Abstract: This study aims to prepare ultrasound-targeted glial cell-derived neurotrophic factor (GDNF) retrovirus-loaded microbubbles (M pLXSN-GDNF) to verify the properties of the microbubbles and to study the therapeutic effect of the GDNF retrovirus-loaded microbubbles combined with ultrasound (U) to open the blood–brain barrier (BBB) in a Parkinson’s disease (PD) model in rats, allowing the retrovirus to pass through the BBB and transfect neurons in the substantia nigra of the midbrain, thereby increasing the expression of GDNF. The results of western blot analysis revealed significant differences between U + M plXSN-EGFP, U + M + plXSN-GDNF, and M plXSN-GDNF (P < 0.05) groups. After 8 weeks of treatment, the evaluation of the effect of increased GDNF expression on behavioral deficits in PD model rats was conducted. The rotation symptom was significantly improved in the U + M plXSN-GDNF group, and the difference before and after treatment was significant (P < 0.05). Also, the content of dopamine and the number of tyrosine hydroxylase-positive (dopaminergic) neurons were found to be higher in the brain of PD rats in the U + M plXSN-GDNF group than in the control groups. Ultrasound combined with GDNF retrovirus-loaded microbubbles can enhance the transfection efficiency of neurons in vivo and highly express the exogenous GDNF gene to play a therapeutic role in PD model rats.

Keywords: cationic microbubble, Parkinson’s disease, retrovirus, GDNF

1 Introduction

The main pathological feature of Parkinson’s disease (PD) is the progressive degeneration of dopaminergic neurons in the substantia nigra of the midbrain, leading to decreased dopamine (DA) content in the striatum [1]. Recently, with advances in cell and molecular biology, scientists have begun to pay attention to finding a way to delay and reverse the degeneration process of substantia nigra dopaminergic neurons. Glial cell-derived neurotrophic factor (GDNF) has been shown to specifically promote the survival of dopaminergic neurons and protect neurons from the effect of neurotoxins, and thus represents a potential therapy for PD. It has been confirmed that increasing GDNF protein content in the nigrostriatal dopaminergic system can significantly improve PD symptoms in animal models [2,3]. Therefore, it has been proposed that GDNF and the regulation of its signaling pathway may be an effective molecular target for the treatment of PD [4–6]. However, due to the relatively large molecular weight of GDNF (25 kDa), it is difficult for it to cross the blood–brain barrier (BBB). For example, direct
intracerebral injection or direct injection of a viral vector carrying the GDNF gene into the brain will damage the brain tissue. Accordingly, the development of a non-invasive method for GDNF to cross the BBB, leading to an increase in its effective concentration in the central nervous system, is key for the application of GDNF in the treatment of PD [7]. Ultrasound with microbubbles has been demonstrated to open the BBB locally, reversibly, and noninvasively at energy levels that do not cause cellular damage, which provides the possibility of successfully treating brain diseases, such as PD. Some scientists have used focused ultrasound with a microbubble contrast agent to open the BBB and treat central nervous system diseases by targeted release of drugs and biomolecules [8–10]. Acoustic microbubbles could be used as gene carriers to treat diseases. Ultrasound targeted microbubble destruction (UTMD) technology uses microbubble contrast agents as carriers to make an adherent surface or to encapsulate target genes [11,12]. UTMD is definitely a promising strategy to improve the efficiency of gene delivery for multiple applications, and it is proven by increasing evidence that organs can be targeted with its high specificity [13]. Targeted acoustic microbubbles containing target genes are injected intravenously to reach target tissues, where the bubbles are broken to release the genes using ultrasonic irradiation.

With the in-depth study of ultrasound drug-loaded microbubbles and application of ultrasound with microbubbles to open the BBB, microbubbles carrying drugs or genes targeted to treat brain diseases provide a new strategy for the treatment of these diseases. In this study, we prepared targeted cationic microbubbles carrying GDNF retrovirus to enhance the transfection efficiency of neurons in vivo and highly express the exogenous GDNF gene to play a therapeutic role in PD model rats.

2 Materials and methods

2.1 Materials and equipment

Distearoylphosphatidylcholine (DSPC), polyethylene glycol–distearoyl phosphatidylethanolamine (DSPE-PEG2000), and polyethylene diamine-600 (PEI600) (Avanti Polar Lipids Inc., Alabaster, AL, USA) were used; an automatic dilution AccuSizer particle counter (Particle Sizing Systems, Santa Barbara, CA, USA), Quintessential Stereotactic Injector (Stoelting, Wood Dale, IL, USA), inverted fluorescence microscope (Olympus Corp., Tokyo, Japan), and centrifuge (Eppendorf, GmbH, Hamburg, Germany) were the instruments used. Retroviral vectors, pLXSN-GDNF and pLXSN-EGFP, were purchased from HanBio Technology Co. Ltd (Shanghai, China). Sprague Dawley (SD) rats were obtained from Guangdong Medical Laboratory Animal Center (Foshan, China). The system used to generate ultrasound energy in all the experiments comprised a function generator (AGF3022B; Tektronix, USA), an RF amplifier (DC2500A; AR, Souderton PA, USA), and a custom-made passive L–C matching circuit. Ultrasound waves were generated using a single-element focused ultrasound transducer (Valpey Fisher, Hopkinton, MA, USA). The subsequent experiments were performed under these conditions as our previous research (frequency, 1 MHz; MB dosage, 0.5 mL; exposure time, 1 min; pressure amplitude, 0.8 MPa; delay time, 60 s) [14].

2.2 Preparation of cationic microbubbles

DSPC, DSPE-PEG2000, and PEI 600 (Avanti Polar Lipids Inc., Alabaster, AL, USA) were added into a tube at a molar ratio of 9:0.5:0.5. A layer of phospholipid film was formed on the tube wall under nitrogen gas flow (0.1 MPa). The tube mouth was sealed with a sealing film with a few holes punched using a needle. The tube was put into a 500 mL suction filter bottle and subjected to a vacuum for 2–3 h. Subsequently, after adding 5 mL of Tris buffer, the tube was oscillated in an ultrasonic oscillator at 55–60°C for 20 min. The phospholipid suspension in the tube was subpackaged into 2.5 mL penicillin vials with 1 mL in each for gas exchange to fill the vial with perfluoropropane [15]. Normal lipid microbubbles and biotinylated lipid microbubbles were prepared by the above methods.

2.3 Preparation of cationic microbubbles loaded with GDNF virus

After the microbubbles were prepared by the mechanical vibration method, they were washed 3–4 times with phosphate-buffered saline (PBS) by the centrifugal floating method to remove the unreacted phospholipid not incorporated into microbubbles [16]. A total of 4 × 10⁷ microbubbles were added to 1 mL of a pLXSN-GDNF virus solution (4.2 × 10⁷ particles/mL). After incubation at room temperature for 30 min, the microbubbles were washed twice by the floating method to wash off the unbound virus particles and obtain the pLXSN-GDNF microbubble contrast agent. Four sample aliquots were
taken from the virus solution, washing the supernatant each time and microbubble solution binding virus, in which the virus quantity was detected by real-time (fluorescence) quantitative PCR, and the adhesion efficiency of the virus and microbubbles was determined [17]. The preparation and detection methods for pLXSN-EGFP microbubbles were the same as those above.

2.4 Establishment of PD rat models

All procedures in the animal experiments were conducted in accordance with the guidelines developed by the National Institutes of Health and approved by the Institutional Animal Care and Use Committee of Xinxiang Medical University (Permit No. 18-216). Before injection of 6-hydroxydopamine (6-OHDA), the PD rats with memory impairment were excluded using the Morris water maze test, and those with rotational behavior were excluded using an apomorphine-induced rotation test after intraperitoneal injection of apomorphine (0.5 mg/kg). The rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (4 mL/kg). A 2μL volume of 6-OHDA (4 g/L) was injected at two injection sites, namely in the right dense part of the substantia nigra (4.4 mm behind the anterior fontanelle, 1.2 mm to the right of the sagittal suture, and 7.8 mm under the dura) and the ventral tegmental area of the midbrain (4.8 mm behind the anterior fontanelle, 1.0 mm to the right of the sagittal suture, and 7.8 mm under the dura). The 6-OHDA was injected slowly at a rate of 0.5 μL/min, and stopped 10 min after injection, when the needle was slowly withdrawn. Postoperatively, the rats were kept warm and hydrated, and given a daily intraperitoneal injection of penicillin 30,000 U/d for 1 week to prevent infection. On the 21st day postoperatively, the rats were injected with 0.5 mg/kg apomorphine intraperitoneally to induce left rotational behavior. The rats with an average rotation speed of more than 7 rpm on the 21st day were considered as successful PD models.

2.5 Grouping and treatment

The rats were assigned to four groups: (1) control group (normal group), including normal rats that were not treated; (2) ultrasound + pLXSN-EGFP microbubble group (U + MpLXSN-EGFP group), injected with 4 × 10⁷ pLXSN-EGFP microbubbles into the tail vein and sonicated with ultrasound in the damaged brain region; (3) ultrasound + pLXSN-GDNF microbubble group (U + MpLXSN-GDNF group), injected with 4 × 10⁷ pLXSN-GDNF microbubbles into the tail vein and sonicated with ultrasound in the damaged brain region; and (4) ultrasound + microbubbles + pLXSN-GDNF group (U + M + pLXSN-GDNF group), injected with 4 × 10⁷ microbubbles and pLXSN-GDNF virus into the tail vein and sonicated with ultrasound in the damaged brain region.

After locally opening the BBB by using magnetic resonance imaging (MRI)-guided low-frequency focused ultrasound with microbubbles (with or without virus), the transfection efficiency of neurons in the striatum of each group was determined. The level of GDNF in the brain was detected by western blot analysis, and the behavioral changes of PD rats were evaluated.

2.6 Detection of GDNF expression in the brain

Western blot analysis was used to detect the expression of GDNF in the substantia nigra in each group. Total proteins in the substantia nigra were extracted using conventional methods and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. After transferring to a membrane, 5% bovine serum albumin was used to block the membrane overnight at 4°C, and rabbit anti-rat GDNF protein primary antibodies were added. Glass dishes with the membranes were placed on a shaking table and gently shaken at room temperature for 1 h. Secondary antibodies were added and shaken gently at room temperature for 45 min, and then the membrane was rinsed three times with TBS-T, with gentle shaking at room temperature for 5 min each time. The expression of GDNF was measured after exposure, development, and fixation using an ECL kit (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.7 Detection of dopaminergic neurons

Immunohistochemistry analysis was used to detect the changes in the number of dopaminergic neurons by determining the number of tyrosine hydroxylase (TH)-positive neurons in each group. Paraffin-embedded sections of rat brain tissues were prepared on microscope slides. The sections were then immersed in 0.5% hydrogen peroxide for 20 min at room temperature and incubated with goat serum for 30 min to block specific antigens. Rabbit anti-TH antibodies were added, and the
slides were incubated at 4°C overnight. Afterward, the slides were washed three times with PBS, then secondary sheep anti-rabbit IgG was added and incubated in a dark box at room temperature for 1 h. After washing three times with PBS, the slides were sealed, observed under a microscope, and photographed. ImagePro image analysis software was used to analyze and count the number of TH-positive neurons in the ultrasound-irradiated area from three slices of each rat.

2.8 Effects of different treatments on the content of DA in brain

For measuring the content of DA, samples were prepared with the substantia nigra. The striatum was dissected in accordance with the rat brain atlas. The brain tissues were homogenized in 0.20 M HClO₄. Homogenates were centrifuged for 10 min at 4°C and 12,000 g. The supernatant was filtered using a 0.20 µm membrane filter and injected into the high-performance liquid chromatography (HPLC). The levels of AD were expressed as ng per µg of brain tissues.

2.9 Statistical analysis

Our normality test indicated that the data belong to normal distribution, and so the measurement data were expressed as mean ± SD. Statistical analyses were performed using Student’s t test for two-group comparisons, and one-way ANOVA followed by post-hoc tests for multiple comparisons among more than two groups. All data analyses were performed with SPSS 19.0 statistical software (IBM Corp., Armonk, NY, USA). P < 0.05 was considered as statistically significant.
3 Results

3.1 Detection of ultrasound microbubbles

The average size of the cationic microbubbles was 1.02 ± 0.4 µm, and the concentration was \((1.2 ± 0.3) \times 10^{10}\) bubbles/mL. After loading the virus, the particle size was 1.06 ± 0.4 µm and the concentration was \((1.0 ± 0.2) \times 10^{10}\) bubbles/mL. The average surface potential of the cationic microbubbles was 32.0 ± 2.0 mV measured with a Malvern laser potentiometer, and it changed to −8.67 ± 1.52 mV after adding the virus. The adhesion efficiency of the microbubbles, measured by real-time (fluorescence) quantitative PCR, was 37.2 ± 2.2% (Figure 1).

3.2 Detection of GDNF expression in the brain

After 48 h of ultrasonic irradiation, the rats in each group were sacrificed. The brain tissue from the irradiated area was harvested, and total protein was extracted. The content of GDNF protein in the ultrasound-irradiated area was determined by western blot analysis. The results showed significant difference between the U+MpLXSN-EGFP group and U+M+PLXSN-GDNF group \((P < 0.05)\) (Figure 2).

3.3 Behavioral tests

After 8 weeks of treatment, the rats were subjected to an apomorphine-induced rotation test. The rotation test results demonstrated that after 8 weeks of treatment, the difference between before and after treatment in the \(\text{U + M + pLXSN-GDNF}\) group was statistically significant \((P < 0.05)\), and in other groups before and after treatment, it was not statistically significant \((P > 0.05)\). Moreover, there was significant rotational behavior (>7 rpm) (Figure 3).

3.4 Quantification of the number of TH-positive neurons

Targeted opening of the BBB promotes the release of pLXSN-GDNF into the lesion area, and the virus transfects the cells in this area. Immunohistochemistry analysis was used to determine the number of TH-positive cells in the substantia nigra of rats from each group, and the protective effect on dopaminergic neurons in the targeted transfection area was assessed. Compared with the control group, the number of TH-positive neurons in the \(\text{U + MpLXSN-EGFP}\) group and the \(\text{U + M + pLXSN-GDNF}\) group decreased significantly \((P < 0.01)\). Additionally, the number of dopaminergic TH-positive neurons was significantly increased in the \(\text{U + MpLXSN-GDNF}\) group compared with the \(\text{U + MpLXSN-EGFP}\) group and the \(\text{U + M + pLXSN-GDNF}\) group \((P < 0.05)\) (Figure 4).

Figure 2: Detection of GDNF protein expression in the substantia nigra of rats from each group by western blot analysis.
(a) Expression of GDNF protein in the substantia nigra of rats from each group; (b) relative optical density analysis; compared with the normal group and \(\text{U + MpLXSN-EGFP}\) group, \(^*P < 0.05\).

Figure 3: Rotation test after 8 weeks of treatment; the rats were subjected to an apomorphine-induced rotation test to the left by intraperitoneal injection of 0.5 mg/kg apomorphine. The \(\text{U + M + pLXSN-GDNF}\) group showed a significant difference between before and after treatment, \(^*P < 0.05\).
3.5 Effects of the treatment with GDNF on DA concentration in the substantia nigra of rats

The DA content in the substantia nigra of rats was determined by HPLC, and the data were analyzed by a one-way ANOVA test. The results showed that compared with the normal group, there were significant differences in the other groups (U + MpLXSN-EGFP and U + M + pLXSN-GDNF groups, \( P < 0.01 \); U + MpLXSN-GDNF group, \( P < 0.05 \)). Additionally, compared with the U + MpLXSN-EGFP and the U + M + pLXSN-GDNF groups, the DA concentration in the substantia nigra of rats from the U + MpLXSN-GDNF group was significantly increased (\( P < 0.05 \)) (Figure 5).

4 Discussion

The main pathological feature of PD is the progressive decrease in substantia nigra dopaminergic neurons in the midbrain, leading to reduced DA content in the striatum [18,19]. With recent advances in cell and molecular biology, scientists have started to pay attention to finding a way to delay and reverse the degeneration process of substantia nigra dopaminergic neurons. GDNF has been shown to protect neurons from the effect of neurotoxins, and specifically promote survival of dopaminergic neurons, thereby representing a potential therapeutic approach for patients with PD [20–22].

However, due to the relatively large molecular weight of GDNF, it is difficult for it to cross the BBB. Direct intracerebral injection of GDNF or direct injection of viral vectors carrying the GDNF gene into the brain will damage the brain tissue. Thus, getting GDNF non-invasively...
across the BBB, increasing its effective concentration in the central nervous system, is key for the successful treatment of PD with GDNF [23,24]. Combining ultrasound with microbubbles has been shown to open the BBB locally, reversibly, and noninvasively, thereby offering the possibility for the effective treatment of brain diseases.

In this study, real-time MRI-guided focused ultrasound with microbubbles carrying a retrovirus containing the GDNF gene was used to irradiate the substantia nigra of rats to open the BBB, allow the retrovirus to cross the BBB, transfect dopaminergic neurons in this area, and increase the expression of GDNF in these neurons. The expression of GDNF in the brain was analyzed by western blotting. The difference between the U + MpLXSN-EGFP group, the U + M + pLXSN-GDNF group, and the normal group was statistically significant ($P < 0.05$) and that between the U + MpLXSN-GDNF group and the normal group was not ($P > 0.05$). The effect of the increased GDNF content on 6-OHDA PD rats was evaluated by behavioral tests. After 8 weeks of treatment, the rats in each group were subjected to an apomorphine-induced left rotation test. Compared with the behavior before treatment, the rotational behavior of rats in the U + M + pLXSN-GDNF group was significantly improved ($P < 0.05$), while that in other groups was not ($P > 0.05$). In addition, immunohistochemistry analysis of the number of TH-positive neurons in the rat brain revealed that after the BBB was partially opened, retrovirus entered the brain to transfect neurons specifically in the substantia nigra, thereby increasing the expression of GDNF and affecting the number of central dopaminergic neurons in this brain region. The results showed significantly increased ($P < 0.05$) TH-positive neurons in the U + MpLXSN-EGFP group and the U + M + pLXSN-GDNF group. Moreover, U + MpLXSN-GDNF showed a significant therapeutic effect on PD model rats. Therefore, it is confirmed that ultrasound with GDNF virus microbubbles can increase the transfection efficiency of nerve cells in targeted regions of the brain and result in high expression of the exogenous gene GDNF, demonstrating its potential for application as a therapeutic approach to treat PD.

In addition, the mechanism of the GDNF effect on PD was also examined in this study. The pharmacological study of the long-term expression of the target gene in the brain after non-invasive and reversible opening of the BBB by low-frequency focused ultrasound with microbubbles and gene transfection will contribute to applying the advantages of GDNF in the treatment of brain diseases, and provide scientific basis for the use of GDNF in the treatment of PD.

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**References**

[1] Beal MF. Experimental models of Parkinson’s disease. Nat Rev Neurosci. 2001;2:325–34.

[2] Bohilhalter S. Update on parkinson disease. Rev Med Suisse. 2013;9:247–8.

[3] Bjorklund A, Lindvall O. Parkinson disease gene therapy moves toward the clinic. Nat Med. 2000;6:1207–8.

[4] Penttinen AM, Parkkinnen I, Voutilainen MH, Koskela M, Back S, Their A, et al. Pre-alpha-pro-GDNF and pre-beta-pro-GDNF isoforms are neuroprotective in the 6-hydroxydopamine rat model of Parkinson’s disease. Front Neurol. 2018;9:457.

[5] Sun S, Zhang Q, Li M, Gao P, Huang K, Beejadhursing R, et al. GDNF promotes survival and therapeutic efficacy of human adipose-derived mesenchymal stem cells in a mouse model of Parkinson’s disease. Cell Transplant. 2020;29:963689720908512.

[6] Kirik D, Cederfjäll E, Halliday G, Petersén Å. Gene therapy for Parkinson’s disease: disease modification by GDNF family of ligands. Neurobiol Dis. 2017;97:179–88.

[7] Biju K, Zhou Q, Li G, Imam SZ, Roberts JL, Morgan WW, et al. Macrophage-mediated GDNF delivery protects against dopaminergic neurodegeneration: a therapeutic strategy for Parkinson’s disease. Mol Ther. 2010;18:1536–44.

[8] Kobus T, Zervantonakis IK, Zhang YZ, McDaniel NJ. Growth inhibition in a brain metastasis model by antibody delivery using focused ultrasound-mediated blood–brain barrier disruption. J Controlled Release. 2016;238:281–8.

[9] Alii S, Figueiredo CA, Golbourn B, Sabha N, Wu MY, Bondoc A, et al. Brainstem blood brain barrier disruption using focused ultrasound: a demonstration of feasibility and enhanced doxorubicin delivery. J Controlled Release. 2018;281:29–41.

[10] Zhao GJ, Huang Q, Wang F, Zhang X, Hu JG, Tan Y, et al. Targeted shRNA-loaded liposome complex combined with focused ultrasound for blood brain barrier disruption and suppressing glioma growth. Cancer Lett. 2018;418:147–58.

[11] Tan JK, Pham B, Zong Y, Perez C, Maris DO, Hempill A, et al. Microbubbles and ultrasound increase intraventricular polyplex gene transfer to the brain. J Controlled Release. 2016;231:86–93.
Chang EL, Ting CY, Hsu PH, Lin YC, Liao EC, Huang CY, et al. Angiogenesis-targeting microbubbles combined with ultrasound-mediated gene therapy in brain tumors. J Controlled Release. 2017;255:164–75.

Danialou G, Comtois AS, Dudley RWR, Nalbantoglu J, Gilbert R, Karpati G, et al. Ultrasound increases plasmid-mediated gene transfer to dystrophic muscles without collateral damage. Mol Ther. 2002;6:687–93.

Wang F, Shi Y, Lu L, Liu L, Cai Y, Zheng H, et al. Targeted delivery of GDNF through the blood–brain barrier by MRI-guided focused ultrasound. PLoS One. 2012;7:e52925.

Jin Q, Wang Z, Yan F, Deng Z, Ni F, Wu J, et al. A novel cationic microbubble coated with stearic acid-modified polyethylenimine to enhance DNA loading and gene delivery by ultrasound. PLoS One. 2013;8(9):e76544.

Zhu J, Yang L, Zhang Q, Meng J, Lu ZL, Rong R. Autophagy induced by Simian Retrovirus infection controls viral replication and apoptosis of Jurkat T lymphocytes. Viruses. 2020;12(4):381.

Xu G, Xiong Z, Yong Y, Wang Z, Ke Z, Xia Z, et al. Catalpol attenuates Mptp induced neuronal degeneration of Nigral-Striatal dopaminergic pathway in mice through elevating Glial cell derived neurotrophic factor in striatum. Neuroscience. 2010;167:174–84.

Sonntag KC, Simantov R, Isacson O. Stem cells may reshape the prospect of Parkinson’s disease therapy. Mol Brain Res. 2005;134:34–51.

Whone AL, Boca M, Luz M, Woolley M, Mooney L, Dharia S, et al. Extended treatment with Glial cell line-derived neurotrophic factor in Parkinson’s disease. J Parkinsons Dis. 2019;9:301–13.

Salvatore MF, Zhang JL, Large DM, Wilson PE, Gash CR, Thomas TC, et al. Striatal GDNF administration increases tyrosine hydroxylase phosphorylation in the rat striatum and substantia nigra. J Neurochem. 2004;90:245–54.

Wu SS, Frucht SJ. Treatment of Parkinson’s disease: what’s on the horizon? CNS Drugs. 2005;19:723–43.

Whone A, Luz M, Boca M, Woolley M, Mooney L, Dharia S, et al. Randomized trial of intermittent intraputamenal glial cell line-derived neurotrophic factor in Parkinson’s disease. Brain. 2019;142:512–25.

Bohn MC, Kozlowski DA, Connor B. Glial cell line-derived neurotrophic factor (GDNF) as a defensive molecule for neurodegenerative disease: a tribute to the studies of Antonia Vernadakis on neuronal-glial interactions. Int J Dev Neurosci. 2000;18:679–84.