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Original Article

Experimental study of vein subvolution combined with neural stem cells to repair sciatic neurologic defects in rats

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Abstract: This study aims to explore the effects of vein subvolution combined with neural stem cells on nerve regeneration. Animal model of sciatic nerve defects was established with SD rats. A total of 63 SD rats were divided into control group, vein subvolution group (treatment 1 group) and vein subvolution combined with neural stem cells group (treatment 2 group). The recovery of neurological function after 12 weeks was evaluated by nerve electrophysiological technique, histological observation and other methods. The recovery degree of sciatic nerve defect in treatment 2 group was better than that of other groups; The SFI values significantly decreased in treatment 2 group after 8 weeks; the regenerative nerve fiber numbers in treatment 2 group were significant higher than that of other groups; the recovery rates of treatment 1 and 2 groups were significant higher than that of control group. The effects of vein subvolution combined with neural stem cells on repairing peripheral nerve defects were better than that of vein subvolution method.

Keywords: Vein subvolution, neural stem cells, tissue engineering, neurologic defect

Introduction

Peripheral nerve defects are common and it is still a great challenge for their clinical treatment because of the specific characteristics of peripheral nerve defects [1]. At present, peripheral nerve defect is still mainly repaired with autologous nerve graft, which is limited because of the microsurgery operation technology and the limited source of autologous nerve. It has become an inevitable trend for the use of tissue engineering technology instead of nerve graft to promote the regeneration of peripheral nerve for the treatment of peripheral nerve defect [2]. There were many studies on the nerve conduit bridging technique for repair of peripheral nerve defects. The nerve conduit bridging technology is an ideal method to repair nerve and neural physiological function can recovery rapidly. Vein membrane has good viscoelasticity because nerve fiber rich in it and it is considered as a good nerve bridging material. There are number of veins and it is easy to draw with little damage. It can guide nerve regeneration more effectively through inside-out vein graft and bridging nerve [3]. The autologous vein bridging subvolution has mature clinic application in the repair of peripheral nerve defects.

Neural stem cells (NSCs) are a kind of multipotent stem cell with multiple differentiation potential and self replication update, they mainly distributed in the cerebral cortex, spinal cord and along the ventricle [4]. NSCs were paid more attention in cell transplantation because of their excellent properties [5, 6]. They had been successfully applied in the treatment of central nervous system diseases [7-9]. It was reported that NSCs were also used to repair sciatic nerve defect in rats. There were a large number of myelinated fibers after implantation of NSCs for 6-10 weeks [10], which suggested that NSCs were very promising in the repair of peripheral nerve defects.

The regeneration of peripheral nerve requires a suitable micro-environment, autologous vein

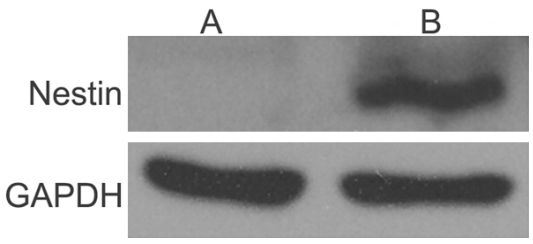

Figure 1. Determination of specific protein nestin of NSC by Western blotting method. A: C6; B: Neural stem.

Table 1. Results of electrophysiological examination

Group	Nerve conduction velocity (m/s)	Action potential (mV)
Control	4.80±0.73	1.44±0.19
Treatment 1	17.71±2.61**	3.73±0.89**
Treatment 2	24.05±1.46**,#	6.84±0.75**,#

*P<0.05; **P<0.01 *vs* control; #P<0.01; *vs* Treatment 1.

subvolution bridging cannot provide good regeneration microenvironment. Therefore, we explored the effects of vein subvolution combined with neural stem cells on nerve regeneration in rats' model, which could provide experimental basis for the treatment of peripheral nerve defects.

Materials and methods

Experimental animals

A total of 63 SPF SD male rats (weight 250±50 g) were purchased from Shanghai experimental animal center. The rats were raised in cages with good ventilation and natural day and night lighting at 18~25°C. The rats were raised for one week for adaptation and then anaesthetized with intraperitoneal injection of 2% pentobarbital sodium (30 mg/kg weight). The sciatic nerve was exposed under sterile conditions and 10 mm sciatic nerve was resected from piriform margin to establish the sciatic nerve defect model. They were divided into 3 groups: control group without treatment after modeling; treatment 1 group with vein subvolution after modeling and treatment 2 group with vein subvolution combined with neural stem cells after modeling. In treatment 1 group, 14 mm vein segments were obtained from the left jugular vein of rats under aseptic conditions, the inside of vein turned out and sutured at the nervous broken ends. In treatment 2 group, the flipped vein was injected 100 μl mixture of ECM matrix and cultured NSCs and then sutured at the nervous broken ends.

Reagents and instruments

Pentobarbital sodium (SIGMA, Cat No. P3761); Medium (GIBCO, Cat No. 11330-057); Fetal bovine serum (GIBCO, Cat No. 10099-133); B-27 (GIBCO, Cat No. 17504-001); EGF Recombinant Mouse Protein (GIBCO, Cat No. PMG8043); bFGF Recombinant Mouse Protein (GIBCO, Cat No. PMG0031); ECM Matrigel (SIGMA, Cat No. E0282); Nestin monoclonal antibody (Santa Cruz Biotechnology, Cat No. sc-21248); Horseradish peroxidase (HRP) labeled antibody; Optical microscope (Olympus BX53); NeuroCare-CT/DT/ET (Shanghai).

Preparation of neural stem cells from newborn mouse

The newborn 0-3 day mice were killed by cervical vertebra dislocation and disinfected by 75% alcohol for 2 minutes, the fetal rat cerebral cortex was taken out by using a dissecting microscope and the meninges and blood vessels were stripped. The cleared cerebral cortex was digested with 0.25% trypsin and 0.02% EDTA in Eppendorf tube at 37°C for 5 min. They were centrifuged and the supernatant was removed. The cell precipitation was re-suspensed with DMEM/F12 medium (containing 2% B27, 20 ng/ml EGF and 20 ng/ml bFGF) and cultured in flask at 37°C with 5% CO_2.

The determination of specific protein Nestin of NSC by Western blotting

The cells were lysed using lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40 and 0.1% sodium dodecylsulfate (SDS) at pH 7.4, supplemented with protease inhibitors cocktail (10 g/ml leupeptin, 10 g/ml pepstatin A, 10 g/ml aprotinin and 1 mM of 4-(2-aminoethyl)-benzenesulfonyl fluoride) and phosphatase inhibitors (1 mM NaF and 1 mM Na3VO4). The cell lysates were centrifuged at 1000 g for 20 min at 4°C. The proteins were size-fractionated by SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane. The membrane was then blocked with 5% skimmed dry milk in Tris-buffered saline. The primary antibody anti-Nestin (1:1000) was added and incubated for 2 h at room temperature. They were washed and developed using a peroxidase-conjugated secondary antibody.

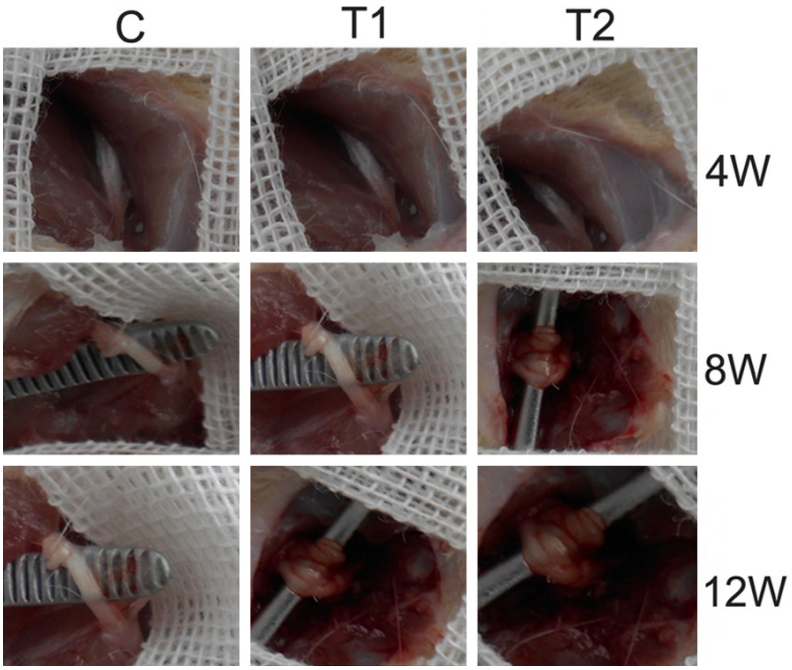

Figure 2. The regeneration of sciatic nerve in different groups. C: control group; T1: treatment 1 group; T2: treatment 2 group; W: weeks.

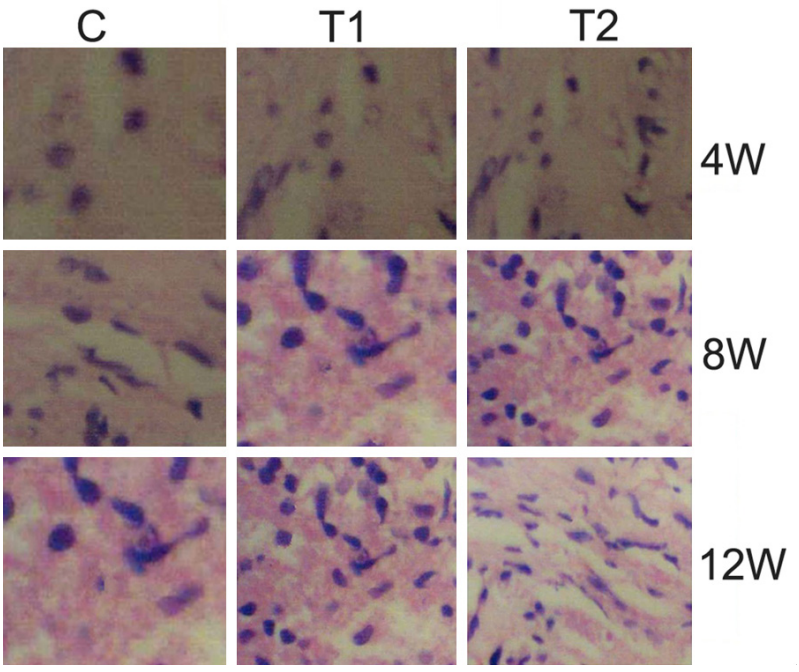

Figure 3. HE staining of the regeneration of sciatic nerve in different groups. C: control group; T1: treatment 1 group; T2: treatment 2 group; W: weeks.

Electrophysiological examination of sciatic nerve

The nerve conduction velocity (NCV) and action potential (AP) of rats' sciatic nerve were determined with NeuroCare-CT/DT/ET detector after operation for 12 weeks. The rats were anaesthetized with intraperitoneal injection of 2% pentobarbital sodium (30 mg/kg weight) and cut the original wound, the electrode clipped at both ends of plants and the waveform of electrodes was recorded. NCV and AP were calculated.

The determination of sciatic nerve function index

After operation for 4 weeks, 8 weeks and 12 weeks, the sciatic nerve function index (SFI) was detected according to the Bain calculation formula. SFI=-100% suggested complete loss of nerve function, SFI=0~-11% suggested normal nerve function, SFI=-11%~-100% suggested part nerve function restored.

Histological observation

The middle samples of flipped vein were taken after operation for 4 weeks, 8 weeks and 12 weeks for conventional HE staining. The regenerative nerve fiber numbers were calculated under microscope to evaluate the growth of nerve fibers.

Detection of recovery rate of gastrocnemius wet weight

To compare the recovery of muscle atrophy in operation side, the hibateral gastrocnemius muscles of rats were separated after operation for 12 weeks. Their wet weights were weighed and the recovery rates were calculated. Recovery rate of gastrocnemius wet weight (%) = gastrocnemius wet weight of operation side/gastrocnemius wet weight of normal side ×100%.

Table 2. The results of sciatic nerve function index determination

Group \ Time	Sciatic nerve function index (SFI/%)		
	4 weeks	8 weeks	12 weeks
Control	-91.3±5.24	-70.8±2.83	-68.1±5.31
Treatment 1	-89.6±3.77	-67.7±2.67	-62.6±1.80*
Treatment 2	-89.8±1.84	-66.3±2.29**	-60.5±1.83**

*P<0.05; **P<0.01 vs control.

Table 3. The number determination of regenerative nerve fibers in different groups

Group \ Time	The number of regenerative nerve fibers (Mean ± SD)		
	4 weeks	8 weeks	12 weeks
Control	619±119	1471±344	1821±442
Treatment 1	820±101	2203±243**	3967±176**
Treatment 2	885±95	2954±315**	4040±824**,#

*P<0.05; **P<0.01 vs control; #P<0.01; *vs* Treatment 1.

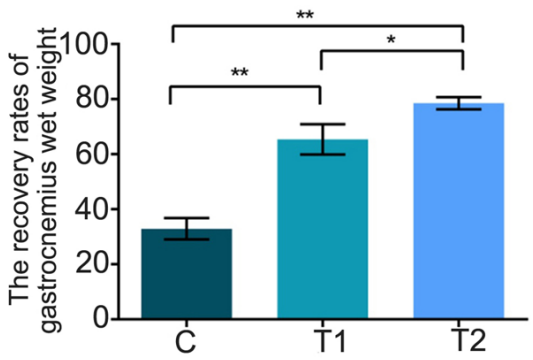

Figure 4. The recovery rates of gastrocnemius wet weight in different groups. C: control group; T1: treatment 1 group; T2: treatment 2 group. *P<0.05; **P<0.01.

Statistic analysis

All results were expressed as the mean ± SD. SPSS 15.0 software was used to do statistic analysis. A Student's t-test was used to compare individual treatments with their respective control values. P<0.05 was considered to indicate a statistically significant difference.

Results

Nestin expressed in NSCs

As shown in **Figure 1**, western blotting results showed that nestin specifically expressed in neural stem cells isolated from the brain tissue of newborn mouse, while it did not express in control cells.

The results of nerve electrophysiological examination

The results of NCV and AP after operation for 12 weeks were shown in **Table 1**. It showed that both single vein subvolution and vein subvolution combined with neural stem cells could repair the damaged nerve. The recovery degree of sciatic nerve defect in treatment 2 group was better than that of other groups (P<0.01).

The determination of sciatic nerve function index

The regeneration of sciatic nerve in different groups was shown in **Figure 2** and the HE staining of the regeneration of sciatic nerve in different groups was shown in **Figure 3**. The results of sciatic nerve function index determination were shown in **Table 2**. There was no obvious difference among the groups in SFI values after treatment for 4 weeks, which suggested that the function of new nerve cells has not been fully restored. The SFI values significantly decreased in treatment 2 group after 8 weeks, which suggested that the repair effects of treatment 2 group were better than that of other groups (P<0.01).

The determination results of regenerative nerve fiber numbers

The determination results of regenerative nerve fiber numbers were shown in **Table 3**. It showed that the regenerative nerve fiber numbers in treatment 2 group were significant higher than that of other groups (P<0.01).

Detection of recovery rate of gastrocnemius wet weight

The results of recovery rate of gastrocnemius wet weight were shown in **Figure 4**. It showed that the recovery rates of treatment 1 and 2 groups were significant higher than that of control group, and the recovery rate of treatment 2 group was significant higher than that of treatment 1 group (P<0.05).

Discussion

Repair treatment of peripheral nerve defects was a hot research field of surgery, it greatly

improved with the progress of microsurgical technique. Autologous nerve graft was considered to be the best choice of peripheral nerve repair for a long time. However, it was limited by difficult source, trauma and complications. At present, the researches focus on the choice of vein and the regeneration microenvironment. Nerve repair and regeneration need a good microenvironment [11, 12], good regeneration microenvironment close to the nature physiological state is the key to the success in the repair of peripheral nerve defect.

Tissue engineering played an important role in the repair of peripheral nerve defects. There is not a compliant material to fully achieve the repairing effect of autogenous nerve transplantation so far [13, 14], at the same time, the new materials were limited by immunoreactions. Autogenous vein had many good characteristics, there were many Schwann cells in it which promote the growth of new nerve [15, 16]. The flipped vein is a kind of ideal nerve conduit. However, the difficult of autografts limited its application. In this study we explored the creation of good regeneration microenvironment with adding seed cells in vain.

NSCs mainly used in the treatment of central nervous system diseases, there were a few reports of the application in the repair of peripheral nerve injury [10]. In this study we isolated NSCs from the brain tissue of newborn mice. Nestin is the specific protein markers of NSCs and could express in NSCs specifically. Nestin was only expressed in the neural epithelium in early embryonic development and stopped after birth. The NSCs we obtained could express nestin. The NCV in vein subvolution combined with neural stem cells treatment group was higher than that of vein subvolution group. The SFI in vein subvolution combined with neural stem cells treatment group decreased significantly after operation for 8 weeks, which suggested that the effects of vein subvolution combined with neural stem cells on repairing peripheral nerve defects were better than that of vein subvolution method.

Disclosure of conflict of interest

None.

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