TOXICITY OF ATRAZINE ON ACETYLCHOLINESTERASE ACTIVITIES AND HISTOLOGICAL PROFILE (BRAIN) OF *CLARIAS GARIEPINUS* (BURCHELL, 1822): THE NEURO-PROTECTIVE ROLE OF VITAMIN C

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Abstract: The harmful effects of atrazine on fish have been well-documented. Consequently, it is imperative to explore alternative methods to safeguard the health of aquaculture species such as *Clarias gariepinus*. This study investigated the toxicological impacts of atrazine on Acetylcholinesterase (AChE) activities and the histological characteristics of the brain and the potential ameliorative effects of dietary supplementation of Vitamin C to atrazine toxicity. Four-week-old *C. gariepinus* juveniles were exposed to 0, 10, 20, and 30 µg/L of atrazine and 0.08g of vitamin C for twenty-eight days in a static renewal bioassay. Atrazine exposure significantly (p<0.05) inhibited the activities of AChE in the brain, ranging from 47.86 (control) to 32.39 U/L in the 30 µg/L treatment. Histopathological assessment of the brain showed the cerebrum with enlarged disorganized cells with vascular spaces around them and the appearance of blood congestion in the lowest treatment (10 µg/L). Dissociation of Purkinje's cell layer from the granular cell layer was observed mostly at the 20 µg/L group, while severe cell infiltration occurred at 30 µg/L. Some unusual behaviors, such as heightened opercular rate, erratic swimming, mucous secretion, and air gulping, were observed across treatment groups in a dose-dependent pattern. These results indicate that the influence of atrazine might be linked to changes in the cholinergic system of the central nervous system, potentially impacting the observed behavioral trends. Dietary supplementation of fish feed with Vitamin C in this study has neuro-protective potential against atrazine-induced toxicity.

Keywords: *Clarias gariepinus*, atrazine, Vitamin C, Acetylcholinesterase, histology, behaviour

Introduction

The primary method for weed control in agronomic crops is the application of herbicides, playing a crucial role in enhancing crop yield and global food security (Daramola *et al.*, 2022). However, after application, herbicides enter various aquatic ecosystems, proving to be highly toxic to non-target organisms, particularly aquatic life and their surroundings (Roh *et al.*, 2023). Herbicides have been identified as significant pollutants of soil, surface water, and groundwater (Owolabi and Abdulkareem, 2021). Over the last four decades, Atrazine, an herbicide used to control broad-leaf weeds in crops like corn, sorghum, sugar cane, pineapple, and soybeans, has been considered a persistent pollutant due to its long half-life in the environment (from 10 to 5,824 days in soils and sediments) and high mobility between environmental compartments (soil, water, and sediment) (Torres and Coelho, 2023). The changes in water quality negatively affect organisms, leading to mortalities in acute concentrations and severe exposures, primarily due to herbicide use in agriculture and industry (Kwairanga *et al.*, 2022). The primary water sources for urban-rural communities in developing countries, including Nigeria, are surface waters (such as rivers, streams, ponds, and lakes) and groundwater (in the form of boreholes and hand-dug wells). However, in Nigeria, surface waters are often contaminated with domestic, agricultural, and industrial waste, leading to numerous waterborne diseases and adverse health effects on living organisms (Oyetunji and Obikoya, 2023). The pollution of water bodies poses a significant threat to fish and other aquatic organisms. Pesticide accumulation in these organisms is a global public health concern, and fish are extensively used in environmental monitoring due to their direct absorption of pollutants from water and food, allowing the assessment of pollutant transfer through the food chain (Ezekwe *et al.*, 2022).

Fish are among the well-understood species in the aquatic environment and are widely used to assess the health of aquatic ecosystems. Physiological changes in fish serve as biomarkers of environmental pollution (Adadu and Ochugwo, 2020). Fish, being particularly sensitive to environmental contamination of waters, may experience significant interference with various biochemical processes due to pollutants (Ribeiro *et al.*, 2013). Toxicants in the aquatic environment have diverse effects on fish physiology, especially during vulnerable life stages such as larvae, fingerlings, and juveniles, which can be severely impacted by pesticides and heavy metal pollution. Fish contain Vitamin C, a potent antioxidant defense catalyst, which plays a crucial role in the fish's immune response and resistance to infectious diseases. As many fish species cannot produce Vitamin C, it must be provided through their diet. This vitamin acts as a reducing agent and antioxidant, with high levels of Vitamin C reported to be efficient in reducing toxicity, preventing disease, and enhancing fish tolerance to environmental stress (Ikeogu *et al.*, 2020; Afisu *et al.*, 2022; Gomes *et al*., 2022).

Biochemical parameters serve as valuable indicators for monitoring physiological and histopathological alterations in fish exposed to contaminants, both in laboratory and field studies (Sayed and Hamed, 2016). Essential antioxidant defense enzymes, including superoxide dismutase, catalase, Glutathione S-transferases (GSTs), and Acetylcholinesterase (AchE), play a crucial role in mitigating the biochemical changes induced in fish (Eleyele *et al*., 2017). Examination of fish tissues exposed to toxicants through histopathological analysis reveals

evidence of chronic cellular, tissue, and organ damage. This approach has been employed as biomarkers to assess the overall health of fish. Consequently, a qualitative assessment of histopathological and biochemical parameters in the target organs and tissues within this study will unveil the potential effects of atrazine and shed light on the potential protective role of Vitamin C in juveniles of *Clarias gariepinus*.

MATERIALS AND METHODS

Test Chemicals

Commercial formulation of atrazine, AtraForce ® 50% SC (New Haven, CT, USA), containing the herbicide atrazine (50%), was purchased from the manufacturer. Single concentration stock of 7.5 µg/mL of atrazine in water were freshly prepared every two (2) days. From the stock, 20, 40 and 60 mL were transferred into 15 L of water, resulting in target concentrations of 10, 20 and 30 µg/L, respectively. Vitamin C, Em-Vit- C ® (Emzor Pharmaceuticals, Nigeria), was also purchased for this study.

Experimental Design and Treatment

Five hundred (500), 4-week-old *Clarias gariepinu*s juveniles were purchased from the Department of Fisheries, Faculty of Agriculture, University of Benin and maintained in the animal house at the Department of Animal and Environmental Biology. The fishes were acclimatized for a week in a 60L holding tank filled with dechlorinated water. After acclimatization, twenty juvenile fish were randomly allocated to each treatment and control tank. The juvenile fish had an average weight of 6.93 ± 1.52 g and an average standard length of 8.32 ± 0.81 cm. They were fed 4g (2mm) of commercial fish feed (Blue crown, Olam Group, Kwara, Nigeria) twice a day at eight hours intervals and the test water was renewed every two days to remove debris and possible contaminants. The fish were exposed to three concentrations (10, 20 and 30 µg/L) of atrazine and control (0 µg/L) in a semi-static renewal bioassay for a period of 28 days. The experimental tanks were labeled as AT_I, AT_{II}, AT_{III}, AT_IVT, $AT_{II}VT$, $AT_{III}VT$, CVT and C, representing the three atrazine concentrations, atrazine concentrations with vitamin C, control (with vitamin C) and control (without vitamin C), respectively. At the end of the 28-day period, the brain and liver tissues were harvested from both the control and treatment groups for laboratory analysis.

Extraction of Tissue Samples

At the end of the 28 days exposure period, the fish specimens from control and treatment tanks were dissected carefully using a dissecting kit and the liver and brain were carefully collected. Harvested tissues were placed in different sample bottles, well labelled and preserved in an ice chest and transported to the laboratory for analysis. The organs for histopathology were properly fixed with 10% formalin solution in EDTA Bottles.

Biochemical Analysis

Acetylcholinesterase (AChE)

Freshly harvested brain tissues were prepared by homogenization in 0.1 M phosphate buffer, pH 7.5, followed by centrifugation at 5,000 rpm for 5 minutes. Clear supernatants were used for the assay. One (1) ml of distilled water (assay blank) and 1ml of calibrator were transferred into cuvette and adsorbance was read at 412nm. Fifty (50) µl sample was transferred into a clean cuvette and 800µl buffer was added to the samples only and mixed. Thereafter, 100µl chromogen was added to the samples only, mixed and incubated for 2 mins, while the initial absorbance at 412nm was read. Then 50µl substrate was added to the samples and final absorbance was read at 412nm. This assay is an optimized version of the Ellman method in which thiocholine, produced by AChE, reacts with 5,5° dithiobis (2-nitrobenzoic acid) to form a colorimetric (412 nm) product, proportional to the AChE activity present (Ellman, 1961).

Histopathological Analysis

The tissues were subject to automatic tissue processing using the Leica TP2010 automatic tissue processor for eighteen hours passing them through the four stages of tissue processing namely, Fixation (using 10% Neutral buffered formalin), Dehydration (using ascending grades of isopropyl alcohol), Clearing or dealcoholisation (using xylene) and finally impregnation or infiltration (using molten paraffin wax). The tissues were then embedded in paraffin wax using the Leica automated tissue embedder and sectioned to get ultra-thin sections at five (5) microns, using the thermo scientific semi-automated rotary microtome. Tissues were floated out from the thermo scientific digital floating bath on frosted end pre-labelled slides and dried on the thermo scientific digital slimline hot plate. Tissues were further dried in the hot air oven for one hour at 60 ◦C and subjected to heamatoxylin and eosin staining to demonstrate the general tissue structure. Stained slides were mounted in DPX and allowed to dry before viewing under the microscope using x40 magnification.

Statistical analysis

Data across exposure concentrations were analyzed using computer software, Statistical Package for Social Sciences (SPSS version 21) for levels of the enzyme (AChE). Data were expressed as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) was used to test for significance followed by a posthoc Tukey's multiple tests, for statistical comparisons among the groups. Differences between means were considered significant at $p<0.05$.

Results and Discussion

Physico-chemical Parameters of Test Water

The water physico-chemical characteristics of the test water did not significantly vary from recommended limits for different parameters set by relevant environmental guidelines (Badiru, 2005). The test water had a temperature of 28°C, pH of 6, electrical conductivity of 44µS/cm, alkalinity, hardness, dissolved oxygen, and B.O.D of 6mg/l, 8mg/l, 5.8mg/l and 2.1mg/l, respectively. The concentration of atrazine in water over a 28-day period has previously been reported to remain relatively stable throughout the exposure duration (Opute and Oboh, 2021).

Behavioural Analysis

The behavioural characteristics of *C. gariepinus* juveniles following exposure to atrazine included heightened opercular rate, erratic swimming, mucus secretion, and air gulping (Table 1). The presence of Vitamin C significantly inhibited the toxic effects of the pesticides evidenced by the abated unusual behaviours observed in treatment groups

without vitamin C supplements. The behavioural changes were dose dependent.

Table 1: Behavioural analysis of *Clarias gariepinus* juveniles exposed to sub-lethal atrazine concentration for 28days with and without Vitamin C supplementation.

Behaviour	Control	AT_I	AT_2	AT_3	CVT	AT_1VT	AT_2VT	AT_3VT
Erratic swimming	-	-	+	++	-	-	+	+
Loss of Balance	-	+	++	+++	-	-	+	+
Mucous secretion	-	+	++	+++	-	-	+	++
Heightened opercular rate	-	+	+++	+++	-	-	+	++
Air gulping	-	+	++	+++	-	-	-	+

(+) low response, (++) moderate response, (+++) high response, (-) no response

Biochemical Assessment

Acetylcholinesterase (AChE) Activities on the Brain

Acetylcholinesterase is an enzyme typically tasked with deactivating the neurotransmitter acetylcholine at the synaptic and neuro-effector endings of cholinergic motor and secreto-motor neurons within the enteric nervous system (Opute and Oboh, 2021). The assessment of AChE on the brain of *Clarias gariepinus* exposure to atrazine (Figure 1) revealed a dose-dependent decrease in activity across all treatment concentrations when compared to the control, except for $AT_{III}VT$, which demonstrated an increase in activity. The control had an average AChE value of 47.86 while the group with highest treatment concentration (AT_{III}) presented a lower value of 32.3. Atrazine has been earlier reported to inhibit this enzyme, resulting in overstimulation of muscle fibers and potentially causing paralysis or even death in animals (Al-Sawafi and Yan, 2013). Furthermore, atrazine has also been reported to significantly inhibit levels of brain AChE activities rather than muscular AChE when exposed to 1000 µg/L of atrazine, however, exposure to 10 µg/L atrazine did not affect behaviour or AChE activity (Schmidel *et al*., 2014). AChE serves as a crucial biomarker for detecting neurotoxic agents and given that AChE can be influenced by chemical pollutants (Schmidel *et al*., 2014), The decline in AChE activity in C. gariepinus after exposure to atrazine in this study may be linked to the oxidative stress caused by atrazine exposure (Salbego *et al.*, 2010). Numerous studies have highlighted the inhibitory impacts of pesticides on AChE levels in various fish species (Abdel-Tawwab & Hamed, 2018; Hamed & El-Sayed, 2018). Inhibition of acetylcholinesterase (AChE), a key enzyme in controlling acetylcholine's function at the neuromuscular junction, can result in several outcomes. For instance, it can hinder swimming behavior (Perez *et al.*, 2013), affect feeding capabilities, and potentially cause the eventual death of the organism (Mit *et al.*, 2021). Inhibition of AChE in this study resulted in significant behavioural abnormalities including erratic swimming, loss of equilibrium, and lethargy. Endosulfan exposure has been previously reported to inhibit brain AChE activity and impair swimming performance in adult zebrafish (Danio rerio) (Pereira *et al.*, 2012). However, in treatment concentrations supplemented with vitamin C, there was an observed increase in AChE levels compared to the same concentrations without vitamin C except $AT_{II}VT$. This shows that Vitamin C may play a role in mitigating the effects of atrazine. The neuroprotective properties of dietary supplementation of aqueous *Carica papaya* and *Mangifera indica* leaf extracts have been demonstrated to counteract the atrazine-induced suppression of AChE activity in fish (Owolabi and Abdulkareem, 2021). Additionally, the protective effects of certain plant extracts against the decline of AChE activity due to xenobiotic exposure have been documented in fish (Hamed & El-Sayed, 2018; Gombeau et al., 2019). Therefore, dietary supplementation of fish feed with Vitamin C in this study has amelioration potential against atrazine induced toxicity.

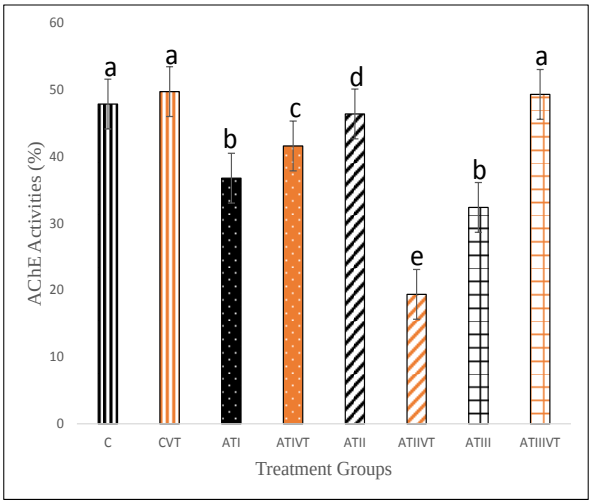

Figure.1. Activities of AChE enzyme in the brain of *Clarias gariepinus* exposure to concentrations of atrazine and vitamin C for 28 days

Qualitative Histopathological Assessment

Significant alterations were observed in the brain which were not present in the control groups (Figure 3 - 10).

Brain Histopathology

The histopathological alterations observed in the exposed juveniles of *C. gariepinus* provide insights into the extent of damage inflicted on their brains by atrazine. The progressive nature of the damage and degenerative alterations in the brain of the fish resulting from atrazine toxicity indicate that histopathological responses are influenced not only by the pesticide concentration but also by the duration of exposure. Histopathological assessment of microscope slides from the control group revealed a normal brain tissue structure (Figure 3). Additionally, the control group supplemented

with Vitamin C exhibited normal cell sizes with no indications of hemorrhages with a normal appearance of nerve cells (Figure 4). However, brain tissues from juvenile specimens across treatment groups revealed histopathological alterations, including disorganization and enlargement of cells with vascular spaces (Figure 5 - Figure 7), the appearance of blood congestion (Figure 5), dissociation of Purkinje's cell layer from the granular cell layer (Figure 7), indications of hemorrhage and cellular infiltration (Figure 8), severe cell infiltration (Figure 9), and complete disorganization of cells (Figure 9 - Figure 10).

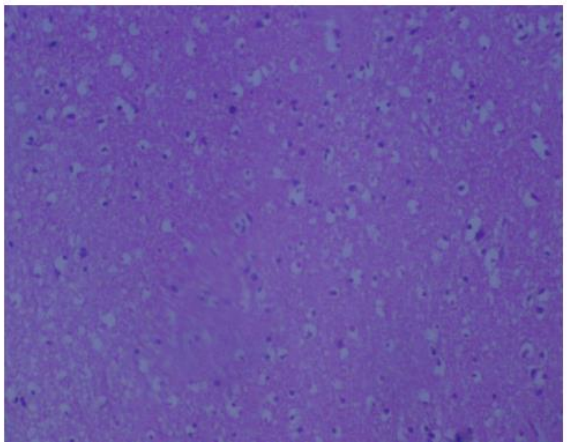

Figure 4.3. Transverse section cerebrum (Control); Cells are of normal sizes with no indication of haemorrhages and normal appearance of the nerve cells (H&E, x40).

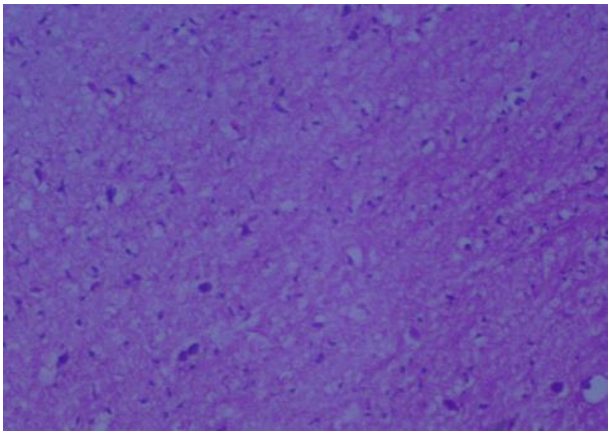

Figure 4.4. Transverse section cerebrum (Control + Vitamin C); Cells are of normal sizes with no indication of haemorrhages and normal appearance of the nerve cells (H&E, x40).

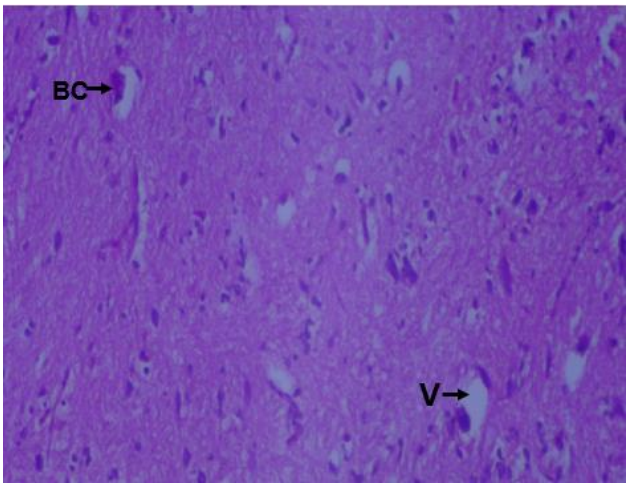

Figure 4.5. Transverse section cerebrum of C. gariepinus juveniles exposed to atrazine (AT_1); Cells are disorganized and are of large sizes with vascular spaces around them (V); appearance of blood congestion (BC) (H&E, x40).

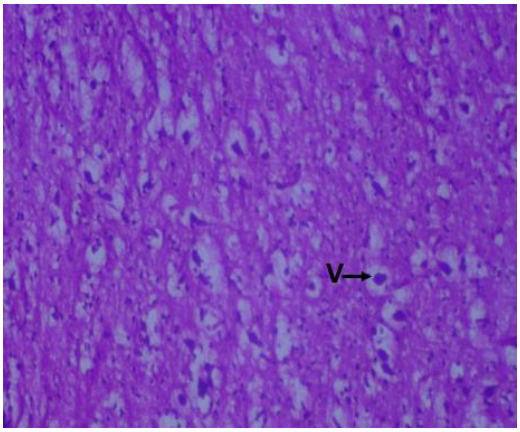

Figure 4.6. Transverse section cerebrum of *C. gariepinus* juveniles exposed to atrazine + Vitamin C (AT_1V); Cells are disorganized and are of large sizes with vascular spaces around them (V) (H&E, x40).

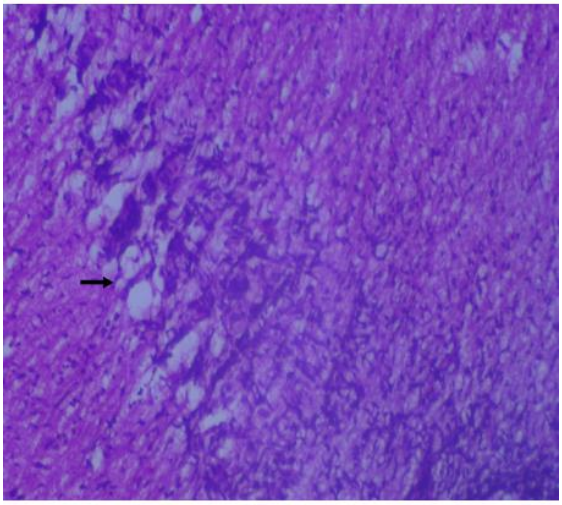

Figure 4.7. Transverse section cerebrum of *C. gariepinus* juveniles exposed to atrazine (AT_2); Cells are disorganized and are of large sizes; Dissociation of Purkinje's cell layer (DPC) from the granular cell layer (H&E, x40).

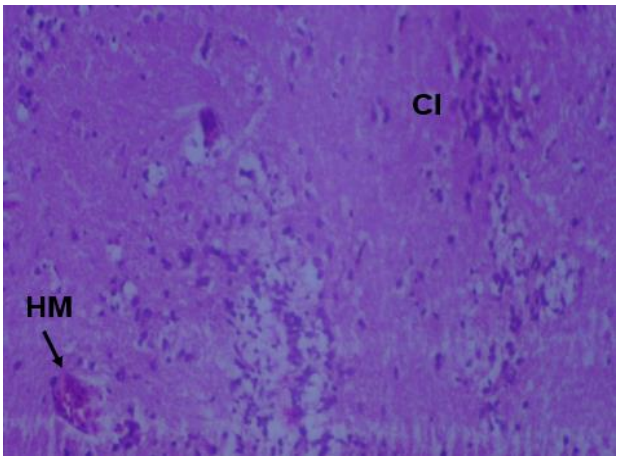

Figure 4.8. Transverse section cerebrum of *C. gariepinus* juveniles exposed to atrazine + vitamin C (AT_2Vc); Cells are disorganized and are of large sizes with vascular spaces; indication of haemorrhage (HM) and cellular infiltration (CI) (H&E, x40).

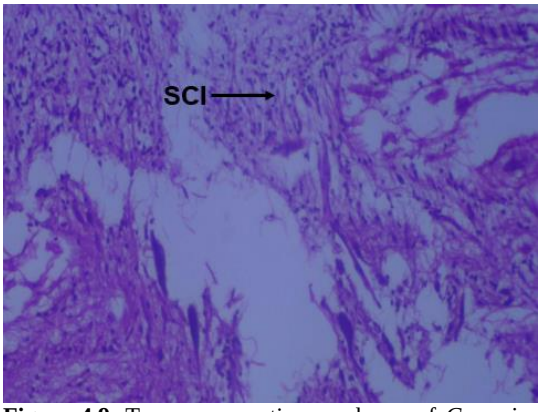

Figure 4.9. Transverse section cerebrum of *C. gariepinus* juveniles exposed to atrazine (AT_3); Cells are completely disorganized; severe cell infiltration (SCI) (H&E, x40).

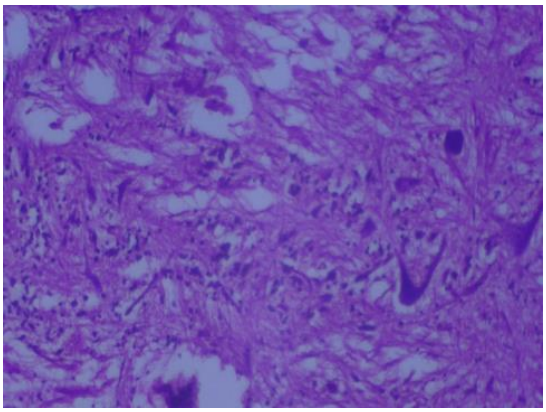

Figure 4.10. Transverse section cerebrum of *C. gariepinus* juveniles exposed to atrazine + vitamin C (AT_3Vc); Cells are completely disorganized (H&E, x40).

Several authors have documented diverse histopathological alterations in the brains of fish exposed to various chemical substances. Das *et al.* (2000) noted mild vacuolar changes in the cerebrum with empty spaces after exposure to 0.35 ppm hexachlorocyclohexane. In contrast, at 1.73 ppm, they observed severe necrosis of neuronal cells in the cerebrum and loss of Nissl substance in the brains of Indian major carp (*Labeo rohita*) exposed to hexachlorocyclohexane. Ayoola and Ajani (2008) reported mononuclear infiltration, neuronal degeneration, and severe spongiosis in the brain of *C. gariepinus* after exposure to lethal concentrations of cypermethrin. Another report by Ayoola (2008) indicated severe congestion, mononuclear infiltration, hemorrhage, and generalized spongiosis in the brains of *Oreochromis niloticus* exposed to lethal concentrations of glyphosate. The brain of the freshwater fish *Ophiocephalus punctatus* exhibited disintegration and severe damage in brain cells, as well as the breakdown of neural bundles, after exposure to different concentrations of malathion (Pugazhvendan *et al.*, 2009). Different regions in the fish brain are associated with various functions, and the impairment of tissue in a specific region due to these pathological changes may result in the restriction of particular functions in fishes. This, in turn, can alter the physiological and behavioral functions of the fish (Lakshmaiah, 2017), as evidenced by significant behavioral abnormalities observed in this study, including erratic swimming, loss of equilibrium, and lethargy. The nervous system especially the brain is one of the most important organs in the body, because it controls many functions, as such, the atrazine-induced histopathological lesion in the brain of *C. gariepinus* observed in this study offers possible explanation for the reduced feeding activity and other unusual behaviours following a likely nervous system failure caused by pesticide poisoning, which affected physiological and biochemical activities.

Conclusion

The results of this study concluded that the exposure of fish juveniles to atrazine disrupts normal behaviour and neurodevelopment. Atrazine exposure also resulted in histopathological alterations. Dietary supplementation with Vitamin C plays a significant role in mitigating the effects induced by atrazine toxicity. It is recommended to conduct further scientific investigations at the molecular and cellular levels to validate the existing research findings on the effects of atrazine.

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