Research on the repairing effect of polylactic acid-trimethylene carbonate/GNDF slow-release catheter on the injured femoral nerve fiber

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Abstract: Present research aims to investigate the repairing effect of polylactic acid-trimethylene carbonate/GNDF slow-release catheter on the injured femoral nerve fiber. Adult SD male rats as the subjects were divided into two groups, the GDNF group and the control group, and received the surgery to remove the nerve from the exposed left femoral nerves. Thereafter, rats in the GNDF group and the control group received the GNDF or normal saline, and we evaluated the changes in rats, including the morphological, functional and electrophysiological changes of regenerated nerves. Regenerated axons were found in each group, but enormous regeneration of axons was only identified in GDNF group. Further analysis showed that: At the 4^{th}, 8^{th} and 12^{th} weeks, areas of the regenerated nerves in GDNF group were (0.95±0.06) mm^2, (1.14±0.07) mm^2 and (1.22±0.06) mm^2, respectively; in the control group, these were (0.15±0.01) mm^2, (0.25±0.07) mm^2 and (0.52±0.05) mm^2, respectively. These showed that the outcome of GDNF group was superior to that of control group. In GDNF group, quantities of the myelinated fiber were ($0.8119\times10^4\pm0.0637\times10^4$), ($1.3371\times10^4\pm0.0460\times10^4$) and ($1.7669\times10^4\pm0.0542\times10^4$); while in control group, these were ($0.2179\times10^4\pm0.0097\times10^4$), ($0.3490\times10^4\pm0.0329\times10^4$) and ($0.7737\times10^4\pm0.0788\times10^4$). Again, these results also indicated that the outcome of GDNF group was superior to that of the control group ($p<0.05$). In GDNF group, the average diameters of myelinated fibers were (2.25±0.17) μm, (2.42±0.14) μm and (2.80±0.10) μm, which were significantly better than (1.24±0.08) μm, (1.43±0.14) μm and (1.82±0.14) μm in the control group. Degrees of fiber myelination in the GDNF group were (0.71±0.03), (0.64±0.03) and (0.6l±0.0l), respectively, which were also significantly higher than (0.02±0.01), (0.04±0.01) and (0.06±0.02) in the control group ($p<0.01$). At the 12^{th} week after surgery, HE staining was performed to observe the histological changes in quadriceps femoris for evaluation of atrophy in each group. In the GDNF group, significant amelioration was found in the atrophy of quadriceps femoris with an average area of myofiber of (84.95±3.92) %, while the area of the control group was (57.95±5.78) %, suggesting that the outcome of the GDNF group was better than that of the control group ($p<0.05$). Electrophysiological examination of nerves was employed to detect the recovery of neurological functions after repair of nerve defect. At the 4^{th}, 8^{th} and 12^{th} weeks after surgery, CMAP amplitudes in the GDNF group were (9.34±0.52) mV, (14.40±0.69) mV and (19.18±0.48) mV, significantly better than (0.39±0.07) mV, (1.44±0.41) mV and (9.27±0.40) in the control group ($p<0.01$). Polylactic acid-trimethylene carbonate/GNDF slow-release catheter can accelerate the functional recovery of injured nerves, thus promoting the regeneration efficiency of femoral nerves.

Keywords: Polylactic acid-trimethylene carbonate/GNDF slow-release catheter, injured, femoral nerve fiber, rat.

INTRODUCTION

Frequently seen in clinical practice, peripheral neuropathy can result in dysfunction of muscle that is dominated by the affected nerves. Although various methods have been developed so far, function recovery remains poor, which is mainly caused by a prolonged regeneration of nerves: For instance of long-nerve defect, irreversible atrophy or degeneration of motor end-plate has been developed before the regenerated axon reaches to the muscle that is dominated by itself, which makes recovery of motor function of muscle impossible [1]. Thus, it is necessary to search for a method to facilitate the regeneration of motor nerves.

Glial cell line-derived neurotrophic factor (GDNF), firstly found in 1993 by Lin LF, is a member of transforming growth factor-β superfamily, and the mature GDNF comprises of 134 amino acid residues and 3 pairs of disulfide bonds [2]. GDNF can phosphorylate the receptor tyrosine kinase (RET) by binding to α receptor of GDNF to transfer the intracellular signal, thereby nourishing the dopaminergic neuron, motor neuron, sensory neuron and sympathetic neuron. Thus, GDNF has been identified as one of the most active neurotrophic factors for motor neurons [3]. In this study, attempts were made to repair the injured femoral nerve branch in rats with polylactic acid-trimethylene carbonate/GNDF slow-release catheter, aiming to provide new ideas for development of therapy of peripheral neuropathy and theoretical evidence for clinical application.

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MATERIALS AND METHODS

Experiment apparatus and material

Adult Sprague-Dawley male rats (provided by Experiment Animal Center of Sichuan University) between 220 and 250 g; polylactic acid (Sigma, USA); trimethylene carbonate (Sigma, USA); GNDF (Sigma, USA); paraformaldehyde (Sigma, USA); electromyogram (Keypoint 4C, Dantec, Germany); fluorescent microscope (BX-60, Olympus, Japan); transmission electron microscope (H600, Hitachi, Japan).

Preparation of polylactic acid-trimethylene carbonate catheter

250 mg polylactic acid and 50 mg trimethylene carbonate were dissolved in 5 mL glacial acetic acid (3 mg/mL) for 24 hours at 4°C and the mixture was then added into GNDF or normal saline (10 mmol/L); mixture was dissolved evenly at 12000 r/min, followed by vacuuming and incubation at 4°C overnight. Thereafter, mold was slowly placed in liquid nitrogen, followed by cooling in different temperatures and drying at 60°C and 13.3 Pa for 24 hours. Then, it was cut into nerve catheter in length of 14 mm, crosslinked by genipin and shaped. Catheter was dried and sanitized through exposing at ^{60}Co and sealed for later use.

Animal experiment

20 SD rats were divided into two groups: GDNF group (n=10) and control group (n=10). Anesthetized by intraperitoneal injection of pentobarbital sodium (1%), rats underwent surgeries under septic environment to remove the nerve in length of 10 mm from the exposed left femoral nerves.

A 7 mm-long slow release catheter was inserted between two ends of nerves to guarantee the length of nerve defect to reach 5 mm. After surgery, rats were fed in separated cages.

Observation indexes

Morphological analysis of regenerated nerves

At 12 weeks after surgery, 5 rats in each group were anesthetized by intraperitoneal injection of 1% pentobarbital sodium, and 4% paraformaldehyde was used to perfuse the animals. Thereafter, 5 mm-long samples were collected from the proximal, middle and distal ends of nerve scaffold, with the outer membrane being dissected. Samples were then cut into pieces which were then fixed in 2.5% glutaraldehyde for 6 h, followed by rinsing with PBS and fixing in 1% (v/v) osmium tetroxide. Then, samples were embedded into paraffin for preparation of ultrathin slices in thickness of 50 nm.

Under the transmission electron microscope, regeneration of axons was evaluated from the following aspects: a) area of regeneration; b) quantity of myelinated fiber; c) average diameter of myelinated fiber; d) myelination of myelinated fiber.

Functional detection of regenerated nerves

Histological observation of the targeted area of quadriceps femoris Twelve weeks after surgery, samples were collected from quadriceps femoris of 5 rats in each group, and then fixed sequentially in paraformaldehyde for 48 h and 30% saccharose for 12 h at 4°C. Fixed samples were washed using PBS and frozen for sectioning along the cross section in thickness of 25 μm. Then, slices were stained using hematoxylin eosin staining method, and images were collected for calculation of the ratio of the area of myofiber to the vision area.

Electrophysiological examination

Respectively, at 4th, 8th and 12th weeks after surgery, the electrophysiological examination was carried out on rats that were anesthetized using 1% (v/v) pentobarbital sodium with the nerves of the operative limb being exposed sufficiently. The bridging area was isolated from the surrounding tissues. Stimulating electrode was inserted at 12 mm from the proximal end of bridging segment in the defect area, while the recording electrode was placed at the distal end of quadriceps femoris. Stimulating signals were delivered to each animal in all groups, during which we recorded the amplitude and latency of compound muscle action potential (CMAP) and nerve conduction velocity (NCV). CMAPs of the normal side was taken as the reference to evaluate the recovery of motor function of rats.

Ethical approval

The research conducted by the Ethics Committee of the Institute met the requirements of relevant laws and ethics, and was discussed at the Hospital Medical Ethics Conference. All subjects had informed consent.

STATISTICAL ANALYSIS

Statistical analysis was carried out with SPSS 17.0 software. All measurement data were presented as the mean ± standard deviation. T test was applied for inter group comparison, while r test for comparison between enumeration data. $p<0.05$ suggested that the difference had statistical significance.

RESULTS

Morphological analysis of the regenerated nerves

Twelve weeks after surgery, the results of observation using transmission electron microscope for each group are shown as follows: Regenerated axons were found in each group, but enormous regeneration of axons was only identified in the GDNF group. Further analysis showed that: At the 4th, 8th and 12th weeks, areas of the regenerated nerves in the GDNF group were (0.95±0.06)

mm^2, (1.14±0.07) mm^2 and (1.22±0.06) mm^2, respectively; in the control group, these were (0.15±0.01) mm^2, (0.25±0.07) mm^2 and (0.52±0.05) mm^2, respectively. These showed that the outcome of GDNF group was superior to that of the control group ($p<0.01$; fig. 1A).

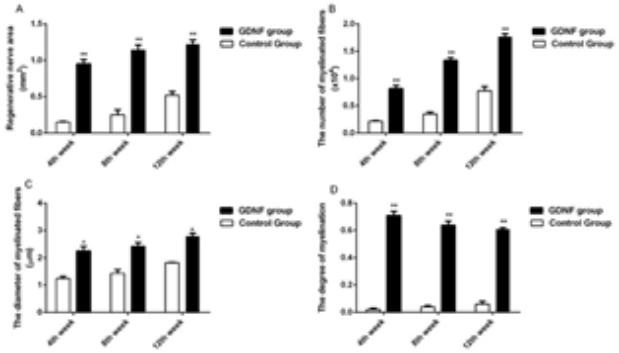

Fig. 1: Morphological analysis of regenerated nerves: (A) area of regenerated nerves; (B) quantity of myelinated nerves; (C) average diameter of myelinated fibers; (D) degree of myelination of fibers. *p<0.05; **p<0.01

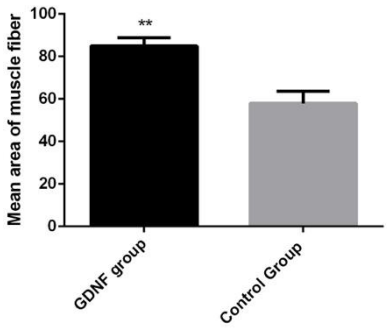

Fig. 2: Average area of myofiber of quadriceps femoris; **p<0.01.

In GDNF group, quantities of the myelinated fiber were ($0.8119\times10^4\pm0.0637\times10^4$), ($1.3371\times10^4\pm0.0460\times10^4$) and ($1.7669\times10^4\pm0.0542\times10^4$); while in control group, these were ($0.2179\times10^4\pm0.0097\times10^4$), ($0.3490\times10^4\pm0.0329\times10^4$) and ($0.7737\times10^4\pm0.0788\times10^4$). Again, these results also indicated that the outcome of GDNF group was superior to that of the control group ($p<0.05$; fig. 1B).

In GDNF group, the average diameters of myelinated fibers were (2.25±0.17) μm, (2.42±0.14) μm and (2.80±0.10) μm, which were significantly better than (1.24±0.08) μm, (1.43±0.14) μm and (1.82±0.14) μm in the control group.

Degrees of fiber myelination in the GDNF group were (0.71±0.03), (0.64±0.03) and (0.6l±0.0l), respectively, which were also significantly higher than (0.02±0.01), (0.04±0.01) and (0.06±0.02) in the control group ($p<0.01$; fig. 1D).

All results above indicated that polylactic acid-trimethylene carbonate/GNDF slow-release catheter can effectively improve the quality of regenerated nerves.

Histological observation of the targeted area of quadriceps femoris

At the 12th week after surgery, HE staining was performed to observe the histological changes in quadriceps femoris for evaluation of atrophy in each group. In the GDNF group, significant amelioration was found in the atrophy of quadriceps femoris with an average area of myofiber of (84.95±3.92) %, while the area of the control group was (57.95±5.78) %, suggesting that the outcome of the GDNF group was better than that of the control group ($p<0.05$). All these results suggested that polylactic acid-trimethylene carbonate/GNDF slow-release catheter can relieve the atrophy of quadriceps femoris caused by loss of innervation, thereby accelerating the innervation of regenerated nerves to targeted organs (fig. 2).

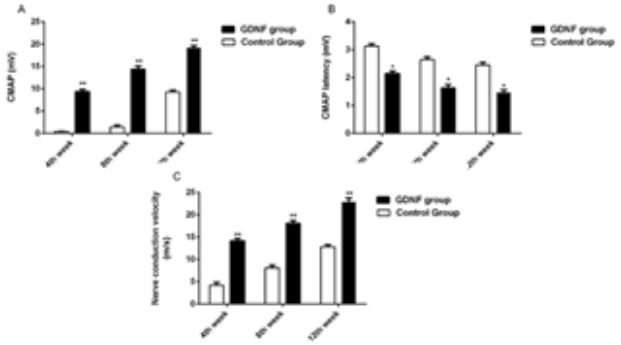

Fig. 3: Electrophysiological examination of nerve: (A) CMAP amplitude; (B) CMAP latency; (C) NCV. *p<0.05; **p<0.01

Electrophysiological examination of nerves

Electrophysiological examination of nerves was employed to detect the recovery of neurological functions after repair of nerve defect. At the 4th, 8th and 12th weeks after surgery, CMAP amplitudes in the GDNF group were (9.34±0.52) mV, (14.40±0.69) mV and (19.18±0.48) mV, significantly better than (0.39±0.07) mV, (1.44±0.41) mV and (9.27±0.40) in the control group ($p<0.01$; fig. 3A).

In the GDNF group, latencies of CMAP were (2.14±0.09) ms, (1.64±0.11) ms and (1.46±0.10) ms, which were significantly lower than (3.13±0.08) ms, (4.64±0.10) ms and (5.45±0.10) ms in the control group ($p<0.05$; fig. 3B).

In the GDNF group, NCVs were (14.13±0.52) m/s, (18.12±0.68) m/s and (22.86±0.87) m/s, significantly faster than (4.17±0.67) m/s, (8.12±0.57) m/s and (12.83±0.48) m/s in the control group ($p<0.01$; fig. 3C), which showed that polylactic acid-trimethylene carbonate/GNDF slow-release catheter can ameliorate the functions of quadriceps femoris after surgery, thereby facilitating the recovery of neurological function after defect.

DISCUSSION

In clinical practice of orthopedics department and neurosurgery department, peripheral neuropathy is quite frequent, which can lead to dysfunction or total loss of function of muscle dominated by peripheral nerves, resulting in muscle atrophy. The poor outcome has been obtained in functional recovery of nerves and muscle, albeit the various methods for treatment of peripheral neuropathy, which is mainly contributed by a certain duration of regeneration of nerve. Excessively long nerves affected by the neuropathy gives rise to the irreversible atrophy of motor end-plate of muscle before the regenerated nerves reach the nerve-dominated muscle; thus, complete recovery can hardly be achieved for the motor function of the nerve-dominated muscle [1]. Therefore, it is quite necessary to search for an effective method to facilitate the regeneration of the nerves.

With the development of molecular biology, some neurotrophic factors (NTFs) that can directly act on the motor neurons have been cloned, and their trophic and protective effects on the injured motor neurons have been proved in various studies, which are involved in the growth, differentiation, death, repair of nerve injury and regeneration of nerves. GDNF, as one of the NTFs, has a more prominent effect which was originally intended to be applied in treatment of amyotrophic lateral sclerosis (ALS) and Parkinson disease, but recent studies have also discovered its potent trophic and protective effects on motor neurons so as to accelerate the recovery of injured peripheral nerves (Cao *et a1*., 2013; Ruan *et a1*., 2012).

Firstly been found in 1993 by Lin LF, GDNF is a member of transforming growth factor-β superfamily, and the mature GDNF comprises 134 amino acid residues and 3 pairs of disulfide bonds (Liu, *et al*, 2015). GDNF can phosphorylate the receptor tyrosine kinase (RET) by binding to α receptor of GDNF to transfer the intracellular signal, thereby nourishing the dopaminergic neuron, motor neuron, sensory neuron and sympathetic neuron. Thus, GDNF has been identified as one of the most active neurotrophic factors for motor neurons (Cao, *et a1*., 2013). In this study, attempts were made to repair the injured femoral nerve branch in rats with polylactic acid-trimethylene carbonate/GNDF slow-release catheter.

In previous studies of injury of sciatic nerve, some studies have revealed that GDNF can increase the quantity of nerve fibers, and generate the nervous tissues, which are similar to the effect of nerve-grafting (Yu, *et a1*.,2011; Huang, *et a1*., 2010; Huang *et a1*., 2010). However, sensing and motor nerve fibers in the sciatic nerve serve as the targets of GDNF. Thus, though it has been confirmed that GDNF can accelerate the regeneration of peripheral nerves, whether it can facilitate the regeneration of motor neurons and axons remains unknown (Allen, *et a1*., 2013; Mickiewicz *et a1*., 2011; Hofer, *et a1*., 2016). Besides, it is quite important for enhancing the regeneration and the functional recovery of motor neurons (Voutilainen *et a1*., 2015; Razavi *et a1*., 2015;1 Zarbin *et a1*., 2013; Forostyak *et a1*., 2013; Awad *et a1*., 2015). In this study, the results showed that at the 12th week after surgery, observation through the TEM indicated the regeneration of axons, but enormous regeneration of axon was only identified in the GDNF group, which was significantly better than the control group. Besides, the quantity of myelinated fibers in the GDNF group was more than that in the control group, and similar changes were also found in the average diameter of myelinated fibers; moreover, myelination of fibers in the GDNF group was also significantly better than the control group. All these results indicated that polylactic acid-trimethylene carbonate/GNDF slow-release catheter can effectively improve the quality of regenerated fibers.

Furthermore, muscle atrophy was also evaluated through HE staining in the target area of quadriceps femoris at the 12th week after surgery, and found that in the GDNF group, significant amelioration was identified in the atrophy of quadriceps femoris with a larger average area of myofiber than that of the control group, suggesting that polylactic acid-trimethylene carbonate/GNDF slow-release catheter can alleviate the atrophy caused by loss of innervation to quadriceps femoris, thus recovering the innervation of regenerated nerves to the targeted organs. In this study, we also adopted the electrophysiological examination to evaluate the recovery of neurological functions after defect of the femoral nerve. At 4th, 8th and 12th weeks after surgery, CMAP amplitude in the GDNF group was significantly larger than that of the control group, while the latency was shorter than that of the control group; and NCV of the GDNF group remained faster than that of the control group. These results suggested that polylactic acid-trimethylene carbonate/ GNDF slow-release catheter can ameliorate the functions of quadriceps femoris after surgery, so as to promote the functional recovery after nerve injury.

CONCLUSION

Polylactic acid-trimethylene carbonate/ GNDF slow-release catheter can accelerate the functional recovery of injured nerves, thus promoting the regeneration efficiency of the femoral nerves.

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