Abstract. The pathological alterations of Parkinson's disease (PD) predominantly manifest as a loss of dopaminergic neurons in the substantia nigra, which may be caused by oxidative stress damage. Proanthocyanidins (PCs) are a class of compounds found in various plants, which have significant antioxidant and free radical-scavenging activity. The present study investigated the protective effects and underlying mechanisms of PCs in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD model in vitro and in vivo. MTT assays were used to detect cell viability, and flow cytometry and TUNEL assays were used to detect cell apoptosis. Mitochondrial membrane potential (MMP) alterations were investigated using a JC-1 MMP Assay kit. The pole test was used to measure motor behavior in a mouse model of PD. Levels of reactive oxygen species (ROS) were measured using the fluorescent probe, 2',7'-dichlorodihydrofluorescein diacetate. Immunohistochemistry and western blotting were performed to detect the expression levels of proteins associated with PD.

In vitro, it was demonstrated that in MPTP-treated PC12 cells, PCs increased cell viability and reduced cell apoptosis in a dose-dependent manner. In vivo, it was revealed that PC treatment inhibited striatal dopamine depletion, which resulted in significant improvements in PD-like movement impairment. Reactive oxygen species (ROS) production and MPTP-induced apoptosis were also inhibited. Furthermore, the results demonstrated that the neuroprotective activity of PCs may be mediated via the inhibition of ROS generation, as well as modulation of c-Jun N-terminal kinase activation. Taken together, these data revealed that PCs may exert neuroprotective effects in in vivo and in vitro PD models, and may have potential in the prevention or treatment of PD.

Introduction

Parkinson's disease (PD) is a common chronic degenerative disease of the nervous system, which is primarily characterized by a substantial loss of substantia nigra dopaminergic neurons, leading to a reduction of dopamine (DA) levels in the striata, accompanied by cognitive impairment and functional defects (1). DA replacement therapy is the predominant treatment for PD, although this does not prevent or reduce dopaminergic neuron degeneration. Therefore, the development of novel drugs that protect dopaminergic neurons without causing dyskinesia is urgently required.

Oxidative stress is thought to be a main cause of dopaminergic neuron degeneration in PD (2,3). In vivo and in vitro, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is often used to establish a model of PD. MPTP traverses the blood-brain barrier and is decomposed into 1-methyl-4-phenylpyridinium ion (MPP⁺) by monoamine oxidase B (4). MPP⁺ subsequently damages the neurons in the substantia nigra, resulting in decreased formation of DA and the production of superoxide anions. It has been reported that MPTP induces a decline in tyrosine hydroxylase (TH) production in PC12 cells and other cellular models, which is the rate-limiting enzyme for the biosynthesis of DA (5-7). In addition, MPTP induces apoptosis and the production of intracellular reactive oxygen species (ROS) in a mouse model (8).

Proanthocyanidins (PCs) are natural phenolic compounds that are present in various plants. PCs have gained increasing attention in the fields of nutrition and medicine, due to their antioxidative, anti-inflammatory (9) and anticancer (10) effects. Epidemiological research has suggested that PCs may reduce the risk of PD (11). Levels of antioxidative indicators,
including superoxide dismutase (SOD), catalase, glutathione and glutathione peroxidase, as well as total antioxidant capacity, are increased by PC intervention, whereas malondialdehyde (MDA) concentration is decreased, in mouse models of oxidative damage (12). In addition, it has been reported that PCs protect rats from cisplatin-induced renal injury and reduce toxic damage through its antioxidative effects (13). Basli et al (14) provided evidence suggesting that the neuroprotective effects of PCs are associated with their antioxidative activity (14). Strathearn et al (12) reported that neurodegeneration in a cellular model of PD is reduced by anthocyanin- and PC-rich botanical extracts, via the improvement of mitochondrial function (12). Therefore, it may be hypothesized that PCs exert neuroprotective functions against the neurodegenerative process in PD. The present study explored the effects of PC pretreatment on MPTP-induced PD in vitro and in vivo.

Materials and methods

Cell and drug treatments. PC12 cells (American Type Culture Collection, Manassas, VA, USA) were maintained at 37°C in an atmosphere containing 5% CO₂ in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 5% horse serum (Gibco; Thermo Fisher Scientific, Inc.), 100 µg/ml streptomycin and 100 U/ml penicillin. Nerve growth factor (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), at a final concentration of 100 ng/ml, was added to the medium 3 days prior to drug treatment to induce neuronal differentiation. Cells were treated with MPTP (Sigma-Aldrich; Merck KGaA) and/or PCs (cat. no. T2849; Target Molecule Corp., Boston, MA, USA).

Cell survival. The viability of cells was measured using the MTT assay. PC12 cells were cultured in 96-well plates at a density of 1x10⁴ cells/well. Cells were exposed to 150 µmol/l MPTP following treatment with 0.5, 1 or 5 µg/ml PCs for 24, 48, 72 or 96 h at 37°C. The cells were then incubated with MTT (0.25 mg/ml) at 37°C for 4 h, after which, MTT formazan products were dissolved in dimethyl sulfoxide and the absorbance was measured at 570 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Assessment of apoptosis by flow cytometry. Cell apoptosis was detected using an Annexin V/Propidium Iodide (PI) Apoptosis Detection kit (Sigma-Aldrich; Merck KGaA), according to the manufacturer's protocol. Cells were exposed to 150 µmol/l MPTP following treatment with 0.5, 1 or 5 µg/ml PCs for 48 h at 37°C. Following this, these cells were harvested and 1x10⁶ cells were fixed using 4% polyformaldehyde for 30 min at 4°C. Following this, the cells were resuspended in 300 ml PBS and were stained with Annexin V-fluorescein isothiocyanate and PI (5 µg/ml each) in the dark for 15 min at 37°C. Apoptotic cells were analyzed by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA). FlowJo software (version 10; FlowJo LLC, Ashland, OR, USA) was used to calculate the apoptosis rate.

Mitochondrial membrane potential (MMP) detection. MMP alterations were measured using a JC-1 MMP Assay kit (Beyotime Institute of Biotechnology, Shanghai, China), according to the manufacturer's protocol. Briefly, cells were exposed to 150 µmol/l MPTP following treatment with 0.5, 1 or 5 µg/ml PCs for 48 h at 37°C. The medium was then replaced with PBS, and 1x10⁶ cells were incubated for 24 h with the JC-1 probe (10 µg/ml) at room temperature. JC-1 fluorescence was subsequently detected using a microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA) with an excitation and emission wavelength of 536-620 nm.

Behavioral tests. The pole test was used to measure motor behavior in the mouse model of PD. The pole test was performed as previously described (15), and began following 7 days of MPTP administration. Briefly, the mice were held on top of the pole (diameter, 8 mm; height, 55 cm; rough surface), and the time taken for the mice to climb down and place four feet on the floor was recorded as the time for locomotion activity (T-LA). Each trial had a cut-off limit of 30 sec. All measurements were performed three times to ensure accuracy.

Brain tissue preparation. Brain tissue preparation was performed as previously described (15,16). Briefly, 24 h after the last injection of MPTP, brains were obtained from the four mice in each group. One side of the brain was fixed in 10% buffered formaldehyde (pH 7.4) containing 25% sucrose at 4°C for 2-3 days. Following this, the brain tissues were frozen, and then substantia nigra tissues were then cut into 25 µm sections and stored in cryoprotectant at 4°C until further use in the immunohistochemistry (IHC) and terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) experiments. For ROS and MMP assays, as well as western blotting, the other side of the substantia nigra was isolated and stored at -80°C until use.

TH IHC. Following three 10 min washes in PBS with 0.05% Tween-20 (PBST), sections were incubated for 1 h at 37°C with...
PBST containing 2% bovine serum albumin (Sigma-Aldrich; Merck KGaA). Sections were subsequently incubated overnight at 4°C with anti-TH antibody (1:1,000; cat. no. 25859-1-AP; ProteinTech Group, Inc., Chicago, IL, USA), followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) secondary antibody (1:5,000; cat. no. 10285-1-AP; ProteinTech Group, Inc.) for 1 h at 37°C and amplification with a DAB Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA, USA), which was performed according to the manufacturer’s instructions. Finally, sections were analyzed using a light Leica DM2700 P microscope (magnification, x40; Leica Microsystems, Inc., Buffalo Grove, IL, USA). Quantification of TH activity was performed by counting the number of TH-immunoreactive (TH-IR) cells in 10 independent visual fields in the SNpc, and by measuring the optical density of TH-IR fibers in the ST using ImageJ software (version 1.48; National Institutes of Health, Bethesda, MD, USA).

**TUNEL staining.** Tissue sections were washed in PBS and subsequently fixed for 30 min with 4% paraformaldehyde at room temperature. Following one wash with PBS, PBS containing 0.1% Triton X-100 was added to the sections for 2 min in order to lyse the cells at room temperature. Sections were subsequently washed once with PBS and mounted onto slides, and 3% H₂O₂ was added to the slides for 5 min at room temperature. Slides were then rinsed and then incubated with 50 µl TUNEL detection solution (Roche Diagnostics, Basel, Switzerland) for 60 min at room temperature. The TUNEL reaction was visualized by chromogenic staining with DAB (0.75 mg/ml; Sigma-Aldrich; Merck KGaA) at room temperature for 20 min. Sections were imaged and ten visual fields were analyzed using a light Leica DM2700 P microscope (Leica Microsystems, Inc.). The percentage of cell death was determined by calculating the number of TUNEL-positive cells within a total of 100 cells in one visual field using ImageJ software (version 1.48; National Institutes of Health, Bethesda, MD, USA).

**Measurement of ROS formation.** ROS was measured with the fluorescent probe 2’7’-dichlorodihydrofluorescein diacetate (H₂DCFDA; Sigma-Aldrich; Merck KGaA). Cells were exposed to 150 µmol/l MPTP following treatment with 0.5, 1 or 5 µg/ml PCs for 48 h at 37°C. The medium was then replaced with PBS, and 1x10⁵ cells were incubated with 10 µmol/l H₂DCFDA at 37°C for 30 min. Substantia nigra tissues were treated with collagenase (5 mg/ml) and then the cells were dislodged in the solution using a pipette. PC12 cells or single cell suspension of substantia nigra homogenate was incubated with 10 µmol/l H₂DCFDA at 37°C for 30 min. The cells were subsequently washed twice with PBS and dissolved in 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.

**Western blotting.** PC12 cells were exposed to 150 µmol/l MPTP following treatment with 0.5, 1 or 5 µg/ml PCs for 48 h at 37°C. Proteins from PC12 cells or substantia nigra were prepared as described previously (17). Protein concentration was measured using bicinchoninic acid assays (Beyotime Institute of Biotechnology, Shanghai, China) and adjusted to the same final concentration. Protein samples (20 µg/lane) were separated by 12% SDS-PAGE and transferred onto polyvinylidene fluoride membranes. Membranes were blocked with 5% skim milk in 50 mM Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h at room temperature, and membranes were incubated overnight at 4°C with the following primary antibodies in the same blocking solution: TH (cat. no. 2792), c-Jun N-terminal kinase (JNK; cat. no. 9252), phosphorylated (p)-JNK (cat. no. 9255), c-Jun (cat. no. 9165), p-c-Jun (cat. no. 3270), B-cell lymphoma 2-like protein 11 (Bim; cat. no. 2933), cleaved caspase-3 (cat. no. 9654), cleaved poly (ADP-ribose) polymerase (PARP; cat. no. 94885) and GAPDH (1:2,000; cat. no. 2118) (all 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA). Subsequently, membranes were washed with TBST and incubated with horseradish peroxidase-conjugated goat-anti-rabbit IgG (1:10,000; cat. no. 7074; Cell Signaling Technology, Inc.) or goat-anti-mouse IgG (1:10,000; cat. no. 7076; Cell Signaling Technology, Inc.) for 1 h at room temperature in TBST containing 5% skim milk. Cross-reactivity was visualized using enhanced chemiluminescence western blotting detection reagents (Sangon Biotech Co., Ltd., Shanghai, China) and was analyzed by densitometry using Tanon 5200 software (Tanon Science and Technology Co., Ltd., Shanghai, China).

**Statistical analysis.** All data were analyzed using Prism software 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Data are expressed as the mean ± standard error of the mean. All experiments were performed in triplicate. Statistical evaluation of the results was performed by one-way analysis of variance followed by Bonferroni’s correction. *P* < 0.05 was considered to indicate a statistically significant difference.

**Results**

**Effects of PCs on the proliferation and apoptosis of MPTP-treated PC12 cells.** The simplest structure of PCs is a dimer formed by catechin, L-Epicatechin or catechin and L-Epicatechin, which is highly soluble in water and may be easily absorbed. Furthermore, PCs also have an important role in scavenging free radicals (18). The chemical structure of a PCs dimer formed from catechin is presented in Fig. 1A. To exclude the possibility that PCs induced PC12 cell toxicity, cell viability was determined in response to various concentrations of PCs at 24, 48, 72 and 96 h using the MTT assay, and the results revealed that PCs did not induce toxicity in PC12 cells (data not shown). The data demonstrated that MPTP markedly inhibited PC12 cell proliferation compared with the control, and this effect was gradually counteracted by increasing concentrations of PCs (Fig. 1B). Furthermore, the apoptotic rate for each group was assessed by flow cytometry. The typical quadrant analysis results obtained from PC12 cells, treated with or without PCs prior to MPTP treatment, are presented in Fig. 1C. Compared with the control group (3.0%), the percentage of apoptotic cells was significantly increased in the MPTP treatment group (22.5%). Conversely, PC pretreatment reduced the apoptotic percentage from 22.5 to 15.2% (0.5 µg/ml), 12.7% (1 µg/ml) and 7.5% (5 µg/ml). It was therefore concluded that PCs may reduce MPTP-induced apoptosis.
PCs inhibit the reduction of MMP and accumulation of ROS induced by MPTP. Mitochondria are the major source of ROS in various mammalian cells, and excessive production of ROS in the mitochondria disrupts normal redox signaling. In addition, MMP is a marker of mitochondrial function, which is also involved in apoptosis (19). The production of ROS in PC12 cells was analyzed using a H$_2$DCFDA fluorescence assay. As presented in Fig. 2A, exposure to MPTP increased ROS levels in PC12 cells. Pretreatment with PCs significantly inhibited the accumulation of ROS induced by MPTP. These results suggested that PCs protected mitochondrial function and suppressed ROS production in PC12 cells. Furthermore, enhanced MMP was observed in the control group and treatment with MPTP significantly reduced MMP in PC12 cells; however, pretreatment with PCs markedly restored reduced MMP (Fig. 2B and C).

Effects of PCs on JNK/c-Jun signaling. JNK/c-Jun signaling is commonly activated by various stress stimuli, and is a known mediator of cell apoptosis under various pathophysiological conditions (20). Therefore, MPTP-induced cell apoptosis and the potential protective effects of PCs were examined by western blotting. Administration of MPTP significantly increased p-JNK/JNK and p-c-Jun/c-Jun expression ratios. Furthermore, proapoptotic proteins Bim, cleaved caspase-3 and cleaved PARP were detected; MPTP significantly increased the expression of these proteins, whereas PC pretreatment inhibited this increase (Fig. 3).

Effects of PCs against MPTP-induced movement impairment in the pole test. As presented in Fig. 4A, the PD mouse model group were of a lower weight compared with the control group; however, this effect was reduced following treatment with PCs. To determine the effects of PCs on MPTP-induced bradykinesia, a pole test was performed on day 7 after MPTP injection. In the MPTP group, T-LA was significantly prolonged to 7.2 sec on day 7, compared with the control group. However, on day 7, T-LA was significantly shortened in the 300, 400 and 500 mg/kg PC-treated groups to 5.9, 6.1 and 4.0 sec, respectively, compared with MPTP alone (Fig. 4B).

PC treatment partially protects dopaminergic neurons. The neuroprotective action of PCs and the functional viability of dopaminergic neurons in the substantia nigra pars compacta were further assessed by determining the expression of the rate-limiting enzyme for DA biosynthesis, TH. As evidenced...
Figure 2. Effects of PCs on MPTP-mediated ROS generation and mitochondrial dysfunction in PC12 cells. Cells were treated with MPTP in the absence or presence of 0.5, 1 or 5 µg/ml PCs for 24 h. (A) ROS levels were detected with the fluorescent probe, 2',7'-dichlorodihydrofluorescein diacetate. (B) Mitochondrial membrane potential was measured using the fluorescent probe JC-1. (C) Increased MMP was observed in the control group and treatment with MPTP significantly suppressed the MMP in PC12 cells; however, pretreatment with PCs significantly attenuated suppressed levels of MMP. Three independent experiments were performed. **P<0.01 vs. untreated control cells; #P<0.05, ##P<0.01 vs. the MPTP group. FITC, fluorescein isothiocyanate; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PCs, proanthocyanidins; ROS, reactive oxygen species.

Figure 3. Western blot analysis of the JNK/c-Jun signaling pathway in PC12 cells. Cells were treated with MPTP in the absence or presence of 0.5, 1 or 5 µg/ml PCs for 24 h. Three independent experiments were performed. **P<0.01 vs. untreated control cells; #P<0.05, ##P<0.01 vs. the MPTP group. Bim, B-cell lymphoma 2-like protein 11; JNK, c-Jun N-terminal kinase 1; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; p, phosphorylated; PARP, poly(ADP-ribose) polymerase; PC, proanthocyanidins.
by IHC and western blot analysis (Fig. 5A and B), the expression of TH was reduced in MPTP mice compared with the control group. Conversely, TH expression in PC-pretreated mice was more pronounced compared with in MPTP-induced PD model mice. The number of TH-positive neurons in the substantia nigra was counted (magnification, x200) and TH protein expression was detected. Three independent experiments were performed. \(^*\)P<0.05 vs. the untreated control group; \(^\#\)P<0.05 and \(^\##\)P<0.01 vs. the MPTP group. MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PCs, proanthocyanidins; TH, tyrosine hydroxylase.
mice. TH is a specific marker protein for the identification of midbrain dopaminergic neurons (21). Therefore, these results demonstrated that PCs may protect against neuronal loss in a mouse model of PD, thus suggesting that PCs exert a neuroprotective effect in vivo.

**PCs reduce MPTP-induced apoptosis via ROS-JNK signaling.** Analysis of TUNEL staining in the substantia nigra further suggested that the control and PC-pretreated groups presented with fewer TUNEL-positive cells compared with the MPTP group (Fig. 6A). ROS levels were subsequently detected. As presented in Fig. 6B, MPTP exposure led to a significant elevation in ROS levels in primary mice substantia nigra cells compared with the control group. Pretreatment with PCs inhibited ROS generation in the MPTP group. Furthermore, western blot analysis demonstrated that MPTP increased
JNK/c-Jun signaling pathway protein expression, whereas PCs reversed this increase (Fig. 6C).

Discussion

In order to investigate the effects of PCs on dopaminergic neurons, an MPTP-induced experimental model of PD was established in vitro and in vivo. The results demonstrated that, in vitro, PCs significantly protected PC12 cells against MPTP-induced toxicity, apoptosis and high ROS levels. In vivo, the data revealed that treatment with PCs prevented neuronal loss in the substantia nigra and prevented apoptosis in a dose-dependent manner. Furthermore, western blotting and immunohistochemical analysis for dopaminergic TH expression revealed that PCs prevented the decrease in TH induced by MPTP. Western blot analysis also revealed that the ROS/JNK signaling pathway was involved in the action of PCs. The results of the present study consistently demonstrated that PCs protected neurons from the impairments induced by MPTP treatment via the ROS/JNK signaling pathway. PD is a movement disorder characterized by progressive loss of nigrostriatal dopaminergic neurons. Therapeutic strategies that slow or stop the neurodegenerative processes of PD are urgently required. The identification of polyphenolic compounds or polyphenols with potential neuroprotective properties has increased considerably during the last few years. Catechins, such as epigallocatechin-3-gallate, have been reported to exert several actions on the CNS, including anxiolytic, sedative and neuroprotective effects on animal models of Alzheimer's disease and PD. Notably, PCs are composed of catechin and epicatechin oligomers (22,23). Hartley et al (24) reported that PCs prevent the early motor and non-motor symptoms of PD, and may represent a promising therapeutic tool in PD via their neuroprotective potential (14). The neuroprotective effects of PCs are exerted via decreasing MDA and SOD levels, in vitro and in vivo (25). Recently, PCs have been reported to possess neuroprotective effects by targeting β-amyloid fibrillization and neurotoxicity (26). The results of the present study also revealed that PCs exerted neuroprotective effects in vitro and in vivo.

Overwhelming evidence has indicated that the apoptotic death of nigrostriatal dopaminergic neurons is initiated by oxidative stress (27). Oxidative stress is self-propagating, in that initial oxidative damage creates additional free radicals and damages mitochondria, leading to further ROS production (28,29). Mitochondrial dysfunction and the overproduction of ROS may also enhance neuronal excitability and increase seizure susceptibility (13). Mitochondrial dysfunction is a common trigger for apoptosis, by inducing the sequential activation of proapoptotic caspase-3 and PARP (30).

Evidence indicates that activation of JNK regulates ROS-induced neuronal apoptosis (31,32). MPP⁺ is selectively transported to the cell through the high affinity DA transporter, and is absorbed by the mitochondria within dopaminergic neurons. By inhibiting the mitochondria electron transfer complex I, it destroys the process of phosphoric oxide phosphorylation and increases cellular ROS expression levels (24). Large amounts of ROS in the mitochondria are released into the cytoplasm, which stimulates JNK phosphorylation and activates signal cascades. The activated JNK subsequently enters the nucleus to activate c-Jun, which further regulates Bim, caspase-3 and PARP to promote the apoptosis of cells (33,34), eventually leading to the death of dopaminergic neurons.

In conclusion, PCs may represent a safe and affordable intervention for the clinical treatment of PD. PCs effectively prevented mitochondrial apoptosis, ROS production and JNK activation in neurons. The results of the present study provided experimental evidence to support the potential use of PCs as a therapeutic agent in PD.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

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

Authors' contributions

XX, HC and JX conceived and designed the study. HC, JX, YL, PH and CL performed the experiments. JJ, XM and SL analyzed the data. XX and XM wrote the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Animal Care and Use Committee of Nanjing Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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