Dog sciatic nerve gap repaired by artificial tissue nerve graft*

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Abstract The feasibility of repairing dog sciatic nerve damage by using a biodegradable artificial tissue nerve graft enriched with neuroregenerating factors is investigated. The artificial nerve graft was implanted to a 30 mm gap of the sciatic nerve damage in 7 dogs. The dogs with the same nerve damage that were repaired by interposition of the autologous nerve or were given no treatment served as control group 1 or 2, respectively. The observations include gross and morphological observations, immune reaction, electrophysiological examination, fluorescence tracing of the neuron formation and the number of the neurons at the experimental sites, etc. Results showed that 6 months after the implantation of the graft, the regenerated nerve repaired the damage of the sciatic nerve without occurrence of rejection and obvious inflammatory reaction in all 7 dogs, and the function of the sciatic nerve recovered with the nerve conduction velocity of (23.91 ± 11.35)m/s. The regenerated neurons and the forming of axon could be observed under an electron microscope. This proves that artificial tissue nerve graft transplantation can bridge the damaged nerve ends and promote the nerve regeneration.

Keywords: **artificial tissue nerve, medical biodegradable material, peripheral nerve regeneration.**

Although the modern microsurgical techniques have achieved a high precision in repairing of peripheral nerve damage they still leave the difficulties in complete repair of the nerve damage in clinical of orthopedics, hand surgery, microsurgery and surgery of warfare. In searching for the methods of repairing peripheral nerve damage, scientists have made effort to increase the length of peripheral nerve itself, which allows a tensionless, end to end anastomosis. But its application in clinical practice needs to be further perfected[1~3]. It is generally agreed that for a damage more than 30 mm long a transplant for bridging repair should be adopted. Auto-transplantation of nerve taken from other sites of the body gives rise to a better result, but the use is limited by many factors. Up to now there are different artificial grafts to substitute for autologous transplantation, such as homologues nerve tissue graft, non-nervous biogenic material for bridging and non-biotransplant[4~6]. The experiments proved that there were a lot of drawbacks in single functional transplant, lacking practical prospect.

The present study used a method combining neurosurgery with the application of nerve regenerating factors to induce the damaged nerve to grow. Such combination was termed as "compound bridging transplant—artificial tissue nerve"[7]. The method has the advantage of promoting material exchange between the tissues at the location of damage and accelerating blood vessel growth.

1 Materials and methods

1.1 Animal group

16 male healthy dogs weighing 6 ~ 9 kg were used in this study. These dogs were randomly divided into 3 groups, 7 in the experimental group; 5 in control group Ⅰ, and 4 in control group Ⅱ.

1.2 Operation performance

Dogs were anesthetized with 30% sodium pentobarbital. A median incision was made at dorsal surface of left gluteus region. The sciatic nerve of left side was exposed. A segment of 26 mm sciatic nerve was resected at lower margin of piriform muscle. The two cutting ends retracted freely creating a gap of 30 mm. For the experimental group the two cutting ends of the nerve bridged with a 30 mm long artificial tissue nerve graft enriched with nerve regenerating factor (180 μg)[8,9] and sutured perineurial three stitches (Plate I-A), and this nerve graft was replaced by an autograft taken from the sciatic nerve on the same dog for control group Ⅰ, and control group Ⅱ only received the 30 mm nerve gap without any treatment. All of the dogs were carefully kept and fed for a 6-month observation.

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1.3 Observations

1.3.1 Gross observation The gait, body weight, appetite, surgical wound and the appearance of the operated leg were observed weekly after operation. Six months later, appearance of the regenerated nerve, weight of peroneal muscle on both legs were examined.

1.3.2 Immunological assay (1) E rosette formation. A 2 mL of venous blood was taken before and 6 months after the operation respectively. The lymphocytes were separated from the whole blood and mixed with 1% sheep red blood cell (RBC) in an equal volume. After an overnight at 4 ℃, the rate of rosette formation was counted. (2) Lymphocyte transformation test. At the same time points, transformation of lymphocytes was determined by a routine method.

1.3.3 Electrophysiological examination After 6 months of the surgery, the proximal and distal ends of the bridged or damaged sciatic nerves were exposed through the previous incision. The conduction velocity of the nerve and their response to nerve stimulation were detected and recorded, and electromyography was carried out on both operated and normal legs to observe and record compound muscular action potential of anterior tibial muscle, by using an MYTO-electromyography instrument (ESAOTE Group Company, Italy).

1.3.4 Fluorescence tracing Six months after operation, 20 μL of 2% Fb (sigma) solution was injected into the sciatic nerve trunk 10 mm below distal end of the bridged nerve. Fluorescence labeled neurons in anterior horn and dorsal root of spinal cord was observed under a fluorescence microscope.

1.3.5 Morphological observation and quantitative analysis of neurons In succession to nerve electrophysiological determination, the perfusion through heart with normal saline, 1.25% glutaraldehyde, 1% polyformaldehyde, 5% sucrose, 0.1 mol/L phosphate buffer was performed sequentially and the lumbar enlargement and dorsal root ganglia (L3-L6) were taken. After fixation in 30% sucrose solution overnight, cross sections were made and stained with Nisser's dye. The samples for fluorescence tracing test were also stained. The number of neurons was counted (calculated as the mean of neuron numbers in spinal cord transection on 10 slides for each dog). The regenerated nerve was embedded in paraffin and sliced then stained by triple stain and HE stain. The nerve regeneration was observed under a light microscope. The effective area of transection (the area containing regenerating nerve fiber) and total number of axon in the proximal and distal segment of bridged portion and distal end of sciatic nerve in different groups were analyzed by an image processing system.

The distal segment of the regenerated bridged sciatic nerve of 3 dogs in each group were obtained and ultrathin slices were made for electron microscopic observation of myelin sheath formation and calculation of diameter of axon and thickness of myelin sheath. Moreover fresh gastrocnemius muscle was frozen and the sections were made under −20 ℃ in triplicate: one used for tissue cholinesterase staining and morphological observation; the other two series used for ATPase staining at pH 4.3 or 10 to investigate type Ⅱ myofiber changes and dystrophy of gastrocnemius during the course of nerve regeneration.

2 Results

2.1 Gross observation

All the dogs in these 3 groups walked with three legs touching the ground and in the difficulty of walking within the following week after operation. Two to three weeks after that all dogs in control group Ⅱ developed different degrees of skin ulcer in paw surface due to the operated leg haul on the ground, while only one dog in the experimental group and in control group Ⅰ developed ulceration, showing mild swell on the operated leg. About 4 weeks after operation, there was muscle atrophy of the operated legs in all the dogs. During the period of 1～2 months after the operation leg swelling tended to disappear, the dogs walked with 4 legs touching the ground (Plate I-B (b)). Muscle atrophy of the operated leg of the dogs in experimental and control group Ⅰ recovered to the same extent 3 months after surgery. Ulcers essentially healed. The dogs walking and running gesture improved, occasionally they could stand by hind legs. At 6 months the operated leg of experimental and control group Ⅰ dogs could walk and run freely and their posture tended to normal, standing on two hind legs (Plate I-B (c)). For the dogs in control group Ⅱ, their operated leg developed ulcer and serious muscle atrophy, and they difficultly walked with three legs touching on the ground. Dogs in experimental and control group Ⅰ had good appetite gain-

ing body weight to 1～2 kg on average. There was no body weight gain in control group Ⅱ. There was no obvious systemic inflammatory symptom in dogs of all three groups.

2.2 Immunological assay

Results of E-rosette formation and lymphocyte transformation showed that no rejection reaction happened in all dogs.

2.3 Electrophysiological examination of the regenerated nerve

2.3.1 Conduction velocity of nerve The conduction velocity of sciatic nerve of the experimental and control groups is shown in Table 1.

Table 1. Conduction velocity of sciatic nerve ($\bar{x} \pm SD$)

Group	Conduction velocity of sciatic nerve (ms^{-1})
Experimental group	23.91 ± 11.35
Control group Ⅰ	11.93 ± 13.04
Control group Ⅱ	—
Normal control group[a)]	101.35 ± 11.39

a) Dogs received no treatment
— no corresponding structure can be found

The results indicated that there was no significant difference between experimental and control group Ⅰ ($P > 0.05$). But these two groups had a much slower conduction velocity of their sciatic nerve when compared with the normal dogs ($P < 0.01$).

2.3.2 Myoelectrogram detection The compound muscle action potential (CMAP) of anterior tibia muscle could be recorded at proximal or distal end of sciatic nerve that had been bridged by artificial tissue nerve enriched with neuroregenerating factor, which gave rise to the mean of (20.15 ± 4.82) m/s. It was slower than CMAP of the normal side (102.93 ± 16.00) m/s.

2.4 Fluorescene tracing test

At sixth month after operation, the Fb positive neurons of spinal cord were visible in the experimental group. The number of positive cells in transection of anterior horn of gray matter reached as many as 4 (Plate Ⅱ-A(a)). The number of labeled neuron body in cross section of dorsal root ganglia was higher and might attain as high as about 20 (Plate Ⅱ-A(b)). The observations in control group Ⅰ was similar to that of experimental group.

2.5 Change of motor neuron in anterior horn of spinal cord

The changes of motor neuron number in anterior horn of spinal cord are listed in Table 2.

Table 2. Count of motor neuron in anterior horn of spinal cord ($\bar{x} \pm SD$)

Group	Total number
Experimental group	21.90 ± 4.60
Control group Ⅰ	21.00 ± 0.71*
Control group Ⅱ	17.56 ± 2.87**
Normal control group	23.35 ± 5.27

vs. normal control group: * $p < 0.05$; ** $p < 0.01$

2.6 Morphological observation and quantitative analysis of regenerated nerve

The bridging graft are well connected to two ends of the original nerve in dogs of both experimental and control group Ⅰ. No edema, hematoma or abscess and inflammatory lesion could be seen. Light adhesion between bridging graft and surrounding tissue occurred. There were connective tissue membranes over the surface of regenerated nerve and abundant blood vessels growth. Thickness of the regenerated nerve appeared slightly thicker than the proximal end of nerve trunk in 5 dogs of experimental group, while the other 2 dogs there were regenerated nerve branches reaching to muscle and the main stem still connecting to distal end. The regenerated nerve of control group Ⅰ looked thicker than normal nerve with basically no branch. One dog in control group Ⅱ, her sciatic nerve of the operated side still kept a 30 mm gap, the remaining 3 dogs in this group had some fine, soft connective tissue filled the gap which possessed no definite structures.

White blood cell count in bridged nerve tissue of the different groups ranged from 0～10 per viewfield, without distinct inflammatory reaction observed. Regenerated nerve trunk of experimental and control group Ⅰ was wrapped by an integrated membrane. Quantity and distribution of nerve fibers were similar to the proximal end of the sciatic nerve. The membrane of tract consisted of thinner connective tissue. Regenerated axon within the fiber passed through whole length of bridged nerve graft with denser and more regular arrangement. More than half of the proximal segments of the regenerated nerve in experimental and control group Ⅰ showed red color myelin sheath in the peripheral of regenerated axon after a specific triple stain but they were thinner than normal

myelin sheath (Plate Ⅱ-B(a)). At the distal side of the regenerated nerve, there were more regenerated axons enclosed by a thin layer sheath. Regenerated axons ran though whole defected site and extended to the distal end in the longitudinal section of regenerated nerve. Only a little nerve proliferation in the proximal end of sciatic nerve was observed in control group Ⅱ.

The measured area of cross section, effective area and the number of axons at proximal end of sciatic nerve, proximal segment and distal segment of bridge, distal end of sciatic nerve trunk are listed in Tables 3 and 4.

Table 3. Area of cross section of regenerated nerve ($\bar{x} \pm SD$)

	Exp. group	Contl. group Ⅰ	Contl. group Ⅱ	Normal contl.
Total area of nerve trunk(mm^2)				
Proximal stump of sciatic nerve	2.52 ± 0.69	3.06 ± 2.32	2.38 ± 1.83	1.53 ± 0.23*
Proximal segment of bridge	3.32 ± 1.46	2.58 ± 0.42	—	1.53 ± 0.23*
Distal segment of bridge	2.26 ± 0.75	2.54 ± 1.33	—	1.53 ± 0.23*
Distal stump of sciatic nerve	2.27 ± 0.69	2.45 ± 1.48	3.00 ± 1.72	1.48 ± 0.08*
Effective area of nerve trunk(mm^2)				
Proximal stump of sciatic nerve	1.22 ± 0.63	1.50 ± 1.17	0.59 ± 0.73	0.64 ± 0.16**
Proximal segment of bridge	1.20 ± 0.65	0.62 ± 0.19	—	0.64 ± 0.16**
Distal segment of bridge	0.65 ± 0.24	0.53 ± 0.28	—	0.64 ± 0.16
Distal stump of sciatic nerve	0.85 ± 0.52	0.35 ± 0.18	0.41 ± 0.20	0.63 ± 0.14

vs. experimental group: * $p<0.01$; ** $p<0.05$
— Corresponding structure can not be observed

Table 4. Count of axons of regenerated nerve ($\bar{x} \pm SD$)

	Exp. group	Contl. group Ⅰ	Contl. group Ⅱ	Normal contl.
Total number of regeneration axons				
Proximal stump of sciatic nerve	1997 ± 795	2621 ± 107	1240 ± 1276	1941 ± 425
Proximal segment of bridge	2027 ± 888	1472 ± 511	—	1941 ± 425
Distal segment of bridge	1473 ± 769	1125 ± 85	—	1941 ± 425
Distal stump of sciatic nerve	1315 ± 584	1177 ± 518	1060 ± 666	1943 ± 40*
Diameter of axon (distal segment, μm)	3.03 ± 1.48	3.06 ± 1.39	—	4.89 ± 2.65*
Thickness of myelin sheath (distal segment, μg)	0.82 ± 0.32	0.79 ± 0.31	—	1.64 ± 0.93*

vs. experimental group: * $p<0.01$
— Corresponding structure can not be observed

2.6.3 Electron microscopic observation Ultra-micro-structure of the regenerated nerve in the experimental group was similar to that of control group Ⅰ at 6 months after operation (Figs. 1 and 2). Density of regenerated axons was higher and their diameter was bigger with an irregular shape in transection. Part of single axons was wrapped by a single layer Schwann cells forming myelin sheath at different maturity, thickness but regularly arranged with high electron density. At the same time a single Schwann cell enclosed multiple regenerated axons, forming a non-myelin nerve fiber.

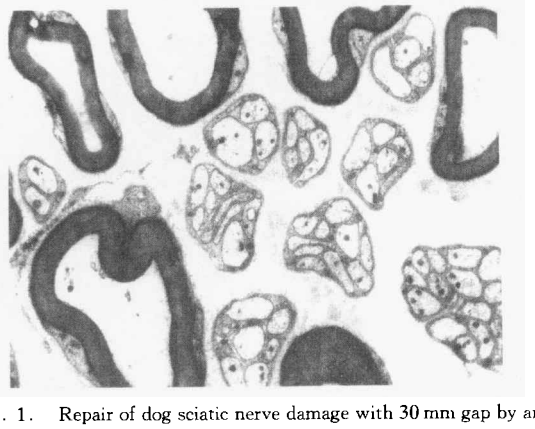

Fig. 1. Repair of dog sciatic nerve damage with 30 mm gap by artificial tissue nerve graft enriched with NRF (nerve regeneration factor) at 6 months after operation. Transmitting electron microscopic observation of distal segment of regenerated sciatic nerve (3000×).

2.7 Observation of gastrocnemius muscle and motor plate

Muscular atrophy of gastrocnemius of the operated side was seen both in dogs of experimental group and control group Ⅰ, but it was less severe than that of control group Ⅱ. Transection area of muscle fibers of gastrocnemius in dogs of experimental group and

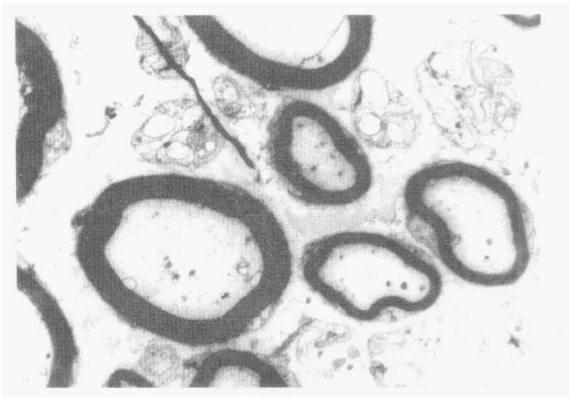

Fig. 2. Repair of dog sciatic nerve damage with 30 mm gap by auto graft, transmitting electron microscopic observation of distal segment of regenerated sciatic nerve at 6 months after operation (3000 ×).

control group Ⅰ was significantly larger than that of control group Ⅱ. Proportion of the ATPase type Ⅱ muscle fiber (quick fiber) in experimental and control group Ⅱ did not change significantly, while the amount of type Ⅱ muscle fiber increased in control group Ⅱ. The number of motor plates of the dogs in control group Ⅰ and Ⅱ decreased obviously when compared with the experimental and normal control groups. Size of motor plate in experimental group and control group Ⅰ was basically uniform, more deeply stained, showing more clear structures and connected to nerve fiber (Plate Ⅱ-C(a)). Motor plate of control group Ⅱ was irregular, smaller and lightly stained (Plate Ⅱ-C(b)). The results of the count are listed in Table 5.

Table 5. Count of gastrocnemius muscle and motor plate ($\bar{x} \pm SD$)

	Exp. group	Contl. group Ⅰ	Contl. group Ⅱ	Normal contl.
Ratio of weight of gastrocnemius muscle	0.89 ± 0.03	0.82 ± 0.10	0.42 ± 0.15*	
Transection area of muscle fiber (μm^2)	840.22 ± 764.71	648.1 ± 332.15	527.49 ± 435.04**	1052.59 ± 666.33*
Ratio of type Ⅱ muscle fiber	0.61 ± 0.08	0.64 ± 0.09	0.73 ± 0.08*	0.58 ± 0.09
Area of motor plate (μm^2)	573.08 ± 394.93	570.26 ± 374.15	429.38 ± 176.94*	867.65 ± 428.46**
Av. Optic density of motor plate	0.30 ± 0.08	0.27 ± 0.06	0.26 ± 0.04*	0.49 ± 0.05**

vs. experimental group: * $p < 0.05$; ** $p < 0.01$.

3 Discussion

Study on the mechanism of repairing nerve injury involves bridging, chemotactic, and microenvironmental factors. Bridging action requires providing the condition for the regenerating axons to cross over the gap site. Bridging materials ever used include nerve tissue bridging graft, non-nervous tissue bridging graft and bio-medical compatible materials. Autograft transplantation can only be obtained from functionally less important small cutaneous nerves, and it will bring new trauma to the recipitant, therefore, clinical application of this technique is limited. Autoisotransplantation will encounter rejection of the graft and no successful result has ever been reported in animal experiment. Non-nervous tissue graft including artery, vein, amnion conduit, muscular bridge, tendon bridge etc. have been tested but no satisfactory results could be achieved so far[10,11].

In searching for non-biological materials suitable for restoring the function of damaged nerves, Gou et al. repaired rat sciatic nerve defect by using a chitin conduit bridging graft[12] and started a preliminary clinical trail. Makinnon et al. repaired rat sciatic nerve defect by the polyglycolic conduit bridge[13], and Suzuki et al. used alginate gel conduit to repair cat sciatic nerve defect[14].

Looking back on the nearly hundred-year history of nerve regeneration and repairing research, we consider that a more ideal nerve implant should possess the following characteristics: (1) more abundant blood supply; (2) biodegradable; (3) without or with only slight antigenicity; (4) compatible to human tissues; (5) inducing a connecting matrix action; (6) containing nerve growth promoting substances; (7) with little scar tissue formation; (8) the substance being resourceful.

Based on the previous studies, we propose using chitin and polyglycolic acids as the conduit and matrix and supplying with some nerve growth promoting substances. This integrated compound is named "artificial nerve graft" which favors nerve growth. In one aspect, this chitin and polyglycolic filament integrated compound is biologically compatible to and degradable in human and also suitable for touch-grow adhesion. In another aspect, this compound has porosity that allows material exchange easily. Chitin-made conduit favors blood vessel growing and intramurally let Shwann cells more orderly approach the supporter

(polyglycolic filament)[15,16]. Supplementarily added nerve growth promoting substance (nerve regenerating factor) administered into the body helps bridging repair of sciatic nerve defect. The graft in the body is also benefitial to nerve growth which makes regenerated nerve fibers mend sciatic nerve defect. As the conduit gradually degraded and absorbed, the regenerated new nerve can finally form which will repair the nerve defect and restore its function.

Our experimental results showed that the dogs received sciatic nerve damage and repaired by means of artificial tissue nerve graft remained healthy and grew steadily during a 6-month observation. No systemic rejection reaction or distinct inflammatory reactions were seen prior to or after bridging implantation operation. Immunologic detection and daily raising observation indicated that the artificial tissue nerve graft was compatible to the animal and nervous tissue, thus it is a favorable artificial tissue-engineering material. The electrophysiological and fluorescene tracing results proved that axons of proximal stump of damaged sciatic nerve had regenerated passing through artificial tissue graft, and at the same time a portion of distal nerve fibers resumed the contact with central neurons. Although the conduction velocity of the regenerated nerve was much slower as compared with a normal nerve due to the incomplete regenerative development of myelin sheath, dogs in experimental group occasionally stood on two hind legs about three months after operation and could walk freely at the 6th month.

Viewing from morphology and quantitative count of regenerated nerve at bridging site explored that chitin and polyglycolic acid filaments had degraded and disappeared essentially. There were abundant blood vessels growing in the bridging site. The area of transection and effective area of the repaired nerve, the number of axons of sciatic nerve trunk in bridging site reached or was higher than those of normal dogs. The diameter and thickness of distal segment of regenerated nerve were less than that of normal control group, but similar to that of autograft implanted group.

In addition, the quantity, size and optical density of motor plate in gastrocnemius muscle of the artificial graft-implanted dogs were similar to that of the autograft implanted dogs, indicating that sciatic nerve had reached to the target organ, i.e. gastrocnemius muscle, and formed new motor plate to innervate and nourish the muscle. There was distinct gastrocnemius muscle atrophy of the operated side in early stage of post operation, but muscle atrophy began to subside from 3rd month due to the regenerated nerve attained and nourished the muscles. Again, the weight and cross section area of muscle fiber and ratio of Type Ⅱ muscle fiber (quick fiber) of the dogs in experimental group resembled those of the autograft-implanted dogs and were significantly superior to nerve damage group.

All of the observations proved that the artificial tissue nerve graft bridged and promoted nerve growth at the 30 mm resected site of the nerve. Its action led proximal nerve fibers elongated along bridging graft to the distal stump, therefore achieved its nerve repairing effect.

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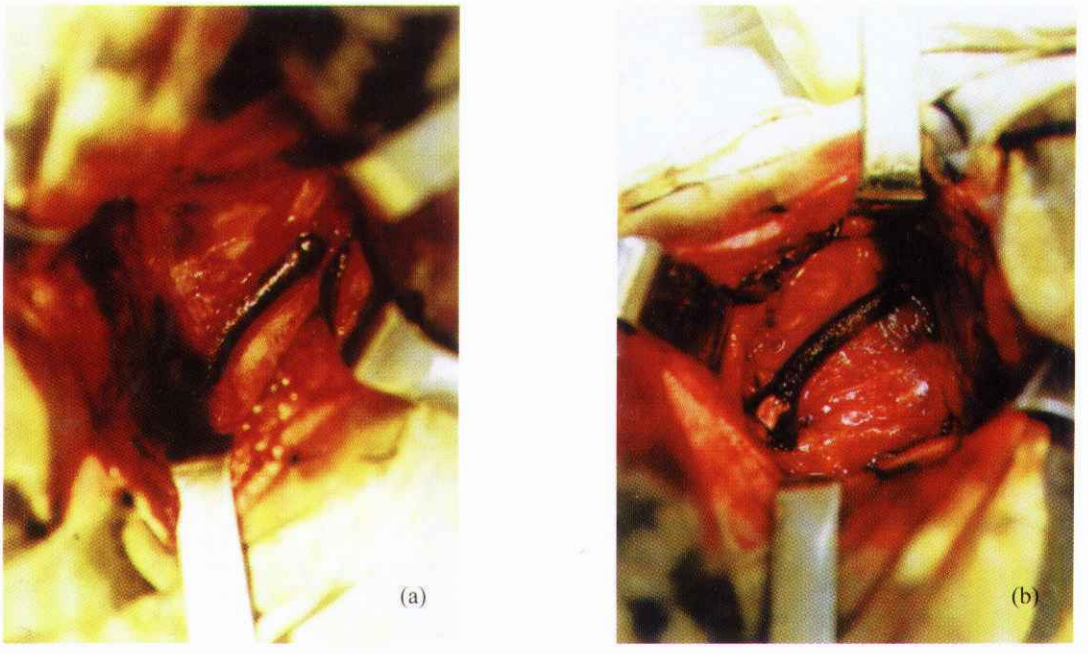

Plate 1—A. Dog sciatic nerve gap of 30 mm repaired by the artificial nerve graft. (a) Before operation, (b) post operation.

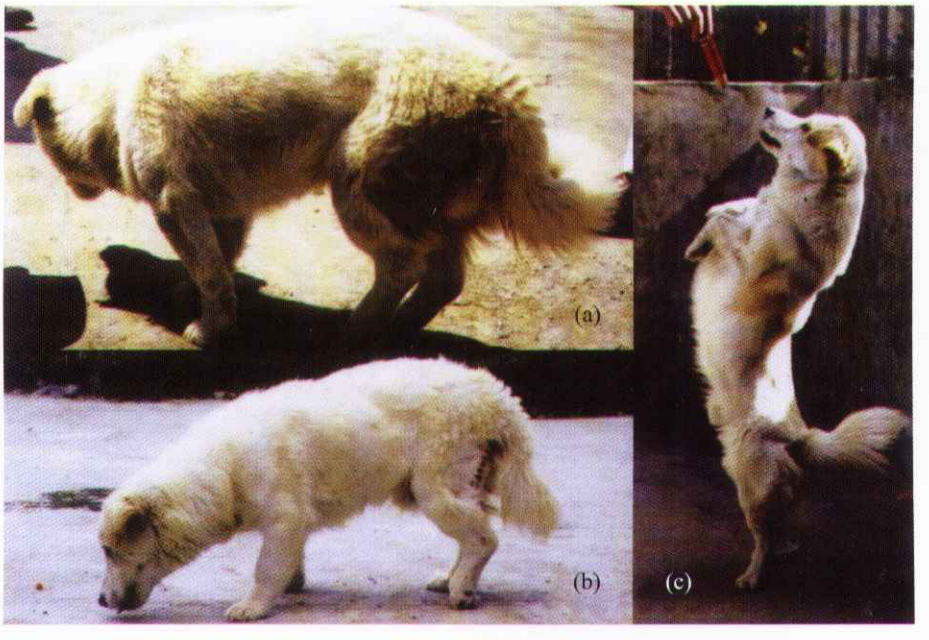

Plate 1—B. Effect of the implantation of the graft. (a) One week post operation, (b) one-month post operation, (c) six-month post operation.

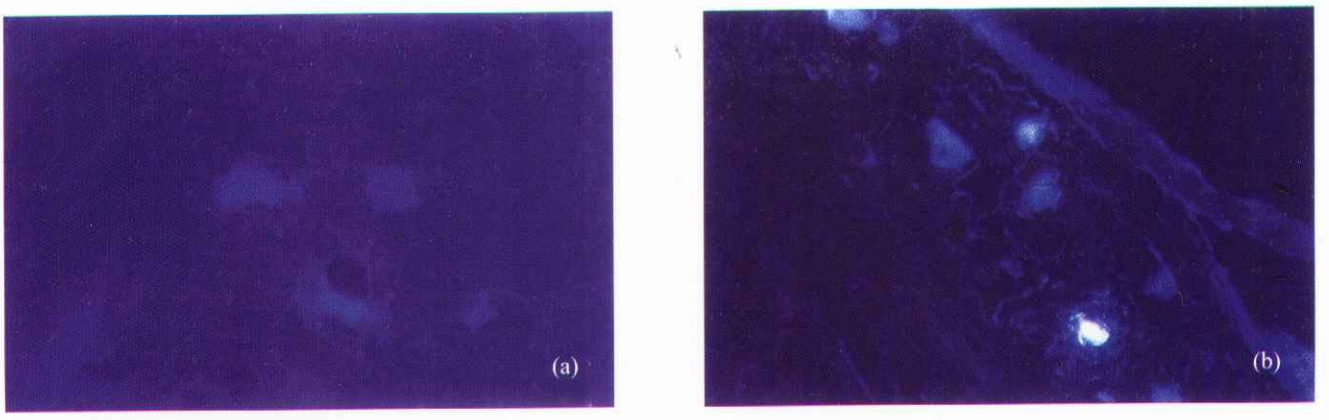

Plate II—A. The results of Fb fluorescent tracing. (a) Fb positive cells in transection of anterior horn of gray matter, (b) Fb positive cells in dorsal root ganglia.

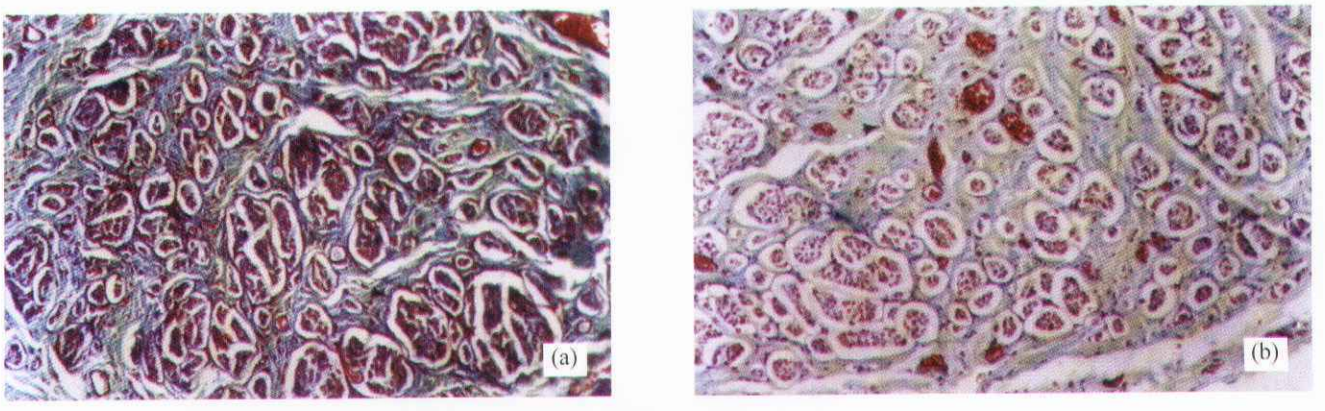

Plate II—B. Trichrome staining. (a) Regenerated cluster in proximal segment in bridge of artificial nerve graft, (b) regenerated cluster in distal segment in bridge of artificial nerve graft.

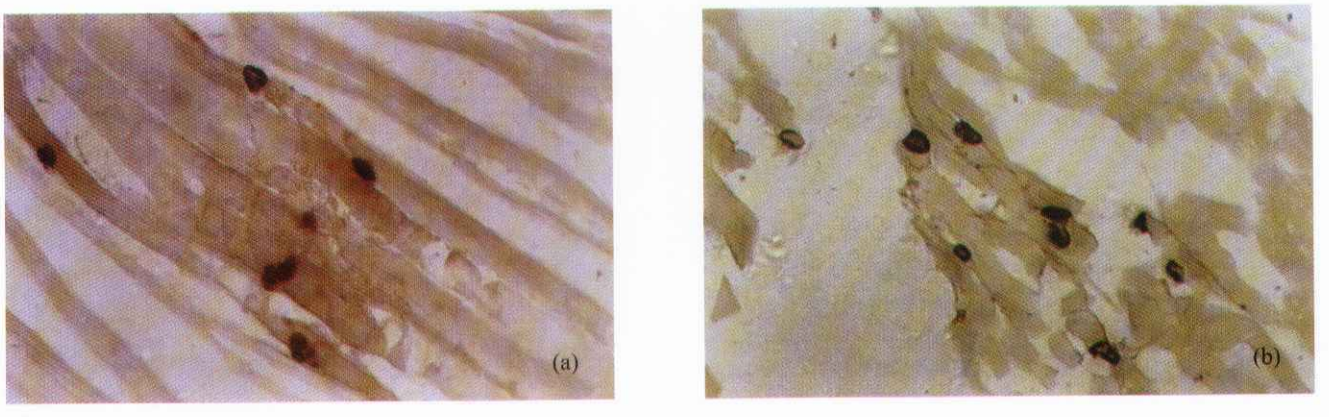

Plate II—C. Motor end plate in gastrocnemius musele on the operated leg six-month ofter operation. (a) Experimental gronp, (b) control group II.