, 1 and 2 week(s) post initial denaturation ($p > 0.05$). The recovery rate of the cross sectional area of muscle fibre is decreasing with prolonging time of initial denaturation among the groups of 2, 4, 8 and 16 weeks post initial denaturation. A significant difference was shown between the 2-week group and 4-week group, 4-week group and 8-week group, and 8-week group and 16-week group ($p < 0.05$) (Tab. 3 and Fig. 2).

Examination of motor end plates of rats for the experiment on end-to-side anastomosis of peripheral nerves

Compared to various groups with longer initial denaturation time, the area of the motor end plate on the experimental side is large, the structure is clear, the staining colour is dark and close to that on the normal side in the groups of 0, 1 and 2 week(s) post initial denaturation. The statistical result shows no significant difference in the recovery rates of the area and staining of motor end plate among the groups of 0, 1 and 2 week(s) post initial denaturation ($p > 0.05$). The recovery rate of the area and staining of motor end plate is decreasing with prolonging time of initial denaturation among the groups of 2, 4, 8 and 16 weeks post initial denaturation. A significant difference was shown between the 2-week group and 4-week group, 4-week group and 8-week group, and 8-week group and 16-week group ($p < 0.05$) (Tab. 4 and Fig. 3).

Electrophysiological Examination of Rats for the Experiment on End-To-Side Anastomosis of Peripheral Nerves

Compared to various groups with longer initial denaturation time, the recovery rates of complex muscle action potential, single muscle contraction force and tetanic contraction force are higher in the groups of 0, 1 and 2 week(s) post initial denaturation. The statistical result shows no significant difference in the recovery rates of complex muscle action potential, single muscle contraction force and tetanic contraction force among the groups of 0, 1 and 2 week(s) post initial denaturation ($p > 0.05$). The recovery rates of complex muscle action potential, single muscle contraction force

and tetanic contraction force are decreasing with prolonging time of initial denaturation among the groups of 2, 4, 8 and 16 weeks post initial denaturation. A significant difference was shown between the 2-week group and 4-week group, 4-week group and 8-week group, and 8-week group and 16-week group ($p < 0.05$) (Tab. 5).

Histological examination of muscle and determination of cross sectional area of muscle fibre of experimental animals undergoing anastomosis of peripheral nerves with FK506

Compared with the normal saline control group, the structure of cross section of tibialis anterior muscle is clear and muscle fibre is thick and close to the morphological structure of normal muscles on the FK506 experimental side. The ratio of cross sectional area of muscle fibre shows that there is significant difference between the FK506 experimental group and the normal saline control group ($p < 0.05$). There is no significant difference among the groups with a dose more than 2 mg/kg/d ($p > 0.05$) of various FK506 experimental groups but they are significantly higher than groups with a dose of 1 mg/kg/d ($p < 0.05$) (Tab. 6 and Fig. 4).

Electrophysiological test for experimental animals undergoing end-to-side anastomosis with FK506

All recovery rates of complex muscle action potential, single muscle contraction force and tetanic contraction force of various FK506 experimental groups are significantly higher than those of the normal saline control group ($p < 0.05$). There is no significant difference among the groups with a dose more than 2 mg/kg/d ($p > 0.05$) of various FK506 experimental groups but they are significantly higher than groups with a dose of 1 mg/kg/d ($p < 0.05$) (Tab. 7).

Discussion

The most significant changes of the nerve fibre are disintegration of myelin sheaths and axons and proliferation of Schwann cells. The substance resulting from disintegration and disruption of myelin sheaths and axons is removed by macrophages in the early stage after nerve injury (10). A large number of karyons can be observed after nerve injury. With the high level of karyons can neurotmesis last until 2–3 weeks after neurotmesis. Most of these karyons are considered to be Schwann karyons and the remaining ones may be intima cells and a small number of macrophage karyons (11, 12). With increasing number of Schwann cells, Schwann cells are arranged regularly and close to the internal surface of the intima passage to form typical Bungner's bands, which play a basic role in nerve regeneration and impose mechanical chemotactic effects on nerve regeneration (13). From this perspective, most scholars think that the best restoration period shall be 7-14days after nerve injury (14).

The muscle loses innervation leading to amyotrophy and fibrosis, particularly intrinsic muscles of hand, of which the rate of degeneration and fibrosis is higher than that of other parts (15, 16). The motor end plate will also degenerate after denervation. Song et al. have found that there is no significant difference in staining and number between the motor end plates and the normal ones 2–4 weeks after the muscles was denervated; the changes of the motor end plates are more significant, i.e. the staining colour becomes lighter, the staining is not uniform and the number of the motor end plates is decreasing, the acetylcholine esterase content of the motor end plates is only 54.4 % of the normal ones, 15.1 % of the normal ones after 4 months and the motor end plates cannot be stained after 5 months based on a quantitative analysis on degeneration of the motor end plates. It is still controversial whether the motor end plate can regenerate following nerve regeneration with disappearance of the motor end plate (17). We can learn from the above that the motor end plate is maintained stable, which may be related to Wallerian degeneration and Schwann cell proliferation within 1 month after muscle denervation. Certain chemical substance may be released thus delaying regression of the motor end plate during the process of Wallerian degeneration and Schwann cell proliferation (18). Completion of Wallerian degeneration and Schwann cell proliferation may lead to acceleration of motor end plate degeneration (19). From this perspective, most scholars think that neural repair shall occur as early as possible after nerve injury (20).

The long-distance tissue defect resulting injury of peripheral nerves is a difficult for clinical treatment and repaired by the method of autologous neural transplantation (21). However, autologous neural transplantation also has such disadvantages as limited sources and dysfunction of innervated region etc. and is not applicable to the condition where the proximal nerve trunk is severely damaged or defective, e.g. brachial plexus avulsion and the like (22). To treat such injury, the distal and lateral end of the injured nerve can be anastomosed to the adjacent healthy nerves by means of end-to-side to obtain reconstruction of sensory and motor functions (23, 24). The experiment further studies the effects of initially-denatured nerve end-to-side anastomosis at different times to seek the best time for the nerve end-to-side anastomosis based on relevant experimental researches. The experimental results show that nervous lateral branch growth indexes, target muscle and motor end plate histological indexes and neuromuscular electrophysiological indexes on the experimental side of the groups of 0, 1 and 2 week(s) post initial denaturation are superior to various groups with longer time and the effect of nerve end-to-side anastomosis is becoming poorer with prolonging time. The experimental results indicate that the best time for nerve end-to-side anastomosis is within 2 weeks after injury and a surgery shall be performed as early as possible after 2 weeks to achieve the best repair effects.

The neurotrophic effects of FK506 and its derivatives are stronger than such polypeptide neurotrophic factors as epidermal growth factors, nerve growth factors, ciliary cellular neurotrophic factors etc. (25). Yan et al have found that GPI-1046 can significantly promote dopaminergic neurons of the corpus striatum of rats to

sprout neurites while ciliary cellular neurotrophic factors do not have such effects based on a comparison between the GPI-1046 and ciliary cellular neurotrophic factors with the strongest effects among various neurotrophic factors [(26). When polypeptide neurotrophic factors act on normal animals, neurons can produce many scattered neurites. Neurones can sprout scattered neurites thus leading to hyperesthesia if the neurotrophic factors act on normal sensory neurones (27). No such phenomenon was observed in neither peripheral nerves nor nervus centralis after use of FK506 (28). In addition, all polypeptide neurotrophic factors only function after being intrathecally injected and FK506 and derivatives can penetrate the blood. For nerve barrier, it will function by oral administration or injection (29). Obviously, using FK506 as nerve nutrient is advantageous (30).

Based on the pharmacologic action of FK506, its mechanism of action may be that it accelerates the pathological process of Wallerian degeneration, promotes proliferation of Schwann cells and secretion of neurotrophic factors, protects neurons and promotes sprouting of axons and lateral branches, inhibits interneural proliferation and autoimmune response and reduces obstruction in nerve regeneration thus raising the quality and rate of nerve regeneration and growth of lateral branches and promoting functional rehabilitation of nerves (31). The mechanism of dose-effect relationship is expected to be further studied. As a new method for repairing nerve defect and nerve injury with missing proximal end, the end-to-side anastomosis of peripheral nerves has achieved satisfactory effects in clinical application. In the research, the immunosuppressive agent FK506 is injected within the target muscle to promote growth and functional rehabilitation of lateral nervous branches. The method used is simple and reliable and provides an experimental basis for applying FK506 after repair of injured peripheral nerves by scholars in the same profession.

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