Tectorigenin attenuates the MPP+-induced SH-SY5Y cell damage, indicating a potential beneficial role in Parkinson's disease by oxidative stress inhibition

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Abstract. Tectorigenin is a plant isoflavonoid primarily derived from the flowers of *Pueraria thomsonii* Benth. Although various biological properties of tectorigenin have been reported, such as its antioxidant activity, the effects of tectorigenin on the cellular models of Parkinson's disease have not yet been elucidated. The aims of the current study were to investigate whether tectorigenin prevents neurotoxicity induced by MPP+ (also known as 1-methyl-4-phenylpyridinium) in SH-SY5Y cells and to elucidate the underlying protective mechanism. Cell viability and lactate dehydrogenase release were measured. The morphological changes of apoptotic cells were observed by Hoechst 33258 staining. Caspase-3, superoxide dismutase, catalase and glutathione peroxidase activity was measured using commercially available ELISA kits. The expression of cytochrome *c*, Bax, Bcl-2 and NADPH oxidase were detected by western blot analysis. The results indicated that treatment with MPP+ causes a significant decrease in the viability of cells and an increase in apoptosis, as evidenced by the upregulation of apoptotic cells, caspase-3 activity and cytochrome *c* expression. By contrast, these effects were all reversed by pretreatment with tectorigenin in SH-SY5Y cells. Tectorigenin also inhibited the MPP+-induced changes of Bax and Bcl-2 levels. In addition, pretreatment with tectorigenin mitigated the MPP+-caused increases in the levels of reactive oxygen species and NADPH oxidase protein in SH-SY5Y cells. Simultaneously, tectorigenin abolished the downregulation of antioxidant enzymes, including superoxide dismutase, catalase and glutathione peroxidase, that was induced by MPP+. In conclusion, the present study data indicate that the neuroprotective effect of tectorigenin against MPP+-induced cytotoxicity and apoptosis may be involved in attenuating oxidative stress and enhancing antioxidant defense.

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Introduction

Parkinson's disease (PD) is a common age-dependent neurodegenerative disorder characterized by selective loss of the substantia nigra dopaminergic neurons, which results in significant reduction of the dopamine content in the striatum. The incidence of this disease is increasing year by year and affects 1% of the population over the age of 65 years (1). Although the exact etiology and underlying mechanisms of PD remain unclear, the contributions of oxidative stress, mitochondrial dysfunction, accumulation of iron ions, activation of the apoptotic cascade, and the degeneration and death of dopaminergic neurons in the development of PD have been reported (2,3). The formation of reactive oxygen species (ROS), loss of mitochondrial membrane potential, depletion of ATP, and activation of caspase-3 and caspase-9 have been observed in the substantia nigra and cerebrospinal fluid of PD patients (4-6). Multiple stimuli-induced oxidative stress, free radical damage and ubiquitin proteasome also serve an important role in the pathogenesis of PD (7). In addition, increasing evidence demonstrated that antioxidants, as scavengers of ROS and free radicals, are vital in the prevention of PD (8). The MPP+ ion, also known as 1-methyl-4-phenylpyridinium, is the neurotoxic form of methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPP+ can been taken up by dopaminergic neurons, leading to mitochondrial dysfunction, oxidative stress and programmed cell death, which simulates the parkinsonian syndrome in cell and animal models (9).

Isoflavones are bioactive compounds with different physiological and pharmacological properties, including antioxidation, anti-free radical, anticancer, cardiovascular protection and neuroprotective effects, and are widely applied in the medicinal, food and cosmetic sectors (10-12). Tectorigenin is a type of natural isoflavone, derived from the flower of *Pueraria thunbergiana* (Leguminosae) which is used in traditional Chinese medicine. The antioxidant, anticancer and anti-inflammatory effects of tectorigenin have

been reported in a variety of disease model (13-15). A research performed by Kang *et al* revealed that tectorigenin protects hamster lung fibroblast V79-4 cells against hydrogen peroxide (H_2O_2)-induced damage by activating the extracellular signal regulated kinase pathway, while it simultaneously enhances the activities of antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) (16). In addition, Park *et al* demonstrated that tectorigenin exerts antioxidant effects characterized by inhibition of ROS production and upregulation of antioxidant enzyme activity in H_2O_2-treated C6 glioma cells and rat primary astrocytes (17). These findings suggest that tectorigenin may have beneficial effects on MPP^+-induced neurotoxicity through regulation of oxidative stress.

In present study, it was initially demonstrated that tectorigenin exerted a neuroprotective effect against MPP^+-elicited cytotoxicity and apoptosis in SH-SY5Y cells. Furthermore, tectorigenin was observed to reverse the MPP^+-induced enhancement of the oxidative stress system and inhibition of the antioxidant defense system. These results suggest that the antioxidant effect of tectorigenin mediates its protective role against MPP^+-induced neurotoxicity.

Materials and methods

Materials. Tectorigenin, MPP^+ iodide, 2',7'-dichlorofluorescein diacetate (DCFH-DA), Dulbecco's modified Eagle's medium (DMEM) and heat-inactivated fetal bovine serum (FBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Streptomycin/penicillin, BCA protein assay kit (P0009), Hoechst 33258 staining (C0003), BeyoECL Plus western blotting assay system (P0018), RIPA lysis buffer (P0013B), Cell Counting kit-8 (CCK-8, C0038) and lactate dehydrogenase (LDH) cytotoxicity detection kit (C0017) were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Caspase-3 ELISA kit (SEA626Mu) was obtained from USCN Business Co., Ltd. (Wuhan, China). The commercial SOD (A001-1), CAT (A007-1) and GSH-Px (A005) bioluminescence ELISA kits were provided by Jiancheng Bioengineering Institute (Nanjing, China). An antibody against cytochrome *c* was purchased from Abcam (Cambridge, UK, ab13575), and antibodies against NADPH oxidase (NOX, no. 4301), Bax (no. 2774) and Bcl-2 (no. 15071) were purchased from Cell Signaling Technology (Carlsbad, CA, USA). Tubulin (60008-1-Ig) and secondary antibodies were purchased from Proteintech (Danvers, MA, USA). All reagents were of high purity.

Cell culture and treatment. Human neuroblastoma SH-SY5Y cells were obtained from the Institute of Biochemistry and Cell Biology (Shanghai, China). Cells were maintained in DMEM supplemented with 10% FBS and 1% streptomycin/penicillin at 37˚C in a humidified incubator with 5% CO_2. The cell culture medium was changed every 2-3 days. The experimental groups were as follows: Untreated control, PD model (treated with 0.5 mM MPP^+), tectorigenin treatment alone (10 μM), and tectorigenin treatment (0.1, 1 and 10 μM) in MPP^+ model.

Cell viability assay. SH-SY5Y cells that were grown in the logarithmic growth phase were seeded in 96-well plates at a density of $3x10^3$ cells/well. When 70-80% confluence was reached, cells were treated as described earlier and the viability of cells was examined by CCK-8 assay. In brief, after treatment for 24 h, 10 μl CCK-8 reagent was added into the culture medium of each well and incubated for 2-3 h at 37˚C. The absorbance at 570 nm was recorded using a microplate reader (Varioskan; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and the viability of cells was recorded as the percentage relative to the control.

LHD release assay. The effect of tectorigenin on MPP^+-induced cytotoxicity was evaluated based on the degree of cytosolic LDH released into the culture medium (18), using an LDH Cytotoxicity Detection kit according to the manufacturer's protocol. Briefly, cultured SH-SY5Y cells were incubated with tectorigenin or MPP^+ as previously described for 24 h; subsequently, 50 μl of medium containing the released LDH was transferred to a new 96-well plate and mixed with 50 μl reaction mixture. After 30 min of incubation at room temperature, stop solution (50 μl) was added to terminate the reactions, and the absorbance at 490 and 680 nm was measured using a microplate reader to determine the LDH activity. LDH release was represented as the percentage vs. the control cells.

Hoechst 33258 nuclear staining assay. SH-SY5Y cells (in logarithmic growth phase) were seeded in 24-well plates at a density of $1x10^4$ cells/well. When grown to 70-80% confluence, cells were treated with MPP^+ and/or tectorigenin as described earlier for 24 h. After washing three times with phosphate-buffered saline (PBS) at 4˚C, SH-SY5Y cells were fixed in 4% paraformaldehyde for 10 min at room temperature and then washed again. The cells were subsequently incubated with 500 μl Hoechst 33258 at room temperature for 10 min. Following further washing with PBS, the fluorescence was detected using a fluorescence microscope (Olympus FV1000; Olympus Corp., Tokyo, Japan).

ROS measurement. The level of cytosolic ROS was quantified using DCFH-DA as a fluorescent probe. Following treatment with aforementioned indicated reagents for 24 h, SH-SY5Y cells were washed with PBS three times and incubated with 10 μmol/l DCFH-DA for 30 min at 37˚C. Subsequently, the cells were washed twice with Hank's buffer salt solution (Invitrogen; Thermo Fisher Scientific, Inc.), and the fluorescence of DCF was recorded at 485±10 nm excitation and 530±12.5 nm emission wavelengths using an EnSpire Multimode plate reader (PerkinElmer, Inc., Waltham, MA, USA).

Caspase-3 and antioxidant enzyme activity detection. Logarithmic growth phase SH-SY5Y cells were seeded in 6-well plates at a density of $1x10^5$ cells/well. After incubation with the aforementioned indicated treatments for 24 h, the cells were harvested and lysed. The concentration of protein was quantified by a BCA assay kit. The activities of caspase-3, SOC, CAT and GSH-Px were measured using the corresponding commercially available ELISA kits, according to manufacturer's protocol. Caspase-3 activity was expressed as the fold of the control group, while SOD, CAT and GSH-Px activities were expressed as U/mg protein.

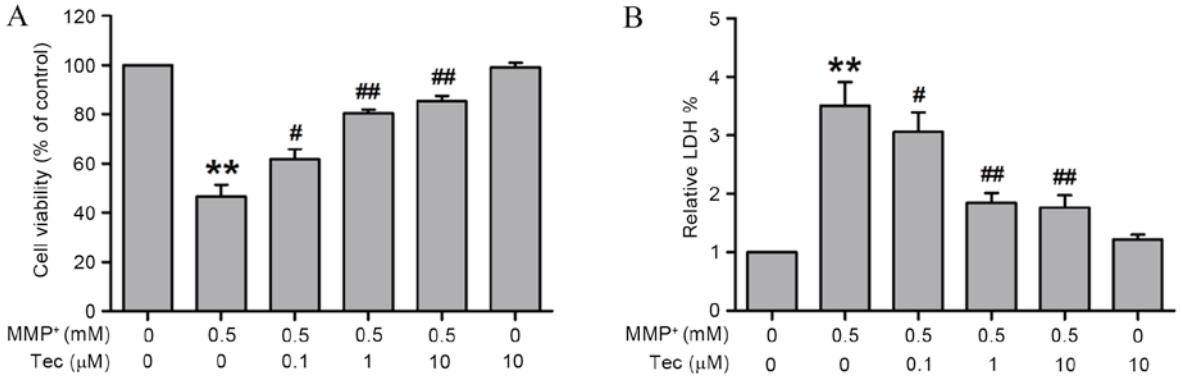

Figure 1. Inhibitive effects of Tec on MPP+-induced cell death. SH-SY5Y cells were pretreated with various concentrations of Tec (0.1, 1 and 10 μM) for 30 min prior to exposure to MPP+ (0.5 mM) for 24 h. (A) Viability of SH-SY5Y cells was assayed by cell counting kit-8 assay, and (B) LDH release was detected by an LDH release assay kit. Data are represented as the mean ± standard error from three experiments. **P<0.01 vs. control group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. MPP+ alone group. Tec, tectorigenin; LDH, lactate dehydrogenase.

Western blot assay. After reaching ~70% conference, SH-SY5Y cells were incubated with the indicated reagents for 24 h and then lysed using RIPA lysis buffer. After centrifugation at 10,000 x g for 10 min at 4˚C, the supernatant was collected and protein concentration was quantified by the BCA protein assay kit following the manufacturer's guide. An equal amount of protein (30-50 μg) was separated by 10-12% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane. The membranes were blocked with Tris-buffered saline/Tween 20 (TBST; containing 3.03 g Tris base, 18.8 g glycocine, 1 g SDS and 1 ml Tween-20 in 1,000 ml distilled water, pH 7.6) containing 5% non-fat milk for 2 h at room temperature. Next, samples were incubated with 1:1,000 dilution of cytochrome *c*, Bax, Bcl-2 and NOX primary antibodies, respectively. After incubation overnight at 4˚C, the membranes were washed with TBST three times and incubated with a horseradish peroxidase-conjugated secondary antibody (1:5,000 dilution in TBST) for 2 h at room temperature, followed by detection with a BeyoECL Plus western blotting detection kit. Protein blot images were quantized by ImageJ2x software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Each experiment was performed in triplicates, independently. The statistical significance of differences was evaluated with one-way analysis of variance test, followed by least-significant difference test, using GraphPad Prism 5 software (GraphPad Inc., San Diego, CA, USA). All values are presented as the mean ± standard error of the mean. P<0.05 was considered to demonstrate differences that were statistically significant.

Results

Tectorigenin reverses MPP+-induced cytotoxicity in SH-SY5Y cells. Firstly, in order to explore the protective effect of tectorigenin on the MPP+-exhibited cell injury, SH-SY5Y cells were pretreated with different concentrations of tectorigenin (0.1, 1 and 10 μM) for 30 min and then cultured with MPP+ (0.5 mM) for 24 h. The cell viability and the LDH release were detected by CCK-8 assay and LDH release assay kit, respectively. As shown in Fig. 1, pretreatment of SH-SY5Y cells with tectorigenin significantly upregulated the viability of cells in a concentration-dependent manner as compared with the viability in the MPP+-treated group (Fig. 1A). In addition, MPP+ alone for 24 h markedly increased the LDH release, while pretreatment with tectorigenin attenuated this LDH release in SH-SY5Y cells (Fig. 1B). Tectorigenin alone has no effects on the viability of cells or the release of LDH in SH-SY5Y cells (Fig. 1). These results suggest that tectorigenin protects against MPP+-induced cell damage.

Tectorigenin alleviates MPP+-induced apoptosis in SH-SY5Y cells. To further investigate the effects of tectorigenin on MPP+-induced neurotoxicity, the changes in apoptosis under MPP+ treatment in the presence or absence of tectorigenin were investigated. The Hoechst 33258 staining results revealed that the cells undergoing apoptosis, as indicated by dense granular fluorescence, in the MPP+ (0.5 mM)-treated alone group were increased in comparison with the control group. However, this phenomenon was reversed by pretreatment with 1 μM tectorigenin (Fig. 2A). Furthermore, tectorigenin abolished the increase in the activity of caspase-3 (a critical executioner of apoptosis) that was induced by MPP+ incubation in SH-SY5Y cells (Fig. 2B). Western blot analysis results also revealed that tectorigenin (1 μM) attenuated the upregulation of cytochrome *c* levels induced by MPP+ (Fig. 2C). These findings indicate that tectorigenin, which alone had no significant effect, produces antiapoptotic effects in MPP+-treated SH-SY5Y cells.

Tectorigenin reverses MPP+-induced changes of Bax and Bcl-2 expression levels in SH-SY5Y cells. The present study also examined the expression levels of apoptosis-associated proteins in SH-SY5Y cells that were exposed to MPP+ (0.5 mM) in the presence or absence of tectorigenin (1 μM). The Bax (pro-apoptotic protein) and Bcl-2 (anti-apoptotic protein) levels were determined by western blot assay. As shown in Fig. 3, MPP+ clearly promoted the expression of Bax (Fig. 3A) and inhibited the expression of Bcl-2 (Fig. 3B), compared with that of the control group. However, co-incubation of tectorigenin (1 μM) and MPP+ (0.5 mM) evidently reversed the alterations of Bax and Bcl-2 expression induced by treatment with MPP+ alone. These results suggest that

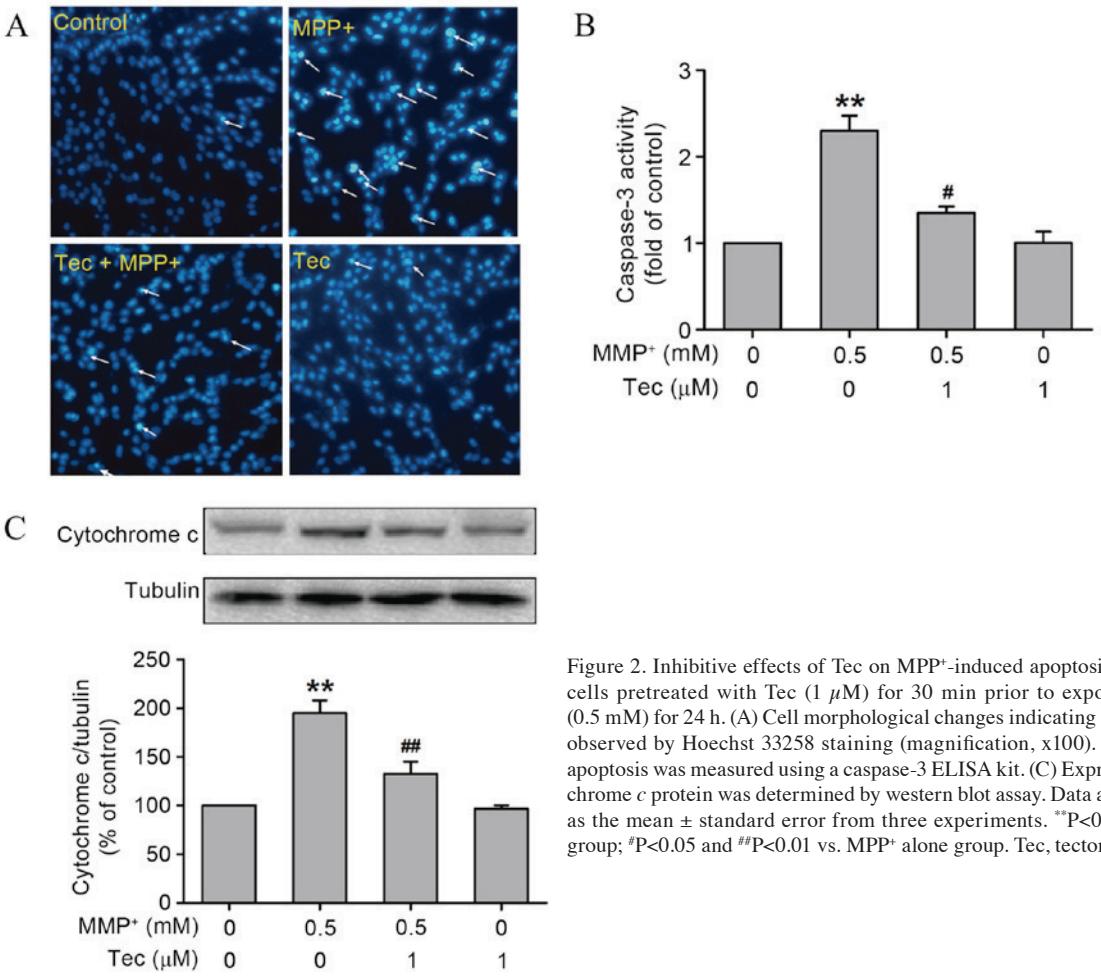

Figure 2. Inhibitive effects of Tec on MPP+-induced apoptosis in SH-SY5Y cells pretreated with Tec (1 μM) for 30 min prior to exposure to MPP+ (0.5 mM) for 24 h. (A) Cell morphological changes indicating apoptosis were observed by Hoechst 33258 staining (magnification, x100). (B) Caspase-3 apoptosis was measured using a caspase-3 ELISA kit. (C) Expression of cytochrome *c* protein was determined by western blot assay. Data are represented as the mean ± standard error from three experiments. **P<0.01 vs. control group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. MPP+ alone group. Tec, tectorigenin.

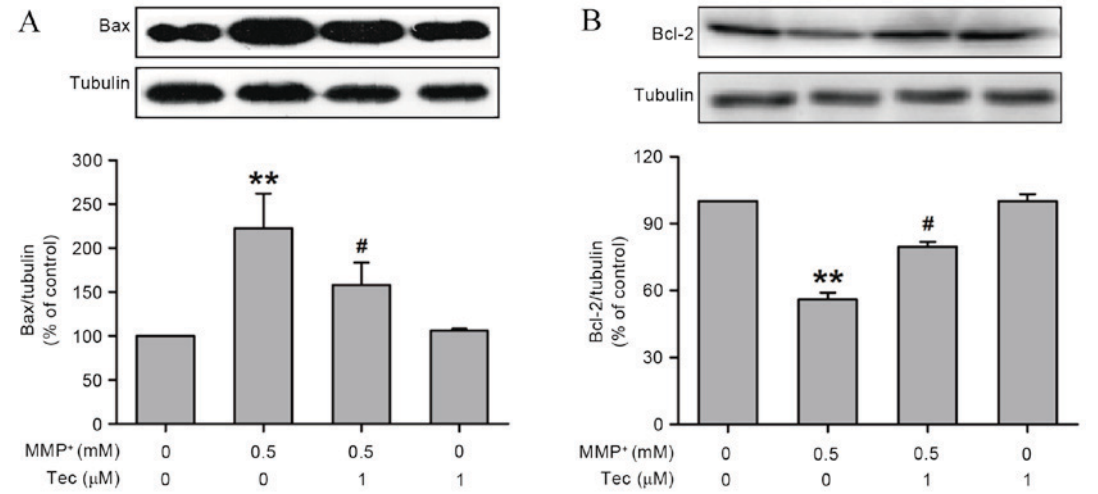

Figure 3. Effects of Tec on MPP+-induced alterations of Bax and Bcl-2 expression levels. SH-SY5Y cells were pretreated with Tec (1 μM) for 30 min prior to exposure to MPP+ (0.5 mM) for 24 h. Protein expression levels of (A) Bax and (B) Bcl-2 were detected by western blot assay. Data are presented as the mean ± standard error from three experiments. **P<0.01 vs. control group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. MPP+ alone group. Tec, tectorigenin.

tectorigenin decreases the ratio of Bax/Bcl-2 and prevents MPP+-induced apoptosis.

Tectorigenin blocks MPP+-induced ROS formation and NOX expression in SH-SY5Y cells. Oxidative stress is known to serve an important role in the pathological process of PD (19). To further investigate the underlying protective mechanism of tectorigenin in MPP+-induced neurotoxicity, the changes in oxidative stress under MPP+ treatment in the presence or absence of tectorigenin were examined. As shown in Fig. 4, compared with the control group, MPP+ (0.5 mM) treatment alone led to an increase in ROS formation in SH-SY5Y cells;

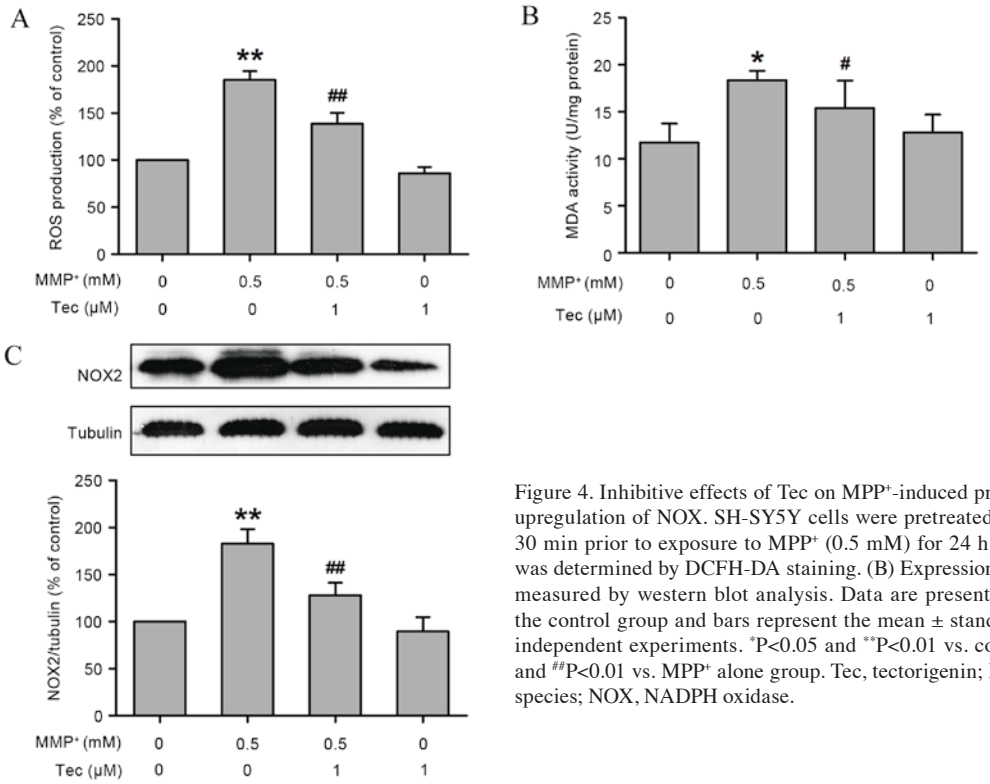

Figure 4. Inhibitive effects of Tec on MPP+-induced production of ROS and upregulation of NOX. SH-SY5Y cells were pretreated with Tec (1 μM) for 30 min prior to exposure to MPP+ (0.5 mM) for 24 h. (A) ROS generation was determined by DCFH-DA staining. (B) Expression of NOX protein was measured by western blot analysis. Data are presented as percentages of the control group and bars represent the mean ± standard error from three independent experiments. *P<0.05 and **P<0.01 vs. control group; #P<0.05 and ##P<0.01 vs. MPP+ alone group. Tec, tectorigenin; ROS, reactive oxygen species; NOX, NADPH oxidase.

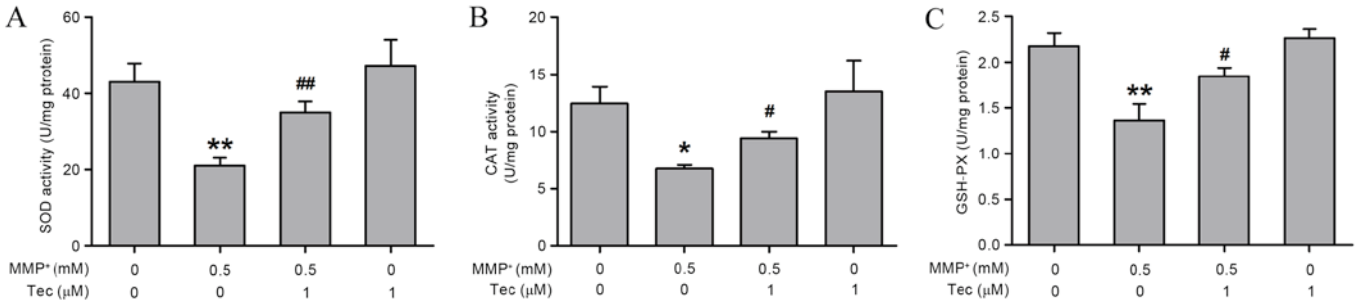

Figure 5. Inhibitive effects of Tec on MPP+-induced attenuation of antioxidant defense. SH-SY5Y cells were pretreated with Tec (1 μM) for 30 min prior to exposure to MPP+ (0.5 mM) for 24 h. (A) SOD activity, (B) CAT activity and (C) GSH-Px level were measured by bioluminescence ELISA kits. Data are presented as the mean ± standard error from three independent experiments. *P<0.05 and **P<0.01 vs. control group; #P<0.05 and ##P<0.01 vs. MPP+ alone group. Tec, tectorigenin; SOD, superoxide dismutase; CAT, catalase; GSH-Px, glutathione peroxidase.

however, this effect was evidently reversed by pretreatment with tectorigenin (1 μM; Fig. 4A).

NOX is the most important enzyme in cells resulting in generation of ROS in the central nervous system (20). Following exposure of SH-SY5Y cells to MPP+ for 24 h, the expression of NOX was significantly increased (Fig. 4B). However, pretreatment with tectorigenin (1 μM) blocked the MPP+-caused upregulation of NOX levels in SH-SY5Y cells (Fig. 4B). Notably, tectorigenin alone had no effect on oxidative stress (Fig. 4). These results reveal that tectorigenin prevents MPP+-induced oxidative stress, which may contribute to the protective functions of tectorigenin.

Tectorigenin restores MPP+-induced decreases in antioxidant enzyme activities in SH-SY5Y cells. Subsequently, the current study evaluated the effects of tectorigenin on the activity of endogenous antioxidant enzymes, which regulate the levels of ROS and free radicals in the cells. As shown in Fig. 5, treatment of SH-SY5Y cells with MPP+ (0.5 mM) significantly decreased the activities of SOD (Fig. 5A) and CAT (Fig. 5B), as well as the level of GSH-Px (Fig. 5C). However, pretreatment with tectorigenin (1 μM) for 30 min, which alone had no significant effect, prevented the MPP+-induced decreases in antioxidant enzyme activities (Fig. 5). These results indicate that enhancement of antioxidant defense by tectorigenin may be involved in the protective effects against MPP+-induced neurotoxicity.

Discussion

The incidence of progressive neurodegeneration in PD with increasing age involves the occurrence of oxidative stress.

However, there are currently no effective therapeutic drugs to eliminate the excessive production of ROS and free radicals in the nigrostriatal pathway of this disease. In the present study, the *in vitro* PD model demonstrated that tectorigenin, an antioxidant compound, abolished the neurotoxicity caused by MPP^+ treatment in SH-SY5Y cells. Tectorigenin reversed the MPP^+-induced reduction of cell viability and increase of cell apoptosis. Notably, the underlying protective mechanism of tectorigenin may be mediated through the attenuation of ROS production and NOX expression, and the enhancement of antioxidant defense, as evidenced by the downregulation of SOD, CAT and GSH-Px levels.

In the 1980s, MPTP was reported to cause Parkinsonian symptoms (21), and it was later observed that it is biotransformed into toxic MPP^+ by glial cells, which can cross the blood-brain barrier and enter dopaminergic neurons, resulting in mitochondrial damage, the generation of oxidative stress and the activation of pro-apoptotic pathways (22). In agreement with these previous studies, the present study observed that treatment of SH-SY5Y cells with MPP^+ induced loss of cell viability, occurrence of apoptotic features, enhancement of caspase-3 activity and cytochrome *c*, which was associated with the increases in the ratio of Bax/Bcl-2 and the elevation of ROS levels in SH-SY5Y cells. Increasing evidence revealed that oxidative stress serves an important role in the pathogenesis of PD and causes death of dopamine neurons in the substantia nigra in various ways (23). In recent years, the beneficial effects of naturally occurring phytochemicals with potent antioxidant properties on neurodegenerative disorders have received increasing attention (24,25). It has been reported that traditional Chinese medicines, particularly plant-derived constituents, may have a potential clinical value in alleviating the pathologic processes of PD (26). In the present study, tectorigenin, which is a natural isoflavone derived from the flower of *Pueraria thunbergiana* (Leguminosae), was found to attenuate MPP^+-induced cytotoxicity, apoptosis and ROS production in SH-SY5Y cells. These results are in agreement with the observations of a previous study, reporting that pretreatment with *Puerariae flos* decreased the viability of cells, the occurrence of apoptotic features and the mRNA expression of caspase-3 in ethanol-treated human neuroblastoma SK-N-MC cell line (27).

Excessive accumulation of ROS and free radicals, as well as an imbalance of the oxidative stress and antioxidative stress system, induce lipid peroxidation and damage DNA, subsequently resulting in cell functional disorder and even apoptosis (28,29). In addition, oxidative stress-induced apoptosis participates in the development of nervous system diseases, whereas intervention with antioxidants reduces the apoptosis via inhibiting the generation of ROS and thereby resulting in neuroprotective effects (19). Multiple studies have reported that ROS contributes to the apoptosis-associated mechanism of MPP^+-mediated neurotoxicity (30). Therefore, in the present study, it is hypothesized that the protective effects of tectorigenin against MPP^+-induced apoptosis is associated with modulation of the oxidative stress and antioxidative stress system. Furthermore, emerging evidence has demonstrated that NOX enzymes transport electrons across the plasma membrane to promote the generation of ROS, contributing to neuronal death in the pathological processes of PD (20). In the present study, it was also observed that pretreatment with tectorigenin significantly mitigated the MPP^+-caused upregulation of NOX protein in SH-SY5Y cells, further suggesting the inhibitory effect of tectorigenin on the oxidative stress system, which may contribute to the neuroprotective effects of tectorigenin. However, in order to verify this hypothesis, further investigations involving the use of NOX enzyme inhibitors need to be conducted.

Kang *et al* revealed that tectorigenin enhances the activities of cellular antioxidant enzymes, including SOD, CAT and GSH-Px, while it also increases the expression of their protein levels, thus protecting V79-4 cells against H_2O_2-mediated damage (16). Similarly, the present study indicated that pretreatment with tectorigenin markedly abolished the MPP^+-induced inhibition of SOD, CAT and GSH-Px activities in SH-SY5Y cells, which indicates that the protective effect of tectorigenin against MPP^+-induced neurotoxicity may be mediated by upregulation of the antioxidant defense system. However, a limitation of the present study is that the inhibition of antioxidant enzymes was not used to further verify this hypothesis.

In conclusion, using MPP^+-treated SH-SY5Y cells to simulate PD symptoms, the present study determined that tectorigenin exerted a neuroprotective effect against PD. The mechanism underlying this protective effect was further investigated and found to be associated with the antioxidant activity of tectorigenin, as indicated by the tectorigenin-induced downregulation of oxidative stress and enhancement of antioxidant defense.

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