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Original Article

Ganoderma Lucidum polysaccharides protect against MPP^+ and rotenone-induced apoptosis in primary dopaminergic cell cultures through inhibiting oxidative stress

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Abstract: Oxidative stress plays a pivotal role in the progressive neurodegeneration in Parkinson's disease (PD) which is responsible for disabling motor abnormalities in more than 6.5 million people worldwide. Polysaccharides are the main active constituents from *Ganoderma lucidum* which is characterized with anti-oxidant, antitumor and immunostimulant properties. In the present study, primary dopaminergic cell cultures prepared from embryonic mouse mesencephala were used to investigate the neuroprotective effects and the potential mechanisms of *Ganoderma lucidum* polysaccharides (GLP) on the degeneration of dopaminergic neurons induced by the neurotoxins methyl-4-phenylpyridine (MPP^+) and rotenone. Results revealed that GLP can protect dopamine neurons against MPP^+ and rotenone at the concentrations of 100, 50 and 25 μg/ml in primary mesencephalic cultures in a dose-dependent manner. Interestingly, either with or without neurotoxin treatment, GLP treatment elevated the survival of THir neurons, and increased the length of neurites of dopaminergic neurons. The Trolox equivalent anti-oxidant capacity (TEAC) of GLP was determined to be 199.53 μmol Trolox/g extract, and the decrease of mitochondrial complex I activity induced by MPP^+ and rotenone was elevated by GLP treatment (100, 50, 25 and 12.5 μg/ml) in a dose dependent manner. Furthermore, GLP dramatically decreased the relative number of apoptotic cells and increased the declining mitochondrial membrane potential ($\Delta\Psi m$) induced by MPP^+ and rotenone in a dose-dependent manner. In addition, GLP treatment reduced the ROS formation induced by MPP^+ and rotenone at the concentrations of 100, 50 and 25 μg/ml in a dose-dependent manner. Our study indicates that GLP possesses neuroprotective properties against MPP^+ and rotenone neurotoxicity through suppressing oxidative stress in primary mesencephalic dopaminergic cell culture owning to its antioxidant activities.

Keywords: Parkinson's disease, Ganoderma Lucidum polysaccharides, oxidative stress, MPP^+, rotenone

Introduction

Parkinson's disease (PD) is a complex neurodegenerative disorder characterized mainly by the selective loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc), which leads to disabling motor abnormalities such as rigidity, resting tremor and disturbance in balance [1, 2]. It is the second most common neurodegenerative disease, affecting more than 6.5 million people worldwide [3]. Although the exact etiology and pathogenesis of PD still remain unknown, oxidative stress is confirmed as a pivotal contributing factor to the selective degeneration of dopaminergic neurons in SNpc [4-6]. It has been shown that excessive production of the reactive oxygen species (ROS) induces mitochondrial dysfunctions, including decrease in mitochondrial membrane potential ($\Delta\Psi m$) and respiratory chain complex I activity, and mitochondrial DNA abnormality [7, 8]. Moreover, selective susceptibility to oxidative stress of the nigrostriatal pathway contributes to DNA damage in dopaminergic neurons of PD patients [9].

The neurotoxin methyl-4-phenylpyridine (MPP^+) and rotenone can selectively damage dopaminergic neurons in a dose- and time-dependent manner, by way of inhibiting the activity of mito-

chondrial electron transport chain complex I, one of the enzymes of the inner mitochondrial membrane involved in oxidative phosphorylation [10, 11]. MPP^+ is the active Parkinsonian neuro toxin metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which selectively destroys dopaminergic pathways in a pattern similar to PD, and induces Parkinsonian syndrome in humans, monkeys and mice [12-14]. It has been proved that MPP^+ exerts oxidative stress on mesencephalic dopaminergic neurons [15]. The high-affinity binding of MPP^+ to the mitochondrial complex I results in decreased production of ATP, excessive generation of ROS, and subsequently cell apoptosis or necrosis [16]. Another Parkinsonian neurotoxin rotenone is confirmed to be a classical, well-characterized and high affinity specific inhibitor of mitochondrial respiratory chain complex I [17]. In both *in vivo* and *in vitro* models, rotenone has been observed to impose an oxidative burden on dopaminergic neurons, which results in dysregulation of dopamine release and uptake [18, 19]. Furthermore, excessive ROS production induced the opening of the mitochondrial permeability transition pore (PTP), contributing to cell death in primary mesencephalic culture [20].

Previous studies have demonstrated that compounds interfering with impairment of mitochondrial complex I or with the accumulation of ROS might be protective in preventing the neurodegenerative process of PD [21, 22]. *Ganoderma lucidum*, a powerful medicinal mushroom, has been widely used for treating autoimmune disorders, cancer and, viral infection in China [23-25]. Some studies have also revealed that *Ganoderma lucidum* extracts can help to prevent dopaminergic neuron degeneration of cell lines [26]. Polysaccharides (GLP) are the major antioxidative components in *Ganoderma lucidum* [27], although studies evaluating the neuroprotective effect of GLP in primary dopaminergic culture model induced by MPP^+ and rotenone have not yet been conducted.

In the present study, we investigated the neuroprotective potential and probable mechanisms of GLP against the degeneration of dopaminergic neurons induced by MPP^+ and rotenone. Firstly, we evaluated the antioxidant activity of GLP. Then we observed the neuroprotective effect of GLP in primary dopaminergic cell cultures from embryonic mouse mesencephala treated with 10 μM MPP and 10 nM rotenone respectively. Immunocytochemical staining was applied to detect the direct toxicity to neurons. Furthermore, apoptosis, mitochondrial membrane potential ($\Delta\Psi m$) and ROS formation in the overall cell culture were determined by fluorescence staining methods. In addition, mitochondrial complex I activity in culture medium was measured by spectrophotometric determination.

Material and methods

Preparation of Ganoderma Lucidum polysaccharides

Ganoderma Lucidum was provided and authenticated by Professor Wolf Dieter Rausch at the University of Veterinary Medicine in Vienna, and *Ganoderma Lucidum* polysaccharides were prepared according to the method of a previous study [28]. The powder of dried *Ganoderma Lucidum* was extracted with 90°C hot water three times, and then precipitated with 96% ethanol. The sample was stored at 4°C and dissolved in phosphate buffered saline until time of use. The content of polysaccharides was detected by a phenol-H_2SO_4 test [29].

Anti-oxidant activity analysis of GLP

Anti-oxidant activity of GLP was measured by the ferric reducing antioxidant power (FRAP) method [30], 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) being used as positive control, and the OD value was detected by a spectro-photometer (Pharmacia Biotech, England) at 593 nm.

Animals

Pregnant OF1/SPF mice at gestation day (GD) 14 were provided by the Institute of Laboratory Zoology and Veterinary Genetics (Himberg, Austria). The study was carried out in accordance with the guidelines of the European Union Council (86/609/EU) for care and use of laboratory animals.

Preparation of primary mesencephalic dopaminergic cell culture and treatment

At gestation day 14, pregnant mice were sacrificed and embryos were transferred to Petri dishes containing sterile Dulbecco's phosphate

buffered saline (DPBS, Invitrogen, Germany). Under a stereoscope (10× magnification Nikon, SMZ-1B), brains were dissected, ventral mesencephala excised and primary cultures were prepared according to the methods in previous studies [31]. In brief, mesencephala were cut into small pieces in DPBS and collected in a sterile test tube containing 2 ml of 0.1% trypsin (Invitrogen, Germany) and 2 ml 0.02% DNase I (Roche, Germany) in DPBS. The tube was incubated in a 37°C water bath for seven min, then 2 ml of trypsin inhibitor (0.125 mg/ml in DPBS) (Invitrogen, Germany) were added. The tissues were centrifuged (Hettich, ROTIXA/AP) at 100xg for 4 min and the supernatant was discarded. Then the tissues pellets were triturated in Dulbecco's modified Eagle's medium (DMEM, Sigma, Germany) containing 0.02% DNase I. Dissociated cells were collected in basic medium (BM), DMEM supplied with 10% fetal calf serum (FCS), glucose (30 mM), glutamine (2 mM), 1% penicillin-streptomycin, Hepes buffer and amphotericin. The cells were plated into multidishes (Nunclon, Germany) pre-coated with poly-D-lysine (PDL) and incubated at 37°C, 5% CO_2. From the 6th day in vitro (DIV), BM was replaced by serum-free DMEM containing 0.02 ml B27/ml DMEM. At DIV 10, either MPP^+ (10 μM) or rotenone (10 nM) was added to primary cell cultures, and various concentrations of GLP (100, 50, 25, 12.5 μg/ml) were sequentially added and incubated for 48h at 37°C, 5% CO_2.

Identification of tyrosine hydroxylase immunoreactive (THir) neurons

On DIV 12, cultures were fixed with 4% paraformaldehyde (PFA) at 4°C for 30 min and permeabilized with 0.4% Triton X-100 for 30 minutes at room temperature. Cultures were washed three times with PBS, and then incubated with horse serum block solution (Vectastain ABC Kit, USA) for 90 minutes. Cells were incubated overnight at 4°C with mouse first anti-tyrosinhydroxylase (TH) antibody, and subsequently biotinylated with secondary antibody (Vectastain) and AB solution (Vectastain) for 90 minutes at room temperature. The color was developed with 3,3'-diaminobenzidine (DAB, 5 mg/ml) containing hydrogen peroxide (H_2O_2). THir neurons were counted in ten randomly selected fields at 100× magnification with a Nikon inverted microscope. The experiment was conducted in triplicate.

Measurement of mitochondrial complex I activity

Complex I activity in primary cell cultures was detected according to the method described in previous studies [32, 33]. The reaction was initiated by addition of 0.1 mM NADH, and complex I activity was measured spectrophotometrically by monitoring the rate of decrease in absorbance at 334 nm for 3 min (Pharmacia Biotech, NovaspecII).

PI uptake

Propidium iodide (PI) is membrane impermeable and generally excluded from viable cells, but when cell membranes disintegrate, PI intercalates into DNA rendering nuclei highly fluorescent [34]. PI uptake was therefore conducted to determine the effect of GLP on cell necrosis induced by MPP^+ and rotenone toxicity. Cultures were incubated with MPP^+ or rotenone for 48h, and then PI (invitrogen) was added to the cultures, with a final concentration of 10 μg/ml in colorless DMEM. After incubation at 37°C for 5 min, the cultured cells were photographed by a fluorescence microscope (Nikon, Japan), using 488 nm excitation. Four photos were taken randomly from each well. The photos were analyzed by Image J 1.47v software (National Institutes of Health).

DAPI staining

DAPI (4',6'-diamidino-2-phenylindole dihydrochloride) is a permeable DNA-binding dye that can distinguish necrotic and apoptotic cells, so we adopted this method to investigate the effect of GLP on cell apoptosis induced by MPP^+ and rotenone. After incubation with MPP^+ or rotenone for 48 h, cells were fixed with 4% PFA for 30 min, and then permeabilized with 0.4% Triton X-100 for 30 min. DAPI (final concentration 1 μg/ml in DPBS, invitrogen) was added to the cultures and incubated at room temperature for 5 min. Four photos were taken randomly from each well using 340 nm excitation. Nuclei with condensed and fragmented chromatin were counted with Image J 1.47 software.

Measurement of mitochondrial membrane potential with JC-1 staining

JC-1 dye can be used as an indicator of mitochondrial membrane potential in a variety of

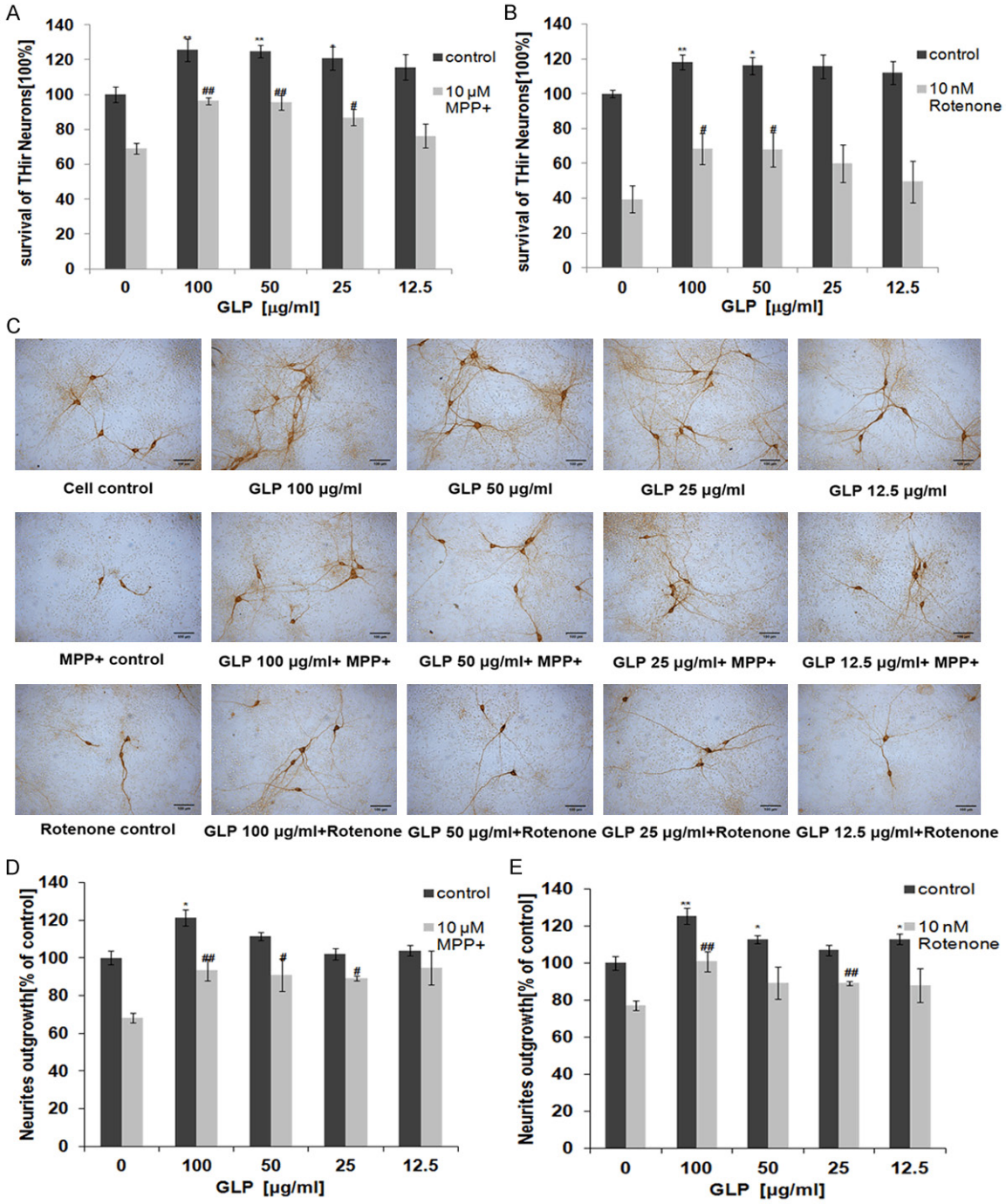

Figure 1. Neuroprotective effect of GLP on the MPP^+ and rotenone-induced cytotoxicity in primary mesencephalic dopaminergic cell cultures. Values were expressed as mean ± SEM with 3 wells in each group. In each well 10 randomly selected fields were analyzed. A. The mean relative survival rate of THir neurons induced by MPP^+. B. The mean relative survival rate of THir neurons induced by rotenone. C. Microscope images of THir neurons stained with DAB in each group. D. The average length of neuritis growth of THir neurons induced by MPP^+. E. The average length of neuritis growth of THir neurons induced by rotenone. $^{**}p < 0.01$, $^{*}p < 0.05$ compared to cell control group, $^{\#\#}p < 0.01$, $^{\#}p < 0.05$ compared to neurotoxin (MPP^+ or rotenone) control group.

cell types. In live cells, JC-1 exists either as a green-fluorescent monomer at depolarized membrane potentials or as an orange-fluorescent JC-1 aggregate at hyperpolarized membrane potentials. Incubated with MPP^+ or rotenone for 48 h, primary cell cultures were loaded

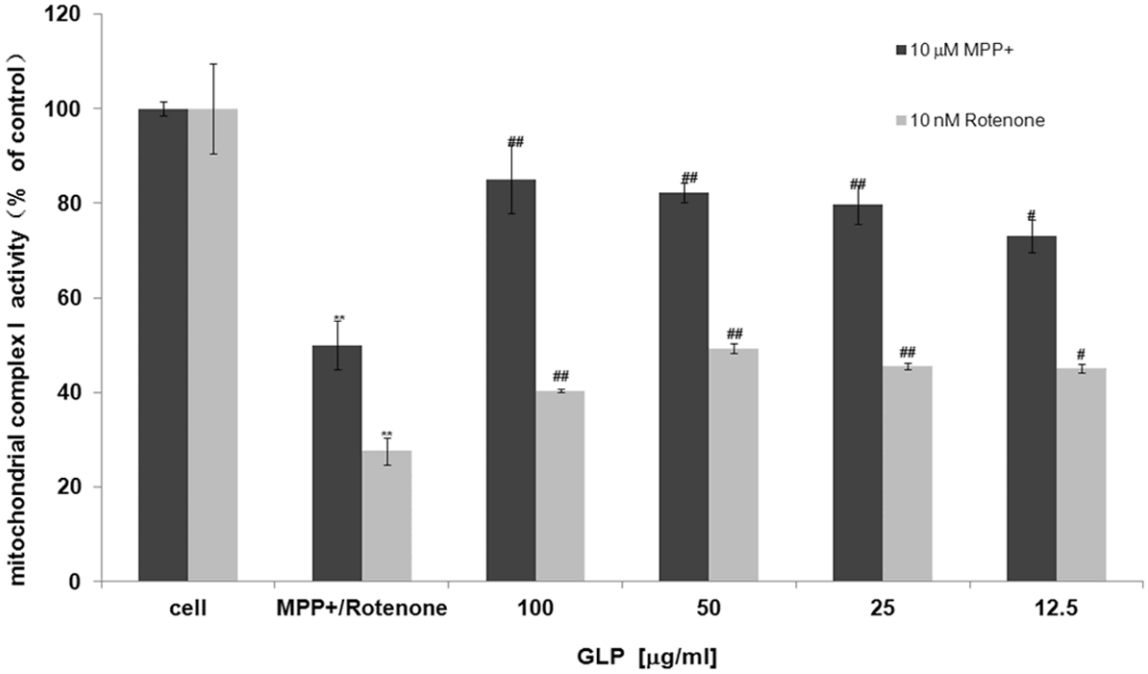

Figure 2. Effect of GLP on mitochondrial complex I activity of primary dopaminergic cell cultures treated with MPP^+ and rotenone. Values were expressed as mean mitochondrial complex I activity with 4 samples in each group (n = 4). $^{**}p < 0.01$ compared to cell control group, $^{\#\#}p < 0.01$, $^{\#}p < 0.05$ compared to neurotoxin (MPP^+ or rotenone) control group.

with JC-1 (final concentration 10 μg/ml, invitrogen) at 37°C for 15 min, and photographed on a Nikon fluorescence microscope with 488 nm and 568 nm excitation for green and red fluorescence, respectively. Four photos were taken randomly from each well. Fluorescence intensity of the red/green ratio was determined semiquantitatively by Image J 1.47 software.

Measurement of ROS generation with C-DCDHFDA

In the study, the fluorescence dye C-DCDHF-DA was used to detect the inhibitory effect of GLP on ROS generation for determination of the degree of overall oxidative stress. After incubation with MPP^+ or rotenone for 48 h, cells were loaded with C-DCDHF-DA (final concentration 10 mM in colorless DMEM) for 30 min at 37°C. Cultures were rinsed two times with colorless medium and photographed on a Nikon fluorescence microscope with 488 nm excitation. Four photos were taken randomly from each well and analyzed by Image J 1.47 software. In order to minimize the autofluorescence of C-DCDHF-DA during photographing, the cells were exposed to the light for no longer than 5 s.

Statistical analysis

All data were presented as mean ± SEM (standard error of the mean). Results were analyzed by one-way analysis of variance (ANOVA), and significant differences were determined by the Bonferroni Test. Statistical differences were considered significant when P values < 0.05.

Results

Anti-oxidant activity

In the anti-oxidant activity assay, the Trolox equivalent anti-oxidant capacity (TEAC) of GLP was determined to be 199.53 μmol Trolox/g extract.

Neuroprotective effect of GLP

In primary dopaminergic cell cultures induced by MPP^+ or rotenone, the number of THir neurons was counted, and the average length of neurite growth was measured. In MPP^+ treatment groups, the survival of THir neurons was markedly decreased to 69.25%. GLP treatment (100, 50, 25, 12.5 μg/ml) increased the survival of THir neurons to 96.32%, 95.42%,

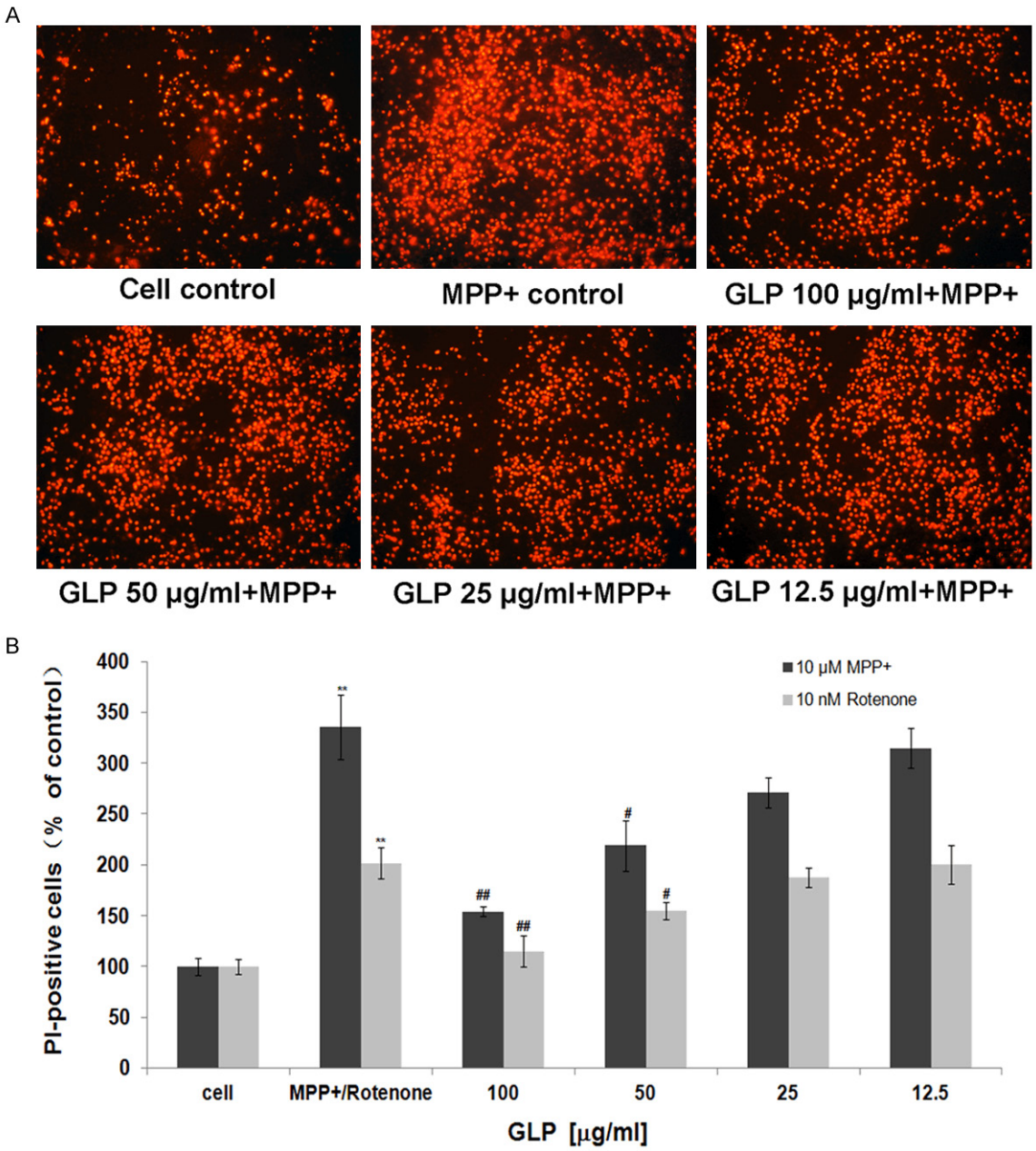

Figure 3. Effect of GLP on necrosis of primary dopaminergic cell cultures treated with MPP^+ and rotenone. A. Fluorescence images of primary dopaminergic cells stained with PI fluorescent at 488 nm in MPP^+ treatment groups. B. GLP treatment reduced number of PI-positive cells in a dose-dependent manner. Values were expressed as relative proportion of PI-positive cells with 4 wells in each group. In each well 4 randomly selected fields were analyzed. $^{**}p < 0.01$ compared to cell control group, $^{\#\#}p < 0.01$, $^{\#}p < 0.05$ compared to neurotoxin (MPP^+ or rotenone) control group.

86.97%, 76.38%, and 125.64%, 124.83%, 120.79%, 115.62% with and without MPP^+ treatment respectively (**Figure 1A**, **1C**). Moreover, in the rotenone treatment groups, the survival of THir neurons was dramatically decreased to 39.42%. GLP treatment (100, 50, 25, 12.5 μg/ml) increased the survival of THir neurons to 68.36%, 67.87%, 59.96%, 49.46%, and 118.11%, 116.16%, 115.63%, 111.96%, with and without rotenone treatment respectively (**Figure 1B**, **1C**).

The average length of neurite growth of dopaminergic neurons was extensively decreased to 68.12% and 77.08% after exposure to MPP^+ and rotenone respectively. GLP treatment (100,

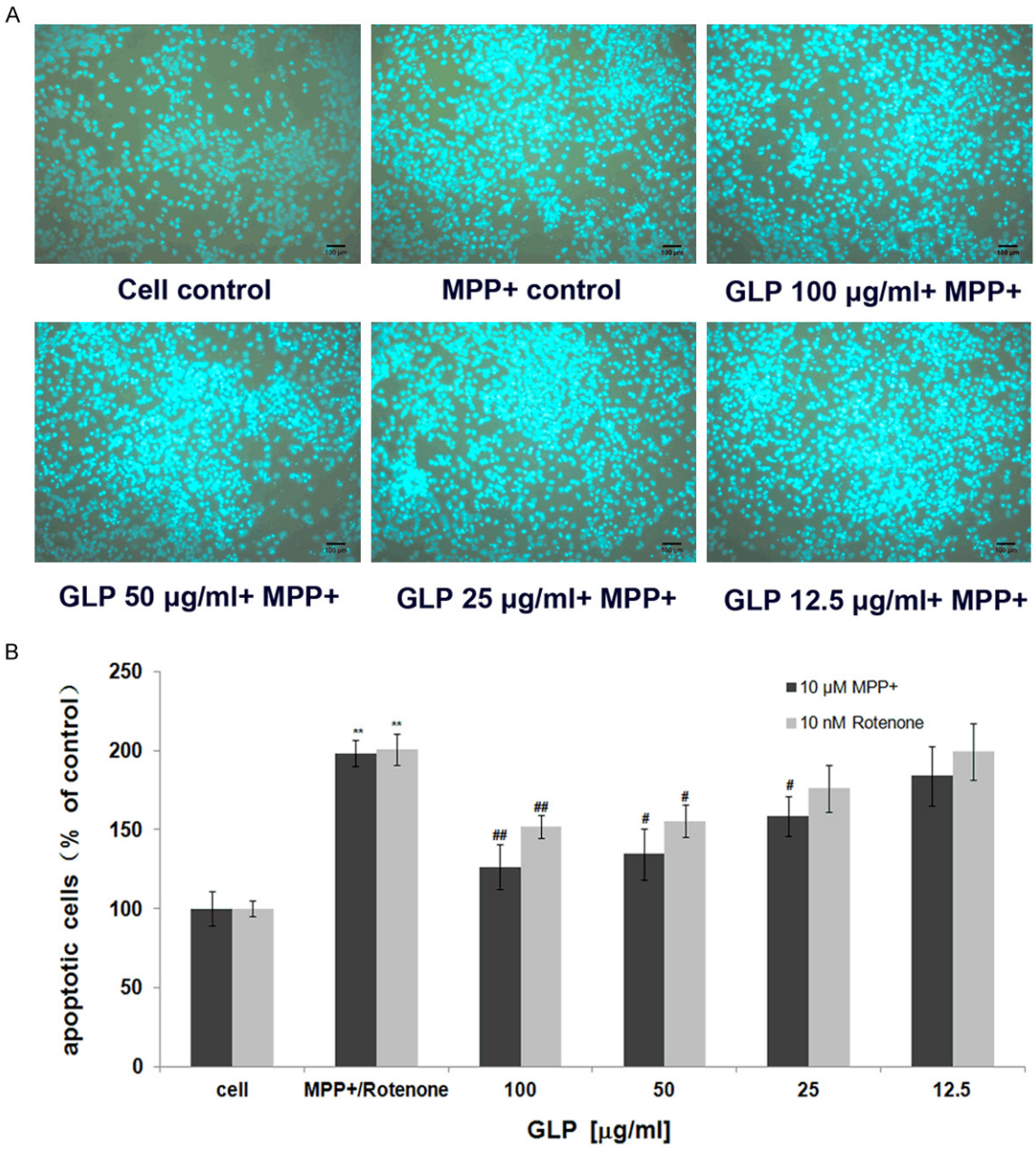

Figure 4. Effect of GLP on apoptosis of primary dopaminergic cell cultures treated with MPP^+ and rotenone. A. Fluorescence images of primary dopaminergic cells stained with DAPI fluorescent at 488 nm in MPP^+ treatment groups. Nuclei of apoptotic cells showed the features of condensed and fragmented chromatin. B. GLP treatment reduced relative number of apoptotic cells in a dose-dependent manner. Values were expressed as relative proportion of apoptotic cells with 4 wells in each group. In each well 4 randomly selected fields were analyzed. $^{**}p < 0.01$ compared to cell control group, $^{\#\#}p < 0.01$, $^{\#}p < 0.05$ compared to neurotoxin (MPP^+ or rotenone) control group.

50, 25, 12.5 μg/ml) increased the length of neurites of dopaminergic neurons to 93.41%, 90.83%, 89.15%, 94.68%, and 121.43%, 111.48%, 102.05%, 103.81%, with and without MPP^+ exposure respectively (**Figure 1D**). Furthermore, GLP treatment (100, 50, 25, 12.5 μg/ml) increased the length of neurites of dopaminergic neurons to 100.82%, 89.23%, 89.08%, 87.98%, and 125.39%, 112.59%, 107.08%, 112.82% with and without rotenone exposure respectively (**Figure 1E**).

These results demonstrated that GLP exerted a neuroprotective effect on the MPP^+ and rote-

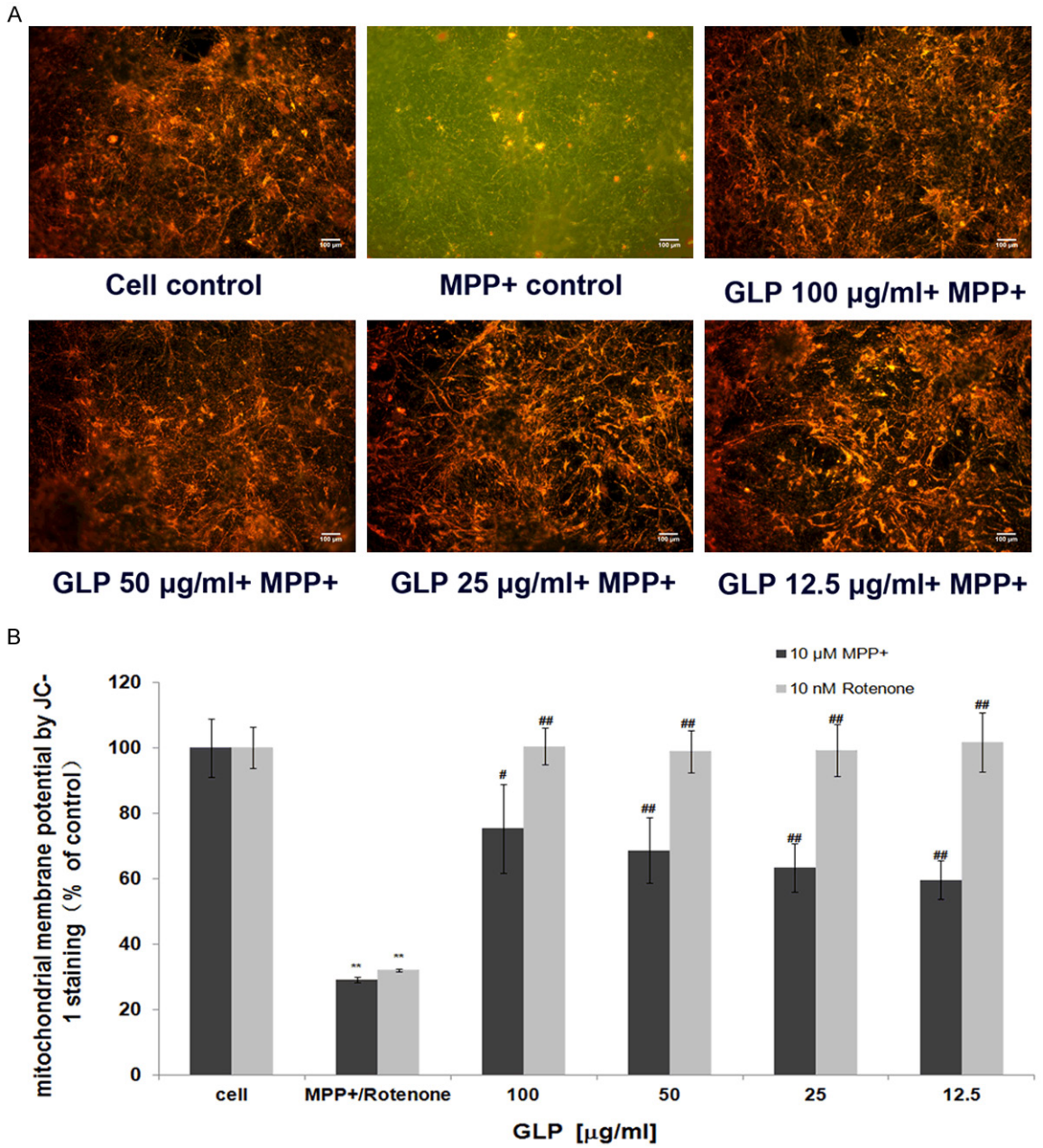

Figure 5. Effect of GLP on mitochondrial membrane potential of primary dopaminergic cell cultures treated with MPP^+ and rotenone. A. Fluorescence images of primary dopaminergic cells were merged by images taken with 488 nm and 568 nm excitation for green and red JC-1 fluorescence staining respectively in MPP^+ treatment groups. B. GLP treatment reversed depolarized membrane potential induced by MPP^+ and rotenone in a dose-dependent manner. Values were expressed as relative mitochondrial membrane potential with 4 wells in each group. In each well 4 randomly selected fields were analyzed. $^{**}p < 0.01$ compared to cell control group, $^{\#\#}p < 0.01$, $^{\#}p < 0.05$ compared to neurotoxin (MPP^+ or rotenone) control group.

none-induced cytotoxicity in a dose-dependent manner.

GLP increased mitochondrial complex I activity

MPP^+ and rotenone caused a significant decrease in complex I activity to 50% and 27.61% respectively. GLP treatment (100, 50, 25, 12.5 μg/ml) reversed the effect of MPP^+ and rotenone in a dose dependent manner (**Figure 2**), which demonstrated that the decrease of mitochondrial complex I activity induced by MPP^+ and rotenone could be reverted by GLP.

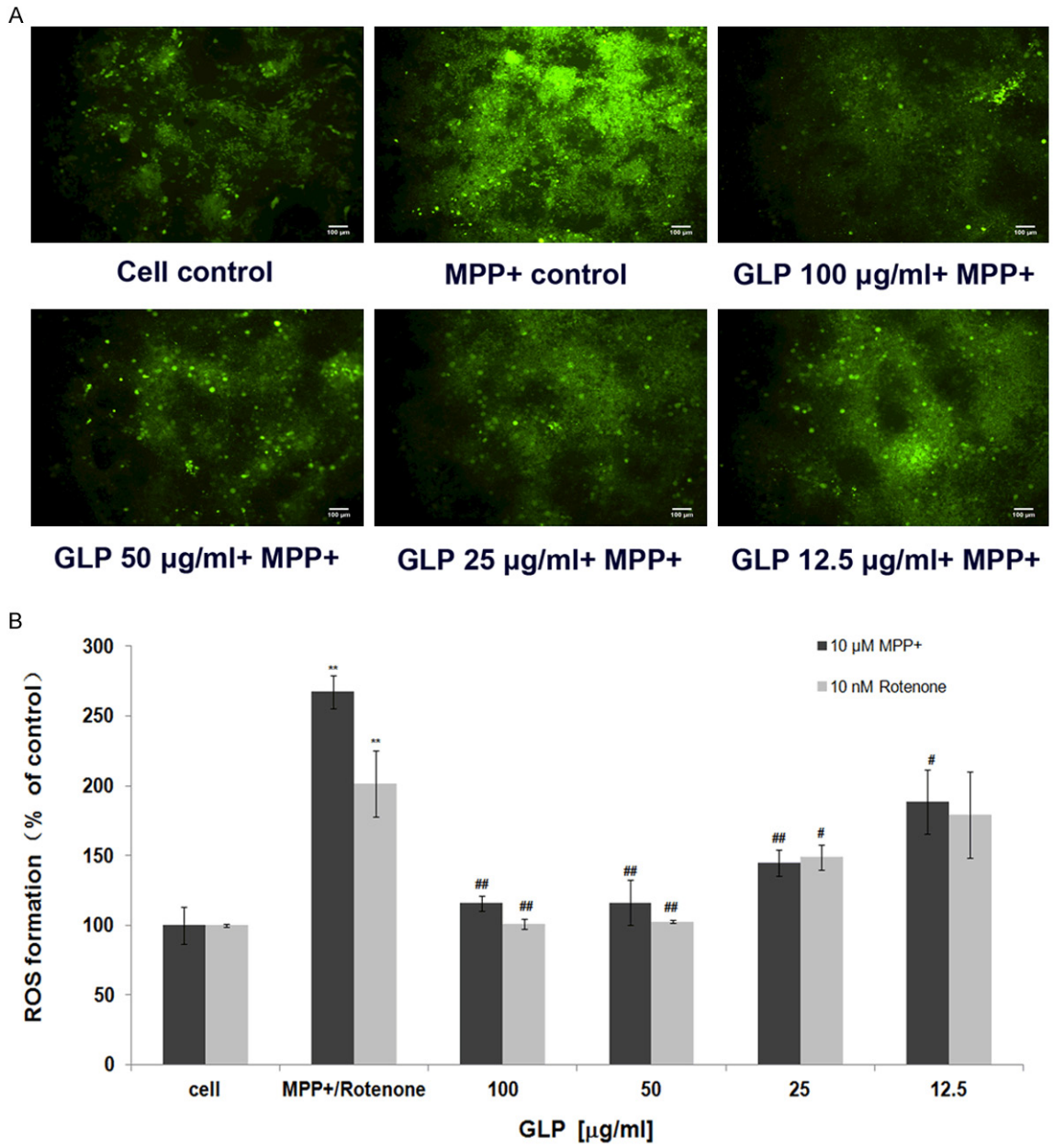

Figure 6. Effect of GLP on ROS formation of primary dopaminergic cell cultures treated with MPP^+ and rotenone. A. Fluorescence images of primary dopaminergic cells stained with C-DCDHF-DA fluorescent at 488 nm in MPP^+ treatment groups. B. GLP treatment decreased relative ROS formation in a dose-dependent manner. Values were expressed as relative ROS fluorescence intensity with 4 wells in each group. In each well 4 randomly selected fields were analyzed. $^{**}p < 0.01$ compared to cell control group, $^{\#\#}p < 0.01$, $^{\#}p < 0.05$ compared to neurotoxin (MPP^+ or rotenone) control group.

GLP decreased PI uptake by cultured cells

PI marked cells undergoing necrosis or late apoptosis. Compared with cell control, MPP^+ and rotenone clearly increased the relative proportion of PI-positive cells to 335.35% and 201.76% respectively. Compared with the MPP^+ control group, the numbers of PI-positive cells were significantly decreased to 154.05% and 218.78% by GLP (100, 50 μg/ml) respectively. Furthermore, proportions of PI-positive cells were significantly decreased to 115.12% and 154.39% by GLP (100, 50 μg/ml) compared with the rotenone control group (**Figure 3**).

These results demonstrated that GLP can prevent cell death induced by MPP^+ and rotenone in a dose-dependent manner.

Effect of GLP on apoptotic features

Apoptotic nuclei with highly condensed and fragmented chromatin were detected by DAPI fluorescence staining. MPP^+ and rotenone exposure increased the relative number of apoptotic cells to 198.42% and 201.06% in comparison to cell control, respectively. The relative number of apoptotic cells was significantly reduced to 126.50%, 134.59% and 158.52%, respectively in the case of GLP treatment (100, 50, 25 μg/ml) as compared to the MPP^+ control group in a dose-dependent manner. Moreover GLP dramatically decreased the relative number of apoptotic cells to 151.85% and 155.57% respectively at the concentrations of 100 and 50 μg/ml, compared with the rotenone control group (**Figure 4**). These results demonstrated that GLP can inhibit the cell apoptosis induced by MPP^+ and rotenone in a dose-dependent manner.

GLP increased the mitochondrial membrane potential (ΔΨm)

The changes in relative ΔΨm were determined by comparing the red/green ratio of JC-1 fluorescence intensity. A higher red/green ratio correlates with a much higher ΔΨm. MPP^+ and rotenone exposure decreased relative mitochondrial membrane potential to 29.10% and 31.96% in comparison to the cell control, respectively. GLP treatment (100, 50, 25, 12.5 μg/ml) dramatically increased relative ΔΨm to 75.32%, 68.59%, 63.33% and 59.55%, compared with MPP^+ control respectively. Compared with the rotenone control, GLP treatment (100, 50, 25, 12.5 μg/ml) significantly increased relative ΔΨm to 100.46%, 98.85%, 99.26% and 101.82% respectively (**Figure 5**). These results demonstrated that GLP can reverse the decrease of mitochondrial membrane potential induced by MPP^+ and rotenone in a dose-dependent manner.

GLP inhibited ROS formation

The formation of ROS in primary dopaminergic cell cultures was measured using C-DCDHF-DA fluorescence staining. Compared with the cell control, MPP^+ and rotenone exposure increased relative ROS formation to 267.26% and 201.21% respectively. GLP treatment (100, 50, 25, 12.5 μg/ml) significantly decreased the relative ROS formation to 115.92%, 116.31%, 144.68% and 188.70%, compared with MPP^+ control respectively. Compared with the rotenone control, GLP treatment (100, 50, 25 μg/ml) dramatically decreased relative ROS formation to 101.05%, 102.66% and 148.80% respectively (**Figure 6**). These results demonstrated that GLP can reduce the ROS formation induced by MPP^+ and rotenone in a dose-dependent manner.

Discussion

Accumulating evidence indicates that oxidative stress plays a key role in the pathogenesis and pathophysiology of Parkinson's disease [35, 36]. Oxidative stress is an imbalance between the production of free radicals and reactive metabolites, which is intimately linked to other components of the degenerative process, such as mitochondrial dysfunction, excitotoxicity, nitric oxide toxicity and inflammation [37, 38]. Oxidative stress mainly contributes to the vulnerability of DAergic cells, mitochondrial dysfunction and chronic neuroinflammation in the process of PD [39]. Extensive clinicopathological studies have provided evidence that oxidative stress induced by iron leads to oxidative injury in substantia nigra pars compacta [40]. Increased content of iron in nigral DAergic neurons initiates the Fenton reaction which stimulates free radical formation, lipid peroxidation and ultimately leads to cell demise. Furthermore hydrogen peroxide and superoxide radicals in the Fenton reaction can further increase oxidative stress [41].

Ganoderma lucidum (Ling Zhi) is a basidiomycete white rot fungus, which has been used widely for therapeutic use in Asian countries for thousands of years [42]. *Ganoderma lucidum* polysaccharides (GLP) are the major secondary metabolites of *Ganoderma lucidum*, and they have been shown to possess antioxidant, immunoregulatory and anti-inflammatory properties [43, 44]. Emerging evidence has suggested that *Ganoderma lucidum* extract has shown a potential neuroprotective effect in an MPTP animal model and co-cultures of dopaminergic neurons and microglia [45, 46].

The present study was designed to demonstrate the neuroprotective effect of polysaccharides extracted from *Ganoderma lucidum*

(GLP), and to discover the potential mechanism of the pharmacodynamic action. The results demonstrate for the first time that GLP can protect dopamine neurons against MPP^+ and rotenone at the concentrations of 100, 50 and 25 μg/ml in primary mesencephalic cultures. Morphological inspection suggested that both MPP^+ and rotenone treatment not only decreased the number of TH-positive neurons, but also significantly inhibited the outgrowth of neuritis. These toxin effects were reversed by GLP in a dose-dependent manner. Furthermore, treatment with GLP alone not only elevated the survival of THir neurons, but also increased the length of neuritis of dopaminergic neurons.

A number of Chinese herbs, extracts, and herbal formula exert neuroprotection effects against Parkinson's toxin, which may involve their anti-oxidant or anti-oxidative stress activity [47-49]. In accordance with the FRAP test, GLP was proved to possess potent anti-oxidant activity.

MPP^+ and rotenone were both powerful inhibitors of complex I activity in isolated brain mitochondria, and they can induce parkinsonism both *in vitro* and *in vivo*. Impairment to mitochondrial complex I in the electron transport chain will reduce mitochondrial respiration and cause ROS production [50]. Reduced complex I activity has been found in cells or tissues derived from subjects with parkin gene mutation [51]. Accumulating evidence suggests that dopaminergic cell death of DAergic neurons in Parkinson's disease involves damage to mitochondrial complex I, oxidative stress, and microglial activation [52]. It has been proved that mice mutations in parkin gene such as PINK, DJ-1 and α-synuclein showed reduced complex I activity along with oxidative damage [53]. Previous studies have demonstrated that *Ganoderma lucidum* extract protects dopaminergic neuron degeneration in the cell line MES 23.5 through inhibition of microglial activation [26]. However, the precise mechanism of GLP against dopaminergic neurodegeneration induced by MPP^+ and rotenone in primary mesencephalic cell culture is still unknown. Our study showed that GLP significantly elevated mitochondrial complex I activity inhibited by MPP^+ or rotenone in primary mesencephalic dopaminergic cell culture. This effect of GLP may be related to its antioxidant activity.

Mitochondrial dysfunction is implicated in some complex disorders such as neurodegenerative diseases, cardiac dysfunction, diabetes or cancer [54]. In Parkinson's disease, the role of mitochondrial dysfunction is dependent on oxidative stress. Injury induced by oxidative stress to mitochondrial macromolecules can lead to an apoptosis mechanism [55]. Additionally, mitochondria are the target of parkinsonian neurotoxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in the brain, and its metabolite 1-methyl-4-phenylpyridinium (MPP^+), and the pesticides rotenone, and paraquat [56, 57]. Therefore, the mitochondrial-targeted antioxidants are regarded as promising therapeutic agents for PD patients. In our study, MPP^+ and rotenone induced cell death labeled by PI fluorescence staining in primary dopaminergic culture, and cell nuclei showed apoptotic features detected by DAPI fluorescence staining. In addition, MPP^+ and rotenone exposure decreased relative mitochondrial membrane potential with JC-1 fluorescence staining. These results suggested that two major parkinsonian toxins caused apoptosis in primary dopaminergic culture, which are consistent with findings in previous studies [58, 59]. Of greater interest in the present study is that the increased relative number of apoptotic cells and the decreased mitochondrial membrane potential induced by MPP^+ or rotenone were dramatically reversed by GLP treatment. These results indicate that apoptosis triggered by MPP^+ or rotenone could be suppressed by GLP.

To our knowledge, mitochondrial complex I inhibition by MPTP or rotenone triggers oxidative stress by stimulating excessive intracellular reactive oxygen species (ROS), which finally results in mitochondrial-dependent apoptosis [60, 61]. These findings were supported by the results in the present study. Multiple lines of evidence have suggested that the scavenging of ROS mediated by antioxidants may be a potential strategy for deferring the progression of Parkinson's disease [62]. GLP effectively attenuated the ROS formation induced by MPP^+ and rotenone with C-DCDHFDA fluorescent staining in a dose-dependent manner in a primary dopaminergic cell culture. The results indicated that GLP protected against MPP^+ and rotenone-induced apoptosis in primary dopaminergic cultures through inhibiting oxidative stress. Nevertheless, we need to investigate whether GLP could protect against nigrostriatal degeneration after MPTP or rotenone intoxication to model PD in mice or rats in the next

step. Moreover, further investigations are required to determine the mitochondrial apoptotic or antioxidant pathways involved in the neuroprotective effect of GLP against Parkinson's disease.

In conclusion, our study shows that GLP possesses neuroprotective properties against MPP^+ and rotenone neurotoxicity in a primary mesencephalic dopaminergic cell culture. Owing to its antioxidant activities, GLP inhibits cell apoptosis through suppressing oxidative stress in primary cell culture during dopaminergic neurons degeneration. These results showed that GLP is promising as a potential therapeutic drug for patients suffering from neurodegenerative diseases induced by oxidative damage, and so further research and development should be encouraged. Further studies should be focused on evaluating the neuroprotective effect of GLP in the animal experimental PD model and in discovering the signal pathways related to antioxidant or anti-apoptotic behaviour.

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Disclosure of conflict of interest

None.

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