Gastrodin inhibits neuroinflammation in rotenone-induced Parkinson's disease model rats★

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Abstract

The present study showed that the latency of rats moving on a vertical grid was significantly prolonged, and the number of rats sliding down from the declined plane was increased remarkably, in rotenone-induced Parkinson's disease model rats compared with control rats. The moving latency recovered to normal levels, but the number of slides was significantly increased at 28 days after model establishment. The slope test is a meaningful approach to evaluate the symptoms of Parkinson's disease model rats treated with rotenone. In addition, loss of substantia nigral dopaminergic neurons in model rats was observed at 1 day after the model was established, and continued gradually at 14 and 28 days. The expression of tyrosine hydroxylase-positive cells was significantly increased in gastrodin-treated rats at 14 days. Significant numbers of activated microglia cells were observed in model rats at 14 and 28 days; treatment of rats with Madopar at 28 days suppressed microglial activation. Treatment of rats with gastrodin or Madopar at 28 days significantly reduced interleukin-1β expression. The loss of substantia nigral dopaminergic neurons paralleled the microglial activation in Parkinson's disease model rats treated with rotenone. The inflammatory factors tumor necrosis factor-α and interleukin-1β are involved in the substantia nigral damage. Gastrodin could protect dopaminergic neurons *via* inhibition of interleukin-1β expression and neuroinflammation in the substantia nigra.
Key Words: gastrodin; rotenone; neuroinflammation; dopamine; microglia cells; interleukin-1β; Parkinson's disease

INTRODUCTION

Neuroinflammation processes have been shown to be associated with the pathogenesis of Parkinson's disease (PD)[1-3]. Both postmortem analysis and various animal models of PD[4] have detected massive amounts of activated microglia, as well as reactive astrocytes around degenerated neurons. Microglial activation might participate in the development of PD, as suggested by the results of an epidemiological investigation[5]. Activated microglia and astrocytes produce a wide array of prostanoids, free radicals and cytokines, which work in concert to induce neurodegeneration[6-7]. Rotenone-induced models reproduce effectively the pathological features of PD[8-12]. The loss of substantia nigral dopaminergic neurons and microglial activation in rotenone-induced rats were observed in our previous study. However, little has been reported about the changes to the numerous cytokines in the brains of model rats and the relationship between neuroinflammation and loss of dopaminergic neurons.
Complex prescription of Chinese drugs, with

Rhizoma Gastrodiae as a monarch or ministerial drug, has been widely used in the treatment of PD[13-17]. However, the effects of these drugs on PD animals and treatment-related mechanism are still unknown[18]. Gastrodin is the major active component of *Rhizoma Gastrodiae*, with a molecular weight of 286 Da, a molecular formula of $C_{13}H_{18}O_7$, and the following chemical structural formula:
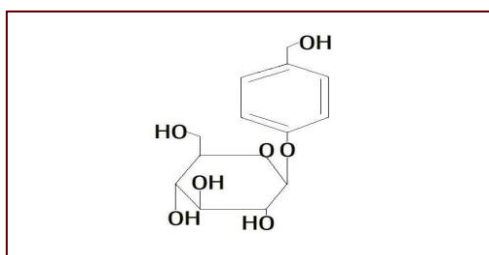
The present study sought to elucidate the effects of gastrodin on substantia nigra dopaminergic neurons in rotenone-induced PD rats, as well as its effects on microglial activity and cytokine expression in rat models of PD, using Madopar as a control. Moreover, this study also demonstrates the relationships among microglial activation, inflammatory cytokines and loss of dopaminergic neurons in the substantia nigra of model rats.

RESULTS

Quantitative analysis of experimental animals

A total of 90 Wistar rats were used in this study; 11 of them were selected as controls, while the remaining rats were used to make PD models induced by rotenone. A total of 49 successful model rats were treated with Madopar (Madopar group, n = 15), gastrodin (gastrodin group, n = 15) or saline (model group, n = 19) by gavage. In total, 60 rats were included in the final analysis.

Effect of gastrodin on the neurobehavior of rotenone-induced PD rats

Grid tests showed that the moving latency in rats at 1 day after model establishment was lengthened remarkably compared with that in normal rats (P < 0.01), but that it recovered to the normal latency by 28 days after model establishment (P > 0.05). There was no significant difference between model rats and rats treated with gastrodin or Madopar (Table 1).

Slope tests showed that the number of sliding model rats at 1 day after model establishment was significantly higher than the number of sliding normal rats (P < 0.05), and that it was higher still at 28 days (P < 0.05). There was a trend toward fewer sliding gastrodin- or Madopar-treated rats, but there was no significant difference between the models and drug-treated rats (Table 1).

Gastrodin increases tyrosine hydroxylase (TH) expression in the substantia nigra of PD rats

TH-positive expression was significantly decreased in the model rats at 4 days after model establishment compared with normal rats (P < 0.05; Table 2, Figures 1A, B), and this decrease was even greater at 14 and 28 days (P < 0.01; Figures 1C, D). TH-positive expression at 14 days after model establishment was significantly increased in gastrodin-treated rats compared with model rats (P < 0.05; Figures 1C, E, F), but the level of TH-positive expression in Madopar-treated rats was not significantly greater than that in model rats, despite a trend toward an increase.

Table 1   Effect of gastrodin on behavioral changes in rotenone-treated Parkinson's disease rats

Group	n	Moving latency (second)	Number of slide (times/30 s)
Normal	15	1.15±0.90	2.49±1.34
Model 1 day	15	12.99±6.84[b]	4.85±3.05[a]
Model 28 days	11	1.51±1.20[d]	10.25±5.75[bc]
Madopar 28 days	11	1.70±1.13	6.85±5.75
Gastrodin 28 days	11	0.97±0.75	6.49±5.05

Data are expressed as mean ± SD. The greater the number of slides, the worse the neurological function of the rats was. [a]P < 0.05, [b]P < 0.01, *vs.* normal group; [c]P < 0.05, [d]P < 0.01, *vs.* model rats at 1 day (one-way analysis of variance).

Table 2   Area of tyrosine hydroxylase-positive cells ($\times 10^3$ μm^2) in the substantia nigra of rats

Group	Time after model establishment (day)		
	4	14	28
Normal	193.76±40.60	–	–
Model	139.13±20.43[a]	89.84±14.93[bc]	108.47±22.02[b]
Madopar	–	107.96±17.21	136.97±23.82
Gastrodin	–	117.75±16.06[d]	157.83±35.34

Data are expressed as mean ± SD of four rats in each group at every time point. [a]P < 0.05, [b]P < 0.01, *vs.* normal group; [c]P < 0.05, *vs.* model rats at 4 days; [d]P < 0.05, *vs.* model rats at 14 days (one-way analysis of variance).
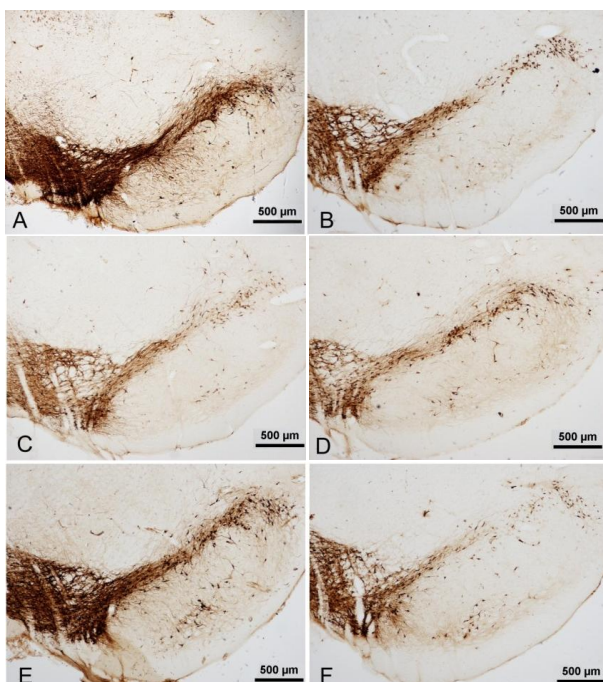

Figure 1   Tyrosine hydroxylase (TH) expression in the substantia nigra of rats (immunohistochemical staining, light microscope, scale bars: 500 μm).

A large number of TH-positive cells with deeply colored cell bodies and abundant processes were observed in the substantia nigra of normal rats (A).

Compared with normal rats (A), the numbers of TH-positive cells and processes in model rats at 4 days (B) were clearly decreased, and lighter colored cell bodies were seen.

The loss of TH-positive cells was aggravated in model rats at 14 days (C) and 28 days (D).

Gastrodin at 14 days increased the numbers of TH-positive cells and processes (E) compared with the model group at 14 days (C).

The numbers of TH-positive cells and processes of Madopar-treated rats also tended towards a decrease at 14 days (F).

Effect of gastrodin on complement receptor (OX$_{42}$) expression in the substantia nigra of PD rats

Microglial activation was observed in model rats at 14 and 28 days, and the area of OX$_{42}$-positive cells was significantly lower in model rats than in controls ($P < 0.05$; Table 3, Figures 2A–D). Microglial activation in rats treated with gastrodin and Madopar at 28 days showed decreased microglial activation, with significant differences between the Madopar group and the model group ($P < 0.05$; Figures 2E, F).

Table 3　Area of OX$_{42}$-positive cells ($\times 10^3$ μm^2) in the substantia nigra of rats

Group	Time after model establishment (day)		
	4	14	28
Normal	75.02±9.99	–	–
Model	50.25±62.64	58.19±4.32[a]	56.92±10.96[a]
Madopar	–	49.73±23.30	77.17±11.29[b]
Gastrodin	–	47.22±23.88	68.43±4.66

Data are expressed as mean ± SD of four rats in each group at every time point. [a]$P < 0.05$, *vs.* normal group; [b]$P < 0.05$, *vs.* model rats at 28 days (one-way analysis of variance).

The smaller the area of positive cells is, the greater the activation of microglial cells is.

Gastrodin inhibits tumor necrosis factor (TNF)-α expression in the substantia nigra of PD rats

TNF-α expression decreased sharply in model rats at 4 days after model establishment compared with the normal group ($P < 0.05$; Table 4, Figures 3A, B). It showed a tendency toward an increase at 14 and 28 days, but was not significantly different from the expression levels in normal control rats. Treatment of rats with gastrodin and Madopar at 14 days significantly downregulated TNF-α expression ($P < 0.05$; Figures 3C, E). TNF-α expression in the Madopar group at 28 days was higher than that at 14 days ($P < 0.05$; Figures 3E, F), but the expression level continued to decrease in the gastrodin-treated group.

Table 4　Area of tumor necrosis factor-α-positive cells ($\times 10^3$ μm^2) in the substantia nigra of rats

Group	Time after model establishment (day)		
	4	14	28
Normal	225.05±28.91	–	–
Model	159.91±29.38[a]	248.37±34.21	282.81±87.65[b]
Madopar	–	167.23±17.93[c]	259.26±71.52[d]
Gastrodin	–	176.41±59.46[c]	203.12±31.28

Data are expressed as mean ± SD of four rats in each group at every time point.

[a]$P < 0.05$, *vs.* normal group; [b]$P < 0.05$, *vs.* model rats at 4 days; [c]$P < 0.05$, *vs.* model rats at 14 days; [d]$P < 0.05$, *vs.* Madopar-treated rats at 14 days (one-way analysis of variance).

Gastrodin inhibits interleukin (IL)-1β expression in the substantia nigra of PD rats

IL-1β expression showed a tendency to increase continuously in the substantia nigra of model rats. IL-1β expression was clearly increased in the model group at 14 days ($P < 0.05$; Table 5, Figures 4A, B), and it was significantly higher at 28 days ($P < 0.01$; Figure 4C). There was a tendency for IL-1β expression in rats treated with gastrodin and Madopar to be slightly lower than that in model rats at 14 days, and this difference was significant at 28 days ($P < 0.05$; Figures 4D, E).

Table 5　Area of interleukin-1β-positive cells ($\times 10^3$ μm^2) in the substantia nigra of rats

Group	Time after model establishment (day)		
	4	14	28
Normal	198.71±53.47	–	–
Model	252.09±17.53	293.67±64.59[a]	320.85±5.61[b]
Madopar	–	251.47±31.25	252.04±3.22[c]
Gastrodin	–	246.50±18.45	243.58±10.63[c]

Data are expressed as mean ± SD of four rats in each group at every time point. [a]$P < 0.05$, [b]$P < 0.01$, *vs.* normal group; [c]$P < 0.05$, *vs.* model rats at 28 days (one-way analysis of variance).
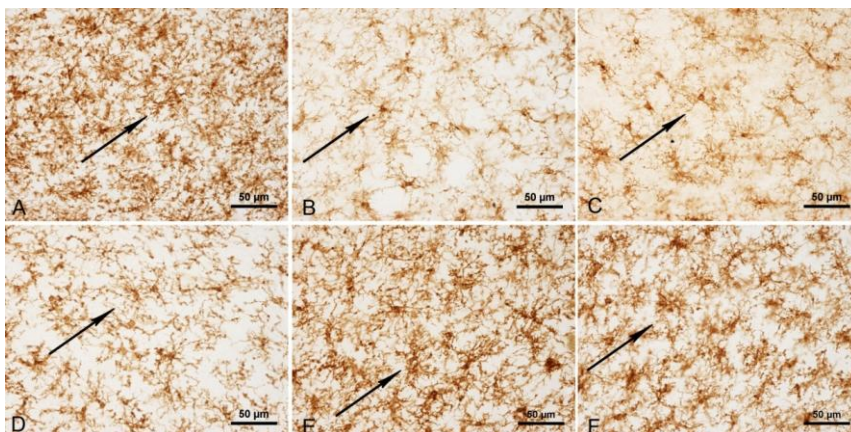

Figure 2　Complement receptor (OX$_{42}$) staining in the midbrains of rats (immunohistochemical staining, light microscope, scale bars: 50 μm).

Resting microglia had branches in the substantia nigra of normal controls that stained for OX$_{42}$ (A).

Microglia cells showed an amoeba-like morphology in the model at 4 days (B), 14 days (C) and 28 days (D). Microglial activation was reduced in gastrodin-(E) and Madopar-treated rats (F).

Arrows represent OX$_{42}$ positive expression.
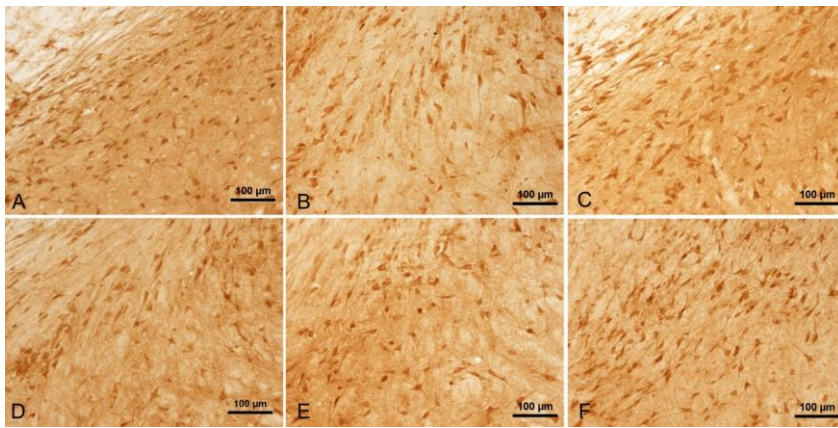
Figure 3   Tumor necrosis factor-α staining in the midbrains of rats (immunohistochemical staining, light microscope, scale bars: 100 μm).

Many dark brown-colored cells can be observed in the control rats (A).

The number of positive cells was reduced in the model rats at 4 days (B) and 14 days (C). This was also the case in the gastrodin (D) and Madopar (E) groups at 14 days.

The number of positive cells in the Madopar group at 28 days (F) was greater than that at 14 days (E).
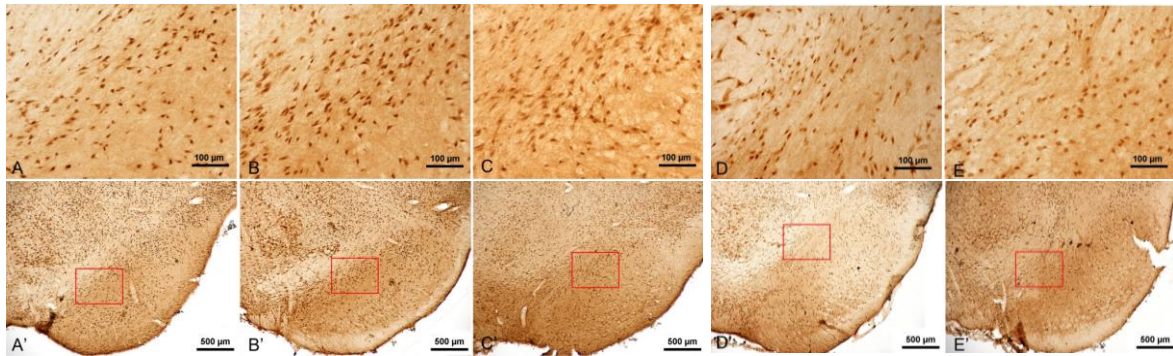
Figure 4   Interleukin-1β staining in the midbrains of rats (immunohistochemical staining, light microscopy; scale bars in A–E: 100 μm; scale bars in A'–E': 500 μm). (A, A') Normal group; (B, B'): Model group at 14 days; (C, C'): Model group at 28 days; (D, D'): Gastrodin group at 28 days; (E, E'): Madopar group at 28 days. Dark brown-colored cells were observed in the control (A), and the number of these was increased in the model at 14 days (B) and 28 days (C). The number of positive cells was reduced in the gastrodin (D) and Madopar groups at 28 days (E).

Effect of gastrodin on IL-6 expression in the substantia nigra of PD rats

IL-6 expression in the substantia nigra of model rats tended to be up-regulated at first and then down-regulated; there was no significant difference in the level of IL-6 expression in model rats compared with the normal group. Treatment of rats with gastrodin or Madopar produced a trend towards a decrease in the level of IL-6 expression, but no significant difference was observed compared with the expression level in the normal group (Table 6; supplementary Figure 1 online).

Table 6   Area of interleukin-6-positive cells ($\times 10^3$ μm^2) in the substantia nigra of rats

Group	Time after model establishment (day)		
	4	14	28
Normal	218.68±13.90	–	–
Model	250.24±19.85	250.77±74.95	215.92±21.33
Madopar	–	209.80±24.34	199.11±32.37
Gastrodin	–	202.25±20.17	195.11±27.07

Data are expressed as mean ± SD of four rats in each group at every time point. One-way analysis of variance revealed no significant difference among groups at each time point.

Temporal correlation between the loss of TH cells, microglial activation, and expression of TNF-α, IL-1β and IL-6

As shown in Table 7, the loss of dopaminergic neurons was associated with microglial activation and expression of the inflammatory factor IL-1β in PD model rats.

Table 7   Relationship between substantia nigra dopaminergic neuronal loss and neuroinflammation in rotenone-treated Parkinson's disease rats

Cells or cytokines	Time after model establishment (day)		
	4	14	28
Dopaminergic neurons	--	---	---
Microglia	+	++	++
Tumor necrosis factor-α	--	+	+
Interleukin-1β	+	++	+++
Interleukin-6	+	+	0

"+": increase; "++": $P < 0.05$; "+++": $P < 0.01$, activated or increased; "--": $P < 0.05$; "---": $P < 0.01$, lost or decreased; 0: no change.

DISCUSSION

In the present study, moving latency was significantly

prolonged in model rats with slow movement, and the number of slides increased significantly; combined with behavioral findings, these findings indicated successful establishment of the model. The sluggish symptoms improved greatly in model rats by 28 days, which suggested that it would be difficult to assess the effects of drugs on neurobehaviour based on sluggish symptoms that only occurred at an early stage. By contrast, the number of slides in model rats at 28 days was higher than that at the initial stage of model establishment, and the rats' muscular strength was clearly weakened, suggesting that the inclined slope plane test could be applied to evaluate the effects of interventions. Additionally, the loss of dopaminergic neurons was aggravated in model rats at 28 days. Both behavioral and immunohistochemical analyses indicated that damage to dopaminergic neurons developed following model establishment, consistent with previous findings. However, the degree of damage and the time when it occurred varied among individuals (data not shown); thus, model animals with the same scores should be chosen for studies on interferences. Microglia are the brain's resident immune cells. As an important line of defense in the brain, microglia become readily activated in response to injuries to the brain or to immunologic stimuli[19-20]. Activated microglia undergo dramatic morphologic changes, metamorphosing from a ramified morphology into an amoeboid morphology[21]. They exhibit functionally increased expression of surface molecules for antigen recognition and presentation, such as major histocompatibility complex molecules and complement receptor 3, and show up-regulated secretion of a large number of cytokines, such as TNF-α, IL-1β and IL-6, which magnify and perpetuate inflammation and immunological reactions[22]. At the same time, activated microglia also produce large amounts of free radicals like superoxide, eicosanoids and nitric oxide, which mediate neural damage[23].

The present study showed that microglia continue to be activated in model rats from 14 days to 28 days after model establishment, and massive loss of dopaminergic neurons was observed at 14 days. These findings suggest that microglial activation likely parallels dopaminergic neural damage, similar to previously described findings. Activated microglial cells are extensively found in 1-Methy-4-phenyl-1, 2, 3, 6-tetrahydropyridine-[1], 6-hydroxydopamine-[2] and rotenone-induced[3] PD models, and microglial inhibitors could relieve dopaminergic neuronal degeneration. All of these results suggest that loss of dopaminergic neurons is closely associated with microglial activation, even though the temporal relations between the two vary in different studies, probably being related to the type of nerve agents used and observation points.

TNF-α expression was reduced in conjunction with distinct loss of TH cells in model rats at 4 days, suggesting that TNF-α might not be the key factor involved in the initial dopaminergic loss. However, there was a significant difference between minimum (in the model at 4 days) and maximum (at 28 days) TNF-α expression levels, which indicated that TNF-α underwent a bigger change after the model was successfully established. IL-1β expression increased consistently from 14 days to 28 days, in parallel with continuous microglial activation and further loss of TH cells, suggesting that microglial activation and upregulation of IL-1β expression are major components in concert with dopaminergic neural inflammatory injury. IL-6, a secondary inflammatory factor was not a key player in the development of dopaminergic neural damage at this stage. These results have not previously been reported in PD models induced by rotenone. The rotenone model of PD with the features of non-invasion and chronic formation reveal a likely connection between dopaminergic neuronal loss and neuroinflammation during the development of PD.

Gastrodin possess the following activities: anti-inflammation[18], antioxidation[24], antagonistic neurotoxicity of excitatory amino acid[24-25], immunomodulation[26-27] and anti-epilepsy[28]. Research has shown that gastrodin has a therapeutic effect in 6-OHDA-induced PD rats, and that it can improve behavioral abnormalities, decrease the loss of nigral TH-positive cells, and inhibit nigral TNF-α expression[18]. In the present study, it was observed for the first time that gastrodin was able to reduce the loss of TH-positive cells in a rotenone-induced model of PD: it had a protective action on dopaminergic neurons, down-regulated nigral IL-1β expression resulting in a restraining of neuroinflammation during the damage, and improved muscle rigidity and endurance in PD rats. These activities might provide a pharmacological basis for improving the clinical symptoms of PD patients. Similar to gastrodin, Madopar could also ameliorate muscle rigidity and endurance loss, depress IL-1β expression, and inhibit microglial activation. However, it could not reduce the loss of TH cells, and had no protective effect on dopaminergic neurons. Collectively, the results presented here show that gastrodin is superior to Madopar in improving clinical symptoms and protecting dopaminergic neurons in rotenone-induced PD rats.

MATERIALS AND METHODS

Design
A randomized controlled animal experiment pertaining to the neuropathology.

Time and setting
This experiment was performed at the School of Traditional Chinese Medicine, Capital Medical University, China, from April to July in 2009.

Materials
A total of 90 male Wistar rats, aged 3 months, weighing 220−250 g, of clean grade, were purchased from the Vital River Laboratory Animal Technology Co., Ltd., China (Certification number SCXK (Jing) 2002-0003).

Experimental procedures were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[29].

Methods

Rotenone-induced PD model establishment and drug treatment

Rotenone oily emulsion (2 mg/kg per day; Sigma, St. Louis, MO, USA) was injected into the backs of the rats for 3−5 weeks, and rats with behavioral scores ≥ 2 (see the Behavioral scoring system section below for details) were regarded as successful PD models[10]. Rats that scored 2 were chosen for the present study, and divided into three groups: a Madopar group (intragastric injection of Madopar, 7.8 mg/kg per day; F. Hoffmann-La Roche Ltd., Basel, Switzerland); a gastrodin group[18, 30] (intragastric injection of gastrodin, 0.2 g/kg per day; Youcare Pharmaceutical Group Co., Ltd., Beijing, China); and a model group (intragastric injection of saline).

Behavioral scoring system

Behavioral scores were measured using a scoring system with seven behavioral features scored. (1) Resistance was suppressed, piloerection, hair dirty, roachback, bradykinesia, score 1. (2) Score 1 plus clear bradykinesia, tremor, instability of gait, score 2. (3) Score 2 plus instability of gait, or cannot go straight, or rotation, score 3. (4) The body inclines towards one side, forelimb or posterior limb of unilateral paralysis, walk and taking food difficulty, score 4. (5) Forelimb and/or posterior limb of unilateral paralysis entirely, muscular constriction, weight loss, decreased food intake, score 8. (6) Impending death or died, score 10. (7) No abnormal behavior, score 0[10].

Neurobehavioral test

Neurobehavioral experiments were carried out on days 1 and 28 after model establishment[31].
Grid test: The experimental installation was an independent metallic meshes upright to the horizon (50 cm × 40 cm, distance between meshes: 0.9 cm × 1.7 cm). The rats were placed upright on the center of the meshes, and grasped the meshes with four paws. Moving latency, that is, the time from a resting state to the moment that facultative paw moved, was noted.
Inclined plane test: Rats were placed head upward at the top of an inclined plane (60 cm × 48 cm) situated at 50° to the ground. It was ensured that the rats could not grasp the edge of the plane or any other objects. The number of slides in a 30-second period was recorded three times.

Sampling

Four rats were selected from the model group on day 4 for scoring, and a total of four rats were selected from each group on days 14 and 28, followed by anesthetization, sacrifice and perfusion of paraformaldehyde. Brains were removed and stored in 70% paraformaldehyde containing 30% sucrose overnight. Frozen sections (50 µm) through the substantia nigra[32] were prepared using a freezing microtome and used for immunohistochemistry.

Immunohistochemical detection of TNF-α, TH, OX$_{42}$, IL-1β and IL-6 expression in the substantia nigra of rats

TNF-α immunohistochemical staining: Frozen sections containing the substantia nigra were blocked with 0.1% trypsin solution for 30 minutes for antigen retrieval. The sections were washed three times in distilled water three times (for 5 minutes each time), treated with 3% hydrogen peroxide for 10 minutes to block endogenous peroxidase, washed in distilled water and 0.01 M PBS, incubated with 5% normal goat serum for 30 minutes to inhibit non-specific binding, and incubated in primary rabbit anti-rat TNF-α monoclonal antibody (1: 50; Wuhan Boster Bio-engineering Co., Ltd, Wuhan, China) for 48 hours at 4°C. They were then washed, incubated in biotinylated-goat anti-rabbit IgG (1: 300; Beijing Zhongshan Goldenbridge Biological Technology, Beijing, China) for 2 hours, washed, incubated in horseradish peroxidase-avidin (1: 300; Beijing Zhongshan Goldenbridge Biological Technology) for 2 hours, and colorized with 3, 3-diaminobenzidine for 10−30 minutes.
TH immunohistochemical staining: The primary antibody was mouse anti-rat TH monoclonal antibody (1: 10 000; Sigma), in which sections were incubated at room temperature for 24 hours. Then, the antibody solution was changed to the biotin-labeled goat anti-mouse immunohistochemical staining kit (Beijing Zhongshan Goldenbridge Biological Technology). Instead of 0.1% trypsin solution, frozen sections were blocked with 1 N hydrochloric acid for 30 minutes for antigen retrieval, and other treatments were the same as for TNF-α immunohistochemical staining.
OX$_{42}$ immunohistochemical staining: The primary antibody was mouse anti-rat OX$_{42}$ monoclonal antibody (1: 2 000; BD), in which sections were incubated at room temperature for 24 hours. Then, the antibody solution was changed to the biotin-labeled goat anti-mouse immunohistochemical staining kit (Beijing Zhongshan Goldenbridge Biological Technology). No antigen retrieval was required; other treatments were the same as for TNF-α immunohistochemical staining.
IL-1β and IL-6 immunohistochemical staining: The primary antibodies were rabbit anti-rat IL-1β monoclonal antibody (1: 50; Wuhan Boster Bio-engineering Co., Ltd.) or rabbit anti-rat IL-6 monoclonal antibody (1: 50; Wuhan Boster Bio-engineering Co., Ltd.). All other treatments were the same as for TNF-α immunohistochemical staining.

Image analysis

The area of positive cells ($\times 10^3$ µm^2) in midbrain sections at one location (three slices from each rat) was quantified using the NIS-Elements pathological image analysis system (NIS-Elements Ver.2.1; Nikon Shanghai Company, Shanghai, China) with a Nikon optical microscope (ECLIPSE80i; Nikon, Tokyo, Japan).

Statistical analysis

SPSS 10.0 (SPSS, Chicago, IL, USA) was used for statistical analysis. Data are expressed as mean ± SD

and evaluated using one-way analysis of variance followed by the Student-Newman-Keuls (SNK-q) test. A value of $P < 0.05$ was considered statistically significant.

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