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Controlled Release Strategy Based on Biodegradable Microspheres for Neurodegenerative Disease Therapy

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1. Introduction

The past decades have seen considerable advances in our understanding of the developmental mechanisms of neurodegenerative disorders, including Parkinson's, Alzheimer's and Huntington's diseases and spinal cord injury [1-3]. The major neuropathological characteristics of neurodegenerative disorders are atrophy or loss of specific neurons in the specific brain areas, such as cholinergic neuron degeneration observed in the basal forebrain, hippocampus and cortex in Alzheimer's disease (AD) and dopaminergic neuron degeneration observed in the striatum in Parkinson's disease [4-6]. As the life span of human beings increases and more people live beyond the age of 60, the number of people with neurodegenerative disorders is also progressively increased. Contrasting with the enormous toll that these disorders put on patients, their caregivers and the whole society is the lack of effective therapy at present.

Neurotrophic factors (NTFs) are secreted peptides essential for the phenotypic development and maintenance of specific neuronal populations in developing and adult vertebrate nervous system [7-14]. These diffusible proteins act via retrograde signaling from target-neurons and by paracrine and autocrine mechanisms, and regulate many aspects of neuronal and glial structure and function. In addition to playing key roles during development, NTFs are also required in the adult brain for maintaining neuronal function and phenotype. Previous studies of intracerebroventricular (ICV) administration of NTFs have shown to reduce or prevent neuronal atrophy or neuronal loss in neurodegenerative diseases [7-9, 12, 13, 15, 16]. The cholinergic neurons of basal forbrain express both the low affinity receptor (p75NTR) and high affinity receptor (Trk receptor), and respond to neurotrophic factors by increased activity levels of the choline acetyltransferase (ChAT). NTFs exert neurotrophic actions on the cholinergic neurons of the basal forebrain and protect them against neurodegeneration [7, 12, 15, 17]. For example, ICV injection of nerve

growth factor (NGF) has shown to improve the learning and memory ability, and the survival of basal forebrain cholinergic neurons in aged or fimbria-fornix lesioned animals. Furthermore, NTFs have been used for the experimental therapies in Parkinson's, Alzheimer's and Huntington's diseases and spinal cord injury [4-14].

Modern biotechnology has enabled the large-scale production of highly purified proteins; therefore, large quantities of growth factors can now be manufactured for clinical use. However, NTFs are large molecular proteins that do not readily cross the blood–brain barrier (BBB), and have short biologic half-life [10-13]. To improve their therapeutic efficacy and patient compliance, local and controlled delivery of NTFs directly to the desired brain area are preferred [3, 18-20]. NGF-releasing and glial cell line-derived neurotrophic factor (GDNF)-releasing microspheres have been developed for the experimental therapy of Alzheimer's and Parkinson's disease (PD) [21-30]. Compared to other exiting approaches, the strategy based on biodegradable microspheres has demonstrated a number of advantages.

- Ease of administration to the targeted area of the brain, thus avoiding open operation and damage to surrounding tissue [31, 32].
- Preservation of drug activity during encapsulation and storage.
- Localized, controlled-release profiles for a desired period, resulting in enhanced therapeutic effect while minimizing side effects.
- Better safety profiles, as compared with gene therapy such as gene delivery and transplantation of genetically modified cells.

In this chapter, to serve as a good example, we presented our previous works focusing on the formulation and characterization of NGF-loaded microspheres. The in vivo efficacy of these microspheres was evaluated in a rat model of AD. The methods described here can be applied broadly to other types of NTF-releasing microspheres.

2. Materials

2.1 Formulation of NGF-releasing microspheres

General regents and instruments for the preparation of NGF-releasing microspheres, the abbreviations and source of these reagents are as follows:

2.1.1 Neurotrophic factors such as human recombinant β-nerve growth factor (NGF) (Calbiochem or Sigma)

2.1.2 Poly(lactide-co-glycolide) (PLGA) (lactic acid/glycolic acid = 75/25) (Birmingham Polymers Inc. or Sigma)

2.1.3 Bovine serum albumin (BSA) or human serum albumin (HSA) (Calbiochem or Sigma).

2.1.4 FITC-labeled BSA (FITC-BSA) (Sigma)

2.1.5 Polyvinyl alcohol (PVA) (Sigma).

2.1.6 Acetone (Sigma)

2.1.7 Methylene chloride (Sigma)

2.1.8 Sonicator (Sonics & materials INC. USA)

2.1.9 Cantilever agitator (IKA. RW20 DZM.n, Guangzhou Scientific Instrument, Ltd Co)

2.2 Characterization of NGF-releasing Microspheres

Instruments for microsphere characterization, the abbreviations and source of these reagents are as follows:

2.2.1 Coulter counter (model ZM, Coulter Electronics Limited, UK)

2.2.2 Scanning electron microscopy (XL30ESEM, Philips, Netherlands)

2.2.3 Rabbit anti-rhNGF-beta polyclonal antibody and CY3-conjugated goat anti-rabbit secondary antibody (Chemicon)

2.2.4 Ultraviolet spectrophotometer (Hach)

2.2.5 Confocal laser scanning microscopy (CLSM; Leica TCS SP5, Leica Mikrosysteme Vertrieb GmbH, Germany)

2.3 Cell culture

General Regents for PC12 cell culture, the abbreviations and source of these reagents are as follows:

2.3.1 Dulbecco Modified Eagle's medium (DMEM) (Invitrogen)

2.3.2 Fetal bovine serum (FBS) (Invitrogen)

2.3.3 Fetal horse serum (FHS) (Invitrogen)

2.3.4 Trypsin-EDTA (1x) (Invitrogen)

2.3.5 Penicillin/Streptomycin (100x) (Invitrogen)

2.3.6 L-glutamine (100x) (Invitrogen)

2.4 Evaluation of in vivo efficacy in a rat model of AD

Normotensive adult male Sprague Dawley (SD) rats weighing 250-300 g are used. The general tools, instruments and reagents for in vivo evaluation in a rat model of AD are as follows:

2.4.1 Narishige stereotaxic instrument (Narishige SR-5, Narishige Scientific Instrument Lab, Tokyo, Japan)

2.4.2 One scalpel size 3 with No. 10 blade

2.4.3 Two small scissors for blunt dissection

2.4.4 One needle holder

2.4.5 One pair of microsurgical scissors

2.4.6 Two pairs of microsurgical forceps

2.4.7 3/0 sutures

2.4.8 Dentistry drill

2.5 Tissue processing

2.5.1 Ice-cold phosphate buffered saline (PBS)

2.5.2 Ice-cold saline.

2.5.3 15% and 30% sucrose in PBS

2.5.4 4% paraformaldehyde

2.5.5 Serum infusion set with a blunted 20-ga needle

2.5.6 OCT Tissue freezing medium

2.5.7 Anti-rhNGF antibody, anti-Choline acetyltransferase (ChAT) antibody and appropriate secondary antibodies

2.5.8 Image J software (National Institutes of Health)

2.6 Assessment of learning and memory impairment

2.6.1 Y-maze test (Chongqing Medical Apparatus Co, China)

3. Methods

3.1 Preparation of NGF-releasing microspheres

NGF-releasing microspheres were formulated using with a modified multiple emulsion solvent extraction-evaporation technique (W/O/W) (see Note 1) [24]. NGF (5-10 mg) (see Note 2) and BSA (1/50-2000, w/w) in 100-200 μL distilled water. The protein solution was emulsified in a solution of PLGA (100-200 mg) (see Note 3) in methylene chloride/acetone (3/1, 4-8 mL) to form the first W/O emulsion (see Note 4), using sonication for 1-2 min at 20-40 W over an ice bath. The first W/O emulsion was then added to 1% PVA aqueous solution (25-50 mL) (see Note 5), and homogenized at 800-2000 rpm for 5-10 min over an ice bath to form W/O/W double emulsion. The W/O/W double emulsion was stirred at room temperature for 3-4 h to allow the evaporation of the organic solvents in the fume hood. The microspheres were collected by centrifugation, and washed three times with distilled water to remove any proteins that were weakly bound onto the surface of microspheres. The harvested microspheres were freeze-dried to obtain a free flowing powder. The NGF/FITC-BSA-releasing microspheres were prepared using in the same way for in vivo release and tracking studies (see Note 6 and 7).

3.2 Characterization of NGF-releasing microspheres

3.2.1 The size distribution of NGF-releasing microspheres was measured using a Coulter counter

3.2.2 The morphology of NGF-releasing microspheres was examined using scanning electron microscopy (SEM)

The freeze-dried NGF-releasing microspheres were mounted on the conductive tap of a metal stub, and sputtered coated with gold before being observed using SEM (Figure 1).

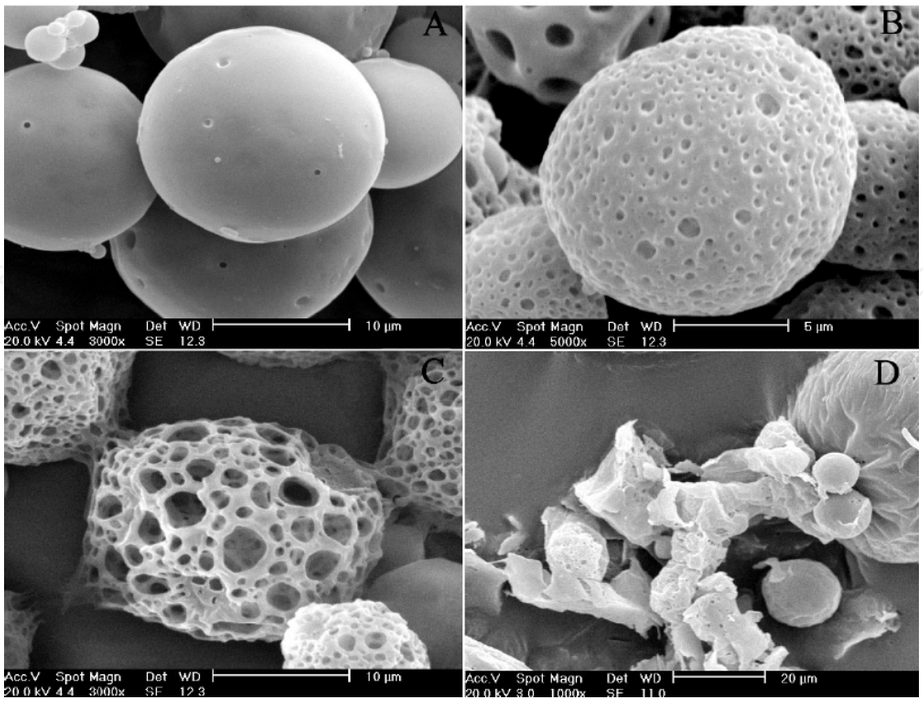


Fig. 1. SEM micrographs of NGF-releasing microspheres after various intervals of incubation in PBS. (A) The microspheres were spherical in shape with smooth surface before incubation with PBS. (B) After 1 week of incubation, shallow pores appeared on the surface of the microspheres that maintained intact spherical shape. (C) After incubation for 3 weeks, the microspheres showed highly porous surfaces and irregular shapes, with eroded inner matrix. (D) After incubation for 5 weeks, most of the microspheres lost their spherical shape and collapsed. This picture was taken from our previous paper and allowed to use here by Polymer International.

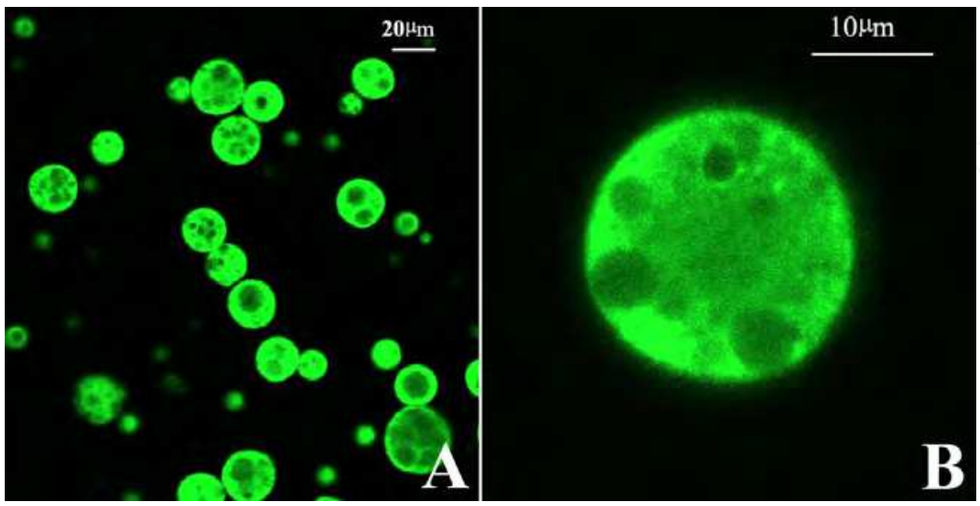


Fig. 2. Confocal Laser Scanning Microscopy (CLSM) micrographs of NGF/FITC-BSA-releasing microspheres at lower magnification (A) and higher magnification (B). This picture was taken from our previous paper and allowed to use here by Polymer International.

3.2.3 The morphology of NGF/FITC-BSA-releasing microspheres was examined by confocal laser scanning microscopy (CLSM)

Freeze-dried NGF/FITC-BSA-releasing microspheres spread on glass coverslips were observed under CLSM (Figure 2).

3.2.4 The amount of the protein encapsulated in the NGF/BSA-releasing microspheres was determined by a protein extraction method described below

Ten to thirty milligrams of microspheres were dissolved in 1-2 mL of chloroform and extracted with 4 mL of deionized water. The mixture was vortex for 5-10 min. The extracted protein in aqueous phase was separated and collected by centrifugation. Three extractions were performed for each sample. The aqueous extracts were pooled together, and the protein concentration was determined by BCA microassay. Experiments were run in triplicate per sample. The protein loading and encapsulation efficiency in microspheres were defined by the following equations:

Protein loading in microspheres (%) = Weight of protein in the aqueous extract/Weight of microspheres extracted × 100

Encapsulation efficiency (%) = Weight of protein in microspheres/Weight of protein in the initial formulation × 100

3.2.5 In vitro release of NGF

In vitro drug release from the NGF-releasing microspheres was monitored in PBS at 37°C, utilizing double-chamber diffusion cells on a shaker stand. Approximately 50 mg of the microspheres were suspended in 4 mL of PBS buffer in the donor chamber. The receiver chamber was filled with 4 mL plain buffer. The donor chamber and receiver chamber were separated with 0.45 μm low-protein absorbable membrane. At predetermined time points, the PBS buffer in the receiver chamber was collected and replaced with same amount of fresh PBS. The protein concentration in the collected buffer solution was analyzed using BCA microassay. The concentration of the released NGF in the buffer solution was measured using ELISA and the amount of released NGF was calculated. Experiments were run in triplicate per sample.

The bioactivity of the released NGF from the microspheres was assessed using PC12 cell culture. PC12 cells were maintained in growth on T-25 cell culture flasks (Corning Inc., Corning, NY) in RPMI 1640 supplemented with 10 wt% horse serum, 5 wt% fetal bovine serum and 50 U mL^{-1} penicillin/streptomycin. For the bioactivity of released NGF testing, PC12 cells were plated on poly (L-lysine) pre-coated 24-well plates. The cells were cultured at 37°C in a 95% water-saturated, 5% CO_2 air atmosphere and plated at a density of approximately 1.0×10^4 cells/cm^2 (in 2 mL). Twenty-four hours, 2 mL solution of in vitro NGF release buffer collected in the first week was sterile-filtered through a 0.22 μm filter (Millipore) and incubated with PC12 cells (30 ng mL^{-1}). Incubating with NGF-supplement medium (30 ng mL^{-1}) alone or with the released buffer from BSA-releasing microspheres (without NGF) served as positive or negative control, separately. To further confirm the bioactivity of the released NGF, anti-NGF antibody was added into the medium to neutralize the bioactivity of the released NGF.

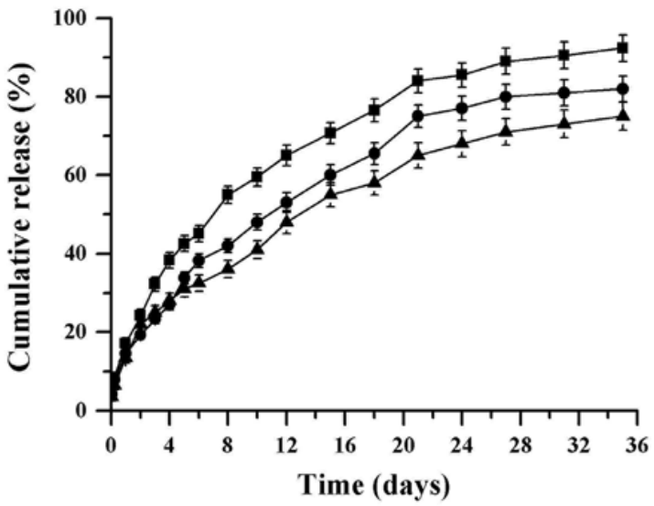


Fig. 3. *In vitro* rhNGF release from rhNGF/BSA-loaded microspheres: with protein/PLGA (w/w) ratio of (▲) 5/100, (●) 10/100, and (■) 15/100. This picture was taken from our previous paper and allowed to use here by Polymer International.

3.2.6 The bioactivity of the released NGF was test using PC 12 cells

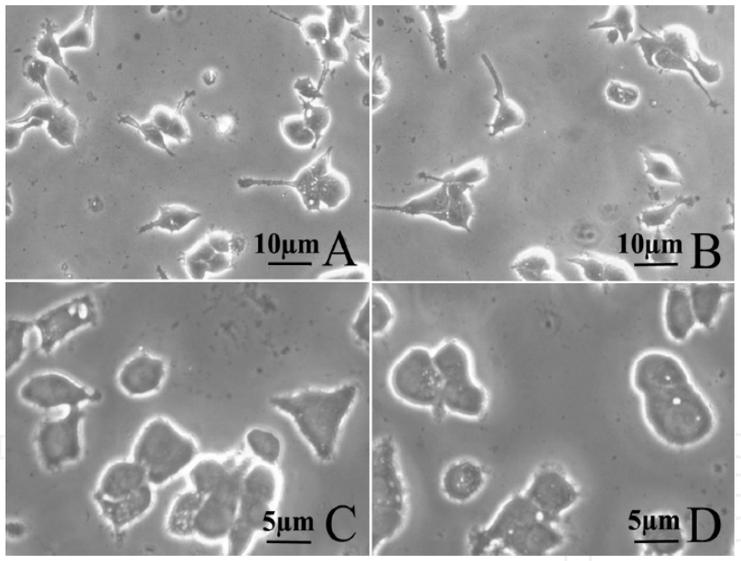


Fig. 4. The bioactivity of the released NGF from the PLGA microspheres as assessed with PC12 cell culture. (A) Positive control cells that were incubated with NGF-supplemented medium at a concentration of 30 ng mL^{-1}; (B) the cells incubated with the released NGF from PLGA microspheres and added to the culture medium at a concentration of 30 ng mL^{-1}; (C) negative control cells incubated with the released BSA from BSA-only PLGA microspheres and added to the culture medium ; (D) cells incubated with the released NGF-supplemented culture medium plus anti-NGF antibody added . The percentage of neurite-bearing cells was determined by counting 100–200 cells in several randomly chosen fields under an optical microscope for each sample after 24 h incubation. This picture was taken from our previous paper and allowed to use here by Polymer International.

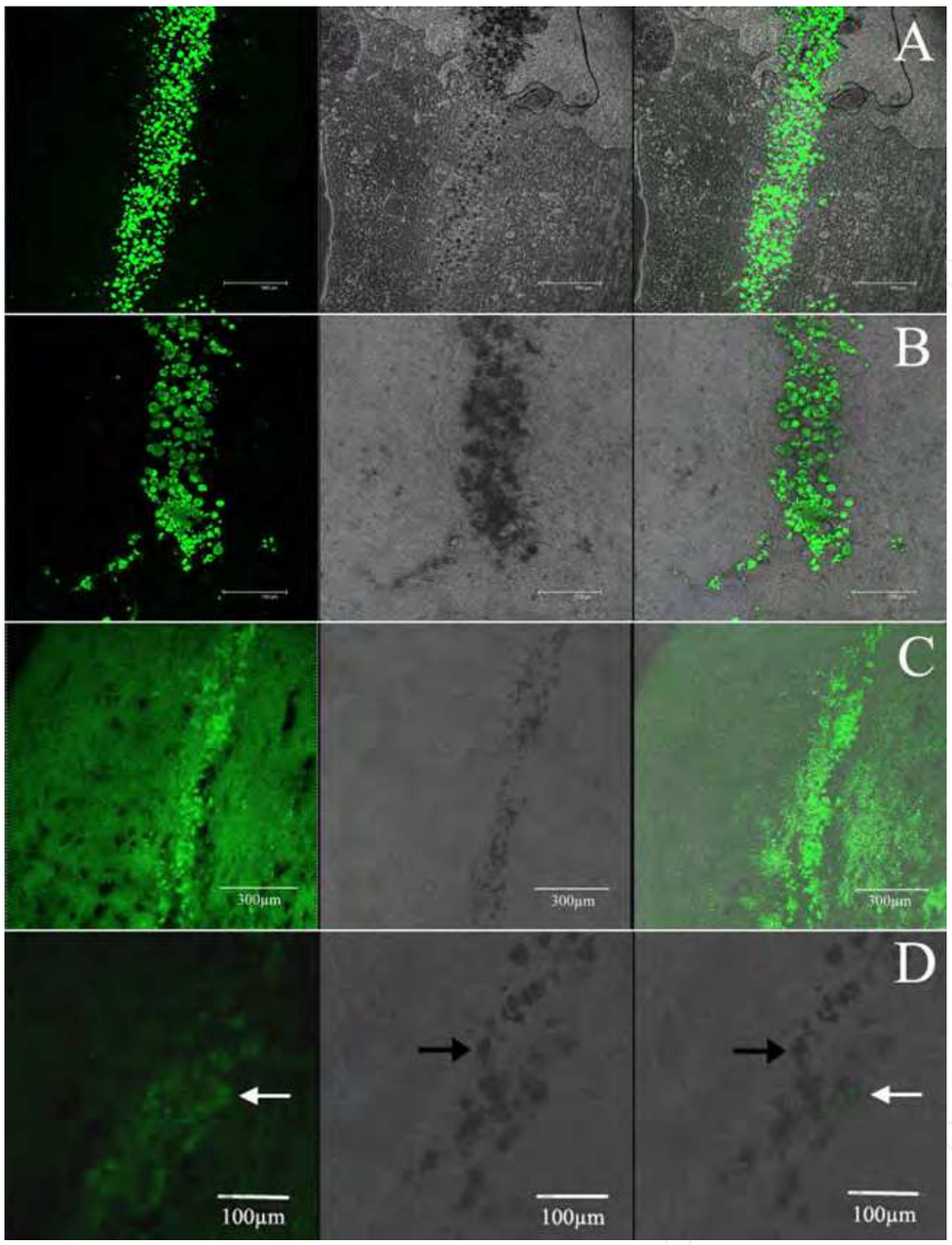


Fig. 5. CLSM micrographs of NGF/FITC-BSA-loaded microspheres implanted in the BF (left column: observed under fluorescent filter; middle column: observed under normal light; right column: merged from left and middle): (A) 1 week after implantation, the fluorescent in implanted NGF/FITC-BSA-loaded microspheres is very strong; (B) 3 weeks after implantation, the fluorescent in implanted NGF/FITC-BSA-loaded microspheres became weak; (C) 4 weeks after implantation, the fluorescent in implanted NGF/FITC-BSA-loaded microspheres was weaker; (D) 5 weeks after implantation; most of the microspheres showed empty microspheres (black and white arrows showing an empty and a still-loaded microsphere, respectively). This picture was taken from our previous paper and allowed to use here by Polymer International.

3.2.7 In vivo tracking and release studies

All experiments were carried out strictly according to the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised in 1996. All experiments were performed on adult male Sprague-Dawley (SD) rats (250–300 g) (see Note 8). The animals were housed in groups of two or three in macrolon cages; food and water were available ad libitum. All the animals were peritoneally anesthetized with sodium pentobarbital (40 mg kg−1 body weight) by peritoneal injection and positioned in a Narishige stereotaxic instrument (Narishige SR-5, Narishige Scientific Instrument Lab, Tokyo, Japan). An amount of 3 mg of NGF/FITC-BSA-loaded microspheres (suspended in 10 μL of dispersing medium) was implanted into the BF (coordinates: anterior–posterior, +0.6 mm; left lateral, +0.6 mm; dorsal–ventral, −5.5 mm from bregma).

At the predetermined time intervals, animals were perfused via the aorta with PBS (pH = 7.4) 200 ml and fixed with 4 wt% paraformaldehyde for 30 min. The brains were removed and further fixed for 3-6 h at 4°C. The fixed brain was incubated in a 15 wt% sucrose buffer solution overnight and in 30 wt% sucrose buffer solution until it was sunk. Brain sections (20 μm) were cut at the transverse plane on a freezing microtome. These sections were mounted on gelatin-coated slides for directly examining the protein release from the NGF/FITC-BSA-loaded microspheres using CLSM.

3.3 Recombinant human NGF-loaded microspheres implantation promotes survival of basal forebrain cholinergic neurons and improve memory impairments of spatial learning in the rat model of Alzheimer's disease (see Note 9)

Unilateral fimbria-fornix (FF) of SD rats was transected to simulate the impairment of cholinergic neurons of AD by the lesion of the septo-hippocampus pathway. The animals were randomly divided into four groups: (1) normal control group; (2) lesion control group; (3) unloaded microspheres group; (4) rhNGF-loaded microspheres group. At the lesion time, three milligrams of rhNGF-loaded microspheres (corresponding to 5 μg of NGF) (suspended in 10 μl of PBS) were stereotaxically implanted into the basal forbrain.

At the predetermined time points, animals were perfused via the aorta with PBS (pH = 7.4) 200 ml and fixed with 4 wt% paraformaldehyde for 30 min. The brains were removed and further fixed for 3-6 h at 4°C. The fixed brain was incubated in a 15 wt% sucrose buffer solution overnight and in 30 wt% sucrose buffer solution until it was sunk. Brain sections (30 μm) were cut at the transverse plane on a freezing microtome. The cholinergic neurons in medial septum (MS) and vertical diagonal band (VDB) were stained by ChAT immunohistochemistry. In brief, brain sections were incubated with 3% H_2O_2 to block endogenous peroxidase 30 min and then incubated in 10% normal goat serum for 1 h before incubation with rabbit anti-rat ChAT antibodies overnight at 4°C. Following primary antibody incubation, brain sections were washed with 1 × PBS, incubated with secondary antibody HRP-conjugated anti-rabbit IgG 1-2 h at room temperature and washed with 1 × PBS. And then brain sections were incubated with DAB. After incubated with gradient ethanol and xylene, the brain sections were mounted neutral permanent mounting medium.

Y-maze test (see Note 10) was used to evaluate the ability of spatial learning and memory in different trial groups.

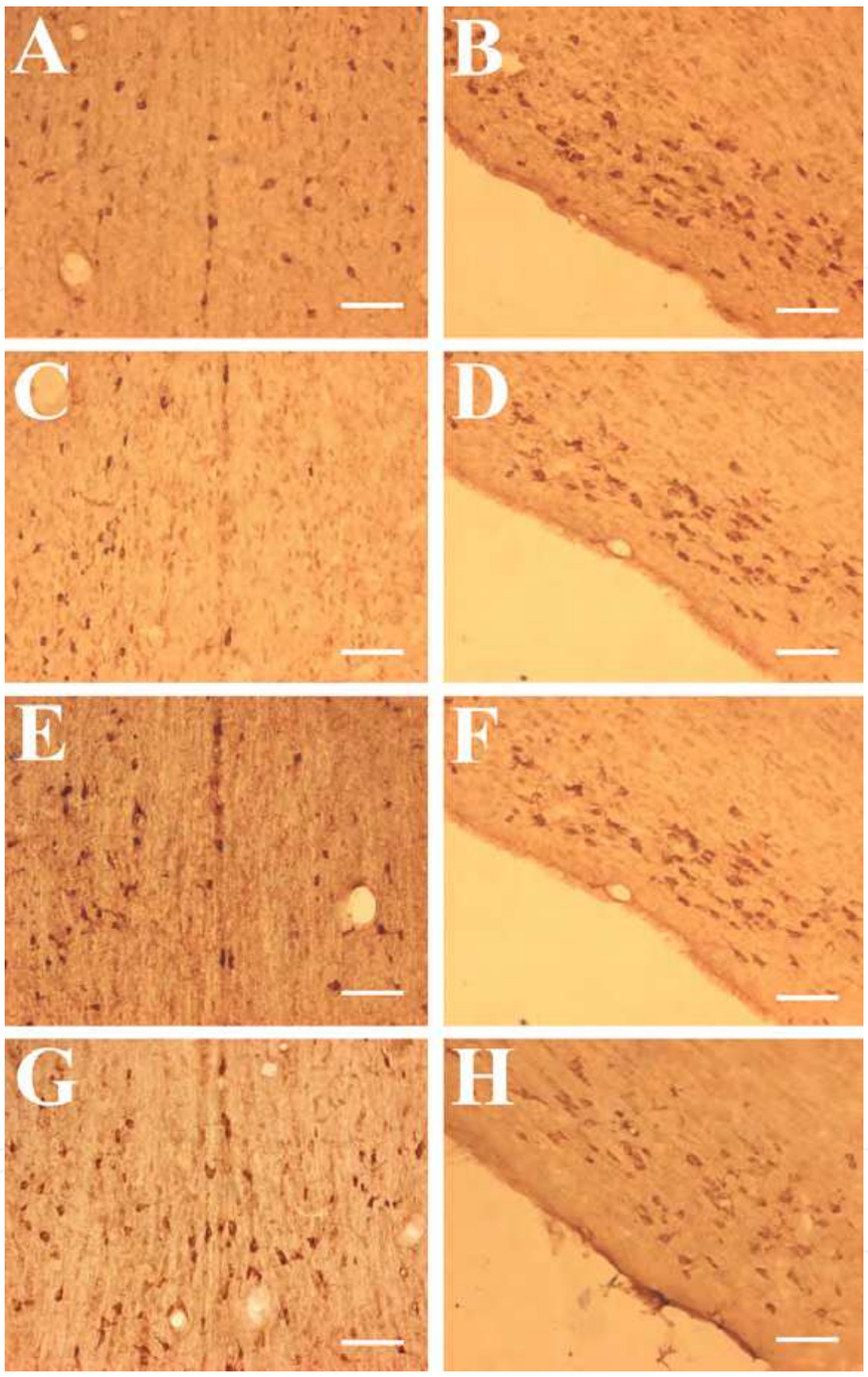


Fig. 6. Immunohistochemistry of cholinergic neurons show ChAT-positive in four groups in basal forebrain 4 weeks after microspheres implantation. (A and B) ChAT-positive in MS and VDB of the normal control group. (C and D) ChAT-positive in MS and VDB of the lesion control group. (E and F) ChAT-positive in MS and VDB of the unloaded microspheres group. (G and H) ChAT-positive in MS and VDB of the rhNGF-loaded microspheres group. Bar = 25 μm. This picture was from our previous paper and allowed to use here by Neuroscience letters.

4. Notes

The protocols given in this chapter are routinely conducted in our laboratory. The following notes may be of interest:

1. Drug-releasing biodegradable polymeric microspheres are commonly prepared by physicochemical processes such as W/O/W emulsion solvent extraction-evaporation method, or mechanical processes such as spray coating, spray drying and spray congealing [33]. It is generally accepted that W/O/W emulsion solvent extraction-evaporation technique is most suitable for encapsulation of proteins and peptides [25].
2. Based on the structure and function, neurotrophic factors (NTFs) are divided into several families: (1) nerve growth factor (NGF)-superfamily, including nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin -4/5 (NT-4/5) and neurotrophin-6 (NT-6); (2) glial cell line-derived neurotrophic factor (GDNF) family, including glial cell line derived neurotrophic factor (GDNF), neurturin (NTN) and persephin (PSP); (3) neurokine superfamily, including ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), interleukin (IL) and cardiotrophin (CT); (4) non-neuronal growth factor-superfamily, including some members of fibroblast growth factor superfamily, epidermal growth factor (EGF) [10], insulin-like growth factor (IGF) [11, 14], bone morphogenetic protein (BMP) [2, 8, 12]. Furthermore, other growth factors also have neurotrophic function, such as vascular endothelial growth factor (VEGF) and erythropoietin (EPO) [34, 35]. Most of these neurotrophic factors are protein or glycoprotein and are suitable to prepare sustained release microspheres [22, 31, 36-39]. GDNF has been used to prepare sustained release microspheres and to use for the treatment of Parkinson's disease [19, 21, 23, 25, 40]. Basic fibroblast growth factor (bFGF) has been used to formulate bFGF-loaded microshperes and use to treat ischemic hindlimb [27, 41, 42]. In central nervous system, different neurons have express different receptors and react with different neurotrophic factors. For example, cholinergic neurons express low affinity growth factor receptor ($p75^{NGFR}$) and Trk receptor [20, 43].
3. PLGA belongs to a family of polyhydroxyalkanonate that have been extensively used in medical and pharmaceutical fields. It is biodegradable and biocompatible with brain tissue [44]. Drug release in PLGA based microspheres is modulated by both diffusion and degradation of polymer. The degradation process involves hydrolysis of the ester bonds, which is then autocatalyzed by the presence of acidic degradation products. The degradation rate of PLGA microspheres can range from less than one month to a few years, depending on the polymer composition (i.e. the molar ratio of lactic and glycolic acid units). This provides a convenient approach to engineering PLGA microspheres in order to provide a sustained and controlled release profile for a desired period to meet the clinical needs. Thus far, PLGA still remains the most popular polymer for the formulation of NTF-releasing microspheres. Several natural polymers have also been employed for the preparation of NTF-loaded microspheres. These includes hyaluronane derivatives [45], alginate-polylysine [46] and chitosan [47] *etc.* The application of these biomacromolecules needs to pay attention to the purity, batch-to-batch variation, and homogeneity of samples according to their origin.

4. A number of studies have indicated that the methylene chloride/water interface in the first emulsion step is detrimental to the structural integrity of protein, causing substantial conformational changes and protein denaturation [48, 49]. This denature effect could be overcome by optimizing the formulation parameters such as co-encapsulation of BSA [49] and/or PEG 400 [50] as stabilizing agents, the use of low temperatures and short periods of sonication time.
5. PVA has been intensively used as an emulsifier for the formulation of microspheres. Other polymeric surfactants such as polyvinylpyrrolidone (PVP) could serve as alternative emulsifier. Conway *et al* reported that microspheres formulated with PVP exhibited much higher protein loading and pronounced reduction in the "burst" release of protein [51]. However, research activity in this aspect is limited so far, especially in the application for neurodegenerative disease therapy.
6. The formulation of NGF-releasing microspheres must be performed under aseptic conditions and using sterile starting materials. The current available sterilization methods such as autoclaving, γ-ray induced irradiation and ethylene oxide gas can induce protein degradation and/or toxicity.
7. Recently nanospheres have attracted increasing attention in the delivery of growth factors [52]. Their much smaller sizes allow more effective internalization by cells via endocytic pathway, compared to microspheres. In the case of bioactive macromolecules that bind the membrane receptors to exert their functions, microspheres are more advantageous.
8. Anesthesia with sodium pentobarbital is easy to apply by peritoneal injection. This anesthesia method is unexpensive and well tolerated by the animals. This method is simple, repeatable and can provide deep anesthesia more than 1 h that is usually enough for most of surgical procedures. The animals can be applied more this anesthesia chemical if they feel uncomfortable during surgical procedures. Other anesthesia reagents can also be used, such as Ketamine, Medetomidine and so on. If the surgical procedures last more than 2 h, better control of the duration of the anesthesia is required, such as inhalation anesthesia method. Whichever method is used, all the animals should be applied the same anesthetics.
9. Sustained release microspheres are good strategy not only for the treatment of Alzheimer's disease, but also for the other neurodegenerative disorders. As mentioned in Note 2, GDNF-loaded microsphere has been used to treat Parkinson's disease.
10. Learning and memory impairment of Alzheimer's rats can be tested with other mazes, such as Morris water maze and 8-arm maze [53, 54]. Investigators should use different behavioral tests to evaluate different animal models. For Alzheimer's disease animal model, the common behavioral test is maze testing. For Parkinson's disease animal model, rotation, reaching task, forelimb asymmetry test and tactile placing are widely used to evaluate the behavioral changes [55].

5. Acknowledgements

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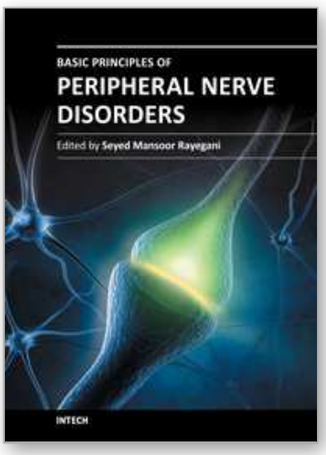
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Peripheral nerve disorders are comprising one of the major clinical topics in neuromusculoskeletal disorders. Sharp nerve injuries, chronic entrapment syndromes, and peripheral neuropathic processes can be classified in this common medical topic. Different aspects of these disorders including anatomy, physiology, pathophysiology, injury mechanisms, and different diagnostic and management methods need to be addressed when discussing this topic. The goal of preparing this book was to gather such pertinent chapters to cover these aspects.

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