

Repair of peripheral nerve defects with chemically extracted acellular nerve allografts loaded with neurotrophic factors-transfected bone marrow mesenchymal stem cells

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Abstract

Chemically extracted acellular nerve allografts loaded with brain-derived neurotrophic factor-transfected or ciliary neurotrophic factor-transfected bone marrow mesenchymal stem cells have been shown to repair sciatic nerve injury better than chemically extracted acellular nerve allografts alone, or chemically extracted acellular nerve allografts loaded with bone marrow mesenchymal stem cells. We hypothesized that these allografts compounded with both brain-derived neurotrophic factor- and ciliary neurotrophic factor-transfected bone marrow mesenchymal stem cells may demonstrate even better effects in the repair of peripheral nerve injury. We cultured bone marrow mesenchymal stem cells expressing brain-derived neurotrophic factor and/or ciliary neurotrophic factor and used them to treat sciatic nerve injury in rats. We observed an increase in sciatic functional index, triceps wet weight recovery rate, myelin thickness, number of myelinated nerve fibers, amplitude of motor-evoked potentials and nerve conduction velocity, and a shortened latency of motor-evoked potentials when allografts loaded with both neurotrophic factors were used, compared with allografts loaded with just one factor. Thus, the combination of both brain-derived neurotrophic factor and ciliary neurotrophic factor-transfected bone marrow mesenchymal stem cells can greatly improve nerve injury.

Key Words: nerve regeneration; peripheral nerve injury; brain-derived neurotrophic factor; ciliary neurotrophic factor; chemically extracted acellular nerve allografts; bone marrow mesenchymal stem cells; peripheral nerve; neural regeneration

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Introduction

The local concentration gradient of nerve growth factor is important for guiding proximal growth cones. Brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF) not only maintain neuronal morphology, but also promote neuronal growth (Krase et al., 2014). In recent years, with the advance in biotechnology, gene and cell-replacement therapy has gradually become an expanding area (Min et al., 2014). However, it is not known whether target genes are efficiently expressed *in vivo* either long-term or permanently. A key issue is the ability to select appropriate target cells in which introduced cells can maintain self-renewal (Tiago et al., 2014).

Bone marrow mesenchymal stem cells (BMSCs) are adult stem cells that possess self-renewal, multi-directional differentiation properties and can be gene-transfected mature

carrier cells (Mamun et al., 2013). Our previous studies investigated chemically extracted acellular nerve allograft (CEANA) compounded with BDNF-transfected BMSCs and CEANA compounded with ciliary neurotrophic factor (CNTF)-transfected BMSCs for repair of rat peripheral nerve injury and resulted in remarkable curative effects (Zhang et al., 2013, 2014a, b). We hypothesized that CEANA compounded with both BDNF- and CNTF-transfected BMSCs for repair of peripheral nerve injury would achieve better curative effects than CEANA compounded just one neurotrophic factor with transfected BMSCs. We constructed eukaryotic expression vectors carrying BDNF and CNTF genes and acquired BMSCs strains containing BDNF-CNTF genes. CEANA compounded with BDNF-CNTF gene-transfected BMSCs were transplanted by end-to-end anastomosis, thus creating space for regeneration. After being transfected

into BMSCs, BDNF and CNTF have been shown to be consistently expressed in the local microenvironment, greatly reducing neuronal death and promoting neurite regeneration after peripheral nerve injury (Hudson et al., 2004). This method also enhanced the promotional effects of CEANA and promoted the improvement of morphological changes of the injured nerve.

Materials and Methods

Animals

This study included 65 male 8-week-old Sprague-Dawley rats weighing 200–300 g, which were provided by Henan Province Laboratory Animal Center, China (license No. SCXK (Yu) 2010-0002). All efforts were made to minimize the number of animals used and the suffering caused by the procedures used in the present study. The whole experimental procedure was approved by the Ethics Committee of Zhengzhou University of China, and in accordance with the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1986). All rats were housed at controlled temperature (22°C) in a 12-hour light-dark cycle with free access to food and water.

Preparation of acellular nerve allograft

Ten Sprague-Dawley rats were anesthetized with 10% chloral hydrate (0.4 mL/100 g) and the skin at the surgical area was shaved and disinfected according to methods described by Zheng et al. (2000). Under sterile conditions, rat bilateral sciatic nerves, 10 mm in length, were cut 3 mm away from the inferior margin of periformis muscle, and adipose tissue on the surface of the nerve was removed under the microscope. Rat sciatic nerves were immersed in distilled water for 13 hours, oscillated in 3.0% Triton X-100 solution for 12 hours at room temperature and in sodium deoxycholate for 24 hours at room temperature (Sondell et al., 1998). The above steps were repeated once. The sciatic nerves were then washed in distilled water for 0.5 hours and preserved in sterile PBS solution (pH 7.4) at 4 °C for later use (Mligiliche et al., 2002).

Preparation of BDNF and/or CNTF-transfected BMSCs

Femoral bone marrow was taken from five Sprague-Dawley rats. The harvested BMSCs were cultured in a 5% CO₂-containing environment at 37 °C. After 24 hours, the culture medium was replaced for the first time. The cells were washed three times with PBS to remove the non-adherent cells, and the culture medium was replaced with an equal amount of fresh L-Dulbecco's Modified Eagle Medium (L-DMEM) and

refreshed once every 3 or 4 days. When cells reached 80–90% confluency after 8–10 days of culture, they were digested with 0.25% trypsin. When the cells became round-shaped and began to depart from the bottom of the flask, the culture was terminated with L-DMEM complete culture solution and prepared into a single cell suspension. The single cell suspension was centrifuged at 1,100 r/min for 5 minutes. After removal of supernatant, cell suspension was seeded at 1×10^5 /mL in a 6-well culture plate and culture medium was refreshed every 3 or 4 days. Cell growth was observed daily. When cells reached nearly complete confluency, they were digested with trypsin. BMSCs could be sub-cultured for several passages.

Passage 3 BMSCs were transfected with different recombinant plasmids to form four groups: pIRES-BDNF, pIRES-CNTF, pIRES-BDNF-CNTF and empty vector pIRES. A blank control group in which cells were not transfected by any plasmid was also designated. Eukaryotic expression vector pIRES (Clontech Laboratories, Mountain View, CA, USA) can highly express two target genes in eukaryotic mammalian cells. Because these two target genes are isolated by IRES sequence, the two target proteins are expressed interdependently. Cytomegalovirus eukaryotic expression promoters were used for the vectors. Kanamycin was used for prokaryotes, but geneticin was required for eukaryotes.

Recombinant plasmids pIRES-BDNF, pIRES-CNTF and pIRES-BDNF-CNTF (School of Basic Medical Sciences, Zhengzhou University, China) were electrically transferred to BMSCs. The BMSC suspension (1 mL) prepared in the above procedure was inserted into a 4-mm electrical transfer glass (Bio-Rad, Hercules, CA, USA), followed by addition of 12 µg pIRES-BDNF (or pIRES-CNTF, pIRES-BDNF-CNTF or pIRES) plasmid. The mixture was electrically transferred using the following electrical transfer parameters: voltage 300 V, capacitance 980 µF, second pulse, and square wave/cyclotron wave. The cells were subjected to electric shock within 5 minutes, immediately placed in an ice bath, and then cultured with DMEM medium containing 15% fetal bovine serum at 37 °C in a 5% CO₂ incubator.

Detection of BDNF and CNTF genes in transfected cells

The plasmid pCMV-SPORT6-BDNF (or CNTF) which encodes full-length cDNA of BDNF or CNTF gene was taken as a PCR template. PCR amplification was performed with primers specific for the BDNF gene.

The primer information is shown as follows:

Gene		Sequence (5'–3')	Product size (bp)
BDNF	Upstream	CCT CGA GAT GTT CCA CCA GGT G AG AAG AGT GA	788
	Downstream	CAC GCG TCT ATC TTC CCC TT T TAA TGG TCA GT	
CNTF	Upstream	GTC TAG AAT GTT CCA CCA GGT GA G AAG AGT GA	611
	Downstream	GGT CGA CCT ATC TTC CCC TTT TA A TGG TCA GT	

BDNF: Brain-derived neurotrophic factor; CNTF: ciliary neurotrophic factor.

Identification of recombinant plasmids

After BDNF (or CNTF) was conjugated with pIRES and transformed on LB agar plates with ampicillin, several positive colonies appeared. Two positive colonies were randomly selected and subjected to PCR amplification using primers specific for sequences of BDNF and CNTF gene. PCR product was electrophoresed on a 1.5% agarose gel to determine whether target genes BDNF and CNTF were inserted into pIRES.

After CNTF was conjugated with pIRES-BDNF and transformed on LB agar plates with ampicillin, a large number of positive colonies appeared. Two positive colonies were randomly selected and inoculated in the LB broth containing ampicillin. PCR amplification of extracted plasmids was performed using primers specific for sequences of BDNF and CNTF gene (dilution 1:100).

Based on PCR amplification, recombinant plasmid DNA was digested by restriction enzymes Xba I, Sal I, Xho I and Mlu I (New England Biolabs, Ipswich, MA, USA). PCR products were visualized by gel electrophoresis using a 1.5% agarose gel. The size of enzyme-digested products was determined by a DNA marker and the products were observed by a gel image system under a wavelength of 256 nm to confirm the recombinant BDNF and CNTF mRNA-expressing vectors pIRES-BDNF-CNTF mRNA. pIRES-BDNF-CNTF protein expression was detected by western blot assay.

Preparation of rat models of sciatic nerve defects

The remaining 50 Sprague-Dawley rats were anesthetized using 10% chloral hydrate (0.4 mL/100 g) for 2–2.5 hours. Under sterile conditions, the right sciatic nerve was exposed and a length of 7 mm sciatic nerve was cut 3 mm lateral to the inferior margin of the piriformis muscle, allowing sciatic nerve retraction (one more sciatic nerve resection was necessary if 10 mm sciatic nerve defect was not achieved). Therefore, a 10 mm sciatic nerve defect model was created.

The 50 models of sciatic nerve defect were randomly divided into five groups, with 10 rats per group: CEANA, CEANA + BDNF/BMSCs, CEANA + CNTF/BMSCs, CEANA + BDNF + CNTF/BMSCs and CEANA/BMSCs. In the CEANA group, sciatic nerve defect was repaired with 10 mm CEANA through end-to-end anastomosis. In the CEANA + BDNF/BMSCs, CEANA + CNTF/BMSCs, CEANA + BDNF + CNTF/BMSCs and CEANA/BMSCs groups, sciatic nerve defect was repaired with 10 mm CEANA through end-to-end anastomosis and then 0.1 mL of 33 mg/L BDNF/BMSCs, CNTF/BMSCs, BDNF + CNTF/BMSCs and BMSCs was injected into the tissue around the transplanted site.

General condition of rats after surgery

The mental state, wound healing, muscle atrophy and ulceration at the surgical site, complications, presence of a dragging or crouching walk were observed.

Morphology of distal anastomotic stoma of nerve allografts

At 8 weeks after surgery, a segment of sciatic nerve comprising the tissue 1 mm away from the proximal and distal

anastomotic stoma of CEANA was resected (mainly to observe whether regenerated axons passed through the anastomotic scar), fixed with 10% formaldehyde, embedded with paraffin, and successively sliced into sections (each section spanning the area 2 mm away from the proximal and distal anastomotic stoma). The transverse sections were routinely stained with hematoxylin-eosin, cut into semi-thin sections, stained with toluidine blue and observed under the optical microscope (Olympus, Tokyo, Japan). A 3 mm-long sciatic nerve segment was cut 3 mm away from distal anastomotic stoma of the sciatic nerve on the surgical side. An identical sciatic nerve segment from the control side was cut as a normal control. The specimen was rinsed, fixed with glutaraldehyde, embedded with epon 812, sliced into ultrathin cross-sections, counterstained with uranium-lead and observed under transmission electron microscope (CM120, Oxoid, Basingstoke, UK). Using the Image-Pro Plus image analysis system (Media Cybernetics, Bethesda, MD, USA), the total number of myelinated nerve fibers was counted and the thickness of myelin sheath was measured.

Rat sciatic functional index (SFI) after surgery

Before surgery and at 2, 4, 6 and 8 weeks after surgery, according to the method described by Nakazato et al. (2007), total spreading (toe spread), print length, and distance between intermediate toes (intermediary toe spread) were measured. Rat SFI was calculated using the Bain formula: $SFI = -38.3 (EPL - NPL) / NPL + 109.5 (ETS - NTS) / NTS + 13.3 (EIT - NIT) / NIT - 8.8$, where EPL is the print length of experimental feet; NPL is the print length of normal feet; ETS is the toe spread of experimental feet; NTS is the toe spread of normal feet; EIT is the distance between intermediary toe spread of experimental feet; and NIT is the distance between intermediary toe spread of normal feet. $SFI = 0$ indicates normal sciatic nerve function and $SFI = -100$ indicates complete loss of sciatic nerve function.

Determination of triceps wet weight recovery rate

At 8 weeks after surgery, rat bilateral triceps surae were harvested and weighed after surface blood blot was removed using gauze. Triceps wet weight recovery rate was calculated as (triceps wet weight on the surgical side / triceps wet weight on the control side) \times 100%.

Electrophysiology tests

At 8 weeks after surgery, rat sciatic nerve on the surgical side was exposed. Gastrocnemius muscle surgery was performed using an SXP-1 operating microscope (Shanghai Medical Equipment Co., Shanghai, China). The recording electrodes were placed in the middle of the calf muscle and pricked into the muscle at an appropriate depth. The upper part of the proximal anastomotic stoma and lower part of the distal anastomotic stoma were stimulated using stimulating electrodes to record latency and motor-evoked potential amplitude (NDI-200P EMG evoked potentiometer; Shanghai Haishen Medical Electronic Instrument Co., Shanghai, China). The distance between the two points of stimulation was measured to calculate nerve conduction velocity.

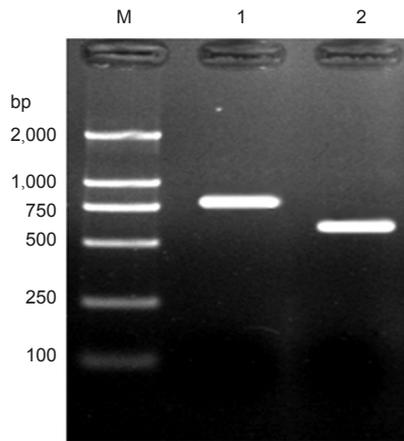


Figure 1 Electrophoretogram of the amplified products of targeted genes brain-derived neurotrophic factor (*BDNF*) and ciliary neurotrophic factor (*CNTF*) in the transfected cells.
M: Marker; 1: *BDNF* product (788 bp); *CNTF* product (611 bp).

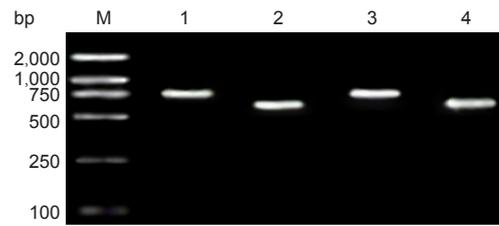


Figure 4 Identification of double-gene recon by PCR.
M: DNA marker; 1: double-gene recon 1 *BDNF* mRNA amplification product (788 bp); 2: double-gene recon 1 *CNTF* mRNA amplification product (611 bp); 3: double-gene recon 2 *BDNF* mRNA amplification product (774 bp); 4: double-gene recon 2 *CNTF* mRNA amplification product (597 bp). Recombinant plasmid pIRES-*BDNF* was digested by *Xho* I and *Mlu* I, and two fragments, a 774-bp-long target gene *BDNF* fragment and an approximately 1.6-kb-long pIRES vector fragment, were acquired, confirming the product was recombinant plasmid pIRES-*BDNF*. *BDNF*: Brain-derived neurotrophic factor. *CNTF*: ciliary neurotrophic factor.

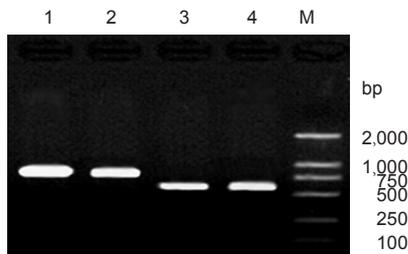


Figure 2 PCR identification of recombinant plasmids pIRES-*BDNF* and pIRES-*CNTF*.
1, 2: pIRES-*BDNF*-positive recon amplification product (788 bp); 3, 4: pIRES-*CNTF*-positive recon amplification product (611 bp); M: DNA marker. *BDNF*: Brain-derived neurotrophic factor; *CNTF*: ciliary neurotrophic factor.

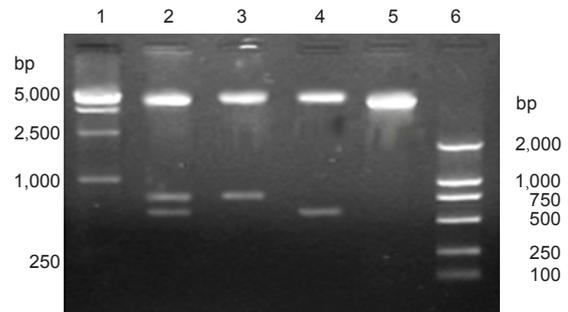


Figure 5 Identification of recombinant plasmid pIRES-*BDNF*-*CNTF* by enzymatic digestion.
1: DNA marker; 2: recombinant plasmid pIRES-*BDNF*-*CNTF* was digested by *Xba* I and *Sal* I, *Xho* I and *Mlu* I simultaneously; 3: recombinant plasmid pIRES-*BDNF*-*CNTF* was digested by *Xho* I and *Mlu* I; 4: recombinant plasmid pIRES-*BDNF*-*CNTF* was digested by *Xba* I and *Sal* I; 5: empty vector pIRES was digested by *Xho* I and *Mlu* I; 6: M: DNA marker. Enzymatic digestion products were visualized by gel electrophoresis using a 1.5% agarose gel. A 6.1-kb-sized vector band, a 774-bp-sized *BDNF* mRNA band and a 597-bp-sized *CNTF* band appeared, confirming the double-gene recombinant plasmid is pIRES-*BDNF*-*CNTF*. *BDNF*: Brain-derived neurotrophic factor; *CNTF*: ciliary neurotrophic factor.

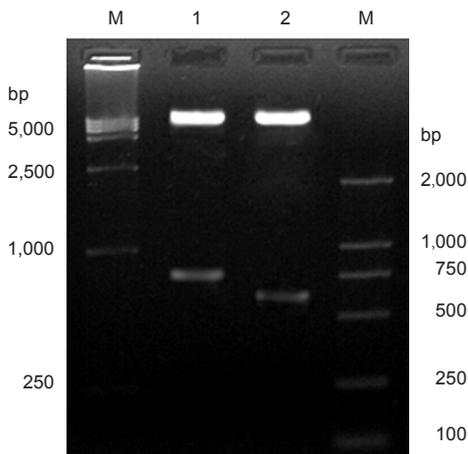


Figure 3 Identification of PCR products by enzymatic digestion.
M: DNA marker; 1: recombinant plasmid pIRES-*BDNF* (774 bp) by *Xho* I and *Mlu* I; 2: recombinant plasmid pIRES-*CNTF* (597 bp) by *Xba* I and *Sal* I; two fragments [*i.e.*, target gene *CNTF* fragment (597 bp) and pIRES vector fragment (approximately 6.1 kb)] were acquired, confirming the product was recombinant plasmid pIRES-*CNTF*. *BDNF*: Brain-derived neurotrophic factor; *CNTF*: ciliary neurotrophic factor.

Table 1 mRNA expression (relative optical density value) of *BDNF* and *CNTF* in BMSCs after transfection (RT-PCR)

Group	<i>BDNF</i>	<i>CNTF</i>
pIRES- <i>BDNF</i>	0.929±0.074**	0.393±0.031
pIRES- <i>CNTF</i>	0.291±0.024	1.143±0.089**
pIRES- <i>BDNF</i> - <i>CNTF</i>	1.072±0.081**	1.241±0.077**
pIRES	0.253±0.018	0.322±0.029
Normal control	0.221±0.015	0.347±0.028

***P* < 0.01, vs. pIRES and normal control groups (mean ± SD, *n* = 10, one-way analysis of variance and the least significance difference test. BMSCs: Bone marrow mesenchymal stem cells; *BDNF*: brain-derived neurotrophic factor; *CNTF*: ciliary neurotrophic factor.

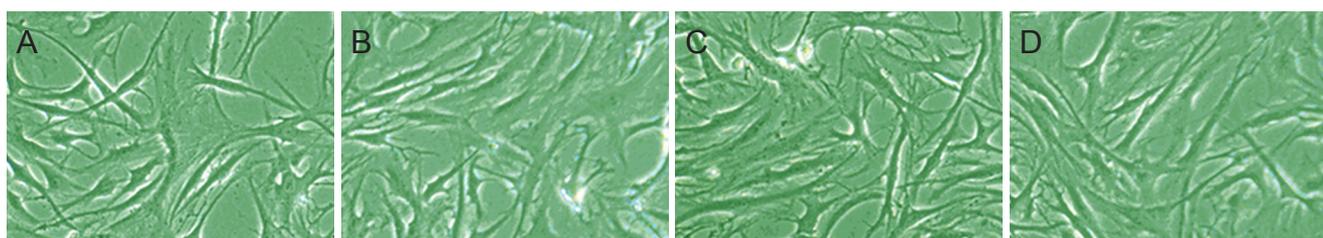


Figure 6 Morphology of BMSCs stably transfected with BDNF and CNTF mRNA (hematoxylin-eosin staining, optical microscope, × 400). (A–D) pIRES-BDNF, pIRES-CNTF, pIRES-BDNF-CNTF and pIRES groups. pIRES-BDNF, pIRES-CNTF, pIRES-BDNF-CNTF and empty vector pIRES were transfected into cells. Cells in each group gradually presented with uniform fusiform and long-shuttle-shaped appearance with a large amount of nucleoplasm, were unevenly distributed and did not grow in colonies. Cell density was increased and cells distributed along the long axis of the soma in a whirlpool. BMSCs: Bone marrow mesenchymal stem cells; CEANA: chemically extracted acellular nerve allograft; BDNF: brain-derived neurotrophic factor; CNTF: ciliary neurotrophic factor.



Figure 7 BDNF and CNTF protein expression in BMSCs after transfection (western blot assay). 1: pIRES-BDNF group; 2: pIRES-CNTF group; 3: pIRES-BDNF-CNTF group; 4: pIRES group; 5: blank control group. Western blot assay showed that BMSCs from the pIRES-CNTF and pIRES-BDNF-CNTF groups expressed high levels of CNTF, while CNTF levels in the BMSCs from the pIRES-BDNF, pIRES and blank control groups were relatively lower. BMSCs: Bone marrow mesenchymal stem cells; BDNF: brain-derived neurotrophic factor; CNTF: ciliary neurotrophic factor. GAPDH: Glycerolaldehyde phosphate dehydrogenase.

Figure 10 BDNF and CNTF protein expression in the rat nerve tissue harvested from transplantation site (western blot assay). 1: CEANA group; 2: CEANA + BDNF/BMSCs group; 3: CEANA + CNTF/BMSCs group; 4: CEANA + BDNF + CNTF/BMSCs group; 5: CEANA/BMSCs group. Recombinant plasmids pIRES-BDNF and pIRES-BDNF-CNTF were normally expressed in the nerve allografts normally expressed in the nerve allografts in the CEANA + BDNF/BMSCs and CEANA + BDNF + CNTF/BMSCs groups. Recombinant plasmids pIRES-CNTF and pIRES-BDNF-CNTF were normally expressed in the nerve allografts in the CEANA + CNTF/BMSCs and CEANA + BDNF + CNTF/BMSCs groups. CEANA: Chemically extracted acellular nerve allografts; BDNF: brain-derived neurotrophic factor; CNTF: ciliary neurotrophic factor; BMSCs: bone marrow mesenchymal stem cells. GAPDH: Glycerolaldehyde phosphate dehydrogenase.

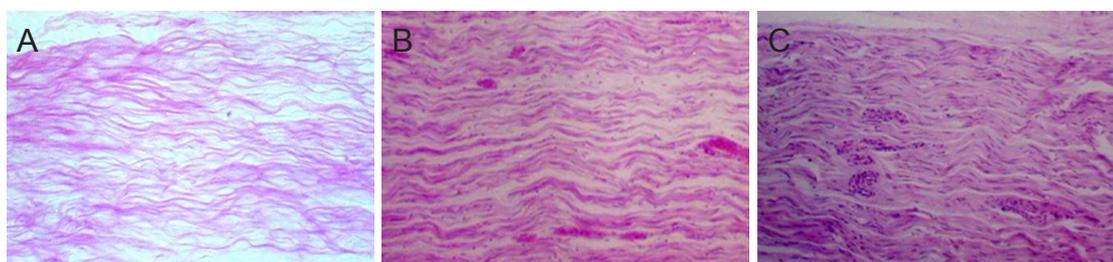


Figure 8 Morphology of distal nerve anastomotic sites at 8 weeks after transplantation of CEANA loaded with neurotrophic factors-transfected BMSCs shown on longitudinal sections (hematoxylin-eosin staining, optical microscope, × 40). (A) CEANA + BDNF/BMSCs group; (B) CEANA + CNTF/BMSCs group; (C) CEANA + BDNF + CNTF/BMSCs group. Myelinated nerve fibers were unevenly distributed in the CEANA + BDNF/BMSCs, CEANA + CNTF/BMSCs, and CEANA + BDNF + CNTF/BMSCs groups. CEANA: Chemically extracted acellular nerve allografts; BDNF: brain-derived neurotrophic factor; CNTF: ciliary neurotrophic factor; BMSCs: bone marrow mesenchymal stem cells.

Table 2 Rat sciatic functional index (SFI) after transplantation of CEANA loaded with neurotrophic factors-transfected BMSCs

Group	Time after surgery (week)			
	2	4	6	8
CEANA	-103.31±1.36	-87.13±2.47	-67.32±1.78	-52.14±1.52
CEANA/BMSCs	-92.17±2.34	-81.21±2.53	-53.42±2.65	-49.62±1.79
CEANA+CNTF/BMSCs	-87.67±3.89	-69.63±4.47	-44.43±4.56	-33.39±3.58
CEANA+BDNF/BMSCs	-76.56±1.28	-50.28±1.67	-31.69±1.56	-20.76±1.37
CEANA+BDNF+CNTF/BMSCs	-32.33±2.52	-21.25±5.35	-19.21±2.98	-18.69±1.47

At 8 weeks after surgery, rat SFI was highest in the CEANA + BDNF + CNTF/BMSCs group, followed by CEANA + CNTF/BMSCs, CEANA + BDNF/BMSCs, CEANA/BMSCs groups and lowest in the CEANA group ($P < 0.05$; mean ± SD, $n = 10$, one-way analysis of variance and the least significance difference test). SFI = 0 indicates normal sciatic nerve function and SFI = -100 indicates complete loss of sciatic nerve function. CEANA: Chemically extracted acellular nerve allografts; BDNF: brain-derived neurotrophic factor; CNTF: ciliary neurotrophic factor; BMSCs: bone marrow mesenchymal stem cells.

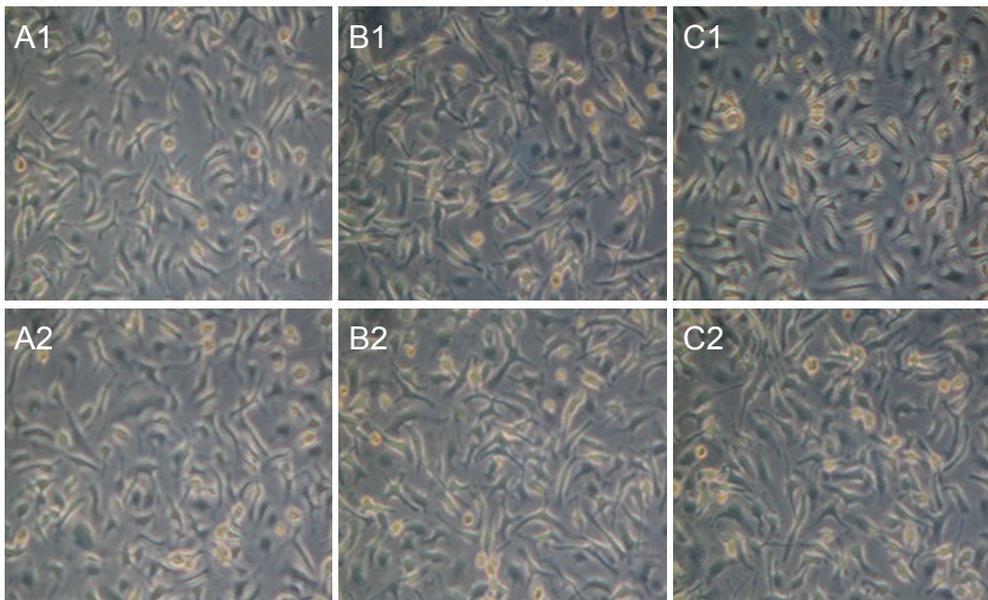


Figure 9 Morphology of distal nerve anastomotic sites at 8 weeks after transplantation of CEANA loaded with neurotrophic factors-transfected BMSCs shown on transverse sections (toluidine blue staining, optical microscope, × 40).

(A1, A2) CEANA + BDNF/BMSCs group; (B1, B2) CEANA + CNTF/BMSCs group; (C1, C2) CEANA + BDNF + CNTF/BMSCs group. In the CEANA + BDNF + CNTF/BMSCs group, there was a large number of large-sized regenerated nerve fibers with thick myelin sheath. The number of myelinated nerve fibers and myelin thickness were greater in the CEANA + BDNF + CNTF/BMSCs group than in the CEANA + BDNF/BMSCs and CEANA + CNTF/BMSCs groups. CEANA: Chemically extracted acellular nerve allografts; BDNF: brain-derived neurotrophic factor; CNTF: ciliary neurotrophic factor; BMSCs: bone marrow mesenchymal stem cells.

Table 3 Triceps wet weight recovery rate, total number of myelinated nerve fibers and myelin thickness at 8 weeks after transplantation of CEANA loaded with neurotrophic factors-transfected BMSCs

Group	Total number of myelinated nerve fibers	Myelin sheath thickness (μm)	Triceps wet weight recovery rate (%)
CEANA	1,021±365	0.27±0.21	31.24±3.65
CEANA/BMSCs	1,203±421	0.32±0.16	37.26±4.12
CEANA+BDNF/BMSCs	1,392±532	0.41±0.12	42.25±3.82
CEANA+CNTF/BMSCs	1,703±468	0.63±0.11	67.21±5.34
CEANA+BDNF+CNTF/BMSCs	1,833±578	0.82±0.13	87.51±4.86

At 8 weeks after surgery, triceps wet weight recovery rate, total number of myelinated nerve fibers, and myelin thickness were highest in the CEANA + BDNF + CNTF/BMSCs group, followed by CEANA + CNTF/BMSCs, CEANA + BDNF/BMSCs, CEANA/BMSCs groups and lowest in the CEANA group ($P < 0.05$; mean ± SD, $n = 10$, one-way analysis of variance and the least significance difference test). CEANA: Chemically extracted acellular nerve allografts; BDNF: brain-derived neurotrophic factor; CNTF: ciliary neurotrophic factor; BMSCs: bone marrow mesenchymal stem cells.

Table 4 Rat gastrocnemius muscle motor-evoked potential at 8 weeks after transplantation of CEANA loaded with neurotrophic factors-transfected BMSCs

Group	Latency (ms)	Peak-peak amplitude (mV)	Nerve conduction velocity (m/s)
CEANA	1.98±0.62	3.65±2.34	11.92±6.21
CEANA/BMSCs	1.94±0.57	4.36±2.04	15.73±5.68
CEANA+BDNF/BMSCs	1.86±0.32	5.10±1.31	18.26±4.64
CEANA+CNTF/BMSCs	1.79±0.35	7.10±2.74	22.11±4.32
CEANA+BDNF+CNTF/BMSCs	1.63±0.15	8.10±2.68	28.15±7.18

In the groups described in the table, motor-evoked potential latency was gradually shortened, amplitude was gradually increased, and nerve conduction velocity was gradually increased ($P < 0.05$; mean ± SD, $n = 10$, one-way analysis of variance and the least significance difference test). CEANA: Chemically extracted acellular nerve allografts; BDNF: brain-derived neurotrophic factor; CNTF: ciliary neurotrophic factor; BMSCs: bone marrow mesenchymal stem cells.

Western blot assay

At 8 weeks after surgery, a 100-g tissue sample was harvested from the transplantation site in each group, homogenized at a low temperature and protein extracted. After protein concentration determination, electrophoresis, and transfer to membrane, protein samples were placed in antibody solution overnight at 4°C. Reagents were added in the following order: rabbit anti-mouse BDNF and rabbit anti-mouse CNTF antibodies (dilution 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat anti-rabbit IgG antibody (dilution 1:500; Santa Cruz Biotechnology) and alkaline phosphatase at appropriate concentration, and protein samples were incubated for 1 hour. There were three TBST washes for 5 minutes each between each step. Thereafter, protein samples were developed with BCIP/NBT for 30–60 minutes, treated with distilled water and naturally dried. Protein expression of BDNF and CNTF in the tissue samples was detected by western blot assay using a gel image system (Bio-Rad).

Statistical analysis

All data were statistically processed using SPSS 13.0 software (SPSS, Chicago, IL, USA). One-way analysis of variance and the least significance difference test were used for intergroup comparison. A level of $P < 0.05$ was considered statistically significant.

Results

BDNF and *CNTF* mRNA expression levels after transfection

PCR products of *BDNF* and *CNTF* target gene bands were consistent with that of the expected size (Figure 1).

Identification of recombinant plasmids pIRES-*BDNF* and pIRES-*CNTF*

Recombinant plasmids pIRES-*BDNF* and pIRES-*CNTF* were preliminarily confirmed by the presence of a 788 bp-sized *BDNF* band and a 611 bp-sized *CNTF* target gene band (Figure 2).

Identification of PCR products by enzymatic digestion

Recombinant plasmid pIRES-*CNTF*, which was preliminarily confirmed positive by PCR, was digested by *Xba* I and *Sal* I. Two fragments, a 597-bp-long target gene *CNTF* fragment and an approximately 6.1-kb-long pIRES carrier fragment, were acquired (Figure 3), which suggests that the recombinant plasmid was pIRES-*CNTF*. Recombinant plasmid pIRES-*BDNF*, which was preliminarily confirmed as positive by PCR, was digested by *Xho* I and *Mlu* I. Two fragments, a 774-bp-long target gene *BDNF* fragment and an approximately 6.1-kb-long pIRES carrier fragment, were acquired (Figure 4), which suggests that the recombinant plasmid was pIRES-*BDNF*.

DNA sequencing

The MCS-A insertion sequence of the recombinant plasmid pIRES-*BDNF*, which was confirmed by PCR amplification and *Xho* I and *Mlu* I digestion, was analyzed and its homology with the sequence of NM_007540 *BDNF* gene in the GenBank was compared. The MCS-A insertion sequence was completely consistent with the designed. This suggests that the acquired recombinant plasmid was pIRES-*BDNF*. The MCS-B insertion sequence of the recombinant plasmid pIRES-*CNTF*, which was confirmed by PCR amplification and *Xba* I and *Sal* I digestion, was analyzed and its homology with the sequence of NM_170786 *CNTF* gene in the GenBank was compared. The MCS-B insertion sequence was completely consistent with the designed. This suggests that the acquired recombinant plasmid was pIRES-*CNTF*.

Reconstruction of recombinant plasmid pIRES-*BDNF*-*CNTF*

PCR identification: In Figure 4, there was a 611-bp-long target gene *CNTF*, indicating that target gene *CNTF* was inserted into the MCS-B sequence of recombinant plasmid pIRES-*BDNF*. At the same time, a 788-bp-long target gene *BDNF* also appeared on the vector pIRES. Therefore, dou-

ble-gene recombinant plasmid pIRES-*BDNF*-*CNTF* was preliminarily confirmed.

Enzymatic digestion: In Figure 5, there was a 6.1-kb-long vector band, a 774-bp-long *BDNF* gene band and a 597-bp-long *CNTF* gene band. This suggests that the double-gene recombinant plasmid is pIRES-*BDNF*-*CNTF*.

Recombinant plasmid expression in BMSCs after transfection

At 24 hours after pIRES-*BDNF*-*CNTF* was electrically transferred to BMSCs, DMEM containing 400 $\mu\text{g}/\text{mL}$ G418 was added. About 3 weeks later, stable *BDNF* and *CNTF* mRNA-transfected BMSCs were acquired (Figure 6) and the cells were maintained with DMEM containing 400 $\mu\text{g}/\text{mL}$ G418.

BDNF and *CNTF* mRNA expression after transfection

RT-PCR results showed that in the pIRES-*BDNF* group (or pIRES-*CNTF* group) and pIRES-*BDNF*-*CNTF* group, pIRES-*BDNF* (or pIRES-*CNTF* group) and pIRES-*BDNF*-*CNTF* expressed by cells can highly transcribe *BDNF* (or *CNTF*) mRNA ($P < 0.01$, vs. pIRES group and blank control group) (Table 1).

BDNF and *CNTF* protein expression after transfection

Western blot assay showed an intense *BDNF* band in BMSCs from the pIRES-*BDNF* and pIRES-*BDNF*-*CNTF* groups, and a much lighter *BDNF* band in the pIRES-*CNTF*, pIRES and blank control groups (Figure 7). Thus pIRES-*BDNF* and pIRES-*BDNF*-*CNTF* transfected in BMSCs from the pIRES-*BDNF* and pIRES-*BDNF*-*CNTF* groups can highly express *BDNF* protein and confirms that BMSCs strains highly expressing *BDNF*, *CNTF* and *BDNF*-*CNTF* were acquired.

General condition of rats after surgery

At 8 weeks after surgery, rat wound healing was good and without infection, and the ulcer at the surgical site disappeared completely. There was no obvious difference in surgical surface before and after surgery in the CEANA + *BDNF*/BMSCs, CEANA + *CNTF*/BMSCs, CEANA + *BDNF* + *CNTF*/BMSCs, and CEANA/BMSCs groups. In the CEANA + *BDNF* + *CNTF*/BMSCs group, rat feet could completely straighten when walking and there was no obvious difference in locomotor activity between the left and right sides. Rats in the CEANA and CEANA/BMSCs groups bent their bodies when walking.

Morphology of distal anastomotic stoma of nerve allografts

Hematoxylin-eosin staining: At 8 weeks after surgery, myelinated nerve fibers were unevenly distributed in the CEANA + *BDNF*/BMSC, CEANA + *CNTF*/BMSCs and CEANA + *BDNF* + *CNTF*/BMSCs groups (Figure 8). Toluidine blue staining showed a high number of large-sized regenerated nerve fibers with a thick myelin sheath. The number of myelinated nerve fibers and myelin thickness were greater in the CEANA + *BDNF* + *CNTF*/BMSCs group than in the CEANA + *BDNF*/BMSCs and CEANA + *CNTF*/BMSCs groups

(Figure 9). The number of myelinated nerve fibers and myelin thickness in the CEANA and CEANA/BMSCs groups were inferior to those in the CEANA + BDNF/BMSC, CEANA + CNTF/BMSCs and CEANA + BDNF + CNTF/BMSCs groups.

Rat SNFI after surgery

Before and 2, 4, 6 and 8 weeks after surgery, wounds healed well. SNFI in the CEANA + BDNF/BMSCs, CEANA + CNTF/BMSCs, and CEANA + BDNF + CNTF/BMSC groups increased with time. At 8 weeks after surgery, SNFI was highest in the CEANA + BDNF + CNTF/BMSCs group, followed by CEANA + CNTF/BMSCs, CEANA + BDNF/BMSCs, CEANA/BMSCs groups and lowest in the CEANA group ($P < 0.05$; Table 2).

Triceps wet weight recovery rate, total number of myelinated nerve fibers and myelin thickness after surgery

At 8 weeks after surgery, triceps wet weight recovery rate, total number of myelinated nerve fibers and myelin thickness in the CEANA + BDNF + CNTF/BMSCs group were significantly higher than in the other groups ($P < 0.05$). A large number of large-sized regenerated nerve fibers were observed in the CEANA + BDNF/BMSCs, CEANA + CNTF/BMSCs and CEANA + BDNF + CNTF/BMSCs groups. Triceps wet weight recovery rate, total number of myelinated nerve fibers, and myelin thickness were highest in the CEANA + BDNF + CNTF/BMSCs group, followed by CEANA + CNTF/BMSCs, CEANA + BDNF/BMSCs, CEANA/BMSCs groups and lowest in the CEANA group ($P < 0.05$; Table 3).

Electrophysiological index changes of injured rat sciatic nerve after surgery

At 8 weeks after surgery, rat gastrocnemius muscle motor-evoked potential amplitude in the CEANA + CNTF/BMSCs group was significantly higher than in the CEANA + BDNF/BMSCs group, but it was significantly lower than in the CEANA + BDNF + CNTF/BMSCs group (both $P < 0.05$). In each group, motor-evoked potential latency was gradually shortened, amplitude was gradually increased, and nerve conduction velocity was gradually increased after surgery (all $P < 0.05$; Table 4).

BDNF and CNTF protein expression in the rat nerve tissue harvested from transplantation site

Western blot assay results of the CEANA, CEANA + BDNF/BMSCs, CEANA + CNTF/BMSCs, CEANA + BDNF + CNTF/BMSCs, and CEANA/BMSCs group are shown in Figure 10. BDNF expression in the rat nerve allografts from CEANA + BDNF/BMSCs and CEANA + BDNF + CNTF/BMSCs was stronger than in the other groups, indicating that recombinant plasmids pIRES-BDNF and pIRES-BDNF-CNTF were normally expressed in the nerve allografts. CNTF expression in the rat nerve allografts from CEANA + CNTF/BMSCs and CEANA + BDNF + CNTF/BMSCs was stronger than in the other groups, indicating that recombinant plasmids pIRES-CNTF and pIRES-BDNF-CNTF were

normally expressed in the nerve allografts.

Discussion

Neurotrophic factors in the microenvironment play an important role in peripheral nerve regeneration (Zhang et al., 2013). Strong evidence exists that soluble protein can promote the synthesis of some nerve regeneration-related enzymes and exhibit effects on neuronal regeneration and survival by inhibiting neuronal apoptosis (Kubo et al., 2001; Zhang et al., 2014a, b). BDNF and CNTF are important neurotrophic factors which affect central and peripheral neurons and not only increase the survival rate of chick embryo sensory neurons, but also promote neurite growth (Mohammad et al., 2000; Mligilich et al., 2002).

BMSCs are a kind of non-hematopoietic adult stem cell that exhibits multidirectional differentiation potential, can express Schwann cell markers after *in vitro* culture and differentiation, and promote the repair of peripheral nerve defects after transplantation (Lassner et al., 1989; Matsumoto et al., 2005). Taken together, BMSCs are characterized by a rich source, strong proliferative capacity and low immunogenicity (Sun et al., 2006). BMSCs can migrate widely and integrate into surrounding tissue easily, and better transfect and express exogenous genes than other stem cells. All these features indicate that BMSCs are ideal carrier cells for gene therapy (Hudson et al., 2004a, b).

CEANA exhibits extremely low immunogenicity, reduces post-transplantation immunological rejection, creates an ideal microenvironment for nerve regeneration, guides the neurite of regenerated peripheral nerves to grow from the proximal disassociated nerve end toward the distal part, reduces the opportunities of aberrant nerve regeneration, ensures selective regeneration, and contributes to growth and functional recovery of regenerated nerves (Zhang et al., 2014a).

In this study, we constructed genetically engineered cells by transfecting BMSCs with BDNF and CNTF using eukaryotic expression vectors. We detected *BDNF* and *CNTF* mRNA expression after transfection and thus, BMSCs strains highly expressed *BDNF* mRNA, *CNTF* mRNA and *BDNF-CNTF* mRNA. We used CEANA compounded with BDNF and CNTF-transfected BMSCs to repair sciatic nerve defects. Results showed that in the CEANA + BDNF + CNTF/BMSCs group, tissue adhesion was milder than that in the other groups, no obvious inflammatory reaction was observed, and only a small amount of scar tissue formed. The number of myelinated nerve fibers, myelin thickness, sciatic functional index, and triceps wet weight recovery rate were highest in the CEANA + BDNF + CNTF/BMSCs group, followed by CEANA + CNTF/BMSCs group, CEANA + BDNF/BMSCs group, CEANA/BMSCs group, and lowest in the CEANA group. In the CEANA + BDNF + CNTF/BMSCs group, neurite density, nerve regeneration velocity, and motor-evoked potential amplitudes in the soleus muscle were greater compared with the other groups. Western blot assay results showed that at 8 weeks after surgery, pIRES-BDNF and pIRES-BDNF-CNTF were normally expressed in the

nerve allografts. The CNTF in the nerve allografts from the CEANA + BDNF + CNTF/BMSCs and CEANA + CNTF/BMSCs groups was stronger than that in the nerve allografts from the CEANA + BDNF/BMSCs, CEANA + BMSCs and CEANA groups. These results indicate that after *CNTF* and *CNTF* mRNA transfection in BMSCs, recombinant plasmids pIRES-CNTF and pIRES-BDNF-CNTF are normally expressed in the nerve allografts.

This suggests that BDNF- and CNTF-transfected BMSCs injected into CEANA exhibit effects on peripheral motor neurons; that is, they first inhibit the irreversible degeneration of soma and maintain neuronal growth, then induce regenerated neurites to extend and pass through the injured site, and lastly promote neurite growth cone to enter the target organ, differentiate into new neurites and advance nerve regeneration. We also found that the treatment with BDNF and CNTF together shows better therapeutic effects than that involving just one neurotrophic factor. Therefore, it is presumed that BDNF and CNTF show synergistic effects. Results from this study also suggest that *BDNF* and *CNTF* mRNA-transfected BMSCs secrete a large amount of BDNF and CNTF and promote the regeneration of peripheral tissue cells through paracrine action. After transplantation, the carrier cells not only secrete a large amount of neurotrophic factors, but also compensate for neurons lost during ischemic injury. CEANA compounded with BDNF and CNTF-transfected BMSCs for repair of sciatic nerve injury is better than CEANA singularly compounded with BDNF or CNTF-transfected BMSCs.

Author contributions: WHH and YRZ designed this study and analyzed experimental data. YRZ, HZ and KK performed experiments. GCZ, GQZ and YS participated in molecular biology experiments. All authors approved the final version of this paper.

Conflicts of interest: None declared.

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