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Cyclotide Offers Neuroprotection Against 6-OHDA-Induced Toxicity in SH-SY5Y Neuroblastoma Cells by Downregulating mRNA Expression of MAO/α-Synuclein/LRRK2/PARK7/PINK1/PTEN Genes

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Abstract:
Dopaminergic loss in the midbrain's substantia nigra affects the patient's movements and causes postural instability in Parkinson's disease (PD). Alpha-synuclein protein accumulation and dementia symptoms are hallmarks of the condition. Monoamine oxidases A and B (MAO A and B), leucine-rich repeat kinase 2 (LRRK2), phosphate and tensin homolog (PTEN), PTEN-induced putative kinase 1 (PINK1), and PARK7 (deglycase 1 (DJ-1)) production are also enhanced by the disease. Cyclotide has been widely cited as a neuroprotective, anti-inflammatory, and antioxidant. As a result, we investigated how downregulation of the mRNA expression of PD pathological proteins like alpha-synuclein, MAO A and B, LRRK2, PTEN, PINK1, and PARK7 (deglycase 1 (DJ-1)) by Cyclotide protected SH-SY5Y neuroblastoma cells from 6-OHDA-induced toxicity. The study found that Cyclotide treatment decreased the pathology marker protein mRNA expression that had been elevated by 6-OHDA. This was in comparison to the positive control, amantadine (AMA), which is now commonly used to treat PD symptoms. Consequently, the study suggests that Cyclotide might be an effective treatment for the neurotoxicity caused by 6-OHDA in SH-SY5Y neuroblastoma cells.

Keywords: Parkinson’s disease. 6-Hydroxydopamine. Neuroblastoma. Oxidative stress. Cyclotide. Amantadine

Introduction

Due to its worsening of the patient's regular movements and stable postural abilities, Parkinson's disease ranks second among all known movement disorders. The patients' extensive loss of dopaminergic cells in the substantia nigra is the exact disease pathology that follows this disease. Because it is thought to be an age-related disorder, incidences were more common in people over 60, at approximately 15 per one lakh. Gourie-Devi (2014) [1] estimates that the prevalence in India ranges from 6 to 53 per 100,000 people. Aside from a few bioactive plant secondary compounds like polyphenols, flavonoids, and coumarins that were used as medications during the disease's preclinical trial stages for pact PD symptoms, there has been no effective treatment for the disease since it was first described. "Medhya medications," a group of home-made prescriptions in the Ayurvedic medication system, are known for their effects on the sensory system [2]. These "Medhya medications," which are mentioned in Ayurvedic texts, are said to improve mental capacities like learning and memory, increase impact, and prevent temperamental issues [3]. Clitoria ternatea is one such potential medicinal herb that is used as a crucial component in a brain tonic called medhya rasayan for treating neurological diseases. Ayurveda is a well-known traditional medicine that has been practised in India since ancient times. By highlighting the plant's importance as a brain medication, this work supports Indian medicine. *Clitoria ternatea,* is distinct among other herbs and has the property of being a good brain drug [4]. Additionally, *C. ternatea* has been widely used in traditional medicine, particularly as a supplement to enhance cognitive functions and alleviate symptoms of numerous ailments including fever, inflammation, pain, and diabetes [5]. Cyclotide, an active compound from *Clitoria ternatea* has been evaluated for antiparkinson’s activity in this study. The analysis of cyclotide insilico study was studied earlier [6] which paved the way for the

present study. Amantadine, an amino adamantane, has improved neuroleptic-induced tardive dyskineias by improving parkinsonian activity [7] served as a positive control in this study. The oxidative stress-induced neuronal death is a major factor in the pathogenesis of Parkinson's disease (PD) [8]. According to Gonzalez-Hernandez et al., [9] the mechanism by which 6-OHDA produces ROS and accumulates in the cytosol results in neurotoxicity.

In addition, 6-OHDA has been identified as a direct respiratory chain inhibitor of mitochondrial complex 1 [10] that caused mitochondrial LDH to be released. Monoamine oxidase A and B equilibrium in the mitochondrial membranes is altered as a result. MAO concentrations in the cytosol rise as a result of this disruption of the mitochondrial membrane. In addition to MAO metabolism, it is hypothesized that PD is caused by many excitatory or inhibitory protein dysfunctions that contribute to the cell signalling pathways. Dopamine is metabolized by intraneuronal monoamine oxidase A (MAO A) and by astrocyte and glial MAO A and MAO B. It has been discovered that proliferative cytotoxicity and blocking intracellular protein clearance are both caused by inefficient ubiquitination that results in unpleasant misfolding or expression of alpha-synuclein as well as degradation by the proteasome and inefficient ubiquitination. PINK1 and PTEN are mitochondrial function-associated PD-associated genes that accelerate protein damage and neurodegeneration by producing an excessive amount of reactive oxygen species (ROS). Although the primary heritable cause of Parkinson's disease (PD) is mutations in the leucine-rich repeat kinase 2 (LRRK2) protein, the function of this multidomain-containing protein is unclear. Despite this, there are still reports that show that control people have more non-mutant LRRK2 proteins. Another PD-related protein, DJ-1 (PARK7), increases its expression levels in neurons and glial cells [11], as a result of an oxidative trauma, and responsive astrocytes in sporadic PD exhibit overexpression of DJ-1 [12]. Furthermore, mitochondrial complex I deregulation and inflammatory responses diminished astrocyte-assisted neuronal protection against oxidative strain in PARK7 knockout mice [13]. Keeping amantadine as a standard, we monitored Cyclotide's effect on mRNA expression of PD-attributing genes, particularly MAO, alpha-synuclein, LRRK2, PARK7, PINK1, and PTEN genes, in SH-SY5Y cells to determine its role in preventing 6-OHDA-induced toxicity.

Materials and Methods

Cell Culture, Maintenance, and Cytoprotective Assays

In brief, the human neuroblastoma cancer cell line SH-SY5Y was plated in a 96-well plate with HAM's medium containing 10% FBS at a concentration of 10^5 cells per well. To make the cells adhere to the surface, they were incubated at 37 °C for 24 hours in 5% CO_2 and 95% O_2. After that, the medium was removed from the cell culture plate, washed with PBS, and pre-treated with fresh medium containing AMA and cyclotide in different concentrations (2, 4, 6, 8, 10, 20, 30, 40, 50 mM, respectively). After 3 h of the expansion of drugs, 250 µM of 6-OHDA (the focus was fixed later a few pretrial of enlistment utilizing 6-OHDA focuses going from 50 to 450 µM, broke up in 0.9% clean saline having 0.02% ascorbic corrosive) was added. The sterile MTT (trimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; 5 mg/ml; 10 µl) arrangement was enhanced to person wells, after 48 h of medication treatment [14]. After a further 4 h of incubation at 37 °C, 100µl of DMSO was added to the cells, and the crystals formed were gently resuspended. Each well's absorbance at 570 nm was measured with a microplate reader. The percentage of growth inhibition was calculated as A_{570} of treated cells divided by A_{570} of control cells x 100.

LDH Assay

According to Wolff et al., [15] the LDH leakage assay was carried out using cell-free supernatant collected from the wells prior to the addition of MTT. This was then well mixed with 2.7 milliliters of potassium phosphate buffer, 0.1 milliliters of 6 M NADH solution, and 0.1 milliliters of sodium pyruvate solution. A spectrophotometer was used to measure the decrease in optical density at 340 nm at 25°C. Activity was calculated as volume of activity (units/mg protein/ml) = (OD_{0min} – OD_{5min}) 3ml dilution factor / 6.2 x 0.1 for the blank that was prepared by substituting enzyme dilution buffer for the sample.

ROS Staining (DCF-DA)

The 96-well plates were seeded with 5 – 10 x 10^{-3} SH-SY5Y cells per well. The intracellular ROS were counted by fluorescence with 2′,7′- dichlorofluorescein diacetate (DCF-DA), which is deacetylated by viable cells in 2′,7′- dichlorofluorescein (DCF) by hydrogen peroxide, making the compound florescent. The cells in the wells were treated with 6-OHDA (250 M), amantadine (4 mM) + 6-OHDA (DCF fluorescence was imaged in a fluorescent microscope (Olympus CK x 41, with Optika Pro5 CCD Camera), with a blue filter to view the green fluorescence, and fluorescence was estimated using flurometry in Qubit 2.0 fluorimeter [16] after the cells were washed with PBS and treated for one hour at 37 °C, 5% CO2 and incubated for 6 hrs.

Flow Cytometric Analysis
Each flask contained 2 x 10^6 SY-SY5Y cells, which were allowed to adhere for 24 hours before being treated with 6-OHDA (250 M), amantadine (4 mM) + 6-OHDA (250 M), and cyclotide (4 mM) + 6-OHDA (250 M) for 48 hours. Following the removal of the medium, approximately 1 x 10^6 cells were washed with binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$). After that, cells were washed with binding buffer, incubated with PI for ten minutes, and kept on ice without being exposed to light to conduct flow cytometry, recording 15,000 events per test. According to Wang et al., [17] annexin V and PI emissions were detected using the FL1-H and FL2-H channels of a Calibur flow cytometer (Becton Dickinson Immunocytometry System, San Jose, CA) at 525 and 575 nm emission filters, respectively with a few alterations.

PCR Analysis
TRIzol was utilized to extract total RNA from the treated and control cells in accordance with the manufacturer's instructions. Using the Prime-Script RT reagent kit (TaKaRa, Tokyo, Japan), total RNA from each sample was reverse transcribed into cDNA. The SYBR Premix ExTaq (TaKaRa, Tokyo, Japan) and mRNA-specific primers for the genes listed in Table 1 were used in the qRT-PCR procedure. For each sample, GAPDH expression was used as a normalization for the relative level of mRNA. The $2^{-\Delta\Delta Ct}$ method was used to study gene expression [18]. Table 1 displays the oligonucleotide product size.

Table 1 The list of primers with their end product length

Gene	Primer details	End product (bp)
MAO A	Forward primer ACTGCTAGGCGGTTTGCTTA Reverse primer CCATTATCCGTTCGCTCACT	190 bp
MAO B	Forward primer GACCGAGTGAAGCTGGAGAG Reverse primer CAGAGGGGGATTGAAGTGAA	156 bp
Synuclein (SNCA)	Forward primer TGACAAATGTTGGAGGAGCA Reverse primer TGTCAGGATCCACAGGCATA	177 bp
LRRK2	Forward primer TCAGCTTGTTGTTGGACAGC Reverse primer ACTGCGTGAGGAAGCTCATT	174 bp
PTEN	Forward primer ACCGCCAAATTTAATTGCAG Reverse primer TTCGTCCCTTTCCAGCTTTA	160 bp
PINK1	Forward primer ACGTTCAGTTACGGGAGTGG Reverse primer GGCTAGTCAGGAGGGAAACC	171 bp
PARK7	Forward primer GGGTGCAGGCTTGTAAACAT Reverse primer GGACAAATGACCACATCACG	192 bp

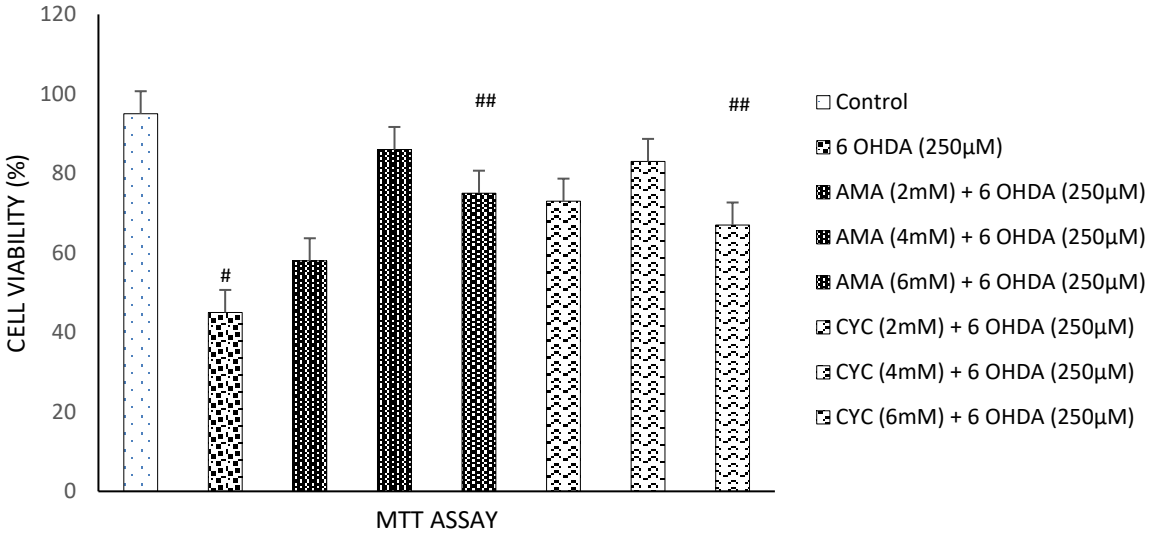
Statistical Analysis
Factual examinations were performed, and every one of the qualities are communicated as mean ± standard deviation (SD) and investigated utilizing one-way investigation of fluctuation (ANOVA) trailed by Tukey's post hoc test (SPSS 20 variant). A value was considered statistically significant when $P < 0.05$.

Results

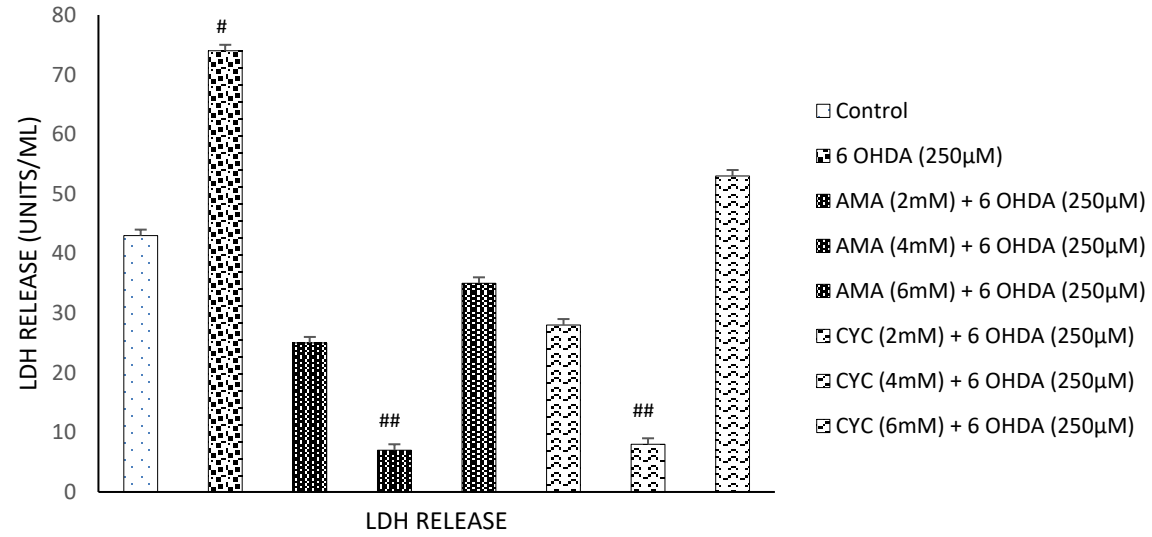
Effect of cyclotide on 6-OHDA-Induced Cytotoxicity in SH-SY5Y Cells
The cytoprotective assay in SH-SY5Y neuroblastoma cells revealed that cyclotide had an IC50 value of 30 mM, whereas AMA had an IC_{50} value of 50 mM, indicating that cyclotide had better therapeutic effects at lower doses than the positive standard (Fig. 1a). Since the inducer (6-OHDA) had an IC_{50} value of 250 M, the MTT assay and the LDH assay used 250 M of inducer in addition to 2, 4, and 6 mM of AMA and cyclotide, respectively. When compared to control cells, the 4 mM treatment of both AMA and cyclotide resulted in cell survival of 83.54% and 80.33%, respectively ($P < 0.01$), while inducer alone-treated cells sustained cell damage of approximately 48.45% ($P < 0.01$) (Fig. 1b).

Fig. 1 Effect of cyclotide on 6-OHDA-induced cytotoxicity in SHSY5Y cells.

a) MTT assay.



b LDH assay.



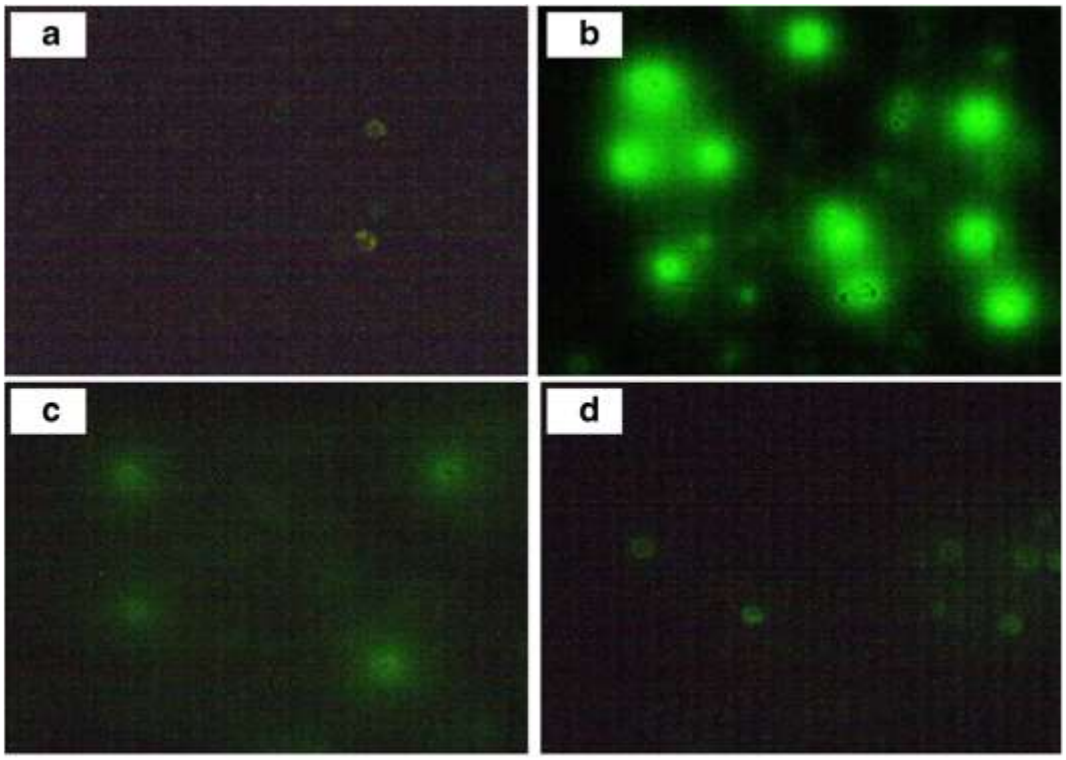
Data represents mean ± SD. #P < 0.01; ##P < 0.01; induced cells were compared with control; 6-OHDA + CYC/AMA (4 mM) were compared with 6-OHDA-induced cells by one-way ANOVA with Tukey's post hoc test.

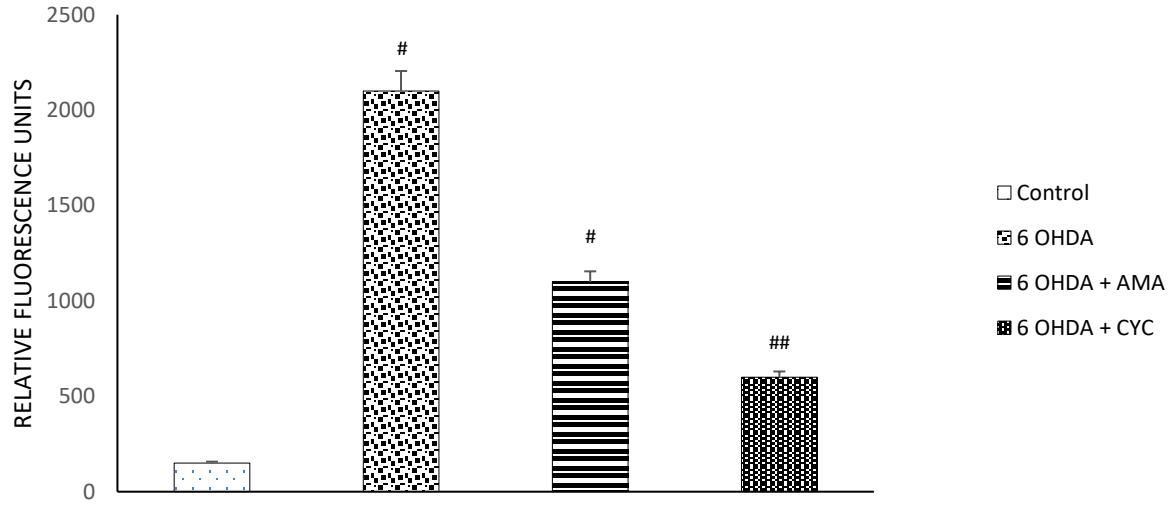
Effect of cyclotide on 6-OHDA-Induced Oxidative Stress in SH-SY5Y Cells

When compared to control cells, the ROS production in 6-OHDA-administered cells was found to be significantly higher (P < 0.001) (Fig. 2). However, when compared to the 6-OHDA-unaided cells, the cyclotide-treated cells experienced a significant decrease in ROS production (P < 0.01) As with cyclotide-treated cells, ROS production was also reduced by the AMA treatment.

Fig. 2 Effect of cyclotide on 6-OHDA-induced oxidative stress in SH-SY5Y cells.

Control cells. b Inducer alone. c. 6-OHDA + 4 mM AMA. D. 6-OHDA + 4 mM CYC. e. Graph showing the relative fluorescence exhibited by the cells after staining with fluorescent dye.



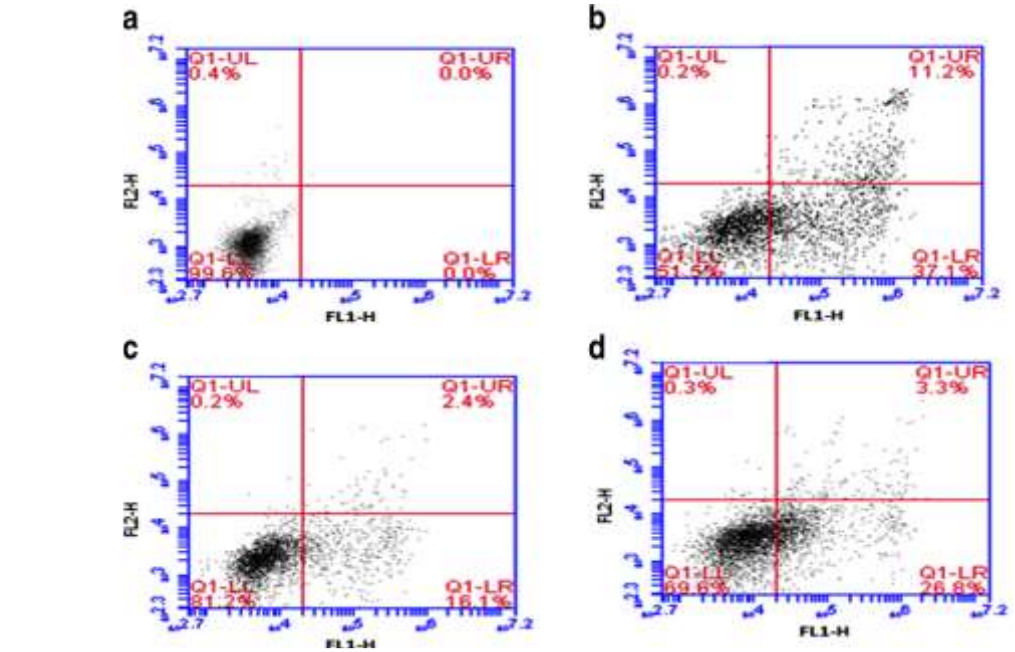


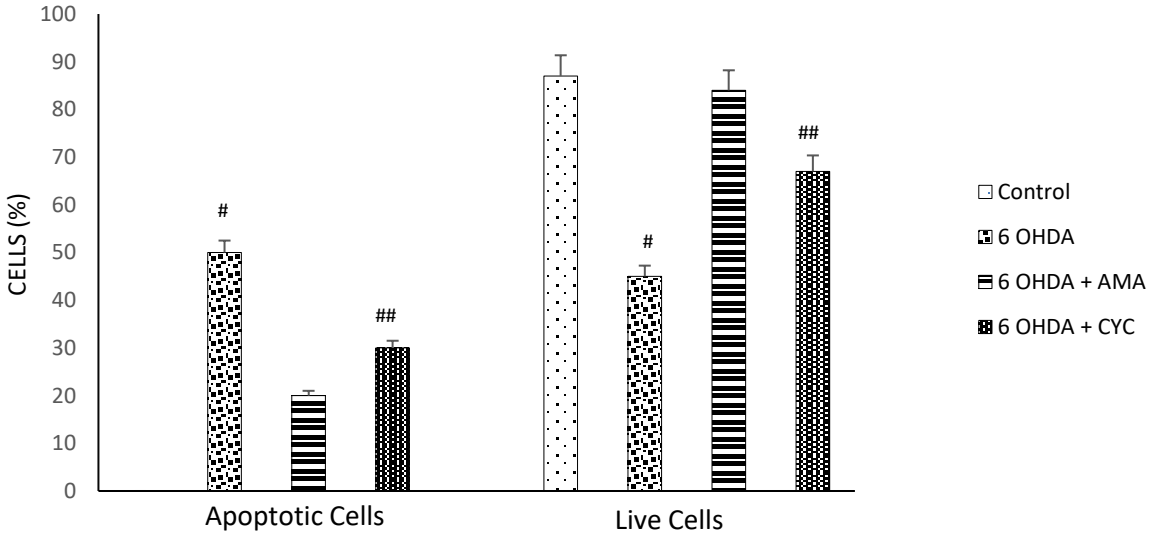
Data represents mean ± SD. #P < 0.01; ##P < 0.01; induced cells were compared with control; 6-OHDA + CYC (4 mM) were compared with 6-OHDA-induced cells by one-way ANOVA with Tukey's post hoc test.

Effect of cyclotide on 6-OHDA-Induced Apoptosis in SH-SY5Y Cells

The results showed that after 48 hours of incubation with 6-OHDA, the proportion of early apoptosis increased significantly ($P < 0.05$), and cyclotide had a beneficial effect by decreasing early apoptosis of cells ($P < 0.05$) as much as AMA treatment (Fig. 3).

Fig. 3 Effect of cyclotide on 6-OHDA-induced apoptosis in SH-SY5Y cells. a Control cells. b Inducer alone. c 6-OHDA + 4mM AMA. d 6-OHDA + 4 mM CYC.





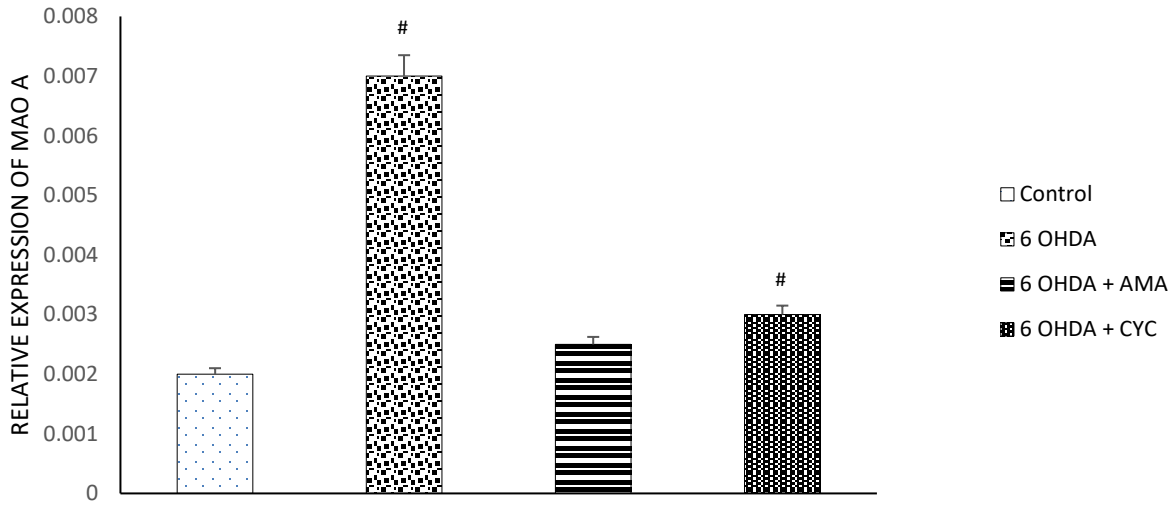
Data represents mean ± SD. #P < 0.01; ##P < 0.01; induced cells were compared with control; 6-OHDA + CYC (4 mM) were compared with 6-OHDA-induced cells by one-way ANOVA with Tukey's post hoc test

Effect of cyclotide on 6-OHDA-Induced Changes in mRNA Expression of Monoamine Oxidases and Alpha-Synuclein in SH-SY5Y Cells

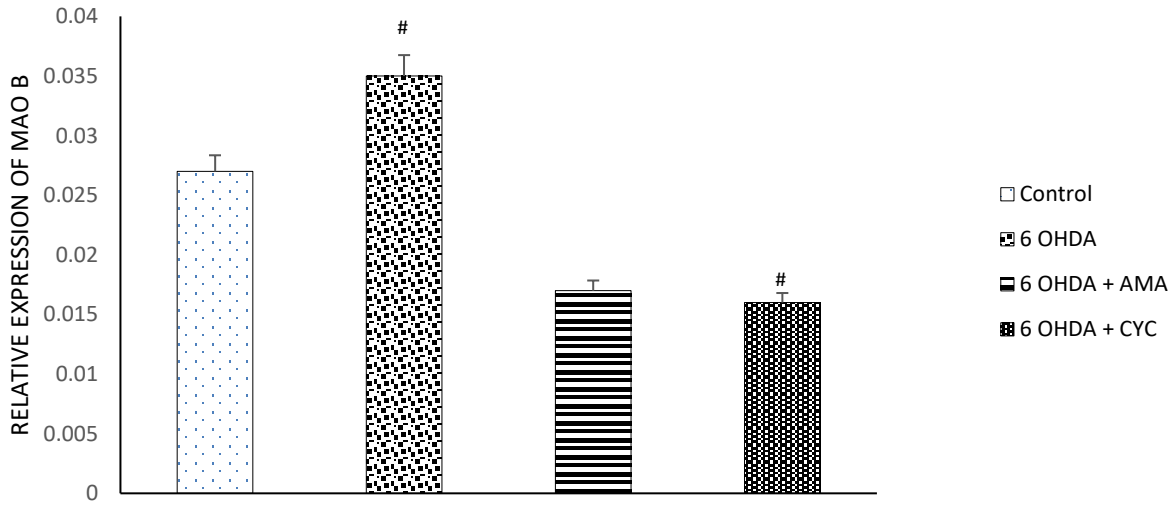
When compared to the control cells, the 6-OHDA-administered cells experienced a significant ($P < 0.01$) increase in monoamine oxidase expression after 48 hours of incubation (Fig. 4). After preexposure to 6-OHDA for three hours, the cells treated with AMA and cyclotide revealed a significant ($P < 0.05$) decrease in the expression of MAO A and B genes (Fig. 4a, b) in comparison to that of 6-OHDA-administered cells administered alone. In a similar manner, cyclotide treatment decreased the upregulated gene expression of alpha synuclein in induced cells in comparison to control cells ($P < 0.05$) (Fig. 4c).

Fig. 4 Effect of cyclotide on 6- OHDA-induced changes in mRNA expression of monoamine oxidases and alpha synuclein in SH-SY5Y cells.

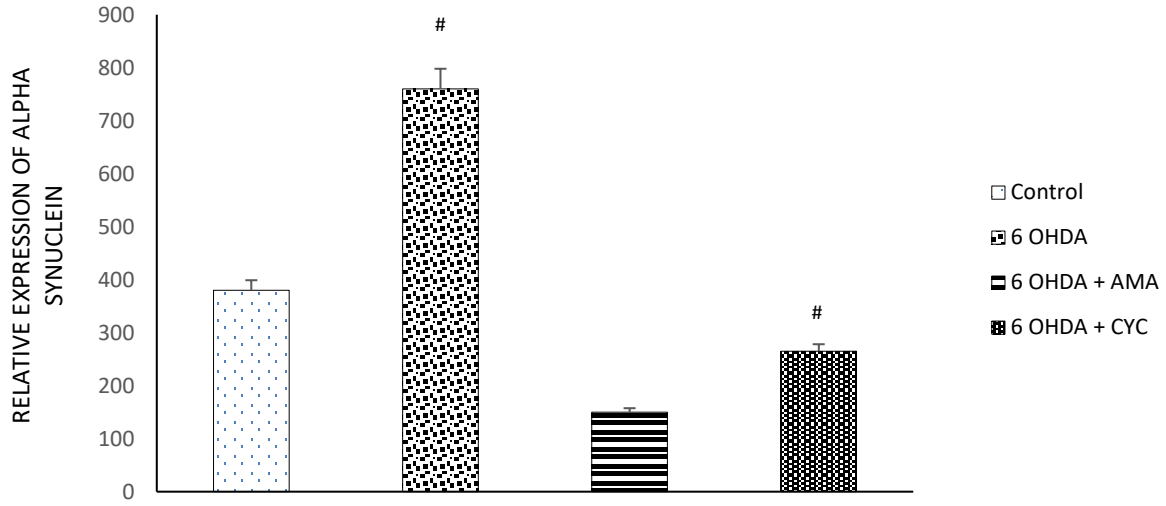
Relative mRNA expression of MAO A.



b Relative mRNA expression of MAO B.



c Relative mRNA expression of alpha synuclein.



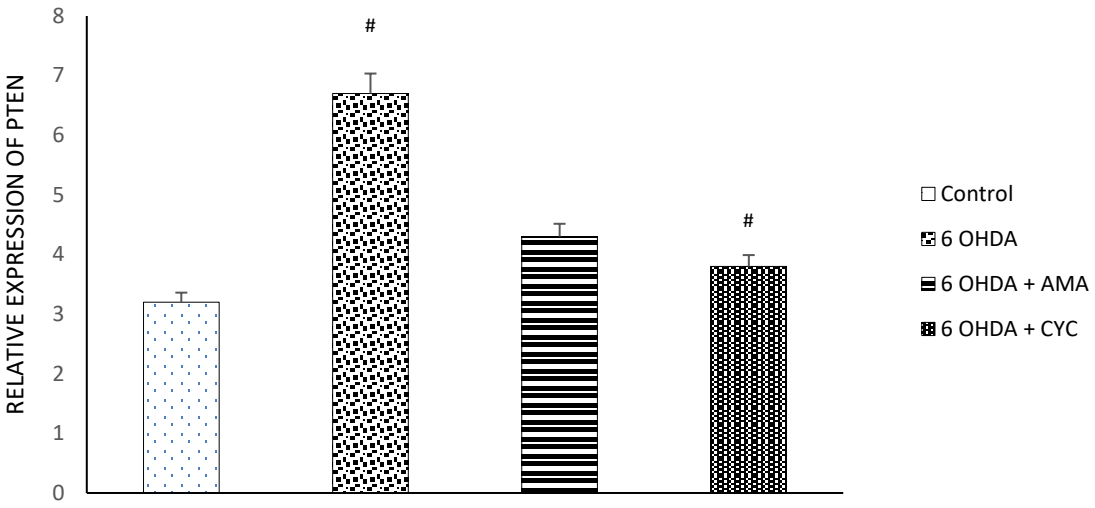
Data represents mean ± SD. #P < 0.01; ##P < 0.01; induced cells were compared with control;
6-OHDA + CYC (4 mM) were compared with 6-OHDA-induced cells by one-way ANOVA with Tukey's post hoc test

Effect of cyclotide on 6-OHDA-Induced Changes in mRNA Expression of PTEN, PINK1, PARK7, and LRRK2 in SH-SY5Y Cells

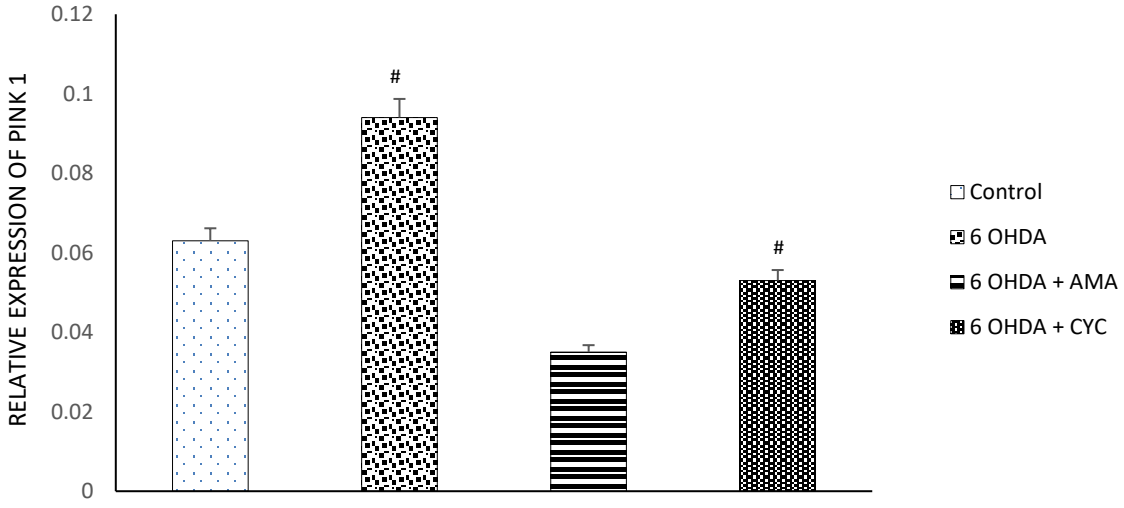
As depicted in Fig. 5, PTEN overexpression was caused by 6-OHDA toxicity (Fig. 5a), and PINK1 (5b), and PARK7 (5c), as well as LRRK2 (5d) than that of control cells that were not treated (P < 0.05). After 48 hours of incubation, co-treatment with cyclotide brought the upregulated gene expressions back to normal, demonstrating its neuroprotective effect.

Fig. 5 Effect of cyclotide on 6-OHDA-induced changes in mRNA expression of PTEN, PINK1, PARK7, and LRRK2 in SH-SY5Y cells.

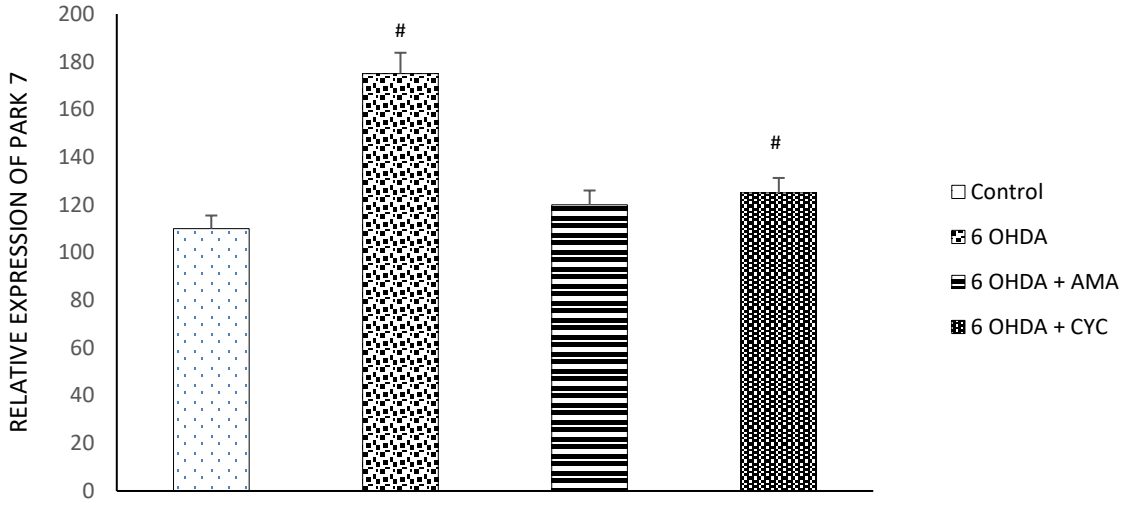
a Relative mRNA expression of PTEN.



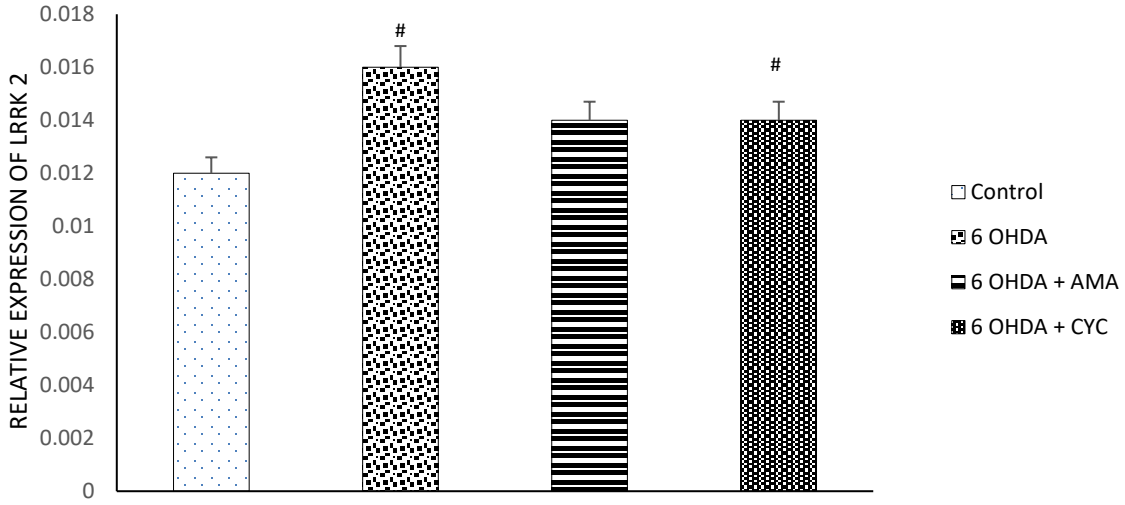
b Relative mRNA expression of PINK-1.



c Relative mRNA expression of PARK7.



d Relative mRNA expression of LRRK 2.



Data represents mean ± SD. #P < 0.01; ##P < 0.01; induced cells were compared with control; 6-OHDA + CYC (4 mM) were compared with 6-OHDA-induced cells by one-way ANOVA with Tukey's post hoc test

Discussion

Neuroprotective therapy is required in Parkinson's disease to stop the neuronal loss and death of dopamine neurons in the substantia nigra. For the first time, we demonstrated in this study that cyclotide, a compound related to tropolone, protected a 6-OHDA-induced cellular PD model. Cell viability decreased when neuroblastoma SHSY5Y cells were exposed to 6-OHDA in this study. AMA, a hepatitis B virus antagonist, was used as a positive control since it had been shown to achieve neuroprotective effect in hepatitis B patients, as those patients develop PD symptoms. The addition of cyclotide significantly increased SH-SY5Y cell viability in a correct therapeutic dosage of 4 mM. As a result, AMA is now being sold as an anti-PD medication [19]. Due to the fact that the effects of 4 mM of cyclotide in the MTT assay were nearly identical to those of 4 mM of AMA, cyclotide was considered an effective protective agent. The estimation of LDH levels revealed 6-OHDA-induced cellular impairment, and it was established that cyclotide at a dose of 4 mM prevented LDH release.

Due to the aforementioned observations, we believe that cyclotide merits additional investigation for its therapeutic potential to shield SH-SY5Y cells from 6-OHDA-induced toxicity.

According to Sayre et al., oxidative stress, also known as the production of an excessive amount of reactive oxygen species (ROS), is a major contributor to the pathology and progression of neurodegenerative diseases [20]. The stability between endogenous antioxidant systems and produced ROS is disrupted by these ROS, which are primarily active in the neuronal tissue as a result of neurotransmitter metabolism and ultimately cause cell death in a specific region of the brain. 6-OHDA has specific neurotoxic effects on the dopaminergic systems in the nigrostriatum [21, 22]. In accordance, the ROS release assay revealed a complex oxidative stress that was reduced by cyclotide treatment in 6-OHDA-exposed cells. However, the antioxidative properties of cyclotide and the counteraction of superoxide dismutase (SOD) and catalase (CAT) enzymes are primarily to blame for the reduction in ROS. Numerous studies have already elucidated the ability of cyclotide to provide cells with antioxidative protection through SOD and CAT when they are subjected to oxidative stress. [23,24,25,26].

In addition, a number of studies have concluded that cyclotide is an effective inhibitor of NRF2 expression in a number of cell lines. Ouyang et al., for instance According to estimates made by (2017), treatment with cyclotide decreased cytosolic NRF2 expression in glioma stem cells. It is important to note here that the NRF2 is a transcription factor that controls the expression of genes that are markers of oxidative stress, like HO-1. As a result, the cell's antioxidative mechanisms may be regulating a decrease in cytosolic NRF2 levels and an increase in nuclear NRF2 levels. According to Zhao et al., [27] the ROS over-release is thought to be involved in PD-related apoptosis and to cause significant damage to cell functions. The modification of the cascade of apoptosis and intracellular ROS regulation may provide novel treatment options for Parkinson's disease (PD) [27]. As a result, the therapeutic antiapoptotic effect of cyclotide was examined using flow cytometry, which may significantly lower the percentage of apoptotic cells.

Both MAO A and MAO B are tightly connected to the outer membrane of the mitochondria. They control the contents of intracellular amine stores, stop the activities of amine neurotransmitters, and shield neurons from exogenous amines. While MAO B preferentially oxidizes phenylethylamine (PEA), MAO A preferentially oxidizes serotonin (5-hydroxytryptamine (5-HT)) and norepinephrine (NE). Dopamine (DA) can be oxidized by either form. Therefore, exposure to 6-OHDA in SH-SY5Y cells may provide cytotoxicity due to the presence of ROS and MAO isoforms. However, due to their capacity to reduce ROS production, MAO that cause neurotransmitter degradation could be utilized as a pharmacotherapy [28]. As a result, cyclotide demonstrated its ability to downregulate MAO A and B expression. This suggests that cyclotide inhibits neurotransmitter degradation through reduced oxidation of dopamine. This would have decreased MAO's action, which would have increased the amount of DA available for dopamine signalling, which would have normalized the molecular events. MAO downregulation would have resulted from this, in turn. Paquette and others [29] demonstrated that AMA inhibited dopamine and serotonin uptake in the synaptosomes of MAO A and B in mouse brain homogenates. As a result, the significance of selecting AMA as a positive drug for reference with cyclotide was demonstrated in this study.

Misfolding and aggregation of α-synuclein (α-syn), which results in the formation of Lewy bodies, may even be the cause of brain degeneration. The rigorous mechanisms that underlie α-syn toxicity have not yet been satisfactorily clarified. Nevertheless, the oxidized α -syn presence is due to dementia-causing sporadic PD [30, 31]. However, cyclotide treatment resulted in a downregulation of the proteins' forming genes in this instance. It has been demonstrated in previous studies that α -syn is susceptible to post-translational modifications and that its interaction with other proteins, neurotransmitters, hormones, and metals can limit its tendency to aggregate [32]. Therefore, more research is required to identify the precise mechanisms.

The PTEN gene plays a crucial role in the inhibition of the PI3K/Akt pathway as well as its subsequent functions, which include activating Akt/protein kinase B, cell proliferation, and survival. PTEN induced overexpression in a variety of cell lines when 6-OHDA was administered, and apoptosis reduced cell proliferation. Another important gene involved in PD pathogenesis is upregulated when PTEN is deleted: the gene for PTEN-induced kinase 1 (PINK1). As PARKIN and PINK1 are attached together in the intact mitochondrial membrane, the release of which is found to be responsible for another type of familial PD, PINK1 coded by the PINK1 gene on chromosome 1p36 is downregulated in the absence of PTEN. PTEN inhibited the PI3K/Akt pathway and upregulated PINK1, suggesting a connection between PTEN and Parkinson's disease (PD). Recessive PD is also caused by PINK1 mutations.

Studies show that mitochondrial stability and quality control are dependent on PINK1. It also protects neurons and maintains mitochondrial homeostasis. According to our research, the cotreatment of cyclotide with 6-OHDA results in a downregulation of PINK1 and PTEN, indicating that ROS production and relative upregulation of the PINK1 gene are occurring to balance cellular oxidative reactions and mitochondrial instability. Wolozin et al., [33] used a novel PD genetics model, Caenorhabditis elegans. to demonstrate how human LRRK2 protects mitochondria from rotenone's toxicity.

According to reports on how LRRK2-PINK1 works in C. elegans, the part of the gene product that makes neuronal polarity has been identified [34]. By lowering the protein levels at its point of action, LRRK2 has a strong connection to the pathological annexations of various neurodegenerative disorders. However, the precise function of LRRK2 in PD pathology remains a mystery. However, LRRK2 expression was high when exposed to 6-OHDA, and cyclotide downregulated it. Since LRRK2 is identified as a pathology-related protein involved in PD, we could draw the conclusion that the medication acts as a neuroprotective agent.

Numerous factors suggest a connection between familial PD and the DJ-1 (PARK7) gene. Multiple roles, including chaperone, protease, mitochondrial regulation, antioxidative stress reaction, and transcriptional regulation, are attributed to PARK7. By oxidizing its cysteine residue at its 106^{th} position, the gene product protein PARK7's activity relates to the state of oxidation. In addition, it was discovered that individuals with sporadic Parkinson's disease had a higher prevalence of excessive PARK7 oxidation, which covered up PARK7 activity. By self-oxidizing its cysteine residues, PARK7 maintains its ROS quenching action [35, 36]. PARK7 was overexpressed in the 6-OHDA-exposed cells in our study, but cyclotide treatment brought it back to normal.

In light of the foregoing, administration of 6-OHDA will cause mitochondrial complex 1 to be damaged, resulting in mitochondrial damage and the production of a significant amount of reactive oxygen species (ROS), a hallmark of neurodegenerative diseases, and cytotoxicity, as demonstrated by the MTT assay. LDH assay demonstrated that this, in turn, causes LDH release from the mitochondria. ROS production even contributes to the cell's apoptosis. MAO levels may rise when oxidative damage and complex 1 inhibition release MAO isoforms from the inner mitochondrial membrane into the cytosol. The precise mechanism by which MAO levels rise remains hazy. Alpha synuclein proteins accumulated and degraded as a result of this imperfect cleavage and ubiquitination, which is the root cause of dementia symptoms in Parkinson's patients. However, other mitochondrial proteins, such as PTEN and PINK1, are also released into the cytosol. PINK1 and PARKIN remain attached when the mitochondrial membrane is intact, reducing the amount of free PINK1 released into the cytosol. Due to mitochondrial damage, 6-OHDA causes PINK1 to separate from PARKIN, resulting in an increase in cytosolic PINK and PTEN levels, which was reversed by cyclotide treatment. LRRK2 and PARK7, on the other hand, shield the cell from oxidative damage. Auto-oxidation enhanced the roles of LRRK2 and PARK7 to treat the load of ROS production when 6-OHDA was administered. ROS levels decreased as a result of cyclotide treatment, which in turn decreased the levels of both protective proteins.

Conclusion

MAO, alpha synuclein, PTEN, PINK1, LRRK2, and PARK7 genes were upregulated in SH-SY5Y cells as a result of the neurodegeneration that was caused by the administration of 6-OHDA. Similar to control cells, downregulation of the MAO, alpha synuclein, PTEN, PINK1, LRRK2, and PARK7 genes occurred when cyclotide was applied to the cells. The neuroprotective effects of cyclotide treatment and positive standard AMA were also found to share a significant mechanism of action. This suggests that cyclotide may have neuroprotective effects against 6-OHDA-induced in vitro neurotoxicity. However, additional research to confirm this is currently underway.

Declarations

Ethical Approval
Not Applicable

Competing interests
The authors declare that there are no competing interests.

Authors' contributions
All authors have equally contributed to the research work.

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Not Applicable

References

1. Gourie-Devi M (2014) Epidemiology of neurological disorders in India: review of background, prevalence and incidence of epilepsy, stroke, Parkinson's disease and tremors. Neurol India 62(6):588–598.https://doi.org/10.4103/0028-3886.149365
2. Singh RH. Neuronutrient impact of Ayurvedic Rasayana therapy in brain aging. *Biogerentology.* 2008;9:369–74.
3. Dias V, Junn E, Mouradian MM (2013) The role of oxidative stress in Parkinson's disease. J Parkinsons Dis 3:461–491. https://doi.org/10.3233/JPD-130230
4. Sivaranjan VV, Balachandran I. *Ayurvedic drugs and their plant sources.* New Delhi: Oxford and IBH Publishing Company; 1994. pp. 425–428.
5. Mukherjee, P. K., Kumar, V., Kumar, N. S., and Heinrich, M. (2008). The Ayurvedic medicine *Clitoria ternatea*-from traditional use to scientific assessment. *J. Ethnopharmacol.* 120, 291–301. doi: 10.1016/j.jep.2008.09.009
6. Muneeswari, M., Gangasani Narasimha Reddy., Arivukodi, D., Usharani, B., Shobana, C., (2022) Analysis of potentiality of Cyclotide, A Major Compound from Clitoria Ternatea as Anti-Parkinsonism Drug: A Pilot In Silico Study. NeuroQuantology 20(10):2758-2773. doi:10.14704/nq.2022.20.10.NQ55237
7. Hideyuki S, Tomoko O, Sadako K, Masahiro N, Kenji Y, Mitsutoshi Y, Kinya H, Takashi K (2010) Amantadine for dyskinesis in Parkinson's disease: a randomized controlled trial. PLoS One 5(12):e15298. https://doi.org/10.1371/journal.pone.0015298
8. Dias V, Junn E, Mouradian MM (2013) The role of oxidative stress in Parkinson's disease. J Parkinsons Dis 3:461–491. https://doi.org/10.3233/JPD-130230
9. Gonzalez-Hernandez T, Barroso-Chinea P, De La Cruz Muros I, Perez-Delgado DMM, Rodriguez M (2004) Expression of dopamine and vesicular monoamine transporters and differential vulnerability of mesostriatal dopaminergic neurons. J Comp Neurol 479:198–215.https://doi.org/10.1002/cne.20323
10. Glinka Y, Gassen M, Youdim MB (1997) Mechanism of 6- hydroxydopamine neurotoxicity. J Neural Transm Suppl 50:55–66.https://www.ncbi.nlm.nih.gov/pubmed/9120425.
11. Bonifati V, Rizzu P, van Baren MJ, Schaap O, Breedveld GJ, Krieger E, Dekker MC, Squitieri F, Ibanez P, Joosse M, van Dongen JW, Vanacore N, van Swieten JC, Brice A, Meco G, van Duijn CM, Oostra BA, Heutink P (2003) Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. Sci 299(5604): 256–259. https://doi.org/10.1126/science.1077209
12. Mullett SJ, Hamilton RL, Hinkle DA (2009) DJ-1 immunoreactivity in human brain astrocytes is dependent on infarct presence and infarct age. Neuropathology 29(2):125–131. https://doi.org/10.1111/j.1440-1789.2008.00955.x
13. Larsen J, Ambrosi G, Mullett SJ, Berman SB Hinkle DA (2011) DJ-1 knock-down impairs astrocyte mitochondrial function. Neuroscience 196:251–264. https://doi.org/10.1016/j.neuroscience.2011.08.016
14. Raszewski G, Lemieszek MK, Łukawski K (2016) Cytotoxicity induced by cypermethrin in human neuroblastoma cell line SH-SY5Y. AnnAgric Environ Med 23(1):106–110. https://doi.org/10.5604/12321966.1196863.
15. Wolff LF, Smith QT, SnyderWK, Bedrick JK, LiljemarkWF, AeppliDA, Bandt CL (1988) Relationship between lactate dehydrogenase and myeloperoxidase levels in human gingival crevicular fluid and clinical and microbial measurements. J Clin Periodontol 15(2):110–115. https://www.ncbi.nlm.nih.gov/pubmed/2831251.
16. Guo S, Bezard E, Zhao B (2005) Protective effect of green tea polyphenols on the SH-SY5Y cells against 6-OHDA induced apoptosis through ROS–NO pathway. Free Radic Biol Med 39:682–695. https://doi.org/10.1016/j.freeradbiomed.2005.04.022
17. Wang Y,Wu C, MorrowWJW (2004) The apoptotic and necrotic effects of tomatine adjuvant. Vaccine 22:2316–2327. https://www.ncbi.nlm.nih.gov/pubmed/15149791.
18. Huang X, Huihui W, Mark RM, Xinghu Q, Jingchuan M, Xiongbing T, Guangchun C, Guangjun W, Xiangqun N, Zehua Z (2016) Quantitative analysis of diet structure by real-time PCR, reveals different

feeding patterns by two dominant grasshopper species. Sci Rep 6:32166. https://doi.org/10.1038/srep32166

19. Hideyuki S, Tomoko O, Sadako K, Masahiro N, Kenji Y, Mitsutoshi Y, Kinya H, Takashi K (2010) Amantadine for dyskinesis in Parkinson's disease: a randomized controlled trial. PLoS One 5(12):e15298. https://doi.org/10.1371/journal.pone.0015298
20. Fukui H, Moraes CT (2008) The mitochondrial impairment, oxidative stress and neurodegeneration connection: reality or just an attractive hypothesis? Trends Neurosci 31:251–256. https://doi.org/10.1016/j.tins.2008.02.008
21. Soto-Otero R,Mendez-Alvarez E, Hermida-Ameijeiras A, Muñoz-Patiño AM, Labandeira-Garcia JL (2000) Autoxidation and neurotoxicity of 6-hydroxydopamine in the presence of some antioxidants: potential implication in relation to the pathogenesis of Parkinson's disease. J Neurochem 74:1605–1612. https://www.ncbi.nlm.nih.gov/pubmed/10737618.
22. Drechsel DA, Patel M (2008) Role of reactive oxygen species in the neurotoxicity of environmental agents implicated in Parkinson's disease. Free Radic BiolMed 44:1873–1886. https://doi.org/10.1016/j.freeradbiomed.2008.02.008
23. Deng H, Le W, Guo Y, Hunter CB, Xie W, Huang M, Jankovic J (2006) Heterogeneous phenotype in a family with compound heterozygous parkin gene mutations. Arch Neurol 63:273–277. https://doi.org/10.1001/archneur.63.2.273
24. Sayre LM, Perry G, Smith MA (2008) Oxidative stress and neurotoxicity. Chem Res Toxicol 21:172–188. https://doi.org/10.1021/tx700210j
25. Yanagida T, Jun T, Yoshihisa K, Daijiro Y, Kazuyuki T, Tomonori S, Atsuko Y, Takashi T, Hiroyuki Y, Takahiro T, Shigehiro M, Toshihiro I, Ikuo T, Hiroyoshi A (2009) Oxidative stress induction of DJ-1 protein in reactive astrocytes scavenges free radicals and reduces cell injury. Oxidative Med Cell Longev 2(1):36–42. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2763229.
26. Zhao DL, Zou LB, Lin S, Shi JG, Zhu HB (2007) Anti-apoptotic effect of esculin on dopamine-induced cytotoxicity in the human neuroblastoma SH-SY5Y cell line. Neuropharmacology 53:724–732. https://doi.org/10.1016/j.neuropharm.2007.07.017
27. Zhao DL, Zou LB, Lin S, Shi JG, Zhu HB (2008) 6,7-di-Oglucopyranosylesculetin protects SH-SY5Y cells from dopamine induced cytotoxicity. Eur J Pharmacol 580:329–338. https://doi.org/10.1016/j.ejphar.2007.11.057
28. O'Carroll AM, Fowler CJ, Phillips J, Tobbia I, Tipton KF (1983) Thedeamination of dopamine by human brain monoamine oxidase. Specificity for the two enzyme forms in seven brain regions. Naunyn Schmiedeberg's Arch Pharmacol 198–202(1983):322.https://www.ncbi.nlm.nih.gov/pubmed/6408492.
29. Paquette MA, Martinez AA, Macheda T, Meshul CK, Johnson SW, Berger SP, Giuffrida A (2012) Anti-dyskinetic mechanisms of amantadine and dextromethorphan in the 6-OHDA rat model of Parkinson's disease: role of NMDA vs. 5-HT1A receptors. Eur J Neurosci 36(9):3224–3234. https://doi.org/10.1111/j.1460-9568.2012.08243.x
30. Settembre C, Fraldi A, Jahreiss L, Spampanato C, Venturi C, Medina D, de Pablo R, Tacchetti C, Rubinsztein DC, BallabioA(2008) A block of autophagy in lysosomal storage disorders. Hum Mol Genet 17: 119–129. https://doi.org/10.1093/hmg/ddm289
31. Song JX, Lu JH, Liu LF, Chen LL, Durairajan SS, Yue Z, Zhang HQ, Li M (2014) HMGB1 is involved in autophagy inhibition caused by SNCA/α-synuclein overexpression: a process modulated by the natural autophagy inducer corynoxine B. Autophagy 10:144–154. https://doi.org/10.4161/auto.26751
32. Cheng F, Li X, Li Y,Wang C,Wang T, Liu G, Baskys A, Uéda K, Chan P, Yu S (2011) α-Synuclein promotes clathrin-mediated NMDA receptor endocytosis and attenuates NMDA-induced dopaminergic cell death. J Neurochem 119:815–825. https://doi.org/10.1111/j.1471- 4159.2011.07460.x
33. Wolozin B, Saha S, Guillily M, Ferree A, Riley M (2008) Investigating convergent actions of genes linked to familial Parkinson's disease. Neurodegener Dis 5:182–185. https://doi.org/10.1159/000113697
34. Sakaguchi-Nakashima A, Meir JY, Jin Y, Matsumoto K, Hisamoto N(2007) LRK-1, a C. elegans PARK8-related kinase, regulates axonaldendritic polarity of SV proteins. Curr Biol 17(7):592–598. https://doi.org/10.1016/j.cub.2007.01.074
35. Mitsumoto A, Nakagawa Y (2001) DJ-1 is an indicator for endogenous reactive oxygen species elicited by endotoxin. Free Radic Res 35(6): 885–893. https://www.ncbi.nlm.nih.gov/pubmed/11811539.
36. Taira T, SaitoY, Niki T, Iguchi-ArigaSMM, TakahashiK, ArigaH (2004) DJ-1 has a role in antioxidative stress to prevent cell death. EMBO Rep 5(2):213–218. https://doi.org/10.1038/sj.embor.7400074