THE EFFECTS OF GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR (GDNF) ON FUNCTIONAL REGENERATION OF TRANSECTED FEMORAL NERVE

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ABSTRACT

Femoral nerve injury is still a challenge because of the lack of effective treatments. In this research, a new drug delivery system is built, which contains a tube of polylactic acid-trimethylene carbonate (PA-TC) equipped with Glial cell line-Derived Neurotrophic Factor (GDNF) and examined its capability to facilitate functional recovery in femoral nerve injury. At the bottom of the transected femoral nerve in rats, a tube injected with GDNF will be installed. Assessment of functional recovery will be made at the fourth, the eight, and twelfth weeks after injury. Compared with animals remedied by antilogous nerve grafting ($p < 0.05$), those animals treated with the PA-TC tube loaded with GDNF showed improvement of recovery in muscle action potentials and regenerated fiber area. As a result, we can conclude that drug delivery system which induced nerve regeneration following femoral nerve transaction takes better effects than which induced by antilogous nerve grafting. It is possible to have great potential to be applied in nerve regeneration of femoral nerve transection injury.

Keywords*: Femoral nerve injury, Glial cell line-Derived Neurotrophic Factor (GDNF), Nerve Regeneration; Functional recovery.*

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Introduction

The femoral nerve is the largest nerve in human leg which is located near the groin. It controls the muscles so that human can straighten his/her leg and to move the hips. This nerve is important and it affects human severely when it gets damaged. The damage of femoral nerve may occur due to direct injury, tumor, growth blocking of nerve, continued pressure on the nerve, a pelvic fracture, radiation on pelvic, etc. Another main reason is the diabetes which cause extensive nerve damage owing to fluctuations in blood sugar and pressure. This results in damage of nerve which thereby affects the legs, feet, toes, hands, and arms which is called as peripheral neuropathy.

This nerve provides a consciousness to major portion of leg, injuries will occur and even may lead to loss of consciousness. It leads to falling. Falls are of particular concern in older adults because they can cause hip fractures, which are very serious injuries. Medication and physical therapy is needed for damage or severe symptoms. The symptoms of femoral nerve are deadness, burning in any part of the leg, dull aching pain in the genital region, lower extremity muscle weakness. The initial checkup will be on the front and middle part of thigh to evaluate the weakness of the nerve.

Physical therapy helps to reduce pain and to regain the activeness of damaged nerve. Removing of any growth will also reduce the pain.

The damage of this nerve can be prevented by keeping sugar and pressure level as normal.

The damage of femoral nerve is due to surgery, lower extremity trauma, and malignancy[1, 2] which leads to muscle paralysis and atrophy and in turn the quality of life is affected[3, 4]. To end a short and large resection gap, end-to-end an astomosis of the transected nerve and Autologous Nerve Grafting (ANG) has been used but this does not

leads to the suitable functional recovery[1, 3, 5-8]. ANG consists of many limitations such as additional trauma affected by harvest of the donor nerve, limited number of donor sites and lengths of available grafts, reduced number of an objective organ function from the donor perspective, and 3-D structural inharmoniousness among the recipient and donor nerves[9-11].

Recently, scaffolding bio-materials has been applied to repair resection gaps and help regeneration[12]. Features such as high cell compatibility and bioactivity, low antigenicity, and the potential are used to offer an enclosed space for directing an axon migration through the resection gap[13-17]. However, non-biodegradable materials, taking silicone for instance, have some adverse reactions by reason of mechanical effects or infection[18]. However, biodegradable resources have their own problems in the same way. The tissues nearby may be affected when the pH level is decreased by the degradation products[19].

Due to the complicated pathophysiological processes of femoral nerve injury, a single treatment method is likely to be insufficient in order to realize satisfactory efficient healing. Therefore, multimodal treatment should be applied. A sustained-release neurotrophic factor delivery system composed of a polylactic acid-trimethylene carbonate (PA-TC) tube containing neurotrophic factor which is Glial cell line-derived neurotrophic factor (GDNF) is used, which is proven to promote motor neuron survival and nerve regeneration[20].

Many studies were conducted in usage of biomaterials to promote femoral nerve repair, though, only limited functional recovery was achieved[11,21,22]. In the present study, we tested a novel combination repair strategy to promote functional nerve regeneration to acquire thorough transaction of the femoral nerve in mice. PA-TC was formed in a tube, which was fitted to the transected femoral nerve to present a positive environment for nerve to regenerate. We also hypothesized that this PA-TC tube may provide a favourable scaffold to deliver the therapeutic substances including neurotrophic factors. In addition, we injected the GDNF into PA-TC tubes. To our best knowledge, this procedure was the first use of PA-TC, as well as GDNF, for the treatment of femoral nerve injury. We proposed that this neurotrophic factor delivery system may be useful for the prevention of misdirected reinnervation during femoral nerve regeneration.

Methods

Preparation of PA-TC tubes

Yantai Zhenghai Biotechnology Co., LTD provide the Polylactic acid-trimethylene carbonate (PA-TC) film (CFDA Certified No. (2015): 3460377) in which the film was hydrated in sterile saline till fully transparent and softened with no bubbles. A metal core is used to roll the film into tubes 7 mm long with a 0.6 mm inner diameter and the tubes were sutured with 10-0 fibre sutures.

Animals

From the Chinese Academy of Science Wuhan Laboratory Animal Center, Ninety (90) male Sprague-Dawley (SD) rats (225 ± 15 g) were bought. The protocols follows the rules of an Institutional Animal Care and Ethic Committee of Huazhong University of Science and Technology and were approved by the Animal Experimental Committee of Tongji Medical College general hospital affiliated with the Huazhong University of Science and Technology on department of hand surgery. All over the study, cautions were taken to minimize the distress.

Surgical procedure

The ketamine hydrochloride (75 mg/kg) and xylazine hydrochloride (10 mg/kg) were administered intraperitoneally to induce anaesthesia. Then, it is a must to cut a plumb midline incision on the right quadriceps femoris muscle. The right femoral nerve was then carefully separated and 5 mm of the right femoral nerve was removed.

The animals were divided into 3 groups, each receiving a different treatment. Among the ANG group, a five-millimeter-long right femoral nerve segment was re-attached in the reverse orientation using 10-0 fibre sutures. In the PA-TC group, both sides of the would have one millimeter length insertion in the PA-TC tube, which was filled with 7.5 μl of normal saline. In the PA-TC+GDNF groups, 7.5 μl of GDNF was injected into the tube. In all groups, the muscle layers and skin were sutured separately. Evaluation were completed at the fourth, the eighth and the twelfth week after operation. (n = 10 rats per group and time point).

Neurofunctional analysis

Once surgery is completed on rat, neurofunctional recovery is evaluated by using an Electromyography (EMG) of the right Quadriceps

Femoris (QF) muscle. First the animals were anaesthetised and the right femoral nerve was out. To connect the right QF muscle, recording electrode was prolonged upward and outward. PowerLab computer-assisted EMG machine is used to inscribe the Compound Muscle Action Potentials (CMAPs) with electrode stimulation which is applied to the proximal end of the PA-TC tube. The stimulation of nerve continues till it found any change. For every rat, three waveforms were observed and recorded. Also the response latencies and maximum amplitudes were noted and measured.

Western blotting

Frigorific liquid nitrogen was used to protect the fresh right QF muscle and regulated in freezing RIPA lysis buffer with 50 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate. For 30 minutes, the incubation with ice exists with the centrifugation of homogenates at 12,000 × g for 10 min at 4 °C were attained. Complete the separation of equivalent amounts of proteins on sodium dodecyl sulphate-polyacrylamide gels and transformation to polyvinylidene difluoride membranes (Millipore, USA) was done. 1:1000 dilution of rabbit polyclonal antibodies was used to incubate the membrane beside GDNF (Santa Cruz Biotechnology) monitored by HRP-conjugated secondary antibodies. Proteins were marked through the procedure of intensive chemiluminescence reagents (Pierce, Rockford, IL).

Statistical analysis

All related statistics were shown with SPSS 18.0 software (IBM). Data are listed by means of ± SD collected from no less than three separate experiments. Diistinction between units would be analyzed by the usage of Student's t test or one-way ANOVA analysis. A flactuation of P-value among 0.05 can be regarded statistically significant.

Results

Surgical outcomes

Till the preselected endpoints, all rats will live with no sign of obstacles like bleeding and infections. Also, no major inflammation, disorder, bulges, or neuroma were found near to the transection nerve ends. The PA-TC tubes endured intact and did not ruin throughout the study.

Electromyographical responses

Figure 1 shows the latency and amplitude of right QF muscles measurement at 4, 8 and 12 weeks after injury demonstrated that ranges in the levels of recovery in the ANG and PA-TC+GDNF groups. After surgery, the response latencies are lowered and amplitudes are increased in case of ANG and PA-TC+GDNF groups from 4 to 12 weeks. In ANG, the response latencies and amplitudes of EMG were clearly reinstated. PA-TC+ GDNF groups were compared to the PA-TC group which show that the PA-TC+ GDNF group has substantial improvements in response latency and amplitude than the ANG group.

Experiments show that there is decrease in response latencies and increase in amplitude from 4 to 12 weeks once the surgery is completed. Figure 1(a) shows the latencies of the right QF were considerably restored in the PA-TC+GDNF group than the PA-TC or ANG group from week 4 to week 12 and compared with that of the PA-TC group at week 12 ($P < 0.05$. iThe latencies were abbreviated in the ANG group. Figure 1(b) shows the amplitudes of the right QF were considerably restored in the PA-TC+GDNF group than the PA-TC or ANG group from week 4 to week 12. The amplitudes were higher in the PA-TC+GDNF group than in the PA-TC or ANG group at week 8 and 12. * $p < 0.05$, compared to the PA-TC group; ** $p < 0.01$, compared to the PA-TC group; # $p < 0.05$, compared to the ANG group.

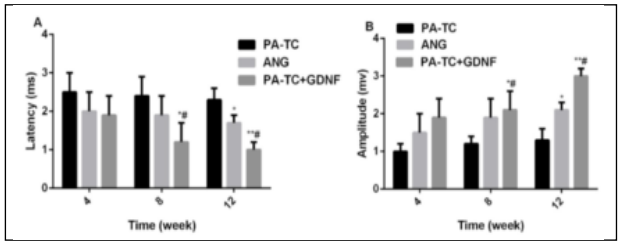

Figure 1: (a) Latency and (b) Amplitude of the right QF muscles.

GDNF expression in the QF muscle

GDNF expression levels in the right QF muscle was measured by Western blot analysis (Fig. 2). Overall, expression of GDNF protein tended to increase from 4 to 12 weeks after surgery in all three groups. GDNF expression was not markedly lower in the PA-TC and ANG groups comparing to that in the PA-TC+GDNF group at 8 and 12 weeks after operation. Meanwhile, GDNF expression was observably lower in the PA-TC group than in the ANG group during week 12 after operation.

Remyelination analysis

Transmission electron microscope (TEM) was used to analyze the cross-sectional area of regenerated femoral nerve fibers as well as the thickness of their myelin sheaths that were generally increased over time. The figure of these two items in the PA-TC group were notably smaller than which in other two groups.

Significant myelin regeneration was observed in the PA-TC+GDNF group. Myelinated nerve fiber cross-sectional area in the PA-TC+GDNF group was larger than the one in the ANG group at 8 and 12 weeks. In addition, we observed only a obviously growth of myelin sheath thickness in the ANG group instead of the PA-TC group at week 8 and 12 after operation.

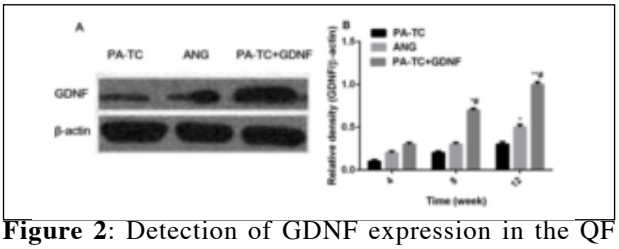

Figure 2: Detection of GDNF expression in the QF muscle. (a) Detection of BDNF expression into the QF musce according to Western blotting analysis. (b) Expression on BDNF tended to increase from 4 to 12 weeks after surgery. BDNF expression in the PA-TC and ANG groups was markedly lower than in the PA-TC+GDNF group than at week 8 and 12. GDNF expression in the ANG group had bigger amount than in the PA-TC group at weeks 12. * $p < 0.05$, contrast to the PA-TC group; ** $p < 0.01$, contrast to the PA-TC group; # $p < 0.05$, contrast to the ANG group.

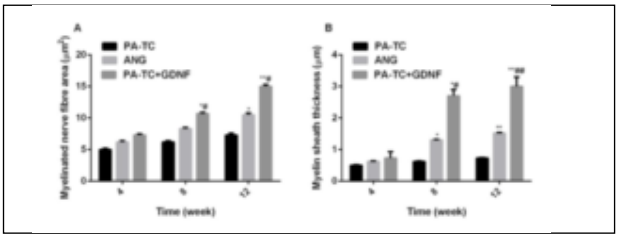

Figure 3: Transmission electron microscopy (TEM) of the middle segment of the regenerated nerve during the twelve weeks after operation. (a) the increase of Myelinated nerve fiber cross sectional area in all experimental groups was observed with the past of time. The area became biger of PA-TC+GDNF group than PATC and ANG groups at week 8 and 12. (b) the growth of myelin sheath thickness in all experimental groups could be soptted similarly. The thickness was thicker in the PA-TC+GDNF group than in the PATC and ANG groups at week 8 and 12. * $p < 0.05$, different from the PA-TC group; ** $p < 0.01$, different from the PA-TC group; # $p < 0.05$, different from the ANG group; ## $p < 0.01$, con trast with the ANG group.

Quantification of TEM results confirmed these observed increases in the two items in these groups (Fig. 3).

Discussion

Transection in femoral nerve fibers results in paralysis of the corresponding structures especially quadriceps femoris (QF) muscle, which severely reduces patients' quality of life. The previous studies have proved that neighboring nerves (PNs) includes ability to revivify after operation. However, regeneration is dependent upon the microenvironment[6]. Therefore, in order to have satisfactory PNs regeneration and functional recovery, the appropriate microenvironment must be provided[24]. The neurotrophic factors, cell transplantation, tissue engineering and other treatments have been widely applied in order to faciliate nerve reborn by researchers as well as clinicians[25].

However, functional recovery is still unsatisfactory. Due to the deleterious effects of femoral nerve injury on patients' life satisfaction and economic barrier on household and society, an effective treatment method to improve axon regeneration and functional recovery is urgently needed.

Usage of tissue-engineering technology to promote PNs repair is attracting increasing attention. In the latest research, we set a rat trial target of femoral nerve injury to test novel neurotrophic factor delivery system including a PA-TC tube combined with GDNF. The previous research has demonstrated that linear ordered structure provides a linear order guidance for spouting axons[6]. The polylactic acid-trimethylene carbonate (PA-TC) film exhibited approximate linear ordered structure. In addition, we used a transection model in which 5 mm of the femoral nerve was removed.

Therefore, our results reflected the real axon regeneration, as no remaining nerve fibres were left after injury. The previous studies have shown that axons and Schwann cells of the proximal PNs stump enter the scaffold and then extend to the distal PNs stump[26]. However, the application of a biological scaffold alone is not sufficient to achieve satisfactory nerve repair and functional recovery. Neurotrophic factors are known to modulate the differentiation, survival and regeneration of peripheral nervous system (PNS) neurons[27].

Furthermore, researchers and clinicians have incorporated neurotrophic factors into biological scaffold in order to prevent the rapid diffusion of

recombinant neurotrophins in the extracellular space[20, 28-30]. To our best knowledge, there were no studies using GDNF published to date.

The movement of QF muscle is controlled by the femoral nerve and the retrieval of the right QF muscle was evaluated as pointers of nerve regeneration and functional recovery but motor function retrieval is not existed in all experiment. This is because of harshness of the PNs injury used and more challenging occurs in case of nerve regeneration succeeding severe injuries. Thus motor recovery has been slow and incomplete.

PNs injury and its functional recovery was serious by Axon regeneration and remyelination. The myelination of regenerated axons was simplified to enhance the functional recovery and PNs injury[31]. After 12 weeks of injury, the PA-TC+GDNF group revealed the considerable improvement in myelinated nerve fiber cross-sectional area than the PA-TC and ANG groups thereby proposing the sustained release GDNF which quicken axon maturation. CMAP recordings replicate the nerve fibers amount by innervating target muscles(32) and deliver an indicator of nerve regeneration, established a similar level of functional recovery in the PA-TC+GDNF group. Thus proposed method merges the PA-TC tube with GDNF improved axonal regeneration and maturation.

The present study also demonstrated the increased expression of GDNF in the QF muscle of rats treated with the PA-TC tubes combined with GDNF. The significant increase in GDNF expression in the PA-TC+GDNF group compared to the PA-TC and ANG groups at week 8 and 12, as revealed by Western blot analysis, may be due to increased both endogenous and exogenous GDNF proteins. At week 4, GDNF expression in PA-TC+GDNF group had the biggest value among the three groups. Which possibly due to the migration of exogenous GDNF to the right QF muscle. The increase in GDNF expression in the PA-TC+GDNF group at week 8 may be due to an increase of endogenous GDNF.

The further increase in GDNF expression in the above group at week 12 may be explained by an increase in endogenous GDNF, as well as decreased retrograde transport efficiency, leading to GDNF accumulation in the right QF muscles.

The promotion of neurite and cone formation increment by PA-TC may account for the superior nerve regeneration observed in the PA-TC+GDNF group. However, present study has shown that only using PA-TC was insufficient and failed to improve nerve regeneration and effective cure. Neurotrophic factor application was of vital importance to achieve revivity of nerves. In addition, combination with PA-TC and GDNF more effectively promoted transected femoral nerve regeneration compared to PA-TC alone.

However, the present study also had several limitations. Firstly, the present study was conducted over 12 weeks using small sample size of rats. Therefore, long-term studies with larger sample size of animals will be needed to verify our data. We speculated the curing consequence in rats treated under PA-TC+GDNF system may approach approximately normal function over longer time. Secondly, it is impossible for a single animal model to reproduce all clinical manifestations of human femoral nerve injury. Therefore, further studies should be attempted to replicate our results using other animal models to reflect human femoral nerve injury as close as possible.

In summary, we have created a biodegradable PA-TC conduit loaded with GDNF to deliver GDNF to sites of femoral nerve injury, where they exert synergistic effects on nerve revivity and functional recovery. After the above research, this system induced nerve regeneration following femoral nerve transection has better effect than that induced by autologous nerve grafting or PA-TC alone. These results demonstrated that the neurotrophic factor delivery system is strikingly effective to cure femoral nerve transection injury. Henceforth, this procedure may be a promising treatment method for the PNs injuries.

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