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Original Article

Neurosteroid dehydroepiandrosterone attenuates 6-hydroxydopamine-induced apoptosis in a cell model of Parkinson's disease

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Abstract

Introduction: Parkinson's disease is a progressive neurodegenerative disorder characterized by progressive death of midbrain dopaminergic neurons. Neurosteroid dehydroepiandrosteone (DHEA) is synthesized de novo in brain glial cells and its concentration is particularly high in the brain, which dramatically decreases by aging. DHEA has neuroprotective activity against different types of neural injuries. In this study, we investigated the effects of DHEA on 6-hydroxydopamine (6-OHDA)-induced toxicity in rat pheochromocytoma (PC12) cells as an *in vitro* model of Parkinson's disease.

Methods: Cell damage was induced by 150µM 6-OHDA and the cell survival rate was examined by MTT assay. The level of intracellular reactive oxygen species (ROS) was determined with a 2,7-dichlorofluorescein diacetate probe. Immunoblotting was also employed to determine the level of biochemical markers of neural apoptosis in PC12 cells.

Results: The data demonstrated toxic effect of 6-OHDA by reducing cell viability in a dose-dependent manner. Furthermore, activated caspase-3 and Bax/Bcl2 ratio were significantly increased in 6-OHDA-treated cells. Incubation of cells with DHEA (400 and 600µg/ml) decreased cell damage.

Conclusion: Our results suggest that DHEA has protective effects against 6-OHDA-induced neural damage. The mechanisms of these effects may be due to the attenuation of neural apoptosis and suggest therapeutic potential of this neurosteroid in the treatment of Parkinson's disease.

Keywords:
Dehydroepiandrosterone;
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6-hydroxydopamine;
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Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder associated with the progressive loss of mesencephalic dopaminergic neurons (Drui et al., 2014); even though, the causality of such neuronal degeneration has not been fully

understood. PD affects mainly the substantia nigra pars compacta, where wide dopaminergic degeneration happens (Fearnley and Lees, 1991). The degeneration of dopaminergic neurons, results in loss of the dopaminergic inputs to the striatum via the nigrostriatal pathway. The etiology of PD has not been fully discovered in majority of the cases. However, risk factors such as genetic factors, oxidative stress, infections, apoptosis, excitotoxicity as well as head trauma and vascular disease have all been implicated (Delamarre and Meissner, 2017). Pharmacologic treatment of PD can be divided into symptomatic and neuroprotective intervention. Symptomatic strategies aim to either contract dopamine deficiency in the basal ganglia or to block muscarinic receptors. Although neuroprotective therapies aim to slow, block or reverse PD progression, at the present time, there is no complete approved neuroprotective or disease-modifying strategy for PD. Increasing evidence suggests that neurosteroids have neuroprotective properties on the central and peripheral nervous systems (Bourque et al., 2009; Ishrat et al., 2009; Goncharov and Katsya, 2013). Dehydroepiandrosterone (DHEA) is an important precursor hormone and is the most abundant circulating steroid present in the human body. In human PD subjects, the serum levels of DHEA, allopregnanolone and testosterone were found to be lower than those in healthy people (di Michele et al., 2003; di Michele et al., 2013; Nitkowska et al., 2015). It seems that a lower level of neurosteroids in brain may promote the progression of degenerative diseases such as PD.

It has been demonstrated that neurosteroids DHEA and alopregnanolone revers the degradation of dopaminergic neurons in MPTP-lesioned rats (Adeosun et al., 2012; Litim et al., 2016). It has also been reported that neurosteroids can affect motor components and cognitive function in rodent models of 6-hydroxydopamine (6-OHDA)-induced Parkinsonism (Nezhadi et al., 2016; Nezhadi et al., 2017). However, the detailed cellular mechanism(s) of such dopaminergic protection has not yet been determined. Since neurosteroids have protective effects against different cellular models of neurodegenerative diseases and dopaminergic cellular damage and apoptosis are involved in the pathogenesis of PD, we decided to examine whether DHEA has a protective role against 6-OHDA-induced cellular model of PD.

Materials and methods

Materials

Cell culture reagents, penicillin-streptomycin solution, trypsin-EDTA and fetal bovine serum (FBS) were obtained from Biosera co. (East Sussex, UK). Culture flasks and dishes were acquired from SPL life science Inc. (Gyeonggi-Do, South Korea). The 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2-tetrazolium bromide (MTT), 6-OHDA and DHEA were obtained from Sigma (St. Louis, MO, USA). Primary polyclonal anti-caspase-3 and primary monoclonal anti-β-actin antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Primary polyclonal anti-Bax and primary monoclonal anti-Bcl-2 antibodies were obtained from Santa Cruz Biotechnology, Inc. (Delaware Ave. Santa Cruz, USA).

Cell culture

Rat pheochromocytoma PC12 cells were obtained from National Cell Bank of Iran (NCBI) Pasteur Institute of Iran (Tehran, Iran). Cells were grown with *Dulbecco's Modified Eagle's Medium* (DMEM) supplemented with 10% FBS, 5% horse serum and penicillin (100 U/ml) and streptomycin (100 µg/ml). They were maintained at 37°C in a 5% CO2 atmosphere.

After two passages, PC12 cells were plated at the density of 5000 per well in a 96 micro plate well for the MTT assay. Control cells were grown in normal DMEM. The cells were incubated with 6-OHDA (150µM) and different concentrations of DHEA (50, 100, 200 and 400µg/ml) for 24 hours. DHEA was added 30 minutes before 6-OHDA. For protein extraction, cells were grown in a 6 plate well and permitted to attach and grow for 24h. Then the cells were incubated with 6-OHDA and different concentration of DHEA for 24h.

Cell viability analysis

Cellular viability was assessed by the reduction of MTT to formazan (Denizot and Lang, 1986). MTT was dissolved in PBS and added to the culture at final concentration of 0.5mg/ml. After additional 2h incubation at 37°C, the media were carefully removed, 100µl DMSO was added to each well and

the absorbance values were determined by spectrophotometry at 490nm with an automatic microplate reader (FLX 8000, Biotek). All experiments were performed six independent times and for each group 6 wells were selected. Results were expressed as percentages of control.

Measurement of intracellular reactive oxygen species (ROS) formation

The level of intracellular ROS was determined with a 2,7-dichlorofluorescein diacetate (DCFH-DA) probe and fluorescencespectrophotometry. In the presence of a properoxidant, DCFH-DA is converted to the highly fluorescentdichlorofluorescein. Different groups of cells were incubated with 1mM DCFH-DA in PBS in the dark for 10min at 37°C. After incubation, the cells were washed three times with PBS and analyzed immediately on the fluorescence plate reader (FLX 800, BioTek, USA). The fluorescence intensity of cells in 96-well plates was quantified at an excitation of 485nm and an emission of 538nm. Each experiment was performed six independent times. Results were expressed as fluorescence percentage of control cells.

Immunoblotting analysis

The PC12 cells were homogenized in ice-cold buffer containing 10mM Tris–HCl (pH 7.4), 1mM EDTA, 0.1% SDS, 0.1% Na-deoxycholate, 1% NP-40 with protease inhibitors (1 mMphenylmethylsulfonyl fluoride, 2.5µg/ml of leupeptin, 10µg/ml of aprotinin) and 1mM sodium orthovanadate. The homogenate was centrifuged at 14000rpm for 15min at 4°C. The resulting supernatant was retained as the whole cell fraction. Protein concentrations were measured using the Bradford method (Bio-Rad Laboratories, Muenchen, Germany). Equal amounts of protein were resolved electrophoretically on a 9% SDS-PAGE gel and transferred to nitrocellulose membranes (Hybond ECL, GE Healthcare Bio-Sciences Corp. NJ, USA). After blocking (overnight at 4°C) with 5% non-fat dried milk in Tris-buffered saline with Tween 20 (blocking buffer, TBS-T, 150mM NaCl, 20mM Tris–HCl, pH 7.5, 0.1% Tween 20), the membranes were probed with rabbit monoclonal antibody to caspase-3 (Cell Signaling Technology, USA, 1:1000 overnight at 4°C), Bax (Δ 21): sc-6236 and Bcl-2 (C-2): sc-7382, Santa Cruz, USA, 1:1000 for three hours at room temperature. After washing in TBS-T (three times, 5min), the blots were incubated for 60min at room temperature with a horseradish peroxidase-conjugated secondary antibody (1:15000, GE Healthcare Bio-Sciences Corp. NJ, USA). All antibodies were diluted in blocking buffer. The antibody-antigen complexes were detected using the ECL system (GE Healthcare Bio-Sciences Corp. NJ, USA) and exposed to Lumi-Film chemiluminescent detection film (Roch, Germany). Lab Work analyzing software (UVP, UK) was used to analyze the intensity of the expression. β-actin immunoblotting (antibody from Cell Signaling Technology, INC. Beverly, MA, USA; 1:1000) was used to control for loading.

Statistical analysis

The results are expressed as mean±SEM. The differences in mean between experimental groups were determined by one-way ANOVA, followed by the Tukey test.

Results

The effect of 6-OHDA on PC12 cells viability

For the induction of neurotoxicity, the cells were treated with different concentration of 6-OHDA and cell viability was analyzed using MTT assay. After the initial 24h attachment/grow period, the cells were exposed to 6-OHDA at the concentration of 50, 100 and 150µM for 24h. Figure 1 shows that 6-OHDA could decrease the viability of PC12 cells in a dose dependent manner. A significant neurotoxic effect was observed in the cells that received 100 $P<0.05$) or 150µM ($P<0.001$) 6-OHDA. Since, 150µM of 6-OHDA resulted in 52.2±1.38% of relative cell viability, this dose was used for inducing neurotoxicity in the next experiment.

The effect of DHEA on 6-OHDA-induced cell toxicity

To examine the possible protective effect of DHEA on 6-OHDA-induced cellular toxicity, the cells were treated with different doses of DHEA, 30min before 150µM 6-OHDA. The data revealed that DHEA in dose of 200 ($P<0.01$) and 400µg/ml ($P<0.001$) significantly attenuated the 6-OHDA-induced cell damage, while 50 and 100µg/ml of DHEA had no protective effect in 6-OHDA-induced PC12 cell toxicity (Fig. 2). Therefore, 200 and 400µg/ml DHEA (the most effective doses in MTT assay) were

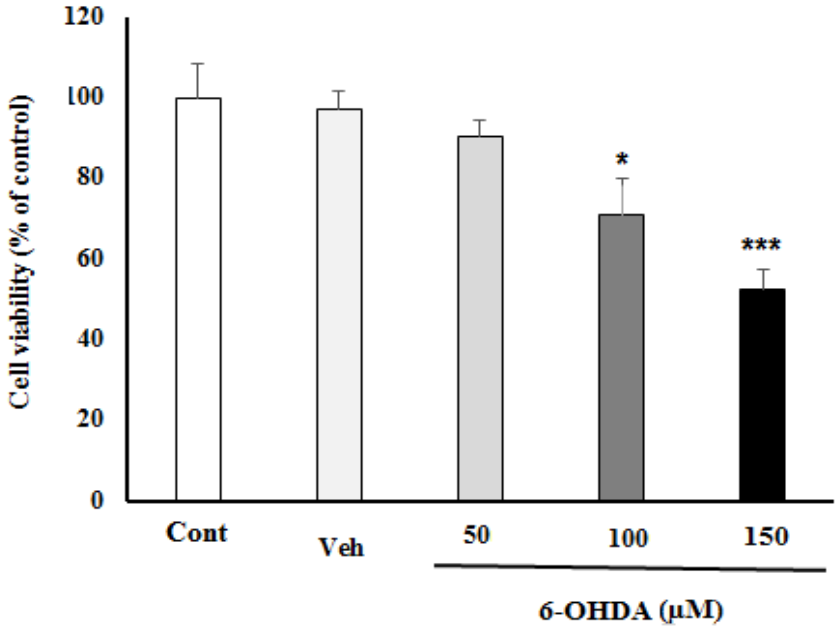

Fig.1. Effects of different concentration of 6-hydroxydopamine (6-OHDA) on PC12 cells viability. Cells were treated with 6-OHDA for 24 hours and then cell viability was measured by MTT assay. Data are expressed as mean±SEM, n=6 wells for each group. $^{*}P<0.05$ and $^{***}P<0.001$ significantly different versus control and vehicle treated cells.

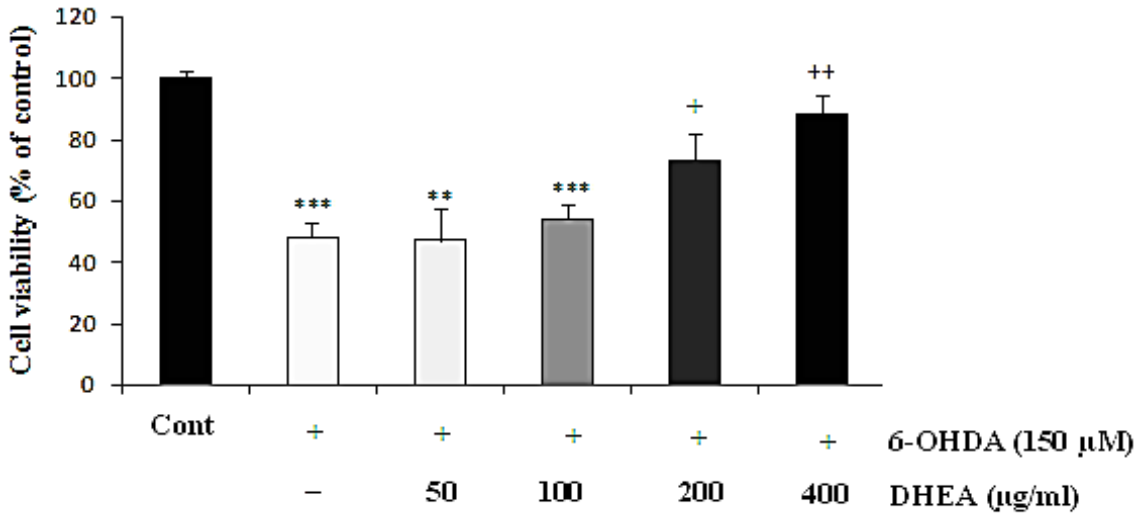

Fig.2. The effects of dehydroepiandrosterone (DHEA) on 150µM 6-OHDA-induced PC12 cell damage. DHEA was added 30min before 6-OHDA. The cells were treated with 6-OHDA and different concentration of DHEA for 24h. Data are expressed as mean±SEM, n=5-6 wells for each group. $^{***}P<0.001$ and $^{**}P<0.01$ versus control cells; $^{+}P<0.05$ and $^{++}P<0.01$ versus 6-OHDA treated cells.

selected for using in the next steps of the experiment.

Effect of DHEA on intracellular ROS formation

The intracellular ROS levels were assessed in control, 6-OHDA and 6-OHDA plus DHEA–treated cells. Exposure of PC12 cells to 6-OHDA led to an increase in ROS level ($P<0.001$) which was significantly attenuated in DHEA- (200 and 400µg/ml) treated cells (Fig. 3).

Western blot analysis of cleaved caspase-3, Bax and Bcl2 in PC12 cells

To examine the potential mediators of 6-OHDA-induced apoptosis, we analyzed caspase-3 activation and Bax:Bcl-2 proteins ratio. The cells were exposed to control, 6-OHDA, 6-OHDA plus different concentrations of DHEA for 24h. Cleaved caspase-3 was found to be increased in 6-OHDA-treated cells ($P<0.001$). The data showed that neurosteroid DHEA

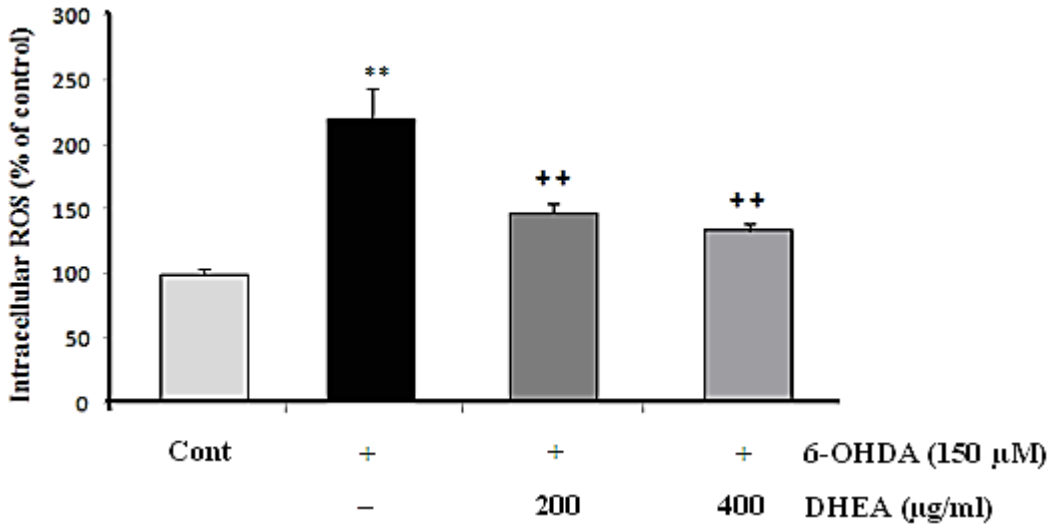

Fig.3. Effect of dehydroepiandrosterone (DHEA) on 6-OHDA-induced increase in intracellular ROS level. Cells were pretreated with DHEA (200 and 400µg/ml) for 30min and then 150µM 6-OHDA were added and incubated for additional 24h. Data are expressed as mean±SEM, n=5-6 wells for each group. $^{**}P<0.01$ versus control cells; $^{++}P<0.01$ versus 6-OHDA treated cells.

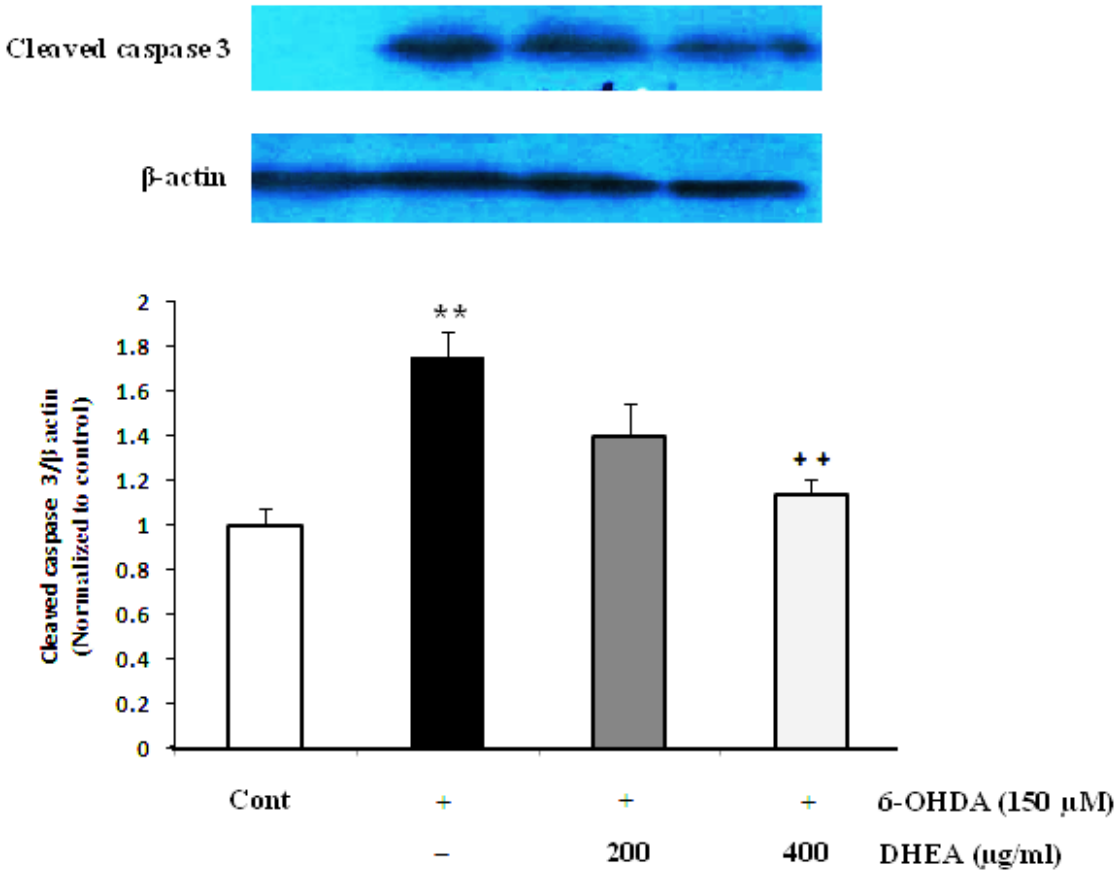

Fig.4. Effect of 6-OHDA alone and in accompanied with dehydroepiandrosterone (DHEA) on activated caspase 3 in PC12 cells. Western blot analysis of caspase 3 was performed in control, 6-OHDA and 6-OHDA plus different concentrations of DHEA for 24h. Each value in the graph represents the mean±SEM band density ratio for each group. $^{**}P<0.01$ compared to control group; $^{++}P<0.01$ compared to 6-OHDA incubated cells.

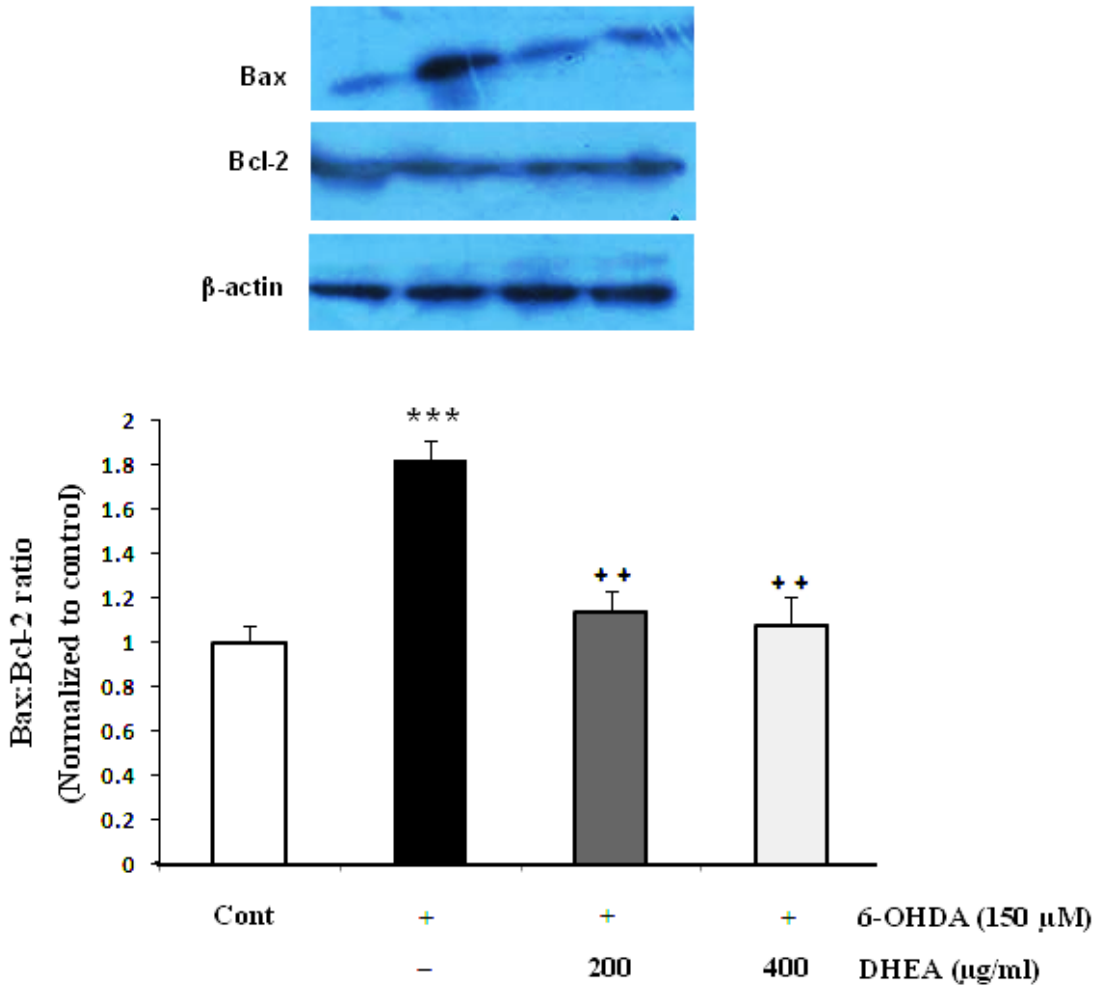

Fig.5. Effect of 6-OHDA alone and in accompanied with dehydroepiandrosterone (DHEA) on Bax and Bcl-2 proteins expression in PC12 cells. DHEA has a decreasing effect on Bax/Bcl-2 proteins ratio. Each value in the graph represents the mean±SEM band density ratio for each group which normalized to control. $^{***}P<0.001$ compared to control cells; $^{++}P<0.01$ compared to 6-OHDA treated cells.

(400µg/ml) significantly ($P>0.01$) antagonized 6-OHDA-induced up-regulation of cleaved caspase-3 (Fig. 4).

Under 6-OHDA treatment a significant increase in Bax protein level was seen. In contrast, 6-OHDA led to a mild reduction in Bcl-2 protein density. Consequently, there was a significantly increase in the Bax: Bcl-2 protein ratio ($P<0.01$) in the cells exposed to 150µM 6-OHDA as compared to those exposed to control medium. However, the increased Bax:Bcl-2 ratio was not observed in the presence of 200 and 400µg/ml DHEA (Fig. 5).

Discussion

Parkinson's disease is a progressive neurodegenerative disorder and its current therapeutic strategies are ineffective. It has been shown that protective strategy became an important part of disease management (Kim, 2017). Although the neuroprotective role of neurosteroids has been studied in different neurodegenerative models, the modulatory activity of DHEA has received less attention. Several studies have shown that DHEA is neuroprotective under different experimental conditions, including models of ischemia, traumatic brain injury, spinal cord injury, glutamate excitotoxicity and neurodegenerative diseases (Arbo et al., 2018). Limited data are available on the neuroprotective activity of DHEA on cellular models of PD and especially its neuroprotective effect. The data indicated that neurosteroid DHEA has a protective effect on cellular model of dopaminergic degeneration and through its antioxidant and anti-apoptotic property.

Previous studies demonstrated that oxidative stress

plays crucial role on the pathogenesis of PD. In addition, oxidative stress has been shown to play an important role in neurodegeneration and apoptosis (Jiang and Dickson, 2018). Injection of 6-OHDA mediates cell death by inhibiting energy production in the cell through disruption of complex I of the electron transport chain in the mitochondria and finally causes the oxidative stress-induced dopaminergic cell death (Hernandez-Baltazar et al., 2017). Thus, 6-OHDA can be used to produce cell damage in *in vitro* model of PD. The data showed that DHEA (200 and 400µM) has potential preventing effects on 6-OHDA-induced oxidative stress and ROS generation. Current evidence suggests that one of the most important effects of neurosteroids might be their antioxidant and anti-apoptotic effect. It seems that DHEA protected neuronal cells against 6-OHDA-mediated oxidative stress and apoptosis.

Since the effect of DHEA on 6-OHDA-induced cell toxicity has not yet been reported, we tried to find the effective doses of DHEA in our pilot study. DHEA was used in nanogram to microgram (per ml) concentrations. The data (according to such trial and error study) showed that DHEA could elicit protection in concentrations of 200 and 400µg/ml and these doses were chosen to complete the next steps of the study. It has been reported that neurosteroids can exert its anti-apoptotic effects through the reduction of the pro-apoptotic proteins (caspase-3) gene expression. Furthermore, neurosteroids exert their neuroprotective effects by controlling the expression of major apoptotic effectors, such as Bcl-2 proteins and cytochrome C (Charalampopoulos et al., 2008). Not surprisingly, neurosteroid DHEA with potent antioxidant and anti-apoptotic property could be protective in cell model of Parkinsonism here.

Conclusion

Present study showed that DHEA strongly preserve dopaminergic PC12 cells from 6-OHDA-induce toxicity. The results provide some interesting cues regarding antioxidant and anti-apoptotic effect of DHEA and its useful area of research in order to study its anti-parkinson effect in animal models as well as clinical trial and the possible utility of allopregnanolone as a neuroprotector agent.

Acknowledgments

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Conflict of interest

The authors declare that there is no conflict of interest.

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