

# Protective effects of apigenin against 1-methyl-4-phenylpyridinium ion-induced neurotoxicity in PC12 cells

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**Abstract.** Parkinson's disease is recognized as the second most common neurodegenerative disorder after Alzheimer's disease, characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta and can be experimentally mimicked by the use of the neurotoxin, 1-methyl-4-phenylpyridinium ion (MPP<sup>+</sup>), in *in vitro* models. In this study, we investigated the potential protective effects of apigenin (AP), galangin and genkwanin, naturally occurring plant flavonoids, on the MPP<sup>+</sup>-induced cytotoxicity in cultured rat adrenal pheochromocytoma cells (PC12 cells). The PC12 cells were pre-treated with various concentrations of the test compounds for 4 h, followed by the challenge with 1,000  $\mu$ M MPP<sup>+</sup> for 48 h. We found that only pre-treatment with AP (3, 6 and 12  $\mu$ M) before injury significantly increased cell viability, decreased the release of lactate dehydrogenase, reduced the level of intra-

cellular reactive oxygen species and elevated mitochondrial membrane potential in the MPP<sup>+</sup>-treated PC12 cells. In addition, AP markedly suppressed the increased rate of apoptosis and the reduced Bcl-2/Bax ratio induced by MPP<sup>+</sup> in the PC12 cells. Taken together, the findings of this study demonstrate that AP exerts neuroprotective effects against MPP<sup>+</sup>-induced neurotoxicity in PC12 cells, at least in part, through the inhibition of oxidative damage and the suppression of apoptosis through the mitochondrial pathway.

## Introduction

It is well recognized that Parkinson's disease (PD), one of the most common neurodegenerative movement disorders, is characterized by the selective degeneration of dopaminergic neurons in the nigrostriatal system (1). Recent statistics show that PD affects approximately 2% of the population over the age of 60 and the incidence is expected to rise dramatically in the next 25 years with the extension of life expectancy by improved health care (2). Although the detailed mechanisms responsible for PD are still under investigation (3), extensive research over the last several decades has indicated that mitochondrial dysfunction and oxidative stress resulting from the excessive production of reactive oxygen species (ROS) play crucial roles in the pathogenesis of PD (4,5). Oxidative stress induced by ROS induces the opening of the mitochondrial permeability transition pore and the dissipation of mitochondrial membrane potential (MMP) (6) and, subsequently, apoptotic activators are released from the mitochondria (7), finally resulting in the apoptosis of dopaminergic neurons observed in PD (8).

Advances in our understanding of dopaminergic neuronal apoptosis in PD have been achieved by studies on Parkinsonism induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 1-methyl-4-phenylpyridinium ion (MPP<sup>+</sup>) (9,10). Moreover, pheochromocytoma cells (PC12 cells), derived from a clonal rat pheochromocytoma cell line, have been widely used as cellular models of PD, as these cells share characteristics with midbrain dopaminergic neurons (11,12). In addition, previous studies have demonstrated that MPP<sup>+</sup>-induced cytotoxicity in PC12 cells, which induces apoptotic characteristics accompa-

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**Abbreviations:** PD, Parkinson's disease; ROS, reactive oxygen species; MMP, mitochondrial membrane potential; MPP<sup>+</sup>, 1-methyl-4-phenylpyridinium ion; PI, propidium iodide; AP, apigenin; GA, galangin; GE, genkwanin; DCFH-DA, 2',7'-dichlorofluorescein diacetate; LDH, lactate dehydrogenase; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazolyl carbocyanine iodide

**Key words:** apigenin, Parkinson's disease, 1-methyl-4-phenylpyridinium ion, rat pheochromocytoma cells, neuroprotection

nied by mitochondrial dysfunction and oxidative stress, is a classic cellular model of PD (13,14). Therefore, in this study, we investigated the effects of three plant flavonoids on cytotoxicity in MPP<sup>+</sup>-treated PC12 cells to determine whether they may be of preventive or potential therapeutic value in PD.

Naturally occurring plant-derived flavonoids have been suggested to play a role in protecting the central nervous system against oxidative and excitotoxic stress, although the mechanisms of action require further study (15). In this study, using MPP<sup>+</sup> as the oxidative insult in PC12 cells, we investigated the mechanisms responsible for neurotoxicity and attempted to identify the possible sites of action of three of the most potent protective flavonoids, apigenin (AP), galangin (GA) and genkwanin (GE) (the chemical structures are shown in Fig. 1). AP (4',5,7-trihydroxyflavone), GA (3,5,7-trihydroxyflavone) and GE (4',5-dihydroxy-7-methoxyflavone), which are very similar in structure, have been reported to possess a number of similar biological activities, including antioxidant/free radical scavenging activity, antitumor effects and anti-inflammatory activity (16-19). Furthermore, AP has been shown to inhibit A $\beta$ -mediated oxidative damage in nerve cells and in animal models of Alzheimer's disease (20,21). Additionally, several antioxidants and free radical scavengers, including luteolin (22), morin (23) and myricetin (24), which also have a similar chemical structure to that of these three flavonoid compounds, have been reported to attenuate the oxidative toxicity induced by MPP<sup>+</sup>. However, to the best of our knowledge, no study has been published to date on the protective effects of these three flavonoid compounds against MPP<sup>+</sup>-induced toxicity *in vivo* or *in vitro*. Therefore, in the present study, we aimed to investigate whether these compounds exert protective effects against MPP<sup>+</sup>-induced neurotoxicity in PC12 cells and to explore the underlying molecular mechanisms of these neuroprotective effects.

## Materials and methods

**Chemicals and reagents.** AP, GA and GE, purchased from Sigma-Aldrich (St. Louis, MO, USA), were dissolved in dimethyl sulfoxide (DMSO) (<0.1%) and diluted with serum-free medium prior to each experiment. MPP<sup>+</sup> and methylthiazolyl-diphenyl-tetrazolium bromide (MTT) were also purchased from Sigma-Aldrich. 2',7'-Dichlorofluorescein diacetate (DCFH-DA) was obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, penicillin and streptomycin were purchased from Gibco (Grand Island, NY, USA). All other reagents and chemicals used in the study were of analytical grade.

**Cell culture and drug treatment.** The PC12 cells, obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), were routinely cultured in DMEM supplemented with heat-inactivated horse serum (5%, v/v), heat-inactivated fetal calf serum (5%, v/v), penicillin (100 IU/ml) and streptomycin (100  $\mu$ g/ml) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>/95% air. The culture medium was changed every 2 days. In all the experiments, apart from the assessment of cell viability, the cells were incubated for 24 h and were then treated for 4 h with or without various concentrations of AP, GA and GE (3,

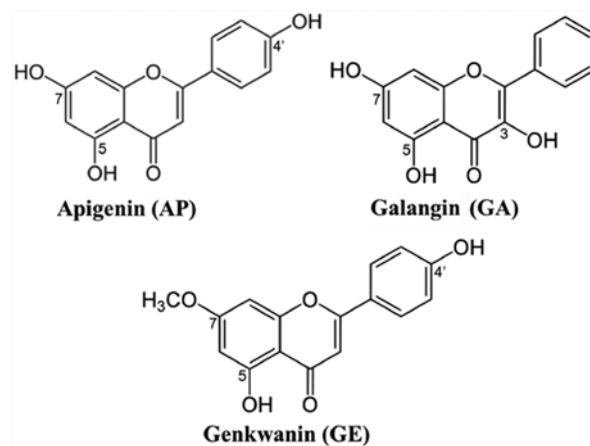


Figure 1. Chemical structures of apigenin, galangin and genkwanin.

6 and 12  $\mu$ M), prior to the addition of MPP<sup>+</sup> (final concentration, 1,000  $\mu$ M) for an additional 48 h. The control cells were treated in the same manner without the addition of the test compounds and MPP<sup>+</sup> to the free serum culture medium. All experiments were repeated at least 3 times for each treatment condition in each experiment.

**MTT assay.** Cell survival was quantified by the colorimetric MTT assay using a previously described protocol (14). Briefly, the PC12 cells were seeded in 96-well culture plates at a density of  $2 \times 10^4$  cells/well; following treatment with the drugs, MTT solution was added to the cell cultures at a final concentration of 1 mg/ml followed by incubation for a further 4 h at 37°C. The supernatant was carefully aspirated and 150  $\mu$ l of DMSO were added to dissolve the formazan crystals. The 96-well microplate was then transferred to a microplate reader (BMG Labtech, Offenbury, Germany) and the absorbance was read at 570 nm. Cell viability was expressed as the percentage of the untreated controls.

**Lactate dehydrogenase (LDH) activity assay.** Cytotoxicity was quantitatively assessed by measuring the activity of LDH released from the damaged cells into the culture medium (25). The PC12 cells were seeded in 96-well culture plates at a density of  $2 \times 10^4$  cells/well and treated according to the procedures as described above. At the end of the treatments, the medium was collected for the measurement of the extracellular LDH level; the cells were then treated with 0.5% Triton X-100, after being centrifuged at 10,000  $\times$  g and the supernatant was used for the measurement of the intracellular LDH level by spectrophotometrical determination at 440 nm following the procedures provided in the assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). LDH release was expressed as the percentage of the total LDH activity (LDH in the medium + LDH in the cells), according to the following equation: LDH release (%) = (LDH activity in the medium/total LDH activity)  $\times$  100. Cultures under normal conditions (control group) represent the basal LDH release.

**Measurement of intracellular ROS production.** Intracellular ROS production was measured using the intracellular peroxide-sensitive fluorescent probe, DCFH-DA, as previously

described (25). Briefly, the PC12 cells were seeded in 96-well black culture plates at a density of  $2 \times 10^4$  cells/well and treated according to the procedures as described above. Following treatment with MPP<sup>+</sup> and the drugs, the cells were washed twice with D-Hank's solution and incubated with DCFH-DA at a final concentration of  $10 \mu\text{M}$  for 30 min at  $37^\circ\text{C}$  in dark. After the cells were washed twice with D-Hank's solution to remove the extracellular DCFH-DA, the fluorescence intensity was measured using a fluorescence microplate reader (Tecan, Groedig, Austria) at an excitation wavelength of 485 nm and an emission wavelength of 538 nm. The measured fluorescence values were expressed as the fold changes relative to the control group.

Additionally, the PC12 cells were seeded in 6-well culture plates at a density of  $1 \times 10^6$  cells/well. At the end of the drug treatment, the cells were washed twice with D-Hank's solution and incubated with DCFH-DA (final concentration,  $10 \mu\text{M}$ ) for 30 min at  $37^\circ\text{C}$  in the dark. Changes in ROS production were then assessed using a fluorescence microscope (Olympus, Tokyo, Japan).

**Measurement of MMP.** A JC-1 kit (Beyotime, Haimen, China) was used to measure the mitochondrial depolarization in the PC12 cells according to the manufacturer's instructions. 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazolyl carbocyanine iodide (JC-1) is a cationic dye for detecting MMP changes, where mitochondrial depolarization is indicated by an increase in the green/red fluorescence intensity ratio. In the mitochondria of healthy cells, JC-1 forms aggregates and fluoresces red. When the MMP collapses, the cationic dye remains in the cytoplasm as green fluorescence, its monomeric form. Briefly, on the one hand, PC12 cells were seeded in 96-well black culture plates at a density of  $2 \times 10^4$  cells/well. At the end of the drug treatment, the cells were washed twice with D-Hank's solution and incubated with JC-1 reagent (final concentration,  $10 \mu\text{g/ml}$ ) for 20 min at  $37^\circ\text{C}$  in the dark. Subsequently, the green (excitation, 490; emission, 530 nm) and red (excitation, 525; emission, 590 nm) fluorescence were measured using a fluorescence plate reader (Tecan). The measured green/red fluorescence ratios were expressed as fold changes relative to the control group.

On the other hand, the PC12 cells were seeded in 6-well culture plates at a density of  $1 \times 10^6$  cells/well. At the end of the drug treatment, the cells were washed twice with D-Hank's solution and incubated with JC-1 (final concentration,  $10 \mu\text{g/ml}$ ) for 20 min at  $37^\circ\text{C}$  in dark. Changes in MMP were assessed using a fluorescence microscope (Olympus).

**Flow cytometric analysis of apoptosis.** The apoptotic rate was measured by flow cytometry according to the protocol provided with the Annexin V-FITC/PI kit (Sigma-Aldrich). Briefly, the PC12 cells were seeded in 6-well culture plates at a density of  $1 \times 10^6$  cells/well. At the end of the drug treatment, the cells were harvested by centrifugation at  $1,000 \times g$ , washed twice with ice-cold phosphate-buffered saline (PBS) and resuspended in binding buffer at a concentration of  $1 \times 10^6$  cells/ml. A total of  $5 \mu\text{l}$  of  $20 \mu\text{g/ml}$  Annexin V-FITC and  $50 \mu\text{g/ml}$  propidium iodide (PI) were added and the tube was incubated for 30 min in the dark. The quantitative analysis of apoptosis was carried out using a flow cytometer (BD Biosciences, San Jose, CA, USA).

Quadrants were positioned on Annexin V-FITC/PI dot plots, allowing living cells (Annexin V-FITC/PI<sup>-</sup>), early/primary apoptotic cells (Annexin V-FITC<sup>+</sup>/PI<sup>-</sup>), late/secondary apoptotic cells (Annexin V-FITC<sup>+</sup>/PI<sup>+</sup>) and necrotic cells (Annexin V-FITC<sup>+</sup>/PI<sup>+</sup>) to be distinguished (26). Data were analyzed using CellQuest™ software (BD Biosciences).

**Western blot analysis.** Western blot analysis was performed to investigate the changes in the protein levels of Bcl-2 and Bax. The PC12 cells were seeded onto 100-mm dishes at  $5 \times 10^6$  cells/dish and allowed to grow until confluent. The cells were then washed twice with ice-cold D-Hank's solution after drug treatment and lysed using protein lysis buffer. The lysates were collected by scraping from the plates and then centrifugation at  $13,000 \times g$  at  $4^\circ\text{C}$  for 15 min. The supernatant was separated and stored at  $-80^\circ\text{C}$  until use.

Western blot analysis was performed according to a procedure described previously (27). Briefly, protein samples were electrophoresed by SDS-PAGE for 2 h at 80 V and then transferred onto polyvinylidene fluoride membranes for 40 min at 200 mA. The blots were blocked for 2 h at room temperature in fresh blocking buffer (0.1% Tween-20 in Tris-buffered saline, pH 7.4, containing 5% non-fat dried milk) and subsequently incubated at  $4^\circ\text{C}$  overnight with primary antibodies against Bcl-2 (1:300; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), Bax (1:200; Santa Cruz Biotechnology, Inc.) or GAPDH (1:500; Santa Cruz Biotechnology, Inc.) in blocking solution. Subsequently, the membranes were washed with TBS-T (Tris-buffer saline containing 0.1% Tween-20) 3 times and incubated with horseradish peroxidase-conjugated secondary antibody at 1:5,000 in PBS with 5% non-fat dry milk at room temperature for 1 h. To verify the equal loading of samples, the membranes were incubated with monoclonal antibody GAPDH, followed by a horseradish peroxidase-conjugated goat anti-mouse IgG. The membrane again was washed with TBS-T 3 times and finally, the protein bands were visualized using ECL western blotting detection reagents (Amersham Biosciences, Buckinghamshire, UK). The intensity of each band was analyzed using Image J software (NIH Image; National Institutes of Health, Bethesda, MD, USA).

**Statistical analysis.** All quantitative data are presented as the means  $\pm$  standard error of the mean (SEM). The changes in variable parameters between the treated groups and the control group were analyzed by one-way ANOVA followed by Dunnett's test as a post hoc comparison. A value of  $p < 0.05$  was considered to indicate a statistically significant difference in all cases.

## Results

**Effects of the test compounds on MPP<sup>+</sup>-induced cytotoxicity in PC12 cells.** To investigate the effects of MPP<sup>+</sup> on PC12 cells, we exposed the cells to a range of concentrations of MPP<sup>+</sup> (250–1,500  $\mu\text{M}$ ) for various periods of time (24, 48 and 72 h). There was a concentration- and time-dependent decrease in cell viability following exposure to MPP<sup>+</sup> (Fig. 2A). The cells exposed to 1,000  $\mu\text{M}$  MPP<sup>+</sup> for 48 h exhibited 59.5% of the cell viability observed in the control cells. Therefore, we used 1,000  $\mu\text{M}$  MPP<sup>+</sup> treatment for 48 h as the optimal standard

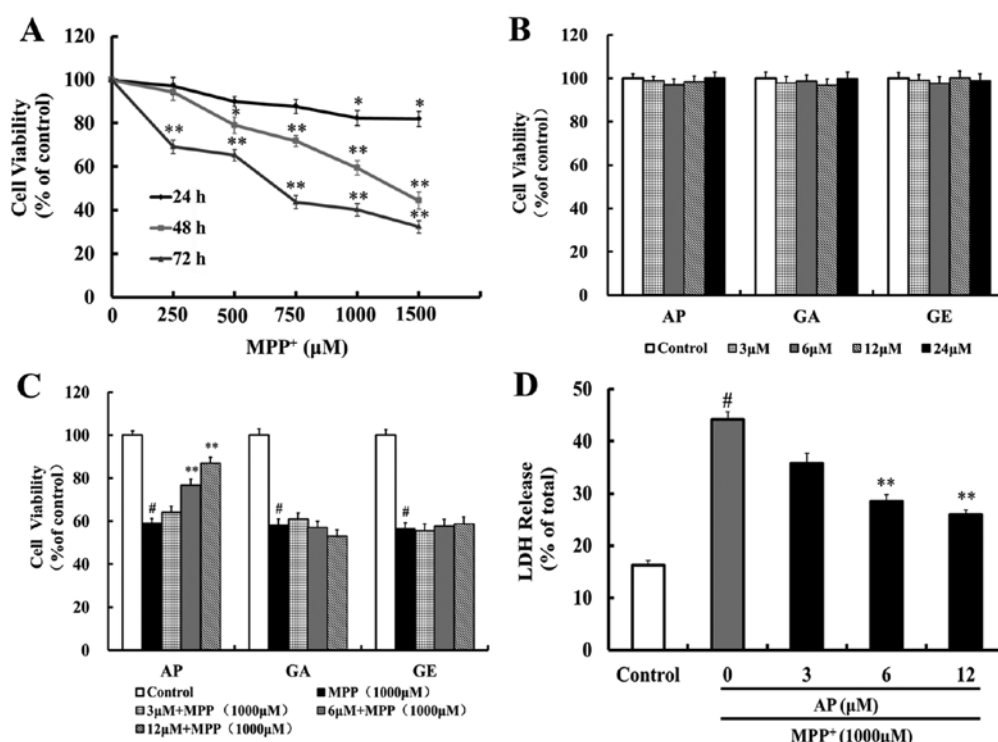


Figure 2. Effects of apigenin (AP), galangin (GA), genkwanin (GE) and 1-methyl-4-phenylpyridinium ion (MPP<sup>+</sup>) on the viability of rat pheochromocytoma cells (PC12 cells). (A) Neurotoxic effects of MPP<sup>+</sup> on the viability of PC12 cells. (B) Effects of treatment with AP, GA and GE on the viability of PC12 cells. (C) Effects of AP, GA and GE on the viability of PC12 cells subjected to MPP<sup>+</sup>-induced injury. (D) Effect of AP on MPP<sup>+</sup>-induced damage in PC12 cells, as indicated by the lactate dehydrogenase (LDH) assay. Data are expressed as the means  $\pm$  SEM (n=6). \*p<0.05, #p<0.05 compared with the control cells; \*\*p<0.01 compared with cells exposed to MPP<sup>+</sup> alone.

concentration and time point for the induction of apoptosis in the subsequent experiments.

We then investigated the neuroprotective effects of the 3 flavonoid compounds (AP, GA and GE). These 3 compounds alone did not have any cytotoxic effects at concentrations ranging between 3–24 μM (Fig. 2B). The PC12 cells were pre-treated with these test compounds (3, 6 and 12 μM) for 4 h, and then exposed to 1,000 μM MPP<sup>+</sup> for 48 h. MTT assays indicated that pre-treatment with AP (6 and 12 μM) markedly increased the cell viability (p<0.01 and p<0.01, respectively) as compared with the MPP<sup>+</sup> group and the survival rate was 76.8 and 86.9% of the controls, respectively (Fig. 2C). However, pre-treatment with GA and GE (3–12 μM) did not increase the cell viability when compared with the MPP<sup>+</sup> group (Fig. 2C). Therefore, in the subsequent experiments, we focused on the protective effects of AP against MPP<sup>+</sup> neurotoxicity.

**Effect of AP on the MPP<sup>+</sup>-induced release of LDH.** To further investigate the protective effects of AP, LDH assay, another indicator of cell toxicity, was performed. LDH is a stable cytoplasmic enzyme present in all cells and is rapidly released into the cell culture supernatant upon damage to the plasma membrane. As shown in Fig. 2D, when the PC12 cells were incubated with 1,000 μM MPP<sup>+</sup> for 48 h, the percentage of LDH being released increased from 16.2 (controls) to 44.1%. Pre-treatment with AP (6 and 12 μM) significantly attenuated the MPP<sup>+</sup>-induced release of LDH to 28.4 and 25.9% (p<0.01 and p<0.01, respectively), as compared to the MPP<sup>+</sup>-treated control group.

**Effect of AP on the intracellular ROS level in MPP<sup>+</sup>-treated PC12 cells.** As shown in Fig. 3, exposure of the PC12 cells to 1,000 μM MPP<sup>+</sup> for 48 h led to a significant increase in the levels of ROS (2.14-fold increase relative to the control value). However, the overproduction of the intracellular ROS level was markedly inhibited by pre-treatment with AP at concentrations of 3, 6 and 12 μM (p<0.05, p<0.01 and p<0.01, respectively), when compared with the cells cultured with MPP<sup>+</sup> only.

**Effect of AP on MMP in MPP<sup>+</sup>-treated PC12 cells.** As shown in Fig. 4, following exposure to 1,000 μM MPP<sup>+</sup> for 48 h, JC-1 aggregates within the normal mitochondria were dispersed to the monomeric form (green fluorescence) and the ratio of green/red fluorescence intensity was significantly increased (p<0.01 vs. the control group), suggesting that MPP<sup>+</sup> induced a significant decrease in MMP. However, pre-treatment with AP (3, 6 and 12 μM) markedly prevented the decrease in MMP induced by MPP<sup>+</sup> (p<0.05, p<0.01 and p<0.01, respectively), as compared to the group treated with MPP<sup>+</sup> alone.

**Effect of AP on MPP<sup>+</sup>-induced apoptosis in PC12 cells.** Annexin V-FITC and PI double staining was used to detect apoptosis. As shown in Fig. 5, following exposure to 1,000 μM MPP<sup>+</sup> for 48 h, the percentage of apoptotic cells was significantly increased (43.2%) in comparison with the control group (5.2%). However, pre-treatment with AP (3, 6 and 12 μM) markedly reduced the cell apoptotic rate (p<0.05, p<0.01 and p<0.01, respectively) as compared with the MPP<sup>+</sup> group and the cell apoptotic rate was 33.6, 27.9 and 9.4%, respectively.

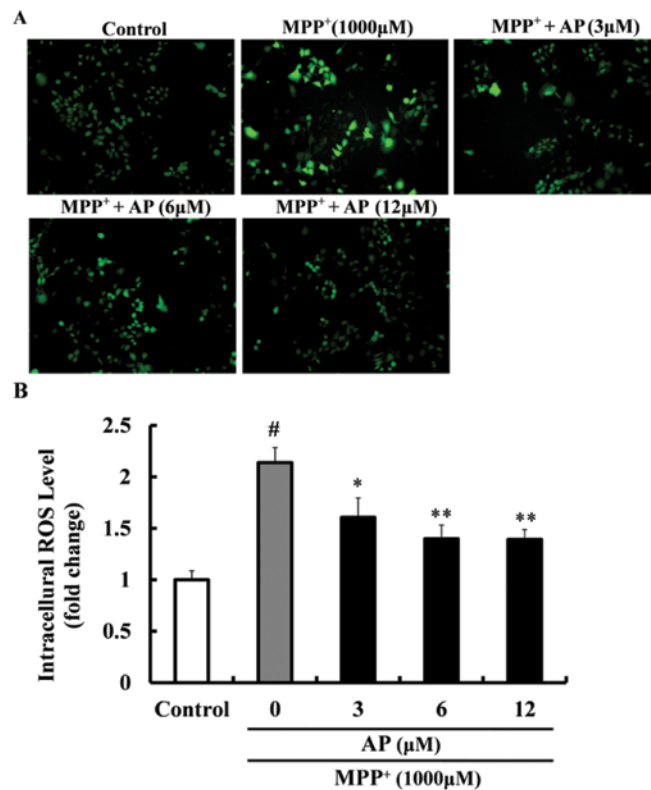


Figure 3. Effect of apigenin (AP) on intracellular reactive oxygen species (ROS) production in 1-methyl-4-phenylpyridinium ion (MPP<sup>+</sup>)-treated rat pheochromocytoma cells (PC12 cells). (A) Representative fluorescence images of PC12 cells stained with an intracellular peroxide-sensitive fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA) (magnification, x200). (B) Quantitative analysis of the fluorescence intensity by using DCFH-DA method. Data are presented as the means  $\pm$  SEM of fold changes relative to the controls (n=3). <sup>#</sup>P<0.01 compared with the control cells; <sup>\*</sup>p<0.05 and <sup>\*\*</sup>p<0.01 compared with cells exposed to MPP<sup>+</sup> alone.

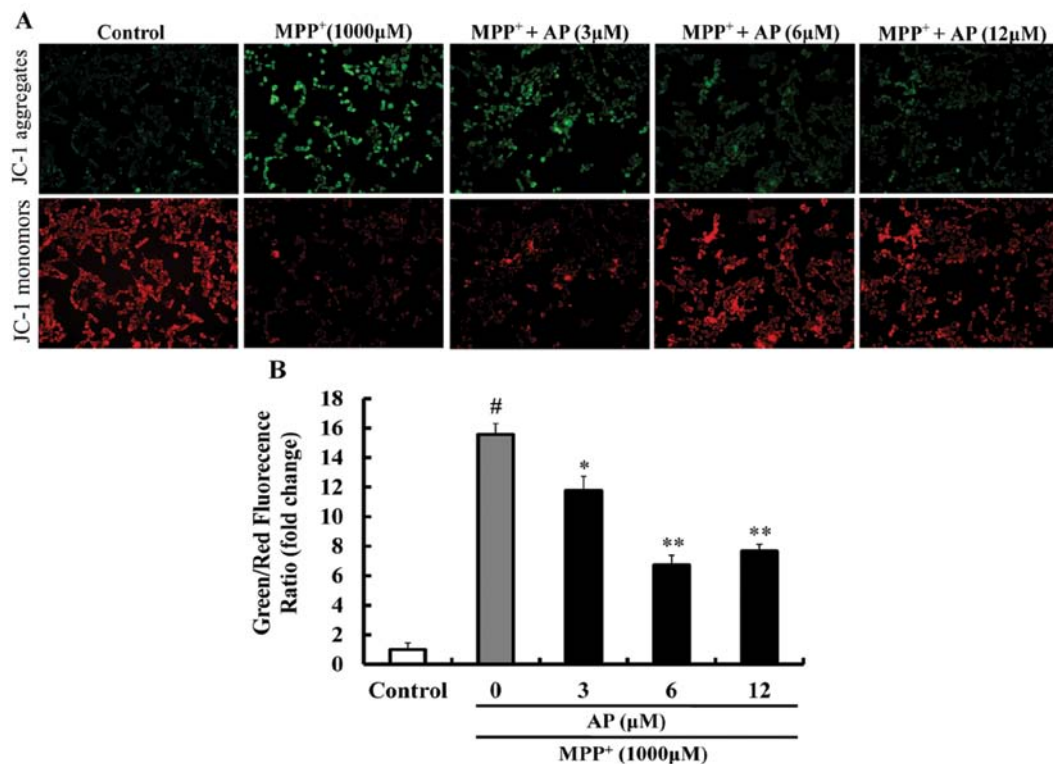


Figure 4. Effect of apigenin (AP) on mitochondrial membrane potential (MMP) in 1-methyl-4-phenylpyridinium ion (MPP<sup>+</sup>)-treated rat pheochromocytoma cells (PC12 cells). (A) Representative fluorescence images of PC12 cells stained with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazolyl carbocyanine iodide (JC-1) (magnification, x200). (B) Quantitative analysis of the ratio of green/red fluorescence intensity by using JC-1 method. Data are presented as the means  $\pm$  SEM of fold changes relative to the controls (n=3). <sup>#</sup>P<0.01 compared with the control cells; <sup>\*</sup>p<0.05 and <sup>\*\*</sup>p<0.01 compared with cells exposed to MPP<sup>+</sup> alone.

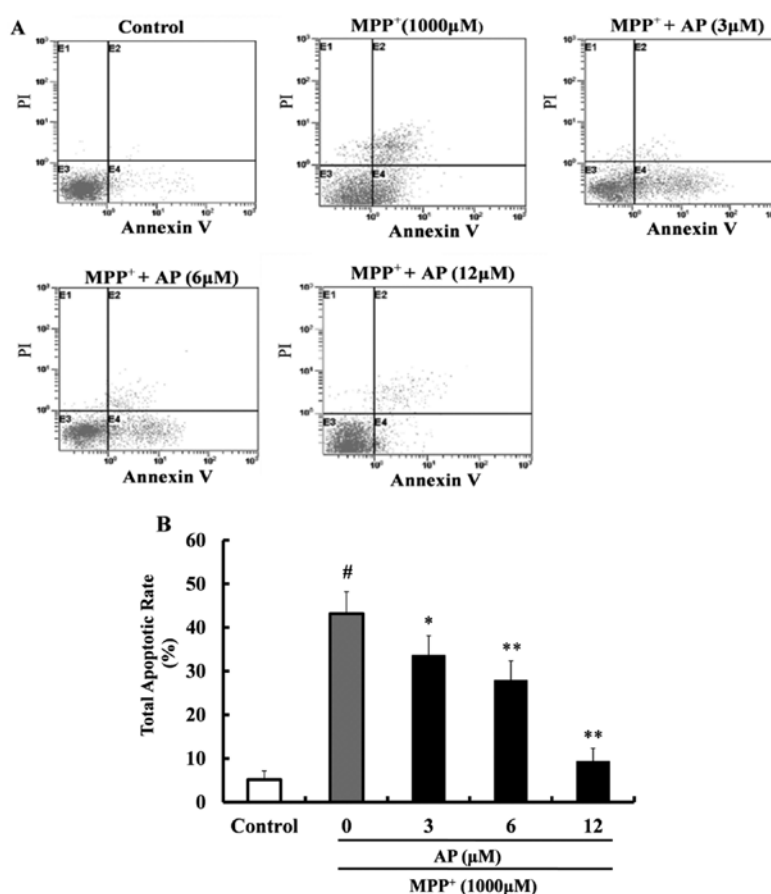


Figure 5. Effect of apigenin (AP) on 1-methyl-4-phenylpyridinium ion (MPP<sup>+</sup>)-induced apoptosis in rat pheochromocytoma cells (PC12 cells). (A) Diagram of flow cytometry of double-staining with Annexin V-FITC/propidium iodide (PI). (B) Graphical representation of the total apoptotic rate. Data are presented as the means  $\pm$  SEM for 3 independent experiments. # $P$ <0.01 compared with the control cells; \* $p$ <0.05 and \*\* $p$ <0.01 compared with the cells exposed to MPP<sup>+</sup> alone.

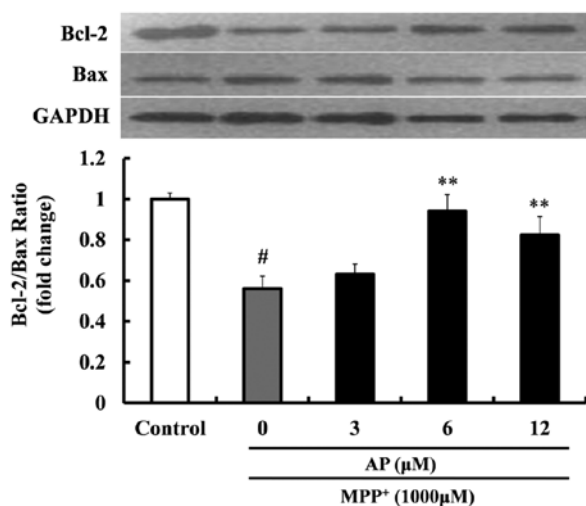


Figure 6. Effect of apigenin (AP) on the expression of Bcl-2 and Bax in 1-methyl-4-phenylpyridinium ion (MPP<sup>+</sup>)-treated rat pheochromocytoma cells (PC12 cells). PC12 cells were pre-treated with or without AP (3-12 μM) for 4 h and then exposed to MPP<sup>+</sup> (1,000 μM) for 48 h. Data are presented as the means  $\pm$  SEM of fold changes relative to the controls ( $n=3$ ). # $P$ <0.01 compared with the control cells; \*\* $p$ <0.01 compared with the cells exposed to MPP<sup>+</sup> alone.

*Effect of AP on the expression of Bcl-2 and Bax in MPP<sup>+</sup>-treated PC12 cells.* To elucidate the molecular mechanisms respon-

sible for the protective effects exerted by AP against PC12 cell apoptosis induced by MPP<sup>+</sup>, we measured the ratio of Bcl-2/Bax protein expression. Following exposure to 1,000 μM MPP<sup>+</sup> for 48 h, as shown in Fig. 6, the ratio of Bcl-2/Bax was sharply decreased (1.79-fold decrease relative to control,  $p$ <0.01). However, when the cells were pre-treated with AP at the concentrations of 6 and 12 μM, the above changes induced by MPP<sup>+</sup> were significantly mitigated. The ratio of Bcl-2/Bax was significantly increased ( $p$ <0.01 and  $p$ <0.01, respectively), compared with the cells treated with MPP<sup>+</sup> alone.

## Discussion

Various pharmacological and surgical treatments have been used in patients with PD; however, some of these have significant adverse effects and most do not halt or retard the degeneration of dopaminergic neurons (28). Thus, in recent years, considerable attention has been paid to the development of neuroprotective drugs from natural origins as a therapeutic strategy for PD (29). Since naturally occurring plant-derived flavonoids have been shown to play a useful role in protecting the central nervous system (15), in this study, we investigated the neuroprotective effects of the three most potent protective natural flavonoids, including AP, GA and GE, using the model of MPP<sup>+</sup>-induced neurotoxicity in PC12 cells. Our data suggested that the neuroprotective effects of these natural

flavonoids varied dramatically, although they are structurally similar. Significant protective effects of AP were observed against MPP<sup>+</sup>-induced cell death, while GA and GE exerted no protective effects. We conclude that this effect may well be due to the position and number of free phenolic hydroxyl groups (OH groups) in their structures. Free OH group is a typical reactive functional group, particularly when it is attached to a C-7 or C-4' of flavonoid structure (16). A comparison of the AP structure (4',5,7-trihydroxyflavone) with that of GA (3,5,7-trihydroxyflavone) (4',5-dihydroxy-7-methoxyflavone) and GE shows that the 5-, 7- and 4'-OH substitutions are important. For GE, one OH group (at the C-7 position) is methoxylated; for GA, a free 4'-OH group is lacking (Fig. 1). Therefore, AP, with three free OH groups at C-5, C-7 and C-4' positions, exerts significant protective effect against MPP<sup>+</sup>-induced neurotoxicity in PC12 cells. Our data further suggest that AP ameliorates the MPP<sup>+</sup>-induced production of ROS, increases the number of viable cells, attenuates the release of LDH, prevents the loss of MMP, reduces the total number of apoptotic cells and elevates the ratio of Bcl-2/Bax. Even in the long-term procedure of MPP<sup>+</sup>-induced cytotoxicity in PC12 cells, significant neuroprotective effects of AP can be observed.

There is convincing evidence that the overproduction of ROS, which leads to a pro-oxidant state known as oxidative stress, plays an important role in the etiology and/or progression of a number of neurological diseases and is responsible for neurodegeneration (5,30). The excessive production of ROS can cause severe impairment of cellular functions, such as peroxidize membrane lipids, oxidize protein and attack cytoplasmic RNA and mitochondrial DNA (31). Moreover, previous data have demonstrated that ROS are involved in the apoptotic mechanism of MPP<sup>+</sup>-mediated neurotoxicity and may contribute to the apoptotic processes that are associated with the development of PD (32). In addition, ROS generated by MPP<sup>+</sup> may be at least partly responsible for the opening of mitochondrial permeability transition pores and the collapse of MMP (33). As mentioned above, our experiment data suggested that treatment with MPP<sup>+</sup> resulted in a significant increase in ROS production which was consistent with previous research papers (14,22) and pre-treatment with AP markedly reduced the generation of intracellular ROS. Based on these findings, it can be concluded that AP ameliorates oxidative damage induced by MPP<sup>+</sup> in PC12 cells at least partly through the scavenging of ROS.

Mitochondria are well known to be the principal source of intracellular ROS production (5). The production of ROS in the mitochondria is accelerated by ROS themselves, termed ROS-induced ROS release and ROS generation in only small numbers of mitochondria can affect neighboring mitochondria, eventually propagating the ROS surge to the whole cell through this positive feedback loop (6). The overproduction of ROS rapidly causes a decrease in MMP and then this depolarization of MMP results in the release of apoptogenic factors, such as cytochrome *c* from the intermembrane space to the cytoplasm. Subsequently, cytochrome *c* and other apoptogenic factors trigger the caspase family and induce cells apoptosis (5,34). In this study, the involvement of the mitochondria in MPP<sup>+</sup>-induced apoptosis was investigated by evaluating the loss of MMP. Our data indicated that the treatment of the PC12 cells with MPP<sup>+</sup> markedly reduced MMP, which was detected by JC-1 staining, and the reduction in MMP induced by MPP<sup>+</sup>

was reversed by pre-treatment with AP. These results strongly suggest that AP stabilizes the MPP<sup>+</sup>-induced MMP disruption and that this may be attributed to the oxidative stress-reducing action of AP.

In addition, the mitochondrial apoptotic pathway is the best known intrinsic apoptotic pathway (35). Although the precise mechanisms through which the Bcl-2 family acts remain unclear, it has been established that the Bcl-2 family does indeed play a pivotal role in the mitochondrial apoptotic pathway (14). The Bcl-2 family proteins consist of two subgroups according to structural homology: the anti-apoptotic proteins, such as Bcl-2 and the pro-apoptotic proteins, including Bax (36). It is now evident that Bcl-2 or Bax control the mitochondrial permeability transition pores or influence other early mitochondrial perturbations (37). Therefore, Bcl-2 or Bax may facilitate the passage of certain important proteins, such as cytochrome *c* or other apoptosis-inducing factors that may trigger the activation of a caspase cascade and result in apoptosis. Cell survival in the early phases of the apoptotic cascade depends mostly on the balance between the anti-apoptotic and pro-apoptotic proteins of the Bcl-2 family (38). In this regard, the Bcl-2/Bax ratio may predict the apoptotic fate of the cell better than the absolute concentrations of either molecule alone (39). In the present study, we investigated the effects of AP on the expression levels of Bcl-2 and Bax in MPP<sup>+</sup>-treated cells by western blot analysis. Our data indicated that MPP<sup>+</sup> significantly decreased the ratio of Bcl-2/Bax, a result that was consistent with that of previous studies (40). However, pre-treatment with AP increased the expression of Bcl-2, while significantly decreasing the expression of Bax, thus ameliorating the MPP<sup>+</sup>-induced reduction in the Bcl-2/Bax ratio in PC12 cells. Therefore, the effects of AP on MPP<sup>+</sup>-induced apoptosis may be at least be partially mediated through the regulation of the expression of Bcl-2 and Bax. These results suggest that the neuroprotective effects of AP are associated with the inhibition of apoptosis through the mitochondrial pathway.

In conclusion, the results from our study demonstrate that AP exerts a protective effect against MPP<sup>+</sup>-induced neurotoxicity in PC12 cells and that the neuroprotective effects of AP are, at least partially mediated through the inhibition of oxidative stress, the stabilization of mitochondrial function and the reduction of neuronal apoptosis via the mitochondrial pathway. To the best of our knowledge, this is the first study to demonstrate that AP rescues neuronal cells from MPP<sup>+</sup>-induced apoptosis *in vitro*. The present results suggest that treatment with AP may prove to be an effective therapeutic approach for neuroprotection in PD. The neuroprotective effects of this compound in PD require further investigation in primary neuronal cultures, as well as in animal models of PD.

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