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•Research article•

Demethylenetetrahydroberberine protects dopaminergic neurons in a mouse model of Parkinson's disease

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[ABSTRACT] Parkinson's disease (PD) is a multifactorial disorder of the nervous system where a progressive loss of dopaminergic neurons exist. However, the pathogenesis of PD remains undefined, which becomes the main limitation for the development of clinical PD treatment. Demethylenetetrahydroberberine (DMTHB) is a novel derivative of natural product berberine. This study was aimed to explore the neuroprotective effects and pharmacological mechanism of DMTHB on Parkinson's disease using C57BL/6 mice. A PD model of mice was induced by administration of MPTP (20 mg·kg^{-1}) and probenecid (200 mg·kg^{-1}) twice per week for five weeks. The mice were administered with DMTHB daily by gavage at the dose of 5 and 50 mg·kg^{-1} for one- week prophylactic treatment and five-week theraputic treatment. The therapeutic effects of DMTHB were evaluated by behavior tests (the open field, rotarod and pole tests), immunohistochemical staining of tyrosine hydroxylase (TH), Nissl staining and biochemical assays. The molecular mechanisms of DMTHB on the key biomarkers of PD pathological states were analyzed by Western blot (WB) and qRT-PCR. DMTHB treatment alleviated the behavioral disorder induced by MPTP-probenecid. Nissl staining and TH staining showed that the damage of dopaminergic neurons in the substantia nigra was remarkably suppressed by DMTHB treatment. Western blot results showed that the ratio of Bcl-2/Bax and TH increased, but the level of *α*-synuclein (*α*-syn) was remarkably reduced, which indicated that the apoptosis of dopaminergic neurons in mice was significantly reduced. The protein phosphorylation of p-PI3K, p-AKT and p-mTOR also increased about 2-fold, compared with the model group. Furthermore, qRT-PCR results demonstrated that the mRNA levels of pro-inflammatory cytokines, IL-1*β* and TNF-*α*, were reduced, but the level of anti-inflammatory cytokine IL-10 increased after DMTHB treatment. Finally, the cellular assay displayed that DMTHB was also a strong antioxidant to protect neuron cell line PC12 by scavenging ROS. In this study, we demonstrated DMTHB alleviates the behavioral disorder and protects dopaminergic neurons through multiple-target effects includubg anti-apoptotic, anti-inflammatory and antioxidant effects.

[KEY WORDS] Demethylenetetrahydroberberine; Parkinson's disease; Dopaminergic neurons; Apoptosis; Neuroinflammation
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Introduction

Parkinson's disease (PD), is the second most common neurodegenerative disease after Alzheimer's disease, and the overwhelming majority of patients are aged 60 and older [1]. With the increase of older adults, the number of PD patients is also increasing year by year [2]. It has been reported that PD is characterized by decreased dopamine (DA) [3], and the remaining dopaminergic neurons generate a large number of Lewy bodies (LBs) containing a lot of insoluble proteins [4-5]. The main reason of LB formation is the misfolding and intercellular aggregation of proteins in brain regions [6-7]. Notably, *in vitro* and *in vivo* studies suggest that *α*-synuclein (*α*-syn) is the main component of LBs, which is an early event in the initiation of PD [8]. However, the pathogenesis of PD remains undefined, which has become the main limitation for the development of PD treatment [9-10]. Previous therapies simply relieved dyskinesia symptoms in a short period of time, through exogenous supplement of DA [11]. Recently, researchers have drawn increasing attention to slowing down or arresting the neurodegenerative process by therapeutic strategies.

PD is a multifactorial disease related to complex molecular mechanisms, including apoptosis, neuroinflammation, oxidative stress, protein misfolding, mitochondrial dysfunction, and iron metabolism disorders. In PD brains, mitochondrial impairment and oxidative stress are the prominent features.

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After systemic administration, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) can cross the blood brain barrier (BBB) and be taken up by glial cells, where it is metabolized to 1-methyl-4-phenylpyridinium (MPP^+). MPP^+ is then released and selectively taken up by DA neurons *via* dopamine transporters (DAT) [12]. After entry into dopaminergic neuron, MPP^+ will inhibit complex I in the electron transport chain, which leads to ATP synthesis blocking, ROS accumulation, and consequent cell apoptosis [13-15]. The cellular mitochondrial dysfunction, as induced by MPP^+, usually includes disturbance in Ca^{2+} homeostasis and oxidative stress [16-17]. Therefore, a PD mouse induced by MPTP is the common experimental model for the development of PD medicinal research. Increased evidence has indicated that the PI3K/Akt pathway is one of vital signal cascades for neuron survival [18]. Activated Akt can increase the transcription and post-transcription of Bcl-2 [19]. Furthermore, mTOR is considered to be an effective regulator for apoptosis and autophagy, and mTOR-related autophagy may be associated with oxidative stress [20-21].

Berberine is an isoquinoline alkaloid that widely exists in different Chinese herbal medicines, especially *Berberis* genus. It is mainly used as antidiarrheal agent, antibacterial agent, antifungal agent and antigenic agent. Current researches focus on its beneficial effects on neurodegenerative diseases, mainly due to its powerful antioxidant effects [22]. For example, berberine was reported to prevent glutamate-induced toxicity, such as protein misfolding, aggregation, mitochondrial fragmentation, and neurodegeneration in astrocyte culture systems [23]. Zhou *et al*. showed that berberine inhibited the generation of intracellular reactive oxygen species induced by hypoxic-glucose deprivation, and then inhibited the release of cytochrome C and apoptotic inducible factors, suggesting its therapeutic significance in the management of stroke [24]. Lee *et al*. investigated the neuroprotective effect of berberine on MK-801-induced cerebral neurodegeneration in rats. They found that berberine protected neuronal cells by enhancing NMDA receptor-mediated activity-dependent cell survival [25]. Jinbum Bae *et al*. found that BBR significantly reduced 6-OHDA-induced generation of reactive oxygen species (ROS), caspase-3 activation, and subsequent cell death in SH-SY5Y. Furthermore, BBR induced PI3K/Akt and p38 activation, which were involved in the induction of Nrf2 expression and neuroprotection [26].

Demethylenetetrahydroberberine (DMTHB) is a novel derivative of natural product berberine, and synthesized by reduction of berberine in our laboratory. DMTHB has higher bioavailability, anti-inflammatory and antioxidant activity, with lower toxicity, compared with berberine [27]. Our previous studies showed that DMTHB significantly reduced the expression of serum protein inflammatory cytokines, such as TNF-α, IL-1β and IL-6, inhibited NOD-like receptor protein 3 (NLRP3) inflammasome signals, and improved the liver fibrosis in MCD-induced NAFLD mice [27]. In the present study, DMTHB improved MPTP-induced Parkinson's disease symptoms, including behavioral disorders and decreased balance, with a decreased number of dopaminergic neurons. DMTHB may be a potential therapeutic agent to prevent neuronal cells in PD mice.

Materials and Methods

Reagents

MPTP from Refmedic Biotech. (Chengdu, China) was dissolved in sterile normal saline. Probenecid from Aladdin (Shanghai, China) was dissolved in DMSO. DMTHB hydrochloride (purity > 98%) was synthesized by our laboratory, and dissolved in pure water followed by ultrasonic dispersion for animal experiments. Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Gland Island, NY, USA). DAPI was purchased from Servicebio (Wuhan, China) and DCFH-DA was purchased from Sigma (St. Louis, MO, USA). All other reagents were of analytical or biological grade.

Establishment of a MPTP-induced chronic PD mouse model

Adult male C57BL/6 mice (8- to 10-week-old) were obtained from the Model Animal Research Center of Yangzhou University (Yangzhou, China). The mice were housed under controlled temperature (22 ± 2 °C) in a 12 h/12 h light–dark cycle. All animals were randomly divided into four groups (n = 8): a control group, a MPTP group (20 mg·kg^{-1}), a low-dose (5 mg·kg^{-1}) DMTHB group, a middle-dose (15 mg·kg^{-1}) DMTHB and a high-dose (50 mg·kg^{-1}) DMTHB group. The MPTP group and DMTHB groups were intraperitoneally injected with MPTP (20 mg·kg^{-1}) 1 h after injection of probenecid (200 mg·kg^{-1}) twice a week, and MPTP injection solution were totally given ten times. During this period, DMTHB was daily administered by gavage (Fig. 1).

Behavior test

Open field test

The floor of open-field arena (ZS, Zhongshi Technology Company in Beijing, China) was divided into 25 equal squares. Each mouse was placed in the center of the floor, and allowed to explore the open field for 5 min. All tests were performed in a closed room and video recorded with a Sony digital camera fixed to the open-field arena. The track and speed of mice were recorded by the software. The open-field apparatus was cleaned with 70% ethanol solution before each test to get rid of the smell of the last mouse.

Rotarod test

Motor coordination was evaluated on a RotaRod (ZS-RDM, Zhongshi Technology Company in Beijing, China) consisting of a five-lane rotating rod (diameter 7.5 cm) designed for mice. Animals were tested with a few runs to familiarize the procedures, where they were positioned on a rotating rod at a speed of 10 r·min^{-1} over 180 s. The mice that completed the task received a final latency time of 180 s. The length of time when the mice were able to stay on the rotating rod was recorded.

Pole test

The pole test was performed to evaluate the movement disorder induced by MPTP. During the test, the mice were

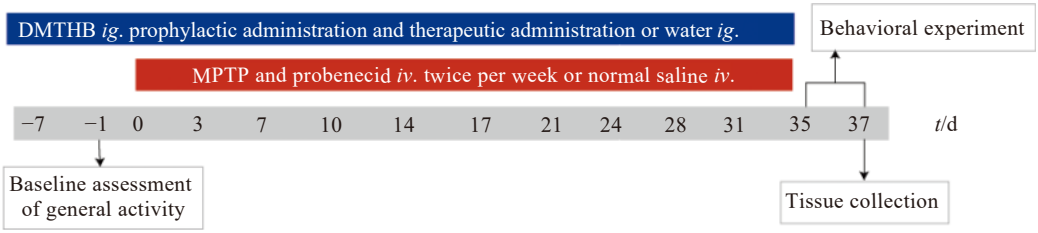

Fig. 1 Time course of the treatment of DMTHB, MPTP/p and behavioral analysis

placed with their head facing upside on top of a rough-surfaced pole. The time required for the mice to turn down completely (T-turn) and climb down to the floor (T-LA) was recorded.

Immunohistochemistry

The brains were collected, fixed overnight in a buffered 4% paraformaldehyde at room temperature, and embedded in paraffin. The sections were rinsed in 0.1 mol·L^{-1} PBS and labeled with primary antibody rabbit antityrosine hydroxylase (TH; 1 ∶ 2000; Pel-Freez, Brown Deer, WI, USA). After incubation overnight, the sections were rinsed and incubated with proper biotinylated secondary antibodies for 1 h. After washing, the sections were stained using an ABC kit at room temperature for 1 h, according to the manufacturer's instructions. After washing with PB, the sections were incubated with 0.05% 3,3′-diaminobenzidine (DAB; Sigma) in 0.1 mol·L^{-1} PB containing 0.003% H_2O_2. Stained samples were washed in PB and mounted on gelatin-coated slides before observation under a bright-field microscope. Cell counting were performed using the Image J software.

Nissl staining

Brain sections were hydrated for 5 min in 95%, 85% and 70% ethanol gradients, followed by washing with running water three times (5 min each time), and stained with 0.1% cresol violet for 10 min. After washing in distilled water, the sections were dehydrated with gradient alcohol, dewaxed using xylene and sealed with neutral resin. Tissues were visualized under a Nikon Eclipse Ni-U microscope. Cell counting were performed using the Image J software.

Western blot

The substantia nigra was homogenized in 1 mL of ice-cold RIPA lysis buffer containing 1% protease inhibitor cocktail (Roche, Switzerland) and centrifuged (12 000 × g, 4 °C) for 15 min. The protein concentration was determined by the bicinchoninic acid (BCA) method (B18020, Yeasen, China). Equal amounts of total protein (100 μg) were subjected to electrophoresis on 10% SDS-PAGE and then transferred to PVDF membranes (Millipore, USA). The levels of Bcl2 (WL01556 1 ∶ 1000 Wanleibio), Bax (WL01637 1 ∶ 1000 Wanleibio), PI3K (WL02849 1 ∶ 1000 Wanleibio), p-PI3K (AF3242 1 ∶ 1500 Affinity), AKT (WL0003b 1 ∶ 1000 Wanleibio), p-AKT (WLP001a 1 ∶ 1000 Wanleibio), mTOR (WL02477 1 ∶ 1000 Wanleibio), p-mTOR (WL03694 1 ∶ 1000 Wanleibio), *α*-syn (10842-1-AP 1 ∶ 1000 proteintech), GRP78 (WL03157 1 ∶ 1000 Wanleibio) and GAPDH (60004-1-Ig 1 ∶ 10000 proteintech) were measured as previously described. ImageJ software was used to analyze the relative density of each band.

qRT-PCR

Total RNA was extracted from brain tissues with Trizol (Invitrogen), and RNA (1 μg) was used for cDNA synthesis with oligo-dT primers and M-MLV reverse transcriptase (Roche). The levels of mRNA were determined by quantitative real-time PCR using high ROX (Roche) on the ABI Step One Plus System (Applied Biosystems) (Table. 1).

Cell culture and drug treatment

PC12 cells were cultured with Dulbecco's modified Eagle's medium (DMEM) under 95% air and 5% CO_2 at 37 °C. The number of the cells was adjusted to 10^5 /mL by cell culture media. MPP^+ was directly dissolved in PBS. The cells were first treated with DMTHB at 12.5 and 25 μmol·L^{-1} for 6 h, followed by MPP^+ (1 mmol·L^{-1}) incubation at 37 °C for 24 h. Then, 20 μL of a 5 mg·mL^{-1} MTT solution was added to each well and the plate was further incubated at 37 °C for 4 h. After the medium was removed, the wells were washed with PBS, and 200 μL of DMSO was added to each well. The microtiter plate was placed on a shaker in order to dissolve the dye. After the formazan crystals was dissolved,

Table 1 Primer sequences for polymerase chain reaction analysis

Name	Forward primer (5′-3′)	Reverse primer (5′-3′)
TH	TGTCAGAGGAGCCCGAGGTC	CCAAGAGCAGCCCATCAAAG
DAT	TGGCTTCGTTGTCTTCTCCT	GCATGAGGAAGAAGACAGCG
IL-10	CGGGAAGACAATAACTGCACCC	CGGTTAGCAGTATGTTGTCCAGC
TNF-α	GGTGCCTATGTCTCAGCCTCTT	GCCATAGAACTGATGAGAGGGAG
IL-1β	GAAATGCCACCTTTTGACAGTG	TGGATGCTCTCATCAGGACAG
β-actin	TGTTCCCTTCCACAGGGTGT	TCCCAGTTGGTAACAATGCCA

the absorbance was spectrophotometrically determined at 490 and 630 nm using a universal microplate reader.

Statistical analysis

Data are presented as mean ± SEM. Statistical comparisons were made using One-way ANOVA with post hoc contrasts by Student-Newman-Keuls test to compare differences between two groups and multiple groups.

Results

DMTHB ameliorated the behavioral disorder caused by MPTP in mice

To investigate the therapeutic effect of DMTHB on behavioral disorder in mice induced by MPTP, the standard behavior test, open field test, rotarod test and pole test were used in the current study. As shown in Fig. 2, the model mice injected with MPTP and probenecid presetned weakened behavioral ability, which indicated PD symptoms. However, DMTHB treatment improved the motor ability of the mice in behavior tests. Compared with the model group, the total traveled distance and average speed were increased by 268.1% ($^{###}P < 0.001$) and 355.7% ($^{###}P < 0.001$) in high dose (50 mg·kg^{-1}) DMTHB group respectively(Figs. 2A and 2B), indicating that DMTHB ameliorated the behavioral disorder caused by MPTP in mice. In the rotarod test, the residence time of rotating rod was increased by 545.7% ($^{###}P < 0.001$) in high-dose DMTHB group, compared with the model group (Fig. 2C). The t-turn was reduced by 37.7% ($^{##}P < 0.01$) in low-dose and 54.2% ($^{###}P < 0.001$) in high-dose groups, respectively, compared with the MPTP group (Fig. 2D). Meanwhile, t-LA was decreased by 26.3% ($^{#}P < 0.05$) in low-dose and 43.8% ($^{###}P < 0.001$) in high-dose group respectively (Fig. 1E). The results of pole tests also demonstrated the improved effect of DMTHB on behaviors.

DMTHB protected against dopaminergic neuron injury induced by MPTP

To explore the protective effects of DMTHB on dopaminergic neuron, Nissl staining and the number of TH-IR neurons were detected in the substantia nigra pars compacta (SNpc). Nissl staining revealed the function and features of neurons in the SNpc, and results indicated that DMTHB treatment at a low dose (5 mg·kg^{-1}) or a high dose (50 mg·kg^{-1}) effectively recovered the Nissl body to normal level in the SNpc of MPTP/p mice (Figs. 3A and 3B). Tyrosine hydroxylase (TH) is a biomarker for normal DA neurons. In the curret study, TH-positive neurons were also structurally damaged in the SNpc of MPTP/p mice, while MPTP treatment resulted in only 43.6% ($^{***}P < 0.001$) survival of the dopaminergic neurons (Figs. 3C and 3D). In contrast, 5 and 50

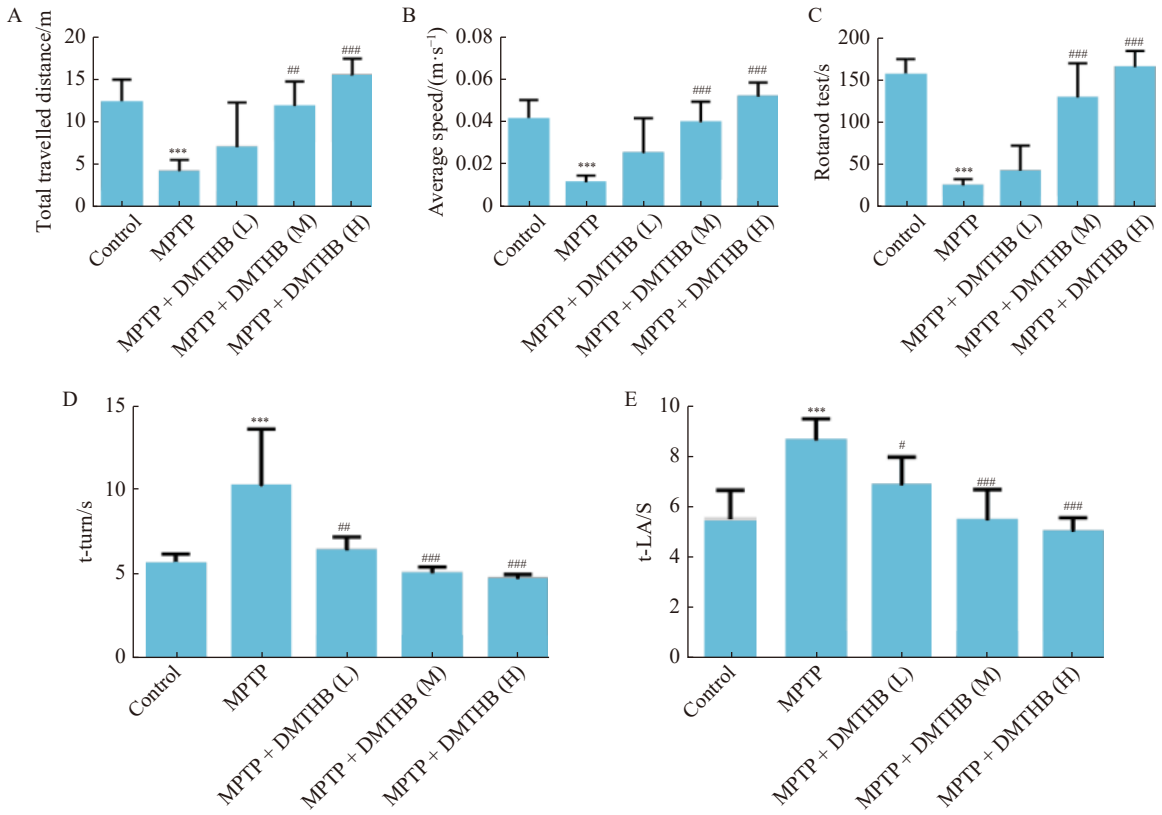

Fig. 2 DMTHB ameliorated the behavioral disorder caused by MPTP in mice. (A) Total traveled distance of mice in the open field test; (B) average speed of mice in open field test; (C) time spent on the rod by mice in each group; (D) t-turn time in the pole test; and (E) t-LA time in the pole test. $^{*}P < 0.05$ and $^{***}P < 0.001$ *vs* control; $^{#}P < 0.05$, $^{##}P < 0.01$, and $^{###}P < 0.001$ *vs* the MPTP-treated group

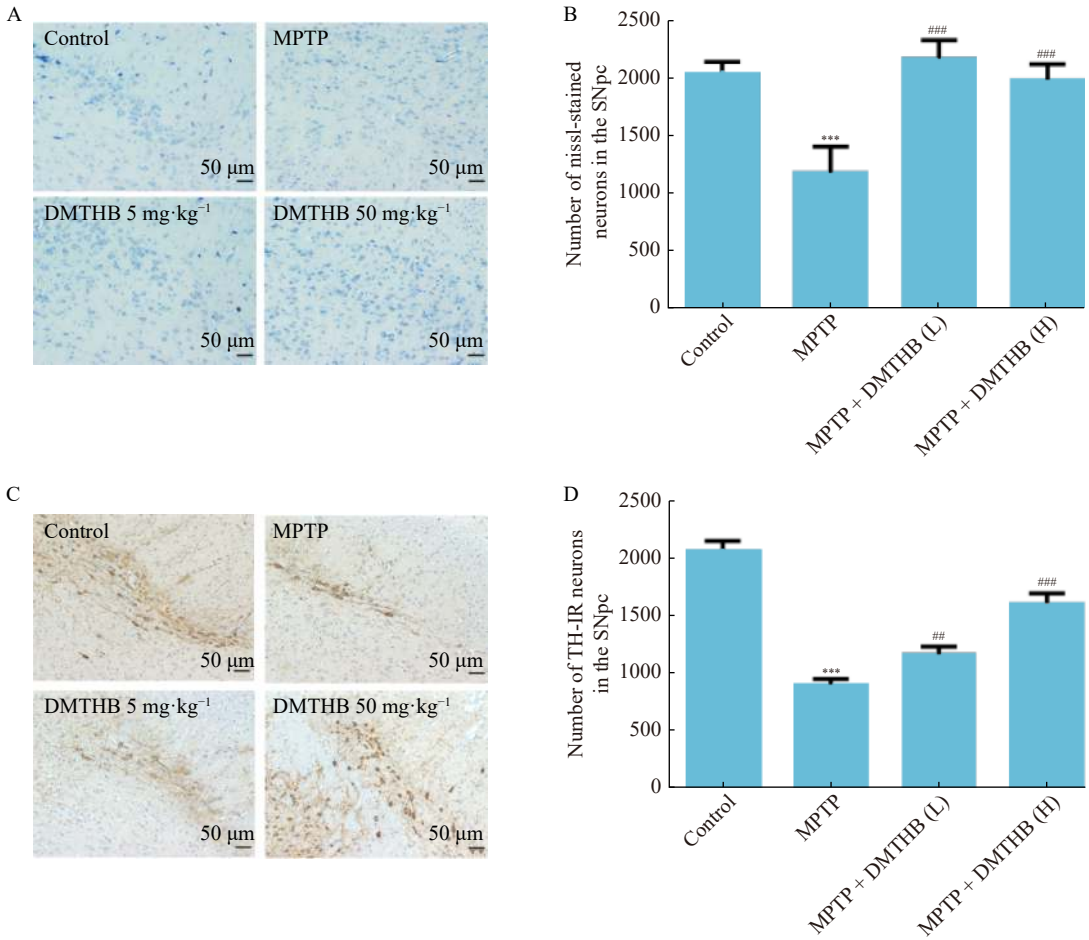

Fig. 3 DMTHB protected against DMTHB-induced dopaminergic neuron damage. (A, B) Representative photomicrographs of Nissl's staining and number of Nissl's stained neurons in the SNpc. (C, D) Representative photomicrographs of TH-immunoreactive neurons and number of TH-IR neurons in the SNpc. $^{***}P < 0.001$ ***vs*** **control;** $^{\#\#}P < 0.01$ **and** $^{\#\#\#}P < 0.001$ ***vs*** **the MPTP-treated**

mg·kg^{-1} DMTHB treatment protected against the neurotoxicity of MPTP, with the survival rates of 56.6% ($^{\#\#}P < 0.01$) and 78.0% ($^{\#\#\#}P < 0.001$) respectively, compared with the control group. These data demonstrated the effect of DMTHB on neuronal protection.

DMTHB reduced α-syn accumulation and neuronal apoptosis induced by MPTP

The Lewy bodies (LBs) and Lewy neurites (LNs) in PD are mainly composed of the aggregated form of a presynaptic protein, *α*-synuclein (*α*-syn). *α*-Syn aggregation and actual aggregated species are responsible for the degeneration of dopaminergic neurons. Western blot results showed that DMTHB alleviated dopaminergic neuron injury by reducing *α*-Syn ($^{***}P < 0.001$) and increasing TH ($^{***}P < 0.001$) (Fig. 4A). These findings were consistent with immunohistochemical images (Fig. 3C). MPTP-induced *α*-syn protein expression markedly decreased after DMTHB treatment. This study also showed that the protein expression of Bcl-2 in the SNpc significantly decreased in the MPTP group, but increased after DMTHB treatment. In contrast, Bax protein expression increased in the MPTP group, but attenuated by DMTHB treatment (Figs. 4D and 4E). The ratio of Bcl-2/Bax increased in the DMTHB treatment groups, which demonstrated the anti-apoptotic effect of DMTHB on dopaminergic neurons.

DMTHB increased the phosphorylation of PI3K/Akt/mTOR in the PD mouse model

The PI3K/Akt pathway plays a vital role for neuron survival, and the signaling cascade can be activated by phosphorylation of key signal intermediates. Western blot data displayed that the protein levels of p-PI3K, p-Akt (Ser473) and p-mTOR markedly decreased in MPTP-treated mice ($^{**}P < 0.01$), while DMTHB treatment increased the phosphorylation of PI3K, Akt (Ser473) and mTOR (Fig. 5, $^{\#\#}P < 0.01$). Therefore, it is possible that DMTHB treatment activates the PI3K/AKT/mTOR pathway to perform anti-apoptotic function and neuron recovery.

DMTHB suppressed the expression of inflammatory cytokines in the PD mouse model

In patients with PD, microglias are always in the state of excessive activation, which cause a series of inflammatory response. qRT-PCR analysis indicated that the major proinflammatory cytokines, TNF-*α* and IL-1*β*, were elevated, but the level of anti-inflammatory cytokine, IL-10, decreased in

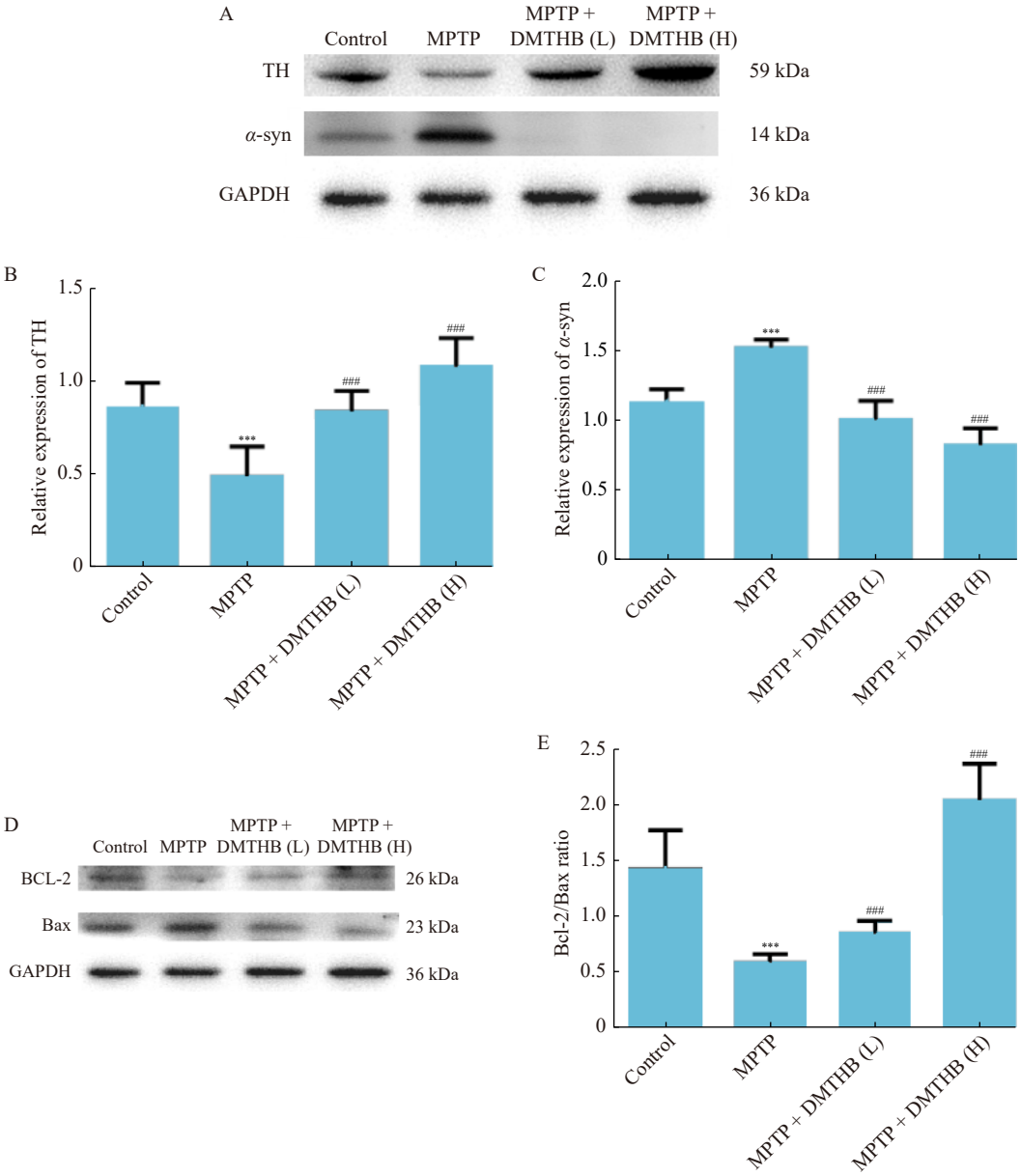

Fig. 4 DMTHB suppressed MPTP -induced *α*-syn accumulation and neuronal apoptosis. (A−C) Relative expression of TH and *α*-syn in the substantia nigra of mice was detected by Western blot. (D−E) Relative expression of Bcl-2 and Bax in the substantia nigra of mice was detected by Western blot. *$P < 0.05$ and ***$P < 0.001$ *vs* control; #$P < 0.05$, ##$P < 0.01$, and ###$P < 0.001$ *vs* the MPTP-treated group

the SNpc of MPTP/p mice. Activated microglias release pro-inflammatory cytokines such as IL-1*β* and TNF-*α*, into the cellular environment. In turn, they can also produce ROS which may be characteristic of neurodegenerative diseases [28]. These events are the result of a neuroinflammatory cascade in which microglias and astrocytes are activated leading to changes in the cross-talk between glia and neurons. However, DMTHB treatment significantly suppressed the mRNA expression of TNF-*α* and IL-1*β* and activated the mRNA expression of IL-10 in PD mice (Fig. 6). Therefore, DMTHB exerted therapeutic effects partially through anti-inflammatory activity in PD mice.

DMTHB protected PC12 cells from cellular damage by scavenging ROS

It has been reported that MPP^+ is specifically taken up by DA neurons *via* dopamine transporters and causes excess ROS generation. The fluorescent probe, DCFH-DA, was used to determine the intracellular ROS generation in this experiment. Our results displayed that DMTHB dramatically reduced ROS generation in the MPP^+-damaged cells (Figs. 7A and 7B). It is possibly that DMTHB exerted its protective effects partially by scavenging ROS. Meanwhile, it was observed that DMTHB did not cause cellular damage when its dosage was not exceeded 125 μmol·L^{-1}, and 100 μmol·L^{-1}

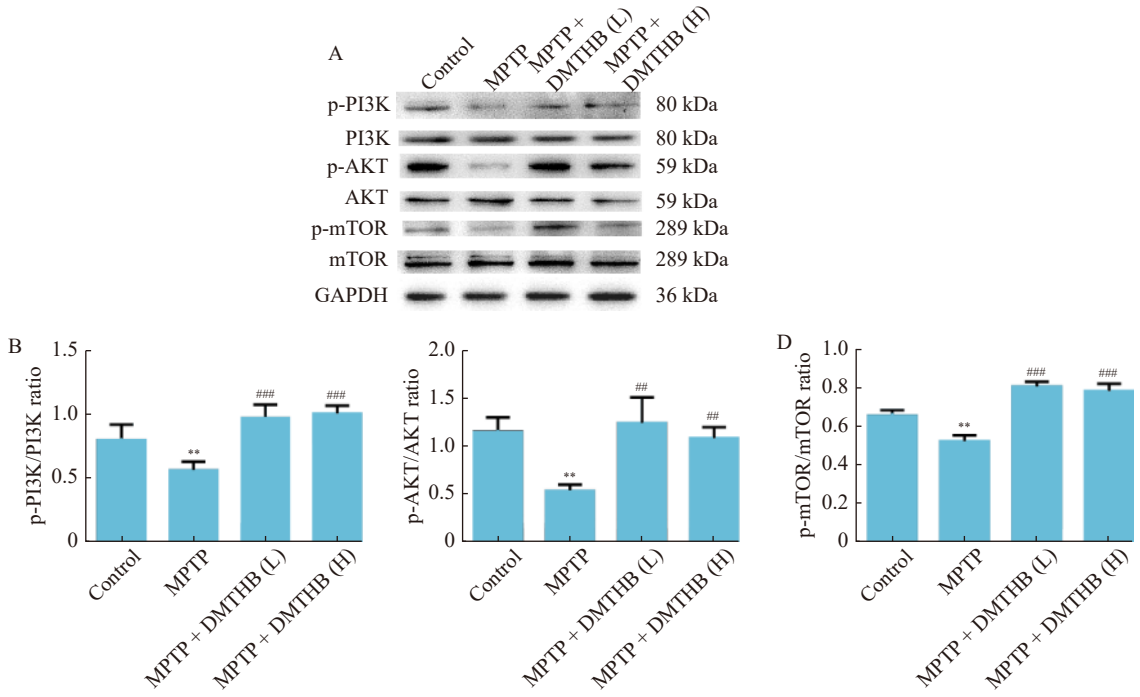

Fig. 5 DMTHB inhibited MPTP -induced apoptosis *via* the PI3K/Akt signaling pathway. (A−D) Relative expression of p-PI3K, PI3K, p-AKT, AKT, p-mTOR, and mTOR in the substantia nigra of mice was detected by Western blot. $^{*}P < 0.05$ and $^{***}P < 0.001$ *vs* control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ *vs* the MPTP-treated group

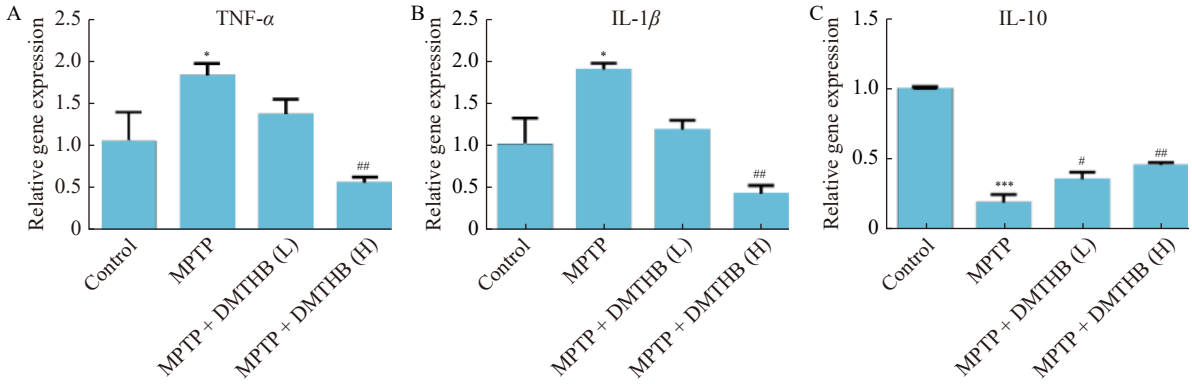

Fig. 6 DMTHB suppressed MPTP-induced neuroinflammatory effects on gene expression. (A-C) Relative gene expression of TNF-*α*, IL-1*β* and IL-10 in the substantia nigra of mice. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ *vs* control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ *vs* the MPTP-treated group

was chosen as the maximum dose for the research (Supplement Fig. 4).

Discussion

Previous studies demonstrated the direct relationship between the loss of DA neurons and motor behavior impairment. Degeneration of DA neurons concurrently decreases the release of DA neurotransmitter that results in Parkinson's disease [29-30]. In our study, DMTHB-treated mice showed improved movement ability in behavioral experiments. DMTHB protected dopaminergic neurons and reduced abnormal accumulation of *α*-syn [31-32]. Our results also showed that DMTHB alleviated the oxidative stress of cells and improved MPTP-induced DA neuron apoptosis. These results suggest that DMTHB is a potential neuroprotective compound for the therapy of PD.

In addition to the chronic PD model of mice mentioned above, a subacute PD model of mice was established to compare the effects of DMTHB with another different BBR dereivative tetrahydroberberine (THB) in this study. THB is similar to DMTHB in structure, which is also one of the metabolites of BBR. It has been reported that THB exerted neuroprotective effects on PD by blocking neuronal K(ATP) channels [33]. This study found that DMTHB improved motor disturbance which were better than tetrahydroberberine (THB) (Supplemental Fig. 3). THB has been reported to

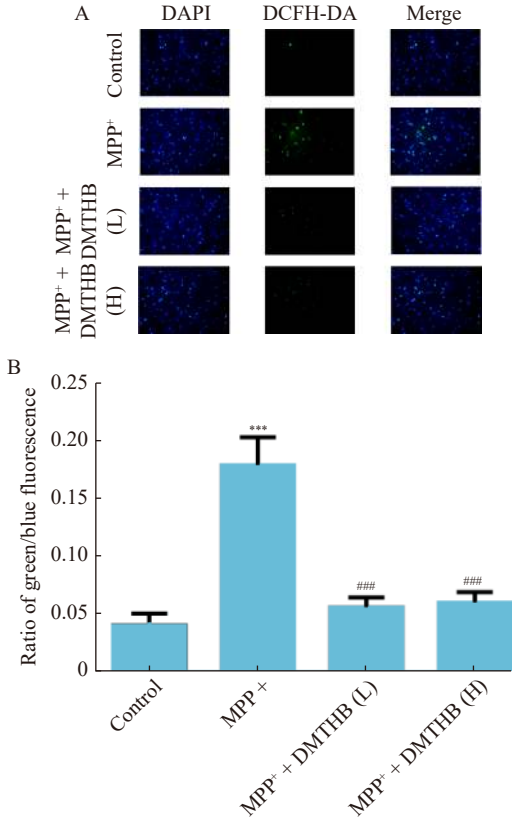

Fig. 7 DMTHB reduced the level of reactive oxygen species induced by MPP^+. (A) Fluorescence micrographs of ROS generation after 24 h MPP^+ incubation and 12.5 or 25 μmol·L^{-1} DMTHB treatment in PC12 cells. (B) Graphical representation of relative fluorescence intensity of DCFH-DA staining in PC12 cells as quantified using Image J_software. $^{***}P < 0.001$ *vs* control, $^{###}P < 0.001$ vs the MPP^+-treated group

show better oral bioavailability than BBR. Levo-tetrahydropalmatine (l-THP) is an active ingredient of Corydalis and also the reduced form of natural medicine palmatine which is an analog of BBR. It has been safely used as an analgesic agent in China for decades [34-35]. The oral bioavailability of l-THP is higher than palmatine due to tertiary amine in l-THP without quaternary ammonium salt. l-THP can be orally administered for patients in clinical settings. This strategy is successful for enhancing the intestinal absorption of isoquinoline alkaloid through reduction of positively charged quaternary ammonium to tertiary amines. It can explain why oral bioavailability of DMTHB is higher than BBR and DMB. The oral bioavailability of DMB should be increased to meet the medication compliance for clinical treatment of PD.

DMTHB is an isoquinoline alkaloid which is a derivative of natural products berberine and demethyleneberberine. DMTHB maintains the characteristics of polypharmacology and multiple targets of natural medicines. Our previous study reported that DMTHB possessed antioxidant, anti-inflammatory and anti-apoptotic properties [27]. Increased evidence has indicated that the PI3K/Akt pathway is one of vital signaling pathways for neuron survival. Activated Akt can increase the transcription and post-transcription of Bcl-2. Furthermore, mTOR is considered to be an effective regulator for apoptosis and autophagy, and mTOR-related autophagy may bee associated with oxidative stress. Dong *et al*. found that TRIM3 up-regulation prominently reduced ROS and MMP and might attenuate apoptosis in PD through activating the PI3K/AKT signal pathway [36]. This study showed that DMTHB also increased the phosphorylation of PI3K/AKT/mTOR to stimulate their activities. Phosphorylated PI3K promoted phosphatidyl inositol (3,4,5)-triphosphate (PIP3) formation [37], Akt were recruited to the plasma membrane, and threonine and serine residues phosphorylation induced Akt activation [38-39]. mTOR activity is important for motor function control, and activation of mTOR and downstream proteins may also promote neuronal survival [40-41]. Thus, DMTHB may regulate cellular apoptosis and relieve oxidative stress.

Abbreviations:

DMB: Demethyleneberberine Hydrochloride; BBR: Berberine Hydrochloride; THB: Tetrahydroberberine; BBB: Blood brain barrier; WB: Western Blot; MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine: MPP^+: 1-Methyl-4-phenylpyridinium iodide; 6-OHDA: 6-hydroxydopamine; PD: Parkinson disease; DA: Dopamine; LB: Lewy body; DR: Dopamine receptor; DAT: Dopamine transporter; TH: Tyrosine hydroxylase; SNpc: Substantia nigra pars compacta; SDS: Sodium Dodecyl Sulfate; *α*-Syn: *α*-Synuclein; Bax: BCL2-Associated X; Bcl-2: B-cell lymphoma-2; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; PI3K: phosphoinositide 3-kinase: AKT: protein kinase B; mTOR: mammalian target of rapamycin; ROS: Reactive oxygen species; TH-IR: Tyrosine hydroxylase immunoreactive.

Supplementary Material

Supplementray information can be acquired by e-mail to corresponding author.

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