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Sublethal Effects of Acute Ammonia Exposure on *Oreochromis niloticus*

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Abstract: Ammonia is the principal nitrogenous waste product of fishes and it is also the main nitrogenous waste material excreted by gills beside urea and amines. This study was planned to determine the effect of acute ammonia exposure on cultured *Oreochromis niloticus*. Fish of nearly the same weight were divided into four groups. The 1st group was served as a control group, the 2ndgroup exposed to 2.5mg/L of total ammonia nitrogen (TAN) (0.16 NH_3 mg/L), the 3rdgroup exposed to 5.0 mg/L of total ammonia nitrogen (TAN) (0.32 NH_3 mg/L) and the 4thgroup exposed to 10.0 mg/L of total ammonia nitrogen (TAN) (0.65 NH_3 mg/L) at water pH 8 and water temperature 28°C for six days.Results revealed that acute ammonia exposure was associated with fish mortalities, clinical abnormalities in the form of convulsions, hyperexcitability, signs of asphyxia and sluggish fish reflexes. Histopathological examination showed lamellar hyperplesia with fusion of the secondary gill lamellae, hydropic degeneration in the liver, hyperactivation of the melanomacrophagecenter of the spleen, glomerulonephritis and cerebral oedema with neuronal degeneration.Also significant increase in lipid peroxidation product (malondialdehyde), nitric oxide, cortisol and micronucleus count were recorded. It was concluded that acute ammonia exposure had several deleterious effects on cultured *Oreochromisniloticus*.

Key words: Toxic Ammonia · Lipid Peroxidatioon · Micronucleus Assay · Clinical Signs · Histopathological Changes · *Oreochromis niloticus*.

INTRODUCTION

Ammonia is a common aquatic pollutant that can enter natural aquatic systems via discharges from wastewater treatment plants, degradation of nitrogen-containing organic matter, fertilizer runoff and industrial sources. Ammonia is also the main nitrogen waste material in teleosts and is generated as a product of protein catabolism [1]. Under intensive culture conditions, if water flow is restricted or inadequate, the presence of uneaten food and organic waste can lead to increased concentrations of ammonia in the water, which may reach levels that adversely affect the physiology of fishes.

Two forms of ammonia occur in water, un-ionized (NH_3) and ionized (NH_4^+) ammonia and the relative proportion of each form is dependent on pH, temperature and salinity [2]. The un-ionized form of ammonia (NH_3) is highly toxic to fish, while the ammonium ion (NH_4^+) is much less so. Un-ionized ammonia mainly enters fish via the gills, as it can readily pass through the gill epithelium [3], which, however, is rather impermeable to ionized ammonia [4].

Ammonia concentrations are usually at their highest rate in the production season when biomass of the cultured species and the amount of protein fed are greatest. Ammonia-nitrogen (NH_3-N) has a more toxic form at high pH and a less toxic form at low pH, un-ionized ammonia (NH_3) and ionized ammonia (NH_4^+), respectively. In addition, ammonia toxicity increases as temperature rise [5].

Exposure of fish to high levels of ammonia therefore results in a rapid increase in plasma levels of the compound [6] and it might result in net accumulation of ammonia at toxic levels in the fish [7].

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The toxic levels of un-ionized ammonia for short-term exposure usually are reported to lie between 0.6 and 2 mg/l, while some consider the maximum tolerable concentration to be 0.1 mg /l [8].

In intensive fish farming, excessively high doses of ammonia, issuing either from excretion or from external pollution can cause reductions in fish growth or even death [9]. For these reasons, toxicity of ammonia to fish has been intensively investigated in numerous of fish species [10].

It has been reported that tilapia can with stand very polluted environment, feeding on animal manure and even sewage sludge [11]. Thus, the intensification of Nile tilapia farming needs to optimize all the production factors, especially the quality of water.

Many studies confirmed the validation of micronucleus assay in fish as a monitor for the occurrence of aquatic genotoxic agents through the observation of close association between the frequency of micronucleus and the degree of pollution [12].

So, this work was planned to study the harmful effects of short-term ammonia poisoning in cultured *O. niloticus* through recording of clinical abnormalities, histopthological changes, biochemical alterations and genotoxicity associated with ammonia poisoning.

MATERIALS AND METHODS

Fish and Rearing Conditions: One hundred and fifty *O. niloticus* fish weighing 90.0±10.0 g was obtained on December 2012 from a private fish farm, Sharkya governorate, Egypt. Fish were transported in plastic buckets supplemented with battery aerators to the lab. of Fish Diseases and Management Dept., Faculty of Vet. Med.,Cairo University, Giza, Egypt. Fish were acclimated in fully prepared glass aquaria supplemented with air pumps containing dechlorinated tap water for 10 days before the experiment commenced. During acclimation, fish were fed on a commercial ration containing 25% protein and dissolved oxygen level was maintained at 6±0.5 mgL^{-1}, while water temperature was 22±2°C. Fish were examined clinically to assure the absence of any abnormalities or external signs according to the methods described by Amlacher [13]. Feeding was stopped 2 days before the starting of the experiment.

Experimental Design: One hundred and twenty *O. niloticus* of almost the same weight and size were categorized into four groups, three replicates of 10 fish for each group. The 1st group was served as a control group, the 2nd group exposed to 2.5mg/l of total ammonia nitrogen (TAN) (0.16 NH_3 mg/l), the 3rd group exposed to 5.0 mg/L of total ammonia nitrogen (TAN) (0.32 NH_3 mg/l) and the 4th group exposed to 10.0 mg/l of total ammonia nitrogen (TAN) (0.65 NH_3 mg/L) at water pH 8 and water temperature 28°C according to USEPA [14] for six days. The experimental technique followed that of APHA and Reish and Oshida and ISO [15-17]. The experimental medium was changed every 24 h with fresh solution. Water was aerated by compressed air to maintain the oxygen concentration at 6±0.5mg/l, water temperature was maintained constant at 28°C using thermostatic water heater and water pH is daily adjusted and maintained at pH 8using NaOH and HCl solutions. Ammonium chloride (NH_4Cl) was used as a source of ammonia. All chemicals used were analytical reagent grade.Serum samples were collected from different groups at the start and at the end of the experiment (0 and 6 days) and stored in deep freezer at -80°C.

Clinicalinvestigation and Postmortem Examination: The exposed fish were kept under proper observation during the period of experiment forany external clinical abnormalities, postmortem (PM) lesions or deaths according to Amlacher [13].

Biochemical Examination: Blood samples were left to clot at room temperature and centrifuged at 3000rpm for 15 min. to separate serum. Also blood samples were collected using sodium citrate as anticoagulant and centrifuged to separate plasma. Serum and plasma samples were used to determine:

Lipid Peroxidation Product: Malondialdahyde was measured according to the method described by Albero *et al.* [18].

Cortisol: The quantification of cortisol in the plasma was carried out by means of a commercial ELISA kit (RADIM Spa, Rome, Italy). Prior to use of this kit, the test was validated for fish blood plasma. The intertest (repeatability) and intratest (reproducibility) coefficients of variation were always below 7% and the mean recovery was approximately 99.3% [19].

Nitric Oxide (NO) Assay: The nitrite level in the collected serum samples was calculated according to the method described by Green *et al.* [20].

Micronucleus Test: A drop of blood from the gills of *O. niloticus* fish exposed to toxic ammonia in water was obtained at 2, 4 and 6 days of experiment. It was mixed with a drop of fetal calf serum on a clean dry slide and air-dried. The specimen was fixed in methanol for 5 minutes. Slides were stained with 10% Giemsa stain for 10 minutes. Giemsa solution stained the nucleus darker than the cytoplasm and the micronuclei appeared beside the normal nuclei. One thousand erythrocytes were examined for every fish to determine the percentage of cells containing micronuclei [21].

Histopathological Studies: Tissue samples from gills, liver, brain, kidneys and spleen were taken from *O. niloticus* that were exposed to toxic ammoniain water at 2 and 4 and 6 days of experiment. The samples were fixed in 10% formal saline while gonads fixed in Bouin's solution. Tissue samples were processed by conventional method, sectioned at 5 *um* and stained with Haematoxylin and Eosin [22].

Statistical Analysis: The results obtained in this study were statistically analyzed according to [23]. All data were subjected to one-way analysis of variance (ANOVA) followed by Duncan's multiple range test for comparison among treatment means using SAS, Version 8.2 [24].

RESULTS AND DISCUSSION

Fish exposed to different concentrations of ammonia moved rapidly, lost equilibrium in water and began to sideways swimming contrary to the control group. An increase in their movements, convulsions, spiral swimming, efforts to swallow air from the surface of water, increase in ventilation and death were observed in varied degrees in a concentration dependent manner (Plate A, Photos 1&2). Postmortem examination revealed an increase in the amount of mucus secretion in the gills and on the body surface, congestion in the gills (Plate A, photo3) and darkening in the eye and on the skin. Mortality was recorded only in 10 ppm exposed group and attained 50 % by the end of the experiment. Acute ammonia toxicity can cause an assortment of clinical signs in fish, the most severe of which include convulsions, coma and death [25], as well as behavioral changes such as hyperexcitability and appetite suppression [26]. The recorded clinical signs were recorded previously by many authors [27-29]. Coughing,hyperventilation followed by sporadic ventilation, twisting, lossof equilibrium, spiral swimming, convulsions and death following a short period in a coma-like state, in which there were no body motions except weak movements of the gills were recorded by Knoph [29]. An increase in mucous secretion in the gills and on the body surface [30] and also appeared as a sign of gill necrosis [31]. The observed clinical signs and PM lesions could be attributed to the histopathological changes recoded in the gills and brain in this study.

The results presented in the Table (1), described the acute exposure of fish to toxic ammonia at different concentrations (0.16 mg/l, 0.32 mg/l and 0.64 mg/l). Toxic ammonia exposure induced an increase in malondialdhyde and nitric oxide levels, similarly to that can be observed in other fish [32, 33]. Acute ammonia intoxication diminishes the activities of antioxidant enzymes and increases superoxide formation. These effects could play a role in the mechanism of ammonia toxicity. It has been shown that ammonia toxicity is mediated by activation of N-methyl-D-aspartate (NMDA) receptors. Ammonia intoxication also induces a depletion of glutathione and an increase in lipid peroxidation [34]. One important molecular consequence of oxidative stress obtained in Table (1) is the activation of intracellular signaling cascades. Numerous studies indicate that reactive oxygen species (ROS) activate protein kinases [35]. In particular, $p38^{MAPK}$, JNK and ERK1/2 are phosphorylated by exposure to exogenous H_2O_2. One possible explanation for the biphasic phosphorylation of ERK1/2 is that the first peak of ERK1/2 phosphorylation might be attributable to ROS generation [36]. The second peak might be attributable to the inactivation of mitogen-activated protein kinase phosphatase, which might occur in the second phase of ERK1/2 phosphorylation [37]. The early phosphorylation of MAPKs by ammonia appears to be critical for the ammonia-induced astrocyte dysfunction. An increase of ammonia in experimental animals or treatment of cultured astrocytes with ammonia generates reactive oxygen and nitrogen species in the target tissues, leading to oxidative/ nitrosative stress (ONS) [38]. Interrelated mechanisms underlying this response include increased nitric oxide (NO) synthesis, which is partly coupled to the activation of NMDA receptors and increased generation of reactive oxygen species by NADPH oxidase. ONS and astrocytic swelling are further augmented by excessive synthesis of glutamine (Gln) which impairs mitochondrial function. Ammonia-induced ONS results in the oxidation of mRNA and nitration/nitrosylation of proteins, which impact intracellular metabolism and potentiate the neurotoxic effects [38].

Table 1: The toxic effects of acute ammonia exposure on Cortisol, Malondialdehyde and Nitric oxide levels of *Oreochromis niloticus* fish

Treatments (ppm/group)		Cortisol (µg/dl)	Malondialdehyde (µMol/l)	Nitric oxide (mMol)
Control		43.77± 3.22[a]	1.36± 0.05[a]	4.97± 0.48[a]
2 days	2.5 ppm TAN	141.14 + 5.21[b]	2.41 ± 0.37[b]	74.07 ± 6.64[b]
	5 ppm TAN	162.03± 6.94[c]	2.71± 0.24[c]	103.12 ± 9.64[c]
	10 ppm TAN	199.48± 6.07[d]	5.26 ±0.36[d]	146.37 ±6.64[d]
4 days	2.5 ppm TAN	103.54 ±3.71[b]	3.47±0.42	62.75±4.04[b]
	5 ppm TAN	185.77± 6.56[c]	3.83± 0.54[c]	129.88± 9.96[c]
	10 ppm TAN	225.46± 6.89[d]	5.14± 0.09[d]	158.73± 9.70[d]
6 days	2.5 ppm TAN	106.15±7.69[b]	2.31± 0.29[b]	67.44 ± 6.08[c]
	5 ppm TAN	160.56 ± 4.26[d]	4.59± 0.40[d]	134.62± 9.70
	10 ppm TAN	206.32 ± 1.45[d]	4.95 ± 0.52[d]	133.61± 9.70[d]

Means with different superscript letters are differed significantly at P<0.05

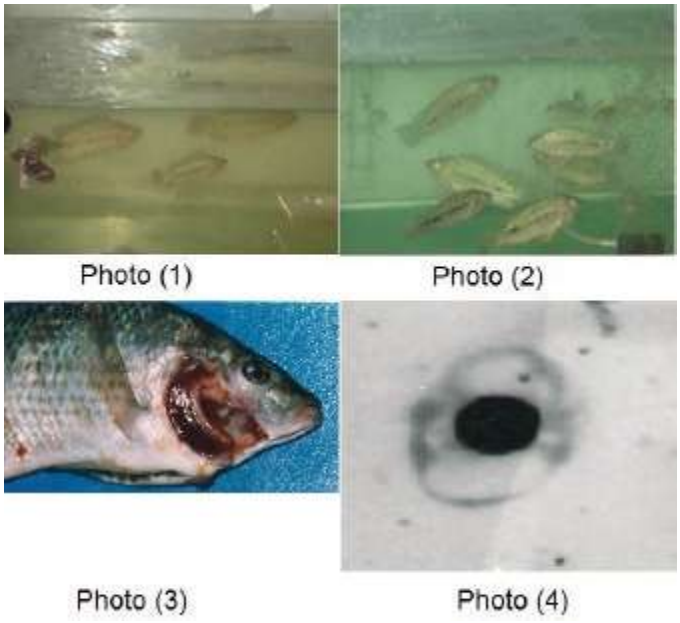

Plate (A):

Photo 1: *O. niloticus* showing surfacing and gasping of air in association with exposure to 5 ppm of TAN for 4 days
Photo 2: *O. niloticus* showing surfacing, gasping of air and accumulation of fish around the source of air in association with exposure to 10 ppm of TAN for 6 days
Photo 3: *O. niloticus* showing gill congestion in association with exposure to 10 ppm of TAN for 6 days
Photo 4: Blood film stained with Giemsa stain showing micronucleus in association with exposure to 10 ppm of TAN for 6 days

The results of this study showed that, acute exposure to toxic ammonia induced an increase in the cortisol levels, similarly to what can be observed for other fish species [32], Person Le Ruyet *et al.* [39]. Given the increase in the levels of cortisol, it is evident that the acute (12 and 24 h) exposure to ammonia activated the hypothalamus-pituitary-interrenal (HPI) axis, initiating a typical stress response, in the Senegalese sole. Taking into account that the cortisol is the main responsible for the effects of stress, it is normal to think that different physiological functions, such as growth, reproduction or the effectiveness of the behavioral responses [40], could be negatively affected in the ammonia-stressed sole. Less data are available for fish, to declare the increase in the cortisol levels although there is some evidence suggesting a possible relationship between the increase in the plasma concentration of cortisol and the increase in the 5-HT activity (serotonin)or in the ratio of 5-HIAA/5-HT [41], or in the 3, 4-dihydroxyphenylacetic acid (DOPAC) and 5-hydroxy-3-indoleacetic acid (5HIAA), DOPAC/DA and 5-HIAA/5-HT ratios [42]. Although the evidence is indirect, we propose that the

Table 2: Mean values and percentages of micronuclei incidence in control and toxic ammonia exposed *O.niloticus* for 2, 4 and 6 days

Treatments	Total No. of examined fish	Total No. of PCE_s	Total No. of Mn.	Micronucleus Mean±SE	Micronucleus %
Control	5	5000	10	2.0±0.14[a]	0.20
2.5 ppm TANfor 2 days	5	5000	30	6.0±0.18[b]	0.60
5ppm TAN for 2 days	5	5000	35	7.0±0.19[b]	0.70
10 ppmTAN for 2 days	5	5000	35	7.0±0.20[b]	0.70
2.5 ppm TANfor 4 days	5	5000	30	6.0±0.24[b]	0.60
5ppm TAN for 4 days	5	5000	40	8.0±0.20[bc]	0.80
10 ppm TAN for 4 days	5	5000	40	8.0±0.18[bc]	0.80
2.5 ppm TANfor 6 days	5	5000	35	7.0±0.19[bc]	0.70
5ppm TAN for 6 days	5	5000	40	8.0±0.26[bc]	0.80
10 ppm TAN for 6 days	5	5000	45	9.0±0.30[c]	0.90

PCE_s = Polychromatic erythrocytes. Mn = Micronuclei.

Means with different superscript letters are differed significantly at $P<0.05$.

increase in the plasma levels of cortisol, induced by exposure to ammonia, may modulate the cerebral activity of the 5-HT and DA systems. Evidently, further studies are required to clarify the possible relationship between cortisol and monoaminergic transmission in fish [43].

The results of micronucleus assay in control and ammonia exposed groups were summarized in Table (2) and Plate A (photo 4). Results showed that there was a significant increase of micronucleus production in different toxic ammonia exposed groups when compared to the control group in a concentration and time dependent manner. Among current cytogenetic techniques, micronuclei and some other nuclear abnormalities are considered to be sensitive indicators of genotoxicity and cytotoxicity. Micronuclei estimation in fish has been shown to be a better parameter than chromosomal aberrations in the environmental studies under laboratory and field conditions [44]. The gill cells were chosen to estimate the micronucleus formation because many studies recorded that gill cells are more sensitive than haemopoieticcells to micronucleus inducing agents [45]. The occurrence of genetic damage in *O. niloticus* exposed to ammonia was illustrated by high frequency of micronucleus in ammonia exposed fish for 2,4 and 6 days compared to the control group in a concentration and time dependent manner. These results are similar to those obtained by Theepharaksapan *et al.* [46] who found that ammonia was the main acute toxic compound and induced DNA damage in ammonia exposed fish. The same findings were reported by Arillo *et al.* and Maurizio *et al.* and Abumourad *et al.* [47-49] who found an existence of mutation and different DNA structural changes such as breaks, transpositions and deletions in *O. niloticus* DNA exposed to ammonia based on DNA patterns obtained after RAPD assay. Also an increase in micronucleus frequency with time was found by Ramirez and Garcia [50] in zebra fish coincide with our results.

The histopathological observations for both control and ammonia exposed Nile tilapia fish with representative images of the tissues displayed in Plates B-D. Control individuals did not show any pathological changes in the tissues examined by the light microscope Plate B (a,b,c,d and e). Most differences between control and fish exposed to 2.5mg/L of total ammonia nitrogen (TAN) (0.16 NH_3 mg/L) (1^{st}gp) were detected. Gills revealed telangiectasis and hyperemia in the branchial and lamellar blood vessels (Plate B, 1), lamellar hyperplasia which leading to fusion of secondary lamellae began from the base to the apex of the lamellae with degenerative changes in the epithelial lining of the secondary lamellae (Plate B, 2). Liver lesions consisted of hydropic degenerations and cloudy swelling in the hepatocytes with focal aggregation of melanomacrophage cells in between the hepatocytes (Plate B, 3). Focal areas of necrosis, mononuclear inflammatory cells and hyperplasia in the wall of the bile duct were also detected (Plate B, 4), while the kidneys displayed glomerulonephritis (Plate B, 5), vacuolar degenerative changes in the tubular epithelium and slight congestion (Plate B, 6). Spleen showed hyperactivation of the melanomacrophagecenters with slight congestion in splenic blood vessels (Plate B, 7). The brain exhibited slight congestion (Plate B, 8). Lateral muscle and the skin of the fish exposed to ammonia showed no histological differences compared to the control groups.

All the pathological alterations showed a relationship with prevalence increasing with increasing ammonia concentration and exposure time.

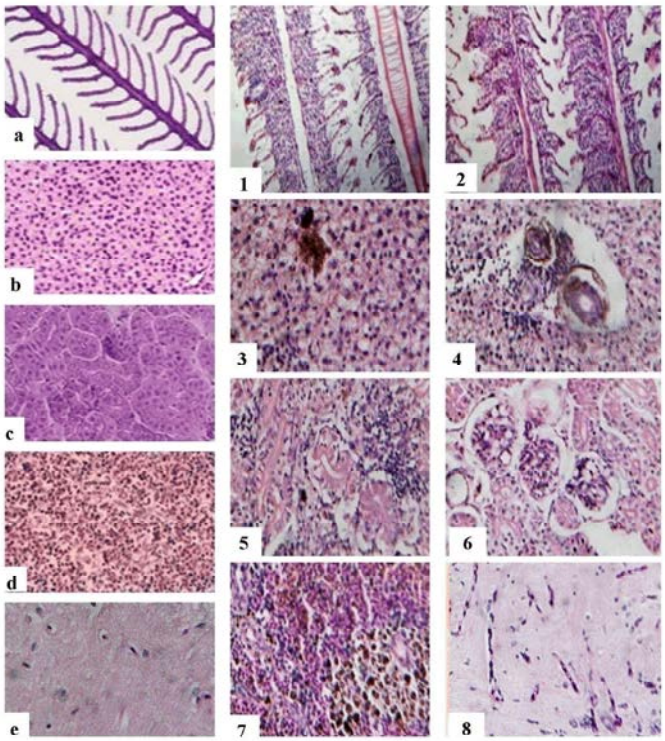

Plate (B): a, b, c, d and e as control, *O. niloticus* of the first group:

Fig. 1: Gills after 4 days of exposure showed telangiectasis and hyperemia in the branchial and lamellar blood vessels H&E, X400

Fig. 2: Gills after 6 days of exposure showed lamellar hyperplasia and fusion of secondary lamellae began from the base to the apex of the lamellae with degenerative changes in the epithelial lining the secondary lamellae H&E, X400

Fig. 3: Liver after 4 days of exposure showed hydropic degenerations, cloudy swelling in the hepatocytes with focal aggregation of melanomacrophage cells in between the hepatocytes H&E,X400

Fig. 4: Liver after 6 days of exposure showed focal areas of necrosis, mononuclear inflammatory cells and hyperplasia in the wall of the bill duct were also detected H&E, X400

Fig. 5: Kidneys after 4 days of exposure showed glomerulonephritis H&E, X400

Fig. 6: Kidneys after 6 days of exposure showed vacuolar degenerative changes in the tubular epithelium and slight congestion H&E, X400

Fig. 7: Spleen after 6 days of exposure showed hyperactivation of the melanomacrophage centers with slight congestion H&E, X400

Fig. 8: Brain after 6 days of exposure showed edema in the cerebellum and slight congestion

More advanced pathological changes than in the 1stgroup were observed in the fish exposed to 5.0 mg/L of total ammonia nitrogen (TAN) (0.32 NH_3 mg/L) (2ndgroup). Gills showed sever congestion (Plate C, 1) associated with severe hyperplasia (Plate C, 2). The liver showed severe congestion and hemorrhages (Plate C, 3) with area of haemolysis in between the hepatic parenchyma (Plate C, 4). The kidney had hyperplasia in the wall of renal blood vessels associated with necrosis in the heamopiotic interstitial tissues, peritubular and periglomerular oedema and degenerative changes in the endothelial lining the glomerular tuft (Plate C, 5&6). The spleen showed severe congestion and hemorrhages, hyperplasia in the wall of spleenic blood vessels, hyperactivation of melanomacrophage centers and focal area of depletion of lymphocytic tissues (Plate C, 7). The brain showed severe oedema in the cerebellum and neuronal degeneration (Plate C, 8).

Macroscopical observation in the fish exposed to 10.0 mg/L of total ammonia nitrogen (TAN) (0.65 NH_3 mg/L) (3rdgroup) showed,moreover the previous changes, the gills exhibited more congestion (Plate D, 1)

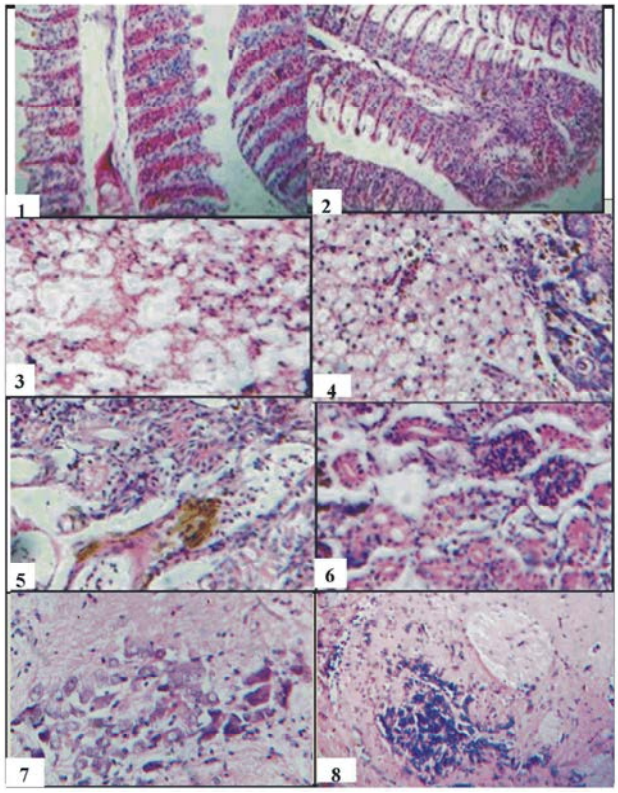

Plate C: Tilapia fish in the 3rd gp. **Fig. 1:** Gills after 4 days of exposure showed more congestion, H&E, X400. **Fig. 2:** Gills after 8 days exposure showed complete fusion of the secondary lamellae, complete obliteration of the inter-lameller space, sever degenerative and necrotic changes in the respiratory epithelium H&E, X400. **Fig. 3:** Liver after 4 days of exposure showed swollen, degenerated and necrotic hepatocytes with losing adenoid structure H&E, X400. Fig. 4: Liver after 8 days of exposure showed infiltration of mononuclear inflammatory cells in between the hepatic parynchyma and vacuolar degeneration and necrosis in acinar cells of pancreas H&E, X400. **Fig. 5:** kidney after 4 days exposure showed showed aggregation of melanomacrophage cells in between the interstitial heamopiotic tissues specially at the necrotic areas with sever degenerative and necrotic changes in the tubular epithelium and heamopiotic tissues H&E, X400. **Fig. 6:** kidney after 8 days of exposure showed with more pronuounced peri-tubular and peri-glomerular edema H&E, X400. **Fig. 7:** brain after 4 days exposure of showed neuronal degeneration H&E, X400). **Fig. 8:** Brain after 8 days exposure of showed aedema and focal gliaosis H&E, X400).

with complete fusion of the secondary lamellae leading to complete obliteration of the inter-lamellar space associated with severe degenerative and necrotic changes in the respiratory epithelium (Plate D, 2). The Liver had swollen, degenerated and necrotic hepatocytes with losing adenoid structure (Plate D, 3), infiltration of mononuclear inflammatory cells in between the hepatic parenchyma and vacuolar degeneration and necrosis in acinar cells of pancreas (Plate D, 4). The kidney showed aggregation of melanomacrophage cells in between the interstitial heamopiotic tissues specially at the necrotic areas with sever degenerative and necrotic changes in the tubular epithelium and heamopiotic tissues (Plate D, 5) with more pronounced peri-tubular and peri-glomerular oedema (Plate D, 6), The spleen showed the same pictures mentioned before with more extent. Brain showed neuronal degeneration (Plate D, 7), oedema and focal glaiosis (Plate D, 8-9).

The results concerning the sublethal effects of ammonia on Nile tilapia with respect to tissue histopathology are presented in Plates B - D. Person Le Ruyet *et al.* [39] have shown that NH_3 entered fish within 15 min of exposure. The first effects of contaminants usually occur at cellular and sub-cellular levels, starting from the first hour of contamination [44]. Chronic ammonia exposure might damage gills, liver and kidney, which may predispose the fish to numerous infections [51].

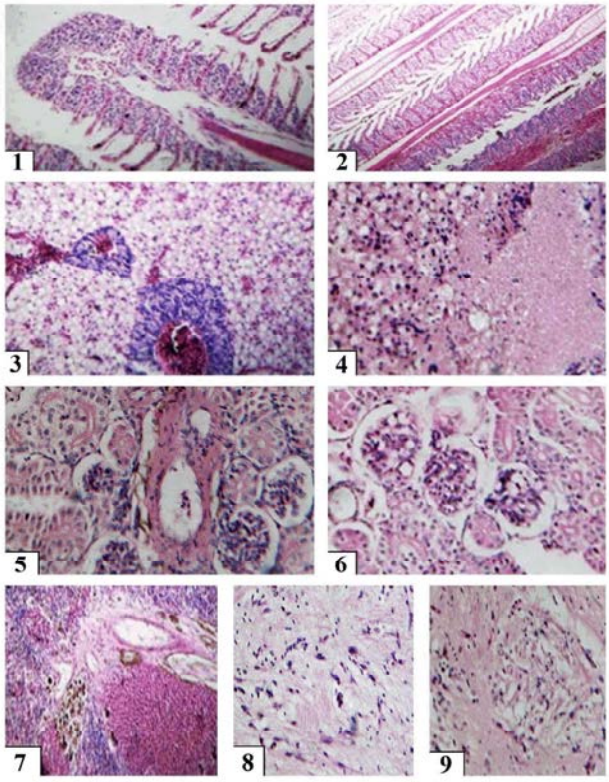

Plate (D): *O. niloticus* in the third group:

Fig. 1: Gills after 4 days of exposure showed more congestion, H&E, X400
Fig. 2: Gills after 8 days exposure showed complete fusion of the secondary lamellae, complete obliteration of the inter-lamellar space, sever degenerative and necrotic changes in the respiratory epithelium H&E, X400
Fig. 3: Liver after 4 days of exposure showed swollen degenerated and necrotic hepatocytes with losing adenoid structure H&E, X400
Fig. 4: Liver after 6 days of exposure showed infiltration of mononuclear inflammatory cells in between the hepatic parenchyma and vacuolar degeneration and necrosis in acinar cells of pancreas H&E, X400
Fig. 5: Kkidney after 4 days ofexposure showed aggregation of melanomacrophage cells in between the interstitial haemopiotic tissues specially at the necrotic areas with sever degenerative and necrotic changes in the tubular epithelium and heamopiotic tissues H&E, X400
Fig. 6: Kidney after 6 days of exposure showed with more pronounced peri-tubular and peri-glomerular edema H&E, X400.
Fig. 7: Brain after 4 days of exposure showed neuronal degeneration H&E, X400
Fig. 8&9: Brain after 6 days of exposure showed oedema and focal gliaosis H&E, X400

The important histopathological effects of sublethal ammonia on the gills were chloride hyperplasia, telangiectasis on lamella and hyperemia on epithelium. Gills are well-known target organs in fish, being the first to react to unfavorable environmental conditions. Several authors have reported similar alterations on the gills of different fish species exposed to ammonia, where [52] observed aneurysms, lamellar-capillary congestion and hemorrhaging of Tilapia after acute (2.4 mg / NH_3–N) and chronic (0.43-0.53 mg /l NH_3–N) ammonia exposure [53]. Also, Larmoyeux and Piper [54] determined aneurysms and fused lamella of rainbow trout (*Salmogairdneri*) gill epithelium cells. Furthermore, Kirk and Lewis [55] reported that the gills of the rainbow trout exposed to 0.1 mg/l ammonia for 2 h exhibited deformation of the lamellae. Salin and Williot [30] observed that Siberian sturgeon *(Acipencerbaeri*) (270 g) exposed to more than 60 mg/1 of ammonia reveal a modification of the epithelium of the secondary lamellae and the base of the filament is slightly turgescent. Similar results were also

confirmed by Mitchell and Cech [56] with channel catfish (*Ictalurus punctatus*), Malik *et al.* [57] with common carp (*Cyprinus carpio*) and Cardoso *et al.* [58] with *Lophiosilurus alexandri*.

Cloudy swelling and hydropic degenerations on the liver were observed where liver being the main organ of various key metabolic pathways, toxic effects of chemicals usually appear primarily in the liver. Ammonia can be carried by the hepatic portal vein to the liver as a nutrient and enter liver metabolic pathways [53]. Ammonia exposure causes liver glycogen vaculation due to disruption of energy production [59]. The most frequently encountered types of degenerative changes are those of hydropic degeneration, cloudy swelling, vacuolization and focal necrosis on fish exposed to different kinds of contaminants [60]. Clear signs of liver pathology in gilthead sea bream (*Sparus auratus*) after 20 days of exposure to 13 mgL^{-1}TA-N (0.7 mgL^{-1} NH_3–N) were observed byWajsbrot *et al.* [61].

Kidney tissues displayed glomerulonephritis and hyperemia after being exposed to different concentrations of sublethal ammonia concentrations where the kidney is a one of the major organs of the toxic effects. Thurston *et al*. [59] observed hydropic degeneration in the kidney of cut throat trout after exposure to 0.34 mg /1 NH_3–N and Larmoyeux and Piper [54] determined glomerular congestion in kidneys of rainbow trout after exposure to 0.8 mg /l NH_3–N. There was hyperactivation of melano-macrophage centers also diffuse infiltration with melanophores within the splenic tissue. The intensity of coring cells increased in melano-macrophage centers because of stress condition where the ACTH (adreno-corticotrophic hormone) released. This hormone produces a rapid dispersion of melanin granules in many fish species, thus pigment changes could be attributed to high level of ACTH associated with stress condition [62].

Concerning the histopathological changes in the brain, which may be resulted from the toxic effect of ammonia or the hypoxic effect, which resulted from gill destruction. These findings accentuates the findings of Ricardo *et al.* [63] who pointed out that fish exposed to ammonia for 96 h, showed proliferation of the glial cells, satellitosis, glial cells proliferation, satellitosis (microglialcells surrounding neurons with swollen and prenecrotic neurons), Virchow-Robin spaces (enlarged perivascular spaces, EPVS) and cellular necrosis. Also he mentioned that these brain histopathologies were severe and probably contributed to fish death. Although this is the first report of brain pathology caused by nitrate toxicity, fish exposed to hypoxic conditions showed the same histopathologies [64]. Therefore, it is not possibleto determine whether the histopathology found in the brain of juvenile cobia exposed to nitrate were caused by the direct action of nitrate or indirectly caused by the possible condition of hypoxia which fish were exposed due to severe branchial alteration observed.

CONCLUSION

The clinical signs, post mortem lesions, histopathological changes and biochemical findings recorded in the present study contribute to understanding the ammonia toxicity mechanism in fish. The present study proved that ammonia also had genotoxic effect.

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