

Original Article

Morin exerts neuroprotective actions in Parkinson disease models *in vitro* and *in vivo*

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Aim: To investigate the neuroprotective effects of morin on 1-methyl-4-phenylpyridinium ion (MPP⁺)-induced apoptosis in neuronal differentiated PC12 cells as well as in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of Parkinson disease (PD).

Methods: PC12 cells were challenged with MPP⁺ in the presence or absence of morin. Cell viability was determined using MTT assay. Cell apoptosis was measured using flow cytometry. Generation of reactive oxygen species (ROS) was assayed using fluorescence assay. In an MPTP mouse model of PD, behavioral deficits, striatal dopamine content, and number of dopaminergic neurons were measured.

Results: MPP⁺ induced apoptosis and ROS formation in PC12 cells. Concomitant treatment with morin (5–50 μmol/L) significantly attenuated the loss of cell viability and apoptosis when compared with MPP⁺ treatment alone. Morin also attenuated ROS formation induced by MPP⁺. MPTP induced permanent behavioral deficits and nigrostriatal lesions in mice. When administered prior to MPTP, morin (20 to 100 mg/kg) attenuated behavioral deficits, dopaminergic neuronal death and striatal dopamine depletion in the MPTP mouse model.

Conclusion: The findings suggest that morin has neuroprotective actions both *in vitro* and *in vivo*, and may provide a novel therapeutic agent for the treatment of PD and other neurodegenerative diseases.

Keywords: morin; apoptosis; PC12 cells; MPTP; Parkinson disease; dopamine

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Introduction

Parkinson disease (PD), one of the most common neurodegenerative disorders, is characterized by the progressive degeneration of dopaminergic neurons in the substantia nigra^[1,2]. Oxidative stress and mitochondrial dysfunction are considered the most important causal factors of neuronal death in PD^[3,4]. During oxidative stress, ROS induce the release of cytochrome *c* from the mitochondria and the consequent activation of the caspase family, which plays a major role in cell apoptosis. Once caspase-3 is activated, it will induce nuclear DNA condensation and fragmentation and, ultimately, apoptosis^[5]. Therefore, drugs that can prevent ROS-induced cell injury might be an appropriate choice for the treatment of PD.

MPTP and its active metabolite MPP⁺ can cause cell death by inhibiting complex I of the electron transport chain and promoting the production of ROS. Administration of these toxins has been used extensively to create models of PD to

screen for neuroprotective agents^[6]. A number of antioxidants, such as polyphenols, have been documented to have a protective effect on vulnerable neurons under neurodegenerative conditions^[7–10]. Morin (3,5,7,2',4'-pentahydroxyflavone, Figure 1) is a natural bioflavonoid that was originally isolated from members of the *Moraceae* family and is a constituent of many herbs, fruits and wine. Previous studies have shown that morin exerts antioxidant, anti-inflammatory, and antiproliferative effects *in vivo* and *in vitro*^[11–13].

Studies investigating the neuroprotective effects of morin have been inconclusive. For example, morin was found to be neuroprotective in a model of ischemic brain damage, and this effect was attributed to its antioxidant and antiapoptotic

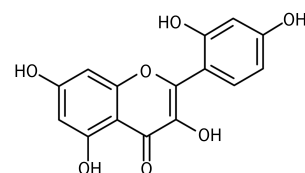


Figure 1. The chemical structure of morin.

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properties^[14]. However, in another *in vitro* study, morin and other antioxidants failed to protect plastic adhering PC12 cells from MPP⁺ toxicity. In that study, the concentration of MPP⁺ was very high (5 mmol/L), and the concentration of morin was not given^[15]. Neuronal differentiated PC12 cells acquire a neuronal phenotype similar to that of dopaminergic neurons, including exit from the cell cycle and extension of long neurites, and thus provide a good model for the study of neuroprotective effects of morin. In this study, we investigated the effects of morin both in neuronal differentiated PC12 cells and in an MPTP mouse model of PD.

Materials and methods

Cell culture and drug treatment

PC12 cells were obtained from the Chinese Type Culture Collection. They were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 5% horse serum (Gibco), 100 µg/mL streptomycin, and 100 units/mL penicillin in a water-saturated atmosphere of 5% CO₂ at 37 °C. One week before drug treatment, nerve growth factor (NGF) was added to the cultures at a final concentration of 50 ng/mL to induce neuronal differentiation. MPP⁺ (Sigma, St Louis, MO) and morin (Sigma) was dissolved in PBS (pH=7.4) or dimethyl sulfoxide (DMSO), respectively, and was prepared as a 10 mmol/L stock immediately before use. The drugs were diluted in culture medium to the indicated final concentrations. The total volume of the reaction system in the following experiments was 5 mL. The final concentration of DMSO in the medium was less than 0.5%.

Measurement of cell viability

PC12 cells were plated at a density of 1×10⁴ cells per well in 96-well plates. Cell viability was determined by the conventional MTT reduction assay. The cells were exposed to different concentrations of MPP⁺ for 24 h in the presence or absence of morin. The cells were then incubated with MTT (0.25 mg/mL) for 4 h at 37 °C, and the reaction was stopped by adding solution containing 50% dimethylformide and 20% sodium dodecyl sulfate (pH 4.8). The amount of MTT formazan product was determined by measuring the absorbance with a microplate reader (Bio-Rad, Hercules, CA) at a test wavelength of 570 nm. Cell viability was expressed as a percentage of the value in untreated control cells.

Assessment of apoptosis by flow cytometry

The Annexin V/PI apoptosis detection kit (Sigma) was used to assess membrane and nuclear events that occurred during apoptosis. After treatment, 2×10⁶ cells were harvested, washed with ice-cold PBS, and then incubated with 5 µL of Annexin V-FITC and 10 µL 20 µg/mL PI for 10 min in the dark at room temperature. The FITC and PI signals were measured with a BD-LSR flow cytometer using the CellQuest software, and 10000 events were acquired. Quadrants were positioned on Annexin V/PI dot plots, allowing live cells (Annexin V-/PI-), early/primary apoptotic cells (Annexin V+/PI-), late/

secondary apoptotic cells (Annexin V+/PI+) and necrotic cells (Annexin V-/PI+) to be distinguished.

Measurement of caspase-3 activity

Apoptosis-induced activation of caspase-3 was assessed using the Caspase-3 Colorimetric Assay Kit (Abcam, Cambridge, UK) according to the manufacturer's instructions. Briefly, cells were collected after drug treatment and then lysed in chilled lysis buffer. Cell homogenates were then incubated with DEVD-pNA substrate (final concentration, 200 µmol/L) in reaction buffer at 37 °C for 2 h. The absorbance of each sample was read at 405 nm in a microplate reader (BioRad). Activity of caspase-3 was presented as the percentage of control.

Measurement of intracellular reactive oxygen species formation

Intracellular ROS was measured using the fluorescent probe 2,7-dichlorofluorescein diacetate (H₂DCFDA). H₂DCFDA is oxidized to the highly fluorescent compound dichlorofluorescein (DCF) by intracellular hydroperoxides. The generation of DCF is proportional to intracellular ROS levels. After exposure to 75 µmol/L MPP⁺ in the presence or absence of morin for 24 h, PC12 cells were incubated with 10 µmol/L H₂DCFDA (Sigma) at 37 °C for 30 min. The cells were washed twice with PBS and then dissolved with 1% Triton X-100. Fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a fluorescence microplate reader.

Animals and treatments

Ten-month-old male B57/BL mice weighting 20–25 g were purchased from the Laboratory Animal Center of Tongji Medical College (Wuhan, China). All animal procedures were approved by the Catholic Ethics Committee of the Tongji Medical College and were carried out in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals. The MPTP mouse model of PD was developed according to the procedures described previously^[16]. Four mice were used for each group. Mice in the MPTP group received one intraperitoneal injection of 30 mg/kg free base MPTP (Sigma) daily for five consecutive days. The control group received an equivalent volume of saline as vehicle. Morin was given at 5, 20, 40, or 100 mg/kg body weight as daily intraperitoneal injections during the whole experiment. Treatment was started 5 d before the first MPTP intoxication and continued to be administered daily, 6 h before the MPTP injections.

Behavioral experiments of the mouse MPTP model

Motor behavior was evaluated using two previously validated behavioral tests at 7 d after the last dose of MPTP. The static and dynamic motor abilities were assessed using the bar test^[17] and the drag test^[18], respectively. In the bar test, each mouse was placed on a table and the forepaws were placed on a horizontal steel bar (diameter 0.3 cm) elevated 3 cm above the tabletop. The duration for which each mouse maintained position with both forepaws on the bar and both hindpaws on the tabletop was recorded as the cataleptic time. In the drag test,

each mouse was lifted by the tail (allowing forepaws on the table) and dragged backward at a constant speed (20 cm/s) for 100 cm. The steps made by each paw were counted.

Determination of striatal dopamine level by HPLC

Mice from each group were sacrificed by cervical dislocation at 7 d after the last dose of MPTP. The striatal samples were sonicated in ice-cold 0.1 mol/L HClO₄ containing 0.01% EDTA and centrifuged at 15000×g for 15 min at 4 °C. Twenty μL supernatant was injected into a high-performance liquid chromatography (HPLC) system equipped with fluorescence detection (Waters, MA). The Lichrosorb Column (C18, 10 μm, 25 cm×4.6 mm, Waters) was employed. The mobile phase consisted of trisodium citrate (0.02 mol/L), sodium dihydrogen phosphate (0.05 mol/L), methanol (40%), EDTA (0.028 g/L), and SDS (0.15 g/L). The solution was adjusted pH 3.0 with H₂SO₄ and filtered through a 0.45 μm membrane and degassed. Flow rate was set to 1.0 mL/min. The standard preparation was bought from the National Institute for the Control of Pharmaceutical and Biological Products. The linear calibration curves were obtained over the range of 6.25–1000 ng/mL ($r=0.9994$). The average recovery rate of dopamine was 98.7%. The precision of the method was good (RSD =0.3%).

Tyrosine hydroxylase immunohistochemistry

Mice were anesthetized with sodium pentobarbital (50 mg/kg) at 7 d after the last dose of MPTP and perfused transcardially with freshly prepared 4% paraformaldehyde in 0.1 mol/L sodium phosphate buffer (pH 7.4), followed by an overnight immersion in the same fixative solution at 4 °C. The sections were cut on a cryostat (35 μm) and collected free-floating. After blocking for 1 h in PBS containing 10% horse serum, the sections were incubated with a mouse monoclonal anti-tyrosine hydroxylase (TH) antibody (1:5000, Sigma) overnight at 4 °C. After a 15-min rinse in fresh PBS, the sections were incubated with biotinylated goat anti-mouse IgG for 2 h, followed by incubation with avidin-biotin peroxidase complex for 30 min at room temperature. Immunoreactivity was visualized with 0.05% diaminobenzidine (DAB). Cell counts were determined from three anatomically matched sections from each of the animals and dividing by three, yielding cell count per section^[19].

Statistical analyses

Data are expressed as the mean±SD of at least four experiments. Comparisons between groups were analyzed by ANOVA (analysis of variance) followed by Dunnett's post-hoc analysis. The level of significance was set at 0.05.

Results

Morin attenuates MPP⁺-induced loss of cell viability

We first determined the neurotoxic effect of MPP⁺ on neuronal differentiated PC12 cells. Our results indicated that MPP⁺ induced a decrease in cell viability in a concentration-dependent manner. Cell viability was 59.75%±6.50% of control cul-

tures after exposure to 75 μmol/L MPP⁺ for 24 h, and further decreased to 42.50%±7.23% after exposure to 150 μmol/L MPP⁺ (Figure 2A). Based on these results, 75 μmol/L MPP⁺ was used in the following experiments to assess the neuroprotective effect of morin.

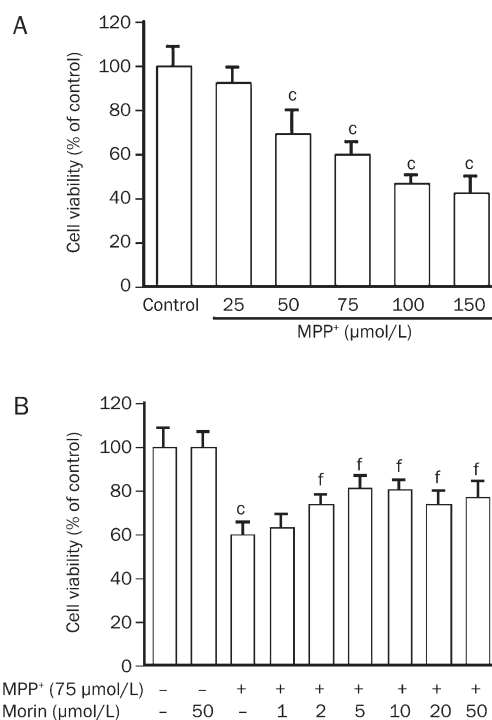


Figure 2. Morin prevents MPP⁺-induced loss of cell viability. (A) Neuronal differentiated PC12 cells were exposed to different concentrations of MPP⁺ (25, 50, 75, 100, and 150 μmol/L) for 24 h. Cell viability was determined by the MTT method. (B) PC12 cells were exposed to 75 μmol/L MPP⁺ for 24 h in the presence or absence of different concentrations of morin (1, 2, 5, 10, 20, and 50 μmol/L) for 24 h. Cell viability was determined using the MTT assay. Data are expressed as percentage of values in untreated control cultures, and are the means±SD of four separate experiments. * $P<0.01$ compared with control. † $P<0.01$ compared with MPP⁺ alone.

Morin (50 μmol/L) alone did not affect the cell viability of differentiated PC12 cells. When incubated together with 75 μmol/L MPP⁺, morin (2, 5, 10, 20, or 50 μmol/L) significantly protected PC12 cells from MPP⁺ toxicity. When the cells were co-incubated with the indicated concentrations of morin for 24 h, the cell viability was 73.78%±4.55%, 81.24%±5.48%, 80.53%±4.60%, 73.89%±5.95%, and 76.98%±7.68% of the control group, respectively. The maximal rescue occurred at a concentration of 5 μmol/L morin. The protective effect of morin did not increase beyond this concentration (Figure 2B).

Morin attenuates MPP⁺-induced apoptosis

Flow cytometric analysis demonstrated that the predominant cell death of PC12 cells after exposure to MPP⁺ occurred via apoptosis. The apoptotic rate after exposure to 75 μmol/L MPP⁺ was 35.04%±3.72% at 24 h. Morin significantly attenu-

ated cell apoptosis induced by MPP⁺ (15.22%±4.14% in the presence of 5 μmol/L morin, Figure 3A).

To further investigate the cellular pathway of morin-induced protection, we assessed the caspase-3 activity. After exposure to 75 μmol/L MPP⁺ for 24 h, caspase-3-like activity was about 1.88-times the control value. However, simultaneous treatment with morin effectively attenuated the increase of caspase-3-like activity. Consistent with the results of the cell viability and apoptotic assays, morin inhibited caspase-3 activity and exerted its maximal effect at 5 μmol/L (1.39 times the control value, Figure 3B).

Effect of morin on ROS formation

After exposure to 75 μmol/L MPP⁺, the fluorescence intensity of DCF increased to about twice the control value. Morin prevented the MPP⁺-induced increase in DCF fluorescence and showed a maximal inhibitory effect at 5 μmol/L (1.37-times of the control value, Figure 4). ROS formation was not further attenuated by higher concentrations of morin (20 and 50 μmol/L).

Effect of morin on MPTP-induced motor behavioral deficits in mice

To determine whether our *in vitro* findings were of relevance to the *in vivo* system, we examined the effect of morin on motor behavior in an MPTP mouse model of PD. MPTP administration resulted in significantly longer cataleptic times in the bar test and a reduced number of steps in the drag test. These results suggest that the mice experienced motor behavioral deficits after MPTP treatment. Morin injection (5 mg/kg) did not significantly affect the behavioral deficits, but morin

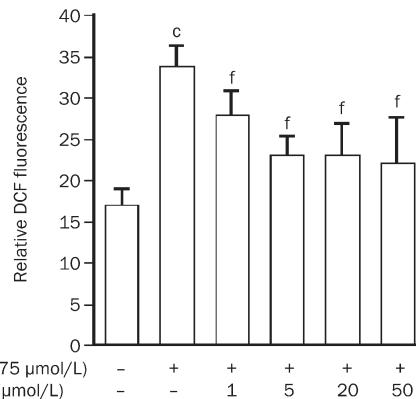


Figure 4. Morin attenuates MPP⁺-induced ROS formation. Formation of ROS was assayed by measuring the fluorescence of dichlorofluorescein (DCF). Data are expressed as the mean±SD of four separate experiments. ^c*P*<0.01 compared with control. ^f*P*<0.01 compared with MPP⁺ alone.

from 20 mg/kg to 100 mg/kg was sufficient to significantly attenuate the behavioral deficits induced by MPTP. Morin 20 mg/kg, 40 mg/kg, and 100 mg/kg had similar effect on motor behavioral deficits (Figure 5).

Effect of morin on striatal dopamine depletion in MPTP mouse model

Seven days after the last MPTP injection, there was a significant decrease of striatal dopamine in MPTP mice (from 8.07±0.40 ng/mg tissue to 3.14±0.34 ng/mg tissue). Pretreatment with 5 mg/kg morin did not affect the striatal dopamine content, but morin at a dose of 20 mg/kg, 40 mg/kg or

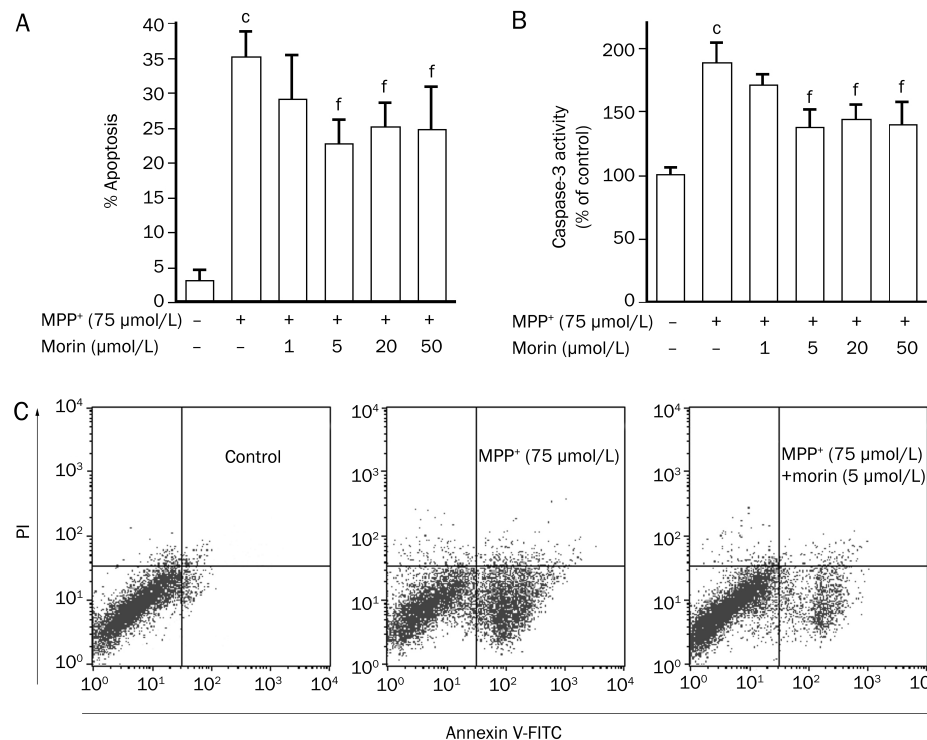


Figure 3. Morin prevents MPP⁺-induced PC12 cell apoptosis. (A) Differentiated PC12 cells were exposed to 75 μmol/L MPP⁺ in the presence or absence of different concentrations of morin (1, 5, 20, 50 μmol/L) for 24 h, double-stained with Annexin V/PI, and analyzed using flow cytometry. The bar chart shows the percentage of apoptotic cells. (B) Cell lysates were assayed for the cleavage of fluorogenic caspase-3 substrates as described in “Materials and methods”. (C) Representative images of apoptotic cells visualized using flow cytometry after Annexin V/PI double-staining. Data are expressed as the mean±SD of four separate experiments. ^c*P*<0.01 compared with control. ^f*P*<0.01 compared with MPP⁺ alone.

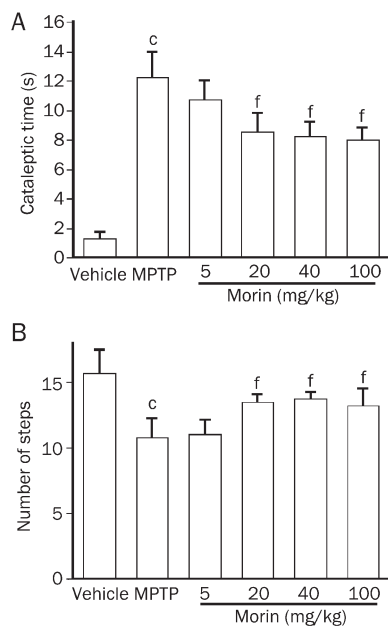


Figure 5. Effect of morin on MPTP-induced behavioral deficits in mice. Mice were pre-treated with morin (5, 20, 40, or 100 mg/kg) for five days and then injected with MPTP (5×30 mg/kg ip, 24-h interval). Motor behavioral deficits were assessed by bar test (A) and drag test (B) at 7 d after the last dose of MPTP. Values are presented as the mean±SD of four separate experiments. ^c*P*<0.01 compared with saline control. ^f*P*<0.01 compared with MPTP alone.

100 mg/kg significantly attenuated striatal dopamine depletion induced by MPTP (4.86±0.20 ng/mg tissue, 5.25±0.29 ng/mg tissue and 5.34±0.29 ng/mg tissue, respectively, Figure 6).

Effect of morin on dopaminergic neuronal survival in the MPTP mouse model

Tyrosine hydroxylase (TH) is the rate-limiting enzyme in dopamine biosynthesis and is considered the definitive marker of dopaminergic neurons. Immunostaining of the substantia nigra demonstrated that TH-positive neurons were abundant in substantia nigra of the saline control mice. MPTP treatment led to a significant loss of TH-positive neurons (from 158.16±9.09 per section to 69.92±13.13 per section). However, pretreatment with morin (40 mg/kg) significantly attenuated the loss of dopaminergic neurons (110.94±18.52 per section, Figure 7).

Discussion

The present study provides *in vitro* and *in vivo* evidence that morin is a potent neuroprotective agent in a model of PD. The pattern of PC12 cell death induced by MPP⁺ is still controversial. Some studies have demonstrated that PC12 cells die by apoptosis after exposure to MPP⁺, particularly at low concentrations^[20], while other studies indicated that MPP⁺-treated PC12 cells die by necrosis^[21]. This discrepancy may be due to different culture conditions. The type of culture

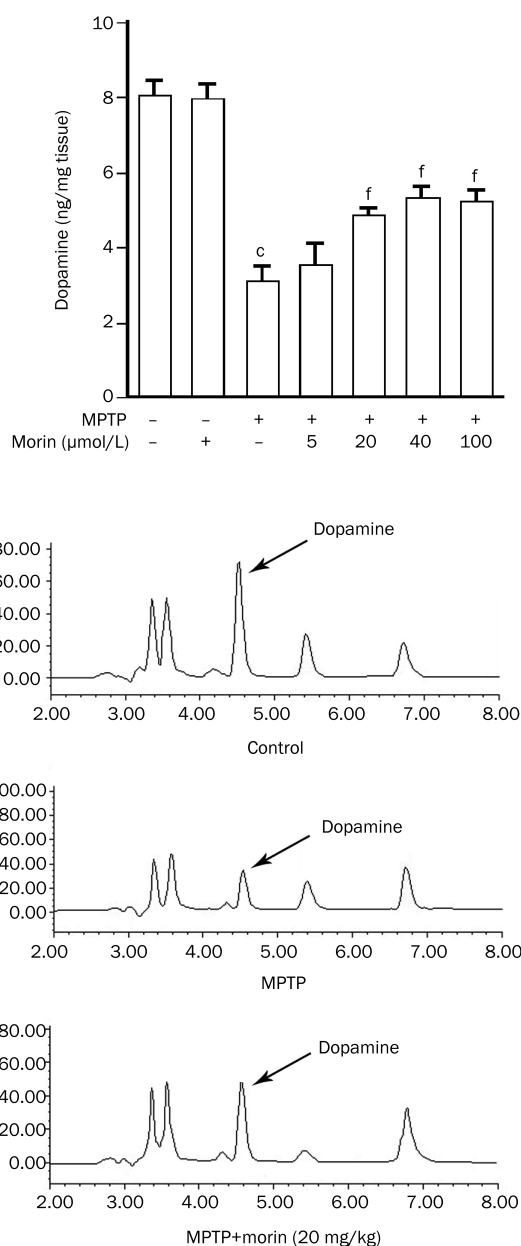


Figure 6. Morin attenuates striatal dopamine depletion in MPTP-lesioned mice. Mice were pre-treated with morin (5, 20, 40, or 100 mg/kg) for five days, and then injected with MPTP (5×30 mg/kg ip, 24-h interval). Striatal dopamine level was detected by HPLC at 7 d after the last dose of MPTP. Values are presented as the mean±SD of four separate experiments. ^c*P*<0.01 compared with saline control. ^f*P*<0.01 compared with MPTP alone.

medium used (DMEM or RPMI 1640) and concentrations of serum may affect the reaction of PC12 cells to MPP⁺. In our study, PC12 cells were first differentiated by NGF, and then MPP⁺ was added to the DMEM culture medium. Flow cytometric analysis demonstrated signs of early apoptotic death. Furthermore, caspase-3 activity was found to increase in MPP⁺-induced neuronal death. These results confirmed that MPP⁺ induces apoptotic death under the current conditions.

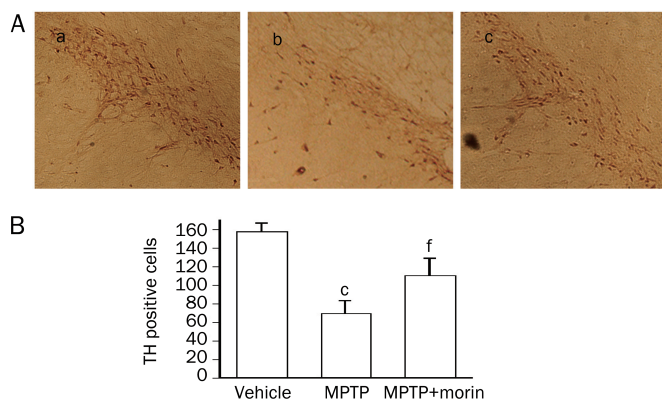


Figure 7. Morin protects dopaminergic neurons from MPTP toxicity in mice. Mice were pre-treated with morin for five days, and then injected with MPTP (5×30 mg/kg ip, 24-h interval). Dopaminergic neurons were immunostained with TH antibody. Photographs were taken at a magnification of 200×. (A) Panels a-c show representative pictures of the substantia nigra pars compacta. a) Control group. b) MPTP-treated group (5×30 mg/kg ip, 24-h interval). c) Morin (20 mg/kg)+MPTP group. (B) Number of dopaminergic cell bodies in the substantia nigra pars compacta. Values are presented as the mean±SD of four separate experiments. ^c*P*<0.01 compared with control. ^f*P*<0.01 compared with MPTP alone.

Morin is a natural polyphenolic compound. Some polyphenols have been shown to exert neuroprotective effects in several models of neurodegenerative diseases. For example, (-)-epigallocatechin-3-gallate, the main polyphenolic constituent of green tea, protects neuroblastoma SH-SY5Y cells from rotenone-induced cytotoxicity^[22]. On the other hand, there is evidence that other polyphenols, such as quercetin, have no beneficial effect on 6-hydroxydopamine-induced rodent parkinsonian models and dopaminergic cell cultures^[23]. These results suggest that the specific chemical structures of individual species of polyphenols are crucial for determining their biological effects. Morin is highly hydroxylated, and it has been shown to exert potent antioxidant effects *in vitro*^[24]. In the present study, we show that morin attenuated MPP⁺-induced loss of cell viability and apoptosis in PC12 cells, suggesting that morin is a potential therapeutic molecule for the treatment of neurodegenerative diseases.

MPP⁺ has been shown to induce oxidative stress *in vitro* and *in vivo*^[25-27]. In agreement with its antioxidant activity^[24, 28], morin attenuated ROS formation. Therefore, the neuroprotective mechanism of morin may be attributed to its antioxidant activity. However, morin also has other bioactivities, such as inhibiting the overactivation of calpain and NF-κB^[14, 29], which have been proposed to participate in the pathogenesis of neuronal death in PD^[30].

In the MPTP mouse model, morin significantly rescued the motor behavioral deficits as assessed by the bar test and drag test. A potential therapeutic drug for PD could be expected to achieve behavioral benefits via two avenues: by direct stimulation of the dopaminergic receptor or by protecting

the dopaminergic neurons from MPTP toxicity. To distinguish between these two possibilities, we further assessed the striatal dopamine level and the amount of dopaminergic cell bodies in the substantia nigra pars compacta. Our results indicated that morin significantly attenuated both striatal dopamine depletion and the loss of dopaminergic neurons. Thus, we propose that morin maintains nigrostriatal pathway function by enhancing the survival of dopaminergic neurons in MPTP-lesioned mice. Besides the antioxidant activity that we detected in the *in vitro* model, the anti-inflammatory activity of morin may also contribute to its neuroprotective effects *in vivo*^[12, 31].

In conclusion, the present study indicates that morin has robust neuroprotective properties. Furthermore, morin is a natural compound that exists widely in dietary supplements and herbs and there is evidence that dietary polyphenols can cross the blood brain barrier^[32, 33]. More importantly, as a natural bioflavonoid, the safety of morin has been well-established by previous studies. No abnormal clinical signs were observed in rodents receiving dietary administration of morin up to 5% (*w/w*) for 13 weeks^[34], or intraperitoneal injection of morin at 75 mg/kg body weight^[35]. These properties make morin a potential neuroprotective agent for the prevention and treatment of PD.

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Author contribution

Tao WANG, Xue-bing CAO, Zhen-tao ZHANG, and Sheng-gang SUN designed the research; Tao WANG, Zhen-tao ZHANG, Nian XIONG, and Hong-cai WANG performed the research; Zhen-tao ZHANG and Jin-sha HUANG analyzed the data; Zhen-tao ZHANG and Tao WANG wrote the paper.

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