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Original Article

Lentivirus vector mediated SOD1 gene silencing targets the PI3K/AKT signaling pathway to protect dopaminergic neurons from oxidative stress in the substantia nigra of Parkinson's disease rats

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Abstract: Objective: To investigate the mechanism of lentivirus vector-mediated superoxide dismutase 1 (SOD1) gene silencing targeting phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway in oxidative stress injury (OSI) of dopaminergic neurons in the substantia nigra of Parkinson's disease (PD) rats. Methods: A total of 80 SD rats were divided into four groups: Normal group, Model group, SOD1 gene silencing group (SOD1-shRNA group) and Negative control group (NC group), with 20 rats in each group. No treatment was performed in the Normal group, and the other three groups were injected with 6-hydroxydopamine (6-OHDA) to induce the PD. Immunohistochemical staining was used to observe the positive expression of tyrosine hydroxylase (TH). The oxidative stress indicators (SOD, MDA and GSH) in the ventral midbrain tissues of rats in each group were determined by corresponding kits. Rat neuronal cell line (PC12) was purchased and divided into four groups: Normal group, Model group, SOD1-shRNA group and NC group. The cell viability was determined by MTT assay. Cell cycle and apoptosis were detected by flow cytometry. The expression of SOD1, cell cycle related gene (cyclin D1), cell apoptosis related genes (Bcl-2, Bax and caspase-3), and PI3K/AKT pathway related genes were detected by qRT-PCR and Western blot. Results: In the animal experiments, compared with the Normal group, the TH positive rate, and activities of SOD and GSH in the other three groups decreased, but those of MDA increased (all $P<0.05$); compared with the Model group and NC group, the SOD1 shRNA group had opposite results (all $P<0.05$). In the cell experiments, compared with the Normal group, the other three groups showed decreased cell viability and lower proportions of S and G2 phase cells, increased cell apoptosis rate and higher proportion of G1/G0 phase cells (all $P<0.05$); compared with the Model group and NC group, the SOD1 shRNA group had opposite results (all $P<0.05$). Compared with the Normal group, the mRNA and protein expression of SOD1 increased in the Model group and the NC group, but they decreased in the SOD1 shRNA group (all $P<0.05$). Compared with the Normal group, the expressions of cyclin D1, Bcl-2, PI3K and AKT decreased, while the expressions of Bax and caspase-3 increased in the other three groups (all $P<0.05$). The expressions of cyclin D1, Bcl-2, PI3K and AKT increased in the SOD1 shRNA group compared with the Model group, and the expressions of SOD1 mRNA and protein, Bax and caspase-3 decreased (all $P<0.05$). No significant difference was seen between the Model group and the NC group in terms of all the above indicators in each experiment (all $P>0.05$). Conclusion: The silencing of SOD1 gene, mediated by lentivirus vector can activate the PI3K/AKT signaling pathway and reduce the OSI of dopaminergic neurons in the substantia nigra of PD rats.

Keywords: SOD1, gene silencing, PI3K/AKT signaling pathway, Parkinson's disease, oxidative stress injury

Introduction

Parkinson's disease (PD) is a common clinical degenerative disease of the nervous system, and middle-aged and elderly people are often affected [1]. The main pathological manifestations of this disease are loss and degeneration of dopaminergic neurons in the substantia nigra pars compacta area [2, 3]. PD has a slow onset, with clinical manifestations including trembling, stiff limbs, thought retardation and the late onset of dementia, which is seriously threatening to human health [4].

Recent studies have found that superoxide dismutase 1 (SOD1) plays an important role in the

Table 1. shRNA sequence

	5'	Sense	Loop	Antisense	3'
shRNA1	GATCC	GCGGATGAAGAGAGGCATGTT	CTCGAG	AACATGCCTCTCTTCATCCGC	TTTTTTG
shRNA2	GATCC	GGATGAAGAGAGGCATGTTGG	CTCGAG	CCAACATGCCTCTCTTCATCC	TTTTTTG
shRNA3	GATCC	GGCCAATGTGTCCATTGAAGA	CTCGAG	TCTTCAATGGACACATTGGCC	TTTTTTG
NC shRNA	GATCC	GGCTCTGTAGTGATCACAAGA	CTCGAG	TCTTCATGATTCAGCCGGACA	TTTTTTG

pathological process of Alzheimer's disease (AD) and PD [5-7]. Pamela et al. found that initially compared with the healthy control group, the activity of SOD1 in PD and AD patients was significantly reduced, but it was 50% higher than that of the control group after 5 years [8]. A variety of studies have confirmed that patients with amyotrophic lateral sclerosis (ALS) have significant SOD1 mutations, and the silencing of SOD1 can effectively improve the neurological function of ALS model mice, this can be used as an important strategy for the treatment of ALS [9, 10]. PD and ALS are both neurodegenerative diseases, and both of them are closely related to environmental and genetic changes. This study aims to further investigate the relationship between SOD1 and PD in rats. In addition, many studies have found that the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway is closely related to PD [11, 12]. The PI3K/AKT signaling pathway is a classical anti-apoptosis and pro-survival signaling pathway. It plays a key role in physiological and pathological processes and also plays an important biological effect via regulating the activation process of related downstream proteins [13, 14]. In addition to PD research, the PI3K/AKT signaling pathway has been widely studied in other common neurodegenerative diseases such as AD, ALS, Huntington's disease and multiple sclerosis [15-18]. Activation of PI3K/AKT signal transduction pathway can play a role in anti-oxidative stress, and effectively prevents and treats oxidative DNA injury and apoptosis of nerve cells [19]. PC12 cells are mainly derived from rat pheochromocytoma cells, and are widely used in neuropathological and pharmacological studies because they secrete dopamine and have the characteristics of neurons [20, 21]. In this paper, the relationship between SOD1, PI3K/AKT signaling pathway and PD were investigated through the combination of tissue experiments and cell experiments.

Materials and methods

Construction of lentivirus vector and animal modeling

SOD1 shRNA sequence was designed and synthesized by Shanghai Genechem Co., Ltd. See **Table 1**. The lentivirus vector systems were composed of pGreenPuro (CMV) vectors.

A total of 80 male SD rats, 7-week old, weighing 220-240 g were purchased from Shanghai Lab Animal Research Center. All rats were raised in a quiet environment with 12 h of light and 12 h of darkness, and were given free access to food and water. The rats were randomly divided into 4 groups: Normal group, Model group, SOD1 gene silencing group (SOD1 shRNA group) and Negative control group (NC group), with 20 rats in each group. The SOD1 shRNA group and NC group were injected with SOD1 shRNA lentivirus and negative control virus (LV3-NC) in the substantia nigra of rats, respectively. The Model group was injected with the same amount of PBS solution. No treatment was performed in Normal group. One week after the injection, the other three groups were injected with 6-hydroxydopamine (6-OHDA; Sigma Company, USA) to induce the PD model. The details are as follows. Intraperitoneal injection of anesthesia was performed by ketamine (80 mg/kg) in the rats, and alcohol was used to disinfect the cranial parietal skin of rats. Rats were injected with virus, PBS and 6-OHDA with the help of a Stereotaxic Instrument (Model 51600; Stoelting Co., Ltd., USA). The location coordinates were A/P + 1.0, L/M + 2.7, and D/v-4.0 [22]. Titers of SOD1 shRNA lentivirus and negative control virus were $2*10^6$ TU/mL, dose of 5 μL. The Model group was injected with the same amount of PBS solution at a speed of 1 μL/min. PD model was induced by the injection of 5 μL mixture of saline containing 0.2% ascorbic acid and 8 μg 6-OHDA at a rate of 0.5 μL/min. After the operation, the rat's scalp was sutured, and the rats were given

appropriate thermal insulation. Thirty days after 6-OHDA injection (Sigma, USA), the rats were intraperitoneally injected with a dose of 0.5 mg/kg apomorphine to induce rotation. After that, the average number of rotations of 360 degrees per minute within 15 min was observed at a fixed time every week for 4 weeks. The success of PD model was defined as the rat consistently rotates to the left and the average rotation speed exceeds 7 rotations/min [23]. The success rates of modeling were 65% (13/20), 60% (12/20), and 65% (13/20) in the Model group, NC group and SOD1 shRNA group, respectively.

All animal experiments in this study were conducted in strict compliance with The Guidelines for the Management and Use of Experimental Animals issued by the American National Institutes of Health. This study was approved by the Laboratory Animal Ethics Committee of Beilun People's Hospital in Ningbo.

TH immunohistochemical staining

A total of 24 rats were selected form the 3 successful modeling groups and the Normal group, 6 rats from each group. After 4 weeks, the rats were subjected to intraperitoneal anesthesia with pentobarbital and perfusion-fixation with 4% paraformaldehyde. The midbrain tissue from each animal was taken and sectioned (thickness of 20 μm) by freezing microtome (CM1520; Leica Biosystems, China). The sections were washed with PBS containing 0.1% Triton X-100 for 5 min, and incubated in 3% H_2O_2 for 15 min at room temperature. Following the 3 PBS washes (3 min each), the sections were blocked with goat serum for 1 h at room temperature. Then, the sections were incubated overnight with rabbit anti-TH antibody (1: 500, Abcam, UK) at 4°C. After being washed with PBS for 3 additional times (3 min each), the sections were incubated with biotin-labeled goat anti-rabbit IgG secondary antibody (1: 1,000, Abcam, UK) and horseradish peroxidase-labeled streptomycin ovalbumin (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., China) for 30 min. After that, the sections were stained with DAB solution, secondly stained with hematoxylin staining solution, dehydrated with alcohol gradients, cleared with xylene, and mounted with permount mounting medium. Five sections of the midbrain substantia nigra near the injection site were taken from each rat, and the TH positive condition of the dark brown substantia nigra was observed under an optical microscope (400×; CX21, Olympus, Japan). Finally, the positive staining rate was calculated.

Oxidative stress measures

Four weeks after the establishment of the model, 24 rats were selected form the 3 successful modeling groups and the Normal group, 6 rats in each group. The rats were decapitated, and the ventral midbrain tissues were separated on the ice. After moisture removal, the brain tissues were homogenized in a homogenizer and were centrifuged at 2,000 rpm for 8 min at low temperature. Then the tissue samples were divided into 3 parts and stored at -80°C. The content of SOD in the brain tissues was determined by colorimetry through SOD assay kit (19160-1KT-F; Sigma, USA). Thibabituric Acid method was used to detect MDA content in the brain tissues through rat lipid peroxide malondialdehyde (MDA) detection kit (A003-1, Nanjing Jiancheng Bioengineering Institute, China). The content of GSH in the brain tissues was determined by visible spectrophotometry via reduced glutathione (GSH) assay kit (BC-1175, Beijing Solarbia Science & Technology Co., Ltd., China). The experiment was repeated 3 times and the average value was obtained.

Cell culture

PC12 cell line was procured from Shanghai Institute of Biochemistry and Cell Biology, and cultured in DEME with 10% fetal bovine serum, 5% horse serum and 1% double-antibody (100 μg/mL streptomycin and 100 U/mL penicillin). The cells were cultured as recommended in a constant temperature incubator with 5% CO_2 at 37°C. Culture media was changed for 2-3 days, and passaging was conducted when the cells reached 80% confluency. Cells in the logarithmic growth phase were selected for the experiments.

Cell transfection and grouping

pGreenPuro vector and lentivirus packaging system were purchased from Invitrogen Corporation (USA). Based on the known mRNA sequence of SOD1 in GenBank, a shRNA (shRNA1-3) sequence and a random negative control sequence (Sigma, USA) were designed and synthesized by the online software RNAi to interfere with the SOD1 gene. See **Table 1**.

Table 2. qRT-PCR primer sequence

	Sequence (5'-3')
SOD1	F: AAAGCTTATGAGTGCCACCAGGAAGAGG
	R: TTTGGATCCGGGTTTCCTCTCCACTGGTT
PI3K	F: GTTCCAGAACAGGCAAGTC
	R: TCGCAGCACCTCAATAAGT
AKT	F: CCACACGTCGCTATTATGCC
	R: ACAGCCTGATGTCCGTTATC
Bcl-2	F: CGTCTTTGCGGAGATGTCCA
	R: ATGCCGCTTCAGGTACTCAG
BAX	F: AGACTGTGGCCTTTTTGTTAC
	R: GAGGACTCCAGCCATAAAGAT
Caspase 3	F: GGACCAGTGGACCGGAAAAA
	R: CCATGTTATATCATCGTCAG
cyclin D1	F: GCGCACACTGACACCAATCT
	R: GCTCCTCAGACAAGATACG
β-actin	F: GCTAACCCTAAGGCCAAC
	R: GTCTGTCATGCACGATTTAC

Note: SOD1, superoxide dismutase 1; PI3K, phosphatidylinositol 3-kinase; AKT, protein kinase B.

After annealing and formation of double chains, the sequences were connected with the pGreenPuro (CMV) vectors, and then were transferred to E.coli DH5α competent cells. BamHI/EcoRI was used for the identification of double-enzyme digestion and then the transformant (pGreenPuro-iSOD1) was obtained. The Phoenix-293 cells were transfected when they reached about 80% confluency. Mixtures of PEI reagent + lentivirus packaging system pGreenPuro (CMV) with empty vector and pGreenPuro-iSOD1 were created, and they were used for transfecting Phoenix-293 cells. The two virus-containing supernatants were collected after 24 h continuous transfection. Culture medium was removed when PC12 cells reached about 60%-70% confluency. The SOD1 shRNA group and NC group were added to the supernatants of pGreenPuro-iSOD1 + transfected Phoneix-293 cells and pGreenPuro-empty vector + transfected Phoneix-293 cells respectively, and the medium was changed to normal medium 12 h later. After 48 h of transfection, the shRNAs with the best inhibitory effect on SOD1 detected by qRT-PCR were selected for subsequent experiments. They were divided into four groups as Normal group (normal cell culture), Model group (PD model was constructed by adding 100 μL 6-OHDA with a final concentration of 50 g/L), shRNA1-3 group (add 100 μL 6-OHDA and transfect with SOD1 shRNA) and NC group (add 100 μL 6-OHDA and transfect with NC shRNA).

Cell viability detection using the MTT method

PC12 cells were seeded into 96-well culture plates, $1*10^4$ cells per well. Each group was established in 5 duplicated wells. The cells were cultured in serum-free medium for 24 h and the fluid was changed every 12 h. In each well we added 20 μL MTT solution (5 g/L; Sigma, USA) 6, 12, and 24 h after the transfection, and it was cultured at 37°C for 3 h. After suctioning of the medium, 100 μL Formazan dissolving solution was added in each well, and the cells were incubated for 4 h to fully dissolve the crystal. Blank group was set as the control group. The optical density (OD) at 490 nm was determined using microplate reader (DG5031; Shanghai Kehuai Instrument Co., Ltd., China). The method for calculating the cell viability is as follows: cell viability = (OD in the blank group - OD in the experimental group)/OD in the blank group * 100%. The experiment was repeated 3 times and the average value was obtained.

Flow cytometry

Aforesaid PC12 cells in the logarithmic growth phase were seeded in 6-well culture plates, 2 mL of each well, $1*10^6$ cells per mL. After continuous culture for 24 h, each group was transfected differently when the cells were reached about 80% confluency. The medium was changed 6 h after transfection. Then the cells were digested and collected with trypsin (without EDTA), and centrifuged at 2,000 rpm for 5 min. Following 2 PBS washes (3 min each), the cells were collected and then fixed overnight with 3 mL of 70% ethanol at 4°C. Twelve hours later, the cells were washed by PBS twice, and 100 μL of cell suspension (≥10^6 cells/mL) was taken and added with 1 mL of PI dye containing RNAase (50 mg/L; P4170; Sigma, USA). After shading for 30 min at 4°C, the cell suspension was filtered with the nylon net (300 meshes). The cell cycle was detected by flow cytometry (6HT; Cellwar Bio-technology Co., Ltd., China). The excitation wavelength was set to Ex = 488 nm.

Annexin V-FITC/PI apoptosis detection kit (APOAF-20TST; Sigma, USA) was used to detected cell apoptosis. The method of cell treatment was the same as above. According to the introductions of the kit, Annexin-V-FITC, PI and

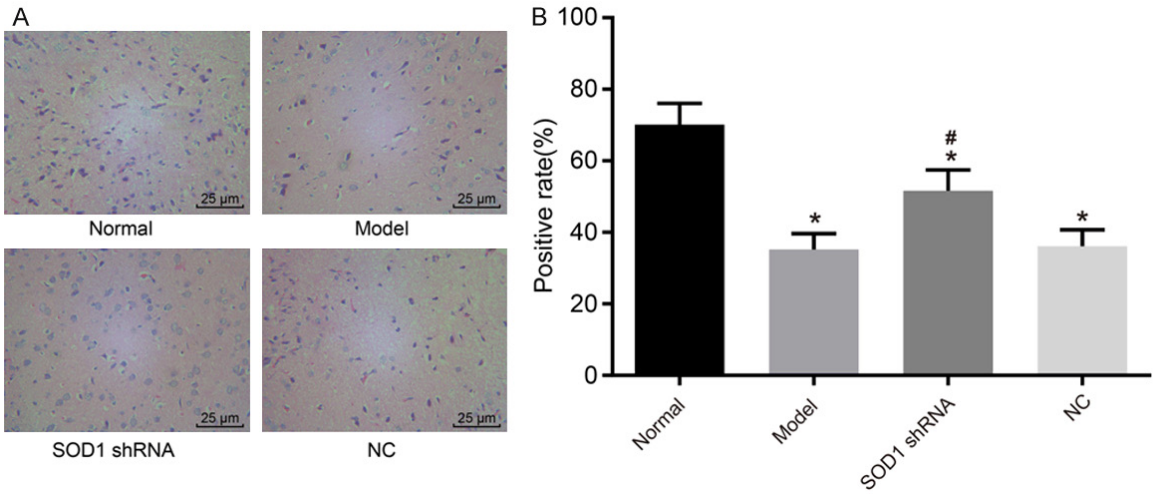

Figure 1. TH immunohistochemistry. A. TH immunohistochemical staining images from rats in each group; B. TH positive rate of rats in each group. Compared with the Normal group, *P<0.05; compared with the Model group, #P<0.05. NC group, negative control group.

HEPES buffer solutions were incorporated into Annexin-V-FITC/PI dye solution at a ratio of 1:2:50. A total of $1*10^6$ cells were resuspended in each 100 μL dye solution, then were incubated at room temperature for 15 min. After that, 1 mL of HEPES buffer solution was added and then oscillated until well combined. FITC and PI-fluorescence were detected with band-pass filters at 528 and 620 nm respectively stimulated by a wavelength of 488 nm, to detect cell apoptosis. Annexin-V-FITC (+) and PI (-) represent cell apoptosis. Apoptotic index = The number of apoptotic cell/total number of cells * 100%. The experiment was repeated 3 times and the average value was obtained.

qRT-PCR

Total RNA of PC12 cells in each group was extracted using Trizol (15596-026; Invitrogen, USA), and its concentration and purity were determined by ultraviolet spectrophotometer (10300101; Thermo Fisher Scientific, USA). RNA was reversely transcribed into cDNA using Primescript™ RT reagent Kit (Takara Biotechnology Dalian Co., Ltd., China) and the cDNA was stored at -20°C. The reverse transcription conditions were: 70°C for 5 min, ice-bath for 3 min, 37°C for 15 min and 95°C for 10 min. The primers in this study were designed and synthesized by Shanghai Genechem Co., Ltd. (China). See **Table 2**. qRT-PCR was performed in ABI7500 quantitative PCR instrument (ABI, USA). The reaction mixture consisted of 10 μL SYBR Premix Ex TaqTM II, 0.8 μL each of PCR forward and reverse primers (10 μM), 0.4 μL ROX Reference Dye, 2.0 μL cDNA template and 6.0 μL sterile purified water. The reaction conditions were: pre-denaturation at 94°C for 5 min, denaturation at 94°C for 1 min, annealing at 58°C for 1 min, elongation at 72°C for 1 min and overall elongation at 72°C for 1 min, for 30 cycles. The relative expression of each gene was calculated by the $2^{-\Delta\Delta Ct}$ method with β-actin gene as the internal reference. ΔΔCt = (Ct value (target gene) - Ct value (reference gene) in experimental group) - (Ct value (target gene) - Ct value (reference gene) in control group). Three experiments were conducted and averaged.

Western blot

After 48 h of cell transfection, the cells were added to protein lysis buffer (Sigma, USA). The cell lysates were centrifuged at 12,000 rpm for 20 min at 4°C, and the supernatant was aspirated. The protein concentration was determined using the BCA protein assay kit (P0012; Beyotime Biotechnology, China) and deionized water was used to adjust the sample size. The protein samples were fractionated by 10% SDS-PAGE and transferred on nitrocellulose membrane by wet phase inversion method. The membrane was blocked with skim milk for 1 h at room temperature, and then incubated with primary antibodies overnight at 4°C. The primary antibodies were SOD1 (1:1,000, Abcam, UK), PI3K (1:2,000, Abcam, UK), p-AKT (1:500, Abcam, UK), Bcl-2 (1:500, Abcam, UK), Bax

Table 3. Activity levels of SOD, MDA and GSH

Group	SOD (NU/mg)	MDA (nmoL/mg)	GSH (mg/g)
Normal group (n = 6)	215.28 ± 13.46	4.89 ± 0.71	168.94 ± 11.83
Model group (n = 6)	165.67 ± 13.75*	7.94 ± 1.08*	86.45 ± 7.58*
NC group (n = 6)	163.32 ± 12.51*	8.15 ± 1.24*	87.03 ± 7.75*
SOD1 shRNA group (n = 6)	183.29 ± 10.21*,#	6.68 ± 1.15*,#	117.53 ± 5.64*,#

Note: Compared with the Normal group, *P<0.05; compared with the Model group, #P<0.05. SOD, superoxide dismutase; MDA, malondialdehyde; GSH, glutathione; NC group, negative control group.

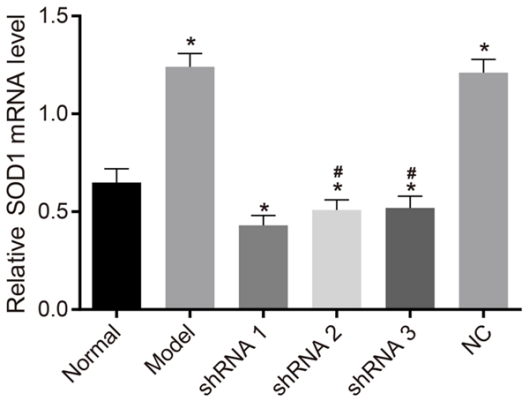

Figure 2. The expression of SOD1 mRNA after transfection. Compared with the Normal group, *P<0.05; compared with the shRNA1 group, #P<0.05. NC group, negative control group.

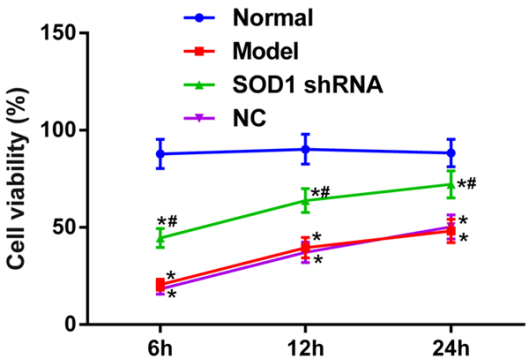

Figure 3. Cell viability. Compared with the Normal group, *P<0.05; compared with the Model group, #P<0.05. NC group, negative control group.

(1:5,000-1:10,000, Abcam, UK), caspase-3 (1:500, Abcam, UK), and cyclin D1 (1:20,000, Abcam, UK). After washing 3 times with PBST, the membrane was incubated with HRP-labeled goat anti-rabbit secondary antibody IgG (1:5,000, Abcam, UK) for 1 h at room temperature. Rinsing with TBST, color development with ECL, X-ray exposure and photographing were performed. β-actin (1:3,000, rabbit polyclonal antibodies, Abcam, UK) was set as the internal reference and Image J software was used to analyze the gray-scale values.

Statistical analysis

SPSS 17.0 statistical software (SPSS Inc., USA) was used to analyze the data. Measurement data were expressed as the mean ± standard deviation. Comparison of data between multiple groups was made using one-way analysis of variance (ANOVA) and post-hoc pairwise comparison was performed using Bonferroni method. P<0.05 is considered statistically significant.

Results

TH immunohistochemistry

The results of TH immunohistochemical staining in each group are shown in **Figure 1**. Compared with the Normal group, TH positive rates of the other three groups significantly decreased (all P<0.05). Compared to that in the Model group, TH positive rate in the SOD1 shRNA group was significantly increased (P< 0.05). There was no significant difference in TH positive rate between the Model and NC groups (P>0.05).

Detection of oxidative stress injury

SOD, MDA and GSH are important indicators of oxidative stress. The indicators of oxidative stress in the ventral midbrain tissues of rats in each group were detected. Compared with the Normal group, the contents of SOD and GSH in the other 3 groups declined, while those of MDA increased (all P<0.05). No significant difference was seen in the contents of the 3 indicators between the Model and NC groups (all P>0.05). Compared with the Model group, the contents of SOD and GSH in the SOD1 shRNA group increased, while that of MDA was decreased (all P<0.05), indicating that the silencing of SOD1 gene plays a protective role in oxidative stress injury of PD rats. See **Table 3**.

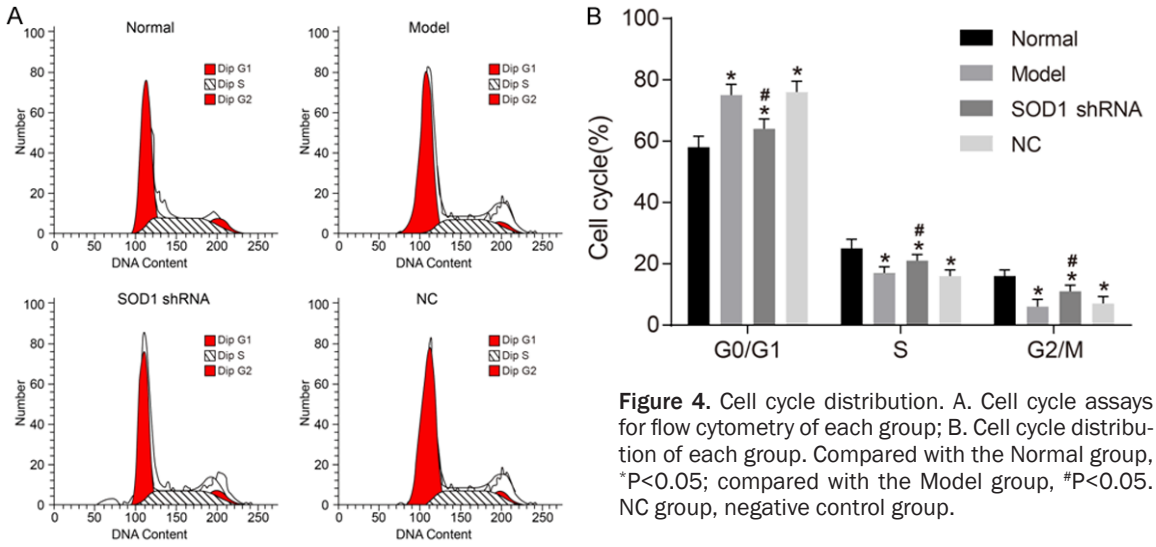

Figure 4. Cell cycle distribution. A. Cell cycle assays for flow cytometry of each group; B. Cell cycle distribution of each group. Compared with the Normal group, *P<0.05; compared with the Model group, #P<0.05. NC group, negative control group.

Effect of siRNA targeted silencing of SOD1 gene

After 48 h of P12 cell transfection, the expression of SOD1 mRNA was detected using qRT-PCR (**Figure 2**). Compared with the Normal group, the expressions of SOD1 shRNA in the Model and NC groups was significantly enhanced (both P<0.05), and those in the SOD1 shRNA group (shRNA1, shRNA2 and shRNA3 groups) significantly reduced (all P<0.05). There was no significant difference in the expression of SOD1 shRNA between the Model and NC groups (both P>0.05). After comparing the inhibition efficiency of shRNA in the three SOD1 shRNA groups, it was found that the inhibition rate of shRNA1 group was the highest. Therefore, the shRNA1 group was selected as the final experimental group (named SOD1 shRNA group) for the follow-up study.

The silencing of SOD1 gene on promotion the viability of PC12 cells

Compared with the Normal group, the viability of cells of the other 3 groups in each time point significantly decreased (all P<0.05). Similar results were obtained in terms of the viabilities of cells in each time point between the Model and NC groups (all P>0.05). Compared with the Model group, the viability of cells of the SOD1 shRNA group in each time point significantly increased (all P<0.05), showing that the silencing of SOD1 gene can promote the viability in PC12 cells. See **Figure 3**.

Silencing of SOD1 affects cell cycle distribution

Flow cytometry was used to detect the cell cycle distribution of rats in each group and the results are shown in **Figure 4**. Compared with the Normal group, the proportion of G0-G1 phase cells increased and cells in S and G2 phase decreased in the other three groups (all P>0.05), indicating that PC12 cells were blocked in G0-G1 phase. There was no obvious difference in the cell cycle distribution between the Model and NC groups (all P>0.05). Compared with the Model group, the proportion of G0-G1 phase cells declined and those of S and G2 phase increased in the SOD1 shRNA group (all P<0.05).

Silencing of SOD1 affects cell apoptosis

Flow cytometry was used to detect cell apoptosis in each group (**Figure 5**). Compared with the Normal group, the amount of cell apoptosis increased significantly in the other 3 groups (all P<0.05). There were no obvious differences in the amount of cell apoptosis between the Model and NC groups (P>0.05). Compared with the Model group, the amount of cell apoptosis decreased significantly in the SOD1 shRNA group (P<0.05).

Gene and protein expression

The expression of mRNA and protein was analyzed by qRT-PCR and Western blot (**Figure 6**). Compared with the Normal group, the mRNA

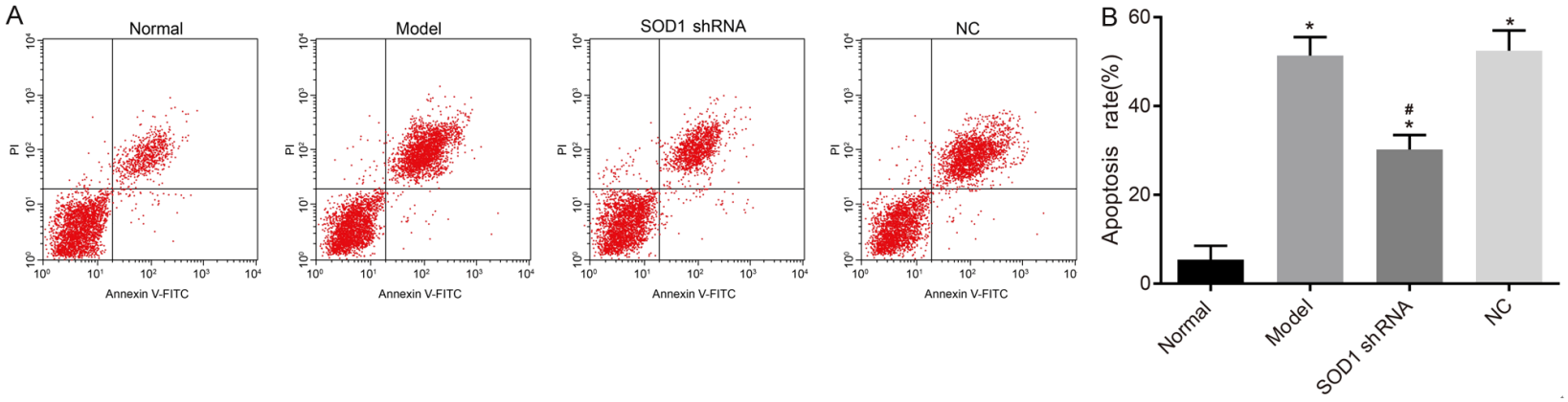

Figure 5. Cell apoptosis. A. PI double staining of each group. B. Cell apoptosis rate of each group. Compared with the Normal group, *P<0.05; compared with the Model group, #P<0.05. NC group, negative control group.

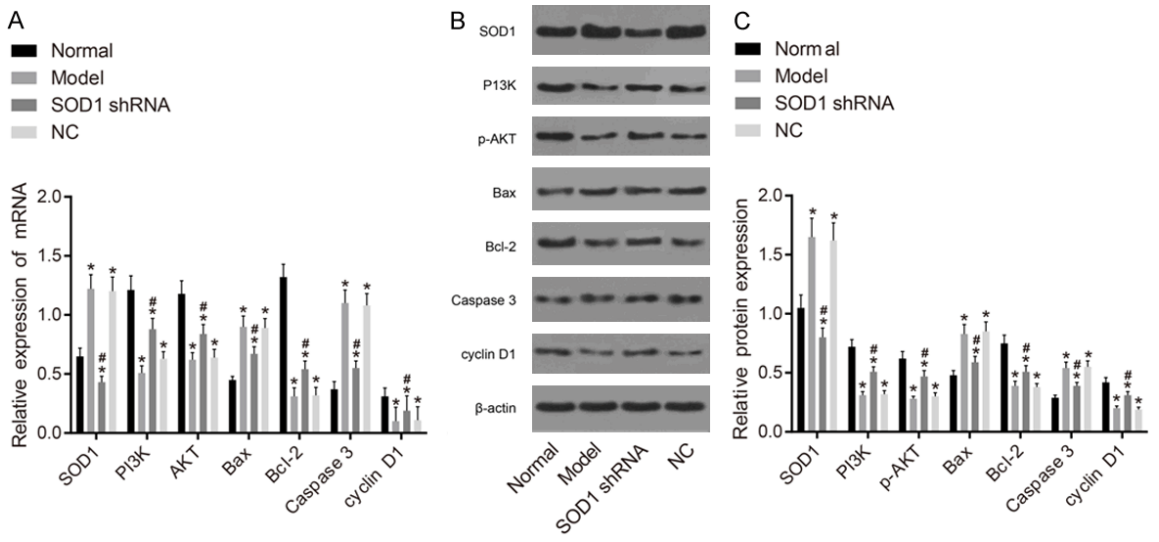

Figure 6. Results of qRT-PCR and Western blot. A. The expressions of mRNA; B. The expressions of protein; C. The histograms of Western blot. Compared with the Normal group, *P<0.05; compared with the Model group, #P<0.05. NC group, negative control group. SOD1, superoxide dismutase 1; PI3K, phosphatidylinositol 3-kinase; AKT, protein kinase B.

and protein expression of SOD1, Bax, and Caspase 3 in the Model and NC groups were increased, while those of cyclin D1, Bcl-2, PI3K and AKT were decreased (all P<0.05). There was no statistical difference in the mRNA expressions between the Model and NC groups (all P>0.05). Compared with the Model group, the mRNA expressions of SOD1, Bax, and Caspase 3 in the SOD1 shRNA group decreased, while those of cyclin D1, Bcl-2, PI3K and AKT increased (all P<0.05).

Discussion

PD is a degenerative disease of the nervous system and many scholars have discussed its pathogenesis and treatment methods [24]. It is mainly caused by the environment, age, and the degeneration and death of dopaminergic neurons in the mesencephalic substantia nigra. Also, oxidative stress plays an important role in dopaminergic neuronal injury [25]. Despite the continuous progress of medical technology, the overall effect of the treatment for PD is poor and it is difficult to cure PD. Therefore, it is urgent to find new treatment methods for PD.

RNA interference (RNAi) refers to the specific degradation of highly conserved evolutionary progress and appearance of homology mRNA, triggered by double stranded RNA. RNAi technology can specifically knock down or eliminate specific genes. Currently, this technology has been widely used in the field of gene therapy for tumors and infectious diseases [26]. SOD1 gene mutation is believed to be a major cause of ALS. Van et al. confirmed that SOD1 gene silencing with RNAi technology is of great significance for the treatment of ALS [10]. Further studies have shown that the SOD1 gene can activate the PI3K/AKT signaling pathway in spinal cord ischemia/reperfusion [27]. This study found that compared with the Model group, the expressions of SOD1, Bax, and Caspase 3 in the SOD1 shRNA group were decreased, while those of cyclin D1, Bcl-2, PI3K and AKT were increased, indicating that SOD1 silencing can activate the PI3K/AKT signaling pathway.

A variety of studies have confirmed the role of PI3K/AKT signaling pathway in neural protection. The PI3K/AKT signaling pathway can promote neuroprotective effects of cerebral ischemia reperfusion injury in rats and protective effects of dopaminergic neurons. Besides, it is related to inflammatory mechanism of PD [28, 29]. It has been found that with the activation of PI3K/AKT signal transduction pathway, the scavenging ability of oxygen free radicals in the body is significantly enhanced, and SOD activity shows an upward trend [30].

The activity of SOD reflects the scavenging ability of oxygen free radicals in the body, with a positive relationship. The higher the activity of SOD is, the better the scavenging ability of oxygen free radicals will be, effectively protecting neurons against oxidative injury [31]. GSH

activity also reflects the body's ability to scavenge oxygen free radicals, while MAD is an important parameter reflecting the body's potential ability to resist lipid peroxidation, indirectly reflecting the degree of tissue cell peroxidation injury [32]. This study found that compared with the Model and NC group, the activities of SOD and MAD in the SOD1 shRNA group were decreased, while the TH positive rate and the activity of GSG were increased. It indicates that SOD1 gene silencing has protective effects on oxidative stress injury of neurons in PD rats. The PI3K/AKT signaling pathway also plays a key role in cell differentiation and apoptosis [33]. This study found that compared with the Model and NC groups, cell activity increased and cell apoptosis decreased in the SOD1 shRNA group.

Although lentivirus vector-mediated gene silencing still has some defects and instability in the treatment of diseases, this method is expected to become an important method for PD treatment, and SOD1 is expected to become a potential target for PD treatment

In conclusion, the expression of silencing of the lentivirus vector SOD1 gene can activate the PI3K/AKT signaling pathway, thus playing a significant protective effect on oxidative stress injury and dopamine neurons in PD rats.

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Disclosure of conflict of interest

None.

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