

## Original Article

# **Radix Ophiopogonis polysaccharide extracts alleviate MPP<sup>+</sup>-induced PC-12 cell injury through inhibition of Notch signaling pathway**

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**Abstract:** *Background:* Parkinson's disease (PD) is a degenerative disease of central nervous system. 1-Methyl-4-phenylpyridine (MPP<sup>+</sup>) is a non-selective dopaminergic neurotoxin that induces cell injury similar to PD. This study aimed to explore the protective effects of *Radix Ophiopogonis* polysaccharide extracts (ROP) on the MPP<sup>+</sup>-induced PC-12 cell injury. *Methods:* PC-12 cells were exposed to MPP<sup>+</sup> with or without ROP treatment. Then the cell viability, apoptosis, reactive oxygen species (ROS) level, calcium (Ca<sup>2+</sup>) concentration, mitochondrial membrane potential (MMP), Cytochrome C release, mitochondrial ATP synthesis, and the expression level of Notch signaling pathway were detected by CellTiter 96 AQ<sub>ueous</sub> One Solution Cell Proliferation assay, fluorescent staining, flow cytometer analysis, and western blotting, respectively. *Results:* MPP<sup>+</sup> treatment obviously induced PC-12 cell injury as evidenced by the cell viability loss and cell apoptosis enhancement. MPP<sup>+</sup> markedly increased the concentrations of ROS and Ca<sup>2+</sup> and the mitochondrial dysfunction in PC-12 cells. Moreover, the activation of Notch signaling pathway was found after MPP<sup>+</sup> treatment. ROP significantly reversed the MPP<sup>+</sup>-induced PC-12 cell viability loss, apoptosis increase, intracellular oxidative stress and endoplasmic reticulum (ER) stress rise, mitochondrial dysfunction and the activation of Notch signaling pathways in PC-12 cells. *Conclusion:* ROP protected PC-12 cells from the MPP<sup>+</sup>-induced injury through suppressing the increase of the intracellular oxidative stress and ER stress and activation of Notch signaling pathway. These findings will be helpful for understanding the protective roles of ROP in nerve cell injury and provide potential therapeutic drug for PD.

**Keywords:** *Radix Ophiopogonis* polysaccharide extracts, 1-Methyl-4-phenylpyridine, Parkinson's disease, oxidative stress, endoplasmic reticulum stress, notch signaling pathway

## Introduction

Parkinson's disease (PD) is one of the most common neural degenerative disease in human, which associates with the selective loss of dopaminergic (DA) neurons in the nigrostriatal pathway [1]. The mainly clinical symptoms of PD are involuntary resting tremor, bradykinesia, rigidity, and postural instability [2]. The increase of oxidative stress in nerve cells is considered to be the main pathogenesis of PD, which disturbs the balance between intracellular reactive oxygen species (ROS) production and ROS elimination system, resulting in lipids, protein, DNA damage, cellular dysfunction and then cell demise [3, 4]. Surgical treatment and pharmacotherapy only improve the clinical symptoms

of PD, but cannot cure PD completely. New therapeutic strategies and more effective drugs are needed urgently.

*Radix Ophiopogonis*, the root tuber of *Ophiopogon Japonicus* (Thunb.), is the one of most widely used Chinese herb in Traditional Chinese Medicine (TCM) [5]. The polysaccharide extracts of *Radix Ophiopogonis* (ROP) have been gained more attention in recent years due to their widely bioactivities. For example, Ding *et al.* reported that ROP showed efficient anti-diabetic effect by decreasing the carbohydrate digestion and glucose absorption [6]. Wang *et al.* demonstrated that ROP displayed antioxidant activity by significantly decreasing the levels of superoxide anion and hydroxyl radical [7]. A

study from Zhao *et al.* pointed out that ROP attenuated myocardial ischemia/reperfusion injury by suppressing mitochondrial mediated apoptosis and oxidative stress and up-regulating liver-X-receptor  $\alpha$  [8].

Notch signaling pathway participants in the regulation of various cell function, such as proliferation, differentiation and cell fate determination [9]. Moreover, it has been reported that Notch signaling pathway involved in the development of the nervous system [10]. Kamarehei *et al.* illustrated that Notch signaling pathway played important role in the protective effects of curcumin on H<sub>2</sub>O<sub>2</sub>-induced SK-N-MC cell injury [11]. Tanriverdi *et al.* demonstrated that resveratrol prevented CCL<sub>4</sub>-induced liver injury by inhibiting Notch signaling pathway [12]. The protective effects of ROP on ischemia/reperfusion injury has been widely studied [13, 14]. It still remains unclear that whether ROP has a similar protective effect on PD cell injury and whether Notch signaling pathway participants in the protective role of ROP on PD cell injury.

Therefore, in this study, we used 1-Methyl-4-phenylpyridine (MPP<sup>+</sup>), a non-selective dopaminergic neurotoxin that induces cell injury similar to PD [15], to induce PC-12 cell injury model. Then the potential protective effects of ROP on MPP<sup>+</sup>-induced PC-12 cell viability loss, apoptosis increase, intracellular oxidative stress rise, mitochondrial dysfunction, and the activation of Notch 2 signaling pathways were detected. Our finding will be helpful for understanding the protective roles of ROP in PC-12 cell injury and provide potential therapeutic drug for PD.

### Materials and methods

#### Cell culture

Rat pheochromocytoma-derived cell line, PC-12, was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown in the Dulbecco's modified Eagle's medium (DMEM, Gibco, Life Technologies, CA, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, UT, USA), 5% horse serum (Hyclone, USA), 100 U/ml benzyl penicillin-100  $\mu$ g/ml streptomycin solution (Gibco, Life Technologies, CA, USA) and incubated at 37°C in humidified atmosphere with 5% CO<sub>2</sub> incubator. 1-Methyl-4-phenylpyridine (MPP<sup>+</sup>, D048, Sigma-Aldrich, MO, USA) was used to induce cell injury.

#### Preparation of *Radix Ophiopogonis polysaccharide extract*

The preparation process of *Radix Ophiopogonis* polysaccharide extracts was similar with the previous study [6]. Briefly, Dried *Radix Ophiopogonis* powder was refluxed with distilled water, filtered through filter paper, concentrated to the density of 1.25 g/ml and added ethanol (95%) to the percentage of the ethanol in the solution was 80%. Then the precipitate in ethanol solution was collected and re-dissolved in distilled water and purified by Sephadex G-75 (G75120, Sigma-Aldrich, MO, USA). The filtrate, which presented a positive reaction to anthrone-H<sub>2</sub>SO<sub>4</sub>, was lyophilized and considered as the polysaccharide extract of *Radix Ophiopogonis* (ROP).

#### Cell viability

Cell viability was measured using CellTiter 96 AQ<sub>ueous</sub> One Solution Cell Proliferation assay (G3582, Promega, WI, USA). PC-12 cells were seeded in 96-well plates (Corning Inc., NY, USA) overnight and then received MPP<sup>+</sup> with or without ROP treatment for different time. After that, 20  $\mu$ l/well of CellTiter 96 AQ<sub>ueous</sub> were added to each well. The absorbance at 490 nm was recorded by a micro-plate reader (ELx800, Bio-Tek Inc., MO, USA). Cell viability (%) was calculated by (1-mean absorbance of treated group/mean absorbance of control group)  $\times$  100%.

#### Lactate dehydrogenase (LDH) release assay

PC-12 cells were seeded in 96-well plates (Corning Inc., NY, USA) overnight and then received MPP<sup>+</sup> with or without ROP treatment for different time. After that, 50  $\mu$ l supernatant of each wells were mixed with 50  $\mu$ l substrate mix of cytoscan-LDH cytotoxicity assay kit (G-Bioscience Inc., MO, USA). The mixtures were incubated at 37°C for 30 min. Then, 50  $\mu$ l stop solution was added to each well to terminate reaction. The absorbance at 490 nm was recorded by a micro-plate reader (ELx800, Bio-Tek Inc., MO, USA).

#### Cell apoptosis

Cell apoptosis was detected using an Annexin V-FITC Apoptosis Detection Kit (Invitrogen, CA, USA) according to the manufacturer's instruction. Briefly, after relevant treatment, PC-12 cells were harvested and suspended into bind-

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ing buffer at 37°C for 15 min in the dark. Then the rate of apoptosis was quantified by flow cytometer (Bender MedSystems, Burlingame, CA, USA). Data was analyzed using FlowJo software (Tree Star, Inc., Ashland, OR, USA).

### *Measurement of caspase-3 activity*

The activity of caspase-3 was measured using the colorimetric assay kit according to the manufacturer's instruction (CASP3C, Sigma-Aldrich, MO, USA). Briefly, after relevant treatment, PC-12 cells were harvested and mixed with buffer in assay kit. The absorbance at 405 nm was recorded by a micro-plate reader (ELx800, Bio-Tek Inc., MO, USA).

### *Intracellular reactive oxygen species (ROS) and calcium (Ca<sup>2+</sup>) concentrations determination*

The concentration of intracellular ROS was detected using 2',7'-dichlorofluorescein diacetate (DCFH-DA, D6883, Sigma-Aldrich, MO, USA) staining and flow cytometer analysis. After relevant treatment, PC-12 cells were collected and washed with phosphate buffered saline (PBS) for three times. Then cells were stained with 10 µM DCFH-DA for 20 min at 37°C in the dark. After that, cells were washed with PBS for three times and the fluorescence was detected using flow cytometer (Bender MedSystems, Burlingame, CA, USA). Data was presented as the mean of the intensity of the recorded fluorescence (NRFU, U/cell).

The levels of intracellular Ca<sup>2+</sup> were detected using fluorescence Ca<sup>2+</sup> indicator Fluo-3-AM (46393, Sigma-Aldrich, MO, USA). After relevant treatment, PC-12 cells were collected and washed with PBS for three times. Then cells were stained with Fluo-3-AM (5 µg/ml) for 60 min at 37°C in the dark. After staining, cells were washed with PBS for three times and the fluorescence was detected using flow cytometer (Bender MedSystems, Burlingame, CA, USA). Ca<sup>2+</sup> levels in cells were expressed as NRFU (U/cell).

### *Quantitative reverse transcription PCR (qRT-PCR)*

Total RNAs in PC-12 cells were isolated using Trizol reagent (Life Technologies, CA, USA). Reverse transcription was performed using the Multiplex One-Step RT-PCR kit (4442135,

Thermo Fisher Scientific, MA, USA). The reverse transcription conditions were 10 min at 25°C, 30 min at 48°C, and a final step of 5 min at 95°C.

### *Mitochondrial membrane potential (MMP) measurement*

MMP of PC-12 cells were detected by JC-10 Mitochondrial Membrane potential kit (MAK-160, Sigma-Aldrich, MO, USA) following the manufacturer's instruction. After relevant treatment, PC-12 cells were collected and washed with PBS for three times. Then 50 µl of JC-10 staining solution were incubated with 500 µl cells for 25 min at 37°C in a CO<sub>2</sub> incubator and measured using flow cytometer (Bender MedSystems, Burlingame, CA, USA).

### *Isolation of mitochondria*

Mitochondria in PC-12 cells were isolated as reported previously [16]. Briefly, PC-12 cells were washed with PBS, mixed with a lysis buffer and centrifuged at 750 g for 10 min at 4°C. The pellets containing the unbroken cells and nuclei were discarded and the supernatant was further centrifuged at 15000 g for 15 min. Then the pellets fraction containing mitochondrial was further mixed with PBS supplement with 0.5% Triton X-100 for 10 min at 4°C. After centrifuged at 16000 g for 10 min, the supernatant was collected as mitochondria fraction.

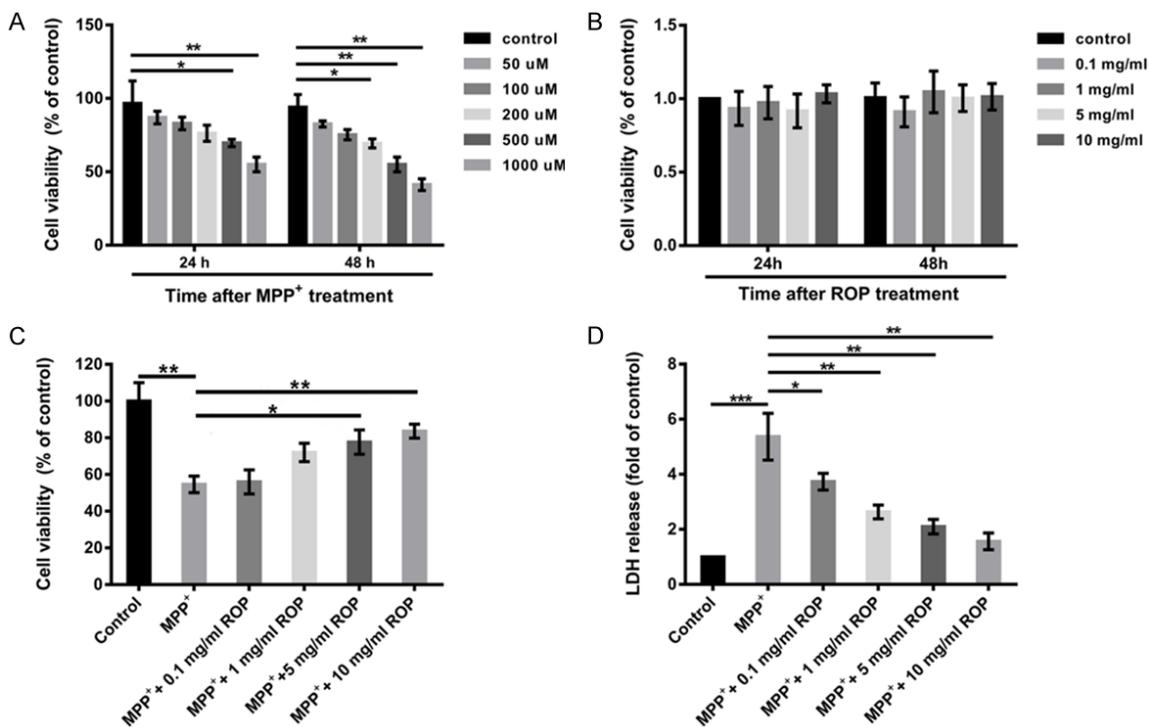
### *Mitochondrial ATP synthesis measurement*

Mitochondrial ATP synthesis was measured using a luciferase/luciferin-based system as previous described [17]. Briefly, 30 µg mitochondrial pellets were mixed with 100 µl buffer A (0.1 nM MgCl<sub>2</sub>, 150 nM KCl, 25 nM Tris-HCl, 2 nM potassium phosphate, PH 7.4), 1 nM malate, 1 nM glutamate, 0.1% BSA, and buffer B (containing 20 mg/ml luciferase and 0.8 nM luciferin in 0.5 M Tris-acetate PH 7.75). The reaction was initiated by addition of 0.1 mM ADP and monitored by a micro-plate reader (ELx800, Bio-Tek Inc.,) for 60 min.

### *Determination of mitochondrial swelling*

Isolated mitochondria were mixed with swelling buffer (containing 10 mM Tris MOPS, 1 mM phosphate, 5 mM succinate, 0.2 mM sucrose, 2 µM rotenone and 1 µM EGTA-Tris, PH 7.4) and 200 µM CaCl<sub>2</sub>. The swelling of mitochondria

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**Figure 1.** ROP reversed the MPP<sup>+</sup>-induced PC-12 cell injury. A and B: Cell viability was measured by CellTiter 96 AQueous One Solution Cell Proliferation assay at 24 or 48 h after MPP<sup>+</sup> (50, 100, 200, 500 or 1000 µM) or ROP (0.1, 1, 5 or 10 mg/ml) treatment. C: Cell viability was measured by CellTiter 96 AQueous One Solution Cell Proliferation assay at 24 h after MPP<sup>+</sup> (500 µM) co-incubation with ROP (0, 0.1, 1, 5 or 10 mg/ml). D: LDH release was detected at 24 h after MPP<sup>+</sup> (500 µM) co-incubation with ROP (0, 0.1, 1, 5 or 10 mg/ml). All data are expressed as means ± SD of triplicates experiments. MPP<sup>+</sup>: 1-Methyl-4-phenylpyridine; ROP: *Radix Ophiopogonis* polysaccharide extracts; LDH: Lactate dehydrogenase. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001.

was measured using an absorbance decrease at 540 nm by micro-plate reader (ELx800, Bio-Tek Inc., MO, USA).

### Western blotting assay

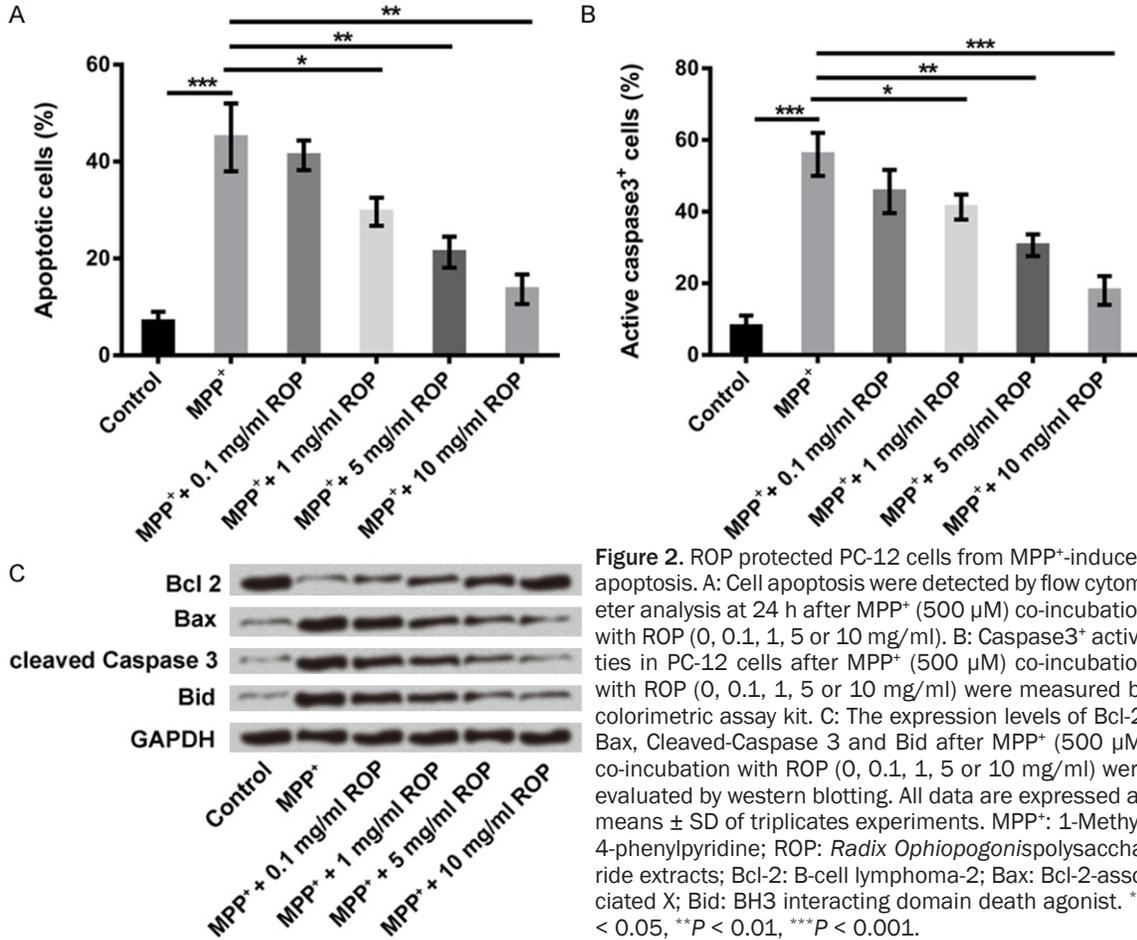
The protein used for western blotting assay was extracted using RIA lysis buffer (Beyotime Biotechnology, Shanghai, China) and quantified by BCA<sup>TM</sup> protein assay kit (Pierce, Appleton, WI, USA). Western blotting assay was established using a Bio-Rad Bis-Tris Gel system (Bio-Rad, Shanghai, China) according to the manufacturer's protocol. The following antibodies were used: B-cell lymphoma-2 (Bcl-2, ab196495), Bcl-2-associated X (Bax, ab32503), Cleaved-Caspase-3 (ab2302), BH3 interacting domain death agonist (Bid, ab191024), Cytochrome C (ab13575), Tubulin (ab7291), Cox V (ab110262), Notch-regulated ankyrin repeat protein (NRARP, ab90859), Hairy/enhancer-of-split related with YRPW motif 1 (HEY1, ab154077), Hairy/enhancer-of-split homologues 6 (HES6, ab218037),

Hairy/enhancer-of-split homologues 1 (HES1, ab108937), GAPDH (ab181602) (Abcam Biotechnology, MA, USA), and Notch 2 (5732, Cell Signaling Technology, MA, USA). After that, the membrane carried blots and antibodies were incubated with horseradish peroxidase-conjugated secondary antibodies (ab6721, ab6788, Abcam Biotechnology) and transferred into Bio-Rad ChemiDoc<sup>TM</sup> XRS system following with the 200 µl Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA, USA) added. The protein signals were captured using Image Lab<sup>TM</sup> Software (Bio-Rad).

### Statistical analysis

All above experiments were repeated three times and data were expressed as means ± standard deviation (SD). Statistical analysis was performed by Graphpad 6.0 statistical software (Graphpad, San Diego, CA, USA). One-way analysis of variance (ANOVA) was utilized for calculating *P*-values. *P* < 0.05 was considered to be significant difference.

## Roles of ROP in MPP<sup>+</sup>-induced PC-12 cell injury



**Figure 2.** ROP protected PC-12 cells from MPP<sup>+</sup>-induced apoptosis. A: Cell apoptosis were detected by flow cytometer analysis at 24 h after MPP<sup>+</sup> (500  $\mu$ M) co-incubation with ROP (0, 0.1, 1, 5 or 10 mg/ml). B: Caspase3<sup>+</sup> activities in PC-12 cells after MPP<sup>+</sup> (500  $\mu$ M) co-incubation with ROP (0, 0.1, 1, 5 or 10 mg/ml) were measured by colorimetric assay kit. C: The expression levels of Bcl-2, Bax, Cleaved-Caspase 3 and Bid after MPP<sup>+</sup> (500  $\mu$ M) co-incubation with ROP (0, 0.1, 1, 5 or 10 mg/ml) were evaluated by western blotting. All data are expressed as means  $\pm$  SD of triplicates experiments. MPP<sup>+</sup>: 1-Methyl-4-phenylpyridine; ROP: *Radix Ophiopogonis* polysaccharide extracts; Bcl-2: B-cell lymphoma-2; Bax: Bcl-2-associated X; Bid: BH3 interacting domain death agonist. \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001.

### Results

#### *ROP significantly reversed the MPP<sup>+</sup>-induced PC-12 cell injury*

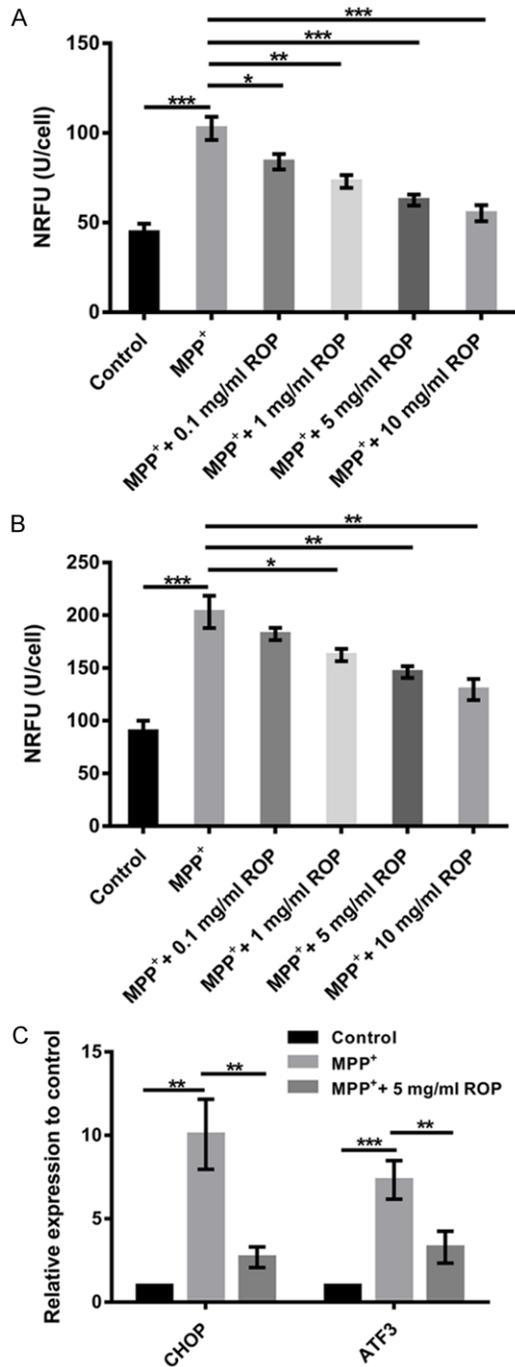
The effects of MPP<sup>+</sup> and/or ROP treatment on cell viability were detected using CellTiter 96 AQ<sub>ueous</sub> One Solution Cell Proliferation assay. **Figure 1A** showed that MPP<sup>+</sup> inhibited PC-12 cell viability in a dose- and time-dependent manner. After 500  $\mu$ M MPP<sup>+</sup> treatment for 24 h, the cell viability reduced to 69.67% ( $P$  < 0.05). After 500  $\mu$ M MPP<sup>+</sup> treatment for 48 h, the cell viability reduced to 55.24% ( $P$  < 0.01). ROP treatment exhibited no significant cytotoxicity to PC-12 cells (**Figure 1B**). When co-treatment with MPP<sup>+</sup> (500  $\mu$ M) and ROP (0.1, 1, 5 or 10 mg/ml) simultaneously, ROP significantly reversed the MPP<sup>+</sup>-induced the PC-12 cell viability loss in a dose-dependent manner (**Figure 1C**,  $P$  < 0.05 or  $P$  < 0.01). In addition, LDH release assay showed that ROP, even in a low dose, remarkably attenuated the MPP<sup>+</sup>-induced LDH

release increase in PC-12 cells (**Figure 1D**,  $P$  < 0.05 or  $P$  < 0.01). These results suggested that ROP reversed the PC-12 cell injury induced by MPP<sup>+</sup>.

#### *ROP protected PC-12 cells from MPP<sup>+</sup>-induced apoptosis*

The effects of MPP<sup>+</sup> and/or ROP treatment on PC-12 cell apoptosis were measured using flow cytometer analysis and western blotting. As shown in **Figure 2A**, MPP<sup>+</sup> treatment obviously enhanced the rate of apoptosis cells ( $P$  < 0.001). After co-incubation with ROP, ROP significantly reduced the cell apoptosis induced by MPP<sup>+</sup> ( $P$  < 0.05 or  $P$  < 0.01). Moreover, ROP remarkably reversed the MPP<sup>+</sup>-induced Caspase 3 activation in PC-12 cells in a dose-dependent manner (**Figure 2B**,  $P$  < 0.05,  $P$  < 0.01 or  $P$  < 0.001). Western blotting assay displayed that the expression levels of Cleaved-Caspase 3, Bax and Bid were all increased after single MPP<sup>+</sup> treatment but decreased

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**Figure 3.** ROP attenuated the MPP<sup>+</sup>-induced oxidative stress and ER stress in PC-12 cells. **A:** The concentrations of intracellular ROS after MPP<sup>+</sup> (500  $\mu$ M) co-incubation with ROP (0, 0.1, 1, 5 or 10 mg/ml) were detected by DCFH-DA staining and flow cytometer analysis. **B:** The levels of Ca<sup>2+</sup> after MPP<sup>+</sup> (500  $\mu$ M) co-incubation with ROP (0, 0.1, 1, 5 or 10 mg/ml) were measured by Fluo-3-AM staining and flow cytometer analysis. **C:** qRT-PCR was performed to evaluate the mRNA levels of CHOP and ATF3 in PC-12 cells after MPP<sup>+</sup> (500  $\mu$ M) co-incubation with ROP (0 or 5 mg/ml). All data are expressed as means  $\pm$  SD of triplicates experiments. MPP<sup>+</sup>: 1-Methyl-4-phe-

nylpyridine; ROP: *Radix Ophiopogonis* polysaccharide extracts; NRFU: The intensity of the recorded fluorescence; CHOP: CCAAT/enhancer-binding protein-homologous protein; ATF: Activating transcription factor 3; ER: Endoplasmic reticulum; ROS: Reactive oxygen species; DCFH-DA: 2',7'-dichlorofluorescein diacetate. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

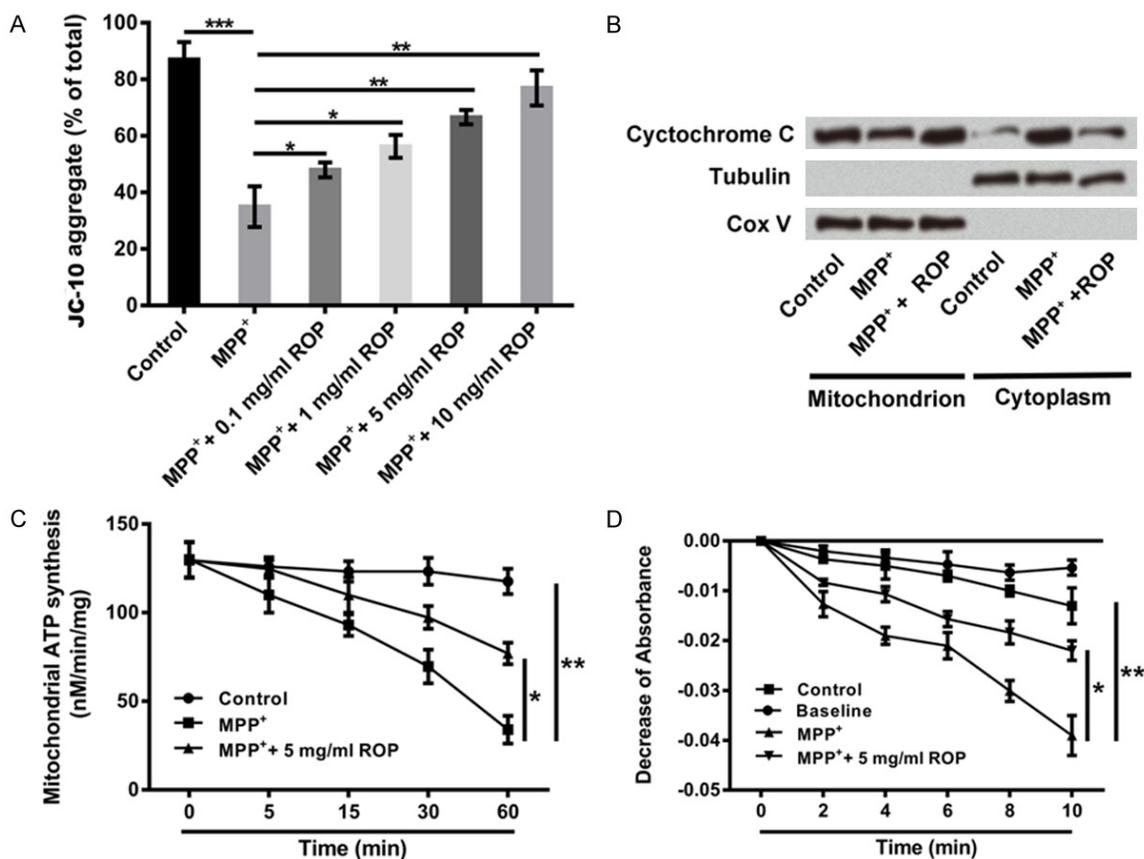
after co-treatment with ROP and MPP<sup>+</sup> (**Figure 2C**). In addition, the expression level of Bcl-2 was decreased in single MPP<sup>+</sup> group but increased in MPP co-treated ROP group. These results suggested that MPP<sup>+</sup> induced PC-12 cell apoptosis, but ROP protected PC-12 cell from MPP<sup>+</sup>-induced apoptosis.

### *ROP attenuated the MPP<sup>+</sup>-induced oxidative stress and endoplasmic reticulum (ER) stress in PC-12 cells*

The concentrations of intracellular ROS and Ca<sup>2+</sup> in PC-12 cells were detected by fluorescent staining and flow cytometer analysis. MPP<sup>+</sup> treatment remarkably increased the oxidative stress and ER stress in PC-12 cells by enhancement the concentrations of ROS and Ca<sup>2+</sup> (**Figure 3A** and **3B**,  $P < 0.001$ ). After co-treatment with ROP, the oxidative stress and ER stress were significantly decreased ( $P < 0.05$ ,  $P < 0.01$  or  $P < 0.001$ ). The mRNA expression levels of CCAAT/enhancer-binding protein-homologous protein (CHOP) and activating transcription factor 3 (ATF3), which involved in intracellular ER stress response [18], were all increased obviously after single MPP<sup>+</sup> treatment but remarkably decreased after MPP<sup>+</sup> + 5 mg/ml ROP treatment (**Figure 3C**,  $P < 0.01$  or  $P < 0.001$ ). These results suggested that ROP attenuated the MPP<sup>+</sup>-induced the increase of oxidative stress and ES stress in PC-12 cells.

### *ROP reversed MPP<sup>+</sup>-induced mitochondrial dysfunction*

To explore whether mitochondrial dysfunction participated in the MPP<sup>+</sup>-induced cell cytotoxicity, the MMP, Cytochrome C release, ATP synthesis and mitochondrial swelling were detected. As shown in **Figure 4A**, MPP<sup>+</sup> significantly decreased the MMP in PC-12 cells by reducing JC-10 aggregation ( $P < 0.001$ ). After co-treatment with different doses of ROP, the MMPs were remarkably increased accompanying with the JC-10 aggregation increase ( $P < 0.05$  or  $P < 0.01$ ). Moreover, ROP co-treatment inhibited the MPP<sup>+</sup>-induced Cytochrome C releasing from



**Figure 4.** ROP reversed MPP<sup>+</sup>-induced mitochondrial dysfunction. A: The changes of MMP after MPP<sup>+</sup> (500  $\mu$ M) co-incubation with ROP (0, 0.1, 1, 5 or 10 mg/ml) were detected by flow cytometer analysis. B: The expression levels of Cytochrome C in mitochondrion and cytoplasm after MPP<sup>+</sup> (500  $\mu$ M) co-incubation with ROP (0 or 5 mg/ml) were evaluated by western blotting. C: The abilities of mitochondrial on ATP synthesis after MPP<sup>+</sup> (500  $\mu$ M) co-incubation with ROP (0 or 5 mg/ml) were measured. D: The swelling of mitochondrial after MPP<sup>+</sup> (500  $\mu$ M) co-incubation with ROP (0 or 5 mg/ml) was detected. All data are expressed as means  $\pm$  SD of triplicates experiments. MPP<sup>+</sup>: 1-Methyl-4-phenylpyridine; ROP: *Radix Ophiopogonis* polysaccharide extracts; MMP: Mitochondrial membrane potential. \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001.

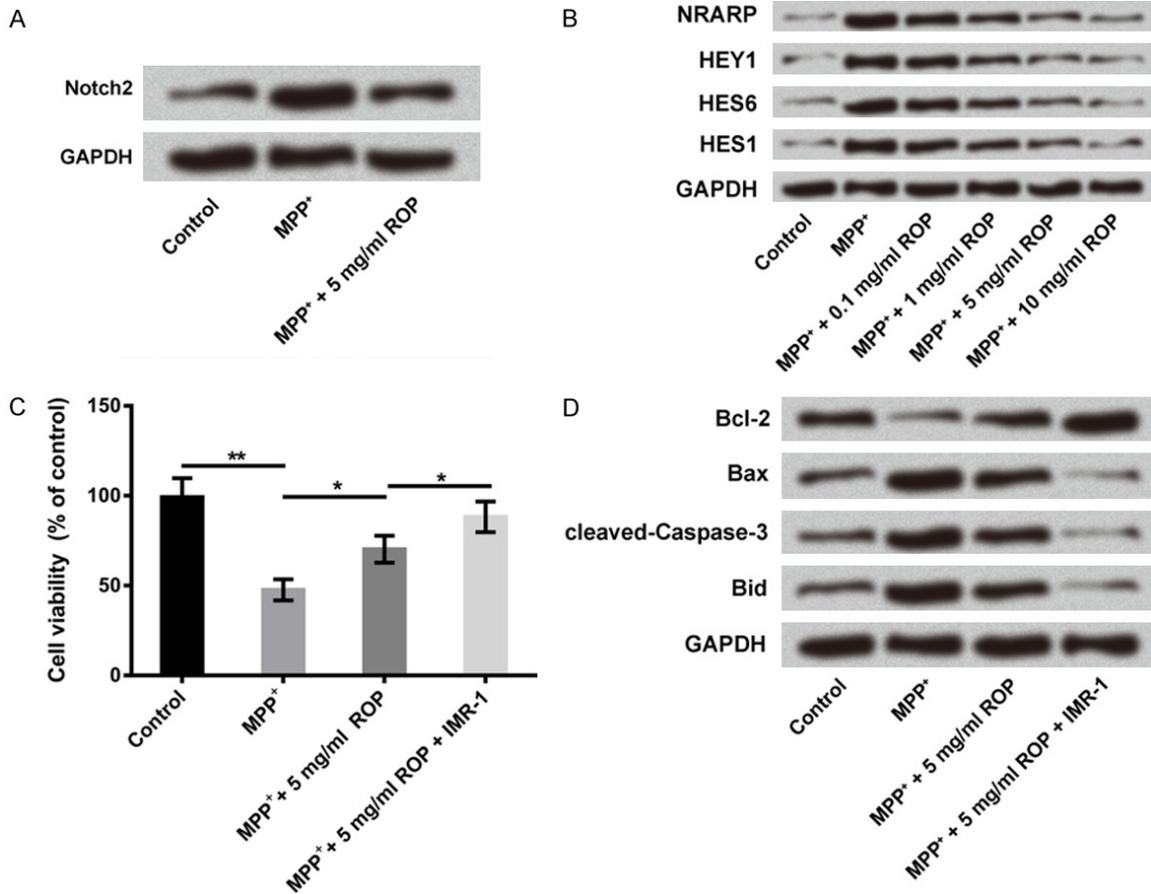
mitochondrion to cytoplasm (Figure 4B). MPP<sup>+</sup> markedly inhibited mitochondrial ATP synthesis and induced mitochondrial swelling (Figure 4C and 4D,  $P$  < 0.01). ROP significantly reversed the effects of MPP<sup>+</sup> treatment on mitochondrial ATP synthesis and mitochondrial swelling ( $P$  < 0.05 or  $P$  < 0.01). These results implied that ROP significantly reversed MPP<sup>+</sup>-induced mitochondrial dysfunction.

#### *Notch signaling pathway participated in the protective effects of ROP on PC-12 cell injury*

The effects of MPP<sup>+</sup> and/or ROP treatment on the Notch signaling pathway in PC-12 cells were detected by western blotting. As shown in Figure 5A and 5B, MPP<sup>+</sup> treatment enhanced the expression levels of Notch 2 and their downstream protein NRARP, HEY1, HES6, HES1

in PC-12 cells. After MPP<sup>+</sup> co-incubated with different doses of ROP, the protein levels of Notch 2, NRARP, HEY1, HES6 and HES1 were all decreased. Inhibitor of Mastermind Recruitment-1 (IMR-1, SML1812, Sigma-Aldrich, MO, USA), an inhibitor of Notch transcription activation complex [19], was used to diminish the expression of Notch and downstream protein in PC-12 cells. Figure 5C showed that compared to MPP<sup>+</sup> + 5 mg/ml ROP treatment group, the cell viability after MPP<sup>+</sup> + 5 mg/ml ROP + IMR-1 co-treatment was effectively increased ( $P$  < 0.05). Moreover, IMR-1 co-treatment with MPP<sup>+</sup> + ROP enhanced the expression level of Bcl-2, but decreased the expression levels of Bax, Cleaved-Caspase 3, Bid, compared to single MPP<sup>+</sup> + ROP treated group (Figure 5D). These results implied that the Notch signaling path-

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**Figure 5.** Notch signaling pathway was involved in the protective effects of ROP on PC-12 cell injury. A: The expression levels of Notch 2 in PC-12 cells after MPP<sup>+</sup> (500  $\mu$ M) co-incubation with ROP (0 or 5 mg/ml) were detected by western blotting. B: The expression levels of NRARP, HEY1, HES6 and HES1 in PC-12 cells after MPP<sup>+</sup> (500  $\mu$ M) co-incubation with ROP (0 or 5 mg/ml) were evaluated by western blotting. C: Cell viability was measured by CellTiter 96 AQ<sub>ueous</sub> One Solution Cell Proliferation assay after added IMR-1 to culture medium simultaneously with MPP<sup>+</sup> (500  $\mu$ M) and ROP (0 or 5 mg/ml). D: The expression levels of Bcl-2, Bax, Cleaved-Caspase 3, Bid in PC-12 cells after added IMR-1 to culture medium simultaneously with MPP<sup>+</sup> (500  $\mu$ M) and ROP (0 or 5 mg/ml) were measured by western blotting. All data are expressed as means  $\pm$  SD of triplicates experiments. MPP<sup>+</sup>: 1-Methyl-4-phenylpyridine; ROP: *Radix Ophiopogonis* polysaccharide extracts; NRARP: Notch-regulated ankyrin repeat protein; HEY1: Hairy/enhancer-of-split related with YRPW motif 1; HES6: Hairy/enhancer-of-split homologues 6; HES1: Hairy/enhancer-of-split homologues 1; Bcl-2: B-cell lymphoma-2; Bax: Bcl-2-associated X; Bid: BH3 interacting domain death agonist. \* $P < 0.05$ , \*\* $P < 0.01$ .

way was involved in the protective effects of ROP on MPP<sup>+</sup>-induced PC-12 cell injury.

*The protective effects of ROP on MPP<sup>+</sup>-induced PC-12 cell injury were depended on the time of ROP incubation*

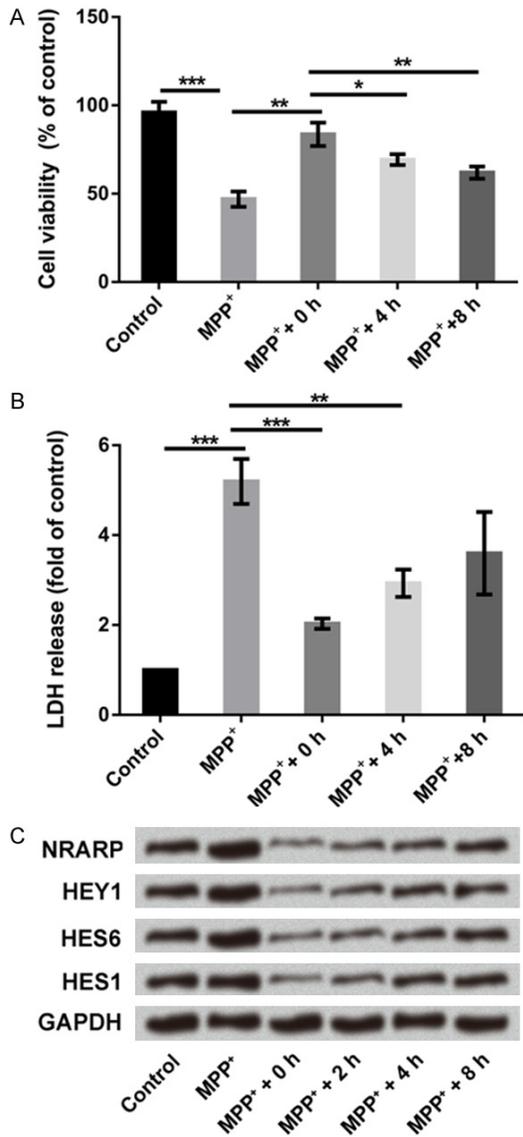
We further investigated the protective effects of ROP on MPP<sup>+</sup>-induced PC-12 cell injury when ROP added to culture medium after MPP<sup>+</sup> treatment different times. CellTiter 96 AQ<sub>ueous</sub> One Solution Cell Proliferation assay and LDH release assay presented that the cell viability decreased with the delay of ROP co-treatment, which suggested that the protective effects of

ROP on MPP<sup>+</sup>-induced PC-12 cell injury were depended on the time of ROP incubation (**Figure 6A** and **6B**,  $P < 0.05$ ,  $P < 0.01$  or  $P < 0.001$ ). These results were further confirmed by western blotting assay, which pointed out that the inactivation effects of ROP on the expression levels of NRARP, HEY1, HES6 and HES1 in PC-12 cells were decreased with the delay of ROP co-treatment (**Figure 6C**).

### Discussion

PD is a chronic and degenerative disease of central nervous system [1, 20]. *Radix Ophiopogonis* has been widely used in TCM. The poly-

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**Figure 6.** The protective effects of ROP on MPP<sup>+</sup>-induced PC-12 cell injury were depended on the time of ROP incubation. A: The protective effects of ROP on cell viability after MPP<sup>+</sup> treatment for 0, 4, or 8 h were detected by CellTiter 96 AQueous One Solution Cell Proliferation assay. B: The protective effects of ROP on LDH release after MPP<sup>+</sup> treatment for 0, 4, or 8 h were measured. C: The activation effects of ROP on the expression levels of NRARP, HEY1, HES6 and HES1 after MPP<sup>+</sup> treatment for 0, 4, or 8 h were evaluated by western blotting. All data are expressed as means  $\pm$  SD of triplicates experiments. MPP<sup>+</sup>: 1-Methyl-4-phenylpyridine; ROP: *Radix Ophiopogonis* polysaccharide extracts; LDH: Lactate dehydrogenase; NRARP: Notch-regulated ankyrin repeat protein; HEY1: Hairy/enhancer-of-split related with YRPW motif 1; HES6: Hairy/enhancer-of-split homologues 6; HES1: Hairy/enhancer-of-split homologues 1. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

saccharide extracts of *Radix Ophiopogonis* (ROP) have a widely bioactivities for treatment diabetes, inflammatory, myocardial ischemia/reperfusion injury and so on [6, 8, 21]. In this study, we investigated the protective roles of ROP in the MPP<sup>+</sup>-induced PD cell model (PC-12 cell injury). The potential signaling pathways participated in the protective process were also detected. Our results found that MPP<sup>+</sup> treatment significantly induced PC-12 cell injury as evidenced by the cell viability loss and cell apoptosis enhancement. MPP<sup>+</sup> markedly increased the concentrations of ROS and Ca<sup>2+</sup> and the mitochondrial dysfunction in PC-12 cells. ROP co-incubation remarkably reversed the MPP<sup>+</sup>-induced PC-12 cell viability loss, apoptosis increase, oxidative stress and ER stress rise, and mitochondrial dysfunction in PC-12 cells. In addition, Notch signaling pathway was involved in the protective effects of ROP on PC-12 cell injury induced by MPP<sup>+</sup>.

Oxidative stress and ES stress have been found to be increased in the patients with PD [22, 23]. The increased of oxidative stress and ES stress induces irreversible damage to cells, and resulting in cell death ultimately [24, 25]. ER is the repository of Ca<sup>2+</sup> in eukaryotic cells [26]. CHOP is a transcription factor which participates in the ER stress induced cellular death by regulating the downstream ATF3 expression [27]. In this research, MPP<sup>+</sup> treatment remarkably increased the concentrations of ROS and Ca<sup>2+</sup> in PC-12 cells, led to the enhancement of oxidative stress and ES stress. The mRNA expression levels of CHOP and ATF3 were also increased after MPP<sup>+</sup> treatment. ROP co-incubation obviously reduced the concentrations of intracellular ROS and Ca<sup>2+</sup> and the mRNA expression levels of CHOP and ATF3 induced by MPP<sup>+</sup> in PC-12 cells, which suggested that the protective effects of ROP on MPP<sup>+</sup>-induced cell injury might be through decreasing the intracellular oxidative stress and ER stress.

Mitochondrial electron transport chain is the major source of ROS in eukaryotic cells [28]. Mitochondrial dysfunction will enhance the level of ROS in cells, resulting in the increase of oxidative stress [29]. In this study, we found that MPP<sup>+</sup> treatment obviously decreased the MMP and mitochondrial ATP synthesis and increased the expression level of Cytochrome C in cyto-

plasm of PC-12 cells. In addition, the mitochondrial swelling was also increased after MPP<sup>+</sup> treatment. These results suggested that MPP<sup>+</sup> treatment markedly induced the mitochondrial dysfunction in PC-12 cells, and then led to oxidative stress. Whereas ROP co-incubation significantly attenuated the effects of MPP<sup>+</sup> on MMP, cytochrome C, mitochondrial ATP synthesis and mitochondrial swelling, which presented a protective roles in mitochondrial function.

To further understand the protective effects of ROP on MPP<sup>+</sup>-induced PC-12 cell injury, the activities of Notch signaling pathway after MPP<sup>+</sup> and/or ROP treatment was detected in this study. Notch signaling pathway was involved in the regulation of the communication between adjacent cells, intracellular signal transduction and gene transcription [9, 30]. Moreover, Notch signaling pathway has been found to be played a regulator role in cellular oxidative stress injury and participated in the development of the nervous system [10, 30]. In this study, we indicated that MPP<sup>+</sup> treatment triggered up-regulation of the expression levels of Notch 2 and the downstream molecular NRARP, HEY1, HES6, HES1, which resulted in the activation of Notch 2 signaling pathway. ROP co-treatment reversed the effects of MPP<sup>+</sup> on Notch 2 signaling pathway in a dose-dependent manner. Moreover, after co-incubation with IMR-1, an inhibitor of Notch signaling pathway, the cell viability was significantly increased accompanied with the apoptosis decreased, which further implied that Notch signaling pathway was involved in the protective effects of ROP on PC-12 cell injury.

At last, we also analyzed the protective effects of ROP after the MPP<sup>+</sup>-induced PC-12 cell injury. Results showed that the cell viability obviously decreased with the delay of ROP co-treatment. Similar results were found in the effects of ROP co-treatment on Notch signaling pathway. These above results presented that the protective effects of ROP on MPP<sup>+</sup>-induced PC-12 cell injury were depended on the time of ROP incubation.

To concluded, in this study, we verified ROP effectively protected PC-12 cells from the MPP<sup>+</sup>-induced injury through inhibition of Notch signaling pathway. We propose that ROP could be an efficient protective and therapeutic drug for PD. Further *in vivo* studies on animal model are still needed to support this proposal.

### Disclosure of conflict of interest

None.

### Abbreviations

PD, Parkinson's disease; DA, Dopaminergic; ROS, Reactive oxygen species; TCM, Traditional Chinese Medicine; ROP, *Radix Ophiopogonis* polysaccharide extracts; MPP<sup>+</sup>, 1-Methyl-4-phenylpyridine; LDH, Lactate dehydrogenase; Ca<sup>2+</sup>, Calcium; PBS, Phosphate buffered saline; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X; Bid, BH3 interacting domain death agonist; NRARP, Notch-regulated ankyrin repeat protein; HEY1, Hairy/enhancer-of-split related with YRPW motif 1; HES6, Hairy/enhancer-of-split homologues 6; HES1, Hairy/enhancer-of-split homologues 1; CHOP, CCAAT/enhancer-binding protein-homologous protein; ATF3, Activating transcription factor 3; ER, Endoplasmic reticulum; MMP, Mitochondrial membrane potential; IMR-1, Inhibitor of Mastermind Recruitment-1.

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