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Histomorphometry of Liver and some Blood Factors of Nile Tilapia, *Oreochromis niloticus* Exposed to Different Concentrations of Ammonia

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ABSTRACT

Most tilapias are microphytes, but some prefer higher plants. Ammonia is one of the most important toxic compounds of nitrogen, which is a serious problem in the environment and aquaculture industry. In the present study, juvenile *Oreochromis niloticus* were exposed to 10, 20, and 30% (96h LC50) of ammonia for two weeks, which are equivalent to 0.9, 1.8, and 2.7 mg / l, respectively. After this period, the fish were anesthetized and blood samples were taken from the caudal stalk with a heparin syringe for evaluating blood indicators. The tissue samples were taken 0.5 cm from the liver, fixed in 10% formalin buffer, and after dehydration with alcohol, clarification with xylol, blocking with paraffin, and cutting 4-6 microns thick with microtome were done. Finally, the stained slides were studied with a light microscope. The results showed phenomena such as hyperemia, nuclear hypertrophy, sinusoidal dilatation, increased melanomacrophage centers, nucleus margination, hepatocyte vacuolation, and cell necrosis in the liver. In the studies of blood serum factors with the increase of ammonia, it has been increased in AST, ALT, and ALP compared to the control and other groups. Also, as the ammonia concentration increased, the severity of the lesions also increased. Therefore, ammonia causes changes in the structure and activity of metabolic enzymes of the liver, which must be controlled by creating the appropriate ammonia and management conditions in the aquatic environment.

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INTRODUCTION

The genus tilapia is a member of the cichlid family and belongs to the Perciformes order, which has a rectangular body (Abdelghany, 2020; Rahmati et al., 2022). Most tilapias are microphytes, but some prefer more organic plants and use organic plants in places where other species of farmed fish feed on plankton (Kim et al., 2019; Gammerdinger et al., 2016). Today, one of the most important human concerns is the increased concentration of contaminants in the environment, especially water, which contaminate world waters in the form of sewage, oil spills, sewage of organic and mineral materials of factories, various chemicals including metals and quasi-metals, etc. One of the most important contaminants is nitrogen compounds, the most dangerous of which is ammonia. Ammonia is widely recognized as a common contaminant in aquatic environments. Ammonia (NH3) consists of nitrogen and hydrogen (El-Sayed and Saad, 2008). It is a common nitrogenous waste, especially among aquatic organisms, and significantly contributes to the nutritional needs of terrestrial organisms by acting as a precursor in fertilizers. Ammonia poisoning is one of the leading causes of fish mortality in aquaculture environments

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(Wang and Walsh, 2000). Ammonia is mainly produced by fish through the catabolism of proteins. It is very toxic to fish, so they need to get rid of this waste product by releasing it into the water. Ammonia damages the liver and other tissues and causes a variety of symptoms. According to research, compounds such as ammonia can easily enter the circulatory system of fish (Kim et al., 2019). Because fishes are permanently immersed in such polluted environments and excretory organs such as the liver and directly exposed to ammonia for a long period of time. As a result, damage to these tissues causes disruption in liver enzymes and factors such as cholesterol. Because after facing stress, fish maintain their body homeostasis in different ways. Liver damage includes spot lesions, necrotic lesions, and degeneration in fish living in infected areas. Considering the role of ammonia in different forms of water contamination and its possible entry into water sources, the aim of the present study was to investigate its effect on *Oreochromis niloticus* as well as liver tissues and enzymes.

MATERIALS AND METHODS

Animals

In the present study, a total of 120 *Oreochromis niloticus* with an average weight (35 ±1g) were prepared, transferred to the laboratory, and kept in a 500-liter tub for two weeks in order to adapt to the new conditions. They had access to proper aeration, feeding by plate (0.02 body weight/day). During the experiment, the mean temperature of water temperature, oxygen concentration, and water hardness were 27 ± 90 °C, 6.2 ± 1 mg / L, and 269 ± 3 mg / L, respectively. After sorting, the fish adapted to the new environmental conditions. Since the lethal concentration (96h LC50) of ammonia is not known, a range-finding test was performed to find the lethal range of ammonia on *O. niloticus*. A static acute toxicity test was performed on *O. niloticus* for 96 hours according to standard instructions (Gao et al., 2020). Ammonia (Merck, Germany) was prepared as an ammonium chloride solution. Feeding of juveniles was stopped 24 hours before the toxicity test. Effective physicochemical parameters of water including pH, dissolved oxygen, and temperature were recorded daily. After determining the lethal range, the final acute ammonia toxicity test was performed in five control treatments for three replications. In each treatment, ten fish were placed in pre-aerated 15-liter aquariums. The dead fish were collected from the aquarium environment immediately and the number of fish losses at 24, 48 , 72, and 96 hours was calculated and recorded. Data obtained from the acute toxicity test was also analyzed by Probit Analysis with a 95% confidence interval (Morovvati et al., 2012). For sub-lethal toxicity studies, 120 *O. niloticus* juveniles were assigned into four treatments, three groups based on different percentages of LC50 96h (10, 20, and 30% LC50 96h) and one control group exposed to ammonia for 14 days at constant temperature and pH. Ten juvenile *O. niloticus* were randomly distributed in 100-liter aquariums and experiments were performed semi-statically according to the standard O.E.C.D method (20% of the aquarium water was changed on a daily basis). The food residues were removed from the aquariums when changing the water. In order to keep the ammonia concentration constant, the same water content, which was removed from the aquarium, was added to the aquarium (Moradkhani et al., 2020).

Tissue sampling and histopathology

Tissue sampling was performed on 5 fishes of each group randomly on days 0 and 14 days after exposure. At the end of the experimental period, fish were randomly caught from each treatment. In order to evaluate the pathological liver impacts of ammonia, liver tissues of each of the treatments were sampled and fixed in 10% formalin buffer solution for 24 hours (Rahmati et al., 2022). Then the fixed samples were transferred to 70% ethanol, then dehydration was performed with a series of 70, 80, 90, and 100% ethanol concentrations (Mohamed et al., 2021). Afterward, clarification was performed by xylene. Liver and kidney tissue samples were then deparaffinized and molded using molten paraffin. All these steps were performed by the tissue

passage device under a pre-defined program. The tissues were then molded and paraffinized on a Tissue-tek mold system at a melting point of 56-58° C. A microtome was used to prepare 4-6 µm thick sections from paraffin molds (Mohamed et al., 2020). After being placed on the slide, molds were placed in an oven at 60°C for half an hour to remove excess paraffin from the tissue. After deparaffinization and replacing it with xylene, the samples were restained by reducing ethanol solutions series. The prepared tissues were transferred back to the oven to dry. Five sections were prepared from each sample and stained and investigated using hematoxylin and eosin and specific stain. The prepared slides were investigated under a light microscope that was connected to a Dinolit lens and a computer system equipped with Dinocapture software. Ten five fields of view from each liver slides were considered (Morovvati et al., 2017).

Serum tests

To study liver enzymes, fish were anesthetized using powdered dianthus, blood was then taken from the tail vein using a 2cc heparin syringe. First, the fish were anesthetized with dianthus extract and blood samples were taken from the tail veins using an insulin syringe (Latif et al., 2014). Finally, the blood was centrifuged at 3000 rpm for 10 minutes and the plasma was transferred to newly labeled microtubes and stored at -80 °C until analysis and used to measure hepatic blood biochemical factors (Savari et al., 2016).

Statistical Analysis

Since we have quantitative and continuous data in the present study, the number of independent groups was evaluated. Considering the normal data distribution, the Kolmogorov-Smirnov test was used. Data analysis was also carried out using SPSS ver. 19 (SPSS Inc, version 19.00, California, USA), and all results were expressed based on the mean ± standard deviation (Hasanzadeh et al., 2018). One-way analysis of variance was used for inter-group comparison and then Tukey's multiple comparison tests were used. $P<0.05$ was considered as the level of significance (Pourkhadje et al., 2014).

RESULTS AND DISCUSSION

General histopathology

The results of the studies did not show any losses in different treatments under total ammonia lethal rate during the adaptation period (0.9, 1.8, and 2.7 mg / l) and control treatment for two weeks. Microscopic studies compared the liver tissues of fish exposed to different ammonia concentrations with control fish and showed tissue changes and lesions that included hyperemia, nuclear hypertrophy, sinusoidal dilation, increased melanomacrophage centers, nuclear margination, hepatocyte vacuolation, and cell necrosis. Overall in histometric studies, the severity of these complications increased with increasing ammonia concentrations (Figure A_{1-} $A_{10;}$ Table 1; Figure 1-4).

Serum findings

In the studies of blood serum factors with the increase of ammonia, it has been increased in AST, ALT, and ALP compared to the control and other groups.

Ammonia is one of the serious problems in the aquaculture industry and causes a series of changes in the tissue indices of the liver and some blood biochemical factors in different fish (Amiripour et al., 2015; Morovvati et al., 2012). One of the fundamental problems is to obtain a standard ammonia concentration that is not dangerous and harmful to aquatic life and to create an environment in which this water toxin causes the least harm to fish. Juvenile fish show higher sensitivity to ammonia and *O. niloticus* was used in the current study (Roshanfekr et al., 2017). Ammonia concentrations are strongly influenced by temperature, pH, and other factors such as dissolved oxygen levels, salinity, animal species, age, and fish size. When the

NH3 concentration in water increases, it enters the cells because it can be infiltrated to the cell membrane, and is plasma ammonia levels increase as its excretion from the fish body decreases. Elevated blood ammonia levels increase oxygen consumption and reduce the blood's ability to transport oxygen in the body. Liver tissue is very suitable for pathological examination because the ammonia poisoning-induced pathological lesions in different fish are often specific to the

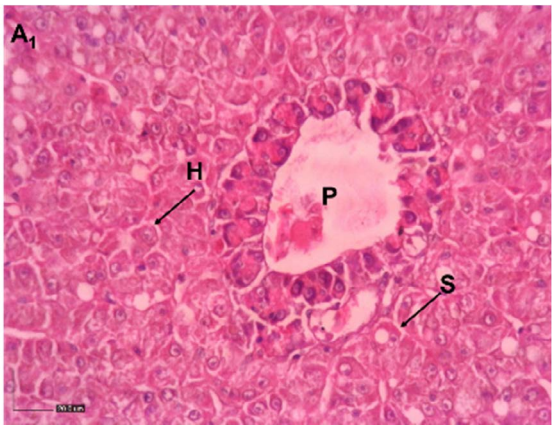

Fig. A_1. Control group liver. Hepatocyte (H), sinusoid (S), and the outer part of the pancreas (P) were shown. H-E x400.

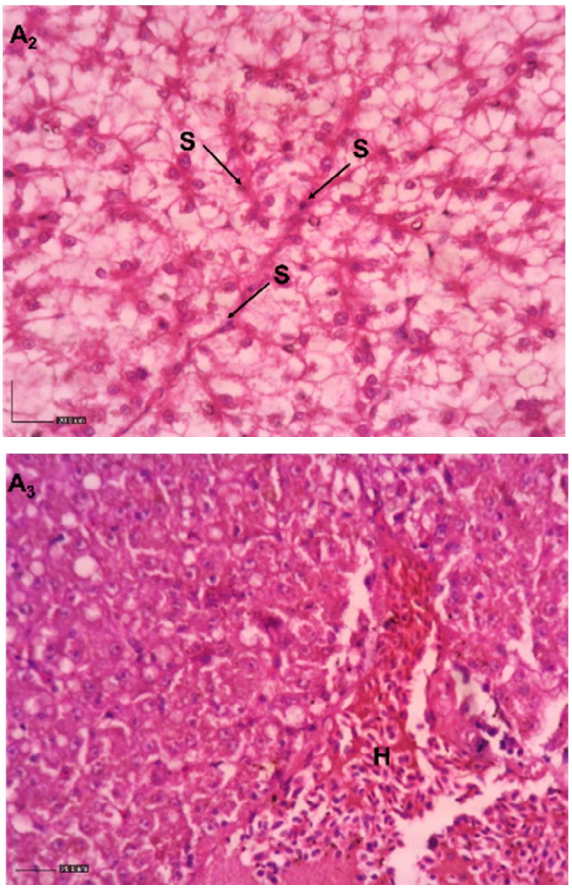

Fig. A_2- A_{10}. The group was exposed to 2.7 mg/l ammonia after two weeks. Sinusoidal dilation (S), Hypermia (H), Melanomacrophages aggregates (Mc), Nucleolar margination (M), Necrosis (N), Hepatocytes vacuolation (A_7), Hypertrophy of hepatocyte nucleus (H). (H-E, x400). Decreased glycogen content of hepatocytes (A_9). (PAS, x400). Increase the view of connective tissue (A_{10}). (Trichrome, x400).

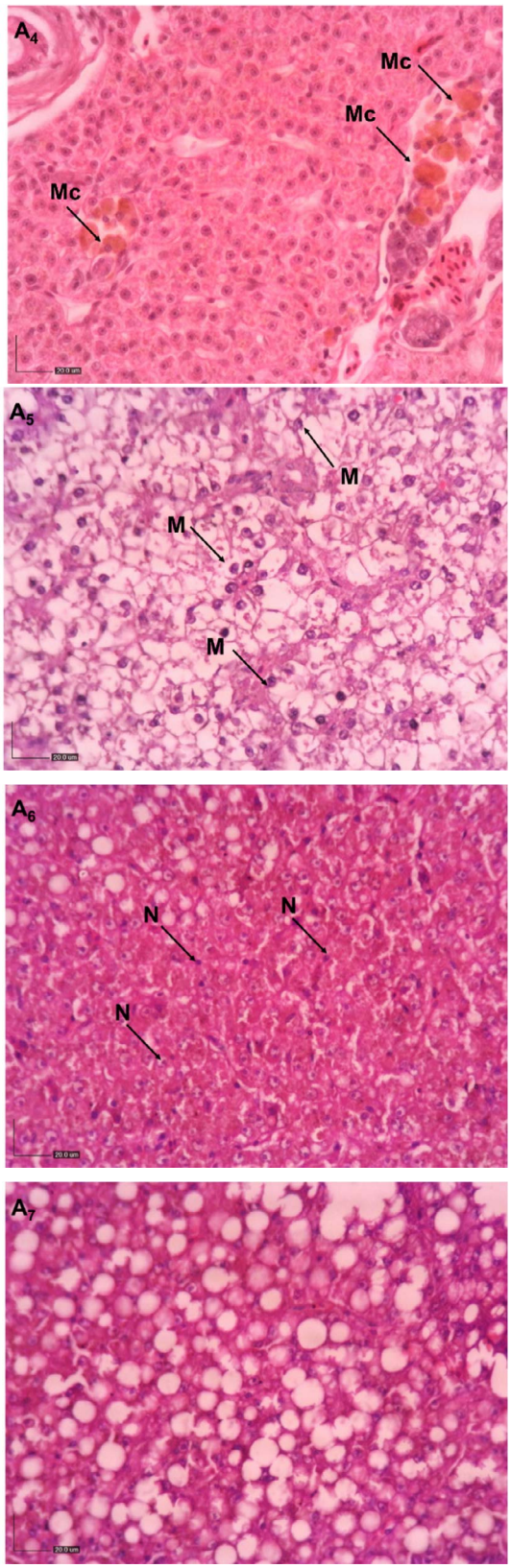

Fig. A_{2-} A_{10}. The group was exposed to 2.7 mg/l ammonia after two weeks. Sinusoidal dilation (S), Hypermia (H), Melanomacrophages aggregates (Mc), Nucleolar margination (M), Necrosis (N), Hepatocytes vacuolation (A_7), Hypertrophy of hepatocyte nucleus (H). (H-E, x400). Decreased glycogen content of hepatocytes (A_9). (PAS, x400). Increase the view of connective tissue (A_{10}). (Trichrome, x400).

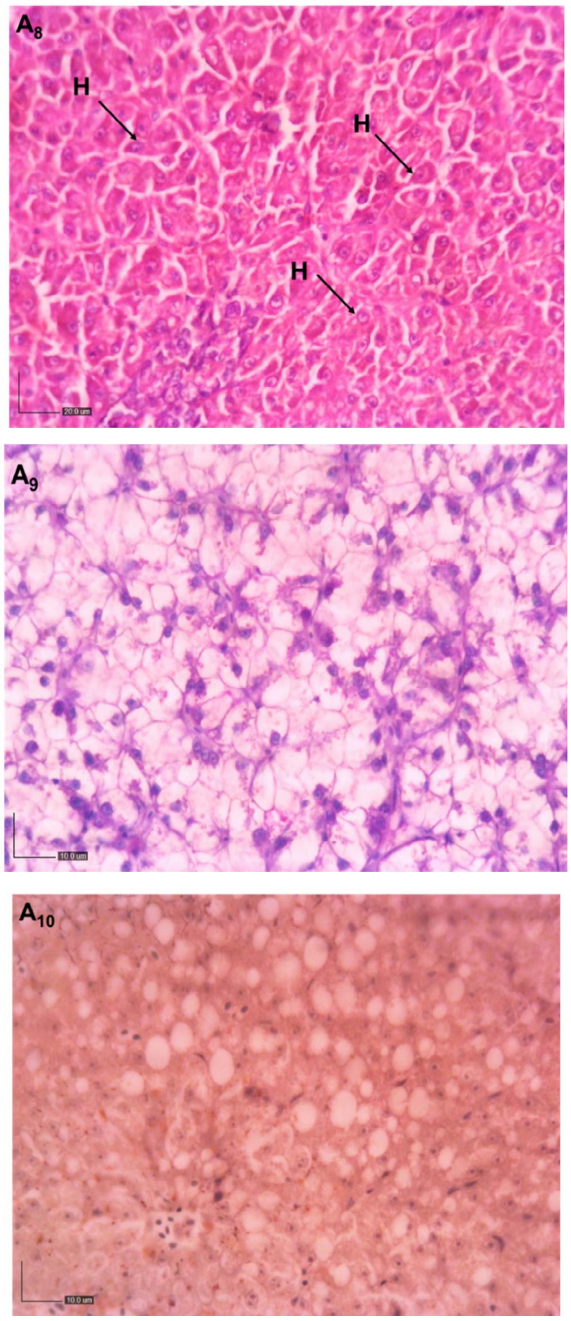

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Table 1. Morphometric analysis of the liver of tilapia after 2 weeks of exposure to ammonia

Changes	Control	0.9 mg/l	1.8 mg/l	2.7 mg/l
Melanomacrophages aggregates	-	+	++	+++
Hypermia	-	+	++	+++
Sinusoidal dilation	-	+	++	+++

Note: absent (-); rare (+); frequency (++); very frequency (+++);Number fish per group =10

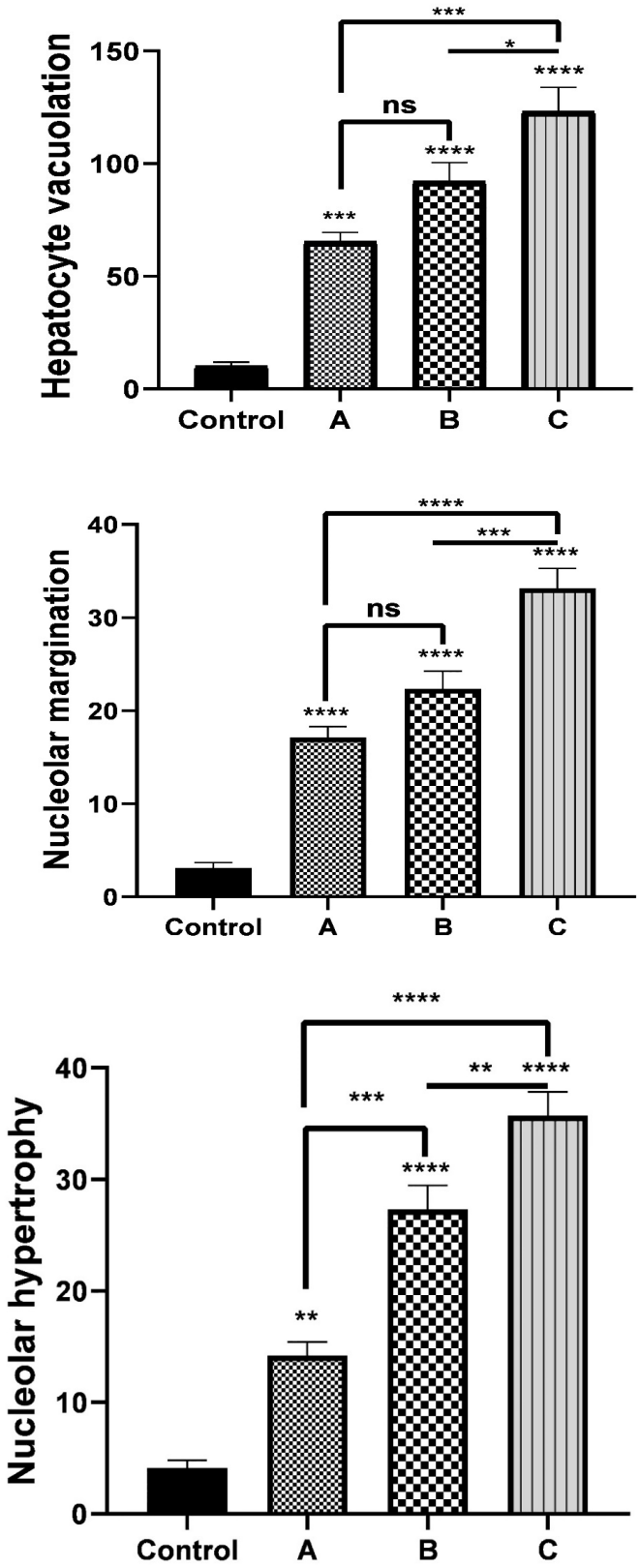

Fig. 1-4. Control and Groups were exposed to 0.9 mg/l (A), 1.8 mg/l (B), and 2.7 mg/l (C) ammonia after two weeks. Increasing the number of vacuolation of hepatocytes, an Increase in the number of hepatocyte nuclei, An increase in the number of hypertrophied nuclei of hepatocytes, Increased number of cell necrosis. The asterisks indicate a significant difference at the level of ($P<0.05$), N=10.

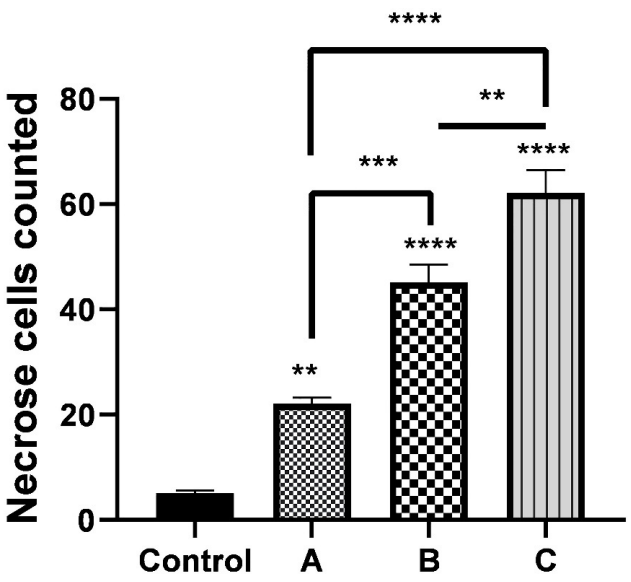

Fig. 1-4. Control and Groups were exposed to 0.9 mg/l (A), 1.8 mg/l (B), and 2.7 mg/l (C) ammonia after two weeks. Increasing the number of vacuolation of hepatocytes, an Increase in the number of hepatocyte nuclei, An increase in the number of hypertrophied nuclei of hepatocytes, Increased number of cell necrosis. The asterisks indicate a significant difference at the level of ($P<0.05$), N=10.

TableS 2. Serum blood factors of tilapia after 2 weeks exposure to ammonia

Factor	Control	A	B	C
AST (U/L)	124.44±21.33 [a]	137.47±11.38 [a]	145.41±13.63 [a]	153.41±16.82 [a]
ALT (U/L)	2.39±0.67 [a]	2.74±0.25 [a]	3.31±0.38 [a]	4.19±0.29 [a]
ALP (U/L)	74.35±7.38 [a]	81.55±7.21 [a]	87.22±6.31 [a]	91.54±7.16 [a]

liver because this tissue plays a major role in ammonia metabolism and excretion. In a study, carp were exposed to lethal ammonia concentrations, and changes in liver enzymes were examined. Results showed a significant increase in AST and ALT enzymes (Kakkar et al., 2011; Jeney et al., 1992). ALT and AST activities are essential in cellular nitrogen metabolism, amino acid oxidation, and hepatic glucogenesis (Zeitoun et al., 2016). In a study, attributed increased AST and ALT levels to the increase in amino acid transaminases and the tricarboxylic acid (TCA) cycle. Besides, increased AST and ALT levels help repair and synthesize this enzyme in order to support glucose production for more energy production (Basir and Abdi, 2016; Roshanfekr et al., 2018). Since aminotransferase is located in the mitochondria and is a biologically beneficial marker for cell damage, changes in blood aminotransferase levels can be associated with mitochondrial disorders and tissue damage. Alkaline phosphatase (ALP) enzyme is present in liver cells, bile duct epithelium, and intestinal and renal mucosa. Obstruction of extrahepatic and intrahepatic bile ducts, cirrhosis, liver disorders, and necrosis of liver tissue leads to increased ALP levels (Becke et al., 2019; Guo et al., 2020). Elevated ALP levels in contact with various contaminants have been introduced as an indicator of liver tissue damage and liver dysfunction in common carp, *O. niloticus*, snakeheads, and mudskippers (Xu et al., 2021; Moallem et al., 2015). Results of the present showed that ammonia has destructive effects on liver tissue. Hepatic complications included hyperemia, nuclear hypertrophy, sinusoidal dilation, increased melanomamacrophage centers, nuclear margination, hepatocyte vacuolation, and cell necrosis (Dastan et al., 2017; Metwally and Wafeek, 2014). Overall, there was an increasing trend in the

severity of these complications with increasing ammonia concentration (Savari et al., 2020). In conclusion, the ammonia susceptibility of fish species in a family is not the same. In the face of chronic ammonia poisoning, growth rate and survival rate decrease and fish become more susceptible to infectious agents (Wood et al., 2017). These conditions are usually associated with gill, liver, and kidney lesions. Overall, it can be concluded that the presence of nitrite and ammonia in water can cause microscopic and macroscopic damage in different tissue of various fish.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

LIFE SCIENCE REPORTING

No life science threat was practiced in this research.

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