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Effects of vitamin C on structural changes in the tissues of the giant African catfish, *Heterobranchus longifilis* exposed to Zinc Oxide nanoparticles

Olufemi D. Owolabi* and Saratu I. Abdulkareem
Fisheries and Hydrobiology Laboratory, Department of Zoology, University of Ilorin, Ilorin 240003, Nigeria.
*Corresponding author: olulabi47@yahoo.com; olulabi@unilorin.edu.ng

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ABSTRACT

Heterobranchus longifilis juveniles (n = 450, Mean length ± SD = 10.28 ± 1.34 cm) were exposed to varying concentrations (0 = control, 6.00, 8.00, 10.00, 12.00 mg/l) of zinc oxide nanoparticles (ZnO-NPs) for 60 days to determine its effects on the histopathology of the gill and liver. To evaluate recovery from ZnO-NPs exposure, fish were subjected to a 30-day depuration. Ascorbic acid (AA) ameliorative potential against ZnO-NPs toxicity was assessed by augmenting fish basal diet with different AA (50.00, 250.00, 500.00, 1000.00 $mg.kg^{-1}$) levels. After each experiment, fish gills and liver were removed for histopathological analysis. Gills of exposed fish revealed different alterations like epithelial hyperplasia, epithelial lifting, lamellar fusion, oedema, aneurysms, and necrosis; while alterations such as nuclear vacuolation, fatty degeneration, cytoplasmic degeneration, focal fibrosis and necrosis were recorded in the liver. The mean degree tissue change (DTC) in both organs of ZnO-NPs-exposed fish gradually increased with increase in ZnO-NPs concentration and exposure period. After 30-day depuration, some of the lesions in both organs attained recovery, while others did not. AA administration to ZnO-exposed fish at 500 $mg.kg^{-1}$ completely reversed the lesions in both organs compared to the normal tissue architecture in the control. The results suggest that ZnO-NPs could trigger structural alterations in both the branchial and hepatic tissues of *H. longifilis*, and full recovery under depuration was slow. However, 500 $mg.kg^{-1}$ AA dietary supplementation have the propensity to ameliorate the ZnO-NPs-induced pathological lesions in *H. longifilis*.

INTRODUCTION

Degradation of aquatic environment due to chemical pollution can be implicated for fish production decline in developing countries, particularly Nigeria. Among the emerging chemical pollutants with the potential of decimating the aquatic ecosystems are the nanoparticles. Nonetheless, owing to the exceptional characteristics of negligible size, reaction activity, and extensive surface area nanoparticles have clearly shown potential beneficial use in the manufacture of consumer and industrial products. Zinc oxide nano-particles (ZnO-NPs) are among the commonly utilised nanoparticles by most industries in the production of commercial and medicinal goods as a result of their distinguished physicochemical properties such as rigidity, photo stability, and biodegradability. Thus,

making them to be useful as photocatalyst, energy generator, solar panel devices, sunscreen, and in the production of ceramics, textiles, rubber and cosmetics (**Wang *et al*., 2005**). They are also used in the manufacture of paints and in the treatment of wastewater (**Zimmermann *et al*., 2011**). ZnO-NPs has also been found useful in the process of meat and vegetable production and packaging (**Asghar *et al*., 2015**). Available literature has revealed that ZnO-NPs have been produced on a large scale in many countries of the world. For example, the percentage industrial use of ZnO-NPs in different regions such as Far East is 38%, Europe: 31%, North America: 18%; South America: 7%; Near East: 4% and Africa: 2% (**Asghar *et al*., 2015**). From this literature, it is apparent that ZnO-NPs production in Africa is still at low ebb; but with time as industries become more aware of its importance and people embrace its use, more of the nanoparticles might be utilised industrially in consumer and industrial products manufacture and in other utilities earlier mentioned.

The increased utilisation and production of ZnO-NPs may lead to increased exposure and vulnerability of aquatic organisms and humans to the risk of toxic effects of the nanoparticles either through discharge from industries, runoff or direct use of ZnO-NPs by man. ZnO-NPs introduction into water bodies may result in ecological disorder and consequent deterioration of water quality, thus threatening the survival of resident fish species and other aquatic organisms. Hence, this has been a cause for concern throughout the world. In Nigeria, the environmental regulation agencies (**NIS, 2007; NESREA, 2011**) saddled with the responsibilities of setting the threshold values for some chemicals used in the daily lives of Nigerians are yet to include information on the threshold limits for nanoparticles in aquatic systems. The lack of such information renders the existing regulations incapable of fully addressing the safety concerns associated with contaminants, particularly nanoparticles. This may have hindered the holistic understanding of the protection and conservation of aquatic ecosystems and their resident fish species.

Environmental contaminants are well known inducers of derangement between antioxidant defense systems and the formation of reactive oxygen species (ROS). In a degraded environment, ROS formation usually causes severe morphological and physiological derangement in the tissues and organs of aquatic animals (**Flores-Lopes and Thomaz, 2011**). The use of histopathology as biomarker is one of the most effective methods of assessing pollutant-induced oxidative stress in aquatic organisms. Several studies have demonstrated the use of histopathology as biomarker of ZnO-NPs effects in fish as well as warning symptoms of health status (**Mansouri *et al*., 2018**). Unfortunately, none of these data mentioned the ZnO-NPs effects on the histopathology of *Heterobranchus longifilis*.

During laboratory studies, fish are usually returned to toxicant-free water after an exposure to initiate ‘self’ detoxification process sufficient to reverse any morphological, biochemical or physiological changes arising from such exposure. Earlier studies have shown that, after exposure, the normal physiology of fish could either be moderately restored (**Adhikari *et al*., 2004; Ramesh *et al*., 2015**) or fully restored (**Maltez *et al*., 2017**) when relocated to water free of toxicant. However, information on fish recovery

after nanoparticles exposure is obscured. **Adhikari *et al.*** (2004) stated that an understanding of fish recovery time after toxicant exposure could help in the maintenance of fish health status and that of its consumers. Fish, like higher vertebrates, are generally endowed with an antioxidant system with which they eliminate free radicals generated by the ROS and recovered from oxidative stress. This system comprises enzymatic (e.g., superoxide dismutase and catalase) and non-enzymatic (e.g., α tocopherol (vitamin E) and ascorbic acid (vitamin C) antioxidants. Vitamin C is a chain-braking, least toxic, most effective and widely used antioxidant that react with free radicals (**Bielski, 1982**), and thus, protects animals against oxidative damage (**El-Keredy *et al*., 2017**). As important as it is, not all fish species are capable of synthesizing vitamin C. They, therefore, depend on exogenous sources to meet their requirements. Several reports are available on dietary efficacy of vitamin C in ameliorating pollutants toxicity to fish (**El-Keredy *et al*., 2017**).

The African giant freshwater catfish, *Heterobranchus longifilis* is a commercially important fish in Nigeria (**Suleiman *et al*., 2015**). However, a dearth of information exists on nanoparticles toxicity with respect to this commercially important species and the sub-lethal effects of ZnO-NPs exposure, recovery responses and potential ameliorative effects of vitamin C are yet to be understood. This study, therefore, attempts to fill the information gap on the histopathology of the gill and liver in *H. longifilis* following exposure to ZnO-NPs, recovery responses after exposure and ameliorative potentials of ascorbic acid (vitamin C).

MATERIALS AND METHODS

Chemicals, Fish collection and maintenance

ZnO-NPs (<100 nm) used were procured from Sigma Aldrich, USA in powdery form (CAS number: 1314-13-2; Product number: 544906; Colour: whitish; Surface area: 15-25 m^2/g; Percentage zinc: 79.1-81.5 %; Shape: rod-shaped) as characterised by **Akanbi-Gada *et al***. (2019). Four hundred and fifty juveniles of *H. longifilis* (Mean ± SD 10.28 ± 1.34 cm) were obtained from a fish farmer in Ilorin metropolis, Nigeria and immediately transferred to the Fisheries and Hydrobiology Laboratory, Department of Zoology, University of Ilorin. In the laboratory, fish were allowed to adjust to a 14-day ambient conditions (at 12h light: 12h dark photoperiod) in an aerated 120 L tank and were fed at 3% body weight two times daily (**Abdulkareem, 2017**). Mortality throughout the acclimation period was less than 2%. The mean physicochemical parameters of the water during the experiment were maintained as follows: temperature 27.00 ± 2.08 ^{0}C, dissolved oxygen 6.45 ± 1.20 mg/l, pH 7.04 ± 0.06 and total hardness as $CaCO_3$ 26.67 mg/l. Deterioration of water quality was prevented by replacement of water every 48 hours.

Experimental design

Following the result of the presumptive test, five nominal concentrations (0, 60, 80, 100 and 120 mg/l of ZnO-NPs were prepared through serial dilution in a static renewal bioassay for acute toxicity test using the Organization of Economic Cooperation and Development (**OECD, 1992**) guidelines. Thirty fish were then randomly selected and subjected to a 60-day exposure regime in four measured sublethal concentrations of the toxicant (6.0, 8.0, 10.0 and 12.0 mg/l); equivalent to 1/10 each of the concentrations used for the acute assay, while the first treatment (0) was the control as it was devoid of the toxicant. The experiment was set up in triplicates and fish were fed at 3% body weight two times daily. The aquaria containing fish were cleaned and toxicant was renewed every day to keep the toxicant concentration constant (**FAO, 1986**). After the 60-day exposure, fish from the different concentrations were transferred each into separate ZnO-NPs-free aquarium (aquarium containing only water) for a 30-day depuration studies. After the 60-day exposure and 30-day depuration periods, five fish from each of the treatments and controls were dissected at 15 days interval to remove the gills and liver for histopathological analyses, respectively.

Ameliorative effect of ascorbic acid (vitamin C) on ZnO-NPs-exposed fish was evaluated by placing the fish on ascorbic acid (AA)-supplemented diet. The diet was formulated with the following ingredients: maize corn (30%), soya bean (20%), groundnut cake (18%), fish meal (30%), bone meal (0.4%), vitamin C (0.2%), methionine (0.4%), lysine (0.4%), salt (0.2%), premix (0.2%) which formed 100% balanced diet. With a meat mincer through 3 mm die, these constituents were ground, crushed, weighed, mixed, pelleted, air dried and kept in moisture-free container prior use (**DeSilva, 1991**). Three hundred and twenty (320) fish with average weight 10.40 ± 1.65g each were distributed randomly into 35-L capacity plastic aquaria of ten fish each. Each aquarium contains ten litres of chlorine-free bore-hole water. The experiment was carried out in triplicate of four different sets with the first three groups (A, B and C) without ZnO-NPs but each was only fed with basal diet, 50 $mg.kg^{-1}$AA (lowest AA concentration) and 1000 $mg.kg^{-1}$AA (highest AA) supplemented diet, respectively. The remaining five groups (D, E, F, G and H) were exposed to 1/10 of the 96 h LC_{50} value (**Narra *et al*., 2011**) estimated to be 10 mg/l. The test solution (1/10 of the 96 h LC_{50} of ZnO-NPs) was renewed on daily basis to maintain the concentration of the toxicant. While group D fed on basal diet, group E, F, G, and H were fed 50, 250, 500 and 1000 $mg.kg^{-1}$AA-supplemented diet, respectively. The ameliorative experiment spanned 30 days, after which fish were sacrificed for histopathological analysis.

Histopathological assay

Following the method of **Woods and Ellis (1994)**, fish from different aquaria with varying concentrations of ZnO-NPs were dissected to excise the gills and liver and fixed in 10% formalin solution to preserve the tissue. The fixed tissues were dehydrated through graded series of alcohol (i.e., 70, 80, 90 and 100%). Tissues were cleared in

xylene and infiltrated with molten paraffin wax under a thermostatically controlled oven (60 ^{0}C) and then embedded in molten paraffin wax for easy sectioning with microtome. The sections (5-µm) obtained were stained with haematoxylin and eosin (H and E). They were then examined under the light microscope and photographed using a digital camera (**Takashima and Hibiya, 1995**). A score ranging from – to +++ contingent on the degree/extent of tissue change: (none), + (mild), ++ (moderate), and +++ (severe) were employed in the qualitative assessment of histopathological changes and a total of 5 slides were observed per treatment (**Kaur and Dua, 2015**). The semi-quantitative evaluation was carried out through a degree of tissue change (DTC) based on the severity of the lesions as described by **Poleksic and Mitrovic-Tutundzic (1994)**. Histopathological disorders displayed in each section were categorised as absent (- 0 % of sections), mild (+< 25 % of sections), moderate (++, 25-50 % of sections) and severe (+++, >50 % of sections) (**Mishra and Mohanty, 2008)**. The degree of tissue (DTC) was evaluated by calculating the histopathological changes that display the stages of damage to the gill and liver. The sum (Σ) of the number of lesion types within each of the stages (*I*, *II* and *III*) was multiplied by the stage coefficient (1, 10 and 100) to give numerical values of the DTC using **Poleksic and Mitrovic-Tutundzic (1994)** modified formula: $DTC = (1 \times \Sigma I) + (10 \times \Sigma II) + (100 \times \Sigma III)$ where *I*, *II* and *III* are the number of lesions of stages I, II and III, respectively. The results were graded, grouped into five categories and interpreted as follows: values of DTC from 0 - 10 = normal functioning organ; 11 - 20 = slightly damaged organ; 21 - 50 = moderately damaged organ; 51 - 100 = severely damaged organ and above 100 = irreparable damaged organ (**Poleksic and Mitrovic-Tutundzic, 1994**).

RESULTS

Histopathology

The primary and secondary lamellae of fish gill from the control group showed normal cellular structure compared to those of the ZnO-NPs-exposed fish which showed diversity of alterations ranging from hyperplasia of epithelial cells, lifting of epithelial, lamellar fusion, lamellar disorganization, curling of secondary lamellae, disruption of cartilaginous core to epithelial oedema, cellular degeneration, cell rupture, aneurysm and epithelial necrosis after 60-d exposure (Fig. 1a-e). Table 1 shows the stages of these alterations during exposure, depuration and amelioration periods. As the concentration and duration of ZnO-NPs exposure increased, the severity of gill damage increased (Table 2). While hyperplasia slightly occurred in all treatments between 15 (Fig. 1d-e) and 45 d exposure and became severe at the highest concentration (12 mg/l) after 60 d (Fig. 1b), epithelial lifting and curling of lamellae were not observed until 45 d exposure at 12 mg/l concentration (Fig. 1e) and 60 d exposure at 8 mg/l concentration (Fig. 1c), respectively. Lamellar fusion occurred moderately in all the treated groups between 15 (Fig. 1a-e) and 45 d (Fig. 1a-e) exposure and became severe at the highest toxicant concentration between 45 (Fig. 1e) and 60 (Fig. 1e) d exposure. Lamellar disorganisation occurred moderately at 12 mg/l within the first 15 d (Fig. 1e) exposure and subsequently

became severe irrespective of the exposure period and toxicant concentration. Disruption of the the cartilaginous core, epithelial oedema, cellular degeneration and rupture of epithelial cells were not exhibited until 30 d exposure; where they appeared as slight or moderate lesions at different concentrations of the toxicant. The lesions, however, became severe at the highest toxicant concentration after 60 d exposure (Fig. 1e). Aneurysm and necrosis were moderately shown at 10 (Fig. 1d) and 12 mg/l (Fig. 1e), respectively after 15 d exposure. Necrosis became severe at the highest toxicant concentration during each exposure regime following the first 15 d (Fig.1e), while aneurysm remained moderate. The occurrence of other lesions observed in the gill did not follow a particular pattern as they occurred slightly/moderately at lower concentration and some severely at higher concentration regardless of exposure duration in both cases.

The liver of fish from the control exhibited normal hepatocytes relative to those of ZnO-NPS-treated fish (Fig. 2). Pathological disorders observed were nuclear alteration, melanomacrophage centre, blood congestion or sinusoids, nuclear vacuolation, fibrosis, necrosis, cellular-, fatty and cytoplasmic degenerations. These disorders either occurred slightly or moderately within the first 15 d exposure irrespective of the concentration except necrosis that became severe at 12.00 mg/l (Table 3). Within 30 and 45 d exposure, nuclear alteration, melanomacrophage centre, blood congestion, nuclear vacuolation, necrosis and fibrosis were either slightly or moderately shown in all the treated groups, while fatty and cytoplasmic degenerations were observed as either slightly or moderately damaged between 6.0 and 10.0 mg/l but became severe at 12.0 mg/l ZnO-NPs concentration. At 60 d exposure, nuclear alteration, melanomacrophage centre, vacuolation, necrosis, fatty and cytoplasmic degenerations exhibited slight to moderate disorder in fish liver from 6-10mg/l ZnO-NPs treatments but became severe at 12.0 mg/l. However, blood congestion appeared as slight disorder at 10.0 mg/l and became severe at 12.0 mg/l, while fibrosis occurred as slight and moderate disorder in all the treated groups. Quantitatively, the mean DTC in the gill of ZnO-NPs-treated fish showed a gradual increase with toxicant concentration and exposure period (Table 4). The mean DTC recorded for the gills of ZnO-NPs-treated fish at 6 (5.70 ± 0.06) and 8 (9.60 ± 0.12) mg/l concentrations indicated normal gill architecture after 15 d exposure, while the values recorded for fish exposed to 10 (25.40 ± 0.64) and 12 (31.30 ± 0.52) mg/l ZnO-NPs showed moderately damaged gill compared to the control (0.00 ± 0.00) that exhibited no damage. At 30 d exposure, fish gill exposed to 8 mg/l of the toxicant was slightly damaged (17.80 ± 0.64) but the damage was moderate in fish treated with 10 (34.80 ± 1.21) and 12 (46.30 ± 0.58) mg/l concentration.

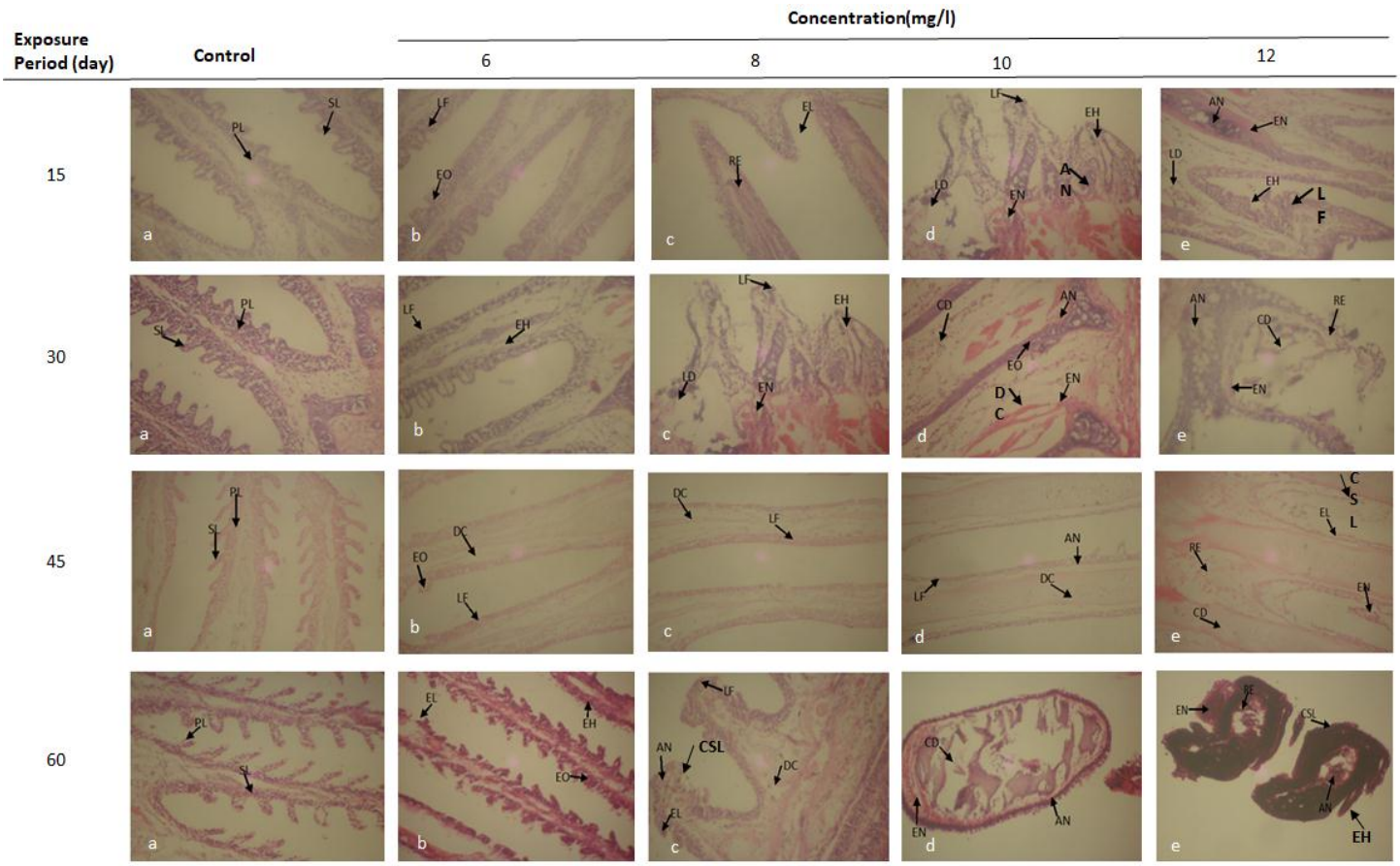

Fig. 1: Histopathological lesions in the gill of *Heterobranchus longifilis* exposed to ZnO-NPs for 60 days (H & E x 100). At 15 days, (a) gill of *H. longifilis* in the control showing normal architecture of normal primary lamellae (PL) and secondary lamellae (SL); (b) lamellar fusion (LF) and epithelial oedema (EO) in the gill of fish exposed to 6 mg/l ZnO-NPs; (c) epithelial lifting (EL) and rupture of epithelial cells (RE) in fish exposed to 8 mg/l ZnO-NPs; (d) epithelial hyperplasia (EH), lamellae fusion, lamellae disorganisation (LD), Epithelial necrosis (EN) and aneurysm (AN) in fish exposed to 10 mg/l ZnO-NPs; (e) epithelial necrosis (EN), aneurysm (AN), lamellae disorganisation (LD), epithelial hyperplasia (EH) and lamellae fusion (LF) in fish exposed to 12 mg/l ZnO-NPs. At 30 days, (a) gill of *H. longifilis* in the control showing normal architecture of normal primary lamellae (PL) and secondary lamellae (SL); (b) lamellae fusion (LF) and epithelial hyperplasia (EH) in fish exposed to 6 mg/l ZnO-NPs; (c) lamellae fusion (LF), lamellae disorganisation (LD), epithelial necrosis (EN) and epithelial hyperplasia (EH) in fish exposed to 8 mg/l ZnO-NPs; (d) cellular degeneration (CD), aneurysm (AN), epithelial oedema (EO), disruption of cartilaginous core (DC) and epithelial necrosis (EN) in fish exposed to 10 mg/l ZnO-NPs; (e) cellular degeneration (CD), aneurysm (AN), epithelial necrosis and rupture of epithelial cell (RE) in fish exposed to 12 mg/l ZnO-NPs. At 45 days, (a) gill of *H. longifilis* in the control showing normal architecture of normal primary lamellae (PL) and secondary lamellae (SL); (b) epithelial oedema (EO), disruption of cartilaginous core (DC), lamellar fusion (LF) in the gill of fish exposed to 6 mg/l ZnO-NPs; (c) disruption of cartilaginous core (DC) an lamellae fusion (LF) in fish exposed to 8 mg/l ZnO-NPs; (d) lamellae fusion (LF), disruption of cartilaginous core (DC) and aneurysm in fish exposed to 10 mg/l ZnO-NPs; (e) cellular degeneration (CD), rupture of epithelial cell (RE), epithelial lifting (EL); curling of secondary lamellae (CSL) and epithelial necrosis (EN) in fish exposed to 12 mg/l ZnO-NPs. At 60 days, (a) gill of *H. longifilis* in the control showing normal architecture of normal primary lamellae (PL) and secondary lamellae (SL); (b) epithelial lifting (EL), epithelial hyperplasia (EH) and epithelial necrosis (EN) in fish exposed to 6 mg/l ZnO-NPs; (c) epithelial lifting (EL); curling of secondary lamellae (CSL); aneurysm (AN) and disruption of cartilaginous core in fish eosed to 8 mg/l ZnO-NPs; (d) cellular degeneration (CD), aneurysm (AN) and epithelial necrosis (EN) in fish exposed to 10 mg/l ZnO-NPs; (e) epithelial necrosis (EN); curling of secondary lamellae (CSL); epithelial hyperplasia (EH) and lamellar fusion (LF) in fish exposed to 12 mg/l ZnO-NPs.

Table 1: Stages of histopathological alterations in gills and liver of *Heterobranchus longifilis* during ZnO-NPs exposure, depuration and amelioration periods

Alterations mostly observed	Stages of lesions	Alterations mostly observed	Stages of lesions
Gill		Liver	
Epithelia hyperplasia	I	Nuclear alteration	I
Epithelia lifting	I	Melanomacrophase hepatocyte	I
Lamellar fusion	I	Blood congestion	II
Lamellar disorganization	I	Nuclear vacuolation	II
Curling of secondary lamellae	I	Fatty degeneration	II
Disruption of cartilaginous core	II	Cytoplasmic degeneration	II
Aneurysm	II	Focal fibrosis	III
Epithelia oedema	II	Necrosis	III
Cellular degeneration	II		
Rupture of epithelial cell with haemorrhage	II		
Epithelia necrosis	III		

Stage I: do not alter normal physiology of tissues; stage II: more severe and causing abnormal physiology of tissue; stage III: marked severe and causing irreparable damage

At 45 d exposure, moderate damage (DTC between 21.90 ± 0.06 and 48.80 ± 0.64) were recorded in all treatments excluding those in 12 mg/l, where the damage was severe (57.20 ± 0.29). Compared to the control, gill damage was moderate in 6 and 8 mg/l treatments (DTC between 36.40 ± 0.64 and 50.30 ± 0.58) but severe in 10 (65.40 ± 0.46) and 12 mg/l (78.50 ± 1.15) treated groups after 60 d exposure. In the liver, DTC of ZnO-NPs-exposed fish also increased with toxicant concentration and exposure period (Table 4) compared to the control. At 15 d exposure, the mean DTC (7.80 ± 0.00) in fish liver from 6.0 mg/l ZnO-NPs treatment showed normal architecture, while those recorded for 8.0 (15.10 ± 0.58), 10.0 (33.30 ± 0.58) and 12.0 (39.40 ± 0.64) mg/l treatments indicated slight and moderate damage of liver cells, respectively. The DTC in fish liver from 6.0 mg/l treatment increased from 11.40 ± 0.12 (slightly damaged) at 30 d exposure to 36.20 ± 0.17 (moderately damaged) at 60 d. Similarly, the liver of ZnO-NPs-treated fish at 8.0 to 12.0 mg/l showed DTC increasing from being moderately damaged after 30 d exposure (DTC ranged from 38.40 ± 0.06 to 49.30 ± 1.21) to severely damaged after 60 d (DTC ranged from 51.80 ± 0.17 to 79.70 ± 0.17).

Recovery

Qualitatively, Table 2 shows that the gill of ZnO-NPs-exposed fish in all the treated groups exhibited depuration with respect to curling of secondary lamellar and rupture of epithelial cell within 15 d recovery period compared to the control where the gill showed a normal architecture with normal primary and secondary lamellae (Fig. 3a). Histopathological lesions such as epithelial lifting which occurred slightly in fish from 10 (Fig. 3d) and 12 mg/l (Fig. 3e) treatments after 15 d depuration were absent after 30 d. Disorganisation of lamellar that appeared severe in fish at 12 mg/l after 15 d depuration, appeared slightly following the extension of recovery period to 30 d. Other lesions such as cellular degeneration (Fig. 3c and e), rupture of epithelial cell, aneurysm

(Fig. 3d), and necrosis (Fig. 3 d) which either occurred slightly, moderately or severely in all the treated groups after 15 d depuration were completely reversed after 30 d recovery period except disruption of cartilaginous core (Fig. 3b). In fish liver from 6.0 to 10.0 mg/l treatments (Table 3, Fig. 4a-d), nuclear alteration, fatty and cytoplasmic degenerations (Fig. 4e) were completely depurated within 15 d recovery, while at 12.0 mg/l these pathological lesions were either slightly or moderately exhibited. Melanomacrophage centre, congestion and nuclear vacuolation were completely depurated at 6.0 and 8.0 mg/l, while fibrosis slightly showed at 6.0 and 8.0 mg/l treated groups and moderately showed at 12.0 mg/l group. At the end of 30 d recovery, five lesions (i.e., nuclear alteration, melanomacrophage centre, fibrosis, fatty and cytoplasmic degenerations) constituting 62.50 % of the pathological lesions in the liver were completely depurated at 6.0 to 10.0 mg/l. Blood congestion showed complete depuration in all the treated groups, while other lesions were either slightly or moderately exhibited at 10.0 and 12.0 mg/l treated groups.

After 15 d depuration, the quantitative evaluation of recovery responses in fish treated with 6, 8 and 10 mg/l concentrations of ZnO-NPs revealed similar gill damage compared to those observed when the fish were subjected to same concentrations for 60 d; as their DTC fell within 21 and 50 (Table 4) categorised as moderate damage, indicating no recovery. The lesions in 12 mg/l treated group after 15 d recovery were also not reversed from being severe as their DTC value was 52.70 compared to the DTC obtained (78.50 ±1.15) during 60-day exposure. At the end of 30 d, however, there was a gradual recovery from moderately damaged to normal organ at 6 mg/l (DTC 5.20 ± 0.05), moderately damaged to slightly damaged at 8 mg/l (DTC 14.30 ± 0.03), and severely damaged to moderately damaged at 10 (DTC 28.20 ± 0.30) and 12 mg/l (31.50 ± 0.06), respectively (Table 4). After 15 d , the DTC in fish liver from 6, 8 and 10 mg/l recovery groups (27.00 ± 0.00 – 49.20 ± 0.05) compared to those observed from the same groups during the 60 d exposure (36.20 ± 0.17–68.50 ± 0.64) indicated recovery from severely damaged organ to moderate, while DTC in the liver of fish in 12.0 mg/l group (62.60 ± 9.56) showed no recovery. At the end of 30 d recovery, the DTC in the liver of fish in 6 mg/l treated group showed slight damage (13.10 ± 0.00), while those in other groups (21.00 ± 0.00 -36.00 ± 0.12) exhibited moderate damage (Table 4).

Table 2: Histopathological alterations in the gill of *Heterobranchus longifilis* exposed to varying concentrations of ZnO-NPs for 60 days and depurated for 30 days

Lesion	Exposure (days)																Recovery (days)							
	Concentration (mg/l)																Concentration (mg/l)							
	15				30				45				60				15				30			
	6	8	10	12	6	8	10	12	6	8	10	12	6	8	10	12	6	8	10	12	6	8	10	12
Epithelial hyperplasia	+	+	+	+	+	+	+	+	+	+	+	+	++	++	+++	+++	+	+	++	+	+	+	+	+
Epithelial lifting	-	++	-	-	-	-	-	-	-	-	-	+++	++	+++	-	+++	-	-	+	+	-	-	-	-
Lamellar fusion	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	+++	++	++	++	+++	-	-	++	++
Lamellar disorganization	-	+	-	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	-	-	-	+++	-	-	-	+
Curling of secondary lamellar	-	-	+	-	-	-	-	-	-	-	-	-	-	++	++	+++	-	-	-	-	-	-	-	-
Disruption of cartilaginous core	-	-	-	-	+	-	-	++	++	+++	+++	+++	+	+++	-	+++	+	+	+++	+++	-	-	++	++
Oedema	-	-	-	-	-	++	++	-	++	-	-	++	++	++	++	+++	-	-	-	++	-	-	-	++
Cellular degeneration	-	-	-	-	++	++	++	++	++	++	++	++	-	++	++	+++	-	+++	+++	+++	-	-	-	-
Rupture of epithelial cell	-	-	-	-	+	+	+	++	-	-	-	++	-	-	-	+++	+	+	-	-	-	-	-	-
Aneurysm	-	-	++	++	-	-	++	++	-	-	++	-	++	-	++	+++	++	++	++	++	-	-	-	-
Necrosis	-	-	++	++	-	++	++	+++	-	++	++	+++	-	++	+++	+++	-	-	+++	+++	-	-	-	-

absent (-); mild (+); moderate (++); severe (+++)

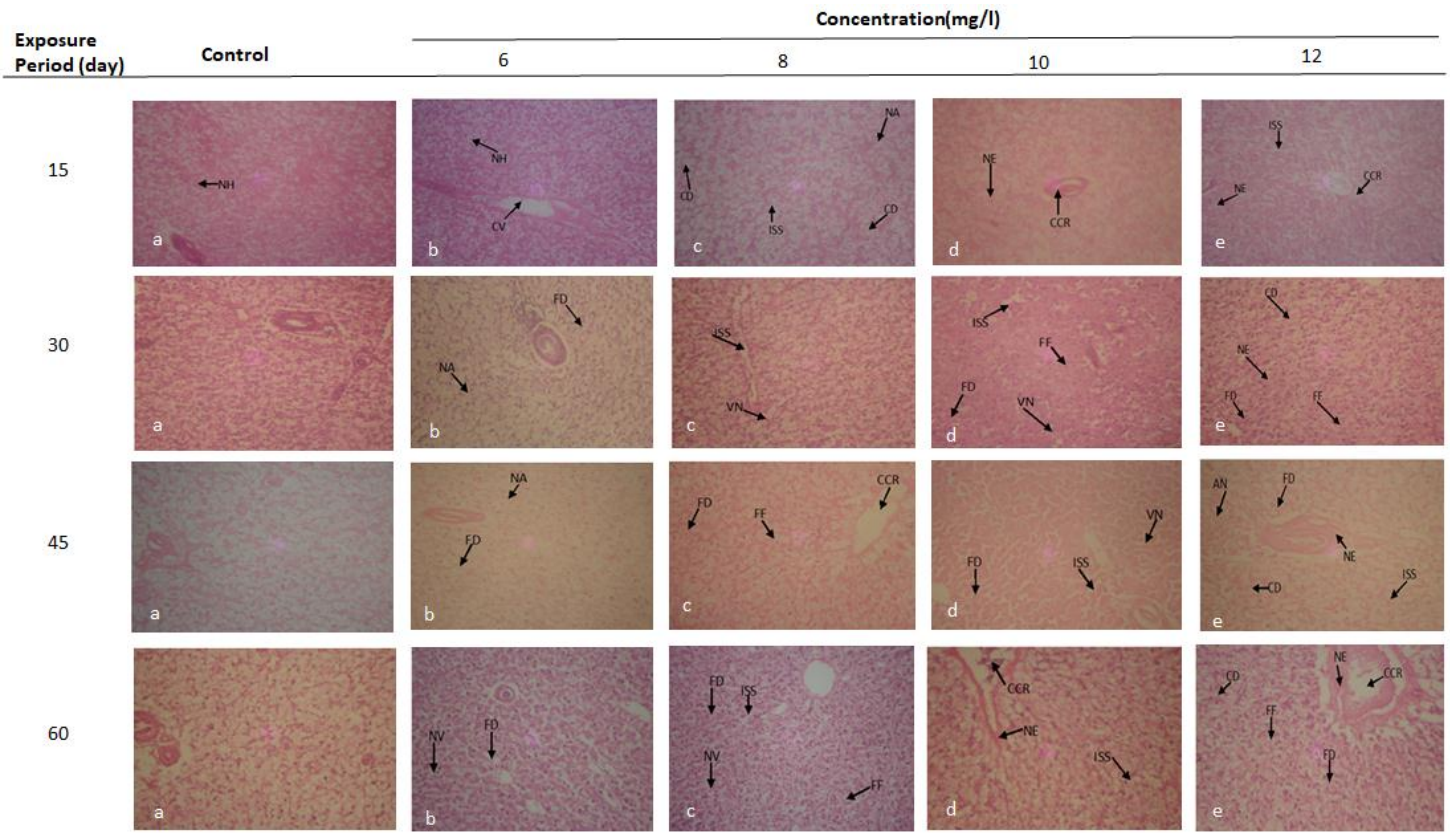

Fig 2: Histopathological alterations in the liver of *Heterobranchus longifilis* exposed to ZnO-NPs (H & E x 100). At 15 days, (a) liver of *H. longifilis* in the control showing normal hepatocytes (NH); (b) central vein (CV) and normal hepatocytes (NH) in fish exposed to 6 mg/l ZnO-NPs; (c) cytoplasmic degeneration (CD); increased sinusoidal space (ISS) and nuclear alteration (NA) in fish exposed to 8 mg/l ZNO-NPs; (d) central vein congested with red blood (CCR) and necrosis (NE) in fish exposed to 10 mg/l ZnO-NPs; (e) increased sinusoidal space (ISS); central vein congested with red blood (CCR) and necrosis (NE) in fish exposed to 12 mg/l ZnO-NPs. At 30 days, (a) liver of *H. longifilis* in the control showing normal architecture; (b) nuclear alteration (NA) and fatty degeneration (FD) in fish exposed to 8 mg/l ZnO-NPs; (c) increased sinusoidal space (ISS) and vacuolation of nucleus (VN) in fish exposed to 8 mg/l ZnO-NPs; (d) increased sinusoidal space (ISS), fatty degeneration (FD); focal fibrosis (FF) and vacuolation of nucleus (VN) in fish exposed to 10 mg/l ZnO-NPs; (e) cytoplasmic degeneration (CD); necrosis (NE); focal fibrosis (FF) and fatty degeneration (FD) in fish exposed to 12 mg/l ZnO-NPs. At 45 days, liver of *H. longifilis* in the control showing normal hepatocytes; (b) fatty degeneration (FD) and nuclear alteration (NA) in fish exposed to 6 mg/l ZnO-NPs; (c) fatty degeneration (FD); focal fibrosis (FF) and central vein congested with red blood (CCR) in fish exposed to 8 mg/l ZnO-NPs; (d) fatty degeneration (FD); increased sinusoidal space (ISS) and vacuolation of nucleus (VN) in fish exposed to 10 mg/l ZnO-NPs; (e) aneurysm (AN); fatty degeneration (FD); cytoplasmic degeneration (CD); necrosis (NE) and increased sinusoidal space (ISS) in fish exposed to 12 mg/l ZnO-NPs. At 60 days, (a) liver of *H. longifilis* in the control showing normal hepatocytes (b) nuclear vacuolation (NV) and fatty degeneration in fish exposed to 6 mg/l ZnO-NPs; (c) fatty degeneration (FD); nuclear vacuolation (NV); increased sinusoidal space (ISS) and focal fibrosis (FF) in fish exposed to 8 mg/l ZnO-NPs; (d) central vein congested with red blood (CCR); increased sinusoidal space (ISS) and necrosis (NE) in fish exposed to 10 mg/l ZnO-NPs; (e) cytoplasmic degeneration (CD); focal fibrosis (FF); fatty degeneration (FD) necrosis (NE) and central vein congested with red blood (CCR) in fish exposed to 12 mg/l ZnO-NPs.

Table 3: Histopathological alterations in the liver of *Heterobranchus longifilis* exposed to varying concentrations of ZnO-NPs and depurated for 30 days

Lesion	Exposure (days)																Recovery (days)							
	Concentration (mg/l)																Concentration (mg/l)							
	15				30				45				60				15				30			
	6	8	10	12	6	8	10	12	6	8	10	12	6	8	10	12	6	8	10	12	6	8	10	12
Nuclear alteration	-	-	-	-	++	++	+	++	+	+	+	+	+	+	++	+++	-	-	-	+	-	-	-	++-
Melanomacrophage hepatocyte	-	-	-	+	+	++	++	+	+	+	+	+++	+	+	++	+++	-	-	+	++	-	-	-	++
Blood congestion	-	-	+	+	+	-	+	-	+	++	++	++	-	-	+	+++	-	-	+	+	-	-	-	-
Nuclear vacuolation	-	+	+	+	+	+	+	-	-	+	+	+	++	++	++	++	-	-	+	+	-	-	+	++
Fatty degeneration	-	-	-	-	++	++	++	+++	++	++	+++	+++	++	++	+++	+++	-	-	-	++	-	-	-	++
Cytoplasmic degeneration	-	+	+	++	+	-	++	+++	-	++	++	+++	++	++	+++	+++	-	-	-	++	-	-	-	++
Focal fibrosis	-	-	-	-	+	+	+	+	-	++	+	+	+	++	++	++	+	+	+	++	-	-	-	+
Necrosis	+	++	+++	+++	-	++	++	++	-	++	+++	+++	+	++	++	+++	+	++	++	++	-	+	+	-

absent (-); mild (+); moderate (++); severe (+++)

Table 3: Histopathological alterations in the liver of *Heterobranchus longifilis* exposed to varying concentrations of ZnO-NPs and depurated for 30 days

Lesion	Exposure (days)																Recovery (days)							
	Concentration (mg/l)																Concentration (mg/l)							
	15				30				45				60				15				30			
	6	8	10	12	6	8	10	12	6	8	10	12	6	8	10	12	6	8	10	12	6	8	10	12
Nuclear alteration	-	-	-	-	++	++	+	++	+	+	+	+	+	+	++	+++	-	-	-	+	-	-	-	++-
Melanomacrophage hepatocyte	-	-	-	+	+	++	++	+	+	+	+	+++	+	+	++	+++	-	-	+	++	-	-	-	++
Blood congestion	-	-	+	+	+	-	+	-	+	++	++	++	-	-	+	+++	-	-	+	+	-	-	-	-
Nuclear vacuolation	-	+	+	+	+	+	+	-	-	+	+	+	++	++	++	++	-	-	+	+	-	-	+	++
Fatty degeneration	-	-	-	-	++	++	++	+++	++	++	+++	+++	++	++	+++	+++	-	-	-	++	-	-	-	++
Cytoplasmic degeneration	-	+	+	++	+	-	++	+++	-	++	++	+++	++	++	+++	+++	-	-	-	++	-	-	-	++
Focal fibrosis	-	-	-	-	+	+	+	+	-	++	+	+	+	++	++	++	+	+	+	++	-	-	-	+
Necrosis	+	++	+++	+++	-	++	++	++	-	++	+++	+++	+	++	++	+++	+	++	++	++	-	+	+	-

Table 4: Degree of tissue change (DTC) in the gills and liver of *Heterobranchus longifilis* exposed to ZnO-NPs for 60 days and depurated for 30 days

Exposure /depuration period /organ	Concentration (mg/l)				
	0.00 (Control)	6.00	8.00	10.00	12.00
15-day exposure					
Gill	0.00 ± 0.00	5.70 ± 0.06	9.60 ± 0.12	25.40 ± 0.64	31.30 ± 0.52
Liver	0.00 ± 0.00	7.80 ± 0.04	15.10. ± 0.58	33.30 ± 0.58	39.40 ± 0.64
30-day exposure					
Gill	0.00 ± 0.00	9.50 ± 0.06	17.80 ± 0.64	34.80 ± 1.21	46.30 ± 0.58
Liver	0.00 ± 0.00	11.40 ± 0.12	38.40 ± 0.06	45.40 ± 0.64	49.30 ± 1.21
45-day exposure					
Gill	0.00 ± 0.00	21.90 ± 0.06	36.50 ± 0.44	48.80 ± 0.64	57.20 ± 0.29
Liver	0.00 ± 0.00	19.60 ± 0.12	42.50 ± 0.64	51.80 ± 0.17	62.20 ± 0.60
60-day exposure					
Gill	0.00 ± 0.00	36.40 ± 0.64	50.30 ± 0.58	65.40 ± 0.46	78.50 ± 1.15
Liver	0.00 ± 0.00	36.20 ± 0.17	56.20 ± 0.12	68.50 ± 0.64	79.70 ± 0.17
15-day depuration					
Gill	0.00 ± 0.00	27.40 ± 0.08	38.20 ± 0.10	44.60 ± 1.12	52.70 ± 4.60
Liver	0.00 ± 0.00	27.00 ± 0.05	42.10 ± 0.14	49.20 ± 0.05	62.60 ± 9.56
30-day depuration					
Gill	0.00 ± 0.00	5.20 ± 0.02	14.30 ± 0.02	28.20 ± 0.30	31.50 ± 0.06
Liver	0.00 ± 0.00	13.10 ± 0.03	21.00 ± 0.12	30.10 ± 0.13	36.00 ± 0.12

Amelioration

Qualitatively, there were no lesions in the gill of fish in groups A, B and C after 15 and 30 d of amelioration with AA, respectively (Table 5, Fig. 5). In group D, lamellar fusion and curling of secondary lamellar appeared severely in the gill after 15 d, while epithelial hyperplasia, epithelial lifting, disruption of cartilaginous core, cellular degeneration, aneurysm and necrosis were moderately exhibited. All other lesions appeared slightly (Table 5). After 30 d treatment, however, group D fish displayed severe cellular degeneration, slight oedema and epithelial cell rupture, while other lesions appeared moderately except lamellar disorganisation that was absent. Each of epithelial hyperplasia, epithelial lifting, lamellar fusion, curling of secondary lamellar, disruption of cartilaginous core, rupture of epithelial cell, aneurysm and necrosis showed similar pattern of exhibition in the gill of group E treated fish after 15 d and 30 d amelioration. Lamellar disorganisation was completely reversed in group E after 30 d depuration. In group F, epithelial hyperplasia, lamellar disorganization and oedema did not appear in the

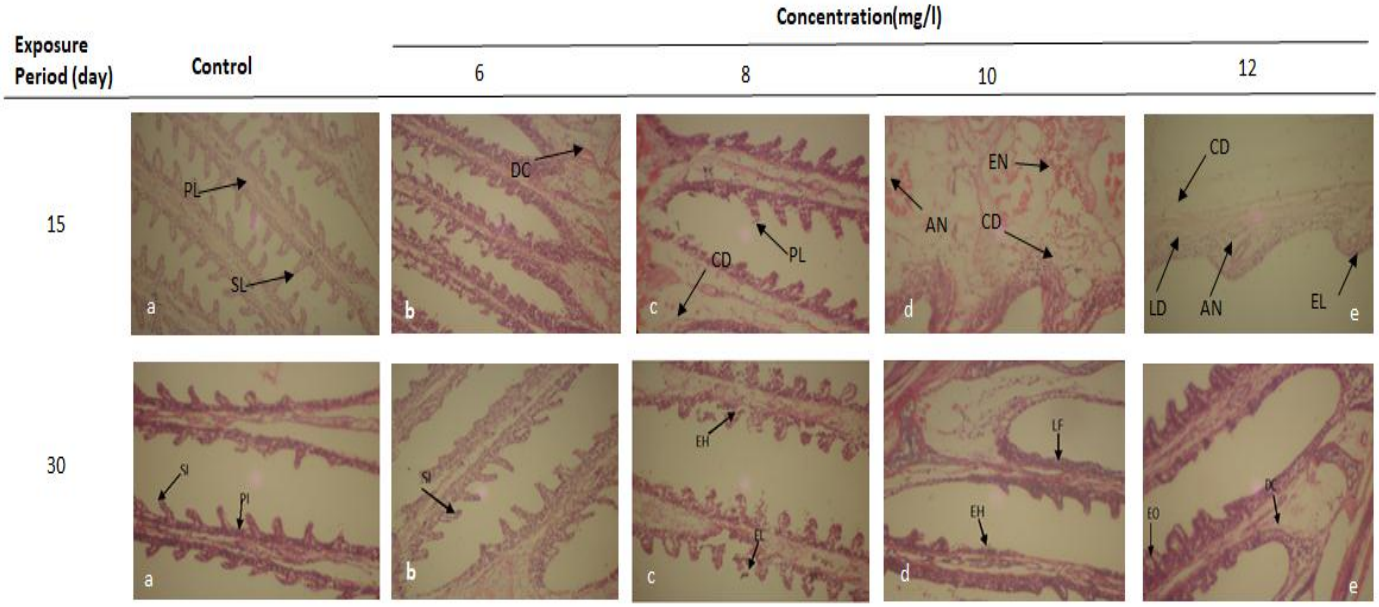

Fig 3: Histopathological lesions in the gills of *Heterobranchus longifilis* exposed to ZnO-NPs for 60 days, depurated for 15 and 30 days (H & E, x 100). At 15 days depuration, (a) gills of *Heterobranchus longifilis* in control showing normal architecture with normal primary lamellae (PL) and secondary lamellae (SL); (b) disruption of cartilaginous core (DC) in fish exposed to 6 mg/l ZnO-NPs; (c) cellular degeneration (CD); Primary lamellar (PL) in fish exposed to 8 mg/l ZnO-NPs; (d) aneurysm (AN); epithelial necrosis (EN) and cellular degeneration (CD) in fish exposed to 10 mg/l ZnO-NPs; (e) cellular degeneration (CD); lamellar disorganisation (LD); aneurysm (AN) and epithelial lifting (EL) in fish exposed to 12 mg/l ZnO-NPs. At 30 days depuration, (a) gills of *Heterobranchus longifilis* in control showing normal architecture with normal primary lamellae (PL) and secondary lamellae (SL); (b) epithelial lifting in fish exposed to 6 mg/l ZnO-NPs; (c) epithelial lifting (EL) and epithelial hyperplasia (EH) in fish exposed to 8 mg/l ZnO-NPs; (d) lamellae fusion (LF) and epithelial hyperplasia (EH) in fish exposed to 10 mg/l ZnO-NPs; (e) epithelial oedema; and disruption of cartilaginous core (DC) in fish exposed to 12 mg/l ZnO-NPs.

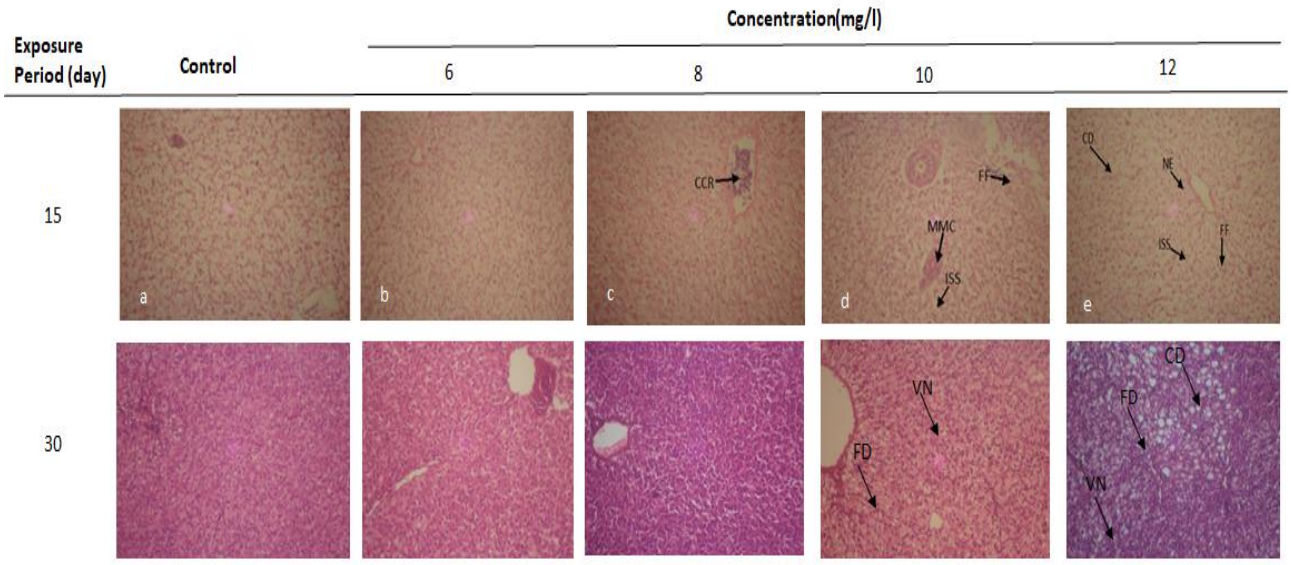

Fig 4: Histopathological lesions in the liver of *Heterobranchus longifilis* exposed to ZnO-NPs for 60 days, depurated for 15 and 30 days (H & E x100). At 15 days depuration, (a) liver of H. longifilis in control showing normal hepatocytes; (b) normal hepatocytes in fish exposed to 6 mg/l ZnO-NPs; (c) central vein congested with red blood (CCR) in fish exposed to 8 mg/l ZnO-NPs; (d) melanomacrophage hepatocytes (MMC), increased sinusoidal space (ISS) and focal fibrosis (FF) in fish exposed to 10 mg/l ZnO-NPs; (e) cytoplasmic degeneration (CD); increased sinusoidal space (ISS); focal fibrosis (FF) and necrosis (NE) in fish exposed to 12 mg/l ZnO-NPs.

gill after 15 d amelioration, while other lesions appeared slightly. Similarly, epithelial hyperplasia, lamellar fusion, lamellar disorganisation and curling of secondary lamellar did not appear after 30 d amelioration, while other lesions appeared slightly. In G and H groups, 54 and 72 % of the lesions did not appear, respectively, and those that appeared (curling of secondary lamellar, cellular degeneration, rupture of epithelial cell, aneurysm and necrosis) which constituted approximately 46 and 28 % for the two groups, respectively, were slightly exhibited after 15 d amelioration. After 30 d amelioration, however, 90.90 % of the lesions in G treated fish did not appear; with only one lesion (aneurysm) appearing slightly, while in H curling of secondary lamellar, rupture of epithelial cell and necrosis were lesions that appeared slightly, and other lesions (72.72 %) did not appear.

In the liver (Table 6, Fig. 6), no lesion was found in A, B and C groups after 15 and 30 d amelioration, respectively. In group D, similar pattern of liver pathological lesions ranging from moderate to slight were observed after 15 and 30 d amelioration period, respectively, except nuclear alteration that showed severe pattern after 15 d. Nuclear alteration and melanomacrophage hepatocyte were moderately shown in the liver of E treated fish after 15 d amelioration, while others were exhibited slightly. This was different from the pattern recorded after 30 d, where fatty and cytoplasmic degenerations, focal fibrosis and necrosis were absent while other lesions appeared slightly. In F treated fish, cytoplasmic degeneration, focal fibrosis and necrosis were absent in the liver, while other lesions were present moderately after 15 d. This pattern was similar to the

observation in the F treated fish after 30 d amelioration except fatty degeneration that did not appear. In G treated fish, 62.50 and 75 % of the lesions in the liver did not appear, while the rest appeared moderately after 15 and 30 d, respectively, while in H treated fish, 62.50 % and 50 % of the lesions did not appear in the liver after 15 and 30 d amelioration, respectively.

Quantitatively, the mean DTC for A, B and C treatments were within the range categorised as normal functioning of gills and liver (Table 7). In group D treated fish, the DTC recorded for both gill (26.3 ± 1.21) and liver (34.2 ± 0.17 after 15 d amelioration and the values recorded (gill: 35.2 ± 0.58; liver: 46.01 ± 1.15) after 30 d fall within the value (21-50) categorised as moderately damaged. In the ameliorated groups of both the gill and the liver from E, F and G, the severity of damage (as reflected by the DTC values) decreased as the concentration of AA increased from 50 to 500 $mg.kg^{-1}$AA diet compared to group D without AA. An increase in the severity of pathological damage were, however, recorded in group H that was ameliorated with the highest concentration of AA (1000 $mg.kg^{-1}$AA).

Table 6: Ameliorative effects of ascorbic acid (AA, vitamin C) on the histopathological lesions in the liver of *Heterobranchus longifilis* exposed to ZnO-NPs for 60 days and ameliorated for 30 days

	Amelioration (days)															
	Treatment group															
	15								30							
Lesion	A	B	C	D	E	F	G	H	A	B	C	D	E	F	G	H
Nuclear alteration	-	-	-	+++	++	+	-	-	-	-	-	++	+	+	-	-
Melanomacrophage hepatocyte	-	-	-	++	++	+	-	-	-	-	-	++	+	+	-	-
Blood congestion/Sinusoids	-	-	-	++	+	+	-	-	-	-	-	++	+	+	-	-
Nuclear vacuolation	-	-	-	+	+	+	-	-	-	-	-	+	+	+	-	+
Fatty degeneration	-		-	+	+	+	-	+	-	-	-	+	-	-	-	-
Cytoplasmic degeneration	-	-	-	+	+	-	+	+	-	-	-	+	-	-	-	+
Focal fibrosis	-	-	-	+	+	-	+	+	-	-	-	+	-	-	+	+
Necrosis	-	-	-	++	+	-	-	-	-	-	-	++	+	-	+	+

absent (–); mild (+); moderate (++); severe (+++); A = basal diet only; B = basal diet + 50 $mg.kg^{-1}$AA (ascorbic acid); C = basal diet + 1000 $mg.kg^{-1}$AA; D = ZnO-NPs + basal diet only; E = ZnO-NPs + basal diet + 50 $mg.kg^{-1}$AA; F = ZnO-NPs + basal diet + 250 $mg.kg^{-1}$AA; G = ZnO-NPs + basal diet + 500 $mg.kg^{-1}$AA; H = ZnO-NPs + basal diet + 1000 $mg.kg^{-1}$AA

Table 7: Degree of tissue change (DTC) in the gills and liver of *Heterobranchus longifilis* exposed to ZnO-NPs and ameliorated with ascorbic acid (AA, vitamin C) for 30 days

	Treatment group							
Duration of amelioration/organ	A	B	C	D	E	F	G	H
15-day amelioration								
Gill	0.00 ± 0.00	0.00 ± 0.00	0.20 ± 0.01	26.30 ± 1.21	23.40 ± 0.52	23.30 ± 0.52	16.60 ± 1.17	24.20 ±1.21
Liver	0.00 ± 0.00	0.00 ± 0.00	0.10 ± 0.00	34.20 ± 0.17	31.50 ± 1.16	23.40 ± 0.48	19.80 ± 0.06	29.10 ± 0.08
30-day amelioration								
Gill	0.00 ± 0.00	0.00 ± 0.00	0.10 ± 0.02	35.20 ± 0.58	32.60 ± 1.14	20.20 ± 0.64	9.30 ± 0.12	22.00 ± 0.45
Liver	0.00 ± 0.00	0.00 ± 0.00	0.30 ± 0.02	46.00 ± 1.15	42.60 ± 0.64	20.40 ± 0.58	10.30 ± 1.21	25.30 ± 0.14

A = basal diet only; B = basal diet + 50 $mg.kg^{-1}$AA (ascorbic acid); C = basal diet + 1000 $mg.kg^{-1}$AA; D = ZnO-NPs + basal diet only; E = ZnO-NPs + basal diet + 50 $mg.kg^{-1}$AA; F = ZnO-NPs + basal diet + 250 $mg.kg^{-1}$AA; G = ZnO-NPs + basal diet + 500 $mg.kg^{-1}$AA; H = ZnO-NPs + basal diet + 1000 $mg.kg^{-1}$AA

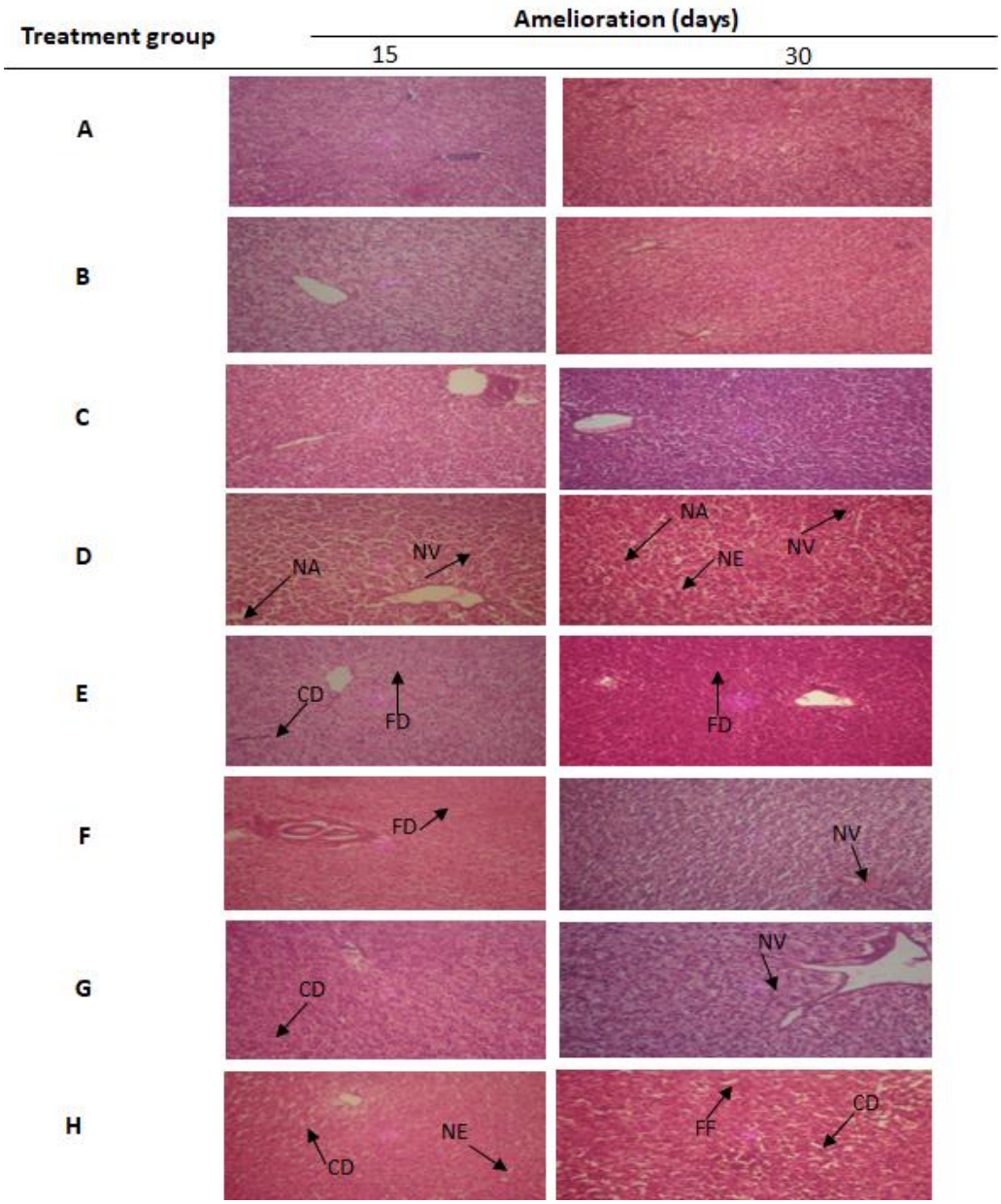

Fig 6: Histopathological lesions in the liver of *Heterobranchus longifilis* exposed to ZnO-NPs for 60 days and ameliorated for 15 and 30 days (H & E x 100). At 15 days amelioration, (A) liver of *H. longifilis* in the group fed normal diet only without ZnO-NPs showing normal architecture with normal hepatocytes; (B) normal hepatocytes in fish fed diet with 50 mg.kg^{-1}AA without ZnO-NPs; (C) normal hepatocytes in fish fed 1000 mg.kg^{-1}AA; (D) nuclear alteration (NA) and nuclear vacuolation (NV) in fish exposed to 10 mg/l ZnO-NPs and fed normal diet; (E) fatty degeneration (FD) and cytoplasmic degeneration (CD) in fish exposed to 10 mg/l ZnO-NPs and fed diet containing 50 mg.kg^{-1}AA; (F) fatty degeneration (FD) in fish exposed to 10 mg/l ZnO-NPs and fed diet containing 250 mg.kg^{-1}AA; (G) cytoplasmic degeneration (CD) in fish exposed to 10 mg/l and fed diet containing 500 mg.kg^{-1}AA; (H) cytoplasmic degeneration (CD) and necrosis (NE) in fish exposed to 10 mg/l ZnO-NPs and fed diet containing 1000 mg.kg^{-1}AA. At 30 days amelioration, (A) liver of *H. longifilis* in the group fed normal diet only without ZnO-NPs showing normal architecture with normal hepatocytes; (B) normal hepatocytes in fish fed diet with 50 mg.kg^{-1}AA without ZnO-NPs; (C) normal hepatocytes in fish fed 1000 mg.kg^{-1}AA; (D) nuclear alteration (NA), necrosis (NE) and nuclear vacuolation (NV) in fish exposed to 10 mg/l ZnO-NPs and fed normal diet; (E) fatty degeneration (FD) in fish exposed to 10 mg/l ZnO-NPs and fed diet containing 50 mg.kg^{-1}AA; (F) nuclear vacuolation (NV) in fish exposed to 10 mg/l ZnO-NPs and fed diet containing 250 mg.kg^{-1}AA; (G) nuclear vacuolation (NV) in fish exposed to 10 mg/l and fed diet containing 500 mg.kg^{-1}AA; (H) focal fibrosis (FF) and cytoplasmic degeneration (CD) in fish exposed to 10 mg/l ZnO-NPs and fed diet containing 1000 mg.kg^{-1}AA.

DISCUSSION

Zinc oxide nanoparticles (ZnO-NPs) may be easily released into water bodies due to their increased utilisation in the production of commercial and medicinal goods, thus constituting a serious risk factor to the aquatic organisms; as well as raising concerns in aquatic monitoring. This is because the health and survival of aquatic organisms have been compromised due to several metabolic and histological alterations caused by pollutants in water bodies **(Shankar *et al*., 2013)**. Pathological effects from ZnO-NPs exposure were examined using the branchial and hepatic tissues because the former plays important roles in gas exchange, osmoregulation and response to environmental perturbations, being the first target organ under direct attack by nanoparticles **(Nnamdi *et al*., 2019)**; while the latter is involved in xenobiotics transformations **(Takashima and Hibiya, 1995)**. These roles make both tissues sensitive to adverse environmental conditions.

H. longifilis exposure to varying concentrations of ZnO-NPs in this work, revealed several histopathological alterations as reflected in abnormal cellular architecture such as lamellar fusion, lamellar disorganisation, epithelial lifting, epithelial hyperplasia, disruption of cartilaginous core, rupture of epithelial lamellae, epithelial oedema, aneurysm and necrosis in the gill; and nuclear alteration, melanomacrophage hepatocytes, blood congestion, nuclear vacuolation, fatty degeneration, cytoplasmic degeneration, focal fibrosis and necrosis in the liver. The qualitative and quantitative assessments of these lesions showed that their severity were concentrations and duration dependent. These histological abnormalities have been reported in the gills **(Rajkumar *et al*., 2015; Nnamdi *et al*., 2019**) and liver **(Ostaszewska et al., 2016; Aghamirkarimi *et al*. 2017; Abdel-Khalek *et al*. 2020)** of different fish species following exposure to varying concentrations of nanoparticles. These histopathological disorders are suggestive of functional impairments of the antioxidant defense systems responsible for maintaining balanced redox potentials, by way of providing structural and metabolic backup/defense to the cells before, during and after chemical insults. The impairment may be attributed to the ZnO-NPs-induced toxicity resulting from excessive reactive oxygen species generation and attendant lipid peroxidation. **Abdulkareem (2017)** reported significant lipid peroxidation caused by ZnO-NPs in the gill and liver of *H. longifilis*. Previous studies had also shown that ZnO-NPs induced toxicity via ROS production and lipid peroxidation in fish **(Firat and Bozat, 2018)**.

Hyperplasia, lamella fusion, epithelial lifting and oedema have been described as defensive mechanisms to wade off pollutants from entering the body of organisms. According to **Yancheva *et al*. (2016)**, these alterations create a barrier between the blood and surrounding milieu to avert the influx of contaminants into the body. Available area for exchange of gases may be diminished due to fusion of lamellae and could lead to hypoxic condition **(Richmond and Dutta, 1989)**. Lamellar fusion in fish gills following introduction to contaminants have been documented **(Nimet *et al*., 2018; Nimet *et al*., 2020)**. Aneurysms, an outward bulging or distension of the blood vessel may have

resulted to the rupture of epithelial lamellae as observed in this study, with attendant consequence of frequent haemorrhagic condition that may predispose the fish to sudden death **(Rajkumar *et al*., 2015)**. Reports by **Subburaj *et al*. (2018)** and **Nimet *et al*. (2020)** have shown a clear correlation between aneurysms in fish and other conventional contaminants such as heavy metals and pesticides. *H. longifilis* are known to be a very active benthopelagic fish, feeding in almost all the niches available in the water system and its wide ecological distribution increases its exposure to xenobiotics **(Teugels *et al*. 1990)**. Against this backdrop, it follows that a lesion such as aneurysms that causes bleeding and loss of vascular integrity could ultimately reduce blood capacity to carry oxygen and adversely affects the fish's respiratory and swimming activities **(Shahzad *et al*., 2018; Nimet *et al*., 2020)**. Eventually, this may compromise the feeding ability, escape from dangers, health and survival of the fish. Vacuolisation, sinusoidal space and nuclear alteration observed in the hepatocytes may also be considered as defense mechanisms deployed to get rid of toxic substances. Vacuoles when formed may collect toxic substances for nullification, so as to avoid disturbance to the functioning of the organ **(Hadi *et al*., 2012)**. Fatty and cytoplasmic degenerations can result into vacuole formation in cells leading to necrosis **(Kelly and Janz, 2009)**. Nuclear alteration may be an attempt to protect the cell from environmental injuries **(Braunbeck *et al*., 1990)**. Necrosis observed in both tissues is known to be one of the most severe causes of cell death **(Aghamirkarimi *et al*., 2017)**, hence inhibiting the functioning capacity of tissues. Many authors have reported inhibition of tissue performance in fish due to cell death **(Ostaswzewska *et al*., 2016)**.

Qualitative and quantitative evaluation of depuration showed that resumption of normal tissue functions was dependent on the severity of lesions, toxicant concentrations and recovery duration. The non-appearance of curling of secondary lamellar and rupture of epithelial cell in the gill of ZnO-NPs-exposed fish in all the treated groups after 15 d depuration is an indication of potential recovery to normal morphology, though other lesions were retained. The slight, moderate and/or severe exhibition of most of the lesions in the gill and liver of the ZnO-NPs-exposed fish at 15 d recovery period is suggestive of the fact that the fish recovery responses after transfer to clean water for 15 d was insufficient for both organs to get rid of most of the lesions and resume their normal functions fully. However, the return of most of the lesions to basal levels after 30 d is an indication that 30 d in good quality water could go a long way to restoring both organs to their normal pathological conditions. The partial or non-recovery of some abnormalities in the treated groups after 30 d depuration suggests that recovery process was slow and a longer time may be required for both organs to return to their normal conditions.

Furthermore, the quantitative assessment of lesions in both tissues/organs validates the results of the qualitative evaluation. The similarity in DTC between the gills of fish transferred from 6, 8 and 10 mg/l treatments to clean water for 15 d and those

challenged with same ZnO-NPs concentrations for 60 d; is indicative of no recovery as DTC values in both cases were within 21-50 categorised as moderate even after fish had been depurated for 15 d, though within this period only slight recovery was recorded in the liver at 6 - 10 mg/l. The retention of severe lesions in both organs at 12 mg/l treated group (DTC = 52.70) even after 15 d depuration is also an indication of no recovery. But after 30 d depuration, however, the return to basal levels for some lesions at lowest concentration (6 mg/l) and partial recovery in other treatments further corroborate the fact that complete depuration in both organs will presumably require an elongated period. In literature, comparable reports on the results of recovery assessment of gill and liver in chronic nanoparticles-exposed fish are not available. However, the observations of **Jonsson et al. (2017)** in which histological lesions persist in fish exposed to herbicides even after recovery are in tandem with the present results. The persistence of lesions after depuration may be due to the inability of organs to regain their antioxidant competence against persistent free radicals due to ZnO-NPs toxicity ever after the fish was transferred to clean water.

The aquacultural implications of this result is that the presence of lesions in ZNO-NPs-treated fish at the lowest concentration suggests that, maintaining safe limits for ZnO-NPs in any production systems is pertinent; because should the safe limit be exceeded it will cause devastating effects to fish. Furthermore, the inability of the fish to recover fully after 15 and 30 d recovery periods is suggestive of the need to develop strategies that could enhance the antioxidant systems such as inclusion of antioxidants, which the body cannot synthesize, in the diets; so as to provide greater resistance to toxicity of xenobiotics. This informed the dietary inclusion of AA in *H. longifilis* to reinforce its antioxidant potency against ZnO-NPs-induced histopathological effects. The importance of dietary inclusion of AA such as improvement in growth performance, immune response, stress elimination and protection against oxidative damage to tissues have widely been reported **(Abdelazim *et al*., 2018; Khan *et al*., 2019)**. However, its importance in modulating histopathological disorder in fish following ZnO-NPs exposure has rarely been known. In this study, an obvious ameliorative potential of AA supplementation in ZnO-NPs-exposed fish was evident in the 'qualitative' disappearance of 54 and 72% of the lesions in the gill; and 75 and 62.50% of the lesions in the liver of fish administered AA-supplemented diet at 500 $mg.kg^{-1}$ and 1000 $mg.kg^{-1}$ after 15 d amelioration, respectively. Furthermore, the 90.90 and 72.72% lesion disappearance in the gill; and 75 and 50% in the liver of fish on 500 $mg.kg^{-1}$ and 1000 $mg.kg^{-1}$AA regimen after 30 d amelioration, respectively also led credence to the ameliorative potency of AA.

Quantitatively, the decrease in severity of damage in both organs as AA supplementation increased also shows that AA probably has additive effect on ZnO-NPs toxicity; the more the dietary AA, the more the additive effect. The groups treated AA-supplemented diet gradually showed reversal of lesions close to the normal cellular architecture in the control; suggesting non-adverse effects on the tissues. In the group

treated 500 mg.kg^{-1} AA diet, clearer details of cellular architecture were observed, as lesions were scanty. This was evident in the reduction of severity of damage from moderate to normal in gill, and from moderate to slight in liver. Thus, indicating that AA had ameliorated the ZnO-NPs-induced pathological effects. However, rather than produce a two-fold reduction in lesion disorders, the doubling of AA caused a gradual increase in the severity of damage; showing that AA supplementation at this rate (1000 mg.kg^{-1}) could have adverse effects on the tissue instead of antagonistic/neutralising effects against ZnO-NPs toxicity (i.e., AA administration effects at 1000 mg.kg^{-1} was becoming prooxidant).

The ability of AA to tackle oxidative stress headlong and reverse haematological and biochemical disorders in pesticide- **(Sarma *et al*., 2009)** and nanoparticles-induced toxicity **(Abdelazim *et al*., 2018)** confirms the ameliorative potency recorded in ZnO-NPs-induced histopathological alterations in this study. AA reduction of histopathological disorders caused by ZnO-NPs exposure may be predicated on its binding efficacy and scavenging of toxic free radicals produced during exposure regime. Thus, the ameliorative effect of AA treatment particularly at 500 mg/kg^{-1} in ZnO-NPS-exposed *H. longifilis* can be attributed to the potent antioxidant effect of AA in modulating the ZnO-NPs-induced oxidative stress. Many studies have widely reported the free radical binding and scavenging properties of AA against ROS production from pollutant-induced oxidative stress in fish **(Abdelhazim *et al*., 2018)**..

Considering the two amelioration periods, it appears that: on one hand, 15 d amelioration at 500 mg.kg^{-1} and 1000 mg/kg^{-1} AA may not be sufficient for effective neutralisation of the free radicals and total recovery of lesions. On the other hand, the 30-d amelioration at 500 mg.kg^{-1} was sufficient to ameliorate almost all the lesions, but duration for amelioration was too long, while amelioration at 1000 mg/kg^{-1} produced adverse effects. This, therefore, suggests that combination of AA with other non-enzymatic antioxidant in appropriate concentration required could potentially reinforce the antioxidant defense systems and completely ameliorates ZnO-NPs adverse effects within few days (i.e., less 15 d). A rapid and efficient ameliorating agent will be more economical to apply in any fish production system exposed to runoffs containing hazardous xenobiotics.

CONCLUSION

Sublethal concentrations of ZnO-NPs induced histopathological alterations in juveniles *H. longifilis*. Treatment with AA was able to ameliorate the lesions though recovery was slow and partial; thus, confirming its efficacy as ameliorating agent against ZnO-NPs-induced structural alterations.

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