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Repair of sciatic nerve defects using tissue engineered nerves

Caishun Zhang, Gang Lv

Department of Hand and Foot Surgery, First Affiliated Hospital of Liaoning Medical University, Jinzhou 121001, Liaoning Province, China

Research Highlights

(1) The acellular nerve allografts prepared in this study were completely decellularized, with an intact structure remaining. The laminin and basilar membrane, which could promote axonal growth, were also retained.
(2) Scanning electron microscope showed that the prepared acellular nerve allografts had a good affinity to the *in vitro* cultured bone marrow mesenchymal stem cells, which were evenly distributed in the grafts.
(3) Under the induction of acellular nerve allografts and the *in vivo* regeneration microenvironment, bone marrow mesenchymal stem cells with multiple differentiation potential could differentiate into Schwann cells, and promote the axons at the proximal end of 15 mm sciatic nerve defects to grow towards the distal end, thus improving the motor functional recovery of rat hind limbs.

Abstract

In this study, we constructed tissue-engineered nerves with acellular nerve allografts in Sprague-Dawley rats, which were prepared using chemical detergents-enzymatic digestion and mechanical methods, in combination with bone marrow mesenchymal stem cells of Wistar rats cultured *in vitro*, to repair 15 mm sciatic bone defects in Wistar rats. At postoperative 12 weeks, electrophysiological detection results showed that the conduction velocity of regenerated nerve after repair with tissue-engineered nerves was similar to that after autologous nerve grafting, and was higher than that after repair with acellular nerve allografts. Immunohistochemical staining revealed that motor endplates with acetylcholinesterase-positive nerve fibers were orderly arranged in the middle and superior parts of the gastrocnemius muscle; regenerated nerve tracts and sprouted branches were connected with motor endplates, as shown by acetylcholinesterase histochemistry combined with silver staining. The wet weight ratio of the tibialis anterior muscle at the affected contralateral hind limb was similar to the sciatic nerve after repair with autologous nerve grafts, and higher than that after repair with acellular nerve allografts. The hind limb motor function at the affected side was significantly improved, indicating that acellular nerve allografts combined with bone marrow mesenchymal stem cell bridging could promote functional recovery of rats with sciatic nerve defects.

Key Words

neural regeneration; peripheral nerve injury; tissue engineering; artificial nerve; sciatic nerve defects; bone marrow mesenchymal stem cells; nerve scaffold; grants-supported paper; neuroregeneration

Caishun Zhang, M.D., Associate professor.

Corresponding author: Gang Lv, Professor, Doctoral supervisor, Department of Hand and Foot Surgery, First Affiliated Hospital of Liaoning Medical University, Jinzhou 121001, Liaoning Province, China, zcs731009@163.com.

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INTRODUCTION

In recent years, repair of long-distance peripheral nerve defects by tissue engineering has become a rapidly growing field[1-3]. Autologous nerve grafts can provide optimal repair effects among current nerve grafts, and are also considered the gold standard for judging other nerve substitutes as they have no immunological reaction and exhibit Schwann cell activity[4]. However, their clinical applications are restricted because of their limited sources, with sensory dysfunction in the residual donor area and tiny nerves in the donor area. Therefore, autologous nerve substitutes for repair nerve defects are currently widely studied[5-6]. Nerve allograft is the most similar to autologous nerve in structure. Antigenicity-free nerve allografts seeded with autologous seed cells are likely to be used for bridging defected nerves and substituting nerve autografts[7-8]. In the repair of long-distance peripheral nerve defects, both nerve stumps should be bridged to recover nerve function[9].

The basic procedure of constructing tissue-engineered nerves used for bridging peripheral nerve defects includes two steps: the first is to seed the *in vitro* cultured seed cells in scaffolds made of natural or artificially synthesized materials, while the second is to make *in vitro* cultured cells adhere to and evenly distribute in the scaffold[10]. Use of an artificial nerve scaffold seeded with Schwann cells is an effective method to repair peripheral nerve defects[11-12]. However, the source of Schwann cells is limited, and xenogenic Schwann cells have the problem of immunological rejection, which influences their repair effects[13-14]. Therefore, it is necessary to find other kinds of seed cells[15].

In this study, we used a tissue-engineered nerve allograft, which was composed of bone marrow mesenchymal stem cells and acellular nerve allografts, to bridge a 15 mm gap in the sciatic nerve, and also preliminarily investigated the effects of artificial nerves in the promotion of motor function recovery.

RESULTS

Histological manifestation of acellular nerve allografts

Hematoxylin-eosin staining showed that normal nerves exhibited intact axons and myelin sheath. The nuclei of Schwann cells and the nodes of Ranvier could be seen in the longitudinal sections of nerves. After chemical decellularization, myelin sheath and axons were not observed in the transverse sections of the nerves, and only a net-shaped structure was left. Wavy arrangement of the fibers in the absence of cellular debris was observed in the longitudinal sections (Figure 1A, B). The immunohistochemistry of nerve grafts exhibited positive staining for laminin (Figure 1C).

Figure 1 Histological manifestation of acellular nerve allografts (× 40).

(A, B) Transverse and longitudinal sections of acellular nerve allografts (hematoxylin-eosin staining). Wavy arrangement of the fibers in the absence of cellular debris was observed in the longitudinal sections. (C) Longitudinal section of acellular nerve allografts (immunohistochemical laminin staining). The immunohistochemistry of nerve grafts exhibited positive staining for laminin (brown).

Transmission electron microscopy showed that in the transverse sections, the chemically decellularized nerve scaffold exhibited a net-shaped structure; the axons, myelin sheath, and Schwann cells disappeared; collagen fiber structure was present on the vascular wall; and the structure of the endoneurial tube was intact (Figure 2A). Under scanning electron microscopy, normal nerves exhibited the presence of myelin sheath and axons. However, myelin sheath and axons disappeared in the chemically decellularized nerve scaffold, with only the vessels of the basilar membrane left, and longitudinal arrangement of collagen fibers was present on the vascular wall (Figure 2B).

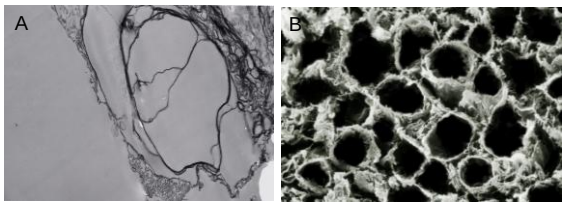

Figure 2 Ultrastructure of acellular nerve allografts in the transverse section.

(A) Transmission electron microscopy (× 5 000): The chemically decellularized nerve scaffold exhibited a net-shaped structure, the axons, myelin sheath, and Schwann cells disappeared. (B) Scanning electron microscopy (× 2 000). The vessel of basilar membrane was left and a longitudinal arrangement of collagen fibers was present on the vascular wall.

Morphology of bone marrow mesenchymal stem cells

Under inverted phase microscopy, bone marrow mesenchymal stem cells cultured *in vitro* adhered to the luminal wall. After three passages, cells were basically purified, exhibited a long-fusiform-shaped appearance, and were spirally arranged on the bottle bottom (Figure 3). After bone marrow mesenchymal stem cells were seeded into the nerve scaffold, there were no obvious changes observed by the naked eye, while expanded and bulged nerves were observed by microscopy.

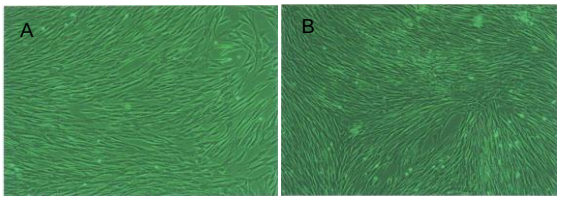

Figure 3 Morphology of bone marrow mesenchymal stem cells at passage 3 (inverted phase contrast microscope, × 10).

(A, B) Cells exhibited a fusiform shape.

Immunohistochemical staining showed that the cultured cells were negative for CD34, but positive for CD44 (Figure 4), indicating that the isolated and cultured cells were bone marrow mesenchymal stem cells, but not hemopoietic stem cells[16].

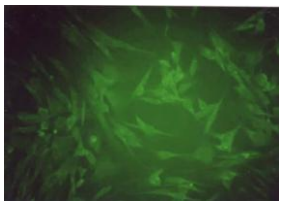

Figure 4 Identification of bone marrow mesenchymal stem cells at passage 3 (fluorescence microscope, ×10).

Green refers to CD44 expression (FITC staining).

Ultrastructure of artificial nerves constructed with acellular nerve allografts and bone marrow mesenchymal stem cells

Scanning electron microscopy showed that after 7 days of culture, bone marrow mesenchymal stem cells exhibited an oval-shaped or multipolar appearance, and were evenly distributed in the basilar membrane vessel of nerve allografts and adhered to the luminal wall (Figure 5).

Quantitative analysis of experimental animals

The 60 Wistar rats used for development of sciatic nerve defect models were equally and randomly divided into three groups: scaffold + cells group (an artificial nerve graft constructed with acellular nerve allografts and bone marrow mesenchymal stem cells was used for repair of sciatic nerve defects in rats); scaffold group (tissue-engineered nerve scaffold was used); and autologous nerve group (autologous nerve graft was used). All 60 rats were included in the final analysis.

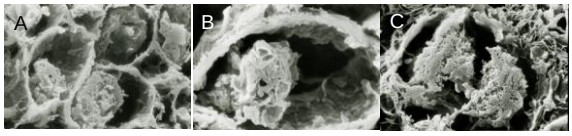

Figure 5 Transverse sections of acellular nerve allografts with bone marrow mesenchymal stem cells after 7 days of culture (scanning electron microscopy).

(A) Cells with an oval-shaped or multipolar appearance were evenly distributed in the luminal cavity (× 5 000). (B) One bone marrow mesenchymal stem cell (arrow) grew in one luminal cavity, exhibited a multipolar appearance, and stretched out the pseudopodia that were connected with the luminal wall (× 10 000). (C) Two bone marrow mesenchymal stem cells were in one luminal cavity (× 12 000).

Tissue-engineered nerves constructed with acellular nerve allografts and bone marrow mesenchymal stem cells improved the behavior of rats with sciatic nerve defects

After surgery, rats dragged their operated limbs and could not touch the ground with their toes. The toes of operated limbs were closed, naturally bended, and could not spread apart. The lower limbs of rats in each group exhibited different extents of swelling and ulcers. At postoperative 4 weeks, swelling gradually subsided, and no toes were lost in the scaffold + cells group and autologous nerve group. However, some toes were lost in the scaffold group. At postoperative 12 weeks, the toes of scaffold + cells group rats could spread apart and touch the ground (Figure 6).

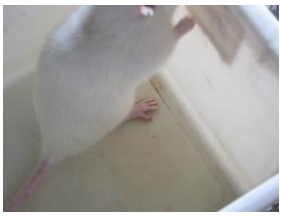

Figure 6 Gross manifestation of the rat with sciatic nerve defects after repair of artificial nerves constructed with acellular nerve allografts and bone marrow mesenchymal stem cells.

At 12 weeks after repair, the rat toes on the operated side could spread apart and touch the ground in the scaffold + cells group.

Tissue-engineered nerves constructed with acellular nerve allografts and bone marrow mesenchymal stem cells improved the electrophysiological function of rats with sciatic nerve defects

At postoperative 12 weeks, the conduction velocity of regenerated nerves in the scaffold + cells group was similar to that in the autologous nerve group (29.37 ± 2.15 m/s *vs*. 31.67 ± 3.54 m/s, $P > 0.05$), but was significantly higher than that in the scaffold group (20.36 ± 4.67 m/s, $P < 0.05$).

Tissue-engineered nerves constructed with acellular nerve allografts and bone marrow mesenchymal stem cells improved the morphology of gastrocnemius muscle motor endplates of rats with sciatic nerve defects

At postoperative 12 weeks, acetylcholinesterase histochemistry showed that acetylcholinesterase-positive nerve fibers in the gastrocnemius muscle motor end-plates were stained light brown. After silver staining, no nerve tracts and nerve terminals were observed. In addition, muscle fiber was thinned and muscle striations were unclear. Motor endplates with acetylcholinesterase-positive nerve fibers were orderly arranged in the middle and superior parts of the gastrocnemius muscle, as shown by acetylcholinesterase histochemistry. Further, regenerated nerve tracts and sprouted branches were connected with motor endplates as shown by acetylcholinesterase histochemistry combined with silver staining (Figure 7).

Figure 7 Gross manifestation of the rat with sciatic nerve defects after repair of artificial nerves constructed with acellular nerve allografts and bone marrow mesenchymal stem cells.

At 12 weeks after repair, the rat toes on the operated side could spread apart and touch the ground in the scaffold + cells group.

Tissue-engineered nerves constructed with acellular nerve allografts and bone marrow mesenchymal stem cells increased the tibialis anterior muscle wet weight ratio of rats with sciatic nerve defects

At postoperative 12 weeks, the tibialis anterior muscle wet weight ratio of the operated side to the contralateral side in the scaffold + cells group was similar to that in the autologous nerve group (0.575 ± 0.148 g, 0.609 ± 0.100 g, $P > 0.05$), but was significantly higher than that in the scaffold group (0.379 ± 0.198 g, $P < 0.05$).

DISCUSSION

In this study, we prepared acellular nerve allografts using a chemical combined with mechanical method. The acellular nerve allografts preserve the laminin and collagen components that can guide and promote axon growth, and retain the natural three-dimensional structure with the absence of stem cells and myelin sheath. Scanning electron microscopy showed that the seeded bone marrow mesenchymal stem cells well grew in the acellular nerve allografts, and they stretched out their pseudopodia, which were closely connected with the luminal wall. These findings suggest that acellular nerve allografts show good affinity to bone marrow mesenchymal stem cells and can provide a structure for adherence, growth, and physiological function performance of bone marrow mesenchymal stem cells[17-18].

With their multi-directional differentiation potential, bone marrow mesenchymal stem cells can promote the axonal growth of peripheral nerve[19-24]. Autologous bone marrow mesenchymal stem cells are easily harvested, cultured *in vitro*, amplified, and purified without drawbacks including limited source, slow amplification, and low purity of Schwann cells, as well as the ethical problem of nerve stem cell application. In addition, bone marrow mesenchymal stem cells can be used as the seed cells of tissue-engineered nerves[25]. Bone marrow mesenchymal stem cells are particularly beneficial as seed cells of tissue construction. Recent studies using bone marrow mesenchymal stem cells for the repair of sciatic nerve defects are shown in Table 1.

We used a tissue-engineered nerve constructed with an acellular nerve allograft and bone marrow mesenchymal stem cells to bridge a 15 mm sciatic nerve defect, and investigated the effects on motor function recovery. Functional recovery after nerve injury is a key measure of nerve regeneration efficacy. Nerve conductivity is a basic function of peripheral nerves, and the conduction velocity of electrical activity in the nerve stem can directly reflect the recovery of nerve conductivity[35]. Nerve conductivity disappears once the peripheral nerve is mutilated, and therefore the conduction velocity of regenerated nerve can be used as a reliable index for judging nerve injury and repair[36].

Table 1 Studies using bone marrow mesenchymal stem cells for repair of sciatic nerve defects

Title	Author	Journal	Publication year	Intervention	Results	Conclusion
Recellularized nerve allografts with differentiated mesenchymal stem cells promote peripheral nerve regeneration[26]	Wang Y, *et al*	*Neurosci Lett*	2012	Immunofluorescence staining was used to evaluate axonal regeneration distance after 2-week implantation. 3-month regeneration of the novel cell-supplemented tissue-engineered nerve graft used to bridge a 15-mm-long sciatic nerve gap in rats.	Introducing bone marrow mesenchymal stem cell (BMSC)-Schwann cells or adipose tissue-derived stromal cells-Schwann cells to the acellular nerve graft promoted sciatic nerve regeneration and functional recovery. Nerve regeneration with BMSC-Schwann cells or adipose tissue-derived stromal cells-Schwann cells was comparable to that with autografting and Schwann cells alone and better than that with acellular nerve allografting alone.	Differentiated BMSCs or adipose-derived stromal cells may be a promising cell source for tissue-engineered nerve grafts and promote functional recovery after peripheral nerve injury.
Sciatic nerve repair by acellular nerve xenografts implanted with BMSCs in rats xenograft combined with BMSCs[27]	Jia H, *et al*	*Synapse*	2012	BMSCs were implanted into the nerve scaffolds and cultured *in vitro*. All the grafts were employed to bridge 1-cm rat sciatic nerve gaps.	Compared with the acellular nerve control groups, nerve regeneration and functional rehabilitation for the xenogenic nerve transplantation integrated with BMSCs were advanced significantly, and the rehabilitation efficacy was comparable with that of the autografting. The expression of neurotrophic factors in the regenerated nerves, together with that of brain-derived neurotrophic factor in the spinal cord and muscles were elevated largely.	Acellular nerve xenografts implanted with BMSCs could replace allografts to promote nerve regeneration effectively, which offers a reliable approach for repairing peripheral nerve defects.
Joint use of a chitosan/PLGA scaffold and BMSCs to bridge an extra large gap in dog sciatic nerve[28]	Xue C, *et al*	*Neurorehabil Neural Repair*	2012	A 60-mm-long sciatic nerve gap in dogs was bridged by tissue-engineered nerve grafts, chitosan/PLGA scaffolds, or nerve autografts.	The outcomes of tissue-engineered nerve grafts were similar to those of autografts and better than those of scaffolds alone.	Introduction of autologous BMSCs to a chitosan/PLGA scaffold improved the repair and rehabilitation of a large gap after peripheral nerve injury in dogs.
Repair of rat sciatic nerve gap by a silk fibroin-based scaffold added with bone marrow mesenchymal stem cells[29]	Yang Y, *et al*	*Tissue Eng Part A*	2011	A new design of tissue-engineered nerve grafts by introducing BMSCs of rats, as support cells, into a silk fibroin-based scaffold was developed, which was composed of a silk fibroin nerve guidance conduit and oriented silk fibroin filaments as the conduit lumen filler.	At 12 weeks after nerve grafting, tissue-engineered nerve grafts yielded an improved outcome of nerve regeneration and functional recovery, which was close to that by autologous nerve grafts. During 1–4 weeks after nerve grafting, BMSCs contained in the tissue-engineered nerve grafts significantly accelerated axonal growth, displaying a positive reaction to S-100 (a Schwann cell marker). During 1–3 weeks after nerve grafting, BMSCs contained in the tissue-engineered nerve grafts led to gene expression upregulation of S100 and several growth factors.	The cell behaviors and neurotrophic functions of BMSCs might be responsible for their promoting effects on peripheral nerve regeneration.
Long-term *in vivo* regeneration of peripheral nerves through bioengineered nerve grafts[30]	di Summa PG, *et al*	*Neuroscience*	2011	Nerve fibrin conduits were seeded with various cell types: primary Schwann cells, Schwann cells-like differentiated BMSCs, Schwann cells-like differentiated adipose-derived stem cells. Two further control groups were fibrin conduits without cells and autografts. Conduits were used to bridge a 1 cm rat sciatic nerve gap in a long term experiment (16 weeks).	A reduction in muscle atrophy was observed in the autograft and in all cell-seeded groups Schwann cells showed significant improvement in axon myelination and average fiber diameter of the regenerated nerves. Differentiated adipose-derived stem cells were the most effective cell population in terms of improvement of axonal and fiber diameter, evoked potentials at the level of the gastrocnemius muscle and regeneration of motoneurons, similar to the autografts.	Differentiated adipose-derived stem cells could be a clinically translatable route towards new methods to enhance peripheral nerve repair.

Table 1 Continued

Title	Author	Journal	Publication year	Intervention	Results	Conclusion
Differentiated mesenchymal stem cells for sciatic nerve injury[31]	Dadon-Nachum M, *et al*	*Stem Cell Rev*	2011	It is examined whether neurotrophic factors (+) cells are capable of rescuing motor neurons in a rat sciatic nerve injury model, where the right hind limb sciatic nerve was crushed. Rats were transplanted with neurotrophic factors (+) cells, BMSCs or PBS into the lesion site.	In rats injected with the neurotrophic factors (+) cells motor function was markedly preserved. Moreover, neurotrophic factors (+) cells significantly inhibited the degeneration of the neuromuscular junctions and preserved the myelinated motor axons.	Autologous therapeutic approach can alleviate signs of sciatic nerve injury and probably other neurological disorders.
Differentiation of mesenchymal stem cells to support peripheral nerve regeneration in a rat model[32]	Ladak A, *et al*	*Exp Neurol*	2011	Longest length of the dorsal root ganglion neuritis and number of sciatic motoneurons regenerating axons through the conduit into the distal nerve stump were determined.	*In vitro*, the mean values of the longest length of the dorsal root ganglion neurites were the same for the differentiated BMSCs and Schwann cells and significantly higher than the undifferentiated BMSCs and dorsal root ganglion mono-culture systems. *In vivo*, compared to an empty conduit, differentiated BMSCs seeded collagen nerve conduits resulted in a greater number of sciatic motoneurons regenerating axons through the conduit into the distal nerve stump.	BMSCs differentiate into a Schwann cell-phenotype that expresses Schwann cell markers transiently and sufficiently to support limited neurite outgrowth *in vitro* and axonal regeneration equivalent to that of Schwann cells *in vitro* and *in vivo*.
The regeneration of transected sciatic nerves of adult rats using chitosan nerve conduits seeded with bone marrow stromal cell-derived Schwann cells[33]	Ao Q, *et al*	*Biomaterials*	2011	Chitosan conduits were seeded them with BMSC-derived Schwann cells as an alternative. The derived Schwann cells used were checked for fate commitment. The conduits were tested for efficacy in bridging the critical gap length of 12 mm in sciatic nerves of adult rats.	By three months post-operation, mid-shank circumference, nerve conduction velocity, average regenerated myelin area, and myelinated axon count, in nerves bridged with BMSC-derived Schwann cells were similar to those treated with sciatic nerve-derived Schwann cells, but significantly higher than those bridged with PBS-filled conduits.	Chitosan conduits seeded with BMSC-derived Schwann cells can be used to treat critical defects in peripheral nerves.
Improvement in nerve regeneration through a decellularized nerve graft by supplementation with bone marrow stromal cells-in-fibrin[34]	Zhao Z, *et al*	*Cell Transplant*	In press	BMSCs were affixed with fibrin glue and injected inside or around the acellular nerve graft. Then the nerve graft was used to repair a 15-mm nerve defect in rat.	15-mm Sprague-Dawley rat sciatic nerve defects was repaired using this nerve graft construction, and MSCs injected around the graft helped improve nerve regeneration and functional recovery of peripheral nerve lesions as determined by functional analysis and histology.	Supplying BMSCs in fibrin glue around acellular nerves is easy, maintains the nerve structure, and can support nerve regeneration like direct injection of BMSCs into the acellular nerve for long nerve defects but may avoid destroying the nerve graft. The technique is simple and is another option for stem cell transplantation.

Our findings showed that at postoperative 12 weeks the conduction velocity of regenerated nerve was similar between the scaffold + cells group and the autologous nerve group, demonstrating that after sciatic nerve defect bridging, the conduction function of regenerated nerve had recovered. The muscle becomes atrophic once it is denervated[39], and reinnervation is the basis for recovery of physiological function of the injured nerve. Results from this study showed that at postoperative 4 weeks, the muscle in the scaffold + cells and scaffold groups was obviously atrophic, muscle fibers were thinned, and there was little connective tissue between muscle tracts, as the regenerated nerve did not reach the target organ. At postoperative 12 weeks, the muscle on the operated side was well stacked, elastic, and showed a similar wet weight. These data suggest that the skeletal muscle has been innervated by the regenerated nerve fibers.

Recovery of motor function after peripheral nerve injury depends on the complete structure of motor endplates and reinnervation. Motor endplate is a specialized structure

located in the conjunction of nerve and muscle, and motor function recovery of the regenerated nerve ultimately depends on the regeneration and reconstruction of motor endplates[38-39]. After peripheral nerve injury, acetylcholinesterase activity in denervated skeletal muscle was significantly decreased[40]. Acetylcholinesterase-positive reaction is considered as a marker of neural regeneration[41]. Acetylcholinesterase histochemistry results from this study showed that acetylcholinesterase-positive reaction of the gastrocnemius muscle appeared at postoperative 12 weeks. These findings suggest that after nerve grafting, the host nerves can regenerate, grow and reach the distal target organs, develop into motor endplates, reinnervate skeletal muscle, and provide a morphological basis for motor function recovery.

After acetylcholinesterase histochemistry combined with silver staining, silver particles precipitated in the acetylcholinesterase-positive region, which can directly display motor endplate and the connected regenerated nerve terminals. This confirms that the regenerated nerve can reinnervate target organs.

In summary, repair of rat sciatic nerve defects using artificial nerves constructed with acellular nerve allografts and bone marrow mesenchymal stem cells can promote the recovery of motor function.

MATERIALS AND METHODS

Design

A randomized, controlled animal study.

Time and setting

This experiment was performed at the Laboratory of Medical Tissue Engineering, the First Affiliated Hospital of Liaoning Medical University, China between June 2008 and June 2009.

Materials

Twenty healthy, clean, male, adult Sprague-Dawley rats, aged 3 months, weighing 180–200 g, were used for preparation of acellular nerve allografts. Ten healthy, clean, male, adult Wistar rats, aged 3 months, weighing 100–120 g, were selected for extraction of bone marrow mesenchymal stem cells. Sixty healthy, clean, male, adult Wistar rats, aged 3 months, weighing 180–200 g, were used for development of sciatic nerve defect models. All animals were provided by the Department of Laboratory Animals, China Medical University, China (certification No. SCXK (Liao) 2003-0009). All experimental protocols were conducted in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China[42].

Methods

Preparation of acellular nerve allografts

Acellular nerve allografts were prepared following a modification of the method provided by Liu *et al* [17]. Following anesthesia, rats were sacrificed by cardiac perfusion, and bilateral sciatic nerve segments of Sprague-Dawley rats were harvested. Both ends of each nerve segment were fixed in the preserving container using 10-0 nylon to prevent nerve retraction. A solution of 0.05 mol/L Tris-HCl (pH 7.4) with proteinase inhibitors (0.1 μg/mL aprotinin, 0.5 μg/mL leupeptin, and 0.6 μg/mL pepstatin A) was added into the fresh-keeping box. Vibration at 4°C was performed for 5–8 days. Then the liquid in the box was replaced with Tris-HCl (pH 7.4) with 3% Triton X-100. The above-mentioned proteinase inhibitors were also added. After 5–8 days of vibration at 4°C and washes with distilled water, the nerve segments were digested for 10–15 hours at room temperature with a mixture of DNAase I and RNase and vibrated for approximately 1 week at 4°C. After washes with distilled water, acellular rat sciatic nerve segments were pre-served in PBS (pH 7.4) containing 100 μg/mL penicillin and 100 μg/mL streptomycin at 4°C.

Hematoxylin-eosin staining for the morphology of nerve scaffolds and normal nerves

The nerve scaffolds and normal nerves were fixed in 10% formalin, embedded with paraffin, and then serially sectioned into 5 μm cross-section and longitudinal slices. For routine histological analysis, paraffin sections were stained with hematoxylin-eosin as previously described[43]. Structural changes in nerves were observed under an optical microscope (Olympus, Tokyo, Japan).

Immunohistochemical staining for laminin expression in nerve scaffold

The nerve graft specimens were dewaxed and hydrated prior to microwave antigen retrieval in citrate buffer solution, followed by 3% H_2O_2 to block endogenous peroxidase. For staining, the specimens were incubated with mouse anti-rat laminin antibody (1:200; Sigma, St. Louis, MO, USA) at 4°C overnight, followed by biotinylated goat anti-mouse IgG (1:100; Sigma) at room temperature for 50 minutes. After each step, the sections were rinsed with 0.01% PBS three times for 20 minutes each. 3,3'-Diaminobenzidine was used as a chromogen for 5–10 minutes to visualize positive staining, and hema-

toxylin was used for counterstaining. The sections were then dehydrated and coverslipped. Immunohistochemical staining in nerves was observed under an optical microscope (Olympus).

Transmission electron microscopy observation for ultrastructure of acellular nerve allografts

Acellular nerve allografts were fixed with 2.5% glutaraldehyde, washed in 0.1 mol/L PBS, fixed with 1% osmic acid, then washed in 0.1 mol/L PBS. Subsequently, alcoholic dehydration, EPON812 soaking, and embedding were performed. Ultrathin sections (70 nm thick) were prepared and stained with uranyl acetate and lead citrate. Finally, the sections were observed by JEM-1200EX transmission electron microscopy (Hitachi, Tokyo, Japan).

In vitro culture and identification of bone marrow mesenchymal stem cells

Following anesthesia by intraperitoneal injection of 1% sodium pentobarbital, under sterile conditions, Wistar rats were decapitated, femur and tibia were separated, and the bone marrow cavity was rinsed with Dulbecco's modified Eagle's medium (DMEM). The rinse solution was centrifuged at 1 000 r/min at 4°C for 10 minutes, and supernatant liquid was discarded. Cells were resuspended with DMEM containing 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 U/mL penicillium, and 100 mg/mL streptomycin, and were incubated in an incubator containing 5% CO_2 at 37°C. After 24 hours, half of the culture medium was re-freshed, and after 48 hours the whole culture medium was changed. The culture medium was refreshed once every 2–3 days. After 5–7 days, cells reached 80% confluency, and were digested with 0.25% trypsin, centrifuged, and passaged at a proportion of 1:2. Passage 3 cells were used for later experiments. Using polyclonal rabbit anti-rat CD44 and CD34 antibodies (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by goat anti-rabbit IgG/FITC antibody (biotinylated goat anti-rabbit IgG; 1:1 000; Beijing Zhongshan Biological Reagent Company, Beijing, China) for 1 hour at 37°C, the bone marrow mesenchymal stem cells were detected by fluorescent microscope (Leica, Wetzlar, Hessen, Germany).

Preparation of tissue engineered nerves

Through the use of an operating microscope (Leica, Heidelberg, Germany), 1 × 10^7/L bone marrow mesenchymal stem cells were injected into the acellular nerve allografts *via* multiple points. The expanded and bulged nerve scaffold was placed into Dulbecco's modified Eagle's medium (containing 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin) for 7 days of culture. The binding of cells and scaffold was observed under scanning electron microscope (Carl Zeiss, Oberkochen, Germany).

Modeling of sciatic nerve defect and treatment of tissue-engineered nerves

Following anesthesia by intraperitoneal injection of 1% sodium pentobarbital (30 mg/kg), a 2 cm arc-shaped incision was made from the right hip to the right thigh in Wistar rats. Under the operating microscope, the sciatic nerve was exposed through a muscle-splitting incision. A 15 mm nerve defect was created by removing the sciatic nerve 5 mm away from the infrapiriform foramen. In the autologous nerve group, a nerve segment was excised, leaving a gap of approximately 15 mm because of natural retraction of the nerve stumps. The excised nerve segment was reversed and used as an autologous nerve graft to bridge the 15 mm gap. In the scaffold + cells group, artificial nerves constructed with acellular nerve allografts and bone marrow mesenchymal stem cells were used to bridge the sciatic nerve defects. In the scaffold group, acellular nerve allografts were used. The nerve stumps were sutured with 9-0 nylon. After surgery, rats were housed in groups of five per cage. Rat consciousness, activities in operated limb, diet, and wound healing were observed.

Gross observation

The behavior and appearance of rats were observed immediately after surgery, and 4 and 12 weeks after surgery.

Electrophysiological determination of regenerated nerve

At postoperative 12 weeks, following anesthesia by intraperitoneal injection of 1% sodium pentobarbital (30 mg/kg), the surgical wounds of rats in all groups were cut open to expose the proximal and distal ends of the nerve grafts. The stimulating electrodes were fixed at the proximal and distal ends of the nerve grafts separately. The recording electrode was inserted into the gastrocnemius muscle and connected to the nerve. The grounding electrode was clamped to the skin. The graphics depicted by the two stimulating electrodes were recorded, and the distance between electrodes was measured. The action potential of the sciatic nerve was measured through the use of NDI-200P+ electrophysiological apparatus (Shanghai Haishen electronic medical Co., Ltd., Shanghai, China)[44]. All acquired data were input into the computer to calculate the conduction velocity of the regenerated nerve.

Measurement of tibialis anterior muscle wet weight

At postoperative 12 weeks, the rat tibialis anterior muscle on the operated side and contralateral sides in each group were excised. After blood stain removal, the wet weight was measured through the use of an analytical balance (Sartorius, Gottingen, Germany). The tibialis anterior muscle wet weight ratio of the operated side to the contralateral side was calculated.

Histological observation of gastrocnemius muscle

Histological changes of the gastrocnemius muscle were observed as described previously[44]. At postoperative 12 weeks, rat gastrocnemius muscle on the operated side was excised for histological observation by acetylcholinesterase histochemistry combined with silver staining.

Statistical analysis

Data were statistically processed using SPSS 13.0 software (SPSS, Chicago, IL, USA) and were expressed as mean ± SD. An independent-samples *t*-test was used for comparison between groups. A $P < 0.05$ value was considered statistically significant.

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