X-box-binding protein 1-modified neural stem cells for treatment of Parkinson’s disease

Lihui Si¹, Tianmin Xu¹, Fengzhang Wang², Qun Liu², Manhua Cui¹

1Department of Gynecology and Obstetrics, Second Hospital of Jilin University, Changchun 130041, Jilin Province, China
2Department of Neurology, Norman Bethune First Hospital of Jilin University, Changchun 130021, Jilin Province, China

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INTRODUCTION

Endoplasmic reticulum stress is one of the major pathogenetic mechanisms of Parkinson’s disease (PD)¹⁻³. X-box-binding protein 1 (XBP1) is an important transcription factor in the endoplasmic reticulum, acting to clear abnormally accumulated proteins and playing critical roles in cell survival and differentiation.⁴⁻⁶ XBP1 can delay the progression of PD through antagonizing endoplasmic reticulum stress⁷. XBP1-transfected neural stem cells (NSCs) with stabilized XBP1 overexpression grew more rapidly and had a higher survival rate after exposure to hypoxia compared to normal NSCs. Moreover, XBP1-NSCs showed stronger ventricular migration and differentiation ability, as well as promoting Bcl-2 expression in infarct areas, inhibiting Bax expression, and significantly ameliorating cerebral infarction in rats with ischemia/reperfusion injury. These results indicate that transplantation of XBP1-NSCs into the lesioned site can maintain stable and continuous XBP1 expression, antagonize endoplasmic reticulum stress in PD, and play a therapeutic role. In the present study, we therefore transplanted XBP1-NSCs into the brains of rats with rotenone-induced PD, to assess survival of transplanted cells and secretion of related proteins.

RESULTS

Quantitative analysis of experimental animals
A total of 45 rats were injected with rotenone microspheres to establish PD models. Seven rats died within 10 days, and 11 rats died as a result of reduced body mass or diarrhea up to 20 days following injection. Of the 34 surviving rats, 27 were identified as successful models, and were randomly assigned to model, NSC, or XBP1-NSCs groups (n = 9 for each), which were injected or transplanted with phosphate-buffered saline (PBS), normal NSCs or XBP1-NSCs, respectively, into the right lateral ventricle. Three rats in the model group died because of reduced food consumption, and the remaining rats were included in the final analysis.

XBP1-NSC transplantation improved PD rat behaviors
Rotation frequency was slightly decreased in NSC group rats, indicating improved rotational behavior (P < 0.05), and rotation frequency was significantly decreased in XBP1-NSCs group rats with increasing...
time, compared to NSC rats, with a significant difference between days 21 and 28 in the XBP1-NSCs group \( (P < 0.05); \) Table 1.

Table 1  Mean rotational speed (rotations/min) in apomorphine-induced rotation test

| Group     | Time after transplantation or injection (day) |
|-----------|---------------------------------------------|
|           | 0   | 7   | 14  | 21  | 28  |
| Model     | 11.1±1.2 | 12.1±2.2 | 10.9±2.1 | 11.8±2.1 | 12.1±1.0 |
| NSC       | 12.0±1.1 | 11.7±1.7 | 10.0±0.8 | 8.4±2.0   | 7.8±1.5   |
| XBP1-NSCs | 12.1±2.2 | 10.9±0.9 | 9.3±2.0  | 7.1±0.8   | 6.2±2.1   |

Lower rotational speed represents better neurological recovery. Data were expressed as mean ± SD. Intergroup differences were compared using analysis of variance and q-test. There were six rats in the model group, and nine rats in the NSC and XBP1-NSCs groups. *P < 0.05, vs. model group; **P < 0.05, vs. NSC group. NSC: Neural stem cell; XBP1: X-box-binding protein 1.

Survival and differentiation of transplanted NSCs

NSCs were labeled with 5-bromodeoxyuridine (BrdU) prior to transplantation. Cells that differentiated into dopaminergic neurons expressed tyrosine hydroxylase (TH). At 28 days following transplantation, immunofluorescence staining of BrdU and TH identified BrdU*, TH*, and BrdU*/TH* cells in the substantia nigra in the NSC and XBP1-NSCs groups. The numbers of BrdU* and TH* cells were greater in the XBP1-NSCs group compared to the NSC group \( (P < 0.05) \). Moreover, the ratio of BrdU*/TH* co-labeled cells to BrdU* cells was significantly greater in the XBP1-NSCs group compared to the NSC group \( (P < 0.01); \) Figure 1, Table 2.

XBP1-NSCs transplantation increased dopamine and 3,4-dihydroxyphenylacetic acid levels in the substantia nigra of PD rats

The dopamine content in the substantia nigra was significantly increased in the NSC group at 28 days following transplantation, compared to the model group, as measured by high performance liquid chromatography \( (P < 0.05) \), while the 3,4-dihydroxyphenylacetic acid content remained unchanged \( (P > 0.05) \). The dopamine and 3,4-dihydroxyphenylacetic acid levels in the substantia nigra were significantly higher in the XBP1-NSCs group compared to the NSC group \( (P < 0.05); \) Table 3.

XBP1-NSCs transplantation decreased α-synuclein expression in the substantia nigra of PD rats

α-synuclein levels were similar in the NSC and model groups at 28 days following transplantation, as shown by western blot analysis \( (P > 0.05) \). However, α-synuclein expression was significantly reduced in the XBP1-NSCs group compared to the NSC and model groups \( (P < 0.01); \) Figure 2.

![Figure 1  Distribution of neural stem cells in the substantia nigra at 28 days following transplantation (immunohistochemical staining, ×400). Tyrosine hydroxylase (TH) cells were stained green following staining with glial fibrillary acidic protein; 5-bromodeoxyuridine (BrdU) cells were stained red following staining with phycoerythrin; and BrdU*/TH* cells were stained green. XBP1: X-box-binding protein 1; NSCs: neural stem cells.](image)
pathogenic mechanisms, pathology, biochemistry and behaviors\cite{9,10}. In the present study, rotenone was delivered to rats using delayed-release microspheres, which can establish a model and maintain a constant blood-drug level in rats. The final success rate of model establishment was 53.3%, indicating that this method provides a good experimental model for studying PD. XBP1 can promote cell differentiation and has been regarded as a crucial transcription factor for the growth, maturation and differentiation of hepatocytes and plasmocytes\cite{11,12}. Moreover, XBP1 can promote NSC differentiation into nerve cells in a rat model of ischemia/reperfusion. The present study marked NSCs with BrdU prior to transplantation to assess the ability of XBP1 to promote NSC survival and differentiation into dopaminergic neurons in a PD environment\cite{13}. There were more BrdU<sup>+</sup> cells in the XBP1-NSCs group compared to the NSC group, indicating a stronger survival ability of XBP1-NSCs under conditions of PD-induced stress, compared to normal NSCs. TH is a specific marker of dopaminergic neurons\cite{9}. Co-labeling of BrdU and TH can thus identify dopaminergic neurons differentiated from transplanted NSCs. The ratio of co-labeled BrdU<sup>+</sup>/TH<sup>+</sup> cells was greater in the XBP1-NSC group compared to the NSC group, indicating that XBP1 promoted NSC differentiation into dopaminergic neurons. In addition, the number of TH<sup>+</sup> cells was also significantly greater in the XBP1-NSCs group compared to the NSC group, which could also reflect the ability of XBP1 to protect existing dopaminergic neurons in the substantia nigra against apoptosis\cite{13}.

Pathologically, α-synuclein can aggregate rapidly to form Lewy bodies, inducing cell apoptosis or death\cite{14,15}. Activation of the XBP1 gene can clear abnormally accumulated α-synuclein to play a neuroprotective role in PD\cite{16}. In the present study, α-synuclein levels in the substantia nigra were similar in the NSC and model groups, suggesting that normal NSCs have no effect on α-synuclein synthesis or degradation. However, transplantation of XBP1-NSCs significantly reduced α-synuclein levels, indicating that NSCs overexpressing XBP1 were able to clear α-synuclein. Moreover, dopamine and 3,4-dihydroxyphenylacetic acid levels in the substantia nigra were significantly greater, and neurological function was significantly improved in the XBP1-NSCs group compared to the model and NSC groups. Overall, the results of this study indicate that transplantation of XBP1-NSCs is more effective than transplantation of normal NSCs for the treatment of PD.

**MATERIALS AND METHODS**

**Design**
A randomized, controlled, animal experiment.

**Time and setting**
The experiment was performed in the Laboratory of Neurology, Medical Transformation Center, Norman...
Rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (3.5 mL/kg) at 28 days following transplantation, followed by cardiac perfusion. Brains were harvested and post-fixed with 4% paraformaldehyde phosphate buffer at 4°C for 24 hours. Serial 4-μm-thick coronal sections were prepared from the olfactory bulb, with one section every 100 μm throughout the substantia nigra.

**Immunohistochemical staining for NSC distribution and differentiation in the substantia nigra**

Paraffin sections of the substantia nigra were dewaxed, hydrated, and re-warmed at room temperature for 0.5 hours, washed three times with 0.01 M PBS (5 minutes each) at 37°C, followed by HCl (2 M) for 0.5 hours. The sections were washed three times with 0.01 M PBS (5 minutes each), mixed with 0.5% Triton X-100 for 30 minutes, followed by a further three washings with 0.01 M PBS (5 minutes each), and blocking with 5% nucleic-acid-stabilizing solution. They were then incubated with mouse anti-rat phycocerythrin-BrdU monoclonal antibody IgG (1:400; BD, San Diego, CA, USA) overnight at 4°C, dried in the dark, incubated with mouse anti-rat glial fibrillary acidic protein-TH monoclonal antibody IgG (1:400; BD) at 37°C for 2 hours. washed three times with PBS (5 minutes each), followed by goat anti-mouse IgM (1:100; BD) at room temperature for 2 hours. The sections were dried and observed under a fluorescence microscope (Olympus, Tokyo, Japan). The mean numbers of TH⁺, BrdU⁺ and BrdU⁺/TH⁺ cells in five 100 x magnification fields of view surrounding an area of substantia nigra ischemia were quantified, and the ratio of BrdU⁺/TH⁺ to BrdU⁺ cells was calculated.

**High performance liquid chromatography analysis of dopamine and 3,4-dihydroxyphenylacetic acid levels in the substantia nigra**

Dopamine and 3,4-dihydroxyphenylacetic acid levels in the substantia nigra were assessed by HPLC coupled to electrochemical detection, according to a previously published method[28]. Briefly, substantia nigra tissues were placed in 1.5-mL Eppendorf tubes (Eppendorf, Hamburg, Germany), weighed, mixed with cold HClO₄ (4 mM; Zhengcheng Chemical Product, Tianjin, China), placed in an ice bath, exposed to ultrasound for 10 seconds (1 Hz), and left for 1 hour. The homogenate was centrifuged at 12 000 r/min at 4°C for 10 minutes. The supernatant was transferred to a clean Eppendorf tube, mixed with potassium citrate (20 mM), K₂HPO₄ (30 mM) and ethylenediamine tetraacetic acid-2Na (2 mM; Zhengcheng Chemical Product) and stored at 4°C. The maximum column pressure for high performance liquid chromatography (J-H Instruments, Shanghai, China) was set to 17 327.5 Pa, with a flow rate of 1.0 mL/minute, and electrochemical detection voltage of 0.65 V. A standard curve was mapped and a linear regression equation was obtained. The supernatant from brain tissues was detected for 35 minutes.

**Western blot assay for substantia nigra α-synuclein expression**

Substantia nigra tissue (50 mg) was cut into pieces,
mixed with ethylenediamine tetraacetic acid-treated tissue lysate on ice for 30 minutes, homogenized for 30 seconds, and centrifuged at 12 000 r/min for 20 minutes. The supernatant was obtained and diluted with lysate. Total protein concentration was determined using the bicinchoninic acid assay[21]. Total protein (15 μg) from each sample was harvested, mixed with 4 μL 6 × loading buffer, placed in boiling water for 5 minutes, and subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 80 V for 40 minutes and 110 V for 90 minutes. It was then electrotransferred to a membrane at 200 mA for 60–90 minutes, blocked with bovine serum albumin (Boster, Wuhan, China) and defatting milk for 2 hours, and eluted six times (10 minutes each) with Tris-buffered saline Tween-20 (Boster). The product was incubated with mouse anti-rat α-synuclein (1:2 000), β-actin (1:2 000) monoclonal antibodies (R&D Systems, Inc., Minneapolis, MN, USA) overnight at 4°C and eluted six times (10 minutes each) with Tris-buffered saline Tween-20. The next day, the samples were incubated with horseradish peroxidase-labeled rabbit anti-mouse polyclonal antibody (1:200; R&D Systems) at room temperature for 2 hours, followed by enhanced chemiluminescence. Results were represented by the absorbance ratio of the target protein to β-actin.

**Statistical analysis**

Data were analyzed using SPSS version 17.0 (SPSS, Chicago, IL, USA). All data were expressed as mean ± SD, except the ratio of TH+/BrdU+ to BrdU+ cells, which was represented by relative number. Intergroup differences were compared using analysis of variance and q-tests. Differences were considered significant at an alpha level of 0.05.

**Author contributions:** All authors designed this study and conducted the experiments.

**Conflicts of interest:** None declared.

**Ethical approval:** This study received permission from the Animal Ethics Committee of Jilin University, China.

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