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Effect of FK506 nanospheres on regeneration of allogeneic nerve after transplant

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ABSTRACT

Objective: To discuss effect of FK506 nanospheres used at different time on the regeneration of allogeneic nerve after transplant. **Methods:** Single emulsion-solvent evaporation method (O/W) was adopted to prepare the FK506 nanospheres and the tibial nerve of rats after allogeneic transplantation. FK506 nanospheres were used in group A after operation immediately, in group B in 24 h after operation, and in group C in 3 d after operation while FK506 nanospheres were not used in group D; in the 4th, 8th and 12th week after operation respectively, general observation of transplanted nerves, histological examination, image analysis of myelinated fibers, wet-weight determination of musculi triceps surae, retrogradely labeling of neurons by the fluorescein and electrophysiological comparison of bilateral tibial nerve were carried out. **Results:** FK506 nanospheres can be degraded and absorbed quickly. The neural regenerations in group A and B were similar, which were both much better than those in group C and D. The difference was statistically significant and so was the difference between group C and D. **Conclusions:** Drug release rate of FK506 nanospheres is accordant with the regeneration law of damaged nerves and the local application can promote the regenerations of nerves. The effect would be better if the drug is used in earlier period (within 24 h).

1. Introduction

The defects of peripheral nerve are very common. Nerve autograft is the first choice in clinic. However, limited resources of nerve autograft become the key factor which restricts the development of it. Currently, allogeneic nerve transplant is paid more and more attention to solve such a problem. It is beneficial to the internal structure of neural network of neural transplant. Besides, it is widely available, which can acquire the transplanted nerve with a similar length to the damaged one. Immunological rejection is the largest obstacle for the application of allogeneic nerve transplant. Through experiments, Bain *et al*[1] argued that axonal regeneration phenomenon appeared after allogeneic nerve transplant and the regeneration distance was only several millimeters. Host cell response occurred around the transplanted nerves and consequently rejection appeared. Immunosuppressive agent FK506 can increase the regeneration rate of peripheral nerves to more than 2 mm/d. It is thought to be the first choice in the field due to its strong promotion of neural regeneration[2,3].

Nanospheres are currently one of the most important products of nano technology, which is widely applied in the fields such as production of pharmaceuticals, disease diagnosis, environmental monitoring, purification treatment and so on[4]. Nanospheres, as the carrier of drugs, possess excellent biocompatibility which makes it not identified by the immune system. It carries the drug directly to the lesion, releases it slowly, increases the local concentration of drugs and prolongs the effect of medicine. Consequently, the aims of slowly releasing drugs and targeted delivery are achieved. We supposed that FK506 nanospheres were applied to increase the drug concentration around the transplanted nerves, played the part in immunologic suppression and promotion of peripheral nerve regeneration and lessened the systemic toxic side effect. Our experiment used FK506 to make nanospheres with the function of slowing the release

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and put them around the segment of transplanted nerves to discuss the relation between the effect of promoting neural regeneration and the optimal medication.

2. Materials and methods

2.1. Preparation of FK506 nanospheres [5]

Single emulsion-solvent evaporation method (O/W) was adopted to prepare the FK506 nanospheres [P (DLLA-co-TMC)]. 100 mg P (DLLA-co-TMC) and a certain sum of FK506 were dissolved in 4 mL dichloromethane. After stirring, the polymer solution was slowly poured into 40 mL aqueous solution of 2% PVA by the injector. To avoid that the polymer settled on the stirrer, the stirring rate was kept at 250 r/min and the rate was adjusted to 600 r/min after about 30 s. After being emulsified for 5 min, the mixed solution was poured into 450 mL stirring distilled water. The solution was volatilized for 4-6 h under the condition of room temperature and pressure release. The acquired nanospheres were centrifuged and cleaned by distilled water for several times to remove the PVA at the surface of nanospheres. Then they were frozen and dried for 48 h (ALPHA2-4 freeze drier, CHRIST). P (DLLA-co-TMC) in the form of white powder was gained and stored in drier at room temperature.

2.2. Animal grouping and operation methods

72 SD male rats weighing 170-190 g were collected (provided by Laboratory Animal Center in Southern Medical University). They were divided into four groups randomly; then according to experimental design, the rats in each group can be divided into new groups again, the group with medication in 4th, 8th and 12th week after operation, and there were 6 rats in each new group. Group A: immediate medication was carried out after allogeneic transplantation; Group B: medication was carried out in the 24 h after allogeneic transplantation; Group C: medication was carried out in the 3 d after allogeneic transplantation; Group D: medication was not carried out after allogeneic transplantation. The use of FK 506 in group A, B and C lasted 30 d. 4% Nembutal (20 mg/kg) was injected in abdominal cavity to achieve anaesthesia. The nervi ischiadicus can be seen in the cut which located in right sciatic lower edge and was paralleled with ischium with the length of about 2 cm and its branches were separated. Nervi tibialis with the length of 0.6 cm in the place which was 0.5 cm away from the crotch of three nervi ischiadicus were cut off and collected. The fresh nerves of the donor were bridged in the place where the nerves were damaged and the nerve stump membrane was sewed up by adopting 11-0 noninvasive suture with ten times larger by means of operating microscope. Then according to the experimental design, FK506 nanospheres were directly placed in the transplanted segments of nerves (the release rate was 1 $mg*kg^{-1}*d^{-1}$ and the release process lasted 30 d). The cut was sewed up with 3-0 noninvasive suture and the rats were fed in single cage.

2.3. General situation and gross observation

The items included diet, mental state, aggressive nature, walking, local infection rate and death rate.

2.4. Detection of wet weight of musculi triceps surae

4% Nembutal (20 mg/kg) was injected in abdominal cavity to achieve anaesthesia respectively in the 8th and 12th week after operation. The rats were fixed and the musculi triceps surae of affected side were separated after routine disinfection and cut off at the starting and ending sites. Then the weight was weighed by means of analytical balance.

2.5. Histological observation and image analysis

4% Nembutal (20 mg/kg) was injected in abdominal cavity to achieve anaesthesia respectively in the 4th, 8th and 12th week after operation in group A, B and C. Then, normal saline was poured into the rats and the transplanted nerve in right side was found through the original cut. Nerve segments with the length of 0.5 cm respectively located in the middle, distal end and proximal end of the transplanted parts were got and then placed into 4% paraformaldehyde phosphate buffer solution and kept for 24 h. Paraffin sections were made and dyed by HE. They were observed and the numbers of medullated fibers were counted respectively in the 4th, 8th and 12th week after operation.

2.6. True blue retrograde tracing and microscopy

4% Nembutal (20 mg/kg) was injected in abdominal cavity to achieve anaesthesia and the transplanted nervi tibialis of right side were found out on the 5th day before taking samples. After the injection of 2 μL 2% true blue in the nerve 5 mm away from distal stoma of transplanted parts, the cuts were closed in turns and the rats were fed in single cage. After five days, anaesthesia was done again and thoracotomy was carried out. 50 mL normal saline and 200 mL 10% formaldehyde were infused by left ventricular puncture through the ascending aorta and the state was kept for one hour. 2-4 spinal cords and corresponding ganglion spinals were collected and fixed by 4% paraform. Then frozen sections were observed with the fluorescence microscope and photographed.

2.7.Electrophysiologic examination

After the anaesthesia by the injection of 4% Nembutal (20 mg/kg) into abdominal cavity of rats in the 12th week after operation, nervi tibialis of both sides and musculi triceps surae were collected. All the branches of the sciatic nerve except for right tibial nerve were cut off at the start. The recording electrode of Medtronic EMG analysis was placed in the muscle belly of musculi triceps surae and the acupuncture needle was cut in the tails of rats and touched the land. The exciting electrode was placed in the distal and proximal end of nervi tibialis. The conduction velocity of the nerves of affected side and the comparison with each own healthy side were acquired.

2.8. Statistical processing

All the data were symbolized by mean±sd. Software SPSS10.0 was used to make statistical description. The comparison of means in multiple samples was analyzed by One-Way ANOVA. According to homogeneity test of variance, L-S-D method was selected when there was overall homogeneity of variance; Tamhane's T2 method was adopted there was no homogeneity of variance. The comparison of means in two samples was examined by the mean of two non-paired samples *t*. When $P<0.05$, the difference was statistically significant.

3. Results

3.1. General situation and gross observation

The rats in group A, B and C were all normal and there was no obvious change in the sleep and diet. No rats died or walked with a limp; in group D, there were two rats with the swelling and ulcer in right lower limbs. In the 4th week after operation, the absorption in group A, B and C which adopted FK506 nanospheres was excellent and there were hardly drug residues or atrophied connective tissue coating or hypertrophic scar and the nervi tibialis were easy to be separated from the tissues; in group D, there were a lot of hypertrophic scars in transplanted parts which adhered to the surrounding tissues and were not easy to be separated from the tissues.

3.2. Detection of wet weight of musculi triceps surae

In the 8th week after operation, the musculi triceps surae of rats in group D atrophied seriously, the following was those in group C and the atrophy in group A and B was not severe; in the 12th week after operation, the musculi triceps surae in group A and B recovered fully and excellently and the secondary was the group C (Figure 1). In the 8th and 12th week after operation, through the comparison of the wet weight of musculi triceps surae in groups, the weights in group A and B were obviously heavier than those in group C and D and the difference was statistically significant ($P<0.05$); the weight in group C was heavier than that in group D and the difference was statistically significant ($P<0.05$, Table 1).

Table 1
Wet weight value of musculi triceps surae of affected side in 8th and 12th week after operation (mean±sd) (mg).

Groups	Sample size	8th week after operation	12th week after operation
Group A	6	753.6±40.2	959.2±41.4
Group B	6	749.8±42.7	936.4±38.6
Group C	6	626.4±35.6	769.6±30.4
Group D	6	525.6±20.3	530.2±28.5

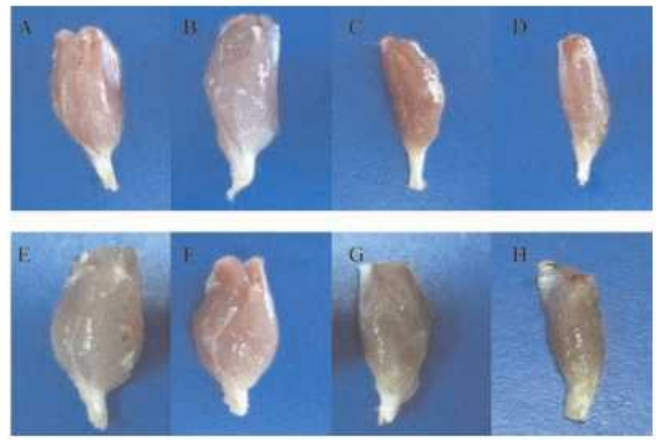

Figure 1. Appearance of rat musculi triceps surae in group a, b, c and d in 8th and 12th week after operation.
A: group A in the 8th week after operation; B: group B in the 8th week after operation; C: group C in the 8th week after operation; D: group D in the 8th week after operation; E: group A in the 12th week after operation; F: group B in the 12th week after operation; G: group C in the 12th week after operation; H: group D in the 12th week after operation.

3.3. Histological observation and image analysis of medullated fibers

In the 4th week after operation, the axons in the four groups were all few. Endothelial cells were swollen and the axons were broken in the form of segments or a string of bead. Besides, some parts were irregularly thickened and vacuolar degeneration appeared. In the 8th week after operation, the nerve fibers in group A and B were still swollen. Compared with those in group C and D, the number of axons increased and the broken ones decreased. The phenomenon of vacuolar degeneration was alleviated. In the 12th week after operation, there were a large number of regenerated nerve fibers and there were few broken axons or the phenomenon of vacuolar degeneration in group A and B. There were also regenerated nerve fibers in group C and D. But compared with those in group A and B, the regenerated nerve fibers

were less and there was still vacuolar degeneration (Figure 2). The results of image analysis indicated that the regenerated medullated fibers in group A and B were much more than those in group C and D. The fibers in group D was the least. The difference was statistically (P<0.05, Table 2).

Table 2
Count of medullated fibers in each group in the 4th, 8th and 12th week after operation (mean±sd).

Groups	Sample size	4th week after operation	8th week after operation	8th week after operation
Group A	6	28.5±2.4	54.8±1.9	86.1±1.5
Group B	6	26.9±3.2	55.0±2.1	84.7±2.5
Group C	6	20.4±1.8	36.3±2.0	58.6±1.7
Group D	6	11.2±2.7	20.6±2.2	34.2±2.1

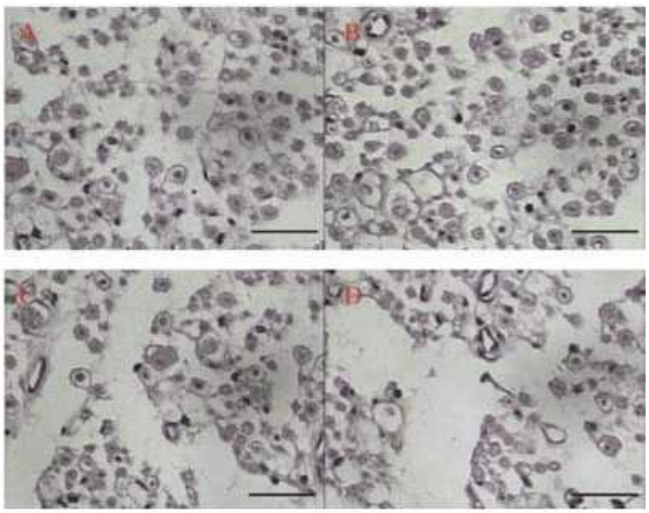

Figure 2. HE dyed pictures of nerve fibers in each group (Scale bars 100 μm).
A: Group A; B: Group B; C: Group C; D: Group D.

3.4. Retrograde labeling of neurons by true blue

Respectively in the 4th, 8th and 12th week after operation, the number of neurons retrogradely labeled by true blue in group A and B was larger than that in group C and D and the difference was statistically significant (P<0.05); that in group C was more than that in group D and the difference was statistically significant (P<0.05, Table 3). Judging from the image of marked neurons in the 12th week after operation, the marker neurons in group A and B were more and lighter than those in group C and D (Figure 3).

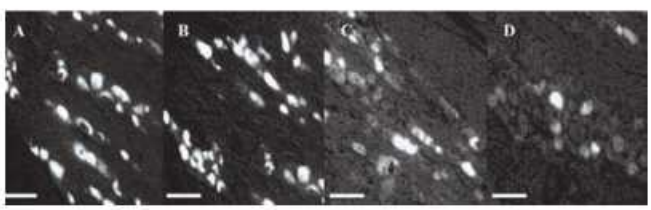

Figure 3. Image of marked neurons in 4 groups in the 12th week after operation (Scale bars 20 μm).
A: Group A; B: Group B; C: Group C; D: Group D.

Table 3
Number of neurons marked by true blue respectively in 4th, 8th and 12th week after operation (mean±sd).

Groups	Sample size	4th week after operation	8th week after operation	8th week after operation
Group A	6	5.6±0.8	16.8±1.2	28.2±1.4
Group B	6	4.9±0.6	16.0±0.9	27.6±1.1
Group C	6	3.2±0.7	10.4±0.6	20.3±0.7
Group D	6	1.6±1.0	5.1±0.8	11.4±0.5

3.5. Electrophysiologic examination of nervi tibialis

The conduction velocity of bilateral tibial nerves in each group was examined in the 12th week after operation. The results showed that the reversion rates of conduction velocity in group A and B were higher than those in group C and D. The conduction velocity in group C was faster than that in group B. The difference was statistically significant (P<0.05, Table 4).

Table 4
Conduction velocity of motor nerve of tibial nerve 12 weeks after operation (mean±sd, m/s).

Group	Sample Size	Affected side	Healthy side
Group A	6	43.57±3.26	49.33±3.47
Group B	6	41.14±4.04	48.15±3.89
Group C	6	31.48±3.45	50.04±3.28
Group D	6	23.61±4.12	49.08±4.02

4. Discussion

Since 1987 when FK506 was firstly reported that it had the function of inhibiting the immune rejection of organ transplantation in rats, people have made a full study on immunosuppressive action and its mechanism is almost clear[6]. Currently, it is widely accepted that FK506–FKBP compound is formed after the combination of FK506 and FK506 binding protein (FKBP). The FK506–FKBP compound combines with the regulatory subunit of calcineurin and inhibits the dephosphorylation of nuclear factor of activated T lymphocytes (NFAT). It also prevents the translocation of NFAT into nucleus and consequently decreases the expressions of a series of cell factors such as IL–2, IL–3, GM–CSF, TNF and so on. Calcineurin is a key enzyme to activate immunologic system. To inhibit its activity is the main mechanism of immunosuppressive function of FK506[7,8]. Besides, FK506 also has the function of promoting the neural regeneration[9,10] and its detailed mechanism is that FK506–FKBP compound makes the phosphorylation of growth–associate protein–43 (GAP–43) enhanced, the increase of expression of GAP–43 mRNA in neuronal cells promotes the formation of neuronal growth cone and the extension of axon and accelerates the growth of nerves[11].

Our experiment directly put the FK506 nanospheres prepared by single emulsion–solvent evaporation method (O/W) around the segment of transplanted nervi tibialis,

increased the medicine adjustment of specific parts and made the drug closer to the targeted cells. The drug concentration was relatively stable and the dose was remarkably less than that of systemic medication. The first pass effect in liver was avoided in systemic medication and the systemic toxic side effect was lessened. The aims of continuous release and lasting effect can be achieved by making FK506 into nanospheres. Moreover, P (DLLA-co-TMC) was degradable copolymer and the degrading period lasted for 30 d and had no effect on the peripheral tissues; it possessed excellent permeability and osmosis and its release was well-distributed, the absorption was complete and there was no residue. The experimental results indicated that in the group with drugs, the wounds in rats were normal and the drug was absorbed well. There were no residues and obvious connective tissue coating and hypertrophic scar. Nervi tibialis was easy to separate from tissues. In the group without drugs, the wounds in the right lower limb of rats were red and swollen and ulcerous. There were many hypertrophic scars in the transplanted parts and the adhesion to peripheral tissues was obvious. It was difficult to separate them from the tissues. So, FK506 nanospheres just adjusted the local environment and inhibited the immune response or the inflammatory process and consequently inhibited the rejection.

In clinic, the diagnosis and treatment of the injury in peripheral optic nerves are usually delayed. Gold *et al*[12] found in the study on the experimental injury of rat sciatic nerve that the effect in the group with immediate medication and with the drugs for 8 d was same to that in the group with medication on 8th day and with the drugs for 8 d. But the effects in the two groups were both worse than that in the group with immediate medication and with the drugs for 17 d. Sobol *et al*[13] found in the tibial nerve transection experiment that FK506 can remarkably improve the effect of neural regeneration. If the medication was given after 3 d, the effect would be lessened. The effect of FK506 weakened remarkably as the medication was carried out at later time. If it was delayed for 5 d, the treatment was hardly effective. Brenner *et al*[14] also had the similar findings in their experiments. In our experiment, the drugs were given respectively immediately, in 24 h after operation and 3 d after operation. The results showed that the effects of promoting neural regeneration in group with immediate medication and group with medication in 24 h after operation were similar and were both more obvious that that in group with medication in 3 d after operation. However, the effect in group with medication in 3 d after operation was better than that in group without medication.

In the several hours after neural injury, changes occurred in nerve cells which were beneficial to the restoration of the cell scaffold of axon. In twenty-four hours, axon formed regeneration unit through budding and the subterminal regenerated bud can become longer than the nerve suture soon in several days after getting damaged. Therefore, researchers thought that FK506 had an effect on the neurons in the early stage and promoted its restoration of the axon scaffold and motivated the axonal regeneration. However, these effects became obviously weak when the medication was delayed.

Conflict of interest statement

We declare that we have no conflict of interest.

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