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A Behavioural and Histological Approach to Dose Dependent Chronic Toxicity Screening of Cyclotide in Zebrafishes

Muneeswari Muniyasamy[1], Rohini Durairaj[2], Usharani Boopathy[3], Arivukodi Deivasigamani[4], Shobana Chandrasekar[5]

[1,2,3,4,5,6]*Department of Biochemistry, Vels Institute of Science, Technology and Advanced Studies, Chennai, Tamil Nadu, India.*

**Corresponding author's E-mail: shobana.sls@velsuniv.ac.in*

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Abstract

Parkinson's disease (PD) is the second most common neurodegenerative illness worldwide. It is an age-related sickness. An illness treatment plan is urgently required. Such secondary metabolites from plants may undoubtedly be the source of the medications with less adverse effects. 6-Hydroxydopamine (6-OHDA) is used experimentally to mimic Parkinson's disease in adult zebrafish. In the realm of medicine, there are no ideal cures for ailments. For the past few decades, a number of plant secondary chemicals have been tested in preclinical settings to cure this illness. Many references have been made to cyclotide's neuroprotective, anti-inflammatory, and antioxidant properties. Furthermore, it has recently been demonstrated that amantadine (AMA), an aminoadamantane well-known for its mild antiparkinsonian action, counteracts central nervous system dysfunction. Apart from oxidative stress and mitochondrial damage, 6-OHDA may also cause decreased cytosolic levels of Tyrosine Hydroxylase (TH). Since this is primarily thought to be in charge of dopamine production in the central nervous system, an antagonist of TH may be the best medication option when treating Parkinson's disease. Since Amantadine (AMA) is the conventional medication, the current study compares the potential of Cyclotide as Tyrosine Hydroxylase Inhibitors to examine the molecular anti-TH interactions in 6-OHDA-induced adult zebrafishes.

Keywords: *6- Hydroxydopamine, Cyclotide, Amantadine, Tyrosine Hydroxylase, Neurodegeneration*

1. Introduction

The second most common movement disorder in the world, Parkinson's disease (PD) impairs a patient's ability to move normally and maintain a stable posture. The significant loss of dopaminergic cells in the substantia nigra of the patient with this disease is its pathophysiology. Since more cases of PD have been observed in those over 60, the condition is regarded as an aging ailment. According to a recent report [1], 15 people per 1 lakh were reported to have Parkinson's disease. Despite not being fatal, this illness significantly reduces a person's quality of life. There is still no precise treatment for the illness. Since the last ten years, several bioactive plant secondary compounds like polyphenols, flavonoids, and coumarins have been utilized as medications in preclinical trials to treat the symptoms of Parkinson's disease (PD), even though there is no specific treatment for the illness's brain [2].

One of these potentially useful herbs is Clitoria ternatea, which is a key ingredient in the brain tonic medhya rasayan used to treat neurological disorders. Since ancient times, Ayurveda has been used as a form of traditional medicine in India. This investigation supports Indian medicine by highlighting the plant's significance as a brain remedy. Clitoria ternatea stands out from other plants and has the quality of being a potent stimulant of the brain [4]. In addition, C. ternatea has long been used in traditional medicine, particularly as a supplement to improve cognitive abilities and lessen the symptoms of a variety of illnesses, such as fever, inflammation, pain, and diabetes [5]. In this study, the antiparkinson's activity of cyclotide, an active ingredient from Clitoria ternatea, was assessed. Earlier research on the examination of cyclotide insilico studies [6] established the way for the current study.

It has now been demonstrated that AMA, an aminoadamantane long recognized for its mild antiparkinsonian action, antagonizes central nervous system dysfunction. When taken at the recommended dosages, AMA may treat Parkinson's disease symptoms more than other anticholinergic medications while also showing fewer negative effects. Additionally, AMA and anticholinergic medications appear to have complementary therapeutic effects [10]. Moreover, tardive dyskinesia caused by neuroleproleptics may be ameliorated by the AMA [11]. Therefore is therefore frequently used as a therapeutic medication for Parkinson's disease symptoms in clinics, and therefore serves as a positive control in our study.

A popular model organism for studying neurological toxicity, particularly 6-hydroxydopamine (6-OHDA) toxicity, is the tropical freshwater zebrafish. Adult zebrafish given 6-OHDA revealed a loss of diencephalic dopaminergic neurons and an odd swimming pattern, revealing behavioral traits resembling those of Parkinson's disease patients. Zebrafish treated with 6-OHDA might therefore reproduce a chemically induced hemi-PD model [12]. Additionally, 6-OHDA lowers cell viability and produces excessive Reactive Oxygen Species (ROS), a defining feature of the initial cellular signature of neurodegenerative illnesses like Parkinson's disease [12]. This is achieved by decreasing the action of mitochondrial complex I.

Owing to 6-OHDA administration, it is shown that there is greater oxidative stress in the substantia nigral sections [13], which lowers the activity of mitochondrial NADH dehydrogenase (complex I) [14, 15]. Therefore, the primary initial mechanism of 6-OHDA toxicity is the persistent suppression of mitochondrial respiration, followed by extremely harmful cellular processes such as increased peroxidation. Since it is an easily oxidizable chemical, free radicals are thought to be the byproduct of its oxidation, which may further increase the toxicity process [16].

Furthermore, the intramuscular injection of 6-OHDA has the potential to increase the expression of Tyrosine Hydroxylase (TH), which is a process that limits the pace at which dopamine is synthesized [17]. On the other hand, rat brains treated with striatal 6-OHDA showed a predicted increase in nigral dopaminergic neuronal death in the substantia nigra [18] together with decreased TH expression, indicating a TH shortage. Tyrosine hydroxylase (TH), the rate-limiting enzyme in the synthesis of dopamine, biochemically catalyzes the generation of L-DOPA, which is thought to be a characteristic of Parkinson's disease pathogenesis. TH agonists, which are DA metabolism inhibitors, may therefore be a useful treatment for Parkinson's disease [17]. This factor's continuous expression improves memory, learning, synaptic plasticity, and neurogenesis [18]. Therefore, using AMA as the usual medication, the molecular interactions of Cyclotide with TH, elevation in mitochondrial complexes, and ensuing neurodegeneration were investigated in 6-OHDA-induced adult zebrafishes to treat PD.

2. Materials And Methods

Animals

The institutional animal ethics committee and the Committee for the Purpose of Control and Supervision of Experiments on Animals, India, have set guidelines that must be followed in order to maintain the wild-type adult zebrafishes that are the subject of this study. Commercial fish feed pellets were used to feed the fish. To keep everything at normal, the water was changed on a regular basis. Twelve mature fish of each sex were kept in groups of ten liter water tanks with constant biofiltration and aeration under a 14/10 light/dark cycle. Temperature, pH, and conductance of the water were kept at 26–28°C, 6.3-2.4, and 300–480 µS, respectively. The fish were kept in 3-liter tanks with varying concentrations and given oral chemical treatment for a duration of 28 days. 13 mg of the chemical was dissolved in 5 ml of 0.1% dimethyl sulfoxide and olive oil to create dilutions of 5–25 mg stock, which were then kept at -20°C until needed. To create the feed, the compound was diluted to a final concentration of 5–25 mg, respectively. After that, this was utilized to make fish meal. Commercial fish feed is mixed with the compound at a specified concentration and allowed to air dry before feeding.

PD Induction

Six mature fish of both sexes (1F:1M) were used in each group. With minor modifications, all fish, with the exception of the control group, were anesthetized using water that was 15 degrees Celsius. The fish were then intraperitoneally injected with 6-OHDA at a dosage of 60 mg/Kg b.w. of fish dissolved in Ringer's solution (6.5g NaCl, 0.42g KCl, 0.25g CaCl2, and 0.2g of sodium bicarbonate per liter) for 14 consecutive days in order to induce Parkinson's disease (PD). For intramuscular injections, an insulin syringe (U-40., 0.25mm (31 G) x 6mm needle) was utilized. The PD induction took place in the morning (between 9:00 and 10:00 a.m.), and three hours later the medications were given orally through the meal.

Dosing of animals

The AMA and CYC were dosed orally by mixing with the fish feed pellets, at fixed concentrations. Being the pioneer work on zebrafish models, the doses of all the three drugs were fixed after various pre-trails with varying concentrations. The AMA mixed food pellets served as positive control. The grouping of animal was done as follows. Group 1: Control fishes administered with Ringers solution and pellets were coated with 0.01% DMSO + olive oil. Group 2: Fishes were administered with 60 mg/kg b.w 6-OHDA intramuscularly (i.m) for 14 days [12]. Group 3: Fishes were induced using 6-OHDA (60 mg/kg b.w) i.m and co-treated with of 5 mg/kg b.w of AMA orally on the same day for 14 days. Group 4: Fishes were induced using 6-OHDA (60 mg/kg b.w) i.m and co-treated with of 10mg/kg b.w of AMA orally on the same day for 14 days. Group 5: Fishes were induced using 6-OHDA (60 mg/kg b.w) i.m and co-treated with of 20mg/kg b.w of AMA orally on the same day for 14 days. Group 6: Fishes were induced using 6-OHDA (60 mg/kg b.w) i.m and co-treated with of 5 mg/kg b.w of CYC orally on the same day for 14 days. Group 7: Fishes were induced using 6-OHDA (60 mg/kg b.w) i.m and co-treated with of 10mg/kg b.w of CYC orally on the same day for 14 days. Group 8: Fishes were induced using 6-OHDA (60 mg/kg b.w) i.m and co-treated with of 20mg/kg b.w of CYC orally on the same day for 14 days.

Behavioral Analysis

The ideas of [19] were taken into consideration when designing behavioral tests, such as Swim-Dive motion tests. By placing the fish in the tank and dividing the whole height of the water zone into top 15 cm and bottom 15 cm, the motor behavior test and anxious behavior were examined. Before the readings were taken, the fish were given 30 minutes to acclimate in the measuring tanks. After the induction and treatments ended on the fourteenth day, measurements were taken right before the animal was sacrificed for smear pathology. A Mac CMOS 5MP motion capture lens for fish was used to record the number of seconds that the fish spent in each zone for a duration of 60 seconds.

Isolation of Mitochondria

The rats were slaughtered and their brains extracted by beheading after the 14-day experiment. Each isolated brain's posterior tuberculin was combined and homogenized in the isolation buffer. In addition, the tissue suspension was centrifuged at 13,000 ×g for five minutes at 4°C. The pellets were centrifuged, allowed to resuspend in the isolation buffer containing ethylene glycol tetra acetic acid (EGTA), then centrifuged again at 13,000×g for approximately 5 minutes at 4°C. Following the transfer of the acquired supernatants to new tubes, the isolation buffer containing EGTA was added, and centrifugation at 13,000×g for approximately 10 min at 4°C was once more permitted [20]. Ultimately, the pellets that were collected and included purified mitochondria were used for additional analysis after being re-suspended in the isolation buffer without EGTA.

Measurement of Mitochondrial Complex Activity

The enzyme activity of complexes I and IV bound to the mitochondria were measured using spectrophotometry in 100 mmol/L of pH 7.4 phosphate buffer at approximately 30°C. Using the methodology described in [21], the activity of NADH-cytochrome c reductase was examined. The absorbance change was measured using spectrophotometry for approximately three minutes at 550 nm. The results were then expressed as units of NADH oxidized/min/mg of protein. The cytochrome oxidase activity was measured using the methodology described in [21]. The results were noted and reported in terms of mg of protein/min/oxidized cytochrome c.

Histological Analysis

The fish were sacrificed by making a cut between the brain and the spinal cord after being put to sleep in water that was 15 degrees Celsius. After the brain was dissected, parts of the midbrain, including the posterior tuberculum, were cut out using a knife and a pin. After spreading the tissue onto a glass slide, it was stained for two minutes each with hematoxylin and eosin and then rinsed with water. Using an Olympus BX 53 light microscope, slides were inspected at a 45X magnification. Three areas per smear were counted for the number of degenerated neurons using a hemocytometer. Degenerative neurons were identified by a high rate of cell lysis during smear preparation, loss of cell structure, enlarged or constricted cells, and irregularly shaped cell membranes [22]. They also stained comparatively lighter. q PCR analysis: Following the manufacturer's instructions, TRIzol was used to extract total RNA from fish posterior tubulum, and the RNA was then reverse-transcribed to cDNA (TaKaRa, Tokyo, Japan). mRNA specific primers for tyrosine hydroxylase (FW- 5'-GCTGTGCTATGATTTCAGCACTT-3' RV- 5'- CGACAAAACTCCAAGCGCAA-3') were created using the Primer 3 program, and the qRT-PCR was carried out using the SYBR Premix ExTaq (TaKaRa, Tokyo, Japan). For every sample, the relative mRNA level was adjusted to GAPDH expression. The 2-△△Ct technique was used to analyze gene expression [23].

Statistical Analysis

After doing statistical analysis, all results were reported as mean ± standard deviation (SD). One way analysis of variance (ANOVA) was used for analysis, and Tukey's Post-hoc test (SPSS 20 version) was used after that. $P < 0.05$ values were regarded as statistically significant.

3. Results and Discussion

Effect of AMA and CYC on 6-OHDA induced degeneration in the posterior tuberculum of control and experimental fishes

The percentage of degenerative neurons in the posterior tuberculum of the experimental and control fish is displayed in Table No. 1. When CYC was administered instead of AMA, the current observation showed that the neural regenerating ability had risen. In fish induced with 6-OHDA, the dosage of AMA and CYC at 5 mg/kg body weight has increased neuronal regeneration by up to 25%. On the other hand, fish that were triggered and given 10 mg/kg b.w of AMA and CYC experienced a 25% and 35% regeneration of neurons, respectively. Treatment groups showed varying capacities of regeneration (i.e., 45% and 20%) at a dosage of 20 mg/Kg b.w. of the AMA and CYC.

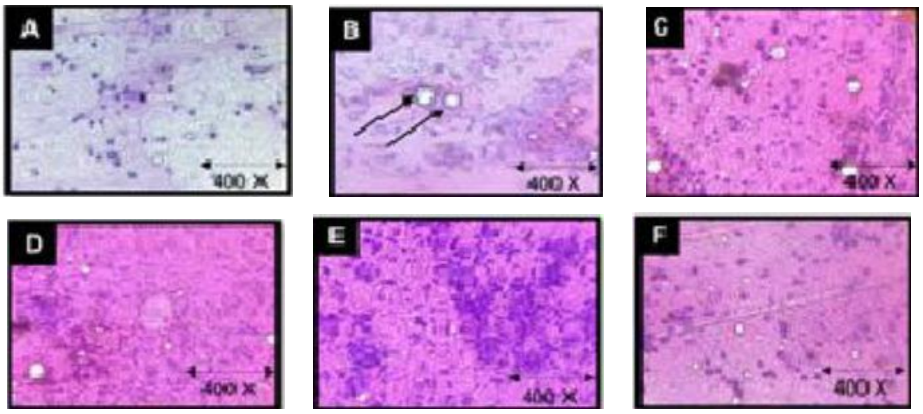
Table 1: The Clinical parameters ensuring the degeneration as well as behavioural changes in control and experimental fishes

Clinical parameters	Control	6-OHDA alone	Amantidine			Cyclotide		
			5	10	20	5	10	20
Percentage of degenerated neurons	5	90	70	70	60	70	60	50
Motor behaviour test	N	S	MS	MS	N	S	N	S
Anxiety behaviour test	N	A	MA	MA	N	A	N	A

N – Normal; S - Slow; MS - Moderately Slow; A – Anxious; MA – Moderately Anxious

Histopathological Analysis

When compared to the control, the posterior tubercular region of the induced fish brain showed significant neuronal degeneration, dramatic loss of neuronal cells, increased inter-neuronal spacing, and loss of glial cells, according to histopathological studies (Fig. 1 A&B). Following AMA therapy, the histological abnormalities have decreased in a dose-dependent manner (5, 10, 20, mg/Kg b.w. of fish). When the AMA was administered at doses of 5 and 10 mg/kg b.w., it caused significant inflammatory lesions and cellular necrosis (Fig. 1 C&D). In contrast, when 30 mg/Kg b.w of AMA was used to cause fish brain cell disease, both neuronal and glial cells were restored (Fig. 1 E). While the administration of CYC at doses of 5 and 20 mg/kg b.w. demonstrated a good therapeutic effect indicating neuronal cellular regeneration, normalized nucleus, and normalized glial cells, the dose of 10 mg/kg b.w. showed severe pathological abnormalities like neuronal cell lysis, nuclear dislocation, cell shrinkage, and inflammation (Fig. 1 F,G&H).



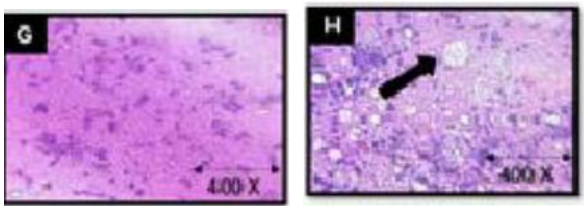


Fig. 1: Effect of CYCLOTIDE on 6-OHDA induced Histopathological alternations in the posterior tuberculum of control and experimental fishes

A) Control fishes, showing normal histological morphology of cells.

B) 6-OHDA alone, showing severe neuronal degeneration and drastic neuronal cell loss.

C) 5 mg/Kg b.w of fishes of AMA + 60mg/Kg b.w of fishes of 6- OHDA showing severe cellular necrosis.

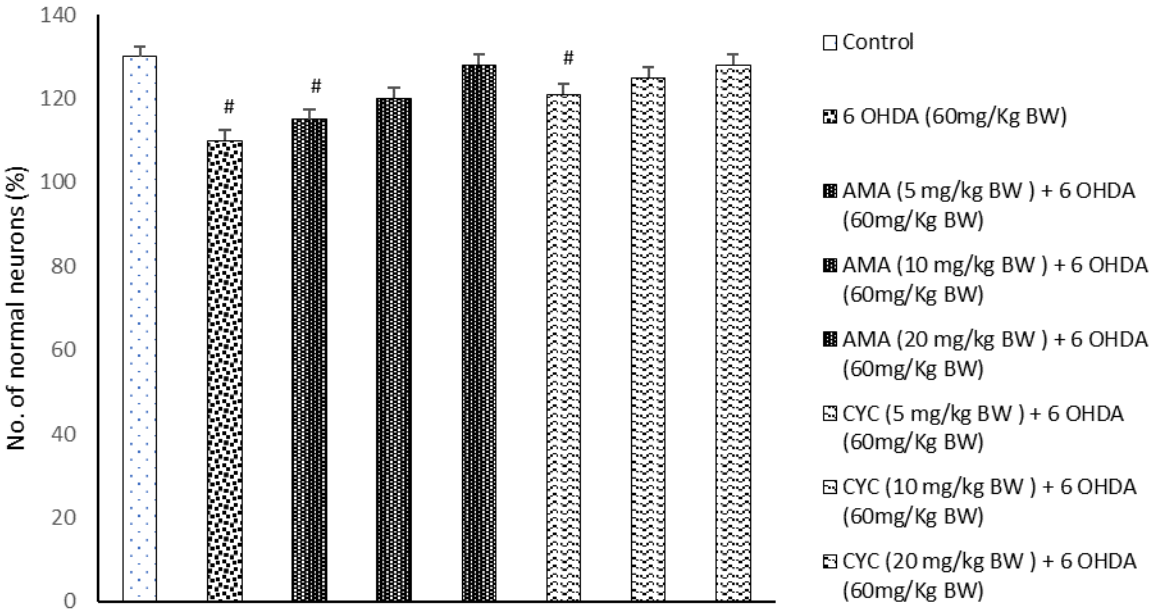
D) 10 mg/Kg b.w of fishes of AMA + 60mg/Kg b.w of fishes of 6- OHDA, showing inflammatory lesions.

E) 20 mg/Kg b.w of fishes of AMA + 60mg/Kg b.w of fishes of 6- OHDA, showing regenerated neuronal cells.

F) 5 mg/Kg b.w of fishes of CYC + 60mg/Kg b.w of fishes of 6- OHDA, severe neuronal cell lysis, nuclear dislocation.

G) 10 mg/Kg b.w of fishes of CYC + 60mg/Kg b.w of fishes of 6- OHDA, showing better therapeutic neuronal plasticity.

H) 20 mg/Kg b.w of fishes of CYC + 60mg/Kg b.w of fishes of 6- OHDA, showing cell shrinkage and inflammation.



Behavioral alterations analysis

When zebrafish were treated with AMA and CYC at dosages of 5, 10, and 20 mg/kg body weight, their behavioral pattern was evaluated using motor behavior analysis. The results showed a substantial ($P<0.05$) improvement in motor activities compared to fish that were given 6-OHDA (Table No. 1). The fish, however, exhibited typical behavior when given dosages of 10 mg/kg b.w. of CYC and 20 mg/kg b.w. of AMA, respectively. Furthermore, similar behaviors were seen in the anxiety motion analysis, indicating that CYC is therapeutically efficacious at lower concentrations than the positive standard.

Mitochondrial Activity

When compared to fish induced with 6-OHDA, the mitochondrial activity of complexes 1 and 4 has been dramatically ($P < 0.05$) reduced (Fig. 2). When compared to control mitochondrial activity, the activity of mitochondrial complexes 1 and 4 has dramatically ($P<0.05$) enhanced in fish treated with CYC and AMA.

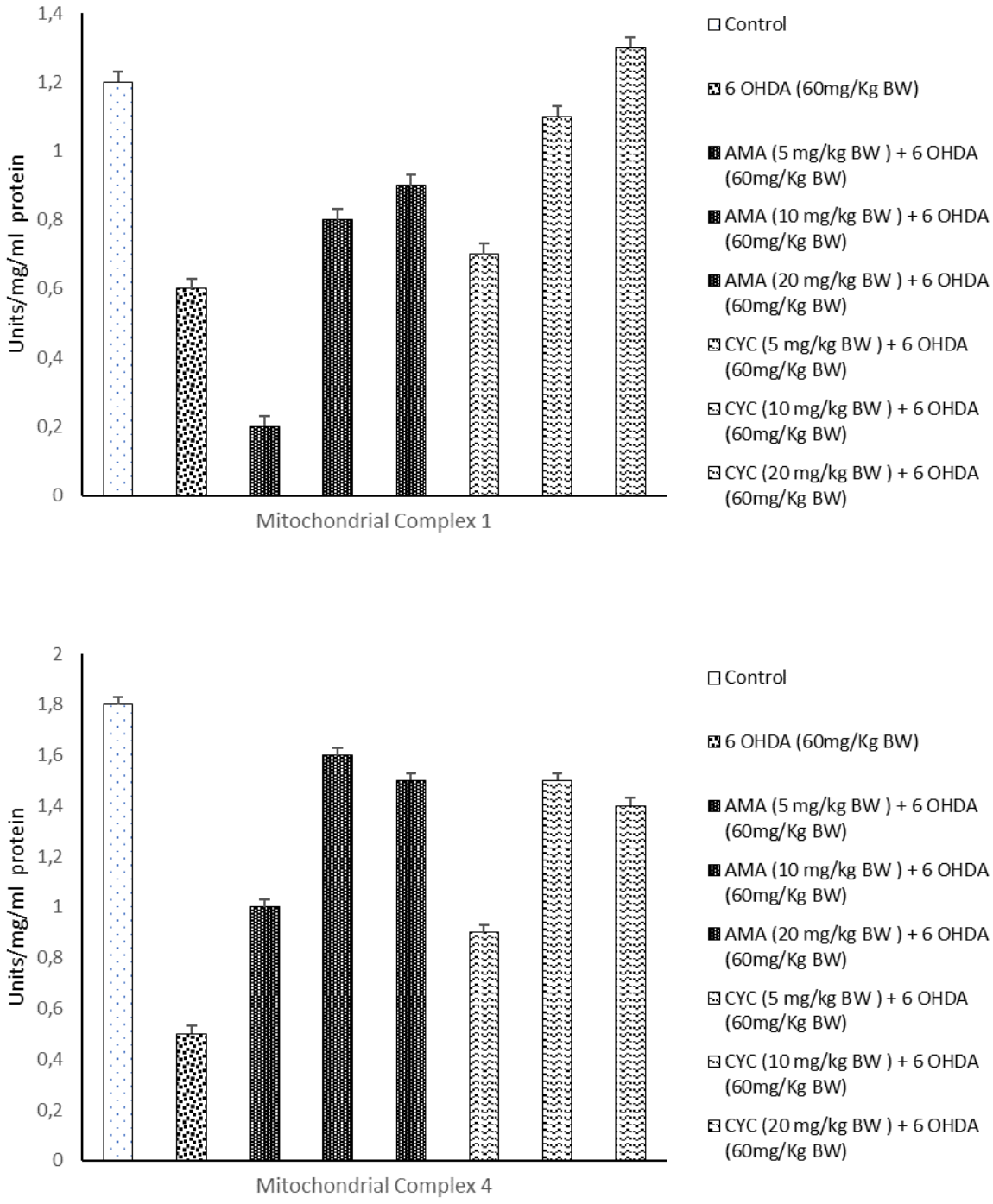


Fig 2: Effect of CYCLOTIDE on 6-OHDA induced alternations in the activities of mitochondrial complexes 1 and 4 in posterior tuberculum of control and experimental fishes

Data represents mean ± SD. a. $P < 0.05$; b. $P< 0.05$; induced fishes were compared with control; Treatment groups were compared with 6-OHDA induced fishes by one way ANNOVA with turkey's post-hoc test

Protein Expression of Tyrosine Hydroxylase

When compared to the protein levels of the control fish, the 6-OHDA alone-administered fish showed a significant ($P<0.05$) decrease in tyrosine protein levels (Fig. 3). The treatment with CYC and AMA led to a considerable ($P<0.05$) increase in protein levels, which in turn reduced the toxicity of 6-

OHDA.The 10 mg/kg dosage of CYC produced roughly comparable neuroprotection as AMA in CYC treatments.

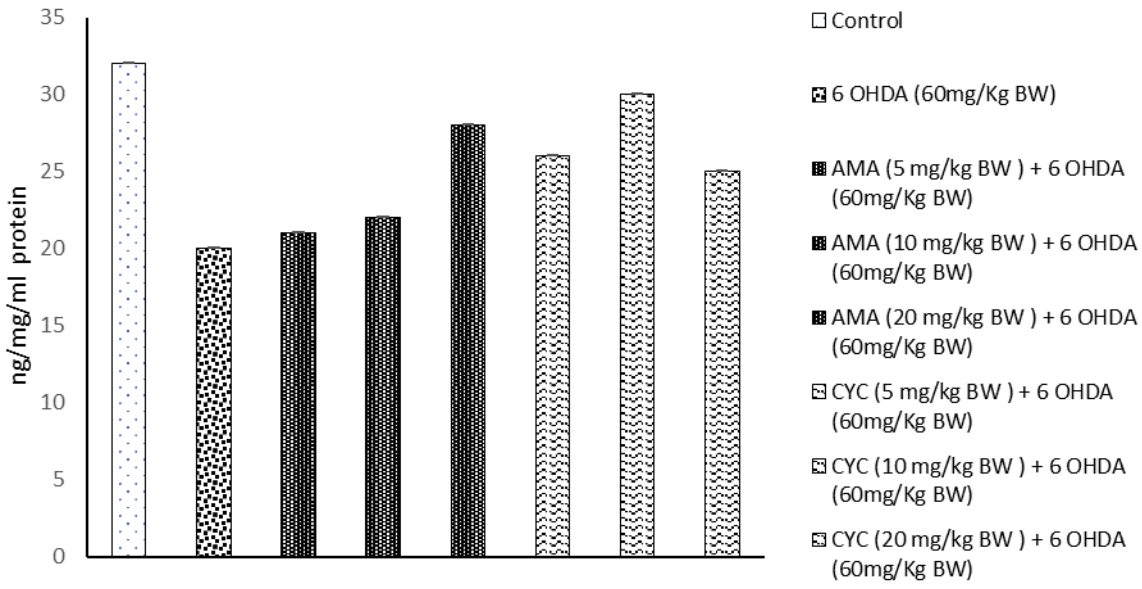


Fig 3: Effect of CYCLOTIDE on 6-OHDA induced alternations in the protein expression of levels of Tyrosine hydroxylase in the posterior tuberculum of control and experimental fishes

Data represents mean ± SD. a. P < 0.05; b. P< 0.05; induced fishes were compared with control; Treatment groups were compared with 6-OHDA induced fishes by one way ANNOVA with turkey's post-hoc test.

The Mrna Expression Of Tyrosine Hydroxylase

Tyrosine hydroxylase mRNA expression levels were significantly (P<0.05) lower in the 6-OHDA-induced zebrafish as compared to the control group (Fig. 4). There seems to have been a considerable (P<0.05) rise in the expression levels following treatment with a 10 mg/kg dosage of CYC. Conversely, in cases when dosages are being compared, it may be more advantageous to use the minimum dose of 10 mg/kg of CYC, as this amount has been shown to cause considerable attenuation.

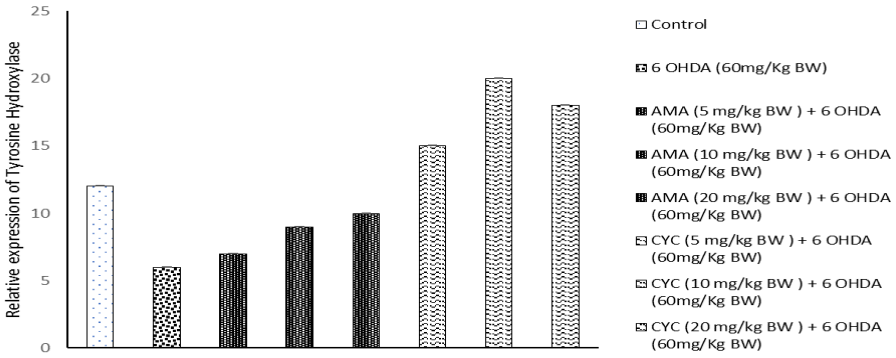


Fig 4: Effect of CYCLOTIDE on 6-OHDA induced alternations in the mRNA expression of Tyrosine hydroxylase in the posterior tuberculum of control and experimental fishes

Data represents mean ± SD. a. P < 0.05; b. P< 0.05; induced fishes were compared with control; Treatment groups were compared with 6-OHDA induced fishes by one way ANNOVA with turkey's post-hoc test.

Parkinsonian patients' reduced dopamine levels are directly caused by the selective loss of neurons in the substantia nigra, also referred to as the posterior tuberculum in fish [24]. Moreover, 6-OHDA is known to promote dopaminergic cell death and is a commonly used chemical to induce Parkinson's disease in experimental animals [25]. Thus, this work examined the neuroprotective effect of CYC on 6-OHDA-induced dopaminergic neuronal injury in adult zebrafishes.

Since then, chordates have been suggested as the most suitable models to shed light on neurodegenerative processes [26]. Zebrafish are one of these models that have drawn interest recently in medication therapy research because they make good models for easy delivery regimens like often adding water to a tank. Similarly, drugs are currently delivered directly to target cells or tissues in fish larvae through the use of microinjection techniques [27].

Current research has focused on the behavioral endpoints of zebrafish, reviewing the fundamental neurological processes related to various kinds of sensory neurons and cognitive function. Numerous behavioral endpoint tests look into a variety of brain functions to assess and correlate particular cognitive, physiological, and perceptual disorders. Furthermore, a number of fish behavioral tests are still being developed.

The majority of recently established methods have very tight comparable ties with the screening of mammalian models. In order to investigate anxious behavior and motor impairment, swim and dive motion analysis were used in this study. This approach is similar to the open field test and rotarod analysis in rats. Zebrafish mobility has also been shown to be a reliable endpoint for identifying neurological deficits during development. For instance, six or more days after being exposed to the environmental toxin chlorpyrifos, embryonic zebrafish exhibited decreased swimming activity [28]. Similar to this, in our investigation, fish given 6-OHDA showed improved swimming patterns after receiving CYC therapy.

Additionally, the rate-limiting enzyme in dopamine metabolism, TH, is hindered by 6-hydroxydopamine [18]. Because of its therapeutic impact, CYC medication consequently increased the levels of TH in the brain's substantia nigra, as previously mentioned. Consequently, control of TH activity is crucial and can be used to inferentially assess how well a given medication works as a treatment. Two methods exist for controlling TH activity: medium- to long-term modulation of gene expression and short-term direct regulation of enzyme activity [33, 34].

Thus, the gene expression of the TH when given orally with CYC and AMA is being investigated in this work as a pioneer analysis. The compound can work as a robust TH enhancer at therapeutic dosage, according to the mRNA expression results, which suggests the medication has the potential to function as a potent ant-PD agent.

Zebrafish models are gaining importance as a brain model, which validates their use in pharmaceutical studies. Treatment with CYC restored the histological changes, behavioral abnormalities, and oxidative mitochondrial stress responses caused by the administration of 6-OHDA to normal, indicating that 10 mg/kg is a potential therapeutic dosage for Parkinsonism. Additionally, the mRNA expression of TH stayed relatively unchanged, indicating that CYC is a strong anti-PD drug.

4. Conclusion

According to the study, CYC can reduce neuronal loss in the posterior tuberculum of 6-OHDA-injected adult zebrafish, which is comparable to the substantia nigra in mammals, and hence function as a better neuroprotective medication against 6-OHDA-induced neurotoxicity. The neuroprotective efficacy of CYC at a dose as low as 10 mg/kg was shown to make it a more effective therapeutic medication, according to the study. Additionally, Tyrosine Hydroxylase's mRNA expression levels followed the same treatment pattern. The adult zebrafish treated with 6-OHDA showed a significant ($P<0.05$) reduction in the lowered levels of TH genes, indicating that CYC is a more effective anti-PD drug for 6-OHDA-induced neurotoxicity

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