Therapeutic effects of paeonol on methyl-4-phenyl-1,2,3,6-tetrahydropyridine/probenecid-induced Parkinson's disease in mice

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Abstract. Paeonol is a major phenolic compound of the Chinese herb, Cortex Moutan, and is known for its antioxidant, anti-inflammatory and antitumor properties. The present study was designed to investigate the therapeutic potential and underlying mechanisms of paeonol on a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine/probenecid (MPTP/p)-induced mouse model of Parkinson's disease (PD). MPTP (25 mg/kg), followed by probenecid (250 mg/kg), was administered via i.p. injection for five consecutive days to induce the mouse model of PD. Paeonol (20 mg/kg) was administrated orally for 21 days. Behavior was assessed using the rotarod performance and open-field tests. Additionally, the levels of tyrosine hydroxylase (TH), microglia, interleukin-1β (IL-1β), and brain-derived neurotrophic factor (BDNF) in the substantia nigra pars compacta (SNpc) were evaluated by immunohistochemical staining. MPTP/p-induced motor deficits were observed to be significantly improved following long-term treatment with paeonol. Paeonol treatment decreased MPTP/p-induced oxidative stress, as determined by the activity levels of superoxide dismutase, catalase and glutathione. Additionally, MPTP/p-induced neuroinflammation was assessed by examining the levels of microglia and IL-1β, which were significantly decreased following paeonol treatment. Paeonol treatment improved the MPTP/p-induced dopaminergic neurodegeneration, as measured by observing the increased TH level in the SNpc. Furthermore, the BDNF level was significantly elevated in the paeonol treatment group compared with mice treated with MPTP/p only. In conclusion, paeonol exerted therapeutic effects in the MPTP/p-induced mouse model of PD, possibly by decreasing the damage from oxidative stress and neuroinflammation, and by enhancing the neurotrophic effect on dopaminergic neurons. The results demonstrate paeonol as a potential novel treatment for PD.

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by the selective degeneration of dopaminergic neurons in the substantia nigra (SN), a region of the brain that controls movement (1). The primary symptoms of PD include tremor at rest, muscular rigidity, bradykinesia, postural abnormalities and instability (2). Although the precise underlying pathogenic mechanism of PD remains to be elucidated, previous studies have suggested that oxidative stress, inflammation, excitotoxicity and mitochondrial dysfunction may be involved in the progression of PD (3,4). Post-mortem analysis of patients with PD have indicated that oxidative stress and inflammatory pathways together cause dopaminergic neurons to undergo apoptosis, ultimately resulting in PD (5). These findings suggest that it may be beneficial to evaluate the therapeutic effects of antioxidant and anti-inflammatory agents on PD.

Although the symptoms of PD may be relieved by dopamine (DA) replacement therapy, the efficacy of drug therapy gradually declines over time (6). Furthermore, long-term treatment with L-dihydroyphenylalanine or DA agonists results in severe adverse effects, which markedly influence patient quality of life (7,8). Therapeutic strategies that unequivocally slow or stop the progression of PD do not currently exist (9). Thus, identifying an agent that provides effective protection against dopaminergic neurodegeneration would be a major breakthrough in the treatment of PD.

Agents that have neurotrophic properties may potentially promote the survival of neuronal cells and slow the progression of PD (10). Due to its neuroprotective and neurotrophic properties, paeonol may provide a new strategy for the treatment of PD (11). The Chinese herb, Cortex Moutan, contains an abundance of phenolic compounds that are effective in protecting against oxidative stress and neuroinflammation (12). Previous studies have suggested that paeonol is an effective antioxidant, anti-inflammatory and anti-tumor agent (13). Furthermore, the neuroprotective properties of paeonol have been confirmed to be beneficial for the treatment of neurodegenerative disease, including Alzheimer’s disease (14), Huntington’s disease (15), and multiple sclerosis (16). In the present study, we evaluated the effects of paeonol on MPTP/p-induced motor deficits, oxidative stress and neuroinflammation, and on the levels of TH, microglia, IL-1β and BDNF in SNpc of mice. These findings may provide a new strategy for the treatment of PD.
Properties, 2'-hydroxy-4'-methoxyacetophenone (paeonol) has been identified as a potential treatment for PD. Paeonol (Fig. 1A) is a flavonoid derivative that exerts numerous physiological activities, including antioxidant (11), anti-inflammatory (12), antidiabetic (13), anticancer, analgesic, and hypnotic (14) action. An in vitro study using PC12 cells suggested that paeonol exerts anti-PD effects, as it prevented oxidative stress and the apoptosis of dopaminergic neurons (15). Another in vitro study on microglia demonstrated that paeonol inhibited the expression of inflammatory mediators, which suggests that paeonol may have therapeutic properties in neurodegenerative diseases (16). However, whether paeonol exerts therapeutic effects against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine/probenecid (MPTP/p)-induced PD in mice remains unknown.

MPTP (Fig. 1B) is a neurotoxin that induces behavioral, biochemical and neuropathological changes that are similar to those observed in patients with idiopathic PD, and it is therefore used to mimic the pathological process of PD in mice (17,18). Furthermore, probenecid blocks the excretion of MPTP, which eventually results in serious neurotoxicity (19). The aim of the present study was to assess the therapeutic and neuroprotective effects of paeonol in an MPTP/p-induced mouse model of PD. Previous studies on the neuroprotective effects of paeonol revealed that doses of 20, 50 and 100 mg/kg body weight significantly improved neuronal damage in experimental mice (20,21); therefore, paeonol was administered at a dose of 20 mg/kg in the present study. The effects of paeonol were determined as follows: i) Evaluating the behavior of mice with rotarod performance and open-field tests; ii) measuring oxidative stress levels, specifically the activity levels of superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) in the midbrain; iii) evaluating dopaminergic neurodegeneration in the SN pars compacta (SNpc) by calculating the number of tyrosine hydroxylase (TH)-positive cells using immunohistochemical staining; iv) examining inflammatory mediators, including microglial cells and interleukin-1β (IL-1β) using immunohistochemical staining; and v) identifying any neuroprotective properties by measuring the level of brain-derived neurotrophic factor (BDNF).

Materials and methods

**Experimental animals.** Adult male C57BL/6 mice (n=40; weight, 18-20 g) were purchased from the Comparative Medicine Center at Yangzhou University (Yangzhou, China). The animals were maintained on a 12-h light/dark cycle at 25±2°C and 60-70% relative humidity with food and water available ad libitum in the Laboratory Animal Center of Bengbu Medical College (Bengbu, China). The study was reviewed and approved by the institutional Animal Ethical Committee of Bengbu Medical College (Bengbu, China). All surgical procedures were performed under anesthesia.

**Chemicals and reagents.** Paeonol (purity, ≥98.0%) and MPTP hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). Probenecid was obtained from Shanghai Jingchun Reagent Co., Ltd. (Shanghai, China). Rabbit anti-TH (cat. no. ab75875; 1:600), rabbit anti-IL-1β (cat. no. ab9722; 1:500), rabbit anti-BDNF (cat. no. ab203573; 1:200) and rabbit anti-ionized calcium-binding adapter molecule 1 (Iba1; cat. no. ab15690; 1:200) were purchased from Abcam (Qatar, Kingdom of Saudi Arabia), and goat-anti-rabbit secondary antibody (cat. no. K5007; 1:1) was purchased from Dako (Glostrup, Denmark).

**Experimental procedure.** The mice were randomly divided into four groups (n=10 per group): i) Control group, mice were treated with saline; ii) MPTP/p only group, mice received MPTP via i.p. injection (25 mg/kg in saline) followed by probenecid (250 mg/kg in 0.03 ml dimethylsulfoxide) once a day for five consecutive days (days 1-5) to induce the PD mouse model (22); iii) MPTP/p+paeonol group, mice received paeonol (20 mg/kg in 5% carboxymethylcellulose sodium) by oral gavage daily for 21 days (days 1-21) and were injected with MPTP/p (as for the MPTP/p only group); and iv) paeonol only group, mice received oral paeonol (as for the MPTP/p+paeonol group) for 21 days. Probencid was injected 30 min prior to MPTP injection, and paeonol was administrated orally 1 h prior to MPTP injection. The rotarod performance and open-field tests were conducted during the course of MPTP/p administration (days 4 and 5) and during the final stages of the study (days 20 and 21). Mice were sacrificed by anesthetic overdose following the behavioral tests, and the brains were harvested for immunohistochemical and biochemical analyses (Fig. 1C).

**Rotarod performance test.** The rotarod performance test is widely used to assess motor and coordination abilities, particularly bradykinesia, in mice (23). In the present study, a rotarod (diameter, 3 cm) at a fixed speed of 30 rpm was utilized. The duration that each mouse spent on the revolving rod was measured. The rotating rod automatically stopped at 60 sec. If the mouse remained on the rod for the duration of the experiment, the value was recorded as 60 sec. Each mouse was trained in a separate lane of the rotarod three times a day for two consecutive days prior to performing the experiment. The rotarod test was performed on days 4 and 20, and the assessment was repeated three times for each mouse, with 30-min rest periods between tests. The mean duration for each mouse was determined and used for comparison (24).

**Open-field test.** The open-field test is an efficient technique for investigating the overall manifestation of motor deficits in animal models of PD, as it is sensitive to dopaminergic neuronal injury (25). In the present study, an open-field composed of a square arena (40x40 cm) and a wall (35-cm high) was divided into 16 sub-squares (4x4). The mouse was placed in the center of the arena and the behavior of the mouse was observed for 5 min; each mouse repeated the assessment three times. Following each test, the mice were allowed to rest for 30 min and the open-field was cleaned completely with a 70% ethanol solution. The number of crossings (a crossing was recorded as each time the mouse crossed the boundary of a sub-square with at least their two forepaws), grooming behaviors (rubbing the body with the paws or mouth and/or rubbing the head with the paws), rearing behaviors (standing on the hind legs), and the duration of immobility, were determined. The open-field test was performed on days 5 and 21, and conducted by an examiner who was blinded to the treatment groups.
Assessment of SOD, CAT and GSH activity. The supernatant of the midbrain tissue homogenate was prepared by centrifugation (Smart R17; Hanil Science Industrial Co., Ltd., Incheon, South Korea) at 9,184 x g for 10 min at 4°C. Total SOD activity was measured by spectrophotometry using a Total Superoxide Dismutase (T-SOD) assay kit (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China) according to the manufacturer's instructions, which contained the experimental steps and calculation formula. SOD activity is expressed as SOD U/mg protein, with one SOD unit being the quantity that reduced the absorbance at 560 nm by 50%, according to a Synergy HT microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

CAT activity in the midbrain was measured by spectrophotometry using a CAT assay kit (Nanjing Jiancheng Bioengineering Research Institute) according to the manufacturer's instructions. The absorbance was measured at 405 nm on a microplate reader, and CAT activity was calculated using the formula provided by the manufacturer, and expressed as units of CAT activity/mg protein.

GSH levels were assessed by spectrophotometry using a reduced GSH assay kit (Nanjing Jiancheng Bioengineering Research Institute) according to the manufacturer's instructions. The absorbance was measured at 420 nm and used to calculate the GSH concentration with the formula provided by the manufacturer, and the results are expressed as GSH/mg protein.

Immunohistochemical staining procedure. Mice were anesthetized with 10% chloral hydrate (3 ml/kg; Sigma-Aldrich) following the behavioral test and their hearts were perfused with saline, followed by 4% paraformaldehyde for 20 min. The brains were embedded in paraffin and coronally sectioned through the SNpc region at a thickness of 4 µm. Sections were fixed on slides, deparaffinized with three changes of xylene, and then dehydrated through decreasing grades of absolute alcohol (95, 75 and 50%). Endogenous peroxidases were blocked by incubating the sections with 3% H2O2 for 10 min. Following antigen retrieval with citrate salt buffer (pH 7.0; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) in microwave oven for 6 min and rinsing with phosphate-buffered saline, the sections were incubated at 37°C overnight with a primary monoclonal antibody (rabbit anti-TH, rabbit anti-IL-1β, rabbit anti-Iba1 or rabbit anti-BDNF), and then with a biotinylated goat anti-rabbit secondary antibody for 50 min. Sections were subsequently washed and developed with a 3,3'-diaminobenzidine staining kit (Dako); the chromogenic reaction was terminated when brown granules (positive cells) were observed. Finally, the sections were counterstained with hematoxylin, rinsed with deionized water, dried and sealed with neutral balsam. The stained sections were analyzed under an optical microscope (Nikon Corporation, Tokyo, Japan).

Statistical analysis. All data are expressed as the mean ± standard deviation. Multiple comparisons analyses were performed using a one-way analysis of variance followed by Tukey's post-hoc tests using the Statistical Package for the Social Sciences (SPSS) software version 16.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of paeonol on the behavior of mice with MPTP/p-induced PD in the rotarod performance test. The MPTP/p group demonstrated significantly reduced retention times compared with the control group on days 4 and 20 (P<0.01). The MPTP/p + paeonol group showed more staining on day 20 when compared with the MPTP/p only group, although this difference was not observed on day 4. The retention times of the control are similar to those of the control group. Data are expressed as the mean ± standard deviation, and statistical analysis was performed using a one-way analysis of variance followed by Tukey's post-hoc test. *P<0.01 vs. control; **P<0.01 vs. MPTP/p only. MPTP, methyl-4-phenyl-1,2,3,6-tetrahydropyridine; p, probenecid. Pae, paeonol.

Effect of paeonol on the behavior of mice with MPTP/p-induced PD in the open-field test. The results of the open-field test are presented in Fig. 3. The MPTP/p only group exhibited significantly reduced locomotor activity (as measured by lines
crossed; Fig. 3A), rearing (Fig. 3B) and grooming (Fig. 3C), and increased duration of immobility (Fig. 3D), compared with the control group on days 5 and 21 (P<0.01). The MPTP/p + paenol group demonstrated significantly improved locomotor activity, rearing and grooming, and decreased immobility time compared with the MPTP/p only group on day 21 (P<0.01); however, no significant differences were observed on day 5. Furthermore, no significant differences were observed between the control and the paenol only groups.

**Paeonol attenuated MPTP/p-induced oxidative stress.** The activity levels of SOD, CAT and GSH in the midbrain, which reflect the level of oxidative stress, are presented in Fig. 4. MPTP/p significantly reduced the levels of SOD, CAT and GSH, and therefore enhanced the level of oxidative stress in the MPTP/p only group compared with the control group (P<0.01). Treatment with paenol for 21 days significantly restored the activity of SOD, CAT and GSH, and therefore alleviated oxidative stress in the MPTP/p + paenol group.
compared with the MPTP/p only group (P<0.01). No significant differences were observed between the control and the paeonol only groups.

**Paeonol attenuated MPTP/p-induced neuroinflammation.** Microglia were stained with an antibody against Iba1 (a microglial marker). As presented in Fig. 5A, immunohistochemical staining for Iba1 and IL-1β in the SNpc revealed large brown-colored regions in the MPTP/p only group. However, the positive cells in the MPTP/p + paeonol group were not only smaller, but were also lighter in color than those in the MPTP/p only group. No or little specific staining of Iba1 or IL-1β was observed in the control and paeonol only groups. The numbers of Iba1-positive (51 vs. 11%; P<0.01) and IL-1β-positive (76 vs. 12%; P<0.01) cells significantly increased in the MPTP/p only group compared with the control group. However, the numbers of Iba1-positive (28 vs. 51%; P<0.01) and IL-1β-positive (39 vs. 76%; P<0.01) cells significantly decreased in the MPTP/p + paeonol group compared with the MPTP/p only group. No significant differences were observed between the paeonol only and control groups.

**Paeonol attenuated the MPTP/P-induced loss of dopaminergic neurons.** The results of the immunohistochemical examinations revealed that the majority of the TH-positive cells (representing dopaminergic neurons) in the SNpc were fusiform or had an irregular triangular shape, and dense neurons with dark-stained cytoplasms were observed in the control and paeonol only groups (Fig. 6A). MPTP administration induced clear fragmentation of the dopaminergic neurons, causing the cellular outline to be ambiguous and the cytoplasmic staining to be faint (Fig. 6B); thus, only 19% of the TH-positive cells were observed in the SN of the MPTP/p only group compared with 100% of the TH-positive cells in the control group (P<0.01; Fig. 6B). However, the MPTP/p + paeonol group demonstrated protection of TH-positive cells when compared with the MPTP/p only group (51 vs. 19%; P<0.01). No significant difference was observed between the paeonol only and the control groups.

**Paeonol attenuated the MPTP/p-induced loss of BDNF.** MPTP/p administration significantly reduced the level of BDNF in the SN of the MPTP/p only group compared with the level in the control group (19 vs. 49%; P<0.01; Fig. 7). Treatment with paeonol for 21 days significantly restored the level of BDNF in the SN of mice with MPTP/p-induced PD compared with the level in the MPTP/p only group (36 vs. 19%; P<0.01). The level of BDNF in the paeonol only group was slightly increased compared with the level in the control group; however, the difference was not statistically significant.

**Discussion**

The discovery of neuroprotective compounds that slow or stop the progression of neurodegeneration is essential for treating various neurodegenerative diseases, including PD (26,27). The results of the present study demonstrate for the first time, to the best of our knowledge, that long-term paeonol treatment
exhibits therapeutic effects in mice with MPTP/p-induced PD. Mice experienced improved motor dysfunction, suppression of oxidative stress and the inflammatory cascade, and protection of TH-positive neurons. In addition, it was observed that BDNF levels increased following paeonol treatment, suggesting that paeonol exerts neurotrophic effects on PD.

In the present study, the mouse model of PD was induced by co-administration of MPTP and probenecid, which was more suitable for investigating the therapeutic effects of long-term drug treatment than mouse models of PD induced with MPTP alone (28). MPTP effectively crosses the blood-brain barrier and accumulated MPTP is metabolized into 1-methyl-4-phenylpyridinium (MPP+) by monoamine oxidase-B. MPP+, which inhibits complex I of the mitochondrial respiratory chain, enters dopaminergic neurons via the dopamine transporter, ultimately inducing their degeneration (29,30).

There is increasing evidence that the motor dysfunctions of MPTP/p-treated mice may be observed using behavioral tests, including the rotarod performance and open-field tests (31). In the present study, the performances of mice with PD significantly improved on the rotarod performance and open-field tests following long-term treatment with paeonol, although no apparent improvement was observed on day 4. This is consistent with the results of previous studies (28,32). The results of the behavioral tests suggested that long-term paeonol treatment has a therapeutic effect on mice with MPTP/p-induced PD.

Oxidative stress is widely considered to be important in the development of PD (33,34). Extensive post-mortem studies have indicated that oxidative stress induces the downregulation of antioxidant protective systems, including...
SOD, GSH and CAT, and damages lipids, proteins and DNA, ultimately resulting in damage to dopaminergic neurons in the SNpc (34-36). SOD, CAT and GSH effectively remove oxygen free radicals and lipid peroxides, which may be used to measure the level of oxidative stress (37). In the present study, the activity levels of SOD, CAT and GSH were significantly decreased in the MPTP/p only group, suggesting that oxidative stress is involved in the pathogenesis of PD. Following long-term paenol treatment, the levels of SOD, CAT, and GSH significantly improved in mice with MPTP/p-induced PD, which is consistent with the findings of a previous study (38). The results of the present study suggest that paenol exerts a therapeutic effect on MPTP/p-induced oxidative stress, perhaps due to its antioxidant activity and ability to reduce the toxicity caused by oxidative stress.

Excessive microglial activation has been observed in the SN of human post-mortem tissue from PD patients and animal models, suggesting that neuroinflammation and activated microglia are crucial in the pathogenesis of PD (39-41). Microglia are involved in immune defense and surveillance, and activate a variety of proinflammatory cytokines, including IL-1β, which may directly induce apoptosis in dopaminergic neurons (42,43). Studies have demonstrated that the overactivation of microglia and the overproduction of proinflammatory cytokines may enhance oxidative stress, eventually leading to neuronal death (44,45). In addition, a previous study has revealed that paenol may be a potential neuroprotective agent, possibly through inhibiting microglia-mediated inflammation and oxidative stress-induced neuronal damage (46). In the present study, the levels of microglia and IL-1β were significantly increased in the SNpc of mice in the MPTP/p only group. However, long-term paenol treatment significantly decreased these levels. These results suggest that paenol was therapeutically, perhaps by downregulating the inflammatory cascade.

TH is the limiting enzyme of dopamine synthesis, and TH immunohistochemical analysis measures the number and function of dopaminergic neurons and fibers in the SN, as reductions in dopaminergic neurons may be the primary reason for the onset of PD (47,48). In the present study, immunohistochemical analysis of TH revealed a significant loss of dopaminergic neurons in the SNpc of mice in the MPTP/p only group, perhaps due to MPTP-induced oxidative stress and inflammation (49). Long-term paenol treatment appeared to protect dopaminergic neurons and fibers in mice with MPTP/p-induced PD, which suggests that its therapeutic effects may be associated with its anti-inflammatory and antioxidant effects.

BDNF is a member of a family of neurotrophic factors that has attracted widespread attention, as it exhibits strong nutritional and protective effects, particularly in DA neurons (50,51). Studies have demonstrated that the level of BDNF significantly decreases in patients with PD (52,53), which is consistent with the results of the present study. These findings suggest that novel treatment strategies for PD should aim to increase the expression levels of BDNF. In the present study, it was observed that treatment with paenol enhanced BDNF expression levels and the number of TH-positive cells in the SN of mice with MPTP/p-induced PD, while no apparent improvement was observed in the paenol only group. These findings suggest that the therapeutic effects of paenol may be associated with its potential neurotrophic effects on dopaminergic neurons.

In conclusion, the results of the present study demonstrate that paenol exerts therapeutic effects against MPTP/p-induced PD in mice, as revealed by improvements in behavioral tests and enhanced TH expression compared with mice that did not receive paenol. In addition, the therapeutic effects may be associated with the ability of paenol to reduce oxidative stress, increase antioxidant defenses, and inhibit the overactivation of microglia along with the release of proinflammatory factors. Furthermore, BDNF levels were improved in mice with MPTP/p-induced PD following long-term treatment with paenol, indicating that paenol has neurotrophic effects on dopaminergic neurons. Therefore, the results of the present study suggest that paenol has potential as a treatment for PD; however, the dose of paenol (20 mg/kg) administered in the present study may not be directly extrapolated to humans. Future studies are required to investigate the dose-dependent effect on MPTP/p-induced PD mice and determine an optimal dose. In addition, further studies are necessary to establish the exact mechanisms underlying the effects of paenol.

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