Nerve growth factor-basic fibroblast growth factor poly-lactide co-glycolid sustained-release microspheres and the small gap sleeve bridging technique to repair peripheral nerve injury

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Graphical Abstract
A new type of microsphere combined with the small gap sleeve bridging technique to repair peripheral nerve injury

Abstract
We previously prepared nerve growth factor poly-lactide co-glycolid sustained-release microspheres to treat rat sciatic nerve injury using the small gap sleeve technique. Multiple growth factors play a synergistic role in promoting the repair of peripheral nerve injury; as a result, in this study, we added basic fibroblast growth factor to the microspheres to further promote nerve regeneration. First, in an in vitro biomimetic microenvironment, we developed and used a drug screening biomimetic microfluidic chip to screen the optimal combination of nerve growth factor/basic fibroblast growth factor to promote the regeneration of Schwann cells. We found that 22.56 ng/mL nerve growth factor combined with 4.29 ng/mL basic fibroblast growth factor exhibited optimal effects on the proliferation of primary rat Schwann cells. The successfully prepared nerve growth factor-basic fibroblast growth factor-poly-lactide-co-glycolid sustained-release microspheres were used to treat rat sciatic nerve transaction injury using the small gap sleeve bridge technique. Compared with epithelium sutures and small gap sleeve bridge alone, the small gap sleeve bridge technique combined with drug-free sustained-release microspheres has a stronger effect on rat sciatic nerve transaction injury repair at the structural and functional levels.

Key Words: biomimetic microfluidic chip; growth factor; in vitro biomimetic microenvironment; nerve function; peripheral nerve injury; sciatic nerve; small gap sleeve bridge; sustained-release microspheres

Introduction
Peripheral nerve injury is a common problem in trauma patients. There are nearly 20 million patients with peripheral nerve injury in China, and the number is increasing by approximately 1 million per year (Zhang et al., 2015a). Sensory and motor dysfunction in the dominating area after peripheral nerve injury seriously affects patient quality of life and imposes a heavy burden on families and society. The treatment of peripheral nerve injury has become a major health problem that must be solved (Wang et al., 2015; Chandran et al., 2016; Cheng and Chen, 2002; Qian et al., 2018; Qu et al., 2021).

At present, peripheral nerve injury repair methods include epineurial neurorrhaphy and autologous nerve transplantation. However, surgical epineurial neurorrhaphy cannot solve the large anastomotic tension and neurotomalosis (Forbes and Rosenthal, 2014). Although autologous nerve transplantation solves the tension problem, it creates damage to the donor area and has low efficacy (Cheng and Chen, 2002), immune rejection, and infection (Ko et al., 2017). As a result, after the repair of peripheral nerve injury, autologous transplants are prone to slow nerve regeneration, twisted ends and scars, incomplete nerve regeneration, and muscle and target organ atrophy that seriously affect the structural and functional recovery of damaged nerves (Bikis et al., 2018; Ma et al., 2018).

Our previous studies found the following. 1) Based on the nerve cannula and surgery, the small gap sleeve bridge repair technique was proposed to replace the peripheral nerve epineurial neurorrhaphy technique that has been used for centuries. The small gap sleeve bridge repair technique has advantages of reducing neuraoma, selective nerve regeneration, and convenient use to peripheral nerve injury (Wang et al., 2014). The sustained-release microspheres combined with the small gap sleeve bridge repair technique provide an innovative solution to peripheral nerve injury; however, there are still urgent issues that need to be addressed, including the selection of the concentration of the sustained-release drugs, the effect of multiple sustained-release drugs (growth factors) used in combination, and how to improve their performance.

In this study, we used a drug-screening biomimetic microfluidic chip to...
simulate the body’s microenvironment in vitro and then screened the optimal combination of NGF/bFGF growth factor (bFGF) to promote Schwann cell regeneration in rats. Based on the selected drug gradients, we prepared the bFGF-sustained-release microspheres loaded with NGF using a water/oil/water (W/O/W) double emulsion solvent evaporation method and evaluated their properties for optimization. We also used the prepared NGF-bFGF-PLGA sustained-release microspheres to repair rat sciatic nerve injury using the small gap sleeve (chitin nerve canula) bridging technique and evaluated the efficacy at the structural and functional levels.

Methods

Construction of the drug screening biomimetic microfluidic chip

The drug screening biomimetic microfluidic chip mold was fabricated using the 3D printer to draw a cell culture chip design (AutoCAD, Version 20.1, Autodesk Inc., San Rafael, CA, USA) and a soft lithography technique (Qin et al., 2010). The biomimetic microfluidic chip was prepared using polydimethylsiloxane and adhesive slides. The prepared drug concentration gradient microfluidic microchip (Olympus Co., Ltd., Suzhou, China) comprises a microfluid pump, syringe, connecting catheters, joints, and microfluidic chips. The chip has a two-layer structure: the first layer is a polydimethylsiloxane material and the second layer is a glass base. The first layer of the chip has two liquid inlets, one liquid outlet, one drug concentration gradient generator, and eight sets of cell culture chambers. The liquid outlet and liquid inlet are connected by a plurality of microfluidic channels. The second layer of the chip is a glass base with the microfluid pump, syringe, connecting conduits, joints, two inlet ports of the first layer pump through which the drug or growth factor is introduced into the chip, and the liquid outlet of the second layer of the chip that receives the waste liquid from the chip and the connecting catheters. After the drug is administered, the growth state of the cells in the cell culture chambers can be observed to investigate the drug efficacy.

Identification of the drug screening biomimetic microfluidic chip

In the biomimetic microfluidic chip, the drug-loaded liquid flows from Inlets 1 and 2, then goes through the CGG structure into the downstream parallel cell culture chamber under the action of an external flow pump, and the liquid first flows through the liquid outlet of Cell Chamber 1 and Liquid 2 form different concentration gradients by constant mixing. According to the Reynolds effect (1883) and Jeon et al. previous work (Li Jeon et al., 2002; Sachette and Lambiase, 2017), when the liquid flows through the microchannel at a lower velocity, it will exist in the form of laminar flow. The fluid particle only follows the flow direction in a one-dimensional movement. There is no macroscopic mixing motion with the surrounding fluid. In the microfluidic device, the flow of the liquid was set at 0.5 μl/min, and the fluids were designed to produce concentration gradients through curved microchannels (100 μm high, 200 μm wide). For example, when Inlet 1 is filled with a drug-free liquid and Inlet 2 is injected with the liquid containing drug liquid, a concentration gradient of microfluidic devices was formed. For a concentration gradient generator, eight sets of cell culture chambers were connected to observe quantitatively using a fluorescence microscope (Olympus, Shinjuku, Tokyo, Japan) and compared with theoretical values. All experimental results were repeated three times.

Isolation, culture, and identification of primary Schwann cells

This study was approved by the Ethics Review Board of Peking University People’s Hospital. The primary Schwann cells were prepared on November 3, 2016. The primary Schwann cells were cultured and obtained by double enzyme digestion. First, ten 24-hour-old specific-pathogen-free Sprague-Dawley rats (Beijing Vital River Laboratory Animal Technology Co., Ltd., animal production license No. SCXK (Jing) 2016-0006, animal usage license No. SYXK (Jing) 2017-0022) were sacrificed by cervical dislocation after inhalational anesthesia with isoflurane (4% isoflurane in 100% medical oxygen, 2 L/min, RWD Life Science Co., Ltd., Shenzhen, Guangdong Province, China) and immersed in 75% alcohol for 15 minutes. The bilateral sciatic nerves were then dissected as a single microfluidic pump (OLYMPUS Co.). The attached soft tissues, blood vessels, and epithelium were removed and then transferred to a pre-cooled PBS (1×, for cell maintenance). The sciatic nerve was cut into 3 cm pieces with fiber scissors, digested with 0.25% trypsin (MilliporeSigma, Burlington, MA, USA) and 0.1% type I collagenase (MilliporeSigma) for 15 minutes, then neutralized with an equal volume of Dulbecco’s modified Eagle’s medium containing 1% fetal bovine serum (GIBCO, Grand Island, USA) and centrifuged (67.2 × g, 5 minutes). After removal of the supernatant, cells were collected, inoculated in a Petri dish, and then cultured in a cell culture incubator (Thermo Fisher Scientific Co., Waltham, MA, USA) at 37°C and 95% humidity. The cells were regularly observed under an inverted microscope. The cells were incubated overnight at 4°C and rewarmed to 37°C the next day, followed by three 5-minute PBS washes. After the addition of fluorescein-conjugated AffiniPure goat anti-rabbit IgG (H + L) (secondary antibody, 1:250, Cat# ZF-0311, RRID: AB_2571576, Zhongshanjinqiao Biotec Co., Beijing, China), cells were incubated at 37°C for 30 minutes in the dark, followed by three 5-minute PBS washes. The cells were stained with Hoechst 33258 (0.5 μg/mL, MilliporeSigma), stored in the dark for 10 minutes, followed by three 5-minute PBS washes. Finally, the slides were stained with immunofluorescence dye, and cells were observed using a fluorescence microscope (Olympus).

Optimal combination of NGF/bFGF for Schwann cell proliferation based on the biomimetic microfluidic chip screening

Primary rat Schwann cells were cultured in the logarithmic growth phase were selected, treated with 0.25% trypsin, and neutralized with the same amount of Dulbecco’s modified Eagle medium complete medium. The supernatant was discarded by centrifugation at 67.2 × g in a high-speed centrifuge (Eppendorf Co., Hamburg, Germany) for 5 minutes. The centrifuged cells were suspended in the appropriate amount of complete medium. The cell number was adjusted to a concentration of 1 × 10^5/mL. A multichannel pipette (Beckman Coulter, Miami, FL, USA) rat tail collagen solution (0.24%, MilliporeSigma) and an appropriate amount of 0.1 M hydrogen oxide sodium were combined to create a three-dimensional gel cell system. The Schwann cell-tail rat collagen mixture was quickly injected into the parallel cell culture channels of the microfluidic chip. The number of cells in each culture chamber was uniform and evenly distributed. The microfluidic chip was placed in a 37°C, 5% CO2 cell culture incubator for 30 minutes to solidify the colloid. The microfluid pump and other ancillary devices were connected, and the NGF complete medium (40 ng/mL) and bFGF-containing medium (10 ng/mL) were pumped into Inlet 1 and Inlet 2 at a rate of 0.1 μl/min. The concentration of the drug generated by the microfluidic chip CGG was as follows (Figure 1). The cells were cultured continuously and the cell growth state was observed periodically. Seventy-two hours later, the culture medium was changed, and acridine orange (AO)/propidium iodide (PI) stain was introduced. Under the fluorescence microscope, different fields of view were selected to investigate the effect of the drug on the cells. The cell proliferation rate was calculated according to the formula: cell proliferation rate = (cell number after culture with NGF and bFGF/initially implanted cell number) × 100%. The above experiments were independently performed. The number of cells in the suspension was calculated using the following formula: the number of cells contained in 1 mL suspension = (total number of cells in four grids/4) × 10^6.

Preparation of the NGF-bFGF-PLGA sustained-release microspheres based on the drug concentrations obtained by chip screening

Primary rat Schwann cells were cultured in the logarithmic growth phase were selected, treated with 0.25% trypsin, and neutralized with the same amount of Dulbecco’s modified Eagle medium complete medium. The supernatant was discarded by centrifugation at 67.2 × g in a high-speed centrifuge (Eppendorf Co., Hamburg, Germany) for 5 minutes. The centrifuged cells were suspended in the appropriate amount of complete medium. The cell number was adjusted to a concentration of 1 × 10^5/mL. A multichannel pipette (Beckman Coulter, Miami, FL, USA) rat tail collagen solution (0.24%, MilliporeSigma) and an appropriate amount of 0.1 M hydrogen oxide sodium were combined to create a three-dimensional gel cell system. The Schwann cell-tail rat collagen mixture was quickly injected into the parallel cell culture channels of the microfluidic chip. The number of cells in each culture chamber was uniform and evenly distributed. The microfluidic chip was placed in a 37°C, 5% CO2 cell culture incubator for 30 minutes to solidify the colloid. The microfluid pump and other ancillary devices were connected, and the NGF complete medium (40 ng/mL) and bFGF-containing medium (10 ng/mL) were pumped into Inlet 1 and Inlet 2 at a rate of 0.1 μl/min. The concentration of the drug generated by the microfluidic chip CGG was as follows (Figure 1). The cells were cultured continuously and the cell growth state was observed periodically. Seventy-two hours later, the culture medium was changed, and acridine orange (AO)/propidium iodide (PI) stain was introduced. Under the fluorescence microscope, different fields of view were selected to investigate the effect of the drug on the cells. The cell proliferation rate was calculated according to the formula: cell proliferation rate = (cell number after culture with NGF and bFGF/initially implanted cell number) × 100%. The above experiments were independently performed. The number of cells in the suspension was calculated using the following formula: the number of cells contained in 1 mL suspension = (total number of cells in four grids/4) × 10^6.

Preparation of the NGF-bFGF-PLGA sustained-release microspheres based on the drug concentrations obtained by chip screening

Neural regeneration research is an area of significant interest in neuroscience. The use of biomimetic microfluidic chips for drug screening offers several advantages, including the ability to mimic the microenvironment of the body and to study the effects of drugs on cells in a controlled manner. This study prepared NGF-bFGF-PLGA sustained-release microspheres to evaluate the efficacy of different drug combinations on Schwann cell proliferation. The microspheres were loaded with a combination of NGF and bFGF, and their concentration gradients were generated using drug screening microfluidic chips. The cells cultured in the microfluidic chip were observed to assess their proliferation rates. The results showed that the optimal combination of NGF/bFGF for Schwann cell proliferation was 1:4, as determined by the microfluidic chip screening. This finding is important for the development of new drugs to promote neural regeneration.

Figure 1 | The schematic diagram of the drug screening biomimetic microfluidic chip. bFGF: Basic fibroblast growth factor; CCU: cell culture unit; CGG: concentration gradient generator; NGF: nerve growth factor.
A small amount of sustained-release microspheres were dispersed in deionized water. The morphology of NGF-bFGF-PLGA sustained-release microspheres was observed under an SEM and a scanning electron microscope (Hitachi, Tokyo, Japan). The average diameter and particle size distribution of the microspheres were analyzed with a laser particle size distribution analyzer (Beckman Coulter).

**Preparation of absorbable artificial biological nerve cannula**

The absorbable artificial biological nerve cannula was prepared using chitosan (Fengrun Biotech, Taizhou, China) as the main raw material, and a hollow fiber spinning and drawing process was performed according to a previous study (Zhang et al., 2015b). Absorbable artificial biological nerve cannula combined with a small amount of sustained-release microspheres releasing different neurotrophic factors causes the bio-cannula to release drugs synchronously during the degradation process. The NGF-bFGF-PLGA sustained-release microspheres were established with a small gap sleeve bridging technique to promote the repair of peripheral nerve damage.

**Evaluation of NGF-bFGF-PLGA sustained-release microspheres combined with the small gap sleeve bridging technique for repairing sciatic nerve injury in rats and its therapeutic effect**

**Experimental animals**

Twenty-four female Sprague-Dawley rats (6–8 weeks old, 200 g body weight, clear genetic background) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. The experimental animals were kept in a specific-pathogen-free cage of the Experimental Animal Center of Peking University People's Hospital, at room temperature 23 ± 2°C, and a relative humidity of 55% ± 5%. Rats were anesthetized with 2% sodium pentobarbital (30 mg/kg, intraperitoneally, Sinopharm Chemical Reagent Co., Ltd., Shanghai, China). Rat sciatic nerve was exposed. The stimulating electrodes (Xi'an Friendship Medical Electronics Co., Ltd., Xi'an, China) were placed at the proximal and distal ends of the sciatic nerve, and the nerve was stimulated with a rectangular pulse (duration 0.1 ms, 0.12 mA, 1 Hz) and the distance between the electrodes was accurately measured. The neural stimulation latency was recorded and the difference was calculated. The sciatic nerve conduction velocity was calculated automatically according to the formula: sciatic nerve conduction velocity = conduction distance/latency difference. At the same time, the action potentials of the rats in each group were recorded, and the amplitudes were compared and evaluated (Figure 4).

**Pathological evaluation**

Neurohistochemistry. Twelve weeks after surgery, bilateral sciatic nerves in each group were isolated for osmic acid staining. First, the sciatic nerve was fixed with 4% formaldehyde for 12 hours, rinsed with running water for 6 hours, stained with 1% osmic acid for 12 hours, and rinsed with running water for 6 hours. The sciatic nerve was then subjected to dehydration and embedded in paraffin. The tissue was cut into 5-μm sections and stained with hematoxylin and eosin and examined under a light microscope (Eppendorf Co.). The standard curve was drawn according to the relationship index = –38.3 \[(EPL – NPL)/NPL\] + 109.5 \[(ETS – NTS)/NTS\] + 13.3 \[(EIT – NIT)/NIT\] – 88.

**Evaluation of sciatic nerve function index** Twelve weeks after surgery, the sciatic functional index in each group was evaluated by a self-made footstep experiment transparent glass plate channel (100 cm long, 15 cm wide, 20 cm high) and a camera with video recording capabilities (Canon 6D0, Canon, Tokyo, Japan) for recording the gait after the surgical plan was completed. The rats were anesthetized with 2% sodium pentobarbital (30 mg/kg, intraperitoneally, Sinopharm Chemical Reagent Co., Ltd., Shanghai, China). Rat sciatic nerve was exposed. The stimulating electrodes (Xi'an Friendship Medical Electronics Co., Ltd., Xi'an, China) were placed at the proximal and distal ends of the sciatic nerve repair site. The recording electrode was inserted into the anterior tibial muscle and the ground electrode was placed on the ipsilateral thigh muscle. The nerve was stimulated with a rectangular pulse (duration 0.1 ms, 0.12 mA, 1 Hz) and the distance between the electrodes was accurately measured. The neural stimulation latency was recorded and the difference was calculated. The sciatic nerve conduction velocity was calculated automatically according to the formula: sciatic nerve conduction velocity = conduction distance/latency difference. At the same time, the action potentials of the rats in each group were recorded, and the amplitudes were compared and evaluated (Figure 4).

**Morphological characterization**

A surgical incision was made on the posterior lateral side of the limb. Rats were anesthetized with 2% sodium pentobarbital (30 mg/kg, intraperitoneally, Sinopharm Chemical Reagent Co., Ltd., Shanghai, China). Rat sciatic nerve was exposed. The stimulating electrodes (Xi'an Friendship Medical Electronics Co., Ltd., Xi'an, China) were placed at the proximal and distal ends of the sciatic nerve repair site. The recording electrode was inserted into the anterior tibial muscle and the ground electrode was placed on the ipsilateral thigh muscle. The nerve was stimulated with a rectangular pulse (duration 0.1 ms, 0.12 mA, 1 Hz) and the distance between the electrodes was accurately measured. The neural stimulation latency was recorded and the difference was calculated. The sciatic nerve conduction velocity was calculated automatically according to the formula: sciatic nerve conduction velocity = conduction distance/latency difference. At the same time, the action potentials of the rats in each group were recorded, and the amplitudes were compared and evaluated (Figure 4).

**Functional evaluation**

Sciatic nerve function index: Twelve weeks after surgery, the sciatic functional index in each group was evaluated by a self-made footstep experiment transparent glass plate channel (100 cm long, 15 cm wide, 20 cm high) and a camera with video recording capabilities (Canon 6D0, Canon, Tokyo, Japan) for recording the gait after the surgical plan was completed. The rats were anesthetized with 2% sodium pentobarbital (30 mg/kg, intraperitoneally, Sinopharm Chemical Reagent Co., Ltd., Shanghai, China). Rat sciatic nerve was exposed. The stimulating electrodes (Xi'an Friendship Medical Electronics Co., Ltd., Xi'an, China) were placed at the proximal and distal ends of the sciatic nerve repair site. The recording electrode was inserted into the anterior tibial muscle and the ground electrode was placed on the ipsilateral thigh muscle. The nerve was stimulated with a rectangular pulse (duration 0.1 ms, 0.12 mA, 1 Hz) and the distance between the electrodes was accurately measured. The neural stimulation latency was recorded and the difference was calculated. The sciatic nerve conduction velocity was calculated automatically according to the formula: sciatic nerve conduction velocity = conduction distance/latency difference. At the same time, the action potentials of the rats in each group were recorded, and the amplitudes were compared and evaluated (Figure 4).

**Nerve conduction velocity:** Twelve weeks after surgery, the rats were sacrificed by cervical dislocation after anesthesia with 2% sodium pentobarbital (30 mg/kg, intraperitoneally, Sinopharm Chemical Reagent Co., Ltd., Shanghai, China). The healthy and operated gastrocnemius of rats in each group were completely separated and immediately weighed (Shimadzu Co., Kyoto, Japan). The overall function of the muscles was evaluated by comparing the wet weight ratio of the experimental side and control side.
an appropriate amount of optimal cutting temperature compound. The box was slowly placed in a small cup containing liquid nitrogen. When the bottom of the box contacted the liquid nitrogen, the sample began to freeze. Caution was taken to avoid the temperature of the cutting surface penetrating the tissue. The tissue quickly froze into a solid after approximately 10–20 seconds. A layer of embedding glue was applied to the sample holder. The tissue was then placed on the sample holder and pre-cooled for 5–10 minutes to achieve the optimal cutting temperature. The combination was then embedded into the tissue (muscle belly) was cut into 5-μm thick sections using the constant temperature ice slice (Leica). Tissue sections were then left at room temperature for 30 minutes, fixed in acetone at 4°C for 10 minutes, washed with PBS, stained with hematoxylin for 3 minutes (to the degree of over-staining), and washed with ethanol for 10 minutes. The tablets were reddish-brown, the tap water was anti-blue, and the nucleus was blue-purple. Subsequently, tissue sections were stained with eosin for 2 minutes, washed with water, dehydrated with alcohol, cleared with xylene I and xylene II, sealed with neutral resin, and observed with the fluorescence microscope.

Statistical analysis
No statistical methods were used to predetermine sample size; however, our sample sizes were similar to those reported in a previous publication (Zhang et al., 2015b). No animals or data points were excluded from the analysis. The evaluator was blinded to grouping. All data were statistically analyzed using SPSS 26.0 software (IBM Corp., Armonk, NY, USA) and expressed as the mean ± standard deviation (SD). One-way analysis of variance followed by the Bonferroni post hoc test was used for comparisons of groups. A level of P < 0.05 indicated statistical difference.

Results
Preparation of NGF-bFGF-PLGA sustained-release microspheres based on the microfluidic chip
Design of the drug screening biomimetic microfluidic chip
The microfluidic virtual model was designed using Autodesk AutoCAD software. The chip comprises an upstream CGG and downstream parallel cell culture unit (CCU). The chip was 45 mm long and 25 mm wide, including two inlet ports, a chip microchannel of 4 mm in diameter, a 6 mm deep bed (drug CGG), and eight cell culture chambers (12 mm × 700 μm × 100 μm). The liquid outlet was connected to the inlet ports by a plurality of microchannels (200 μm in width and 100 μm in height) (Figure 5). The chip base consisted of a clean, adhered glass backsheet.

Preparation of a drug screening biomimetic microfluidic chip
The chip mold was made by soft lithography, and the biomimetic microfluidic chip was replica-molded by polymethylmethacrylate (PMMA) and clean slide. After the preparation, the microflow pump, syringe, connecting conduit, joint, two liquid inlets, and one liquid outlet were connected to form the drug screening biomimetic microfluidic chip system.

Identification of the drug screening biomimetic microfluidic chip
To verify the performance of the microfluidic chip, CGG, PBS, and AO (5 μg/mL) were injected into Inlets 1 and 2, respectively. After a stable concentration gradient was formed, the individual cell culture chambers were viewed under an inverted fluorescence microscope to measure the fluorescence intensity, which was compared with theoretical values. The drug screening microfluidic chip system can form a stable concentration gradient, which met the needs of subsequent experiments (Figure 6).

Isolation, culture, and identification of primary Schwann cells
Schwann cells were successfully cultured by double enzyme digestion. The cells had a normal bipolar long spindle shape, the cells were slender, and the nucleus was oval or round. The immunohistochemical identification showed that the cell body of the Schwann cells was positive for S-100. The cultured primary Schwann cells had good purity and met the needs of subsequent experiments (Figure 7).

Optimal concentration combination screening of NGF/bFGF to promote the proliferation of Schwann cells
Schwann cells were seeded in a biomimetic microfluidic chip, and the NGF concentration was 10 ng/mL and bFGF-containing complete medium (10 ng/mL) were pumped at a constant rate from Inlets 1 and 2 at a rate of 0.1 (μL/min). After 72 hours, the 22.86 ng/mL NGF + 4.29 ng/mL bFGF group had the best proliferation. Therefore, such a growth factor concentration combination strategy was selected for subsequent experiments (Figure 8).

Preparation of the NGF-bFGF-PLGA sustained-release microspheres
According to the drug concentration obtained by the biomimetic microfluidic chip, NGF-bFGF-PLGA sustained-release microspheres were successfully prepared by the W/O/W double emulsion solvent evaporation method. The surface was smooth (Figure 9). The microspheres were spherical in appearance, with a diameter of approximately 29.64 ± 14.03 μm. The release of the drug from the microspheres was 29.64 ± 14.03 μm. We concentrated on the microspheres preparation, evaluation, and improvement of the NGF-bFGF-PLGA sustained-release microsphere appearance, with a normal distribution (Figure 10).

Evaluation and improvement of the NGF-bFGF-PLGA sustained-release microspheres
As shown in Figure 10, the particle size of the NGF-bFGF-PLGA sustained-release microspheres was 29.64 ± 14.03 μm. We concentrated on the sustained-release of NGF drugs and inferred the combined release rate of NGF-bFGF sustained-release microspheres in vitro drug release experiments showed that there was a drug burst release in the NGF-bFGF-PLGA sustained-release microspheres. The amount of drug released was approximately 40% of the total drug for the 1st week, and then gradually increased to approximately 60% in the 4th week. The release curve approached the platform and eventually ended with the degradation of PLGA. Additionally, the in vitro complete degradation time of NGF-bFGF-PLGA sustained-release microspheres showed that the drug encapsulation efficiency of the NGF sustained-release microspheres was approximately 17.04%.

NGF-bFGF-PLGA sustained-release microspheres combined with the small gap sleeve bridging technique for repairing sciatic nerve injury in rats

Postoperative general observation
Twelve weeks after surgery, the rats in each group had excellent growth status; the autophagy occurred in the repaired limbs, all wounds healed well without ulceration, and there was no inflammatory reaction. As shown in Figure 11, after the incision, the epithelium suture group had neuroma formation at the nerve injury repair site, and there was no obvious connective tissue hyperplasia around the nerve. There was no neuroma formation in the small gap sleeve bridging group, and the nerve cannula was not completely degraded. Neonatal axons grew into the center of the cannula and the connective tissue was significantly less in the small gap sleeve bridging group than in the epithelium suture group. There were little differences in appearance among the small gap sleeve bridging + saline, small gap sleeve bridging + drug-free sustained-release microsphere, and small gap sleeve bridging + NGF-bFGF-PLGA sustained-release microsphere groups.

Functional evaluation
Twelve weeks after surgery, the sciatic nerve function index and wet weight of the gastrocnemius in the small gap sleeve bridging + saline, small gap sleeve bridging + drug-free sustained-release microsphere, and small gap sleeve bridging + NGF-bFGF-PLGA sustained-release microsphere groups were larger than those in the epithelium suture + saline group. The wet weight and wet weight of the gastrocnemius were significantly greater in the small gap sleeve bridging + NGF-bFGF-PLGA sustained-release microsphere group than in other groups (P < 0.05; Figures 12 and 13).

Twelve weeks after surgery, the sciatic nerve conduction velocity (SNCV) in the small gap sleeve bridging + saline, small gap sleeve bridging + drug-free sustained-release microsphere, and small gap sleeve bridging + NGF-bFGF-PLGA sustained-release microsphere groups was significantly greater than those in the epithelium suture group and significantly less than those of the normal nerve. The maximal sciatic nerve conduction velocity was faster in the small gap sleeve bridging + NGF-bFGF-PLGA sustained-release microsphere group than in the other groups (all P < 0.05). According to the action potential waveform diagram, the amplitudes of the nerve waves were greater in the small gap sleeve bridging + saline, small gap sleeve bridging + drug-free sustained-release microsphere, and small gap sleeve bridging + NGF-bFGF-PLGA sustained-release microsphere groups than in the epithelium suture + saline group. The amplitude of the nerve waves was less in the small gap sleeve bridging + NGF-bFGF-PLGA sustained-release microsphere group than in the normal control group; however, there was a significant difference compared with the other groups. These findings indicate that the myelinated sciatic nerve conduction function recovered better in the small gap sleeve bridging + NGF-bFGF-PLGA sustained-release microsphere group (Figure 14).

Structural evaluation
Twelve weeks after surgery, the distal nerve in each group was stained with osmic acid. The epithelium suture group had an irregular distribution of myelinated nerve fibers and more connective tissue, and the axons were different in size. Nerve fibers were better in the small gap sleeve bridging group than in the epithelium suture group; however, it was still poorer than the normal nerve group. In the small gap sleeve bridging + NGF- bFGF-PLGA microsphere group, the myelinated nerve fibers were more abundant than in the small gap sleeve bridging + drug-free sustained-release microsphere and small gap sleeve bridging + NGF-bFGF-PLGA sustained-release microsphere groups than in the epithelium suture + saline group. The amplitude of the nerve waves was less in the small gap sleeve bridging + NGF-bFGF-PLGA sustained-release microsphere group than in the normal control group; however, there was a significant difference compared with the other groups. These findings indicate that the myelinated sciatic nerve conduction function recovered better in the small gap sleeve bridging + NGF-bFGF-PLGA sustained-release microsphere group (Figure 15).

The average diameter of the myelinated nerve fibers in each group was smaller than that of the normal sciatic nerve 12 weeks after surgery, and the thickness of the regenerated nerve fibers was not uniform. The fibrous tissue in the nerve was an irregular, fibrous form and no nerve regeneration occurred. The hole was occasionally observed in the small gap sleeve bridging + saline, small gap sleeve bridging + drug-free sustained-release microsphere, and small gap sleeve bridging + NGF-bFGF-PLGA sustained-release microsphere groups, the nerve fiber diameter, and the thickness of the myelinated nerve fibers were less in the epithelium suture group and were less than those of the normal nerve group. The optimal repair effect was observed in the small gap sleeve bridging + NGF-bFGF-PLGA sustained-release microsphere group; the number of myelinated nerve fibers was greater in the small gap sleeve bridging + NGF-bFGF-PLGA sustained-release microsphere group than in the other groups (P < 0.05; Figure 16).

Twelve weeks after surgery, the muscles (gastrocnemius) were stained by hematoxylin and eosin. Our results showed that muscle fibers in the epithelium suture group were severely atrophied, and there was a large amount of collagen fiber. In the small gap sleeve bridging group, there were fewer fibrous tissues, myelinated nerve fibers were more abundant than in the normal nerve group, and the connective tissue was less, which had better repair effect than the other groups (Figure 15).
Establishment and repair of a rat model of sciatic nerve injury.

1. Morphology and identification of primary Schwann cells.

Identification of primary Schwann cells under a fluorescence microscope. Cells appeared to be bipolar long spindle shapes and the nuclei was oval or round. (C, D) Identification of primary Schwann cells under a fluorescence microscope. The cell bodies of Schwann cells were stained green (stained by fluorescein-isothiocyanate) by the S-100 protein antibody, and the nuclei were stained blue by Hoechst33258. Scale bars: 25 μm in A, C; 50 μm in B, D. Red frames indicate the typical morphology of primary Schwann cells.

2. Measurement of the parameters of the sciatic functional index.

(A) Walking trajectory of rats in a closed channel 12 weeks after surgery. (B) Foot length (PL), toe spread (TS), and intermediate toe distance (IT).

3. Particle size (μm) and drug release curve.

(A) Images 1–8 represent eight concentration gradients (more details of concentrations are shown in Figure 1). (B) The difference between the theoretical and experimental data of a concentration gradient generator. Data are expressed as mean ± SD. The study was independently repeated three times.

4. Measurement of nerve conduction velocity and action potential in rats.

The nerve was stimulated with a rectangular pulse (duration 0.1 ms, 0.12 mA, 1 Hz) and the distance between the electrodes was accurately measured. Sciatic nerve conduction velocity = conduction distance/latency difference.

5. Screening of the drug concentration in primary Schwann cells.

(A, B) The morphology of primary Schwann cells viewed with an inverted microscope. Cels appeared to be bipolar long spindle shapes and the nuclei was oval or round. Identification of primary Schwann cells under a fluorescence microscope. The cell bodies of Schwann cells were stained green (stained by fluorescein-isothiocyanate) by the S-100 protein antibody, and the nuclei were stained blue by Hoechst33258. Scale bars: 25 μm in A, C; 50 μm in B, D. Red frames indicate the typical morphology of primary Schwann cells.

6. Virtual model and schematic of a drug screening biomimetic microfluidic chip.

The chip comprises an upstream CGG and downstream parallel CCU, and includes two inlet ports, one liquid outlet, one drug CGG and eight cell culture chambers. CCU: Cell culture unit; CGG: concentration gradient generator.

7. Identification of drug concentration gradients of the biomimetic microfluidic chips.

(A) Images 1–8 represent eight concentration gradients (more details of concentrations are shown in Figure 1). (B) The difference between the theoretical and experimental data of a concentration gradient generator. Data are expressed as mean ± SD. The study was independently repeated three times.

8. Measurement of the parameters of the sciatic functional index.

(A) Walking trajectory of rats in a closed channel 12 weeks after surgery. (B) Foot length (PL), toe spread (TS), and intermediate toe distance (IT).

9. Morphology of the sustained-release microspheres at different magnifications under the electron microscope.

Scale bars: 10 μm in A, 50 μm in B. NGF-bFGF-PLGA: Nerve growth factor basic-fibroblast growth factor poly-lactide co-glycolid.

10. Particle size (μm) and drug release curve.

The red line indicates normal distribution. Data in B are expressed as mean ± SD. The experiments were independently repeated three times. NGF-bFGF-PLGA: Nerve growth factor basic-fibroblast growth factor poly-lactide co-glycolid.
Effect of NGF-bFGF-PLGA sustained-release microspheres on the SNCV

(A) Epithelium suture group. (B) Small gap sleeve bridging + saline group. (C) Small gap sleeve bridging + drug-free sustained release microsphere group. (D) Small gap sleeve bridging + NGF-bFGF-PLGA sustained-release microsphere group. The epithelium suture group had neurona formation at the nerve injury repair site, and there is more connective tissue hyperplasia around the nerve than other groups. Neonatal axons grew into the center of the cannula and the connective tissue around the nerve was significantly less than the epithelium suture group. NGF-bFGF-PLGA: Nerve growth factor basic fibroblast growth factor poly-lactide co-glycolid; SNCV: sciatic nerve conduction velocity.

Figure 11 | Effect of NGF-bFGF-PLGA sustained-release microspheres on the general observation of the repair effect of sciatic nerve injury 12 weeks after surgery.

Figure 12 | Effect of NGF-bFGF-PLGA sustained-release microspheres on the sciatic functional nerve index (SFI) of rats with sciatic nerve injury 12 weeks after surgery.

Figure 13 | Effect of NGF-bFGF-PLGA sustained-release microspheres on the wet weight of the gastrocnemius of rats with sciatic nerve injury 12 weeks after surgery.

Figure 14 | Effect of NGF-bFGF-PLGA sustained-release microspheres on the SNCV action potential of rats with sciatic nerve injury.

Figure 15 | Effects of NGF-bFGF-PLGA sustained-release microspheres on the myelinated nerve fibers in the sciatic nerve of rats with sciatic nerve injury (osmic acid staining).

Figure 16 | Effects of NGF-bFGF-PLGA sustained-release microspheres on the number of nerve fibers (A), myelin thickness (B), and axonal area (C) of the distal sciatic nerve of rats with sciatic nerve injury.

Figure 17 | Effects of NGF-bFGF-PLGA sustained-release microspheres on the gastrointestinal histological changes of rats with sciatic nerve injury (hematoxylin and eosin staining).

Data are expressed as mean ± SD (n = 6). *P < 0.05 (one-way analysis of variance followed by Bonferroni post hoc test). a–d: Epithelium suture group, small gap sleeve bridging + saline group, small gap sleeve bridging + drug-free sustained-release microsphere group, and small gap sleeve bridging + NGF-bFGF-PLGA sustained-release microsphere group. The sciatic nerve function index were significantly greater in the small gap sleeve bridging + NGF-bFGF-PLGA sustained-release microsphere group than in other groups. Data are expressed as mean ± SD (n = 6). NGF-bFGF-PLGA: Nerve growth factor basic fibroblast growth factor poly-lactide co-glycolid.

Figure 18 | Effects of NGF-bFGF-PLGA sustained-release microspheres on the myelinated nerve fibers in the sciatic nerve of rats with sciatic nerve injury (osmic acid staining).

(A) Epithelium suture group. (B) Small gap sleeve bridging + saline group. (C) Small gap sleeve bridging + drug-free sustained-release microsphere group. (D) Small gap sleeve bridging + NGF-bFGF-PLGA sustained-release microsphere group. (E) Normal contralateral sciatic nerve as a negative control. The nerve morphology in the small gap sleeve bridging + NGF-bFGF-PLGA sustained-release microsphere group was more regular and similar to normal nerves with less connective tissue, which had the best repair effect. Scale bars: 25 μm. NGF-bFGF-PLGA: Nerve growth factor basic fibroblast growth factor poly-lactide co-glycolid.
Discussion

Epineural neurotrophism and autologous nerve transplantation are available for the treatment of peripheral nerve injury. However, peripheral neurotrophism has the drawback of fibroma proliferation and poor nerve regeneration. Autologous nerve transplantation has the disadvantages of limited source, donor site destruction, and the graft nerve is single (Jiang et al., 2011; Wang et al., 2017; Liu et al., 2012). Preclinical and clinical studies (Jiang et al., 2010; Kou et al., 2013; Zhang et al., 2015b) demonstrated that the small gap sleeve bridging technique based on a biodegradable bio-cannula for peripheral anastomosis can reduce the operation time, reduce trauma, increase the accuracy of the anastomosis, reduce the incidence of nerve gaps, and prevent the dominating muscle atrophy to improve the structural and functional repair effects after peripheral nerve injury. In this study, we used the small gap sleeve bridging technique to repair the nerve injury in rats with sustained-release microspheres loaded with multiple growth factors to further promote the therapeutic effect of peripheral nerve injury. This was the basic idea and starting point of this study.

The effect of growth factors on the repair of peripheral nerve injury has been recognized worldwide (Lien et al., 2020; Idrisova et al., 2022). Growth factor released locally after nerve injury will participate in the regeneration, migration, proliferation, differentiation, and regulation of various nerve cells (Gong et al., 2022; Idrisova et al., 2022). In the distal injured nerve, Schwann cells deprived of axonal contact proliferate, upregulate the synthesis and release of a variety of neurotrophic factors and basal lamina components that can create an appropriate microenvironment for regenerating axons (Liu et al., 2014). Accumulating evidence indicates that following injury, regenerating axons are unable to cross a peripheral nerve gap without Schwann cell guidance around their migration point (Carrau et al., 2012; Dun and Parkinson, 2015; Chen et al., 2019). Napoli et al. (2012) indicated that Schwann cells dedifferentiate to a progenitor-like state and proliferate, forming a tube structure which allows axons to regenerate after peripheral nerve injury. Studies have demonstrated that multiple growth factors can play a synergistic role in promoting the repair of peripheral nerve injury (Frostie et al., 1998; Önger et al., 2017; Sacchetti and Lambiase, 2017). After peripheral nerve injury, the supply of neurotrophic factors in the distal axon was interrupted, the neuronal cell body degenerated, and the content of proximal axonal neurotrophic factor decreased sharply, while the neurotrophin receptor increased rapidly (Keefe et al., 2017). Therefore, the local administration of various exogenous NGFs in the damaged nerve will satisfy the need for peripheral nerve regeneration, and promote the structural regeneration and functional recovery of peripheral nerves. Currently identified growth factors that promote peripheral nerve regeneration include nerve growth factor, basic fibroblast growth factor, ciliary neurotrophic factor, glial cell line-derived neurotrophic factor, and brain-derived neurotrophic factor (Li et al., 2017). In this study, we selected two growth factors, NGF and bFGF, as loaded drugs. The combined application of growth factors facilitated the regeneration and repair of peripheral nerve injury, which may be related to the following mechanisms. NGF and bFGF have a clear role in the initial stage of nerve injury repair, and NGF mainly manifests in stimulating neuronal synaptic growth, guiding axon directional growth, promoting neurtic fiber differentiation, and neurotrophic chemotaxis. BFGF mainly promotes Schwann cell proliferation, accelerates Schwann cell maturation, and improves angiogenesis and microcirculation. A previous study has shown the synergistic effects of the two factors (Wang et al., 2014).

The traditional intravenous administration method causes the growth factor to be eliminated by the reticuloendothelial system in a short time period (Tria et al., 1994). Therefore, the growth factor can only play a role in promoting nerve regeneration in a short time period (within 1 week), and the therapeutic effect is not satisfactory. Various studies have shown that sustained-release systems of NGF-PLGA drug-loaded sustained-release microspheres prepared by the W/O/W double emulsion solvent evaporation method can increase the number of regenerated nerve fibers, promote the maturation of nerve fibers, and improve the structural and static culture media, which greatly reduces their clinical practical value. As a result, the development of new drug screening methods is crucial. In this study, we used our designed drug screening biomimetic microfluidic chip to test the optimal combination and concentration of NGF and bFGF for promoting peripheral nerve regeneration and repair. The microfluidic chip is a fast and efficient drug screening device. As a result, we successfully obtained the results of eight different drug concentration combinations of NGF and bFGF in vitro. We found that the drug screening results of 22.86 ng/mL + bFGF at 4.29 ng/mL had the best effect on peripheral nerve regeneration. Although the combined concentration of NGF and bFGF was less than the concentration of the single action, it had a better effect and may be related to the synergistic effects of these factors. Based on the drug screening results, we designed NGF-bFGF-PLGA sustained-release microspheres and continued to improve them according to their drug loading and encapsulation efficiency. Our results showed that the self-designed drug screening biomimetic microfluidic chip can continuously supply fresh nutrients and oxygen to the three-dimensional cultured Schwann cells in a controlled fluid medium, and the chip can efficiently screen out the combined concentration of NGF and bFGF, promoting peripheral nerve regeneration.

Our study improved the technique of drug-loaded sustained-release microspheres and the highlights are as follows: 1) The technique promotes a single-factor drug sustained-release microsphere to a multi-factor drug-loaded sustained-release microsphere, which made it easier to improve the microenvironment of peripheral nerve injury, improving the growth rate and accuracy of the axons. 2) The drug screening biomimetic microfluidic chip was designed to simulate the three-dimensional dynamic growth state of Schwann cells in vitro, which provides a new way to select the drug loading of sustained-release microspheres. 3) The preparation of sustained-release microspheres by the small gap sleeve bridging method has the advantage of excellent drug-loading rate and drug release rate, the drug loading during the preparation of the sustained-release microspheres was improved, and the sustained-release amount of the drug-loaded microspheres was closer to the requirement for a better therapeutic effect. 4) Finally, high-performance liquid chromatography was used for measuring the characteristics and drug release of the microspheres, which greatly reduced the cost of testing and may inspire other drug loading research.

In the animal experiment of this study, we compared the repair effect of peripheral nerve injury in rats of NGF-bFGF-PLGA sustained-release microspheres with small gap sleeve bridging, drug-free PLGA microsphere small gap sleeve bridging, simple small gap sleeve bridging, and epineural neurotrophism. After 12 weeks, NGF-bFGF-PLGA microsphere sustained-release microspheres with a small gap sleeve bridging technique exhibited better performance at the structural level (sciatric nerve osmotic staining, nerve fiber count, myelin thickness, axon area, gastrocnemius hematoxylin and eosin staining) and functional level (sciatic nerve function index, muscle weight, nerve conduction velocity, action potential amplitude) than other experimental groups.

There are also shortcomings of this study. For example, considering the complex internal environmental changes and the lack of in vivo observation methods, we could not achieve a one-to-one correspondence between the concentration of sustained-release drugs in vivo and in vitro. The drug-loaded sustained-release drug in vivo, which limits the value of the drug screening biomimetic microfluidic chip. The microfluidic chip is currently using is relatively simple. Future research will include screening the drug combination in a three-dimensional bio-mimetic nerve regeneration chip and various organs. Although the application of NGF-bFGF-PLGA sustained-release microspheres with the small gap sleeve bridging technique to repair peripheral nerve injury in rats had positive results, primate models will have greater clinical value and significance. Overall, this study is an innovative test of repair methods after peripheral nerve injury; however, it lacks the molecular biological mechanism, and we will conduct more molecular biological research.

The optimal combination of NGF/bFGF to promote the proliferation of primary Schwann cells in rats was obtained by a drug screening biomimetic microfluidic chip. The NGF-bFGF-PLGA composite drug-loaded sustained-release microspheres were prepared based on the drug screening results. Animal experiments were used to confirm that the NGF-bFGF-PLGA composite drug-loaded sustained-release microspheres with the small gap sleeve bridging technique to repair peripheral nerve injury in rats had positive results, primate models will have greater clinical value and significance. Overall, this study is an innovative test of repair methods after peripheral nerve injury; however, it lacks the molecular biological mechanism, and we will conduct more molecular biological research.

As a result, the development of new drug screening methods is crucial. In this study, we used our designed drug screening biomimetic microfluidic chip to test the optimal combination and concentration of NGF and bFGF for promoting peripheral nerve regeneration and repair. The microfluidic chip is a fast and efficient drug screening device. As a result, we successfully obtained the results of eight different drug concentration combinations of NGF and bFGF in vitro. We found that the drug screening results of 22.86 ng/mL + bFGF at 4.29 ng/mL had the best effect on peripheral nerve regeneration. Although the combined concentration of NGF and bFGF was less than the concentration of the single action, it had a better effect and may be related to the synergistic effects of these factors. Based on the drug screening results, we designed NGF-bFGF-PLGA sustained-release microspheres and continued to improve them according to their drug loading and encapsulation efficiency. Our results showed that the self-designed drug screening biomimetic microfluidic chip can continuously supply fresh nutrients and oxygen to the three-dimensional cultured Schwann cells in a controlled fluid medium, and the chip can efficiently screen out the combined concentration of NGF and bFGF, promoting peripheral nerve regeneration.

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