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Curcumin attenuates 6-hydroxydopamine-induced cytotoxicity by anti-oxidation and nuclear factor-kappaB modulation in MES23.5 cells

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ABSTRACT

Oxidative stress has been implicated in the degeneration of dopaminergic neurons in the substantia nigra of Parkinson's disease patients, and several anti-oxidants have been shown to be effective on the treatment of Parkinson's disease. Curcumin has been previously reported to possess radical scavenger, iron chelating, anti-inflammatory properties in different tissues. The aim of present study is to explore the cytoprotection of curcumin against 6-hydroxydopamine (6-OHDA)-induced neuronal death, as well as the underlying mechanisms in MES23.5 cells. Our results showed that 6-OHDA significantly reduced the cell viability of MES23.5 cells. Curcumin protected MES23.5 cells against 6-OHDA neurotoxicity by partially restoring the mitochondrial membrane potential, increasing the level of Cu–Zn superoxide dismutase and suppressing an increase in intracellular reactive oxygen species. Furthermore, curcumin pretreatment significantly inhibited 6-OHDA induced nuclear factor-kappaB translocation. These results suggest that the neuroprotective effects of curcumin are attributed to the antioxidative properties and the modulation of nuclear factor-kappaB translocation.

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterized by a selective loss of dopaminergic neurons in the nigrostriatal pathway, resulting in a clinical syndrome characterized by stiffness, tremor, slowness of movement, and postural instability. However, the etiology of PD is not clear up to now. The nigral vulnerability fits well with the strong oxidative stress observed in PD. Increased reactive oxygen species (ROS) generation that is caused by increased oxidative damage and reduced antioxidant level has been identified within the degenerating substantia nigra of PD patients [1,2]. Among the most important defenses against oxygen radicals are the superoxide dismutase (SOD) enzymes. SOD enzymes catalyze the breakdown of superoxide into hydrogen peroxide and water and are therefore central regulators of ROS levels [3]. Moreover, nuclear factor-kappaB (NF-κB) is thought to be a sensor of oxidative stress and is increased in dopaminergic neurons of patients with PD [4]. Some evidence have suggested that the inhibition of NF-κB activation in astrocytes might be useful in the intervention of in PD [5].

The polyphenolic flavonoid curcumin (1,7-bis[4-hydroxy 3-methoxy phenyl]-1,6-heptadiene-3,5-dione) found in turmeric is a yellow curry spice with a long history of use in traditional Indian diets and herbal medicine [6]. Curcumin (diferuloyl methane) is the principle colouring agent present in the rhizomes of *Curcuma longa* (zingiberaceae). Curcumin from *Curcuma longa* has many pharmacological activities including anti-inflammatory properties [7], powerful antioxidant activity [8], iron-chelating activity [9] and some other activities. For example, curcumin can attenuate the incidence of colon cancer and is anti-atherogenic, which are related to its antioxidant activity [7]. Curcumin has cytoprotective effect on PC12 cells against MPP^+ neurotoxicity via anti-apoptosis and antioxidative properties through the Bcl-2-mitochondria-ROS-iNOS pathway [10]. Also some research have revealed that curcumin mediates its anti-inflammatory and antioxidant effects by modulation of several important molecular targets, including transcription factors (e.g., NF-kB and AP-1), enzymes (e.g., COX-2 and iNOS), and cytokines (e.g., TNFa, IL-1b, IL-6, and chemokines) [11,12].

6-Hydroxydopamine (6-OHDA) is a common neurotoxin used as an agent for inducing PD models. We aimed to elucidate whether curcumin could protect dopaminergic cells from 6-OHDA neurotoxicity and the underlying mechanisms. MES23.5 cells was chosen because it was a dopaminergic cell line hybridized from murine neuroblastoma-glioma N18TG2 cells with rat mesencephalic neurons exhibiting several properties similar to the primary neurons originated in the substantia nigra [13]. In the present study, we evaluated the actions of curcumin on 6-OHDA treated MES23.5 cells, observed the effect of curcumin on the mitochon-

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drial membrane potential ($\Delta\Psi$m), ROS generation, Cu/Zn–SOD levels, as well as the expressions of NF-kB nuclear translocation in MES23.5 cells in order to confirm our hypothesis that the neuroprotective effect of curcumin on MES23.5 cells treated with 6-OHDA is due to its anti-oxidation properties and regulating the translocation of NF-κB.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co. (St. Louis. MO, USA). Dulbecco's modified Eagle's medium Nutrient Mixture-F12 (DMEM/F12) was from Gibco (Gibco, Grand Island, NY, USA). The anti-superoxide dismutase 1 (SOD1) antibody was purchased from the BIOS (BIOS, Beijing, China). Other chemicals and regents available were from local commercial sources.

2.2. Cell culture

MES23.5 cells were offered by Dr. Wei-dong Le (Baylor College of Medicine, TX, USA). They were cultured in DMEM/F12 containing Sato's components growth medium supplemented with 5% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/mL streptomycin at 37 °C, in a humid 5% CO_2, 95% air environment. For experiments, cells were seeded at a density of 1×10^5 cm^{-2} in the plastic flasks or on glass coverslips. Curcumin was dissolved in DMSO to the concentration of 2×10^4 μmol/L, stored at −20 °C. Then curcumin was diluted with DMEM/F12 without serum to the final concentrations when used. To study the protective effects of curcumin, cells were pretreated with curcumin or vehicle (DMSO) for 20 min and then incubated with 6-OHDA (100 μmol/L) for 24 h.

2.3. Cell viability assay

MES23.5 cells were seeded in a 96-well plate at a density of 2×10^4 cells/well. After attachment, and the cell viability was determined by the conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The MTT assay relies primarily on mitochondrial metabolic capacity of viable cells and reflects the intracellular redox state. After incubation, then cells were incubated in MTT (5 mg/ml) for 4 h. The medium was removed and 100 μL of DMSO was added to each well. The formazan dye crystals were solubilized for 10 min, and absorbance was measured by colorimetric assay (TECAN, Austria).

2.4. Detection of $\Delta\Psi m$

The changes of the mitochondrial membrane potential with various treatments in MES23.5 cells were measured by rhodamine123 using flow cytometry (Becton Dickinson, USA) as described before [14]. The uptake of rhodamine123 into mitochondria is an indicator of the $\Delta\Psi$m. After pretreated with the concentration of curcumin (10 μmol/L) for 20 min, cells were treated with 6-OHDA (final concentration 100 μmol/L) in ascorbic acid solution (final concentration 2 μg/mL) added to DMEM with serum supplement for the subsequent 24 h, and then incubated with rhodamine123 in a final concentration of 5 μmol/L for 30 min at 37 °C. After washing twice with HBS, fluorescent intensity was recorded at 488 nm excitation and 525 nm emission wavelengths (Fluorescence 1, FL1). Results were demonstrated as FL1-H (Fluorescence 1-Histogram); setting of the gated region M1 and M2 as a marker to observe the changing levels of fluorescence intensity using Cellguest Software.

2.5. ROS assay

Intracellular ROS were examined using H_2DCF-DA as described before [14]. Cells treated as described above were incubated in HBS containing H_2DCF-DA (5 μM) for 30 min at 37 °C. The fluorescent signals were measured with 488 nm excitation and 525 nm emission wavelengths (Fluorescence 1, FL1). Results were demonstrated as FL1-H (Fluorescence 1-Histogram); setting of the gated region M1 and M2 as a marker to observe the changing levels of fluorescence intensity using Cellguest Software.

2.6. Western blots analysis

After pretreatment with curcumin (10 μmol/L), cells were incubated with 6-OHDA (100 μM) for 24 h. After three washes with cold PBS, cells were digested directly on culture plates with RIPA lysis buffer (50 mmol/L Tris–HCl, 150 mmol/L NaCl, 1% Nonidet-40, 0.5% sodium deoxycholate, 1 mmol/L EDTA, 1 mmol/L PMSF) with protease inhibitors (pepstatin 1 μg/mL, aprotinin 1 μg/mL, leupeptin 1 μg/mL) for 30 min on ice and the insoluble material was removed by centrifugation (12,000 rpm, 20 min, 4 °C). The extraction and isolation of nuclear and cytoplasmic protein were performed according to the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, Jiangsu, China). First, cells were centrifuged for 5 min at 1200 rpm at 4 °C and the pellet was dissolved with cytoplasmic protein extraction agent A supplemented with PMSF. After vortex for 5 s, the tubes were incubated for 10–15 min on ice to promote lysis. Next, add the cytoplasmic protein extraction agent B, vortex for 5 s and incubated on ice for 5 s. Then the samples were centrifuged for 5 min at 14,000 × *g* at 4 °C and the supernatant, consisting of the cytosolic fraction, was immediately frozen for further analysis. The pellet was resuspended in nuclear protein extraction agent supplemented with PMSF. After vortexing the tubes 15–20 times for 30 min and centrifuging for 10 min at 14,000 × *g*, the supernatants containing the nuclear extracts were obtained. Sixty microgram total proteins were separated using 10% SDS-polyacrylamide gels and then transferred to PVDF membranes. After overnight blocking with 5% non-fat milk at 4 °C, the membranes were incubated with rabbit anti-rat SOD (1:100, BIOS, China) or NF-κB polyclonal antibody (1:200, Santa Cruz Biotechnology, USA) for 2 h at room temperature. β-Actin was detected by mouse anti-β-actin monoclonal antibody (1:10000, Sigma, Chemical Co., USA) according to similar procedures to ensure equal sample protein loading, and mouse anti-rat PCNA antibody (1:200, Santa Cruz Biotechnology, USA) was used to ensure equal sample protein loading for nuclear. Anti-rabbit and anti-mouse secondary antibodies conjugated to horseradish peroxidase were used at a dilution of 1:10000. Cross-reactivity was visualized using ECL Western blotting detection reagents and then was analyzed through scanning densitometry by Tanon Image System.

2.7. Statistical analysis

Each experiment was performed at least three times, and the results were presented as mean ± S.E.M. One-way analysis of variance (ANOVA) followed by Turkey's test was used to compare the differences between means. A probability value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effect of curcumin on MES23.5 cell viability

Using MTT assay, we detected the viability of MES23.5 cells. The viability of cells was unchanged when they were treated with

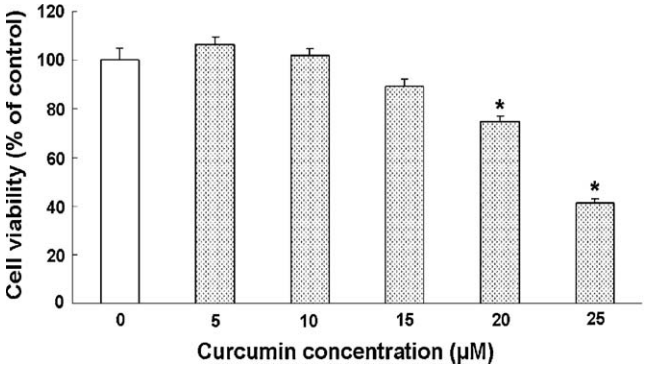

Fig. 1. MTT analysis of cell viability in curcumin treated MES23.5 cells. Cell viability of MES23.5 cells was determined by MTT assay. There was no difference in the three concentrations of curcumin (5 μmol/L, 10 μmol/L and 15 μmol/L). But the cell viability of cells treated with 20 μmol/L and 25 μmol/L curcumin was reduced compared to the control. Data were presented as mean ± S.E.M. of six independent experiments. $^{*}P < 0.01$, compared to the control.

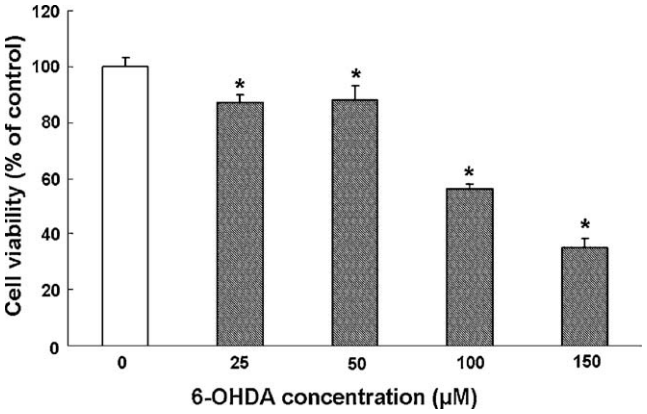

Fig. 2. MTT analysis of cell viability in 6-OHDA treated MES23.5 cells. Cell viability of MES23.5 cells was determined by MTT assay. The cell viability of cells treated with 25 μmol/L and 50 μmol/L 6-OHDA was respectively reduced by 13% and 12%, treated with 100 μmol/L 6-OHDA was reduced by 44% and 150 μmol/L 6-OHDA was reduced by 65%. Data were presented as mean ± S.E.M. of six independent experiments. $^{*}P < 0.01$, compared to the control.

curcumin up to 15 μmol/L compared to that of the control. However, a significant reduction of cell viability was observed when cells were treated with 20 μmol/L and 25 μmol/L curcumin (Fig. 1).

3.2. Effect of 6-OHDA on MES23.5 cell viability

The viability of cells treated with 25 μmol/L and 50 μmol/L 6-OHDA for 24 h reduced by 13% and 12% compared with that of the control. When treated with 100 μmol/L and 150 μmol/L 6-OHDA, cell viability reduced significantly by 44% and 65% (Fig. 2). Therefore, 100 μmol/L 6-OHDA was chosen for the following experiments.

3.3. Curcumin inhibited 6-OHDA-induced cell death in MES23.5 cells

The protective effect of curcumin against 6-OHDA-induced cell death was assessed in cultured MES23.5 cells by MTT test. As shown in Fig. 3, the viability of MES23.5 cells was dramatically reduced in 6-OHDA group, while pretreatment with 10 μmol/L and 15 μmol/L curcumin could significantly increase the cell viability compared to 6-OHDA treatment.

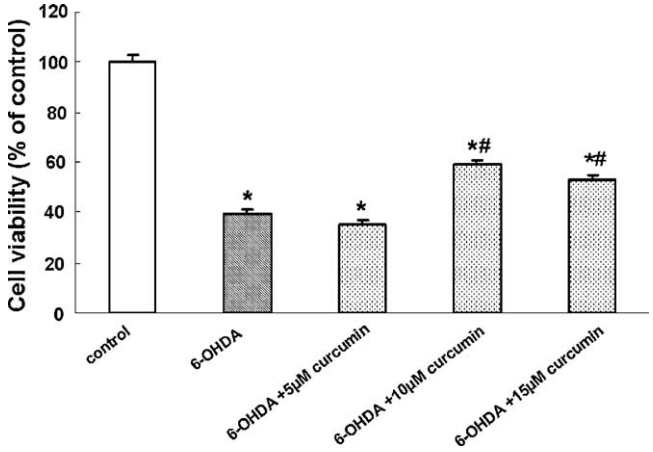

Fig. 3. Changes in cell viability in 6-OHDA treated MES23.5 cells with curcumin pretreatment. Cell viability with different doses of curcumin pretreatment was determined by MTT assay. The cell viability of cells treated with 100 μmol/L 6-OHDA was reduced compared to the control. A significant increase of cell viability was observed when cells were pretreated with 10 μmol/L curcumin for 20 min followed by 100 μmol/L 6-OHDA treatment. $^{*}P < 0.01$, compared to the control and $\#P < 0.05$, compared to 6-OHDA group.

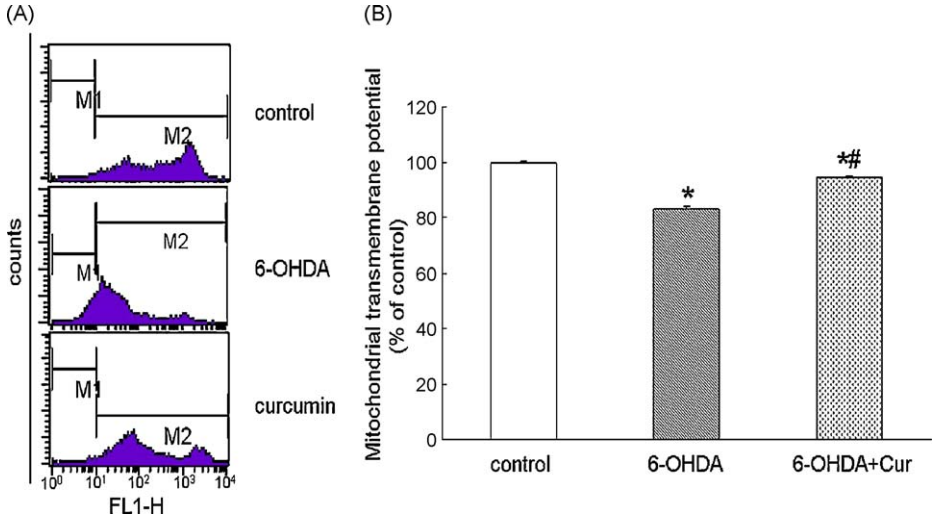

Fig. 4. ΔΨm assessed by flow cytometry in 6-OHDA treated MES23.5 cells with curcumin pretreatment. (A) Representatives of the fluorometric assay on ΔΨm of different groups. 10 μmol/L curcumin pretreatment significantly restored the ΔΨm reduction induced by 6-OHDA and (B) statistical analysis. Data were presented as mean ± S.E.M. of three independent experiments. Fluorescence values of the control were set to 100%. $^{*}P < 0.01$, compared with the control and $^{\#}P < 0.01$, compared to 6-OHDA group.

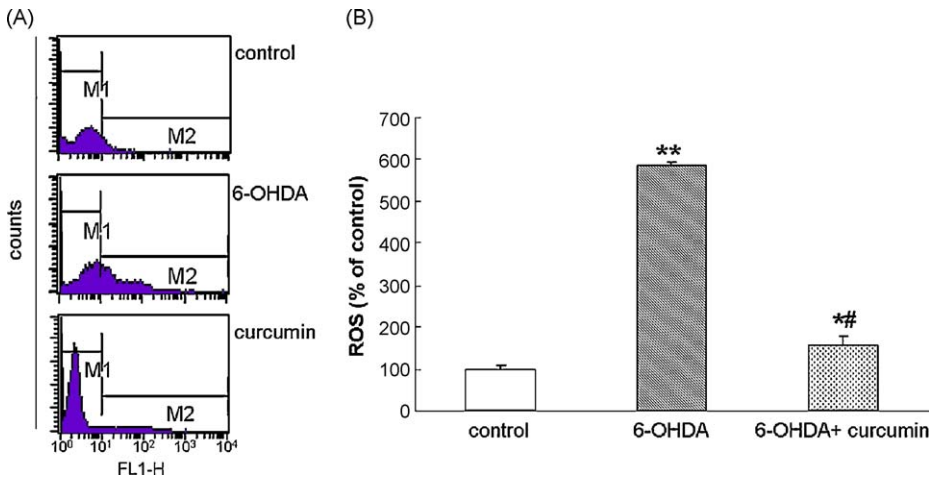

Fig. 5. ROS generation assessed by flow cytometry in 6-OHDA treated MES23.5 cells with curcumin pretreatment. (A) Representatives of the fluorometric assay on ROS of different groups. 6-OHDA treatment caused increased ROS in MES23.5 cells and 10 μmol/L curcumin pretreatment partly prevented the ROS generation and (B) statistical analysis. Data were presented as mean ± S.E.M. of six independent experiments. Fluorescence values of the control were set to 100%. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to the control, and $^{\#}P < 0.01$, compared to 6-OHDA group.

3.4. Curcumin prevents 6-OHDA-induced dissipation of the $\Delta\Psi m$

Mitochondrial membrane potential changes are markers of mitochondria function and are often associated with ROS generation. As shown in Fig. 4, when treated with 6-OHDA, MES23.5 cells showed a significant decrease of $\Delta\Psi m$. Pretreatment with curcumin partially reversed the decrease in mitochondrial potential. This suggested that curcumin could protect cells from 6-OHDA-induced oxidative stress by restoring the mitochondria function.

3.5. Curcumin suppressed 6-OHDA induced an increase of ROS generation in MES23.5 cells

Since ROS played an important role in cell death and changes of mitochondrial membrane potential were considered to be involved in ROS production, we next investigated the intracellular ROS formation using a fluorescent sensitive probe (H2DCF-DA). As presented in Fig. 5, after curcumin pretreatment, the levels of ROS decreased by 72% compared to the 100 μmol/L 6-OHDA treated group.

3.6. Curcumin partially reversed 6-OHDA-induced Cu/Zn–SOD down regulation in MES23.5 cells

Since SOD is a highly potent protective agent against cell injury during oxidative stress, we examined SOD expression in the MES23.5 cells. In 100 μmol/L 6-OHDA-treated cells, Cu/Zn–SOD protein level was down-regulated by 42%. However, after 10 μmol/L curcumin pretreatment for 20 min, the protein level could be up-regulated to 75% of control (Fig. 6).

3.7. Curcumin inhibited 6-OHDA-induced NF-κB nuclear translocation in MES23.5 cells

The mechanism of 6-OHDA-induced cell damage was also investigated by examining NF-κB translocation in MES23.5, using polyclonal antibody of NF-κB (Fig. 7). In control, NF-κB was preferentially located in the cytoplasm. However, upon 6-OHDA administration (100 μmol/L), NF-κB was considerably increased in the nucleus. Curcumin pretreatment inhibited the translocation of NF-κB from cytoplasm to the nucleus.

4. Discussion

In this study we demonstrate that curcumin could effectively protect MES23.5 cells from 6-OHDA neurotoxicity; the protective effects of curcumin were achieved by anti-oxidation activity and the regulation of NF-κB translocation.

6-OHDA lesioning is a well established method for preparing PD models. The hypothetical mechanism of 6-OHDA toxicity is that: reactive oxygen species generated by intra- or extracellular auto-oxidation, hydrogen peroxide formation induced by MAO activity or direct inhibition of the mitochondrial respiratory chain. These events lead to strong oxidative stress [2]. In the present study, we demonstrated that curcumin effectively protected MES23.5 cells from 6-OHDA-induced cell death. Concentrations of curcumin higher than 20 μmol/L (include 20 μmol/L) were toxic to the MES23.5 cells. Therefore, it may not be a simple stoichiometric reaction and the function of curcumin was biphasic: it is nontoxic at concentrations within 20 μmol/L, while higher doses promoted

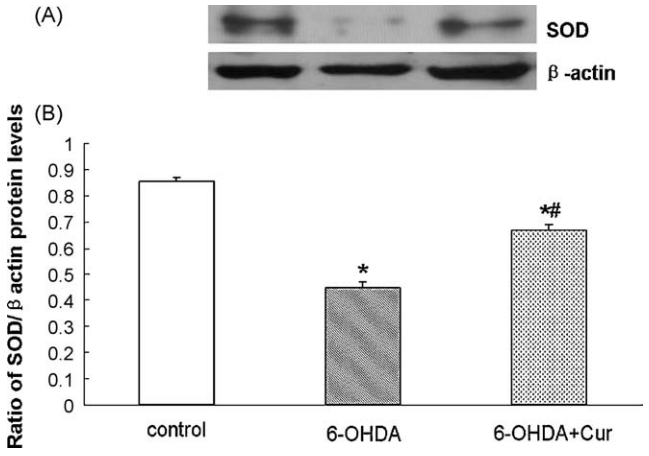

Fig. 6. Cu/Zn–SOD protein levels in 6-OHDA treated MES23.5 cells with curcumin pretreatment. (A) Western blots were applied to detect Cu/Zn–SOD protein levels. Decreased expression of Cu/Zn–SOD was observed in 6-OHDA-treated cells. Curcumin prevented the down regulation of Cu/Zn–SOD partly. β-Actin was used as a loading control and (B) statistical analysis. Data were presented as the ratio of Cu/Zn–SOD to β-actin. Data were presented as the mean ± S.E.M. of six independent experiments. $^{*}P < 0.01$, compared to the control and $^{\#}P < 0.01$, compared to 6-OHDA group.

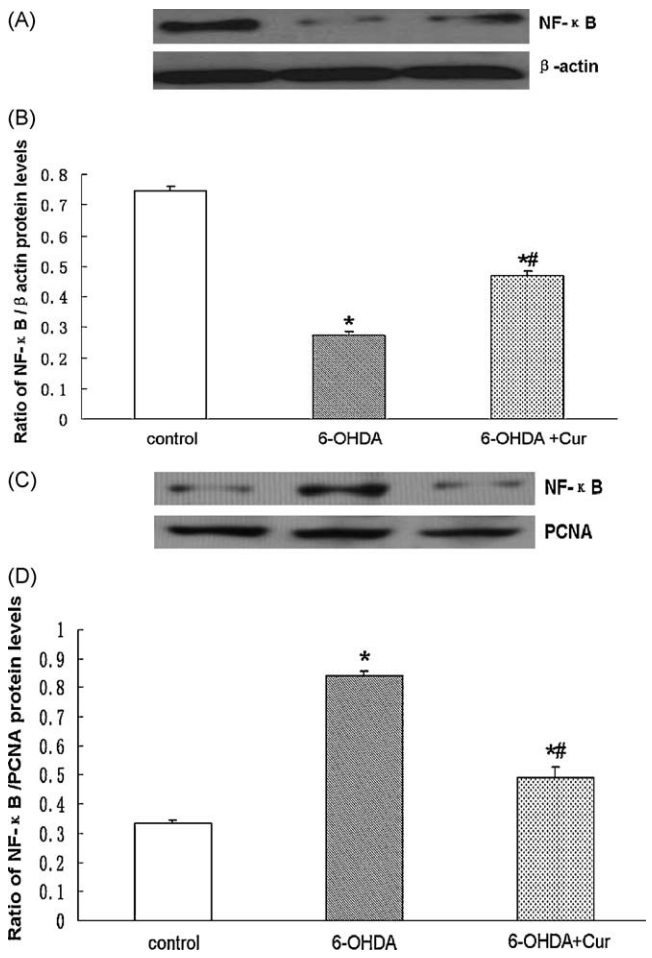

Fig. 7. NF-κB protein levels in 6-OHDA treated MES23.5 cells with curcumin pretreatment. (A) Western blots were applied to detect NF-κB protein levels in the cytoplasm. Decreased expression of NF-κB was observed in 6-OHDA-treated cells. Curcumin prevented the down regulation of NF-κB in the cytoplasm partly. β-Actin was used as a loading control and (B) statistical analysis of NF-κB protein levels in the cytoplasm. Data were presented as the ratio of NF-κB to β-actin. Data were presented as the mean ± S.E.M. of six independent experiments. $^{*}P < 0.01$, compared to the control and $^{\#}P < 0.01$, compared to 6-OHDA group. (C) Western blots were applied to detect NF-κB protein levels in the nucleus. Increased expression of NF-κB was observed in 6-OHDA-treated cells in the nucleus. Curcumin considerably prevented the up regulation of NF-κB in the nucleus. PCNA was used as a loading control. (D) Statistical analysis of NF-κB protein levels in the nucleus. Data were presented as the ratio of NF-κB to PCNA. Data were presented as the mean ± S.E.M. of six independent experiments. $^{*}P < 0.01$, compared to the control and $^{\#}P < 0.01$, compared to 6-OHDA group.

damage. The results were familiar with Levites et al. [15]. Though 1–15 μmol/L of curcumin in our results could prevent the toxic effects of 6-OHDA, 10 μmol/L curcumin which has the best effect against 6-OHDA was chosen.

Oxidative stress plays an important role in PD [16], and dopamine-rich areas of the brain are particularly vulnerable to oxidative stress, because metabolism of dopamine itself leads to the generation of ROS [17]. This pathogenesis is at least partly mimicked by the neurotoxins 6-OHDA [18]. In this study, 6-OHDA injured the mitochondria function and increased ROS generation in MES23.5 cells. When curcumin pretreated, a fall in $\Delta\Psi$m induced by 6-OHDA was blocked and intracellular ROS level was decreased. We know that a very high degree of oxidative stress could overwhelm any endogenous protective antioxidant system, like SOD which is the most important defenses against oxidative stress [19]. Curcumin could increase the level of SOD on 6-OHDA-induced cytotoxicity in MES23.5 cells, which inhibited the oxidative stress. Perhaps it was H-atom donation from phenolic group which was responsible for the "superb antioxidant" properties of curcumin [20].

ROS are known to trigger multiple cellular processes that include a transcriptional response. One of the redox-sensitive transcription factors is NF-κB. Some researches suggest that NF-κB contributes to oxidative stress-induced DA neuron degeneration in PD [21,22]. Intrastriatal administration of DA also produced oxidative damage to striatal neurons and a robust activation of NF-κB [23]. Although NF-κB is activated by multiple factors, ROS are generally thought to play a prominent role in NF-κB activation by 6-OHDA treatment [24]. In the inactive state, NF-κB is retained in the cytosol through complexation with IκB proteins. Upon phosphorylation induced by 6-OHDA, IκBα is degraded, and then NF-κB is released from the cytosolic complex with IκB proteins and translocates to the nucleus, where it initiates inducible nitric oxide synthase (iNOS) transcription that contain an NF-κB binding site [4]. NF-κB, once activated, increases expression of many genes involved in promoting cell death or survival, like p53 and c-Myc in DA neurons [25]. Some evidence has suggested that the inhibition of NF-κB activation in astrocytes might be useful in the intervention of in PD [5]. In our study, we found that the activation of NF-κB by 6-OHDA, was prevented by pretreatment with curcumin. Therefore, curcumin inhibited NF-κB translocation by decreasing ROS generation at least.

In summary, the present study has demonstrated that curcumin exerts neuroprotective effects against 6-OHDA-induced cell death, by a mechanism, believed to increase the level of SOD and the $\Delta\Psi$m, which suppressed an increase in ROS and inhibited the translocation of NF-κB. The present study provides experimental evidence to explore the possible use of curcumin, a very low toxic natural compound as a therapeutic approach in PD.

Acknowledgements

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