

Original Article

Protective effect of gastrodin on dopaminergic neurons by miR-24 targeting of the DJ-1/Nrf2 pathway in Parkinson's disease

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Abstract: *Objective:* DJ-1/Nrf2 plays a certain role in anti-oxidation and protection of brain cells. Gastrodin (Gas) can produce anti-oxidation effects, protect neurons, and improve the pharmacological effects of brain function. Our study aimed to determine the effect as well as the mechanism of gastrodin on Parkinson's disease (PD) via a rat PD model. *Methods:* MPTP (1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine) was used to establish an *in vivo* PD model, which was further separated into the PD group and PD+Gas group. ELISA was used to detect caspase-3 activity, MDA and SOD content in the substantia nigra. The rod exercise test and suspension test were used to evaluate the motor function of the rats. Expression of DJ-1, Nrf2, and miR-24 in brain tissue was detected by western blot and qPCR, respectively. The dopaminergic SH-SY5Y cells were separated into a control group, MPTP group and MPTP+Gas group. The expression of DJ-1, Nrf2, TH and miR-24 in cells and cell apoptosis was detected. *Results:* The content of DJ-1, Nrf2, MDA, caspase-3 activity, in the brain of the PD group were significantly increased, while the SOD activity, the motor function score, and miR-24 were significantly decreased compared to those in control group ($P < 0.05$). Compared to the PD group, the caspase-3 activity and MDA content in the PD+Gas group were significantly decreased, while SOD activity and motor function score were markedly elevated ($P < 0.05$). Of note, bioinformatics analysis showed that miR-24 could target DJ-1 mRNA 3'-UTR. Gas treatment further significantly down-regulated the expression of miR-24 in rat brain and up-regulated the expression of DJ-1, Nrf2 and TH ($P < 0.05$). MPTP treatment significantly induced apoptosis of SH-SY5Y cells, upregulated DJ-1 and Nrf2, and downregulated miR-24 and TH ($P < 0.05$). Gas treatment can down-regulate the level of miR-24 in SH-SY5Y cells and elevate DJ-1, Nrf2 and TH expression, which retarded the regulatory effect of MPTP on SH-SY5Y cells. *Conclusion:* Gas can enhance the anti-oxidation capacity, reduce the damage and apoptosis of dopaminergic neurons through miR-24/DJ-1/Nrf2 pathway.

Keywords: PD, miR-24, DJ-1, gastrodin, apoptosis

Introduction

Parkinson's disease (PD) is a neurodegenerative disease, especially prevalent in the elderly. Oxidative stress (OS) and apoptosis participate in the occurrence and development of PD [1-3]. Nuclear factor erythroid 2-related factor 2 (Nrf2) is an important nuclear transcription factor regulating several cellular activities [4-6]. DJ-1 is involved in antioxidant function and modulation of Nrf2 stability, which promotes Nrf2 protein expression and activation. Multiple studies have found that the antioxidant function of DJ-1 depends to some extent on the

regulation of Nrf2 [5, 6]. Previously, bioinformatics analysis showed a regulation relationship of miR-24 with DJ-1 mRNA 3'-UTR.

Gastrodin (Gas) is an active ingredient of traditional Chinese medicine, which is characterized by anti-oxidation function. It has been found to protect neurons and improve the pharmacological effects of brain function [7-9]. However, it remains unclear whether Gas is likely to regulate miR-24 expression, affect DJ-1/Nrf2 pathway activity, and reduce PD brain damage. Our study sought to determine the effect of Gas on Parkinson's disease as well as the mechanism.

Materials and methods

Reagents

Healthy Sprague-Dawley rats (6-8 weeks, body weight 230-260 g) were bought from Beijing Vital River Laboratory Animal Technology Co., Ltd.. H-cells of SH-SY5Y were from Shanghai Cell Bank. DMEM and FBS were purchased from Gibco, USA. Gastrodin was purchased from Medchemexpress (United States). MPTP (1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine) and DCFH-DA were from Sigma. DJ-1 and Nrf2 antibodies were from Abcam (United States). TH and β -actin antibodies purchased from American Santa Cruz. HRP-conjugated secondary IgG was from Shanghai Sangon Bio. PrimeScript™ RT reagent Kit was purchased from Dalian Takara. Annexin V-FITC/PI was kit purchased from Beijing Solarbio. Luciferase activity assay kit was from Dual-Glo Luciferase Assay System while pGL3 vector was purchased from Promega, USA. miR-24 mimic, miR-24 inhibitor, miR-NC were purchased from Guangzhou Ribobio Bio. Trizol, Lipo 2000 transfection reagents were purchased from Invitrogen, USA.

PD model establishment

SD rats were fed ad libitum for 1 week and divided into 2 groups: PD model group (n=20), which received intraperitoneal injection of 20 mg/Kg of MPTP for continuous 7 days; control group (n=20), with equal volume of saline was injected, continuous injection for 7 days. On the 7th day after the last administration of MPTP, the rats were subjected to the climbing and hanging tests to evaluate the behavioral and neurological function, and the substantia nigra tissues of the rats were collected for further analysis.

The rat brain substantia nigra tissue was ground and passed through a 70 μ m cell strainer for analysis of cell apoptosis using Annexin V-FITC and PI by Gallios flow cytometry.

Gas therapy in model rats

The PD model rats were randomly equally divided into PD group in which saline was administered to the rats 3 days before the model was established once a day for 10 consecutive days until the PD model was completed; and in the PD+20 mg/Kg Gas group, three days before

the model was established, the rats were treated with 20 mg/Kg of Gas via gavage once a day for 10 consecutive days until the PD model was completed. The two groups of rats were subjected to a climbing test and a suspension test to evaluate the behavioral and neurological function of the rats. The rat substantia nigra tissues were collected and the activity and level of mRNA, protein, MDA, SOD and caspase-3 were detected.

Detection of caspase-3 enzyme activity in brain tissue

According to the kit instructions, different concentrations of pNA standards were prepared, and the absorbance at 405 nm was measured. A standard curve was prepared based on the A405 and pNA concentrations. Then, 100 μ L of Caspase Lysis buffer was added to the tissue for centrifugation at 10000 g for 20 min at 4°C. Next, 65 μ L Assay buffer, 25 μ L lysate supernatant, 10 μ L Ac-DEVD-pNA were added to a 96-well plate for 120 min incubation at 37°C. The calculation of relative enzyme activity: $A_{405} \times 100\%$ of the experimental group $A_{405}/100\%$.

Double luciferase activity assay

The PCR product of full-length 3'-UTR fragment of the DJ-1 gene or the fragment containing the mutation was digested and ligated into the pGL3 vector, and named as pGL3-DJ1-WT, and pGL3-DJ1-MUT, respectively, after confirmation by sequencing. pGL3-DJ-WT (or pGL3-DJ1-MUT) was transfected into HEK293T cells with miR-24 mimic (or miR-NC) using the transfection reagent Lipofectamine 2000, and the cells were cultured for 48 h followed by measuring dual luciferase activity with Dual-Glo Luciferase Assay System kit.

Grouping treatment of SH-SH5Y cells

SH-SY5Y dopaminergic neurons were separated into a control group with routine culture, a MPTP treatment group with 100 μ M MPTP while a MPTP+Gas (20 μ M) group with 20 μ M Gas in addition to 100 μ M MPTP after 48 hours of further culture. The cells were collected by trypsin digestion for relevant analysis.

Flow detection of apoptosis

Each treated cell was collected by trypsinization for analysis of cell apoptosis using Annexin

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Table 1. Scores of climbing and suspension test results of the two groups of rats

Test content	Grouping	score	P value	t value
Climbing rod test	Control group	2.96±0.28	<0.0001	23.57
	PD group	1.31±0.14		
Suspension test	Control group	2.85±0.21	<0.0001	20.42
	PD group	1.14±0.13		

V-FITC and PI staining by Gallios flow cytometry.

qRT-PCR detection of gene expression

The RNA was extracted and reversely transcribed to generate cDNA by the PrimeScript™ RT reagent Kit. The PCR amplification reaction was carried out under the Taq DNA polymerase using cDNA as a template in a total of 10 µL of the reaction system, including 2 × SYBR Green Mixture 5.0 µL, 0.5 µL of forward and reverse primers (5 µm/L), 1 µL of cDNA, and ddH₂O with conditions: 50°C 15 min and 85°C 5 min. PCR reaction conditions: 95°C 5 min, 95°C × 15 sec, 60°C × 1 min, 40 cycles on a Bio-Rad CFX96 real-time PCR instrument.

Western Blot for protein expression

RIPA lysate lysed SH-SY5Y cells or substantia nigra tissue. From which 40 µg of total protein from each sample was further processed by electrophoresis (45 V, 3 h) in SDS-PAGE (10% separation gel, 4% concentrated gel), which was then transferred to PVDF membrane (250 mA, 100 min). The membrane was blocked with PBST containing 5% skim milk powder and incubated with primary antibody at 4°C overnight (DJ-1, Nrf2, TH, β-actin dilution ratios were 1:1000, 1:500, 1:1000, 1:5000), the unbound primary antibody was washed away, and then it was incubated with a 1:10000 diluted secondary antibody for 60 min at room temperature, and developed by ECL chemiluminescence. The film was scanned and the data was saved.

Statistical process

SPSS 18.0 software was adopted for analyzing data which were displayed as mean ± standard deviation and assessed by t test or one-way ANOVA and then by Bonferroni method. P<0.05 indicates statistical significance.

Results

Decreased brain function, brain oxidative stress and caspase-3 activity in PD model rats

The result showed that the scores of the climbing and suspension tests in the PD group were significantly lower than those in the Control group (P<0.05) (**Table 1**). The result of spectrophotometry showed that the caspase-3 activity in the substantia nigra of PD rats was significantly elevated compared to that in controls (P<0.05) (**Figure 1A**). Meanwhile, MDA content was significantly elevated in PD rats (**Figure 1B**), along with reduction of SOD activity (**Figure 1C**), compared to those in rats from the control group (P<0.05).

Upregulated DJ-1 and Nrf2 in the substantia nigra of PD model rats and reduced miR-24 level

We found significantly upregulated DJ-1 and Nrf2 mRNA (**Figure 2A**) and downregulated miR-124 (**Figure 2B**) in the substantia nigra of PD rats compared to those in control rats (P<0.05). In addition, DJ-1 and Nrf2 protein was also significantly upregulated in PD rats (P<0.05) (**Figure 2C**).

MiR-24 could target DJ-1 mRNA

Bioinformatics analysis revealed a binding site between miR-21 and DJ-1 3'-UTR (**Figure 3A**). Dual luciferase gene reporter assays showed significantly decreased luciferase activity in pGL3-DJ1-WT transfected HEK293T cells after miR-24 transfection, whereas no significant difference was found in pGL3-DJ1-MUT transfected HEK293T cells (**Figure 3B**), indicating a target relationship of miR-24 with DJ-1 (P<0.05).

Gas down-regulates miR-24 expression and improves brain function in PD rats

Compared with the PD model group, the climbing test and suspension test scores of the Gas treated group were significantly higher (P<0.05) (**Table 2**). The results of spectrophotometry showed that the caspase-3 activity in the substantia nigra of PD rats was significantly reduced after gas treatment (**Figure 4A**) along with decreased MDA content (**Figure 4B**) and

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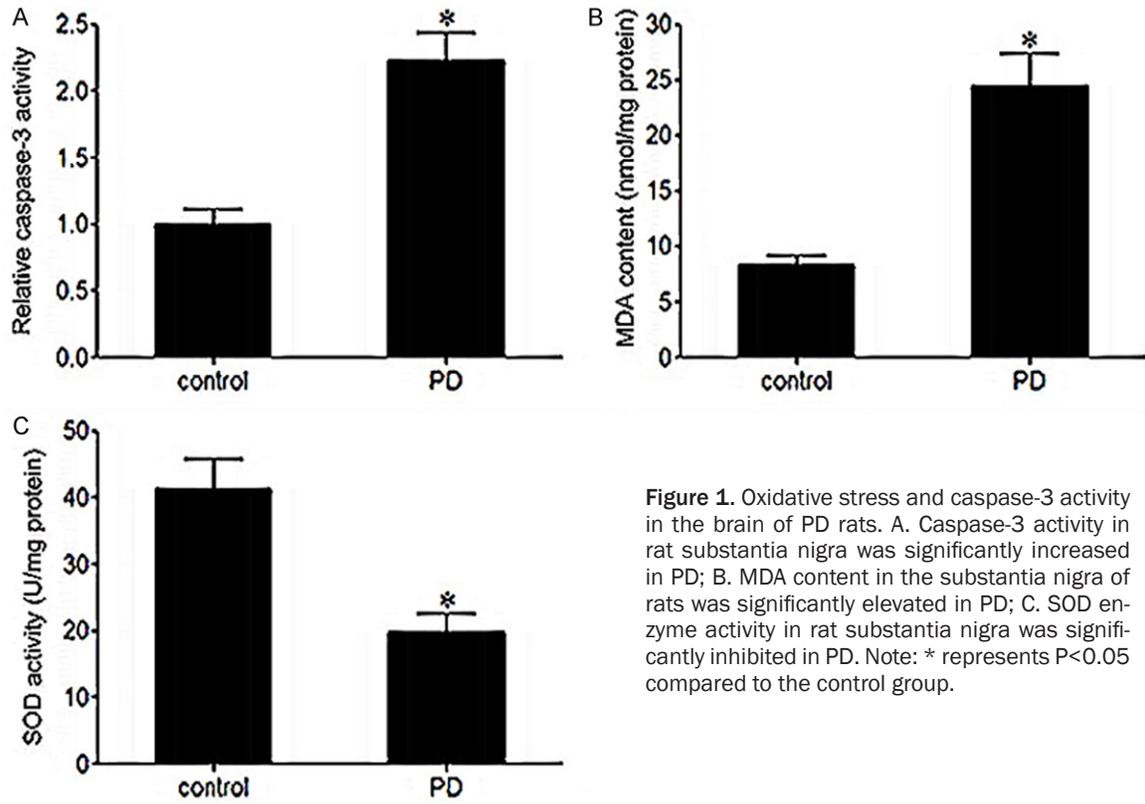


Figure 1. Oxidative stress and caspase-3 activity in the brain of PD rats. A. Caspase-3 activity in rat substantia nigra was significantly increased in PD; B. MDA content in the substantia nigra of rats was significantly elevated in PD; C. SOD enzyme activity in rat substantia nigra was significantly inhibited in PD. Note: * represents P<0.05 compared to the control group.

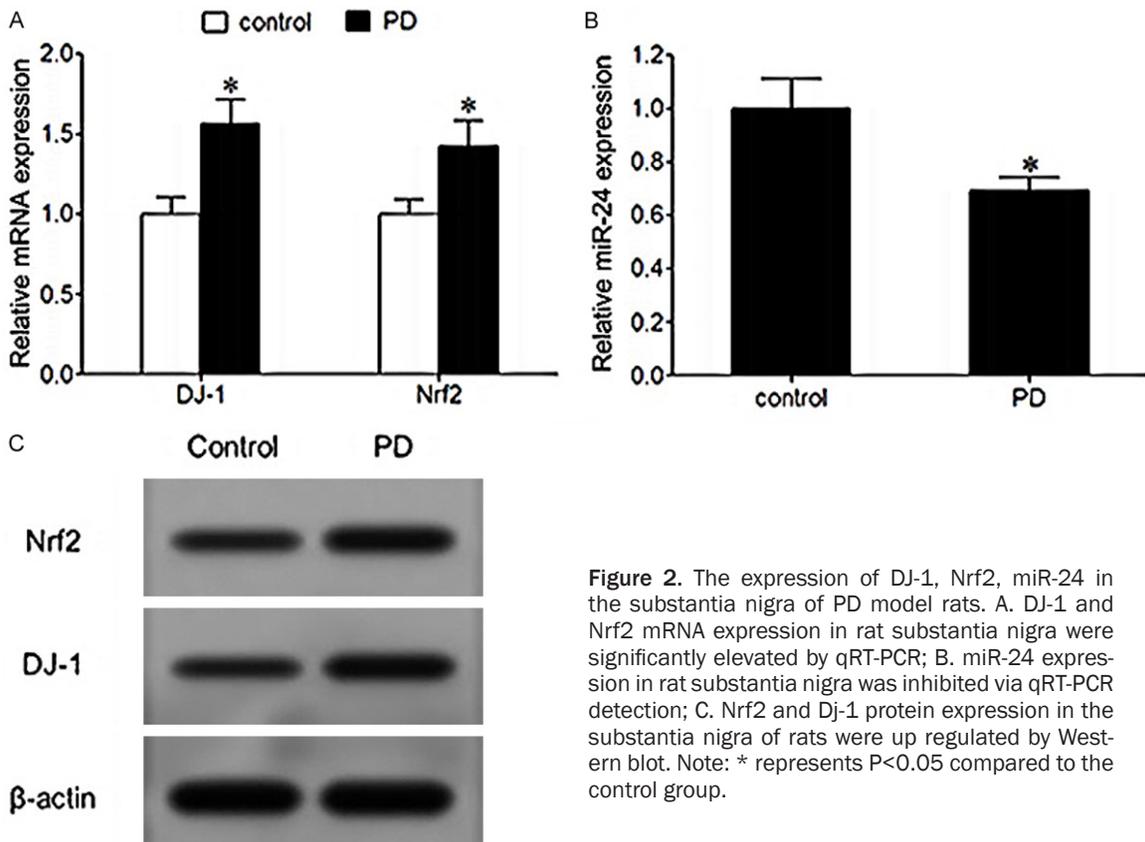


Figure 2. The expression of DJ-1, Nrf2, miR-24 in the substantia nigra of PD model rats. A. DJ-1 and Nrf2 mRNA expression in rat substantia nigra were significantly elevated by qRT-PCR; B. miR-24 expression in rat substantia nigra was inhibited via qRT-PCR detection; C. Nrf2 and Dj-1 protein expression in the substantia nigra of rats were up regulated by Western blot. Note: * represents P<0.05 compared to the control group.

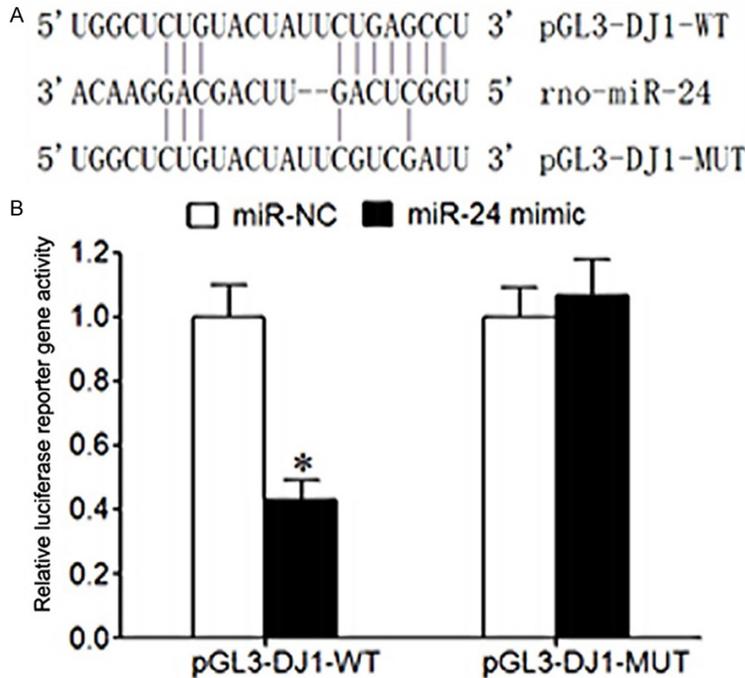


Figure 3. Targeted regulation relationship between miR-24 and DJ-1 mRNA. A. Schematic diagram of the site of action between miR-24 and the 3'-UTR of DJ-1 mRNA; B. Dual luciferase gene reporter assay indicated miR-24 mimic significantly impeded the level of DJ-1; Note: * represents P<0.05 compared to miR-NC.

Table 2. Comparison of scores of climbing and suspension test results of the two groups of rats after operation

Test content	Grouping	score	P value	t value
Climbing rod test	PD	1.22±0.16	<0.0001	8.70
	PD+Gas	1.99±0.23		
Suspension test	PD	1.16±0.13	<0.0001	12.42
	PD+Gas	2.13±0.21		

elevated SOD activity (P<0.05) (Figure 4C). The qRT-PCR results showed significantly upregulated DJ-1 and Nrf2 mRNA (Figure 4D) and downregulated miR-24 (Figure 4E) in the substantia nigra of PD rats after Gas treatment. Consistently, western blot analysis also indicated upregulated DJ-1, Nrf2, and TH protein in the substantia nigra of PD rats after Gas treatment (Figure 4F).

Gas down-regulates miR-24 and up-regulates DJ-1 to attenuate MPTP-induced apoptosis in SH-SY5Y cells

MPTP treatment or Gas treatment significantly upregulated DJ-1 and Nrf2 mRNA in SH-SY5Y cells (P<0.05) (Figure 5A). In addition, miR-24

level in SH-SY5Y cells was significantly reduced after MPTP treatment compared to that in control, and the treatment with Gas further reduced the expression of miR-24 (P<0.05) (Figure 5B). Western blot analysis showed that MPTP treatment significantly up-regulated DJ-1 and Nrf2 protein in SH-SY5Y cells, and Gas treatment on the basis of MPTP could further up-regulate the expression of DJ-1, Nrf2 and TH proteins (Figure 5C). Flow cytometry results showed significantly increased apoptosis of SH-SY5Y cells in the MPTP-treated group, and the administration of Gas significantly attenuated the effect of MPTP on SH-SY5Y cell apoptosis (Figure 5D).

Discussion

PD is a degenerative disease that occurs in the substantia nigra-striatum pathway. Degeneration of dopaminergic (dopamine (DA)) neurons causes a decrease in DA content in the striatum, resulting in a balance disorder of the dopamine-acetylcholine (DA-Ach) system; causing muscle rigidity, reduced movement, and positional disorders.

Nrf2/antioxidant response element (ARE) is the most important endogenous anti-OS signaling pathway discovered to date. Under oxidative stress, Nrf2 is transferred into the nucleus, in which it binds ARE and initiates transcription and expression of downstream phase II detoxification enzymes, antioxidant enzymes, anti-apoptotic genes and other factors, increasing cells for oxidative stress. The resistance of substances such as electrophilic biomacromolecules reduces the oxidative stress damage and apoptosis in the body, thereby protecting the diseased tissues and cells [10-12]. A number of studies have shown that DJ-1 has a wide range of neuroprotective effects, especially in combating oxidative stress, reducing apoptosis, and promoting cell survival, so as to protect

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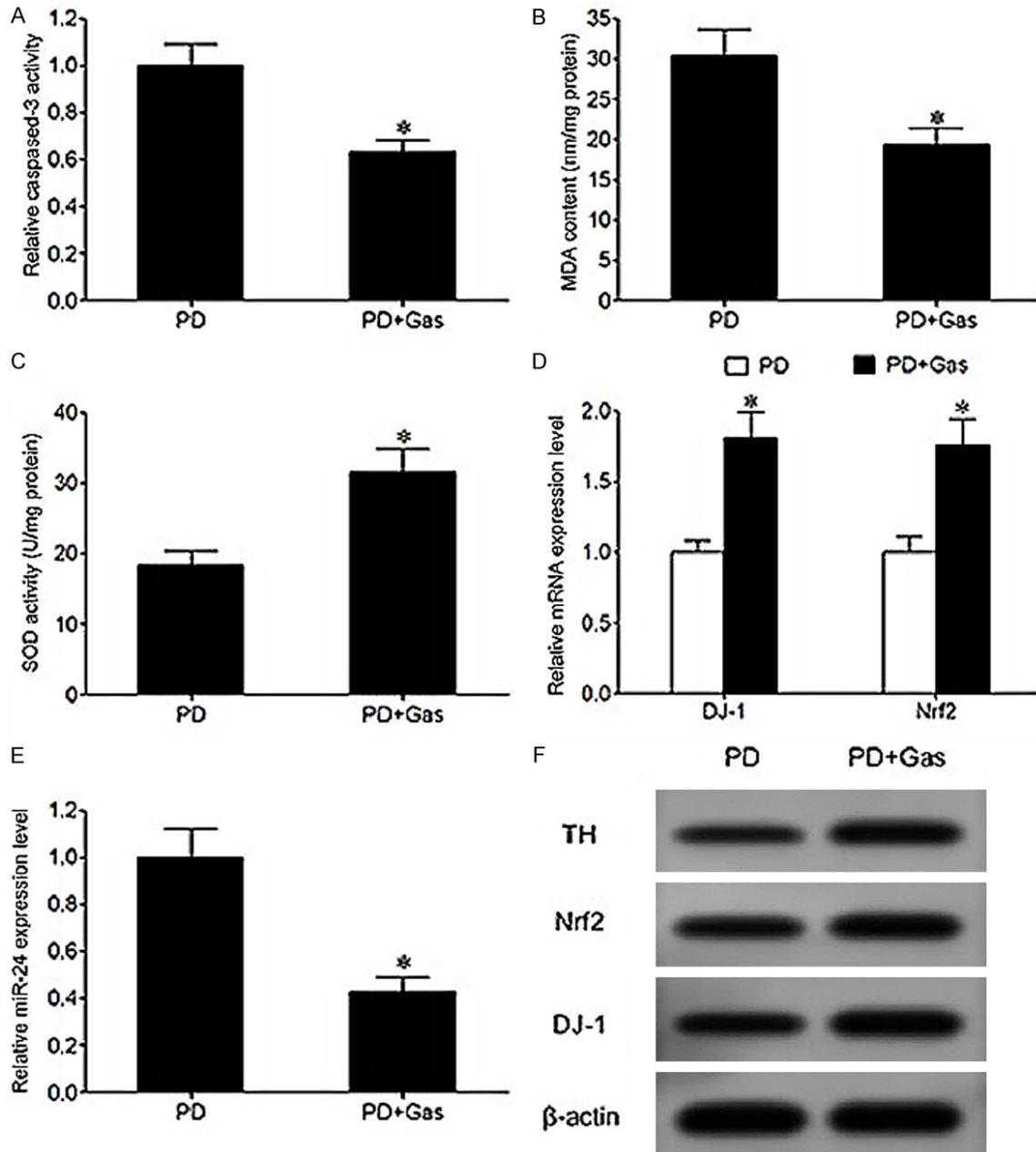


Figure 4. The impact of Gas on miR-24 expression and brain function in PD rats. A. Caspase-3 activity in rat substantia nigra of PD+Gas group was significantly reduced compared to that in PD group; B. The MDA content in the substantia nigra of rats of PD+Gas group was significantly decreased compared to that in PD group; C. SOD enzyme activity in rat substantia nigra of PD+Gas group was significantly enhanced compared to that in PD group; D. DJ-1 and Nrf2 mRNA expression in rat substantia nigra PD+Gas group was significantly up regulated compared to that in PD group; E. miR-24 expression in rat substantia nigra of PD+Gas group was significantly reduced compared to that in PD group by qRT-PCR; F. TH, DJ-1 and Nrf2 protein expression in rat substantia nigra of PD+Gas group was significantly increased compared to that in PD group; Note: * represents the comparison with the PD group, $P < 0.05$.

nerve cells from oxidative stress [13, 14]. Multiple studies have shown that DJ-1 is involved in enhancing Nrf2 stability and promoting Nrf2 protein expression and activation. The antioxidant function of DJ-1 depends to some extent on the regulation of Nrf2 [5, 6].

Gastrodin (Gas) is extracted from the dried roots of the orchid family *Gastrodia elata*, and is an effective monomer of traditional Chinese medicine *Gastrodia elata*. It has the effects of sedation, anticonvulsant, analgesia and increase cerebral blood flow. Numerous studies

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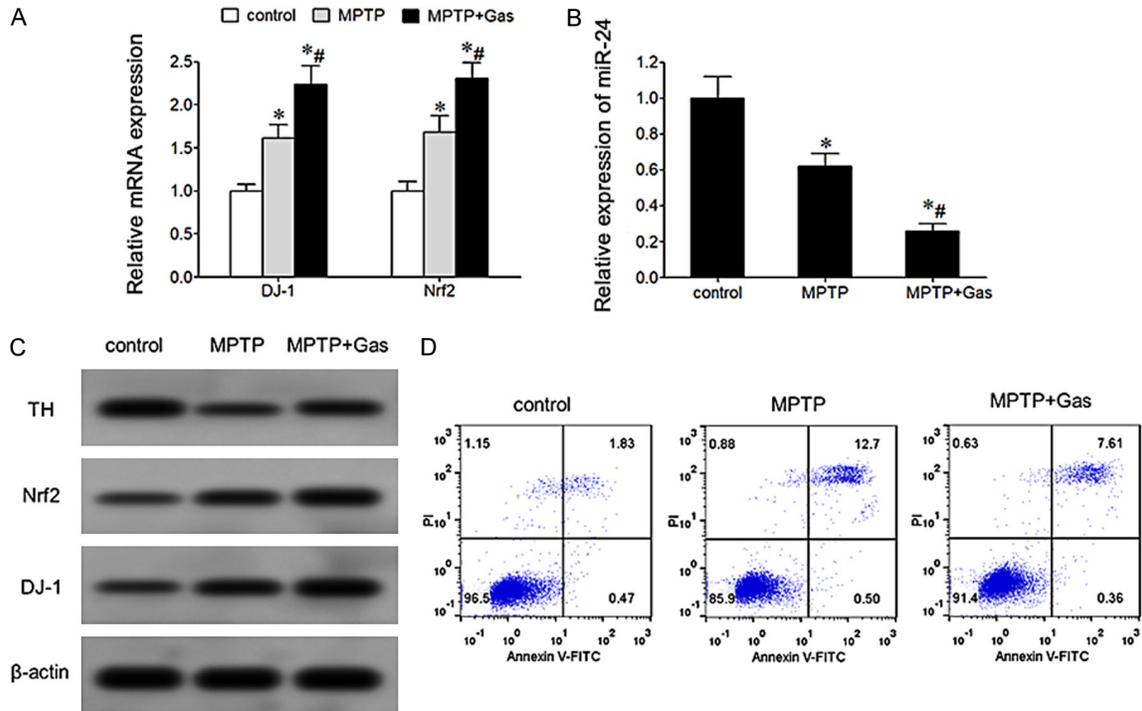


Figure 5. The role of Gas in on MPTP-induced effects in SH-SY5Y cells. A. Gas further enhanced the promoting role of MPTP on DJ-1 and Nrf2 mRNA levels by qRT-PCR; B. The decreasing expression of miR-24 by MPTP was further reduced in SH-SY5Y cells by Gas; C. Gas further enhanced the promoting role of MPTP on TH, DJ-1 and Nrf2 protein levels by Western blot; D. The growing apoptosis of SH-SY5Y cells by MPTP was significantly attenuated by Gas using Flow; Note: * represents $P < 0.05$ compared with the control group; # represents $P < 0.05$ compared with the MPTP group.

have shown that Gas has anti-oxidation effects, protects neurons, and improves the pharmacology of brain function [7-9].

MicroRNA regulates several biological processes such as cell survival, proliferation, apoptosis, migration, etc. [15, 16], and the role of microRNA abnormalities in the pathogenesis of PD has attracted extensive attention [17, 18]. Bioinformatics analysis showed a regulation relationship of miR-24 with DJ-1 mRNA 3'-UTR. Whether Gas is likely to regulate miR-24 expression, affect DJ-1/Nrf2 pathway activity, and reduce PD brain damage is unclear. Our study established PD rat model and adopted Gas treatment intervention to evaluate Gas's role in miR-24 expression, DJ-1/Nrf2 pathway activity, oxidative stress and brain cell apoptosis.

The scores of the climbing rod test and the suspension test in the PD group were significantly reduced, and the motor function and limb coordination were significantly deteriorated, indicating that the PD model was successfully established and could meet the needs of subsequent

experiments. The test results of the kit showed that the caspase-3 activity and MDA content in the substantia nigra of the PD group were significantly increased, while SOD activity was significantly decreased. Further detection showed that DJ-1 and Nrf2 in the substantia nigra of the PD model rats were significantly upregulated, indicating that the body has activated anti-oxidative stress mechanisms by up-regulating DJ-1 and Nrf2, but the antioxidant capacity is still low and the active oxygen species are still significantly elevated. In addition, it is confirmed that miR-24 can target DJ-1 mRNA. The 3'-UTR region inhibits its expression. The detection of miR-24 expression showed that miR-24 in PD modeled rats was significantly reduced compared to controls, indicating that the body may down-regulate the expression of miR-24 by a certain mechanism, thereby up-regulating DJ-1, antagonizing the inhibitory effect of miR-24 on DJ-1 expression, and enhancing antioxidant damage. Gastric administration of Gas in rats with a PD model significantly reduced MDA content and caspase-3 activity in rat substan-

tia nigra tissue, whereas significantly increased the activity of antioxidant enzyme SOD. miR-24 in the substantia nigra of PD rats in the Gas treated group was further decreased, while DJ-1 and Nrf2 were further upregulated. The results showed that further down-regulation of miR-24 expression and elevation of DJ-1 and Nrf2 level are implicated to be mechanism by which Gas exerts anti-oxidative damage, reduces brain cell apoptosis, and protects dopaminergic neurons. In order to directly assess the protective effect of Gas on dopaminergic neurons, we treated Gas-treated SH-SY5Y cells with Gas, and found that Gas can significantly reduce the expression of miR-24 in SH-SY5Y cells under MPTP treatment conditions.

In the study of Gas treatment for PD, Haddadi et al [19] showed that pretreatment with Gas can significantly reduce the neurological damage of the PD modeled rats, as well as improve the motor coordination function, and overall PD treatment. Myeloperoxidase (MPO) activity, decreased lipid peroxidation levels and NO production. Li et al [20] showed that Gas can significantly reduce IL-1 β and TNF- α level in the brains of PD modeled rats, inhibit inflammatory microglia activation, decrease inflammatory responses in the brain, and thus assist in PD treatment. Wang et al [21] showed that antioxidant Nrf2, antioxidant enzymes HO-1 and SOD in the brain of Gas-treated group were significantly increased, while Gas could enhance the phosphorylation of ERK1/2, activate the ERK1/2-Nrf2 pathway, enhance the anti-oxidation in PD mice, and reduce brain damage. All of the above studies have shown that Gas has the effect of protecting nerve cells, alleviating brain nerve function damage, and alleviating PD, which is consistent with our study. In the study of the relationship between miR-24 and PD, Vallelunga et al [22] showed significantly higher miR-24 levels in PD patients. The results of Marques et al [23] also showed significantly higher miR-24 levels in cerebrospinal fluid of PD patients. These studies suggest that elevated miR-24 level is related to PD and may participate in PD pathogenesis. Our *in vivo* and *in vitro* study revealed that treatment of dopaminergic neurons, in both PD rats and cultured cells, can down-regulate the expression of miR-24, enhance the DJ-1/Nrf2 pathway, and increase the antioxidant capacity, thereby alleviating PD disease.

Conclusion

Gas can enhance the DJ-1/Nrf2 pathway by down-regulating the expression of miR-24, increasing the antioxidant capacity, reducing the damage and apoptosis of dopaminergic neurons, which plays a critical role in the treatment of PD.

Disclosure of conflict of interest

None.

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