

Neferine exerts anti-inflammatory activity in BV-2 microglial cells and protects mice with MPTP-induced Parkinson's disease by inhibiting NF- κ B activation

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Abstract. Abnormal activation of microglia and the production of proinflammatory cytokines can lead to chronic neuroinflammation, which is an important pathological characteristic of Parkinson's disease (PD). Neferine is a chemical compound extracted from lotus seed which has previously been reported to exert protective effects on the development of several types of cancer, myocardial injury and hypoxic-ischemic encephalopathy. However, its effect on microglial functions in neuroinflammation remains to be clarified. The present study used network pharmacology and screening in a lipopolysaccharide (LPS) model to demonstrate that neferine suppresses the production of inducible nitric oxide synthase, interleukin-6 and tumor necrosis factor α in LPS-treated BV-2 cells. The working concentration of neferine did not exert cytotoxic effects on BV-2 cells. Mechanistically, neferine attenuated inflammation by inhibiting the phosphorylation and nuclear translocation of the NF- κ B p65 subunit. *In vivo*, neferine protected mice from the inflammatory response in the substantia nigra and inhibited the development of nervous disorders in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced PD model. The present study demonstrated that neferine inhibited LPS-mediated activation of microglia by inhibiting NF- κ B signaling. These findings may provide a new reference for the prevention and future treatment of PD.

Introduction

Neuroinflammation is initially useful for the nervous system to successfully reduce infection and injury via the production of proinflammatory cytokines and other molecules, such as nitric oxide (NO), as it can restore homeostasis of the nervous system (1-3). However, excessive or persistent neuroinflammation is also harmful to the normal function of the nervous system due to nerve cell dysfunction or disruption of the blood-brain barrier (BBB) (4,5). Therefore, neuroinflammation is increasingly recognized as a main disease feature of neurodegenerative diseases, such as Parkinson's disease (PD) (6). Microglia are the resident class of myeloid macrophages in the brain; their activation is considered the first sign of neuroinflammation, and overactivation of these cells contributes to the pathogenesis of PD (7). Pattern recognition receptors, such as the toll-like receptors (TLRs) are present on microglia and can trigger the production of proinflammatory cytokines following the sensing of pathogen-associated molecular patterns or the damage associated molecular patterns in the brain (8). The release of proinflammatory cytokines can trigger the overactivation of protein kinases on neuronal receptors, such as those found in hippocampal and substantia nigra neurons, and subsequently lead to neuronal death and acceleration of neurodegeneration, and there have been numerous reported hypotheses that the inhibition of overactivated microglia and neuro-inflammation is an effective strategy for the treatment of neurodegenerative diseases (9-11).

A prerequisite for neurological drug development is the identification of a therapeutic agent that can be effectively delivered across the BBB (12). Network pharmacology is widely used in the search of pharmaceutical ingredients (13). Therefore, the application of network pharmacology is feasible to identify the candidate components that can cross the BBB and inhibit the activation of microglia.

Neferine is a bis-benzylisoquinoline alkaloid extracted from the seed embryos of *Nelumbo nucifera*, which has multiple types of reported pharmacological activities, such as anticancer, antidiabetic and antiatherosclerotic effects (14-16). Previous studies have also reported the antioxidant and anti-inflammatory properties of neferine on the prevention of

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liver fibrosis and Graves' orbitopathy by suppressing MAPK-, NF- κ B- and autophagy-related inflammation (17,18). In the nervous system, neferine was reported to prevent neurodegeneration in the hippocampal tissue of Alzheimer's disease models by potentially inhibiting the expression levels of inflammatory factors such as tumor necrosis factor α (TNF- α) and interleukin-6 (IL-6) (19). However, limited information is available regarding the effects and mechanisms of neferine on overactivated microglia-associated inflammation and PD. Therefore, the present study explored the function of neferine in the lipopolysaccharide (LPS)-induced microglia activation model and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD mouse model.

In the course of clinical treatment, prophylactic use of neuroprotectants, such as coenzyme Q10 and inosine, is a common option for individuals with a family history of PD and for those with mild symptoms, such as mild tremor or slow movement in a single limb, that have not yet been diagnosed. Furthermore, pretreatment with neferine has been reported to exert a neuroprotective effect in the Kainic Acid-induced seizure model in rats (20), and pretreatment using other neuroprotective drug like salidroside is also used in MPTP models (21). Therefore, pretreatment with neferine was used to assess its neuroprotective effect in MPTP-induced PD mouse models in the present study.

Materials and methods

Cell culture and treatment. BV-2 cells (cat. no. CL-0493A; Procell Life Science & Technology Co., Ltd.) were cultured in Eagle's Minimum Essential Medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 100 U/ml penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.) at 37°C in the presence of 5% CO₂ and saturated humidity. BV-2 cells were treated with LPS (InvivoGen) at a final concentration of 100 ng/ml at 37°C in the presence of 5% CO₂. The duration of treatment is dependent on the specific experiment as declared in figure legends.

Materials. Neferine (cat. no. HY-N0441), nuciferine (cat. no. HY-N0049), methoxsalen (cat. no. HY-30151), 3,4-dimethoxybenzoic acid (cat. no. HY-N2007) and JSH23 (cat. no. HY-13982) were purchased from MedChemExpress. BV-2 cells were pre-treated with these compounds for 30 min before LPS treatment in order to assess their anti-inflammatory activity at a concentration gradient from 0.1-10 μ M at 37°C in the presence of 5% CO₂.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from BV-2 cells using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) and quantified using a nanodrop spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.). cDNA synthesis was performed using PrimeScript RT Master Mix (Takara Bio, Inc.) according to the manufacturer's instructions. Gene amplification was performed using SYBR Green Master Mix (TransGen Biotech Co., Ltd.) on a Roche 480 light cycler instrument (Roche Diagnostics). The thermocycling conditions were as follows: 95°C for 30 sec, followed by 40 cycles at 95°C for 5 sec and 55°C for 30 sec, and

then 95°C for 15 sec and 60°C for 60 sec. The relative expression of IL-6 or TNF α was quantified using the 2^{- $\Delta\Delta$ C_q} method (22), and β -actin was used as an internal control. All reactions were performed in triplicate. Primer Premier 5 software was used for primer design, and the sequences of the primers are as follows: IL-6 forward (F), 5'-GAGTTGTGCAATGGCAATTCTG-3' and reverse (R) 5'-GCAAGTGCATCATCGTTGTTTCAT-3'; TNF- α F, 5'-CCCTCACACTCAGATCATCTTCT-3' and R, 5'-GCTACGACGTGGGCTACAG-3'; and β -actin F, 5'-AGTGTGACGTTGACATCCGT-3' and R 5'-GCAGCTCAGTAA CAGTCCGC-3'.

Cell death assay. Cell death was investigated using the Dead Cell Apoptosis Kits with Annexin V for Flow Cytometry (cat. no. V13242; Thermo Fisher Scientific, Inc.). BV-2 cells were plated at a density of 1x10⁵ cells/well in 24-well plates and treated with neferine (10 μ M) at 37°C for either 24 or 48 h. Trypsin was used to detach the cells from the plate (0.25%; 37°C for 3 min). The cells were centrifuged at 200 x g for 5 min at 4°C. Following removal of the supernatant, the cells were washed twice with PBS buffer. The cells were re-suspended in 100 μ l binding buffer with 1 μ l annexin V and incubated at room temperature (RT) in the dark for 10 min. Subsequently, 5 μ l PI solution was added and incubated with the samples for 5 min in the dark at RT. The cell death percentage was determined using a NovoCyte flow cytometer equipped with the NovoExpress software (version 1.5.6; ACEA Bioscience, Inc.; Agilent).

Cell Counting Kit (CCK)-8 assay. BV-2 cells were seeded in 96-well plates at a density of 5x10³ cells/well and incubated at 37°C with 5% CO₂ on day 0 in the presence of neferine (10 μ M). On days 1 and day 2, 10 μ l CCK-8 solution (Dojindo Laboratories, Inc.) was added to every well and incubated at 37°C. After 2 h, the 96-well plates were removed, and a microplate reader (Bio-Rad Laboratories, Inc.) was used to measure the absorbance at 450 nm. Experiments were performed in triplicate.

Enzyme-linked immunosorbent assay (ELISA). BV-2 cells were seeded in 24-well plates at a density of 2x10⁵ cells/ml and incubated at 37°C with 5% CO₂ on day 0. On day 1, BV-2 cells were pretreated with either DMSO or neferine (10 μ M) for 30 min and subsequently treated with LPS (100 ng/ml) for 0 and 12 h. The levels of the proinflammatory cytokines TNF- α and IL-6 were quantified using Mouse IL-6 Quantikine ELISA Kit (cat. no. M6000B; R&D Systems, Inc.) and Mouse TNF- α Quantikine ELISA Kit (cat. no. MTA00B; R&D Systems, Inc.) according to the manufacturer's instructions.

Western blotting. BV-2 cells were seeded in 6-well plates at a density of 2x10⁵ cells/ml and incubated at 37°C with 5% CO₂ on day 0. On day 1, BV-2 cells were pretreated with either DMSO or neferine (10 μ M) for 30 min and subsequently treated with LPS (100 ng/ml) for 0, 2, 4, 8 or 12 h. The cell lysate was prepared by scraping BV-2 cells or grinding the brain tissue of mice in Pierce Immunoprecipitation Lysis Buffer (Invitrogen; Thermo Fisher Scientific, Inc.). The protein concentration was determined using the bicinchoninic acid assay method. A total 20 μ g protein was separated by PAGE on a 10% SDS gel and

transferred to polyvinylidene membranes (MilliporeSigma). The membranes were incubated with 5% BSA at RT for 60 min to block non-specific binding. Subsequently, the membranes were incubated with the corresponding primary antibodies (Cell Signaling Technology, Inc.) against β -actin (1:1,000; cat. no. 12620), inducible NO synthase (iNOS; 1:1,000; cat. no. 13120), phosphorylated (p)-NF- κ B p65 (Ser536; 1:1,000; cat. no. 3033), p-SAPK/JNK (Thr183/Tyr185; 1:1,000; cat. no. 4668), p-p44/42 MAPK (Erk1/2; Thr202/Tyr204; 1:1,000; cat. no. 4370), p-p38 MAPK (Thr180/Tyr182; 1:1,000; cat. no. 4631), NF- κ B p65 (1:1,000; cat. no. 6956), JNK2 (1:1,000; cat. no. 9258), p44/42 MAPK (Erk1/2; 1:1,000; cat. no. 4695), p38 MAPK (1:1,000; cat. no. 8690), Caveolin-1 (1:500; cat. no. 3267), Lamin A (1:500; cat. no. 86846) and α -synuclein (α -syn; 1:1,000; cat. no. ab212184; Abcam) diluted in 5% BSA at 4°C for 12 h. The membranes were subsequently incubated with anti-rabbit IgG (1:2,000; cat. no. 7074; Cell Signaling Technology, Inc.) and anti-mouse IgG antibody (1:2,000; cat. no. 7076; Cell Signaling Technology, Inc.), linked with HRP for 1 h at RT. The membranes were visualized using SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Inc.). β -actin was used as a whole cell lysate control and Lamin A was used as a nuclear lysate control.

Animal model and drug treatments. Male C57BL/6 mice (10 weeks old; n=32) at SPF level weighing 20–25 g were obtained from the Department of Laboratory Animal Science of China Medical University (Shenyang, China). All mice were housed in standard cages at 22–24°C with relative humidity range of 40–60%, a regular 12 h light/dark cycle and *ad libitum* access to food and water in accordance with mouse care protocols. The mice were divided into four groups (n=8/group) as follows: i) Mice intraperitoneally injected with PBS for 14 days; ii) mice intraperitoneally injected with 15 mg/kg/day neferine dissolved in PBS for 14 days; iii) mice intraperitoneally injected with PBS for 3 days, 30 mg/kg/day MPTP (cat. no. M0896; Sigma-Aldrich; Merck KGaA) for 5 days starting on day 4 and subsequently continuously injected with PBS for 6 days; and iv) mice intraperitoneally injected with 15 mg/kg/day neferine dissolved in PBS for 3 days. On day 4, mice were intraperitoneally injected with MPTP + neferine for 5 days, then neferine was administered for 6 days. The health of mice was monitored daily, and no animals died prior to sacrifice. During this time, none of the animals exhibited symptoms that would require to be euthanized, such as reduced appetite, breathing difficulties and convulsions. Following the behavioral experiments on day 14, the mice were euthanized by cervical dislocation following anesthesia with isoflurane (5% for induction and 3% for maintenance). The death of the mice was confirmed by respiratory and cardiac arrest, and pupil dilation. The tissue samples of the substantia nigra were collected, and the protein lysates were extracted for western blotting to investigate the expression levels of the related molecules. The mice were handled according to the Guide for the Care and Use of Medical Laboratory Animals (Ministry of Health). The experimental protocol was approved by the Laboratory Ethics Committee of China Medical University (approval no. CMU:2020096; Shenyang, China).

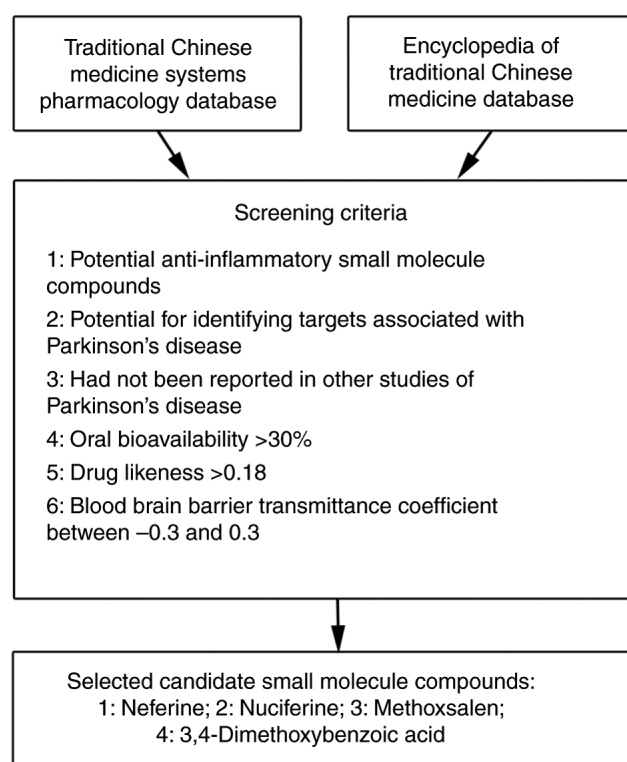


Figure 1. Schematic diagram and criteria for screening candidate drugs using network pharmacology.

Behavioral tests. Behavioral tests were carried out as previously described (23). The pole descent test was performed as follows: A cork ball (diameter, 2.5 cm) was fixed on the top of a wooden pole (50x1 cm), and the pole was wrapped in gauze to prevent slipping. The test mice were placed on the ball, and the time in sec required for the mice to descent back into the cage was recorded. The recording of the time was initiated when the mice started crawling headfirst and ended when their hind legs reached the cage base. The average time was obtained from three replicates for every mouse.

For the traction test, mice were suspended on a horizontal wire (diameter, 5 mm), and their limbs were observed when grasping the wire. Scoring of the test was performed as follows: A total of 3 points were assigned if the two hind legs grabbed the wire; 2 points if one hind leg grabbed the wire; 1 point if the front paw held the wire; and 0 points if the mice fell off the wire. The average score was obtained from three replicates for every mouse.

For the rotor-rod test, training experiments were performed for 3 days to acclimate the mice to the device, and minimize anxiety and exploratory behavior caused by unintentional falls. Animals were placed back on the pole immediately after a fall. Gradual acceleration of the device and rotation of the rod at 4–40 rpm was initiated. In the formal test, the mice were placed on a rotating rod and spun at 15 rpm (rod length, 50 mm; rod diameter, 30 mm). In case the mouse had fallen from the device, the timing end and rotating rod time periods were recorded. Every mouse underwent three trials. The average of the three trials was used for further analysis.

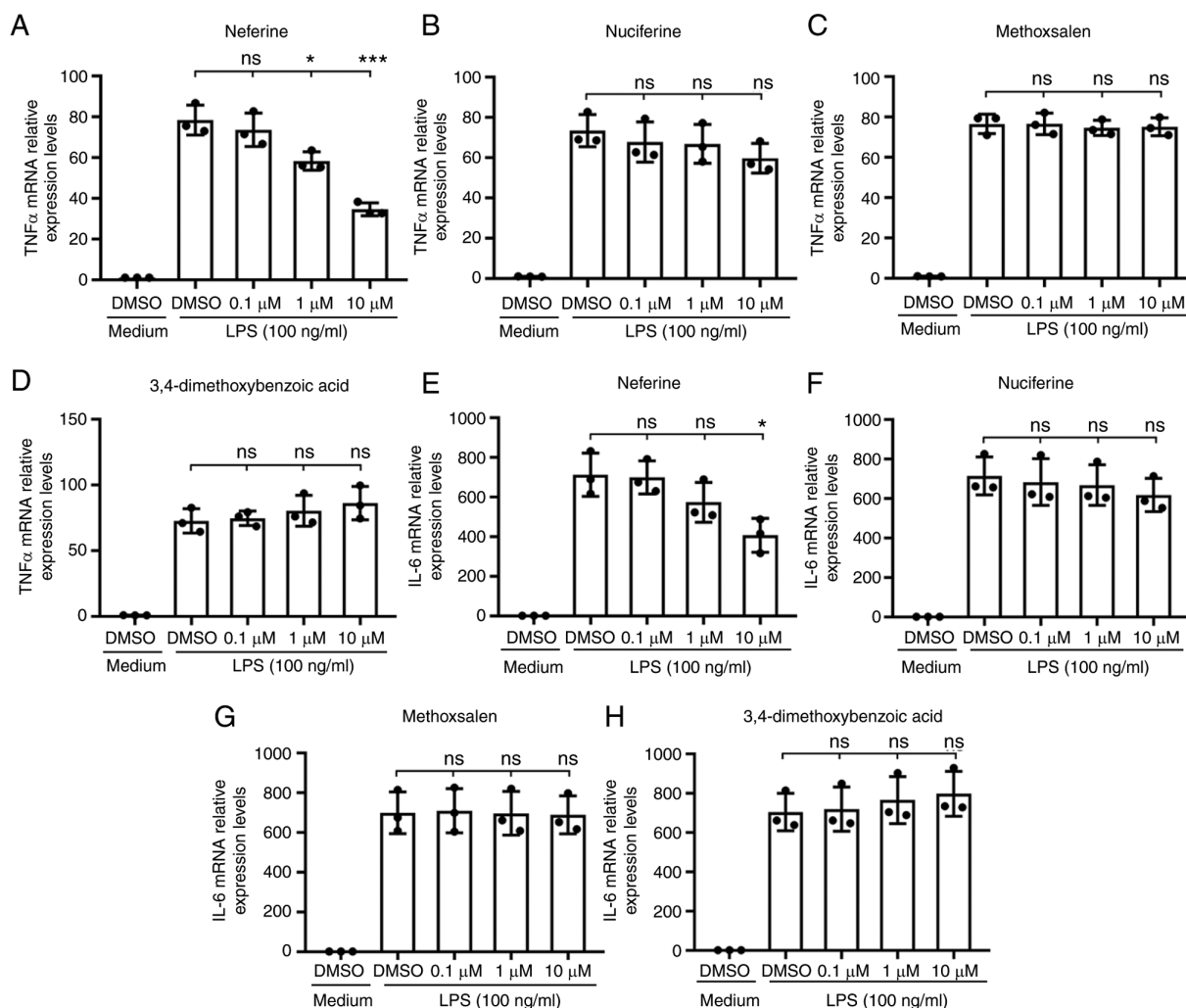


Figure 2. Effects of four drug candidates on LPS-induced proinflammatory cytokines in BV-2 cells. The expression levels of TNF- α were measured after pretreatment of BV-2 cells with (A) neferine, (B) nuciferine, (C) methoxsalen and (D) 3,4-dimethoxybenzoic acid in DMSO with the indicated concentrations for 30 min followed by treatment with LPS (100 ng/ml) for 6 h. The expression levels of IL-6 were measured after pretreatment of BV-2 cells with (E) neferine, (F) nuciferine, (G) methoxsalen and (H) 3,4-dimethoxybenzoic acid in DMSO with the indicated concentrations for 30 min followed by treatment with LPS (100 ng/ml) for 6 h. * $P < 0.05$, *** $P < 0.001$ vs. DMSO. Data are presented as mean $\pm$ standard deviation ($n=3$). LPS, lipopolysaccharide; ns, not significant.

Statistical analysis. The data are presented as mean $\pm$ standard deviation from three independently repeated experiments. A total of three samples were used for every *in vitro* experiment, and eight samples were used for every *in vivo* experiment. SPSS (version 16.0; SPSS, Inc.) was used to assess statistical significance. The differences between different groups were assessed by a Student's unpaired t-test. Multiple comparisons were performed using one-way ANOVA followed by the Bonferroni multiple comparisons test. Traction test data are shown as the median $\pm$ interquartile range, and were analyzed using the Kruskal-Wallis test followed by Dunn's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Neferine inhibits LPS-induced BV-2 activation. The Network pharmacology, the Traditional Chinese Medicine Systems Pharmacology (<https://tcmssp.com/>), and The Encyclopedia of Traditional Chinese Medicine (<http://www.tcmip.cn/ETCM/index.php/Home/>) databases were used to

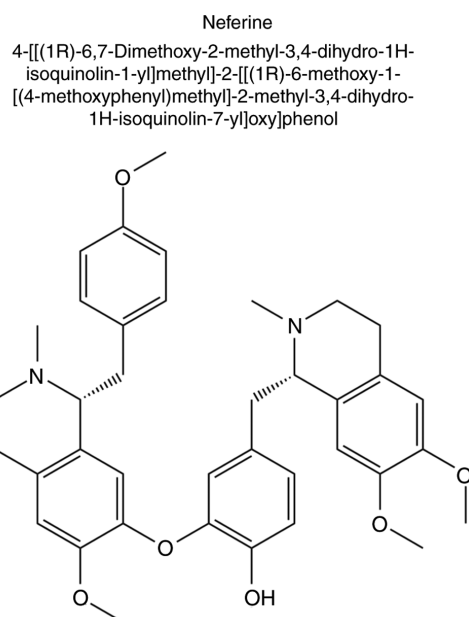


Figure 3. Chemical structure of neferine.

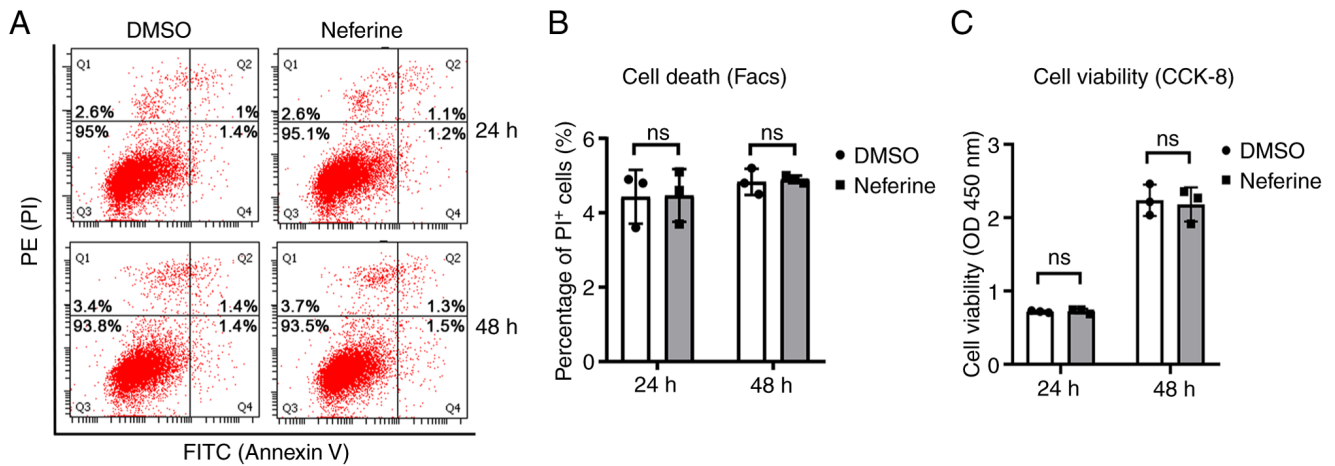


Figure 4. Neferine did not exert a cytotoxic effect on BV-2 cells. BV-2 cells were treated with either DMSO or neferine (10 μ M) for 24 and 48 h, and analyzed using an (A) annexin V/PI assay and (B) the percentage of PI⁺ non-viable cells were quantified. Cell viability was measured using (C) the CCK-8 assay in BV-2 cells following treatment with neferine (10 μ M) for either 24 or 48 h. Data are presented as mean $\pm$ standard deviation (n=3). PI, propidium iodide; CCK-8, Cell Counting Kit-8; ns, not significant.

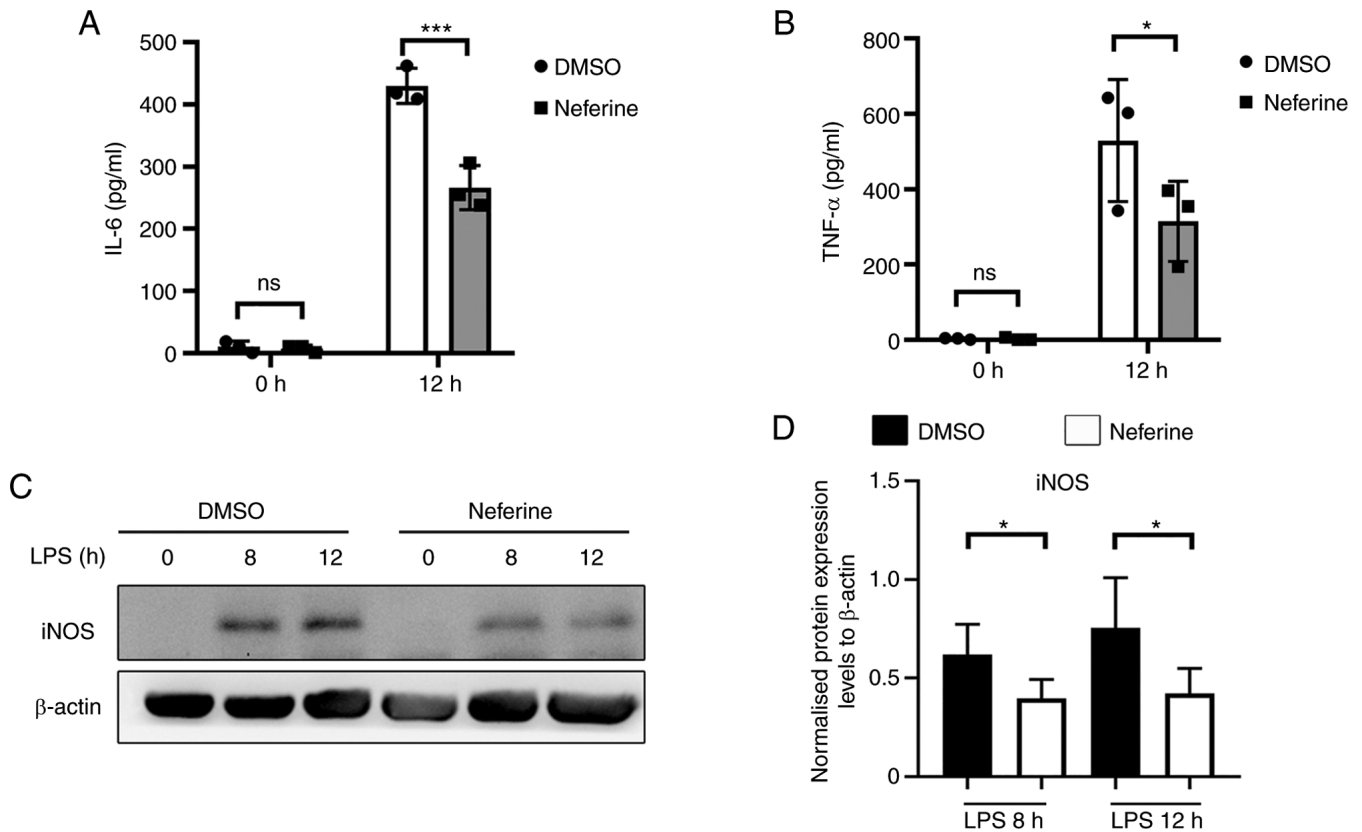


Figure 5. Effects of neferine on LPS-induced proinflammatory cytokine release and iNOS expression in BV-2 cells. BV-2 cells were pretreated with DMSO or neferine (10 μ M) for 30 min and subsequently treated with LPS (100 ng/ml) for 12 h, and the expression levels of (A) IL-6 and (B) TNF- α in the supernatant were measured by ELISA. (C) BV-2 cells were pretreated with DMSO or neferine (10 μ M) for 30 min and subsequently treated with LPS (100 ng/ml) for either 8 or 12 h. iNOS protein expression levels were measured by (C) western blotting, and (D) then they were then semi-quantified. The house keeping protein β -actin was used as a control. *P<0.05, ***P<0.001 vs. DMSO treatment. Data are presented as mean $\pm$ standard deviation (n=3). LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase; ns, not significant.

select potential anti-PD small molecular weight compounds. A total of four candidates were selected, neferine, nuciferine, methoxsalen and 3,4-dimethoxybenzoic acid, which fit the following six selection criteria: i) Potential anti-inflammatory small molecule compounds; ii) potential for identifying targets

associated with Parkinson's disease; iii) not reported in other studies of Parkinson's disease; iv) oral bioavailability >30%; v) drug likeness >0.18; and vi) BBB transmittance coefficient between -0.3 and 0.3 for subsequent functional screening in LPS-induced activated microglia in BV-2 cells (Fig. 1).

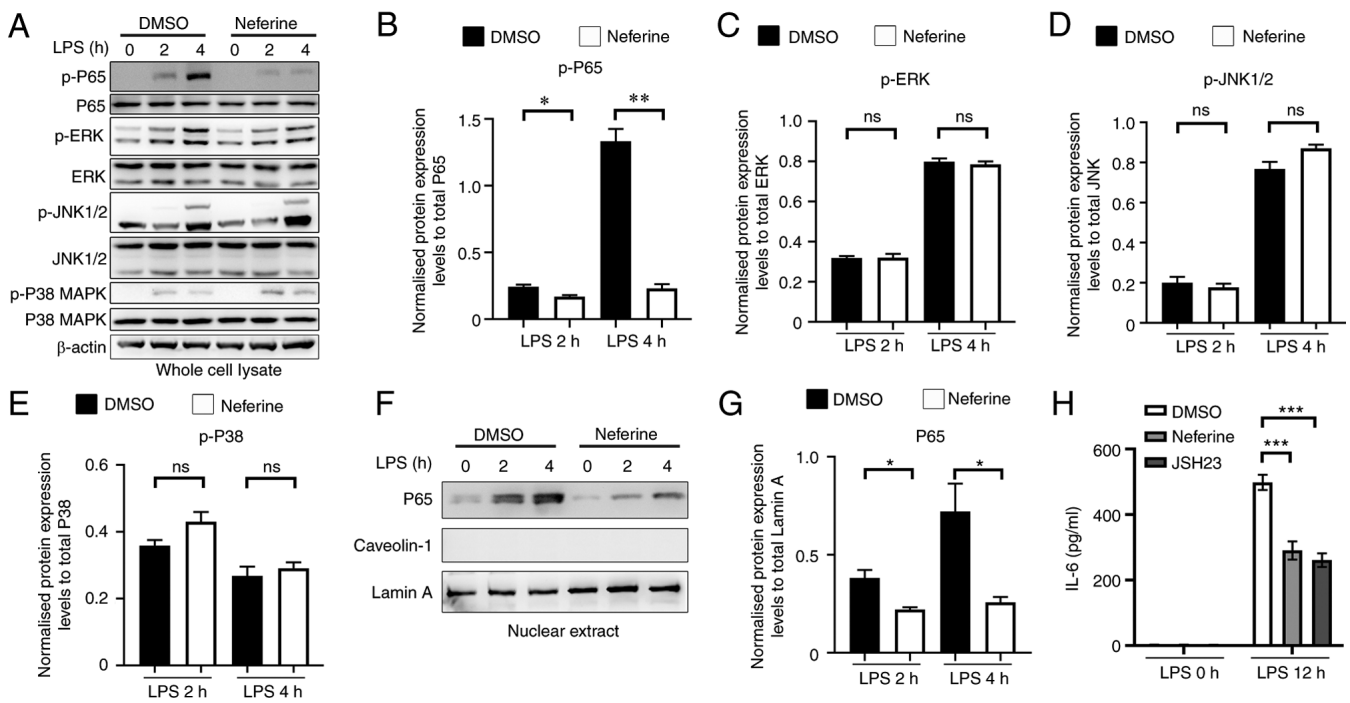


Figure 6. Neferine inhibits NF- κ B nuclear translocation and activation induced by LPS in BV-2 cells. (A) BV-2 cells were pretreated with either DMSO or neferine (10 μ M) for 1 h and subsequently treated with LPS (100 ng/ml) for 2 and 4 h, and (A) western blotting was used to measure the expression levels of various proteins. Protein expression levels of (B) P65, (C) ERK, (D) JNK and (E) P38 were semi-quantified. BV-2 cells were pretreated with either DMSO or neferine (10 μ M) for 1 h and subsequently treated with LPS (100 ng/ml) for 2 and 4 h, and (F) western blotting was used to measure the expression levels of various proteins, while (G) the expression levels of P65 were semi-quantified. BV-2 cells were pretreated with either DMSO, neferine (10 μ M) or JSH23 (10 μ M) for 30 min and subsequently treated with LPS (100 ng/ml) for 12 h, and (H) the expression levels of IL-6 in the supernatant were measured by ELISA. * P <0.05, ** P <0.005, *** P <0.001 vs. DMSO treatment. Data are presented as mean $\pm$ standard deviation (n =3). LPS, lipopolysaccharide; ns, not significant; p-, phosphorylated.

The transcription of TNF- α and IL-6 in BV-2 cells treated with 100 ng/ml LPS was measured by RT-qPCR as an index of microglia activation. Pretreatment with neferine (0.1, 1 and 10 μ M) and DMSO suppressed the activation of the BV-2 microglia cells in a dose-dependent manner, particularly at the highest concentration tested (Fig. 2A and E), while the other three candidates, nuciferine, methoxsalen and 3,4-dimethoxybenzoic acid, did not exert this significant effect (Fig. 2B-D, F-H). These results suggested that neferine was a potential inhibitor of microglia activation.

Neferine inhibits the production of LPS-induced iNOS and proinflammatory cytokines in BV-2 cells. Neferine is a bis-benzylisoquinoline alkaloid derived from lotus seed embryos, which has a wide range of pharmacological activities (Fig. 3). Initially, the cytotoxic effect of neferine was assessed in BV-2 cells. Neferine (10 μ M) did not induce cell death of BV-2 cells (Fig. 4A and B), and no significant difference was observed in the viability between the DMSO- and neferine-treated cells (Fig. 4C). This suggested that neferine exhibited no cytotoxic effect at the concentrations tested.

To further explore the role of neferine in suppressing microglia activation, BV-2 cells were pretreated with 10 μ M neferine, then inflammation was induced by 100 ng/ml LPS treatment for 0, 8 and 12 h (Fig. 5A-C). The protein expression levels of TNF- α and IL-6 in the cell supernatant were significantly decreased in the neferine treatment groups after 12 h (Fig. 5A and B). The production of iNOS was also significantly inhibited by neferine compared with that in the DMSO-treated

group (Fig. 5C and D). Therefore, neferine may suppress the production of LPS-induced TNF- α , IL-6 and iNOS in BV-2 cells in the absence of cytotoxic effects.

Neferine inhibits LPS-induced translocation and activation of NF- κ B in BV-2 cells. The MAPK and NF- κ B signaling pathways have been reported to mediate the activation of microglia cells (24). Therefore, the phosphorylation of relevant signaling proteins was assessed to investigate the ability of neferine to restrain BV-2 activation. Neferine exhibited no significant effect on the phosphorylation of JNK1/2, ERK and p38 MAPK (Fig. 6A-E), whereas it significantly inhibited the phosphorylation of p65 in BV-2 cells treated with 100 ng/ml LPS. Cell treatment with neferine significantly decreased the nuclear translocation of p65, which was consistent with the aforementioned result (Fig. 6F and G). Caveolin-1, a cytoplasmic protein, was used as a negative control for nucleoprotein extraction. JSH23 was previously reported to inhibit NF- κ B transcriptional activity (25). Therefore, JSH23 was selected as the positive control of neferine. It was demonstrated that JSH23 and neferine exhibited similar effects in the inhibition of the release of IL-6 in LPS-activated BV-2 cells (Fig. 6H). These results indicated that neferine inhibited LPS-induced BV-2 cell activation via the inhibition of the NF- κ B signaling pathway.

Neferine ameliorates dyskinesia in a PD mouse model. The release of inflammatory cytokines and NO by microglia leads to the deterioration of neurodegenerative diseases (26). Therefore,

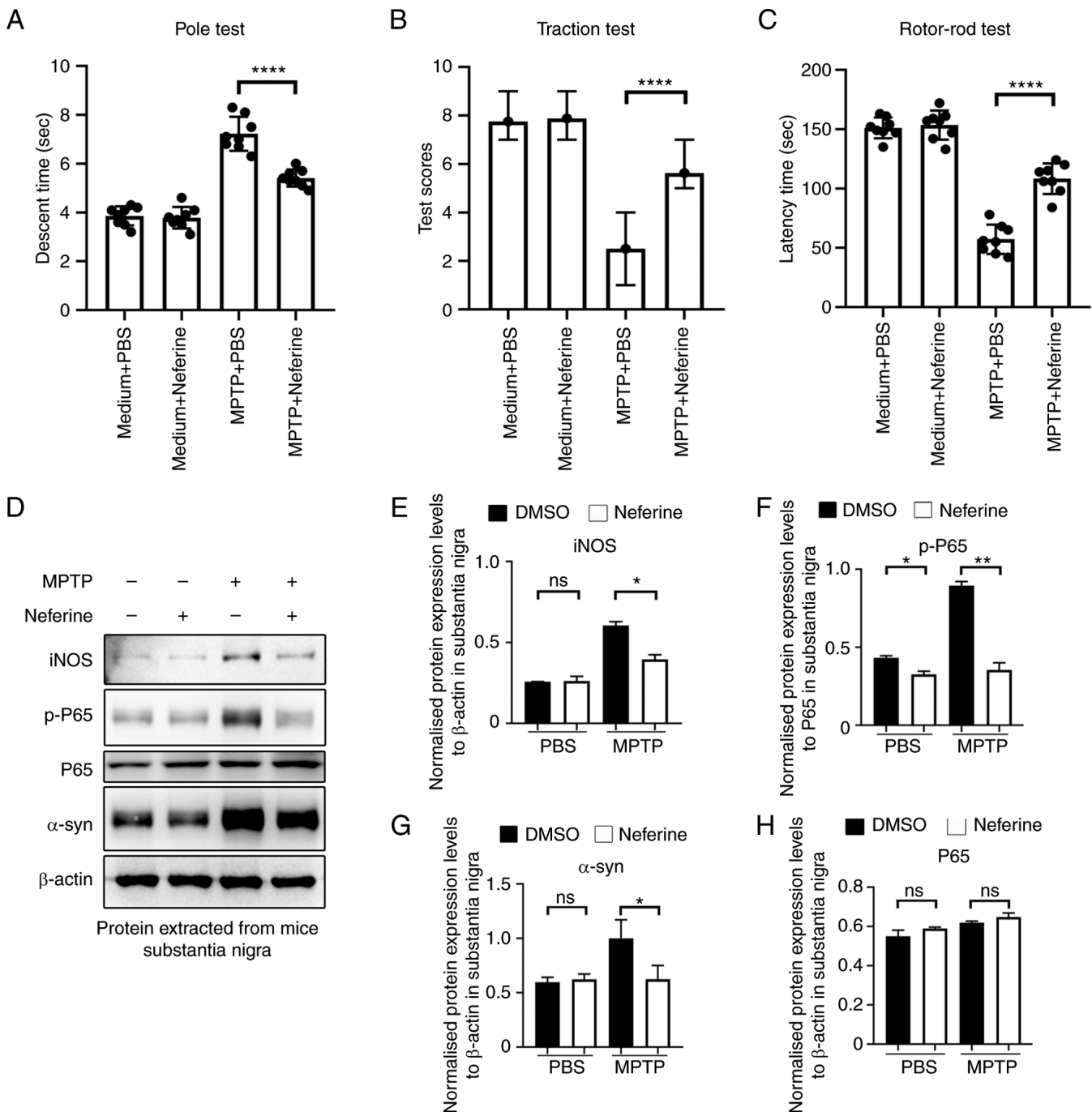


Figure 7. Neferine ameliorates dyskinesia in MPTP-treated mice. (A) Dyskinesia was assessed by a pole test by recording the time required for the mice to descend the pole. Data are presented as mean $\pm$ standard deviation (n=8). (B) Muscle strength and equilibrium were assessed by a traction test by recording the traction reflex score. Data are presented as median $\pm$ interquartile range (n=8). (C) Motor coordination function was assessed by the rotor-rod test by recording the time period required for the mouse to stay on the pole. Data are presented as mean $\pm$ standard deviation (n=8). ****P<0.0001 vs. PBS treatment. Proteins were extracted from the mouse substantia nigra tissue (n=1/treatment group), and their expression was investigated by (D) western blotting. One representative experiment out of a total of four is shown. The total protein expression levels of (E) iNOS, (F) p-P65, (G) α -syn and (H) P65. The house keeping protein β -actin and total protein of P65 was used as a control. *P<0.05, **P<0.005 ****P<0.0001 vs. DMSO treatment. MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; ns, not significant; iNOS, inducible nitric oxide synthase; α -syn, α -synuclein; p-, phosphorylated.

the present study assessed the anti-neuroinflammatory effect of neferine in MPTP-induced PD in mice. Dyskinesia is a typical hallmark of PD, therefore, a pole test was performed to assess bradykinesia, a traction test to assess muscle strength and equilibrium, and a rotor-rod test to assess motor coordination function of PD mice. The mice in the MPTP + neferine group required lower time periods to complete the pole test (Fig. 7A),

exhibited a higher score in the traction test (single treatment; Fig. 7B) and demonstrated the ability to walk longer on the rotors (Fig. 7C) compared with the mice in the MPTP + PBS group. Consistent with these behavioral experiments, activation of NF- κ B indicated by p-p65 and the levels of iNOS and α -syn, which is closely associated with the pathogenesis of PD (8), were significantly lower in the substantia nigra tissues

of MPTP + neferine-treated mice compared with mice treated with MPTP alone (Fig. 7D-G). These results indicated that neferine could exert a neuroprotective role in MPTP-induced PD in mice by inhibiting the activation of NF- κ B.

Discussion

Excessive activation of microglia and the subsequent production of inflammatory cytokines serve critical roles in the pathogenesis and progression of PD (27). Therefore, the identification of novel agents which can inhibit microglia-mediated inflammation is a feasible therapeutic strategy against PD. Neferine has previously been reported to exert its anti-inflammatory capacity in several types of cells and mouse models, such as mast cells (RBL-2H3 cells) (28), PC12 rat pheochromocytoma cells (29), patient-derived orbital fibroblasts (18) and in a carbon tetrachloride (CCl₄)-induced liver fibrosis mouse model (17). The present study reported for the first time that neferine could inhibit microglia activation and subsequent release of TNF- α , IL-6 and production of iNOS. This suggests that neferine exhibits a dual anti-inflammatory effect in inhibiting neuronal and microglia inflammation.

A prerequisite for a drug to be used on the nervous system is its ability to cross the BBB (12). In the present study, neferine was selected by network pharmacology based on the following criterion, appropriate BBB transmittance coefficient. Subsequent *in vivo* experiments in mice suggested that neferine promoted the remission of PD-related symptoms. In addition, it was shown that neferine did not induce cell death of BV-2 cells. These results suggested that neferine meets the basic requirements of a candidate drug for the nervous system. Although preliminary results were obtained indicating that neferine could alleviate the symptoms of PD, further studies are required to investigate the feasibility of the application of this compound as an effective treatment strategy for PD.

LPS-induced activation of BV-2 cells is a widely used cell model to study neurodegenerative diseases (19,30-32). LPS triggers TLR4-mediated signaling, which initiates the activation of NF- κ B and MAPKs via different signalosomes, and ultimately induces the transcription of inflammatory proteins and cytokines (20). Neferine has previously been reported to regulate several types of biological effects. Wang *et al* (17) reported that neferine acted as an antioxidant and anti-inflammatory agent in CCl₄-induced liver fibrosis by inhibiting the MAPK and NF- κ B signaling pathways. Pretreatment of mast cells with neferine also inhibits the phosphorylation of the MAPK/NF- κ B pathway (28). However, neferine can promote the activation of JNK1/2 and p38 MAPK enzymes in melanoma which leads to the induction of apoptosis in melanoma cells (33). Neferine suppresses the differentiation of osteoclasts by inhibiting the NF- κ B signaling pathway rather than the MAPKs, which in turn promotes osteogenesis (34). Neferine was also reported to inhibit the expression of the inflammatory mediators iNOS and COX-2, and the matrix degrading enzymes MMP3 and 13 in IL-1 β -treated rat chondrocytes by suppressing the MAPK and NF- κ B signaling pathways (35). These findings suggest that neferine exhibits different regulatory patterns in different cells. In the present study, the phosphorylation of p65, which represents the activation of NF- κ B, JNK1/2, ERK and p38 MAPK, was examined to analyze the mechanism by which

neferine inhibited LPS-induced BV-2 cell activation. These results indicated that neferine inhibited LPS-induced activation of NF- κ B as opposed to the MAPK-related signaling pathway. However, the differential regulatory mode of neferine requires further investigation.

In summary, the present study indicated that neferine could inhibit microglia activation by suppressing the NF- κ B signaling pathway, which exerted a therapeutic effect in the PD mouse model. The present study provides novel evidence that neferine inhibits microglia overactivation, therefore, this compound may be a potentially effective drug for relieving neurodegenerative diseases such as PD.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TL, YZ, TZ and BX contributed to the study conception and design. Material preparation and data collection were performed by TL and TZ. YZ and BX analyzed the data and confirm the authenticity of all the raw data. The first draft of the manuscript was prepared by TL and BX, and all authors commented on the previous versions of the manuscript. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The mice used in the present study were handled according to the Guide for the Care and Use of Medical Laboratory Animals (Ministry of Health, Beijing, China). The experimental protocol was approved by the Laboratory Ethics Committee of China Medical University, Shenyang, China (approval no. CMU:2020096).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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