

# Experimental study of placental amniotic membrane wrapped acellular nerve bridge repair of peripheral nerve defects

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Received 1 August 2016

Accepted 24 September 2016

## Introduction

Peripheral nerve defect is a clinical common traumatic disease. If by autologous nerve transplantation repair of peripheral nerve defect is inevitable in patients, this increases the surgical trauma resulting in innervation area dysfunction [1]. How to choose the ideal nerve graft to replace autologous nerve, to reduce the patients' secondary trauma is a problem which requires urgent adequate solution for clinical practice, where many have tried to use parts of skeletal muscle, its superficial vein or nerve allograft transplantation substitute for autologous nerve transplantation repair, but the clinical results are still poor [2,3]. In this study, placental amniotic membranes wrapped acellular nerve allograft technology to repair sciatic nerve defects in dogs may play a role in the formation of the basement membrane and thus leave the sheath for the regeneration of nerve fibers, directed to remove the obstacles for nerve recycled fiber to build a good micro-environment in order to provide a theoretical basis for clinical application.

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## ABSTRACT

**Objectives:** To study muscle action potentials, sciatic nerve conduction velocity, and explore placenta amniotic membrane wrapped acellular allogeneic nerve graft.

**Methods:** Animals grouping: groups A and B having 6 dogs each, and group C with only 3 animals. Group A is the allogeneic nerve allograft; Group B: amniotic membrane wrapped acellular nerve transplantation; Group C: allogeneic nerve donor. Groups A and B were anesthetized for bridging nerve allograft. Electrophysiology, HE and immunohistochemistry were used to observe number of axons; and myelin layers in transplanted nerves cross-section. Recorded soleus action potentials and bilateral sciatic nerve conduction velocity.

**Results:** Group A showed poor nerve regeneration. Group B showed more visible schwann cells proliferation and large number of regenerative fibers  $P < 0.05$ . A and B groups' nerve conduction velocity showed  $P > 0.05$ .

**Conclusion:** the study demonstrated Improvement in nerve morphology and graft quality.

## KEY WORDS:

Amniotic membrane  
Allografts  
Nerve repair  
Mongrels

## Materials and Methods

### Ethics Statement

Following the principles outlined in the Declaration of Helsinki 1964 for all human or animal experimental investigations, the authors state that all experimental animals were bred and handled according to the due protocols. All efforts were made to minimize suffering and surgical procedures were performed under 2% sodium pentobarbital anesthesia applied by intraperitoneal 40mg/kg dose, for exposure of the right sciatic nerve grafts and contralateral normal sciatic nerve. Placental tissue was obtained after an informed written consent was signed by participant. Authors then obtained appropriate institutional review board approval from the same Ethical Committee of Henan Province, Zhengzhou University for both the use of the animals and the human placental tissue.

Experimental animals were 15 healthy mongrels, weighing  $20 \pm 4$ kg, no gender difference (purchased from the Experimental Animal Center of Zhengzhou University). Animals were in good condition, shiny fur, safe in their cages, only 1 per cage rearing a liberal normal eating and drinking pattern. The main drugs and reagents: EC glue (Guangzhou Medical Glue Co. Ltd.), Triton X100 (Sigma, USA), immunohistochemical kit (Shanghai Biotechnology Co. Ltd).

### **Animals grouping**

Uniform 15 healthy mongrels were used after being randomly divided into three groups: group A and group B with 6 mongrels per group and group C populated by only 3 mongrels. Group A is the nerve allograft group; Group B: amniotic wrapped acellular nerve transplantation group; Group C: nerve allograft donor groups.

### **Placenta amniotic membrane preparation**

From a healthy natural human labor, extracted amniotic membrane was provided by Luoyang Central Hospital of Obstetrics and Gynecology, and preparation was made in reference to Chen You Gang and Zhu Gu Kai [4] method. The amniotic membrane was rinsed repeatedly until the film is clean to be observed under light microscope, clean and bright and then placed in 0.1% NH<sub>4</sub>OH solution. It was later removed and placed into 250ml phosphate buffer in standby (buffer containing Penicillin 400,000 units, Gentamicin 400,000 units). It was afterwards split into 2-4cm wide and about 4-9cm long strips, and stored at -40°C refrigerator.

### **Preparation of acellular nerves**

Group C having only 3 healthy mongrels, received intramuscularly 100mg anesthesia according to the dose (mg/kg). On the medial side, a longitudinal incision of about 10cm was operated exposing the gap from the vastus medialis and cut the femoral artery sacrificing the animals by bleeding to death. Afterward, cut from behind the biceps femoris to expose the sciatic nerve, and make a bilateral collection of sciatic nerve cut of about 7-11cm long, then; extraction treatment steps: (i) 12h bathing in distilled water, (ii) 5.0 % TritonX-100 decomposition for 12h, (iii) replaced again in distilled water bath 3h (iv) 5.0% sodium deoxycholate digestion for 12h (v) after these processes, once again repeat the cycle time of distilled water for 3h.

After completion of the extraction and purification processes of the sciatic nerve, it was collected and refrigerated at 4°C in a pH 7.2 phosphate buffer solution to be frozen and lyophilized. After the above processing cell-free sciatic nerve, good backup with sterilization processing, the following operations were undertaken: (a) extraction of frozen sciatic nerve (placed on the aluminum plate, frozen at -80°C), (b) freeze-dried for 12 h (at -56 °C in Edwards freeze dryer), (c) repackaging and irradiation sterilization (50 rads  $\gamma$  -rays [gamma rays]), (d) save sample (at 4°C in refrigerator). Then finally, the sterilized acellular sciatic nerve is having a good shape.

### **Preparation of chemical acellular allogeneic nerve**

#### **Shape Comparison**

Appearance comparison of chemically freeze-dried treated acellular nerve with the cellularized non treated one in this way showed no significant change placed in 0.9% saline solution for 20minutes to restore some neuronal elasticity and flexibility.

#### **Weight Comparison**

Chemically treated Freeze-dried acellular nerve weight was reduced.

#### **Morphological comparison**

Specific steps: chemically treated acellular nerve preservation by freeze-drying was after 3 months soaked into 0.9% saline for 30minutes then removed and placed into 10% formalin solution for fixation, after 24 hours, rinsed through distilled water flow, then dehydration into the ethanol gradients, and carried to xylene transparent process, embedded in paraffin wax block to cool for 30 minutes (the specific operation is first placed in paraffin blocks 7°C refrigeration), and then installed for slicing on the fixed microtome surface. Finally stained the sections in HE and in anti-laminin immunohistochemical staining. Viewed under microscope by freeze-drying technique, the chemically treated acellular nerve continuity presented a good basement membrane, although some appeared bent but not broken; clear myelin structure and regular arrangement; immunohistochemical staining displays the chemically treated acellular sciatic nerve basement membrane, in post laminin processing compared with pre-treatment, has not been destroyed or lost.

**Animal Anesthesia:**

2.5% sodium pentobarbital at a dosage of 10mg/kg body weight was intravenously administered and duration of anesthesia been 2hours to 2.5hours.

**Surgical specific steps:**

(i) under the narcotic effect, the animals were placed in prone position, adhesive plaster used at the mouth, limbs separated, and got fixed with a bandage on the cork board respectively, after been disinfected with 8% sodium sulphide and povidone-iodine, hair were shaved on the right hind legs and around 15cm surgical area was treated on the skin, and spread towels ethically. (ii) Six mongrels of group A and equal 6 in group B, their sciatic nerve were collected through the right rear side via a longitudinal incision performed on their retractor biceps femoris, transected with a sharp blade, respectively, resulting in about 5.5cm, 6.0cm, 7.8cm long nerve defect. (iii) A set of operation method: Corresponding length of allogeneic nerve, under microscope ( $\times 10$ ) with 8/0 no damaged membrane sutures anastomosis. (iv) Group B surgical methods: the use of placental amniotic membrane wrapped allogeneic nerve graft bridge was done by measuring the length of nerve defect, take corresponding length of allogeneic nerve, under microscope cut 2 mm toward the end, with 8/0 no damages in the end to end sutures. After nerve cut on both ends with transplanted neural involution, length and diameter matching with the placenta amniotic membrane and no suture anastomosis, apply adhesive medical glue to form a closed chamber bonding between fixed ends, amniotic membrane to be beyond the proximal and distal ends respectively, required anastomosis about 1.5cm, taking care to avoid contamination of the anastomotic glue. (v) protective muscle covering the sciatic nerve, after complete hemostasis, with No.1 silk sutures to close the wound and cover with two layers of gauze then place bandage.

**General situation**

After the dogs were housed separately, 8weeks after surgery they were kept observed and recorded their detailed mental state, physical activity, and limb motor function based on the wounds progress. After 16weeks of detailed observations and recordings, dogs were found suffering from foot ulceration and low recovery and risk of the occurrence of complications of limb function.

**EMG testing**

16weeks after surgery, 2% sodium pentobarbital anesthesia was applied by intraperitoneal 40mg/kg dose, for exposure of the right sciatic nerve grafts and contralateral normal sciatic nerve, the nerve stimulation electrode been placed proximal to the anastomosis to record in the soleus, the signal of the sciatic nerve graft repair, its electrophysiological activity and the soleus muscle action potential. Measurements include: the soleus muscle action potential, bilateral sciatic nerve motor velocity, bilateral sciatic nerve sensory velocity.

**Morphology**

HE staining: 16weeks after taking the sciatic nerve grafts in each experimental group, sciatic nerve transplantation materials proximal and distal to the anastomotic end beyond 1cm, was processed based in 10% formalin after conventional fixation with paraffin embedding, and serial sectioning will consist of a nerve anastomosis in the range of 2mm, both proximal and distal ends. After conventional HE staining, nerve transection sections were stained, and counted the bundles of axons and their areas measured to calculate the density of the axons.

**Statistical Methods**

Experimental data were analyzed using SPSS 13.0 software, data were expressed as mean  $\pm$  Standard deviation and were compared using the t test;  $P < 0.05$  difference was set as statistically significant.

**Results****General situation**

In the two groups of dogs by 2-7weeks after surgery were gradually appearing side foot swelling, ulceration and poor limb strength and cannot fully stand erect on their ankles when walking upright. 7weeks after because of varying degrees of exercise, some functional recovering states were observed; group B was significantly higher than group A in regards to faster foot ulcers healing. After 16 weeks group B recovery of motor function was significantly better than in group A. And group A nerve regeneration results are poorer, long-term foot and leg swelling, ulcers do not heal; Group B began to restore nerve function, muscle strength as calf muscles began to reply, the animals can stand right on their hind limb.

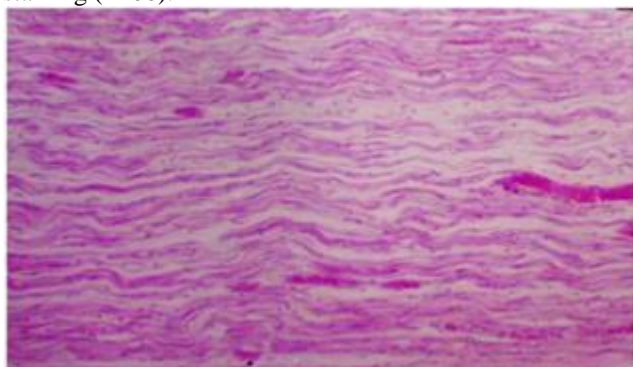
### Groups of nerve grafts general observation:

After 16 weeks, when dogs were with 2% sodium pentobarbital anesthetized through an intraperitoneal dose of 40mg/kg; were exposed the sciatic nerve transplant, showing from dogs in group A good nerve grafts continuity with serious surrounding tissue adhesion localized around the formation of scar tissue, and palpable anastomotic scleroma, with local to pear-shaped change, the degree of difference between the nerve surface vessels, proximal and distal segments of sciatic nerve transplant been thinner and hardened. Group B dogs transplanted nerve segment continuity: no nerve graft segments separated off and not hardened scar tissue surrounding adhesions, inflammation around the nerve graft segment is not obvious, but obvious neurological surface capillary network, two anastomoses slightly enlarged, no palpable in duration and entrapment of pear-shaped change, bridging transplantation of neural stem proximal and distal segments expressed normal appearance.

### Histological observation

**HE staining:** nerve graft after 16 weeks, in A and B groups were seen myelinated nerve fibers, but the density distributions did not show uniformly (Figure 1 and 2).

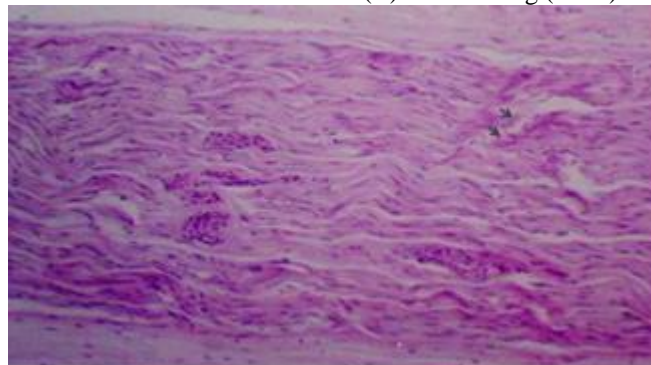
**Figure 1.** Chemical acellularised post-treatment; showing parallel nerve fascicles in undamaged specimens (A). HE staining ( $\times 400$ ).



**HE staining myelin regeneration** After 16 weeks, for both A and B groups were seen regenerating nerve fibers, Schwann cells arranged along the direction of nerve fiber strips. Using light microscope, the sciatic nerve transplantation of group B graft segments were seen more visible large Schwann cells proliferation; nerve regeneration cross section shows a large number of nerve fibers.

**Figure 2.** neurofilament expression demonstrate significantly greater numbers of nerve fibers in the autologous group and disrupted fascicles (arrows in black) of damaged

nerve with thick non-fused areas (B). HE staining ( $\times 400$ ).



### Axon counts

Axon density is the number of nerve axon units within the beam area, reflecting the nerve fiber regeneration. As shown in Table 1, the axon density of group B compared to group A was statistically significant ( $P < 0.05$ ). Prompt wrapped placental amnion bridging nerve allograft transplantation, in sciatic nerve defect repair, is more advantageous to axonal regeneration, restoration of nerve fibers and nerve function.

**Table 1.** Shows axon density 16 weeks postoperatively (units/100  $\mu\text{m}^2$ ) ( $M \pm SD$ ).

Groups	Axon density
A	0.725 $\pm$ 0.032
B	0.913 $\pm$ 0.026*

\*Compared with group A,  $P < 0.05$ .

### EMG measurement and analysis

After 16 weeks, in group B, soleus muscle evoked potential amplitude which was significantly higher than that of group A; after an incubation period the motricity potentials gradually shortened, their amplitude gradually increased; group B compared with group A, difference there was statistically significant ( $P < 0.05$ ) (table 2)

**Table 2.** Soleus EMG comparison ( $M \pm SD$ ).

Groups	Amplitude (mv)	Time (ms)
A	2.58 $\pm$ 0.71	4.09 $\pm$ 1.32
B	5.46 $\pm$ 1.13*	12.26 $\pm$ 2.57*
Control	12.91 $\pm$ 2.58	20.24 $\pm$ 3.49

\*Compared with group A,  $P < 0.05$ .

### Neural electrophysiological testing

After 16 weeks, group A and group B ipsilateral sciatic nerve conduction velocity showed no significant difference

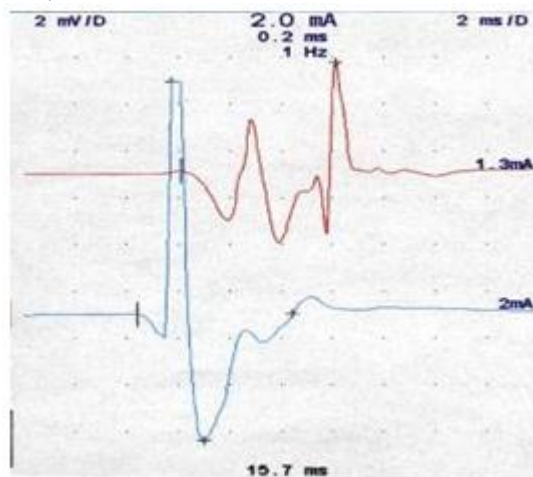
( $P>0.05$ ) (Figure 3), compared with normal sciatic, nerve conduction velocity difference was statistically significant ( $P<0.05$ ) (table 3).

**Table 3.** Comparative 16weeks postoperatively nerve conduction speed (m/s) ( $M\pm SD$ ).

Groups	MNCV	SNCV
A	34.25 $\pm$ 4.69	25.15 $\pm$ 4.48
B	35.71 $\pm$ 3.93*	26.38 $\pm$ 4.27*
Normal control group	39.48 $\pm$ 4.54	28.62 $\pm$ 5.16

\* Compared with group A,  $P<0.05$ .

**Figure 3.** Post 16th week recordings of ipsilateral sciatic nerve conduction velocity ( $P>0.05$ ). Less force is produced (red graph 1.3mA) at a faster stimulus in which muscle shortened; and maximal power at velocities of 30% of maximum shortening velocity (blue graph, 2mA) (NCV = 47.1).



## Discussion

In peripheral nerve allograft surgery defects, its antigenicity is mainly due to the nerve Schwann cells and myelin, and Schwann cell antigenicity of basement membrane was small [5]. In this experiment, we adopted chemical extraction methods to optimize both effective ways to remove the nerve allograft immunogenic components, but also retain the integrity of Schwann cell basement membrane [6] and its organizational structure. Research shows that 16weeks after transplantation [7,8], the Group B experimental animals transplanted nerve segment continuity is good, and there are no scar tissue adhesions around the nerve grafts no shedding and separation and no obvious inflammatory response [9]; while in Group A transplanted nerve segment showed delicate surrounding tissue adhesion, and thinning

of sciatic nerve segment hardening and difference in surface neural vascularization degree. This experiment further confirmed that the optimization of chemical extraction method can reduce the occurrence of postoperative immune rejection as stated by Hudson et al. [10]. Amnion is bounded in a biofilm between mother and fetus, and has the characteristics of immune privilege and good biological compatibility. The amnion immunogenicity did not only favor the nerve regeneration chamber, but can also effectively guide the nerve fiber regeneration for nerve defects repair and provide a good microenvironment [11]. The experimental results show that after 16weeks, the placenta amniotic membrane wrapped allograft segment had a good nerve continuity, there was no loss of nerve grafts neither separation, no adhesion with the surrounding tissues, neural surface has a new capillary network [12], while the untreated ordinary allograft segment got adhesion with the surrounding tissue, proximal and distal segments of sciatic nerve transplant thinning and hardened and got a neural surface vascularization difference. The light microscope view of group B graft segments were seen beyond the large sciatic nerve, Schwann cells proliferation, and regeneration of nerve cross-section shows a large number of nerve fibers. Its axon density compared with group A, the difference was statistically significant. Prompt placental amnion bridging nerve allograft transplantation did not show obvious rejection and is more advantageous to the axon, nerve fiber cells regeneration and recovery of neurological function. Nerve conduction velocity in EMG is the most commonly used detection function of peripheral nerve electrophysiology observation indexes, MNCV and SNCV respectively reflecting both the state of motor and sensory fiber of sciatic nerve function. Supported by the work of Mligiliche et al. [13]; this study found that 16weeks postoperatively, the evoked soleus potential amplitude in the experimental group B is higher than in group A. The two groups of experimental dogs expressed short potential of the incubation period, amplitude increasing gradually. And nerve electrophysiology was not showing any significant difference ( $P>0.05$ ), and this could be due to short observation time [14]. Further observations are needed in upcoming experiments. In this experiment, we observed no obvious surrounding tissue adhesion neither inflammatory reaction,

less scar tissue, nerve regeneration and small resistance [15], the axon density was large, and high- soleus muscle potential amplitude. From the experimental results, allogeneic nerve was extremely of low antigenicity nerve graft substitute materials after chemical extraction, and the immune rejection [7, 16] in clinical application can be prevented by more selective transplanted segment material. In this study, we make full use of placental amniotic membrane, low immunogenicity, and inhibiting the inflammatory response character such as scar hyperplasia, acellular allogeneic nerve to form a relatively closed elongated nerve compartment [17], to avoid interference with the anastomotic suture, combined with medical adhesive application, select diameter, length and nature of allogeneic acellular nerve perfectly matching the dog sciatic nerve cells, give full play to the role of the contact -induced , to guide the growth and regeneration of axons in breaking away from the remote proximal nerve tends, highly selective extension growth, reduce wrong nerve axons to regenerate, while ensuring consistent enough strength to avoid postoperative nerve stump separation, falls-off phenomenon, hence to improve the positive effect of repair of peripheral nerve [14]. Our study demonstrated that we could achieve an increase in nerve fiber number and reduce aberrant axonal regeneration, which is accompanied by an improvement in nerve function. Our data showed improvement in several parameters of functionality, including larger amplitudes of evoked potentials and improved velocity. Therefore, this is a simple and effective method for repairing peripheral nerve defects, with broad prospective application worth of further research. If rehabilitation is achieved for the recovery of neural and limb function, placental amniotic membrane wrapped acellular allogeneic nerve transplantation could be used in the clinic.

### Conflict of Interest

We declare that we have no conflict of interest.

### Acknowledgements

We would like to thank Editage (www.editage.com) for English language editing.

### References

- 1 Zhong H, Chen B, Lu S, Zhao M, Guo Y, Hou S. Nerve regeneration and functional recovery after a sciatic nerve gap is repaired by an acellular nerve allograft made through chemical extraction in canines. *J Reconstr Microsurg*. 2007; 23(8):479-87.
- 2 Guo YZ, Lu SB, Wang Y, Liu XC, Liang YT, Sun MX, et al. Acellular nerve allograft in repair of human long-segment defect of the peripheral nerve. *Chin J Clin Rehab*. 2005; 9(38): 26-27.
- 3 Sondell M, Lundborg G, Kanje M. Regeneration of the rat sciatic nerve into allografts made acellular through chemical extraction. *Brain Res*. 1998; 795(1-2): 44-54.
- 4 Davis GE, Blaker SN, Engvall E, Varon S, Manthorpe M, Gage FH. Human amnion membrane serves as a substratum for growing axons in vitro and in vivo. *Science*; 1987; 29; 236(4805):1106-1109.
- 5 Zhang Yanru, Yao Junna, Zhou Mingwu. Idiopathic Human placenta amniotic membrane package decellularized allogeneic nerve bridge repair dog sciatic nerve defect. *J Zhengzhou University Med Sci*. 2012; 4 (47): 508-512.
- 6 Dubový P, Svízenská I, Klusáková I, Zítková A, Houst'ava L, Haninec P. Laminin molecules in freeze-treated nerve segments are associated with migrating Schwann cells that display the corresponding  $\alpha 6 \beta 1$  integrin receptor. *J Glia*. 2001; 33(1): 36-44.
- 7 Kubo M, Sonoda Y, Muramatsu R, Usui M. Immunogenicity of human amniotic membrane in experimental xenotransplantation. *Inves Opht Vis Sci*. 2001; 42(7): 1539-1546.
- 8 Xu J, Varitimidis SE, Fisher KJ, Tomaino MM, Sotereanos DG. The effect of wrapping scarred nerves with autogenous vein graft to treat recurrent chronic nerve compression. *J Hand Surg Am*. 2000; 25(1): 93-103.
- 9 Zhang Y, Shang Y, Liu HJ, Zhou MW, Wang YS. Effect of chemical extracted acellular nerve on sciatic nerve defect of rats. *Chin J Exp Surg*; 2013; 30(3): 609-611.
- 10 Hudson TW, Zawko S, Deister C, Lundy S, Hu CY, Lee K, et al. Optimized acellular nerve graft is immunologically tolerated and supports regeneration. *Tissue Eng*. 2004; 10(11-12): 1641-1651.
- 11 Matsumoto K, Ohnishi K, Kiyotani T, Sekine T, Ueda H, Nakamura T, et al. Peripheral nerve regeneration across an 80-mm gap bridged by polyglycolic acid (pga)-collagen tube filled with laminated collagen fibers: A histological and electrophysiological evaluation of regenerated nerves. *Brain Res*. 2000; 868 (2): 315-328.
- 12 Harting MT, Baumgartner JE, Worth LL, Ewing-Cobbs L, Gee AP, Day MC, et al. Cell therapies for traumatic brain injury. *Neurosurg Focus*. 2008; 24(3-4): E18.

- 13 Mligiliche N, Endo K, Okamoto K, Fujimoto E, Ide CE. Extracellular matrix of human amnion manufactured into tubes as conduits for peripheral nerve regeneration. *J Biomed Mater Res.* 2002; 63(5): 591-600.
- 14 Lassner F, Schaller E, Steinhoff G, Wonigeit K, Walter GF, Berger A. Rejection and regeneration in peripheral nerve allografts. *Transplantation.* 1989; 48(3): 386-392.
- 15 Hou SY, Zhang HY, Quan DP, Liu XL, Zhu JK. Tissue engineered peripheral nerve grafting by differentiated bone marrow stromal cells. *Neuroscience.* 2006; 140(1): 101-110.
- 16 Hudson TW, Liu SY, Schmidt CE. Engineering an improved acellular nerve graft via optimized chemical processing. *Tissue Eng.* 2004; 10(9-10): 1346-1358.
- 17 Toba T, Nakamura T, Shimizu Y, Matsumoto K, Ohnishi K, Fukuda S, et al. Regeneration of canine peroneal nerve with the use of a polyglycolic acid-collagen tube filled with laminin-soaked collagen sponge: a comparative study of collagen sponge and collagen fibers as filling materials for nerve conduits. *J Biomed Mater Res.* 2001; 58(6):622-630.