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Original Article

The effect of curcumin against 6-hydroxydopamine induced cell death and Akt/GSK disruption in human neuroblastoma cells

Maryam Moosavi[1, 2*], Majid Reza Farrokhi[1], Narges Tafreshi[3]

1. Shiraz Neuroscience Research Center, Shiraz University of Medical Sciences, Shiraz, Iran
2. Nanobiology and Nanomedicine Research Centre, Shiraz University of Medical Sciences, Shiraz, Iran
3. Department of Cancer Imaging and Metabolism, Moffitt Cancer Center and Research Institute, 12902 Magnolia Drive, Tampa, Florida 33612, USA

Abstract

Introduction: Parkinson's disease (PD) is the second most common neurodegenerative disease, characterized by the continuous deficit of dopaminergic neural cells in the substantia nigra pars compacta. The natural compounds from plant extracts, such as turmeric, have been proposed as alternative sources for anti-PD drugs. Human neuroblastoma SH-SY5Y is a dopaminergic neuronal cell line used as an *in vitro* model for the study of dopaminergic cells. The neurotoxin 6-hydroxydopamine (6-OHDA) has been known to induce cell death in dopaminergic neural cells. Curcumin, as the main ingredient of turmeric, has been shown to protect against some animal models of PD. The purpose of the present study was to assess the potential neuroprotective effect of curcumin against the 6-OHDA-induced cell death in SH-SY5Y cells and to delineate its effect on Akt/GSK-3β signaling.

Methods: The cells were exposed to 6-OHDA with/without different doses of curcumin and their viability was examined via MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) and morphological observations. According to the MTT results, the protective doses of curcumin (2 and 2.5μM) were selected for further studies. Western blot assay was done to determine the phosphorylated and total amount of Akt and GSK-3β proteins.

Results: 6-OHDA induced cell death and declined Akt/GSK-3β phosphorylation, while curcumin co-treatment partially restored these effects.

Conclusion: Taken together, these findings suggest that curcumin protects the SH-SY5Y cells from 6-OHDA-induced cell death and Akt/GSK-3β signaling alteration. Thus, our study indicates that curcumin has a partial cytoprotective effect in dopaminergic cell culture systems.

Keywords:
Parkinson's disease;
6-OHDA;
SH-SY5Y;
Curcumin;
Akt;
GSK-3β

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***Correspondence to:**
M. Moosavi

Tel/Fax: +98-7136281517

Email:
marmoosavi@sums.ac.ir

Introduction

Parkinson disease (PD) is the second prevalent neurodegenerative disorder affecting approximately 1% of the population over sixty years and 4% of those over eighty years (Driver et al., 2009). At present, The precursor of dopamine, L-DOPA (L-3,4-dihydroxyphenylalanine) remains the primary therapy of PD. Nevertheless, most patients develop related motor complications like as L-dopa-induced dyskinesia and motor fluctuations after a time

(Morgante et al., 2007). Then, there is an emerging interest in the use of novel therapeutic strategies especially natural molecules with neuroprotective properties.

Curcumin, found in turmeric, is a yellow curry spice with a long history of use in cooking or medicine (Aggarwal et al., 2007). It has a wide pharmacological effects such as anti-inflammatory properties (Srimal and Dhawan, 1973), powerful antioxidant effects (Masuda et al., 1999), anti-protease activity (Sui et al., 1993) and cancer preventive effects (Kim et al., 1998). Recently, some studies have shown the neuroprotective effect of curcumin in experimental models of PD (Wang et al., 2017). In 6-hydroxydopamine (6-OHDA) model of PD, rats pretreated with curcumin showed a protection of substantia nigra and dopamine levels in the striata (Zbarsky et al., 2005). Wang et al. (2009) reported that curcumin has the capability to restore mitochondrial membrane potential and cell viability in 6-OHDA-lesioned mouse embryonic stem cells. Similarly, Rajeswari and colleagues (2008) have shown an increment in striatal dopamine and DOPAC (3,4-Dihydroxyphenylacetic acid) levels after curcumin injection in MPTP (1-methyl-4-phenyl-1, 2,3,6-tetrahydropyridine) injected mice. Accordingly curcumin has been shown to prevent cell death in SH-SY5Y cells (Jaisin et al., 2011; Meesarapee et al., 2014), as the appropriate cellular model of dopaminergic neural cells (Song et al., 2012). 6-OHDA has been shown to alter some signaling molecules; for instance, it affects Akt phosphorylation (Chen et al., 2004) and its downstream element glycogen synthesis kinase 3β (GSK-3β) (Amiri et al., 2016) which is negatively regulated by Akt-mediated phosphorylation at serine 9 (Stambolic and Woodgett, 1994).

As Akt /GSK-3β signaling has been shown to take part in 6-OHDA induced cell death (Chen et al., 2004; Amiri et al., 2016), this study aimed to explore if the protective effect of curcumin against 6-OHDA induced toxicity is accompanied with Akt/GSK-3β signaling alteration.

Materials and methods

Materials

SH-SY5Y cells were purchased from Pasteur institute of Iran. Cell culture materials including Dulbecco's Modified Eagle's Medium (DMEM)-F12, fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Gibco life technologies. Curcumin and 6-OHDA (H4381) were from Sigma-Aldrich. Western blot antibodies including phospho-Akt (Ser473) (4058), Akt (4685), phospho-GSK-3β (Ser9) (5558), GSK-3β (9315), beta-actin (4970) and secondary HRP-conjugated (7074) were bought from Cell Signaling Technology. Amersham ECL select (RPN2235) reagent kit was from GE health care and PVDF membrane was purchased from Millipore. Other reagents were purchased from usual commercial sources.

SH-SY5Y cell culture

Human SH-SY5Y cells were maintained in DMEM containing 10% heat-inactivated FBS and 1% (v/v) penicillin/streptomycin under humidified 5% CO_2 atmosphere at 37°C. For subcultures, SH-SY5Y cells were dissociated with trypsin-EDTA and split into a 1:3 ratio. The medium was changed every 2 days until the cells reached the desired confluence. For MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay, the cells were plated in 96 well plates while for western blot studies they were seeded in 6 well plates. The cells were incubated at 37°C in 95% humidified atmosphere with 5% CO_2. Because differentiated SH-SY5Y cells have been reported to have some alterations in Akt pathway which result in higher tolerance to 6-OHDA (Cheung et al., 2009), undifferentiated SH-SY5Y cells have been reported to be more appropriate to study neurotoxicity or neuroprotection in dopaminergic cell death (Schule et al., 2009). Therefore, this research work used undifferentiated SH-SY5Y cells.

Treatments

The concentrations of both 6-OHDA and curcumin have been chosen according to the initial pilot studies. Accordingly, the dose 50μM of 6-OHDA and the doses 2 and 2.5μM of curcumin were selected for the study. At our first round of experiments, curcumin was used in doses 0.25, 0.5, 1, 2, 3, 5, 7.5, 10, 15 and 20 μM and the results showed the best protection between doses 1-3μM while the higher doses were even toxic possibly due to the pro-oxidant (Banerjee et al., 2008) and cytotoxic effect of curcumin at concentrations more than 10μM (Kim et al., 2008). After narrowing the range of 1-3μM, it was revealed

that curcumin at 2 and 2.5μM are the most protective ones in the present study. Immediately before application, 6-OHDA was diluted in 0.1% ascorbic acid and added to fresh cell culture medium to achieve the required concentration. Curcumin was dissolved in dimethyl sulfoxide (DMSO) at a stock concentration in which the final concentration of DMSO in culture medium did not exceed 0.1%.

Measurement of cell viability

The MTT assay provides a sensitive assessment of the metabolic status of cells, reflecting early cellular redox changes. SH-SY5Y cells (10^4 cells/well in 96-well plates) were incubated at 37°C with 50μM of 6-OHDA for 24h with or without curcumin and then treated with MTT solution (5mg/ml) for 4h. The formazan crystals formed in the cells were dissolved in DMSO and the absorbance at 540 nm was measured with a microplate reader (Synergy HT, Biotek).

Cell morphology

The morphological changes of the cells were assessed 24 hours after treatments. The cell shapes, volumes and numbers were compared.

Western blot analysis

SH-SY5Y cells (10^6 cells/well in 6-well plates) were incubated at 37°C with 50μM of 6-OHDA for the indicated time, with or without curcumin. The cells were washed and harvested with ice-cold PBS and centrifuged at 1000 rpm for 5 min. The cell pellet was resuspended in 70μl of ice-cold RIPA lysis buffer containing protease and phosphatase inhibitor cocktails and incubated on ice for 30min. After centrifugation at 13,000 rpm for 30min at 4°C, the supernatant was separated and stored at −80°C. The protein concentration was determined using Lowry method (Lowry et al., 1951). Proteins were separated on 10% SDS–polyacrylamide gel and then transferred onto a polyvinylidene difluoride transfer membrane which was blocked with 5% BSA containing 0.5mM Tris–HCl (pH 7.5), 150mM NaCl, and 0.1% Tween-20 for 1h at room temperature. The membrane was subsequently incubated with primary antibody overnight at 4°C (pAkt, Akt, pGSK-3β, GSK-3β and β-actin). After the washes with Tris-buffered saline containing 0.1% Tween-20 (TBST), the blots were incubated with horseradish-peroxidase-conjugated secondary antibodies in TBST for 1h at room temperature. The blots were developed using the enhanced chemiluminescence (ECL) detection method by immersing them for 5min in a mixture of ECL reagents A and B at a 1:1 ratio and then exposing them to photographic film for a few minutes (Negintaji et al., 2015). Protein bands were quantified by densitometric analysis using Image J software.

Data analysis

The MTT experiments and western blot analysis were repeated 3 times. The data was analyzed by one way ANOVA followed by Tukey as the post hoc test.

Results

Neurotoxic effects induced by 6-OHDA on SH-SY5Y cells

SH-SY5Y cells were exposed to different concentrations of 6-OHDA (30-100μM) during 24h. As can be observed in the Figure 1, 6-OHDA induced a concentration-dependent effect on the viability of SH-SY5Y cells. The dose 50μM was chosen as it induced about 50% reduction of cell viability.

The neuroprotective effect of curcumin on SH-SY5Y cells exposed to 6-OHDA

The exposition of SH-SY5Y cells to 6-OHDA (50μM) led to a reduction of about 50% of cell's viability. However, when 6-OHDA was incubated with curcumin (2 and 2.5μM), it partially blunted the toxicity induced by 6-OHDA after 24h of incubation (Fig. 2; $P<0.0001$, $F(5, 18) = 15$). Post hoc analysis by Tukey test revealed that curcumin in 2 and 2.5μM prevented 6-OHDA induced cell death. Curcumin by itself had no effect on cell survival comparing to control group.

Changes of cell morphology

Morphological results of SH-SY5Y cells are illustrated in Figure 3. The pictures were captured 24 hours after treatment. As it is shown, 6-OHDA treatment led to the shrinkage of cell body, decreased the number of alive cells and increased the cell debris. These morphological changes attenuated after curcumin co-treatment.

Curcumin inhibits 6-OHDA-induced p-Akt/t-Akt

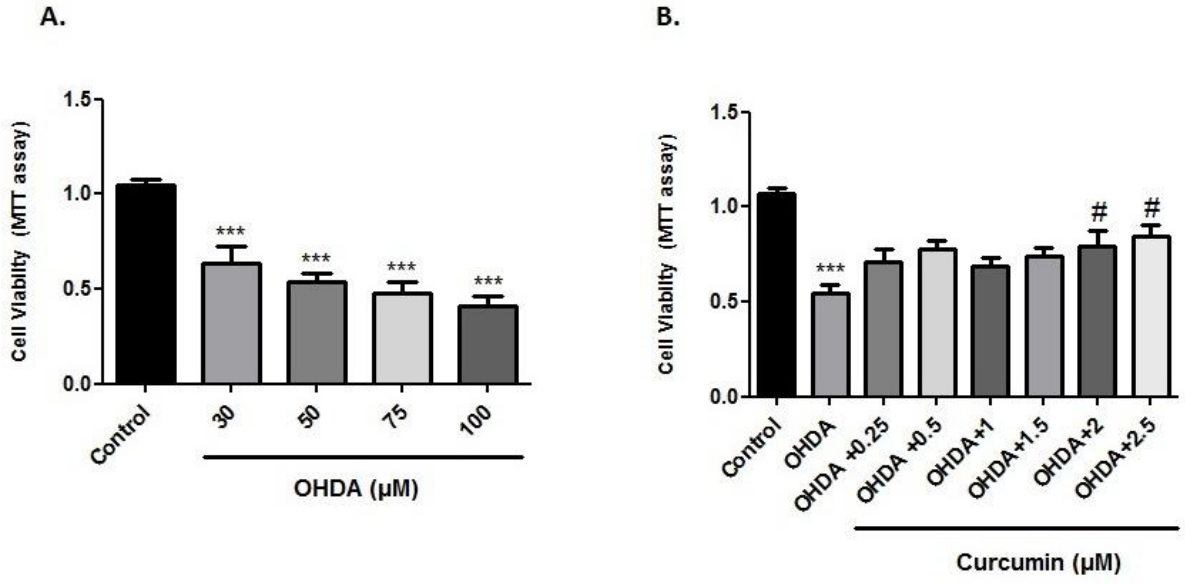

Fig.1. The effect of different doses of 6-OHDA on SH-SY5Y cells viability in MTT assay (A) and the effects of different dose of curcumin against 6-OHDA (B). $^{***}P<0.001$ represents the difference between control and 6-OHDA group. $^{\#}P<0.05$ represents the difference between 6-OHDA and curcumin co-treated groups.

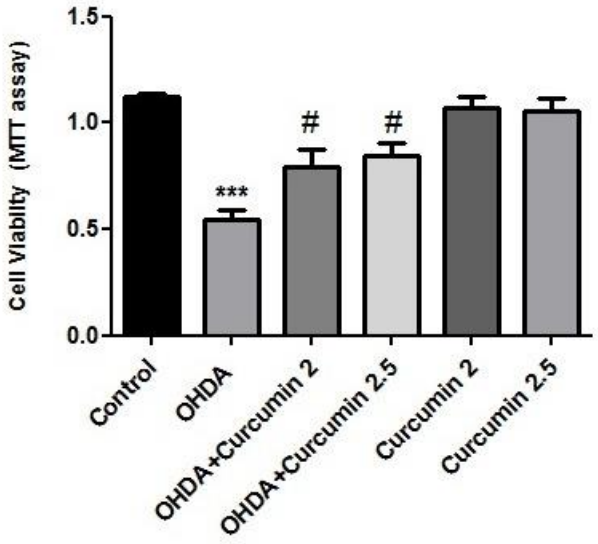

Fig.2. The effect of selected doses of curcumin (2 and 2.5μM) with/without 6-OHDA on cells viability. Data are represented as mean±SEM. $^{***}P<0.001$ represents the difference between control and 6-OHDA group. $^{\#}P<0.05$ represents the difference between 6-OHDA and curcumin co-treated groups.

and p-GSK-3β/t-GSK-3β declin in SH-SY5Y cells

As shown in Figure 4, the evaluation of the phosphorylation statuses of Akt and GSK-3β revealed some differences. One-way ANOVA showed significant difference between groups (P=0.0219, F(5,12)=4.051). The treatment with 50μM 6-OHDA decreases p-Akt/t-Akt ratio, while curcumin treatment in 2 and 2.5μM partially reversed the decrement of p-Akt/t-Akt ratio.

The effect of curcumin on the p-GSK3β and t-GSK3β is shown in Figures 4A and 4C. Antibodies against these proteins, detected the bands at 46 kDa. One-way ANOVA showed a significant difference between groups (P=0.0002, F(5,12)= 13.22). Post hoc by Tukey's test showed that 6-OHDA decreased p-GSK-3β/ t-GSK3β ratio. Curcumin treatment prevented 6-OHDA-induced p-GSK-3β/ t-GSK3β decrement.

Discussion

This study confirmed the results of the previous research works indicating that curcumin has the

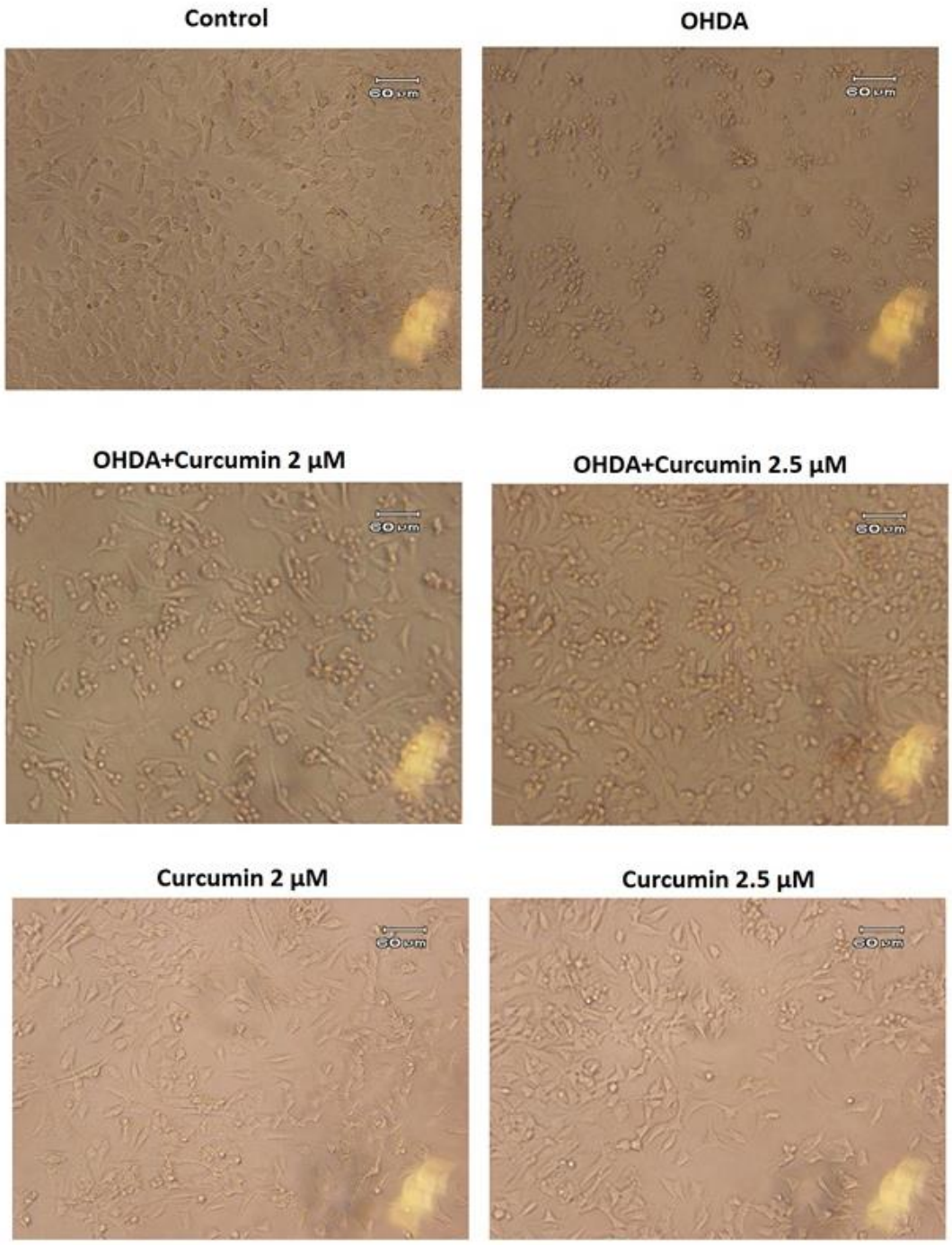

Fig.3. The representative images of cultured SH-SY5Y cells in different groups. Images have been magnified 20 times respectively. As it is figured, after 6-OHDA treatment the cell bodies are shrunk, the number of alive cells is decreased while there is an increment of cell debris. These morphological changes attenuated when curcumin added.

capability to prevent 6-OHDA induced cell death as it partially restored cell viability in MTT assay (Jaisin et al., 2011; Meesarapee et al., 2014). The most characteristic hallmark of PD is dopaminergic neural loss (50-70%) in substantia nigra (Forno, 1996). SH-SY5Y cells as a sub line of human neuroblastoma cells (Xing et al., 2005) have some similar characters with dopaminergic neurons (Song et al., 2012). 6-OHDA enters the cells via a dopamine reuptake transporter (Ljungdahl et al., 1971) and leads to reactive oxygen species (ROS) generation and neural cell death (Hwang, 2013). Curcumin has been shown to be at least ten times more active as an antioxidant than vitamin E (Khopde et al., 2000). Then it is probable that the potent antioxidant effect of curcumin plays a role in this protective effect. Supporting this idea, Curcumin (5µM) has been shown to attenuate paraquat-induced cell death in

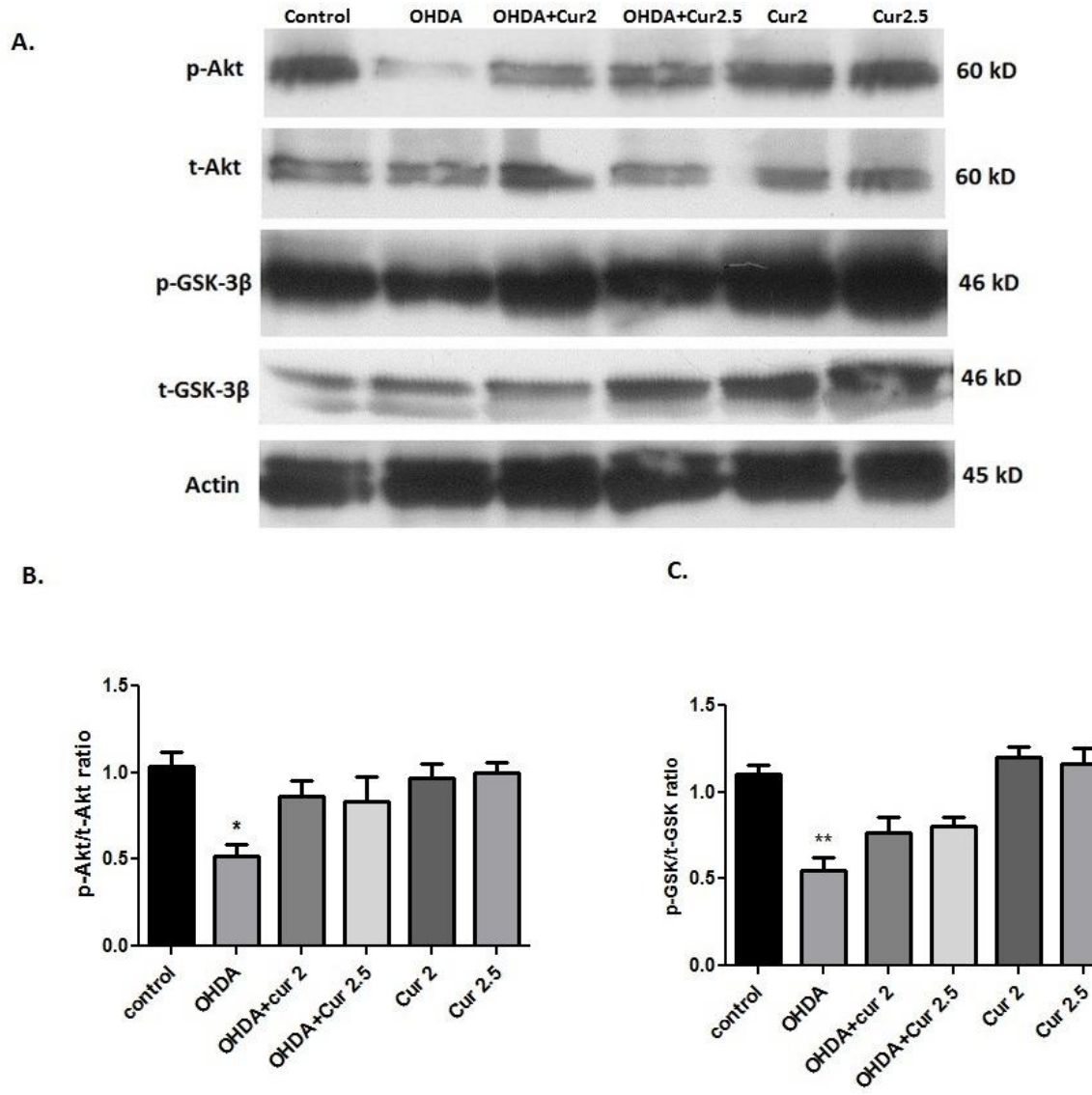

Fig.4. A) Western blot analysis showing the relative the amounts of p-Akt, t-Akt, p- GSK-3β, t-GSK-3β and actin after treatment with 6-OHDA (50µM) and/or curcumin (2 and 2.5µM). B) shows p-Akt/t-Akt ratio and C) depicts p-GSK/t-GSK. $^{*}P<0.05$ and $^{**}P<0.01$ represent the difference between control and 6-OHDA treated groups.

SH-SY5Y cells through modulating oxidative stress (Jaroonwitchawan et al., 2017). Furthermore, curcumin (4µM) was reported to reduce ROS levels induced by α-synuclein aggregates in SH-SY5Y cells (Wang et al., 2010). In addition, curcumin at 1µM concentration alleviated oxidative stress and apoptosis in cultured astrocyte cells (Daverey and Agrawal, 2016). Epidemiological studies have proposed that consumption of turmeric/curcumin by the Asian Indians might relate to the low incidence of PD in India (Ganguli et al., 2000). The protective effect of curcumin against 6-OHDA induced SH-SY5Y cell death which was shown in this study is in line with this suggestion and the recent animal studies (Khatri and Juvekar, 2016; Song et al., 2016) showing the beneficial effect of curcumin in PD models (Wang et al., 2017).

In the striatum of PD patients, the amount of p-Akt, as the active form of this protein, declines (Greene et al., 2011). Similarly, 6-OHDA had been shown to inhibit Akt phosphorylation in SH-SY5Y cells (Chen et al., 2004; Driver et al., 2009; Amiri et al., 2016); the finding which is consistent with the results of the current study. As Akt has been suggested to have a main role in cell survival (Franke and Cantley, 1997; Datta et al., 1999; Ghasemi et al., 2015), the decline of p-Akt in PD patients and dopaminergic cells seems to be involved in cell death. Howbeit, there are some reports proposing that Akt is not always protective because its strong activation increases oxidative stress and cell death (Nogueira et al., 2008). The results of this study showed that the protective effect of curcumin against 6-OHDA induced cell death is accompanied with p-Akt elevation, which suggests

that p-Akt decline has a role in neuroblastoma cell death and Akt signaling is involved in curcumin-induced protection. This finding is in line with previous reports showing the involvement of Akt in the protective effect of curcumin in SH-SY5Y cells against bupivacaine, amyloid-beta ciliary neurotrophic factor APPswe transfection (Yin et al., 2012; Huang et al., 2014; Wang et al., 2014; Fan et al., 2016).

Active PI3K/Akt signaling pathway induces rapid inhibition of GSK-3β by phosphorylation at Ser9 (Stambolic and Woodgett, 1994). Although GSK-3β was originally named according to its glycogen synthase phosphorylation activity and glucose metabolism regulation, it is also known to act as a key regulator of a wide range of cellular functions (Frame and Cohen, 2001). In spite of reports indicating the proapoptotic role of GSK-3β (Bijur et al., 2000; Chen et al., 2004), its inhibition in neuroblastoma Neuro-2A cell line has been shown to promote cell death (Dickey et al., 2011). GSK-3β has been reported to have a critical role in numerous cellular functions such as regulation of cell signaling (Guha et al., 2011), cell division (Diehl et al., 1998) and growth (Shin et al., 2011) as well as apoptosis (Watcharasit et al., 2003). This study revealed that 6-OHDA treatment decreases p-GSK/t-GSK ratio in human neuroblastoma cell, while curcumin prevents this effect of 6-OHDA. Then it seems that GSK inactivation has a role in curcumin protection against 6-OHDA toxicity. Consistently, curcumin was previously shown to restore beta amyloid induced decrement of p-GSK in SH-SY5Y cells (Huang et al., 2012; Huang et al., 2014).

Conclusion

In conclusion, the results of this study showed, for the first time, that the protective effect of curcumin against 6-OHDA toxicity in human neuroblastoma cells is accompanied with Akt/GSK-3β signaling correction. These results are helpful in knowing the protective effects of curcumin in dopaminergic cells.

Acknowledgments

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Conflict of interest

The authors declare that they have no conflict of interests.

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