Original Research (2025) 2(2); 20 - 29
https://doi.org/10.22103/vcbr.2025.24744.1043

Shahid Bahonar University of Kerman

Veterinary and Comparative Biomedical Research

ORIGINAL ARTICLE

Antioxidant and Anti-Apoptotic Properties of Allopregnanolone in Protecting Dopaminergic Neurons from 6-OHDA-Induced Injury

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Online ISSN: 3060-7663 **https://doi.org/10.22103/vcbr.2025.24744.1043**

Article History

Received: *27 January 2025*
Revised: *18 February 2025*
Accepted: *17 April 2025*
Published: *6 June 2025*

Keywords

Dopaminergic neurodegeneration
6-hydroxydopamine
Allopregnanolone
Apoptosis
SH-SY5Y cells

Running Title

Protective Effects of Allopregnanolone on Dopaminergic Neurons

Abstract

Dopaminergic neurodegeneration is associated with oxidative stress, mitochondrial dysfunction, neuroinflammation, and cell death. Allopregnanolone, a multifunctional neurosteroid, is involved in various biological processes within the body. It has been demonstrated that allopregnanolone exhibits protective effects in neurodegenerative conditions. However, its cellular mechanisms in dopaminergic neurons remain incompletely understood. Cell toxicity was induced using 6-hydroxydopamine (6-OHDA), and Cell survival was assessed through the MTT assay. Intracellular reactive oxygen species (ROS) and mitochondrial membrane potential were evaluated using fluorescence probes. Additionally, immunoblotting was employed to measure the levels of apoptosis biomarkers in the cells. Treatment with 6-OHDA significantly decreased cell survival rate and exacerbated the loss of mitochondrial membrane potential. Moreover, there was a notable increase in intracellular ROS levels, the Bax/Bcl-2 ratio, caspase-3, and cytochrome c activity in 6-OHDA-treated cells. Pretreatment with allopregnanolone (250 µM) significantly mitigated these effects in cells exposed to 6-OHDA. Furthermore, the blockade of GABAA receptors by bicuculline significantly reduced the protective effect of allopregnanolone. The data indicate that allopregnanolone's protective effects are attributed to its antioxidant and anti-apoptotic properties, suggesting its therapeutic potential for preventing dopaminergic damage.

How to cite this article: *Hossein Khodadadi, Hossein Jonaidi, Saeed Esmaeili Mahani, Mehran Pourmashayekhi, Parsa Jonaidi. Antioxidant and Anti-Apoptotic Properties of Allopregnanolone in Protecting Dopaminergic Neurons from 6-OHDA-Induced Injury. Veterinary and Comparative Biomedical Research, 2025, 2(2): 20 – 29. http://doi: 10.22103/vcbr.2025.24744.1043*

Introduction

The ongoing loss of nigrostriatal neurons can result in Parkinson's disease (PD), which is characterized by significant movement difficulties. These neurons release the neurotransmitter dopamine (DA) to facilitate communication with their target cells in the striatum. Consequently, the emergence of classic motor symptoms—such as bradykinesia, postural instability, tremor, and rigidity—can be attributed to the degeneration of dopaminergic neurons (1–3).

Even though the cause of PD is only partially understood, various pathological mechanisms such as oxidative stress, neuro-inflammatory processes, mitochondrial and lysosomal dysfunctions, and the formation of pathological inclusions have been proposed as likely contributors (2–4).

The two main divisions of pharmacological treatment for PD are neuroprotective and symptomatic therapies. Neuroprotective therapy aims to slow, block, or reverse disease development. On the other hand, symptomatic strategies try to either counteract DA deficiency in the basal ganglia or block muscarinic receptors (2, 5, 6). However, these therapies slow the underlying loss of dopaminergic neurons. Until now, there has been no definite proof of neuroprotective or disease-modifying therapy (7).

One of the most frequently employed toxins for modeling the degeneration of dopaminergic neurons in PD is 6-hydroxydopamine (6-OHDA). Moreover, 6-OHDA induces the degeneration of dopaminergic neurons, a process that appears to precede the death of these neurons (8). Furthermore, SH-SY5Y cells are human neuroblastoma cells with neural morphological, neurochemical, and electrophysiological properties that have been widely used as an *in vitro* model of PD to examine neuronal damage or death (9,10). Neuroactive steroids, such as progesterone, estrogens, and testosterone, and their metabolites like 5α-dihydroprogesterone and allopregnanolone, play a protective role in the nervous system. These compounds help reduce or prevent the degeneration of neurons and microglial cells, including astrocytes, thereby contributing to decreased mortality (11–13). Mahani *et al*. indicated that dehydroepiandrosterone (DHEA) offers protective benefits against neural damage induced by 6-OHDA. The mechanisms underlying these effects may involve the reduction of neural apoptosis, highlighting the therapeutic potential of this neurosteroid for treating PD (14).

One class of neural messengers is neurosteroids, including allopregnanolone and DHEA. These neurosteroids regulate many neurophysiological processes from ion channel regulation to regeneration of systems (14). Allopregnanolone (5α-pregnane-3α-ol-20-one or Allo) is a natural neurosteroid synthesized through a two-stage transformation. The first stage involves 5α-reductase, which transforms progesterone into 5α-dihydroprogesterone (5α-DHP), and the second stage involves 3α-hydroxysteroid oxidoreductase, which converts 5α-DHP into Allo. It is worth mentioning that the latter stage is also reciprocal, meaning that it can convert Allo into 5α-DHP as well (15, 16).

The analysis of neurosteroid synthesis alterations in the human brain affected by PD has not been fully investigated. It has been reported that there are high levels of Allo in the substantia nigra (SN) and basal hypothalamus of female postmortem subjects (17). It has also been demonstrated that Allo is synthesized and accumulated in the nigrostriatal dopaminergic system (17). In addition, Allo increases the number of dopaminergic neurons in the SN of a triple transgenic mouse model of Alzheimer's disease (AD) (11, 18–22). It has also been reported that the levels of neurosteroids Allo, DHEA, and its sulfate ester DHEA sulfate (DHEAS) decreased with aging or stress (23). Surprisingly, a reduction in the cerebrospinal fluid (CSF) levels of Allo and 5α-DHP has been reported in PD and AD patients (24–26). It seems that such neurosteroids have critical roles in the induction of the disease.

The neurosteroid Allo is a positive allosteric modulator that potently modulates ionotropic GABAA receptors (27). Since neurosteroids can act as neuroprotective agents against various neurodegenerative diseases, they have gained considerable attention lately (27). A variety of beneficial effects of Allo, such as neuroprotective, neurogenic, analgesic, antidepressant, anesthetic, and anxiolytic actions, have been reported in both human and experimental models (28–30). The ability of Allo to modulate different ion channels and receptors, including GABAA, L- or T-type calcium channels, and also the pregnane X receptor (PXR) or membrane progesterone receptors, supports the above-mentioned neuroactive properties (31–33).

It has been reported that Allo has anti-apoptotic and neuroprotective effects in experimental diabetic neuropathy (34). In addition, the pro-cognitive property of Allo has been demonstrated in the 6-OHDA-induced rats' model of PD (34). Allo can inhibit striatal inflammation and apoptosis and preserve pre- and post-synaptic proteins and may maintain the synaptic integrity in the nigrostriatal pathway of PD rats (35).

The present study was designed to examine whether Allo has protective effects against apoptosis mediated by 6-OHDA in SH-SY5Y cells, an *in vitro* model of PD, and to find the possible mechanism of its neuronal protection.

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 Lifesciences Inc. (Gyeonggi-Do, South Korea). 3-[4, 5-dimethyl-2-thiazolyl]-2,5- diphenyl-2-tetrazolium bromide (MTT), 6-OHDA, 2,7-dichlorofluorescein diacetate (DCFH-DA), Rhodamine 123, bicuculline, and Allo were purchased from Sigma-Aldrich (St Louis, MO, USA). Primary monoclonal anti-β-actin and polyclonal anti-caspase-3 antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Primary polyclonal anti-Bax, anti-Bcl-2, and anti-cytochrome c antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cell Culture

Human neuroblastoma SH-SY5Y cells were obtained from the National Cell Bank of Iran (NCBI)-Pasteur Institute of Iran (Tehran, Iran). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing fetal bovine serum (10%), penicillin (100 U/ml), and streptomycin (100 µg/ml). The cells were incubated at 37 ºC in a CO2 incubator (5% CO2 atmosphere). After two passages, SH-SY5Y cells were plated at a density of 5000 cells per well in a 96-well microplate for biochemical assays. Additionally, the cells were cultured in a 6-well plate for 24 hours to allow for attachment and growth. Subsequently, the cells were treated with Allo at concentrations of 50, 100, 250, and 500 µM. After a 30 min incubation, 6-OHDA (150 µM) was added, and the cells were incubated for an additional 24 hours. Cell viability was then evaluated using the MTT test. An effective concentration of Allo (250 µM) was selected for subsequent tests. Bicuculline was added at doses of 50 or 100 µM 10 min before Allo treatment to evaluate its impact on the protective effect of Allo against 6-OHDA-induced cell damage.

Cell Viability Analysis

Cell survival was evaluated by the reduction of 2-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan (30). MTT was dissolved in PBS and added to the culture at a final concentration of 0.5 mg/ml. Following an additional two-hour incubation at 37 ºC, the media were carefully removed and DMSO (100 µl) was added to each well. The optical density (OD) values were determined at 490 nm using an automatic microplate reader (ELX 808, BioTek, USA). Each experiment was performed six times independently. Results were shown as percentages of the control.

Measurement of Intracellular Reactive Oxygen Species (ROS) Formation

Intracellular ROS were assayed using the DCFH-DA probe and fluorescence spectrophotometry. DCFH-DA can convert to the highly fluorescent dichlorofluorescein in the presence of an appropriate oxidant. Different groups of cells were incubated with 1 mM DCFH-DA in the dark for 10 min at 37 ºC. Subsequently, the cells were washed three times with PBS and immediately analyzed on the fluorescence plate reader (FLX 800, BioTek, USA). The fluorescence intensity of cells in 96-well microplates was quantified at an excitation of 485 nm and an emission of 538 nm. Each experiment was performed six times independently. Results were expressed as the fluorescence percentage of control cells (31).

Measurement of Mitochondrial Membrane Potential

The mitochondrial membrane potential was determined with rhodamine 123, which preferentially transfers into the active mitochondria based on the highly negative mitochondrial membrane potential. Depolarization of the mitochondrial membrane potential leads to the loss of rhodamine 123 from the mitochondria and a consequent reduction in intracellular fluorescence. Following treatment of the cells with drugs (as mentioned above), rhodamine 123 (10 µM) was added. After a 30 min incubation at 37°C, the cells were washed and analyzed on the fluorescence plate reader (FLX 800, BioTek, USA). The fluorescence intensity was quantified at an excitation of 540 nm and an emission wavelength of 570 nm (32).

Immunoblot Analysis

SH-SY5Y cells were homogenized in ice-cold buffer containing10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 0.1% SDS, 0.1% Na-deoxycholate, and 1% NP-40 supplemented with protease inhibitors (1 mM phenyl-methyl-sulfonyl fluoride, 2.5 µg/ml of leupeptin, 10 µg/ml of aprotinin), and 1 mM sodium orthovanadate. The homogenate was centrifuged at 14000 × g for 15 min at 4ºC.

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 (40 µg) were separated by electrophoresis on a 9% SDS–PAGE gel and transferred to PVDF membranes (Roche, Germany). After overnight blocking at 4 ºC with 5% non-fat dried milk in tris-buffered saline with Tween 20 (blocking buffer, TBS-T, 150 mM NaCl, 20 mM Tris–HCl, pH 7.5, 0.1% Tween 20), the membranes were probed with primary antibodies (1:1000 dilution overnight at 4 ºC). Following washing in TBS-T (three times, each time 5 min), the blots were incubated for 60 min at room temperature with 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 and exposed to Lumi-Film chemiluminescent detection film (Roche, Germany). ImageJ analyzing software was used to analyze the intensity of the expression. β-Actin immunoblotting (1:1000) was used as a control for loading. The immunoblot experiments for each protein were performed 3–4 times independently.

Immunoblot Analysis

The results were expressed as mean ± SEM using SPSS software (version: 23). The band densities for the proteins Bax, Bcl-2, caspase-3, cytochrome c, and β-actin were obtained via densitometry. These values were normalized to β-actin (each protein/β-actin ratio). Mean differences in these ratios, as well as for factors MTT, intracellular ROS, and mitochondrial membrane potential among experimental groups, were determined by one-way ANOVA, followed by Tukey tests. $P < 0.05$ was considered significant.

Results

Analysis of Cell Viability

To investigate the protective effects of Allo in 6-OHDA-treated SH-SY5Y cells, 150 µM of 6-OHDA was chosen to induce cell damage based on previous studies. The results indicated that treatment with various concentrations of Allo for 24 hours had no significant effect on cultured SH-SY5Y cell survival (Figure 1A), suggesting that Allo exhibits no toxic effect on these cells.

As shown in Fig. 1B, Allo at concentrations of 100, 250, and 500 µM significantly inhibited 6-OHDA-induced toxicity after 24 hours. The maximum effect was observed at a concentration of 250 µM. This drug could not prevent cell damage in a concentration of 50 µM. Therefore, 250 µM was selected for use in the next steps of the experiment.

In addition, to evaluate the role of GABAA receptors, the cells were pretreated with bicuculline as a competitive GABAA receptor antagonist. Notably, the Allo protection was not observed in the presence of 100 µM bicuculline (Figure 2).

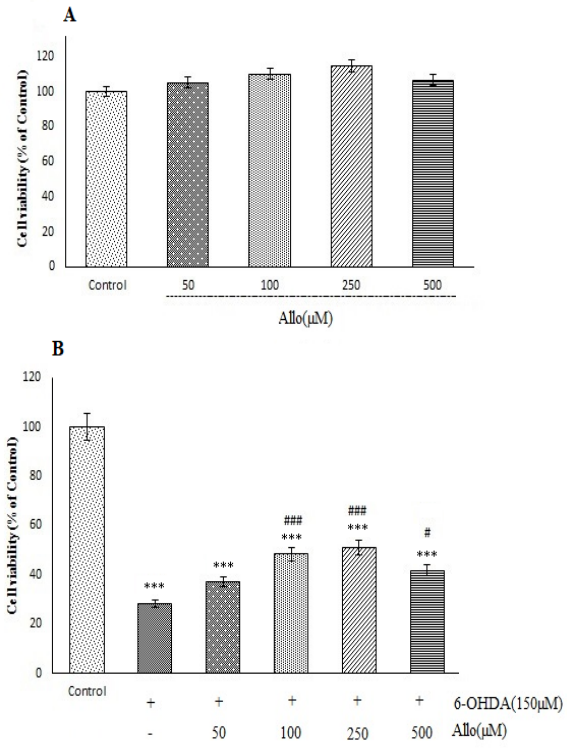

Figure 1. Effects of allopregnanolone (Allo) on naïve (A) and 6-OHDA-treated (B) SH-SY5Y cell viability. Different doses of Allo did not show any toxic effect on SH-SY5Y cells (A). Allo was added 30 min before 6-OHDA (150 µM) treatment. Data are expressed as mean ±SEM; n = 5–6 wells for each group ***$p<0.001$ vs. control cells. $^{\#}p<0.05$ and $^{\#\#\#}p<0.001$ vs. 6-OHDA treated cells.

Measurement of Intracellular ROS in SH-SY5Y Cells

The intracellular ROS levels were measured in control cells, 6-OHDA-treated cells, and 6-OHDA-treated cells with 250 µM Allo. Exposure of SH-SY5Y cells to 6-OHDA led to an increase in ROS levels compared to control cells. The increase in intracellular ROS was significantly attenuated ($p<0.001$) by the Allo treatment (Figure 3).

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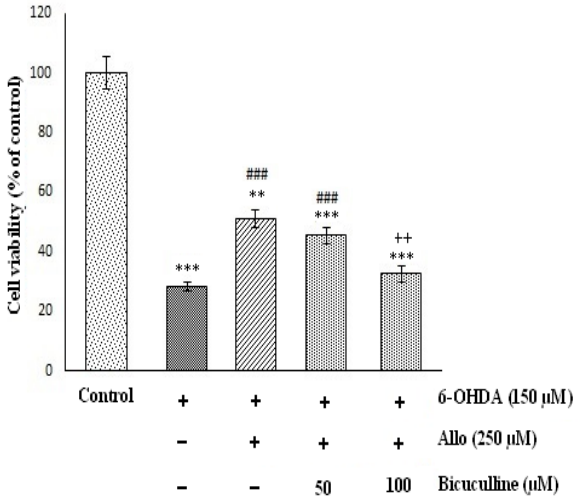

Figure 2. The effects of bicuculline on the protective effect of allopregnanolone (Allo) against 6-OHDA-induced damage in SH-SY5Y cells. Allo was added 30 min before 6-OHDA. Bicuculline was added 10 min before Allo. Data are expressed as mean ± SEM; n = 5–6 wells for each group. **p<0.01 and ***p<0.001 vs. control cells. $^{###}p$<0.001 vs. 6-OHDA-treated cells. ^{++}p<0.01 vs. 6-OHDA+Allo treated cells.

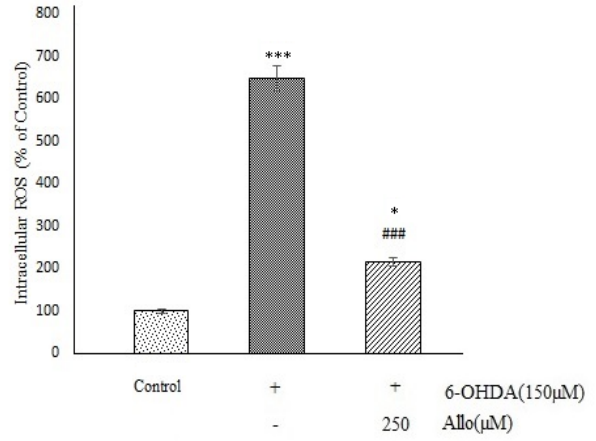

Figure 3. The effect of allopregnanolone (Allo) on 6-OHDA-induced increase in intracellular ROS levels. Cells were pretreated with Allo (250 µM) for 30 min and then incubated with 150 µM 6-OHDA for 24 hours. Data are expressed as mean ± SEM; n = 5–6 wells for each group. *p<0.05 and ***p<0.001 vs. control cells. $^{###}p$<0.001 vs. 6-OHDA-treated cells.

Determination of Mitochondrial Membrane Potential in SH-SY5Y Cells

As shown in Fig. 4, mitochondrial membrane potential was dramatically decreased (p<0.001) in 6-OHDA-treated SH-SY5Y cells. The decrease in mitochondrial membrane potential was attenuated by Allo treatment (Figure 4).

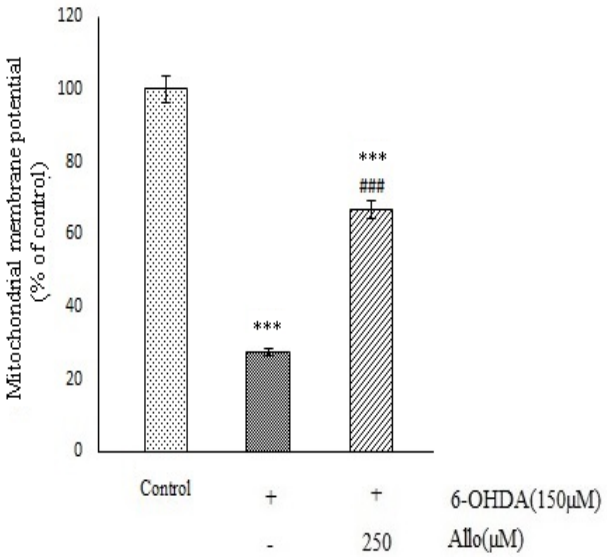

Figure 4. The impact of allopregnanolone (Allo) on 6-OHDA-induced reduction of mitochondrial membrane potential. Data are expressed as mean ± SEM; n =5-6 wells for each group ***p<0.001 vs. control cells. $^{###}p$<0.001 vs. 6-OHDA-treated cells.

Western Blot Analysis of Bax, Bcl-2, Caspase-3 and Cytochrome C in SH-SY5Y Cells

To elucidate the mechanism underlying Allo's protective effects against 6-OHDA-induced cell death in SH-SY5Y cells, the expression levels of Bax, Bcl-2, activated caspase-3, and the release of cytochrome c were analyzed. The cells were exposed to control, 6-OHDA media, and 6-OHDA media plus Allo for 24 hours. The level of Bax protein was significantly increased in 6-OHDA-treated cells, while the Bcl-2 protein decreased. Consequently, there was a significant increase in the Bax/Bcl-2 protein ratio in the cells exposed to 150 µM 6-OHDA compared to control cells (p<0.001). Incubation with Allo (250µM) incubation significantly decreased the Bax/Bcl-2 ratio (Figure 5A).

In addition, caspase-3 levels in 6-OHDA-treated SH-SY5Y cells were increased compared to control cells (p<0.01). Treatment with Allo significantly reduced 6-OHDA-induced activation of caspase-3 (Figure 5B).

As shown in Fig. 5C, there was a significant increase in cytochrome c protein levels in SH-SY5Y cells exposed to 6-OHDA for 24 hours (P < 0.001). The increase in cytochrome c was attenuated in SH-SY5Y cells that received Allo plus toxin (Figure 5C).

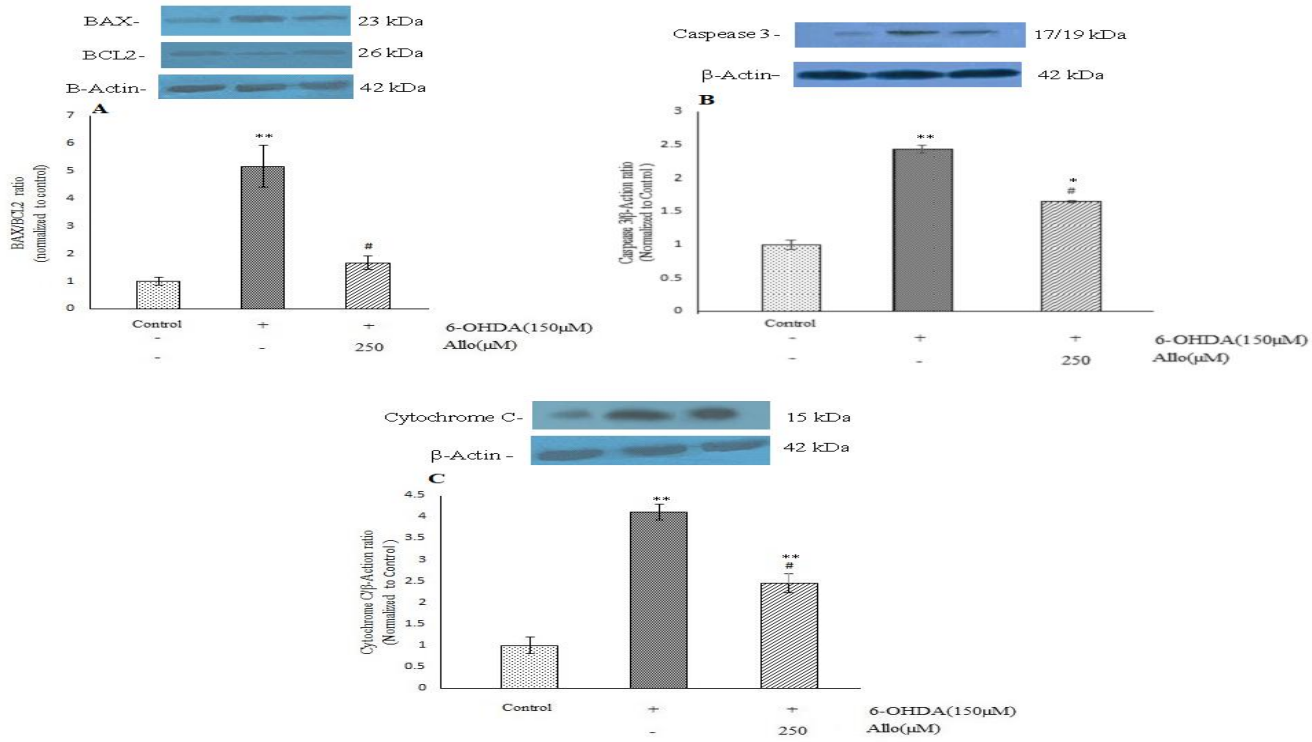

Figure 5. Effect of 250 µM allopregnanolone (Allo) on the 6-OHDA-induced Bax and Bcl-2 protein expression (A), caspase-3 activation (B), and Cytochrome c release (C) in SH-SY5Y cells. The parameters were assayed by immunoblotting. β-actin was used as an internal control. **p*<0.05 and ** *p*<0.01 significantly different vs. control cells. # *p*<0.05 vs. 6-OHDA-treated cells.

Discussion

The neurosteroids are involved in regulating multiple processes in the brain. In the present study, the potential protective effect of Allo was investigated in a cellular model of PD. Allo incubation significantly preserved the viability of SH-SY5Y cells and further down-regulated apoptotic signals in the 6-OHDA-induced cell apoptosis, suggesting that Allo may possess neuroprotective and neuroresorative properties.

It has been documented that oxidative stress plays a crucial role in the pathogenesis of PD (33, 34). The damage to lipids, proteins, and DNA, and the cascade of events that lead to dopaminergic neurodegeneration in PD can be attributed to oxidative stress. The data indicated the suppressive effects of Allo against 6-OHDA-induced intracellular ROS production. Recently, the antioxidant activity of Allo has also been reported in high glucose-induced toxicity in naive and NGF-treated (neuron-like) PC12 cells (27).

Allo has been suggested to have a remarkably protective effect on sleep deprivation-induced anxiety-like behavior and oxidative damage in mice (35). An earlier study yielded noteworthy results regarding Niemann-Pick C disease (NPC). It has been reported that neurons and neuroglia expressing steroidogenic enzymes are lost in NPC mice, and the synthesis of Allo was significantly reduced at birth and further decreased over time in these animals (36). Using Allo to treat the NPC mice led to the following advantages: an increase in lifespan, a delay in the onset of neurological impairment, a significant increase in Purkinje and granular cell survival in the cerebellum, and a reduction in cortical ganglioside accumulation (37).

The neuroprotective effects of steroid hormones could be exerted through both genomic and non-genomic mechanisms by regulating the expression of pro- and anti-apoptotic factors, intracellular signaling pathways, neurotransmission, antioxidative, and anti-inflammatory processes (37). Not surprisingly, the mentioned neurosteroid with antioxidant activity could be protective in an *in vitro* model of PD.

Exogenous Allo as a therapeutic strategy targets DA release modulation, the restoration of neuroprotection, modulation of basal GABAergic tone, and neuroregeneration (38-41).

It is possible to modulate these effects, among others, through GABAergic transmission neuromodulation and less directly by modifying neurotrophin expression.

This concept is reinforced by the evidence that GABA activity can down-regulate inducible neurotrophins (42).

Recent studies have shown that Allo can modulate anxiety, depression, seizure activity, sedative-hypnotic activity, and the immune system within the mammalian brain. Additionally, other studies have proven that Allo not only promotes the neurodegenerative system but also modifies the course of neurodegenerative disease (43).

ROS, such as H2O2, O2−, and •OH, significantly mediate oxidative stress. It has been documented that ROS produced by 6-OHDA can interact with biologically targeted molecules, induce lipid peroxidation, and damage mitochondrial membranes (44). The antioxidant properties of neurosteroids have been demonstrated in several models of oxidative stress.

Considering the important role of caspase-3, as an executor of apoptosis, we decided to investigate the effect of Allo on caspase-3 activation in 6-OHDA-treated SH-SY5Y cells. A variety of scientific reports have concluded that neuronal death in PD is closely associated with caspase-3 activation and consequent apoptosis (36). The activation of caspase-3 can be promoted by mitochondrial damage and cytochrome c release (37, 38). Besides receptor-mediated apoptosis, cytotoxic drugs such as 6-OHDA can activate another pathway. Stress-induced apoptosis involves a cascade of events, beginning with increased mitochondrial membrane permeability, followed by cytochrome c release, apoptosome formation, activation of caspase-9, and ultimately caspase-3 activation, which drives the downstream processes leading to cell death. It has been indicated that Bcl-2 family proteins can regulate the release of cytochrome c. Anti-apoptotic members of the Bcl-2 family, located in the outer membrane of mitochondria, would prevent cytochrome c release, while on the other hand, the translocation of pro-apoptotic members to mitochondria leads to apoptosis induction either by forming pores in mitochondria directly or by antagonizing the anti-apoptotic proteins (39).

According to our results, 6-OHDA-induced cell damage in SH-SY5Y cells increased cytochrome c and caspase-3 levels, and Allo could decrease the mentioned factors. The present study proposes that Allo's protective effects against 6-OHDA may be mediated by its antioxidant and anti-apoptotic activities through the reduction of ROS and inhibition of cytochrome c and caspase-3 activation. We have also previously reported that Allo can ameliorate the 6-OHDA-induced motor impairment and elicit anti-apoptotic and anti-inflammatory effects in rats (35).

Investigating the *in vitro* model of PD raises a significant question regarding the mechanisms by which Allo exerts its protective effects at both cellular and molecular levels. Based on previous research, the action of PKC-ε is dependent on the phosphorylation of the GABAA receptor at the level of Serine 327 of the γ2 subunit (35), which in turn regulates the receptor's response to allosteric modulators. Additionally, PKC-ε kinase controls GABAA receptor trafficking through the N-Ethylmaleimide-Sensitive Factor (NESF) signaling pathway. Variations in Allo efficacy observed in *in vitro* studies suggest that activating PKC-ε can decrease the cell surface expression of these receptors (35). Charalampopoulos *et. al.* reported that Allo activates cAMP response element-binding protein, NF-κB, upstream effectors of anti-apoptotic Bcl-2 protein expression, and PKCα/β, which is a posttranslational activator of Bcl-2 protein (23).

In the current study, the previously mentioned mechanisms might play a role in the observed anti-apoptotic effect of Allo. However, given the limited clinical relevance of cell culture models, complementary in vivo data are needed to strengthen the significance of the in vitro findings.

Some studies have reported that administration of Allo in rats after TBI decreases the apoptotic DNA fragmentation and the expression of caspase-3 and Bax, which are pro-apoptotic proteins. Additionally, similar to progesterone, Allo also enhances animal behavior (40,41). The variations in the GABAA receptor subunit composition determine the effect of neurosteroid Allo on this receptor (42). The protective and anti-apoptotic effects of Allo may be mediated by GABAA receptors. However, there is still a call for more studies to clarify the role of detailed signaling or different signaling pathways within the Allo's protective effect. It has been previously reported that Allo prevents neuropathic pain and motor deficits by inhibiting GABAA receptor down-regulation in the spinal cord of diabetic rats (42).

Conclusion

Our results indicate that Allo can protect SH-SY5Y cells against apoptosis induced by 6-OHDA. It seems that Allo's protective ability might be mediated via its antioxidant properties and modulation of the apoptosis pathway. However, further studies are needed to explore the details of its protective pathway in this phenomenon.

Acknowledgements

The authors would like to express their gratitude to the Faculties of Veterinary Medicine and Basic Sciences at Shahid Bahonar University of Kerman, Kerman, Iran.

Author Contributions

Hossein Khodadadi: Conceptualization, Methodology, Investigation. **Hossein Jonaidi:** Conceptualization, Methodology, Investigation, Supervision, Writing – review & editing **Saeed Esmaeili-Mahani** Supervision, Methodology, Investigation, **Mehran Pourmashayekhi** Methodology, Validation, Visualization, Writing – original draft. **Parsa Jonaidi:** Methodology, Investigation, Data curation

Data Availability

All data generated or analyzed during this study are included in this published article.

Ethical Approval

All animal experiments were assessed and approved by the Research Ethics Committees of Faculty of Shahid Bahonar University of Kerman, Kerman, Iran.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Consent for Publication

Not applicable.

Funding

The present study was carried out with the financial assistance provided by the Research Deputy of Shahid Bahonar University of Kerman.

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