EFFECTS OF DHF ON OXIDATIVE STRESS IN MPTP-INDUCED PARKINSON'S DISEASE MODEL MICE

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ABSTRACT

Objective*: To investigate the effects of 7,8-dihydroxyflavone (DHF) on oxidative stress in Parkinson's disease (PD) model mice induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP).*

Methods*: Forty-five clean-grade, male C57BL/6 mice were randomly selected and fed for 1 week. Mice were randomly divided into a control group, model group, and DHF group, with 15 mice in each group. A PD mouse model was established and the rotating rod experiment, climbing rod experiment, and suspension experiment were conducted on each group. Immunohistochemical staining was performed to evaluate the number of tyrosine hydroxylase (TH)-positive neurons in the substantia nigra of mice in each group. The expression of α-synuclein (α-syn) in the brain tissue was detected by Western blotting. The levels of catalase (CAT), malondialdehyde (MDA), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) were also measured.*

Results*: Compared with the control group, the mice in the model group stayed on the roller for significantly shorter periods and took a significantly longer time to climb down the cylinder and onto the platform ($P < 0.05$). The mice in the DHF group stayed on the roller for significantly longer periods than the model group and required a significantly shorter time to climb down the cylinder and onto the platform ($P < 0.05$). Compared with the control group, the number of TH-positive neurons in the substantia nigra of the model group was significantly reduced ($P < 0.05$) and the corresponding number in the DHF group was significantly increased ($P < 0.05$). The expression level of α-syn in the brain tissue of the model group was significantly higher than that of the control group ($P < 0.05$), and the expression level of α-syn in the brain tissue of the DHF group was significantly lower than that of the model group ($P < 0.05$). Compared with the control group, the levels of CAT, GSH-Px, and SOD in the brain tissue of the model group were significantly lower and the levels of MDA were significantly higher. Compared with the model group, the levels of CAT, GSH-Px, and SOD in the brain tissue of the DHF group were significantly higher, while the levels of MDA were significantly lower ($P < 0.05$).*

Conclusion*: DHF can significantly improve motor dysfunction in PD mice and alleviate the injury caused by oxidative stress.*

Keywords: *DHF, MPTP, Parkinson's disease, oxidative stress.*

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Introduction

Parkinson's disease (PD), also known as tremor paralysis, is the second most common disorder of the central nervous system after Alzheimer's disease. Its main pathological features are progressive loss of dopaminergic neurons in the substantia nigra compact part of the brain, progressive decrease of dopamine content in the striatum, and formation of Lewis corpuscles, the main cause of disability, to which middle-aged individuals and those over the age of 65 years are predisposed[(1)]. At present, the etiology and pathogenesis of PD are not clear. It is believed that PD may be related to oxidative stress, immune responses, excitatory amino acid toxicity, apoptosis, and autophagy[(2)]. Currently, the main treatment method for PD is to improve the symptoms with drugs or intraocular therapy. Levodopa preparations are the most effective drugs for PD. However, long-term use of levodopa may cause symptom fluctuation or neuropsychiatric complications and thus cannot prevent further development of the disease[(3)].

7,8-dihydroxyflavone (DHF) is a tyrosine receptor kinase B (TrKB) agonist that can effectively activate TrKB, inhibit neuronal apoptosis, and has obvious neuroprotective effects[4]. It has been found that the loss of dopaminergic neurons in the substantia nigra of PD mice may be related to the overexpression of α-synuclein (α-syn) in neurons and oxidative stress. (5) This study investigates the effects of DHF on oxidative stress levels in PD mice induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP).

Materials and methods

Experimental animals

Forty-five clean, healthy male C57BL/6 mice (provided by Guangdong Medical Laboratory Animal Center, with production license no. SCXK [Guangdong] 2019-0035) weighing 21 ± 5 g were randomly selected.

Main instruments and reagents

Constant temperature water bath (Shanghai Hetian Scientific Instruments Co., Ltd., specification: HH-US); biomicroscopy (Shanghai Precision Instruments Co., Ltd., model: XSP-8C); low temperature and high-speed centrifuge (Shanghai Hetian Scientific Instruments Co., Ltd., model: TG18G); low-temperature refrigerator (Jinan Creative Biotechnology Co., Ltd., model: MDF-193); rabbit anti-mouse tyrosine hydroxylase (TH) monoclonal antibody (Amy; Jie Technology Co., Ltd.); Catalase (CAT) detection kit (Laier Biomedical Technology Co., Ltd.); superoxide dismutase (SOD) detection kit (Shanghai Jingkang Bioengineering Co., Ltd.); glutathione peroxidase (GSH-Px) detection kit (Shanghai Jining Biotechnology Co., Ltd.); malondialdehyde (MDA) detection kit (Mosak Biotechnology Co., Ltd.); DHF (Wuhan Xinxinjiali Biotechnology Co., Ltd., specifications: 1/kg); MPTP (Beijing Zhongruitai Technology Co., Ltd.).

Establishment of animal models and experimental grouping

The mice had free access to food and drink for 12 hours in the day and were kept at a temperature of 25 ± 1 oC and 55 ± 15% humidity for 1 week. Mice were randomly divided into a control group, model group, and DHF group, with 15 mice per group. The control group mice did not receive any intervention. The model group and DHF group mice were intraperitoneally injected with 25 mg/kg MPTP once a day for 6 consecutive days. The DHF group mice were simultaneously intraperitoneally injected with 5 mg/kg DHF. All mice were sacrificed after the experiment.

Observation indicators

The following behavioral tests were conducted on the mice. 1. Rotary rod experiment: mice were placed onto a roller rotating at a speed of 20 rpm 3 times for 3 minutes each to evaluate their adaptation. The residence time of the mice on the roller was measured 4 times with 60-minute intervals per round. 2. Rod climbing experiment: a 45 cm long cylinder with a diameter of 1 cm was selected and fixed on the ground. Rubber bands were wound around the cylinder at intervals to increase traction force. The mice were placed on top of the cylinder and the time taken to climb down was measured. Average values were obtained from multiple tests. Water and 70% ethanol were used for cleaning between tests. 3. Suspension experiment: an 80 cm long hemp thread was fixed between 2 platforms at a height of 25 cm, the mice were placed in the middle of the 2 platforms, and the time required for the mice to climb to either of the platforms was measured as the average of multiple tests.

After the behavioral tests were conducted, 7 mice in each group were anesthetized. The brain tissues of the mice were collected and fixed with 4% formaldehyde. Paraffin sections were made as per the routine procedure. Each section was sliced to 5 μm thickness using a slicer. Immunohistochemical staining was performed to evaluate the number of TH-positive neurons in the substantia nigra of each group. The expression of α-syn in the brain tissue was detected by Western blotting.

The brain tissues of the remaining 8 mice in each group were extracted. Fresh brain tissues were separated and homogenized. A suitable amount of lysate was added and ground on ice until the tissues were fully broken down. Catalase (CAT), malondialdehyde (MDA), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) levels were then measured after centrifugation at 12,000 rpm using the corresponding test kits.

Statistical methods

SPSS 21.0 software package was used for statistical analysis in this study. The independent sample t-test was used to compare the measurement data between 2 groups, and single-factor multiple sample mean comparison was used for multiple groups. The chi-square test was used to compare the counting

data and the ridit test was used to compare grade data. A value of $P < 0.05$ was considered statistically significant.

Results

Comparison of behavioral test results of mice in each group

Compared with the control group, the residence time of the model group mice on the roller was significantly shorter, and the time needed to climb down the cylinder and onto the platform was significantly longer ($P < 0.05$). The residence time of the DHF group mice on the roller was significantly longer than that of the model group, and the time needed to climb down the cylinder and onto the platform was significantly shorter than that of the model group ($P < 0.05$). The results are presented in Table 1.

Group	n	Rotary rod experiment (s)			
		First	Second	Third	Fourth
Control	15	141.26 ± 32.46	237.71 ± 28.42	241.33 ± 35.65	266.17 ± 25.11
Model	15	50.34 ± 12.53[a]	75.17 ± 25.23[a]	114.51 ± 53.31[a]	151.55 ± 34.73[a]
DHF	15	112.53 ± 13.75[ab]	126.44 ± 27.51[ab]	194.11 ± 15.73[ab]	212.76 ± 27.85[ab]
F		69.441	141.174	42.403	56.673
P		< 0.001	< 0.001	< 0.001	< 0.001

Group	n	Rod climbing experiment (s)	Suspension experiment (s)
Control	15	5.72 ± 0.18	10.56 ± 1.33
Model	15	7.91 ± 0.22[a]	15.27 ± 1.14[a]
DHF	15	6.32 ± 0.53[ab]	12.37 ± 0.75[ab]
F		159.341	108.062
P		< 0.001	< 0.001

Table 1: Comparison of behavioral tests of mice in each group (mean ± SD).

Number of TH-positive neurons in the substantia nigra of mice in each group

Compared with the control group, the number of TH-positive neurons in the substantia nigra of the model group was significantly reduced ($P < 0.05$), and the number of TH-positive neurons in the substantia nigra of the DHF group was significantly increased ($P < 0.05$). See Figure 1 and Table 2..

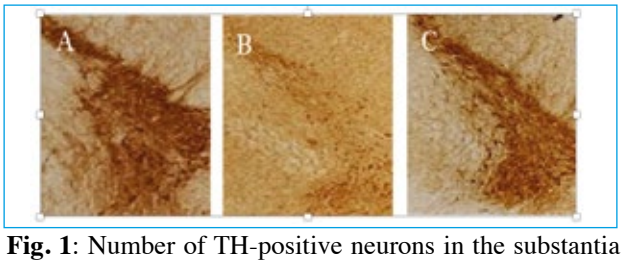

Fig. 1: Number of TH-positive neurons in the substantia nigra of mice in each group. **A**: control group; **B**: model group; **C**: DHF group

Group	n	Number of TH-positive neurons in the substantia nigra (%)
Control group	7	100.01 ± 0.01
Model group	7	60.37 ± 4.51[a]
DHF	7	79.54 ± 3.27[ab]
F		265.923
P		< 0.001

Table 2: Number of TH-positive neurons in substantia nigra of mice in each group (mean ± SD).
a: compared with the control group, aP < 0.05; b: compared with the model group, bP < 0.05

Expression of α-syn in the brain tissue of mice in each group

The expression level of α-syn in the brain tissue of the model group was significantly higher than that of the control group ($P < 0.05$), and the expression level of α-syn in the brain tissue of the DHF group was significantly lower than that of the model group ($P < 0.05$). See Figure 2 and Table 3.

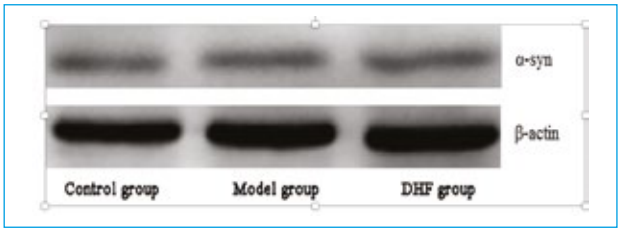

Fig. 2: Expression of α-syn in the brain tissue of mice in each group.

Group	n	Expression of α-syn in brain tissue (%)
Control group	7	100.01 ± 0.02
Model group	7	149.28 ± 18.03[a]
DHF	7	121.09 ± 2.46[ab]
F		38.754
P		< 0.001

Table 3: Expression of α-syn in the brain tissue of mice in each group.
a: compared with the control group, aP < 0.05; b: compared with the model group, bP < 0.05

Comparison of oxidative stress in the brain tissue of mice in each group

Group	n	CAT (U/mg)	MDA (nmol/mg)	GSH-Px (mg/g)	SOD (U/mg)
Control	8	34.74 ± 2.34	3.36 ± 1.09	19.99 ± 1.08	42.28 ± 1.59
Model	8	13.15 ± 1.78[a]	7.81 ± 1.79[a]	6.07 ± 0.92[a]	27.42 ± 1.83[a]
DHF	8	26.38 ± 1.05[ab]	4.98 ± 1.18[ab]	14.34 ± 2.04[ab]	35.13 ± 1.87[ab]
F		291.822	21.052	190.521	141.411
P		< 0.001	< 0.001	< 0.001	< 0.001

Table 4: Comparison of oxidative stress indexes in brain tissue of mice in each group ($\bar{x} \pm s$).
a: compared with the control group, aP < 0.05; b: compared with the model group, bP < 0.05

Compared with the control group, the levels of CAT, GSH-Px, and SOD in the brain tissue of the model group were significantly lower and the levels of MDA were significantly higher. Compared with the model group, the levels of CAT, GSH-Px, and SOD in the brain tissue of the DHF group were significantly higher, while the levels of MDA were significantly lower ($P < 0.05$). The results are presented in Table 4.

Discussion

PD is a chronic degenerative disease of the nervous system in middle-aged and elderly people. Its main manifestations include motor or non-motor symptoms such as quiescent tremor, bradykinesia, muscle rigidity, abnormal posture and gait, cognitive/mental disorders, and sleep disorders. The onset of PD is insidious, and the condition is often progressively aggravated. It has been reported that the degeneration, necrosis, and glial cell proliferation of dopaminergic neurons in the polynigral striatum are directly related to PD[6,7]. MPTP is a neurotoxin commonly used to induce PD animal models. It can significantly enhance the activity of monoamine oxidase, reduce the activity of mitochondrial complex enzyme I, and effectively cross the blood-brain barrier and be converted into 1-methyl-4-phenylpyridine by monoamine oxidase. 1-methyl-4-phenylpyridine can be recognized by dopamine transporters and selectively enter the dopaminergic neurons of the midbrain substantia nigra. The corresponding decrease in ATP levels and increase in oxidative stress lead to the degeneration and apoptosis of dopaminergic neurons in the substantia nigra compact part of the midbrain[8,9]. This study aimed to investigate the effects of DHF on oxidative stress in PD mice induced by MPTP.

DHF is a small-molecule compound with high affinity that can specifically bind to the TrkB receptor through the blood-brain barrier, promote TrkB receptor dimerization and autophosphorylation, and activate the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathway to produce a series of cascade reactions[10]. Wang et al. reported that DHF plays an important role in alleviating dyskinesia symptoms in PD patients.11 Motor symptoms are one of the main clinical symptoms of PD. The rotary rod test, climbing rod test, and suspension test were used to evaluate the motor function of mice in this study. It was found that, compared with the control group, the mice in the model group stayed on the roller for significantly shorter periods, and the time needed to climb down the cylinder and onto the platform was significantly longer ($P < 0.05$). In contrast, the mice in the DHF group stayed on the roller for significantly longer periods than the control group, and compared with the model group, the time required to climb down the cylinder and onto the platform was significantly shorter ($P < 0.05$). These results suggest that DHF can significantly improve MPTP-induced dyskinesia in Parkinson's model mice, supporting the findings of Wang et al[11].

TH is a rate-limiting enzyme for dopamine synthesis in vivo. Tyrosine can be converted into dopamine by tetrahydropterin and molecular oxygen. The main pathological characteristics of PD are the decrease in the number of dopaminergic neurons in the substantia nigra and the decrease in TH-positive cells. Therefore, TH is often used to evaluate the expression level and function of dopaminergic neurons in the substantia nigra[12], In this study, compared with the control group, the number of TH-positive neurons in the substantia nigra of the model group was significantly reduced ($P < 0.05$), and the number of TH-positive neurons in the substantia nigra of the DHF group was significantly increased ($P < 0.05$). These results suggest that DHF can significantly reduce the loss of dopamine neurons in the substantia nigra and has neuroprotective effects on dopaminergic neurons in the substantia nigra.

α-syn is a soluble protein expressed in the presynaptic and perinuclear regions of the central nervous system. It is the main component of Lewis bodies. Its expression level is closely related to the pathogenesis and related dysfunction of PD[13]. It has been found that over-expression of α-syn may lead to oxidative stress and abnormal mitochondrial function in central nervous system tissues[14]. Our results showed that DHF could significantly inhibit the overexpression of α-syn.

It is reported that oxidative stress is closely related to the occurrence and development of PD, and oxidative stress can significantly reduce the level of antioxidants in the midbrain, thus degrading dopaminergic neurons in the substantia nigra of the midbrain[15]. CAT and SOD are the main enzymes that scavenge oxygen free radicals. GSH-Px can protect the results and functions of cell membranes from interference and damage by peroxides. The production of MDA can aggravate membrane damage, and its levels can indirectly measure the degree of damage to the membrane system. The results of this study suggest that DHF can significantly enhance the anti-

oxidant capacity of mice and reduce the toxic reactions caused by oxidative stress.

In conclusion, oxidative stress contributes to the occurrence and development of MPTP-induced PD. DHF has a neuroprotective effect on MPTP-induced PD and can significantly inhibit oxidative stress and α-syn overexpression and improve motor dysfunction in PD mice.

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