Myricetin Attenuates MPP^+-induced Cytotoxicity in MES23.5 Cells *

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ABSTRACT Objective: To investigate the neuroprotective effect of myricetin on 1-methyl-4-phenylpyridinium (MPP^+)-treated MES23.5 cells. Methods: MES23.5 cells were treated with vehicle, MPP^+, MPP^+ and myricetin for 24h respectively. By Hoechst staining and RT-PCR, the nuclear morphological changes and the Bcl-2 and Bax mRNA levels were investigated. Results: The results showed that myricetin treatment significantly attenuate MPP^+-induced cell apoptosis. MPP^+ treatment induced decrease of Bcl-2/Bax mRNA ratio was also inhibited by myricetin. Conclusion: Myricetin may protect against MPP^+-induced cytotoxicity in MES23.5 cells.

Key words: Myricetin; MPP^+; Apoptosis; MES23.5 cells

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Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by selectively loss of dopaminergic neurons in the substantia nigra (SN), giving rise to dopamine (DA) depletion in the striatum. Although the precise pathogenic mechanism leading to neurodegeneration in PD is unknown, a number of factors have been implicated in the pathogenesis of DA neuron loss. The generation of reactive oxygen species (ROS), mitochondrial dysfunction [1-5], excitotoxicity[5,6] and inflammation [7, 8] are all considered important mediators of neuronal death in PD. It is more likely that multiple factors converge to give rise to PD than any single reason.

Myricetin is one of the flavoniods. It has many biological functions, including anti-oxidation, anti-apoptosis, free radical scavenging and anti-inflammation [9]. Recently, several studies demonstrated the neuroprotective effects of myricetin. It has been shown that myricetin protect against rotenone-induced cytotoxicity in SH-SY5Y cells [10], Myricetin also inhibits 6-hydroxydopamine-induced neurotoxicity in rats [11]. The aim of the present study was to inestigate the effect of myricetin on MPP^+-induced neurotoxicity and the underlying mechanisms in MES23.5 cells.

1 Materials and Methods

1.1 Materials

Unless otherwise stated, all chemicals including myricetin were purchased from Sigma Chemical Co. The purity of myricetin used is 95%. Dulbecco's modified Eagle's medium Nutrient Mixture-F12 (DMEM/F12) was from Invitrogen. Hoechst 33258 was from Beyotime (Jiangsu, China) .

1.2 Cell culture

MES23.5 cells were cultured in DMEM/F12 containing Sato's components growth medium supplemented with 5% fetal bovine serum, 100 U/mL of penicillin and 100 mg/mL of streptomycin at 37℃, in a humid 5% CO_2 and 95% air environment., Cells were seeded at a density of $1\times10^5/cm^2$ in the plastic flasks.

1.3 Hoechst 33258 staining

Nuclear morphology was detected by the method previously described in our lab [12, 13]. MES23.5 cells were seeded on sterile cover glasses in 24-well plates and treated with vehicle, MPP^+, MPP^+ and myricetin for 24h respectively. The cells were fixed in 4% paraformaldehyde for 30 min, washed in phosphate-buffered saline, and stained with Hoechst 33258 dye for 30 min at room temperature. After washing 3 times to remove the excessive dye, the cells were examined and photographed under a fluorescence microscope (Olympus, Japan) with an excitation wavelength of 330 380 nm. Apoptotic cells were defined on the basis of nuclear morphological changes, such as chromatin condensation and fragmentation. The total number of condensed cells was counted manually by researchers blinded to the treatment schedule using unbiased stereology. For each well, a 400μm^2 frame was delineated and all condensed and normal nuclei were counted in at least 10 different fields. Average sum of condensed and normal nuclei was calculated per well. The data were presented as the percentage of condensed nuclear number to the total number.

1.4 Reverse transcription-polymerase chain reaction (RT-PCR)

MES23.5 cells were incubated in vehicle, MPP^+, myricetin, MPP^+ and myricetin for 24 h respectively, Total RNA was isolated by Trizol Reagent (Invitrogen). 5μg of total RNA was reversed transcribed in a 20μl reaction using the AMV reverse transcription system (Promega Corporation, Madison, WI, USA). Bcl-2 cDNA

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fragment (amplified products were 409 bp length) was amplified with the primers (forward: 5'- GTC CCG CCT CTT CAC CTT -3'; reverse: 5'- CCC ACT CGT AGC CCC TCT -3'), Bax cDNA fragment (amplified products were 307 bp length) was amplified with (forward: 5'- GGC GAA TTG GAG ATG AAC -3'; reverse: 5'- CCG AAG TAG GAG AGG AGG -3') and GAPDH cDNA fragment (amplified products were 236 bp length) was amplified with (forward: 5'- TTC ACC ACC ATG GAG AAG GC -3'; reverse 5'- GGC ATG GAC TGT GGT CAT GA -3'). The expression of house-keeping gene, GAPDH mRNA, was used as an internal standard. PCRs were run for 36 cycles in an Eppendorf Mastercycler. Denaturing, annealing, and extension reactions were performed at 94℃ for 30 s, 51 ℃ for 30 s, and 72 ℃ for 45 s. The PCR products were electrophoresed in 1% agarose gel, stained with ethidium bromide, and detected by UV irradiation. The levels of Bcl-2 and Bax mRNA were expressed as their respective ratios to GAPDH mRNA.

1.5 Statistical analysis

Data were expressed as mean ± S.E.M. and analyzed by the SPSS11.5 software. One-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test was used to compare the differences between means. A probability value of less than 0.05 was considered to be statistically significant.

2 Results

2.1 Myricetin attenuated MPP$^+$−induced cell apoptosis indicated by Hoechst 33258

Morphological changes of the cells were observed by Hoechst 33258 staining. As shown in Fig.1, 200μmol/L MPP$^+$ treatment caused nuclear condensation in MES23.5 cells, 10^{-9} mol/L myricetin treatment significantly attenuated this effect.

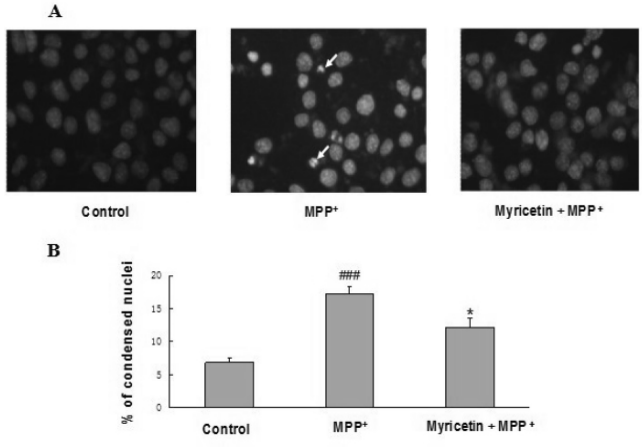

Fig.1 Morphological changes in MES23.5 cells:(A). Representative photographs of Hoechst 33258 staining from vehicle, MPP$^+$, MPP$^+$ and myricetin co-treatment groups. MPP$^+$ treatment resulted in nuclear condensation; however, 10^{-9} mol/L myricetin treatment significantly attenuated MPP$^+$-induced nuclear condensation (Magnification 400×). (B). Statistical analysis. Data were presented as means ± SEM of 6 independent experiments. ($^{###}$P<0.001, compared with the control; *P<0.05, compared with MPP$^+$ treatment group)

2.2 Myricetin increased the ratio of Bcl−2/Bax in the MPP$^+$−treated MES23.5 cells.

MPP$^+$ resulted in a markedly down-regulation of Bcl-2 mRNA and up-regulation of Bax mRNA in MES23.5 cells. Co-treatment with 10^{-9} mol/L myricetin could inhibit the decrease of Bcl-2 and increase of Bax caused by MPP$^+$. Therefore, the Bcl-2/Bax ratio in the MPP$^+$-treated cells significantly increased by myricetin (Fig.2)

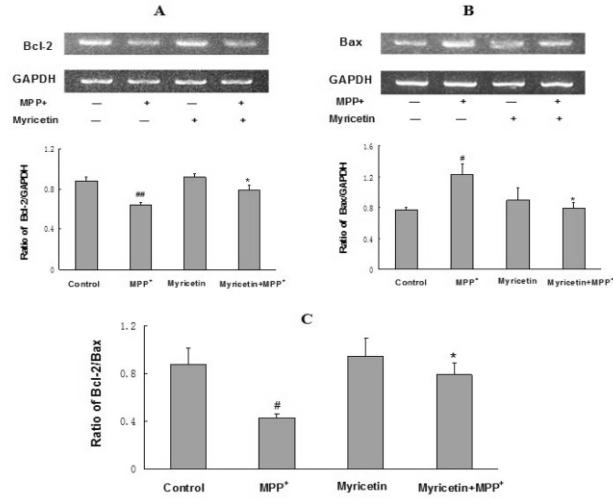

Fig. 2 Changes of Bcl-2 and Bax mRNA level in MES23.5 cells:10^{-9} mol/L myricetin treatment inhibited MPP$^+$-induced Bcl-2 mRNA level down-regulation (A), Bax mRNA level up-regulation(B) and down-regulation of Bcl-2/Bax ratio (C). Bcl-2 and Bax band intensities were normalized with GAPDH band intensity. Each value represented the means ± SEM. ($^{##}$P<0.01, $^{#}$P<0.05, compared with the control; *P<0.05, compared with MPP$^+$ treatment group)

3 Discussion

This study showed the neuroprotective effects of myricetin on MPP$^+$-treated dopaminergic MES23.5 cells. The MES23.5 cells were chosen because it exhibited several properties similar with the primary neurons originated in the SN [14]. MPP$^+$ is a common neurotoxin used as a chemical for inducing PD models. It is well known that MPP$^+$ could enter the cell through the dopamine reuptake system, and then inhibited complex I of the mitochondrial respiratory chain, and induced oxidative stress [15, 16]. The Bcl-2 family is associated with mitochondrial function during apoptosis. Bcl-2, as an anti-apoptotic member of the Bcl-2 family, can bind with Bax to form Bcl-2/Bax heterodimers, thereby attenuating the pro-apoptotic effect of Bax [17]. Mitochondria is the major site of ROS production and also prime target of oxidative molecular damage [18], the consequent formation of ROS further damages the mitochondrial membrane and such damages are implicated as key events in the pathogenic cascades leading to apoptosis. The neuroprotective effect of myricetin might be ascribing to its anti-oxidation, free radical scavenging functions. The Bcl-2 family of proteins plays an important role in intracellular apoptotic signal transduc-

tion by regulating the permeability of the mitochondrial membrane[19]. They are also involved in the regulation of cleavage and activation of effector caspases, such as caspase-3, which is the main executioner caspase inducing cell apoptosis[20]. In the present study, wedemonstrated that pro-apoptotic Bax up-regulation, and anti-apoptotic Bcl-2 down-regulation in MES23.5 cells was partially attenuated with myricetin treatment. Therefore, myricetin effectively protected MES23.5 cells from MPP^+-induced cell apoptosis by partially reversing the MPP^+-induced dysfunction of Bcl/Bax system. Our results suggest that myricetin may be a novel therapeutic strategy for the preventive and/or complementary therapies of Parkinson's disease.

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杨梅黄酮减弱 MPP^+ 诱导的 MES23.5 细胞的细胞毒性 *

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摘要 **目的**：探讨杨梅黄酮对 MPP^+ 处理的 MES23.5 细胞的保护作用。**方法**：实验分为空白对照组，MPP^+ 处理组，MPP^+ 和杨梅黄酮共处理组，分别处理 MES23.5 细胞 24h 后，应用 Hoechst 33258 染色观察各组细胞细胞核形态的改变，应用 RT-PCR 技术观察 Bcl-2 和 Bax mRNA 表达的改变。**结果**：杨梅黄酮能明显抑制 MPP^+ 引起的 MES23.5 细胞核固缩，核碎裂等形态学的改变，杨梅黄酮还可以对抗 MPP^+ 处理造成的 MES23.5 细胞的 Bcl-2/Bax 比率的降低。**结论**：杨梅黄酮对 MPP^+ 处理的 MES23.5 细胞具有保护作用。

关键词 杨梅黄酮；MPP^+；凋亡；MES23.5 cells

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