

EFFECT OF ZINC OXIDE NANO-PARTICLES ON ACTIVITIES OF BIOCHEMICAL ENZYMES IN THE TISSUES OF *Heterobranchus longifilis*

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ABSTRACT

The unregulated release of nano-particles into the environment induces toxicity in living organisms by interfering with normal cell processes. This study investigated the toxicity effects of both lethal and sub-lethal concentrations of ZnO-NPs on biochemical enzymes in the tissues of African catfish, *Heterobranchus longifilis*, by exposing to lethal concentrations (0.00, 60.00, 80.00, 100.00 and 120 mg/l) and sub-lethal concentrations (0.00, 6.00, 8.00, 10.00 and 12.00 mg/l) of ZnO-NPs for 96 h and 45 d in static renewal bioassays with continuous aeration respectively. After the exposure periods, blood was collected through the caudal fin of the fish from control and exposure groups and sacrificed to remove the gills and liver for biochemical assay. The results revealed a significant ($P < 0.05$) increase in the activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), acetyl cholinesterase (AChE), and the levels of total protein and glucose also increased significantly ($P < 0.05$) in the blood, gills and liver with increase in the concentrations and exposure periods compared to the control. The alterations in the biochemical enzymes of the fish indicated that the release of ZnO-NPs into aquatic environment could be dangerous and may cause health risk in man through food chain.

Keywords: Biochemical enzymes, *Heterobranchus longifilis*, zinc oxide nano-particles, lethal, sub-lethal.

INTRODUCTION

Zinc oxide nano-particles (ZnO-NPs) are mostly used in consumer products such as cosmetics, textiles, toothpaste, sunscreen, wall paints and in biomedical applications for the treatment of cancer, nerve injury, etc (Hanley *et al.*, 2008). It is also used in the removal of pollutants (Qiang, 2001). The use of ZnO-NPs in the manufacture of most

products is due to their outstanding catalytic, magnetic, optic, transparency, biocompatibility, efficiency, and isoelectric properties (Qiang, 2001). Despite these advantages, toxicological investigations revealed that zinc oxide nano-particles are harmful to environmental species and human health (Zhang *et al.*, 2010). These impacts could be as a result of the smaller size, greater surface area, and greater number of nano-particles. There is therefore the fear of the risk of these nano-particles on aquatic life when released into water bodies by nano-industries because of their ability to accumulate in the aquatic systems (Moore, 2006).

To understand the potential risk of nano-particles in aquatic animals, fish are used as test organisms, which revealed possible associated ecological and food chain risk. The biological impact of ZnO-NPs released into the aquatic environment is the major concern of every scientist. This therefore attracted investigations on both acute and sub-acute effects of varying nano-metals on biochemical enzymes in the tissues of different types of fish species among which are effects of ZnO-NPs on *Oreochromis niloticus* (Amuta and Subramanian, 2009); ZnO-NPs on zebrafish embryos (Zhang *et al.*, 2009 and Zhu *et al.*, 2009); ZnO-NPs on zebrafish (Shaw and Handy, 2011); Cu-NPs on zebrafish (Griffitt *et al.*, 2009); Fe-NPs on Japanese medaka (Li *et al.*, 2009); TiO₂-NPs on common carp (Zhang *et al.*, 2007); Al-NPs on zebrafish (Griffitt *et al.*, 2008).

These studies showed various degrees of biochemical changes in fish, however, among these, literature on the effect of ZnO-NPs on the biochemical parameters of *Heterobranchus longifilis* is sparse. The present research was carried out to investigate the acute and subacute effects of ZnO-NPs on the biochemical enzymes in the blood, gill, and liver of *Heterobranchus longifilis*.

MATERIALS AND METHODS

Fish and Acclimatization Conditions

The catfish, *Heterobranchus longifilis* (mean body weight 76.40±21.65 SE) and mean total length (24.45±0.13.00 SE) were obtained from a private hatchery in Lagos Nigeria. They were transported in plastic aquaria to a research laboratory in the Department of Zoology of the University of Ilorin, Nigeria. The fish were acclimated to laboratory conditions for a period of two weeks before the experiment. They were maintained in a large aquarium of 1000 litre capacity at ambient laboratory temperature (24.0°C) and water was kept oxygen saturated with aerating pumps. Fish were fed on a commercial diet Coppens (3mm) twice daily at 9:00 am and 5:00 pm at 4% of their body weight. Water was renewed every 24 hours to reduce pollution from metabolic waste. Feeding of the fish was stopped 24 hours before the commencement of the experiment/ Physico-chemical characteristics of water were continuously monitored for temperature, dissolved oxygen (DO), biochemical oxygen demand (BOD), conductivity, chemical oxygen demand (COD) and pH according to the procedure of APHA (1995).

Nano-Particles Suspension Preparation

Zinc oxide nano-particles (< 100 nm) was obtained from Sigma-Aldrich in USA. The nano-powder was dispersed into distilled water and the suspending solution was magnetically stirred with magnetic stirrer for the period of two hours to thoroughly dissolve the particles. A fresh solution of ZnO- NPs was prepared every day for renewal.

Exposure Period

After the period of acclimation, fish were randomly divided into five groups of 10 fish each in an aquarium of 80 litre capacity containing 30 litres of chlorine free borehole water. The first group served as the control without toxicant (0.00 mg/l) while the remaining four groups as the experimental groups with varying dilution of freshly prepared stock solution of ZnO-NPs (60.00 mg/l, 80.00 mg/l, 100.00 mg/l and 120.00 mg/l). Six fish were randomly sampled from each group after 96h of exposure to collect tissues for biochemical analysis. As in the first experiment, new set of five groups of fish were exposed to varying concentrations (0.00 mg/l, 6.00 mg/l, 8.00 mg/l, 10.00 mg/l and 12.00 mg/l) of zinc oxide nano-particles for 45 days based on the results of acute toxicity. The experiments were set up in triplicate with one group serving as the real experiment. At the end of the experimental period, fish were sacrificed to collect blood and remove gills and liver for biochemical assay.

Biochemical Assay

The activities of alamine aminotransferase (ALT) (EC. 2. 6. 1. 2) and aspartate aminotransferase (AST) (EC 2. 6. 1. 1) enzymes in the, serum, gill and liver were analyzed (Bürger *et al.*, 2005). Fresh blood samples were collected into heparinized microhaematocrit tube sealed with plasticine at one end and centrifuged for 5 minutes at 12,000 g to separate serum from red blood cells. One gramme of the tissues was weighed and put into 5 ml of sucrose solution, and homogenized using a warring blender. The homogenate was then centrifuged at 14, 700 g (4°C) for 20 minutes to obtained supernatant. The supernatants were stored at – 80 °C prior analysis. The serum and the supernatants were assayed for ALT, AST, LDH, glucose and total protein using the method described by Bürger *et al.*, (2005). Acetylcholinesterase (AChE) activity was assayed following the procedure modified by Velki *et al.* (2017).

Data Analysis

All data were expressed as mean $\pm$ SE. Analysis of Variance (ANOVA) and post hoc Duncan's Multiple Range Test (DMRT) were performed for differences between each group using SPSS 16.0 version. The significance of differences was considered at 5% level ($P < 0.05$).

RESULTS

The results of water parameters showed that there was a significant ($P < 0.05$) increase in the values of total dissolved solids (TDS), biochemical oxygen demand (BOD), with

TABLE 1
Mean water quality parameters during exposure of *Heterobranchus longifilis* to zinc oxide nano-particles for 45 days

CONC. (mg/l)	TDS (mg/l) Chronic (60 d)	DO (mg/L)	BOD (mg/L)	COD (mg/L)	Conductivity (μ S/cm)	Turbidity	TEMP (°C)	pH
0	1.20 ± 0.58 ^a	8.08 ± 1.13 ^d	4.68 ± 0.58 ^a	30.28 ± 1.13 ^a	58.21 ± 1.22 ^a	1.44 ± 1.06 ^a	25.0 ± 0.58 ^a	7.0 ± 1.15 ^a
6	4.11 ± 0.25 ^a	5.32 ± 0.41 ^c	6.22 ± 1.02 ^b	32.29 ± 1.15 ^b	60.17 ± 1.14 ^b	3.21 ± 0.08 ^b	25.6 ± 0.34 ^a	7.0 ± 0.58 ^a
8	5.20 ± 1.10 ^a	4.54 ± 0.23 ^b	8.43 ± 1.15 ^c	36.24 ± 1.12 ^c	63.35 ± 0.62 ^c	3.49 ± 1.04 ^b	25.9 ± 2.11 ^a	6.5 ± 1.10 ^a
10	5.08 ± 0.14 ^a	3.47 ± 0.05 ^a	10.64 ± 1.23 ^d	40.17 ± 0.14 ^d	74.48 ± 1.05 ^d	5.36 ± 0.58 ^c	25.9 ± 1.01 ^a	6.5 ± 0.61 ^a
12	5.90 ± 0.45 ^a	3.26 ± 0.13 ^a	10.88 ± 1.51 ^d	45.58 ± 0.21 ^e	91.20 ± 1.12 ^e	7.19 ± 1.12 ^d	26.1 ± 0.23 ^a	6.2 ± 0.12 ^a
FEPA	-	5-6	4.00	30.0	-	-	20-33	6.5 – 9.0
WHO	3.00	> 2.00	>6.00	-	-	4.00	-	6.5 – 8.5
NESREA	5.00	4.00	6.00	30.0	53.9	-	-	6.1 – 9.0

Mean (± S.E, n=3) with the same superscript in the same column are not significantly different (P > 0.05)

TDS = Total dissolved solids; DO = Dissolved; Temp. = Temperature; BOD = Biochemical oxygen demand; COD = Chemical oxygen demand.

chemical oxygen demand (COD), and conductivity and turbidity in ZnO- NPs exposed fish, while the value of dissolved oxygen (DO) reduced significantly ($P < 0.05$), with insignificant ($P > 0.05$) difference in the values of temperature and pH compared to control (Table 1).

The activities of investigated enzymes: alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), acetylcholine esterase (AChE), and the levels of total protein and glucose in the blood, gill and liver of *Heterobranchus longifilis* exposed to varying concentrations of zinc oxide nano-particles for 96 h are presented in Figure 1. When the results of fish exposed to zinc nano-particles were compared to the control group, there was significant ($P < 0.05$) increase in the activities of ALT in the gills and liver of the ZnO-NPs-exposed fish, with no significant ($P > 0.05$) difference in the blood. The activities of AST also showed a significant increase in the gills and liver with no significant ($P > 0.05$) difference in the blood. Similar trend was observed in AChE activities with increase ($P < 0.05$) in the gills and liver as the concentration of ZnO-NPs increased, except in the blood where it showed an insignificant difference ($P > 0.05$).

The increase observed in the enzyme activities was concentration dependent with the highest values recorded in fish treated with the highest nano-particle concentration (12.00 mg/l). The levels of total protein in the blood, gills and liver were insignificant ($P > 0.05$) among concentrations with exception of the highest concentration in the liver showing a significant ($P < 0.05$) increase. However, the level of glucose in the blood of ZnO-NPs-exposed fish increased as the concentrations of ZnO-NPs increased.

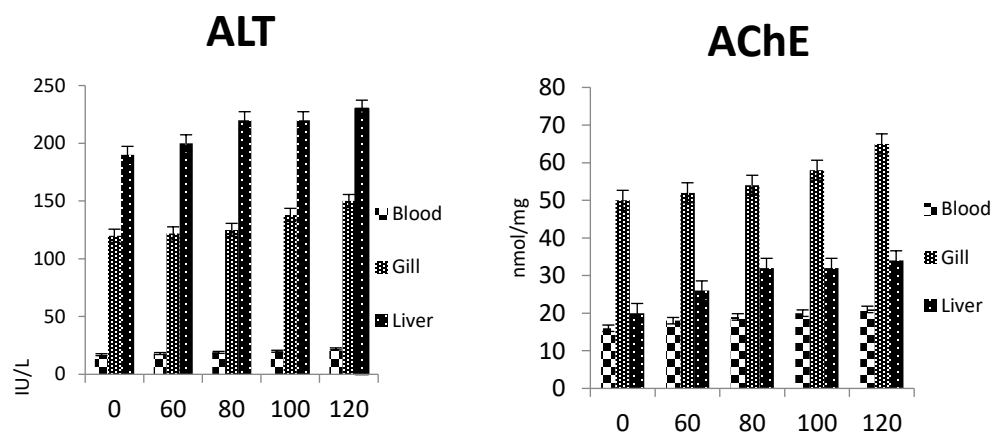


Fig. 1a: Alanine amino transferase (ALT)

Fig. 1b: Acetylcholine esterase (ACHE)

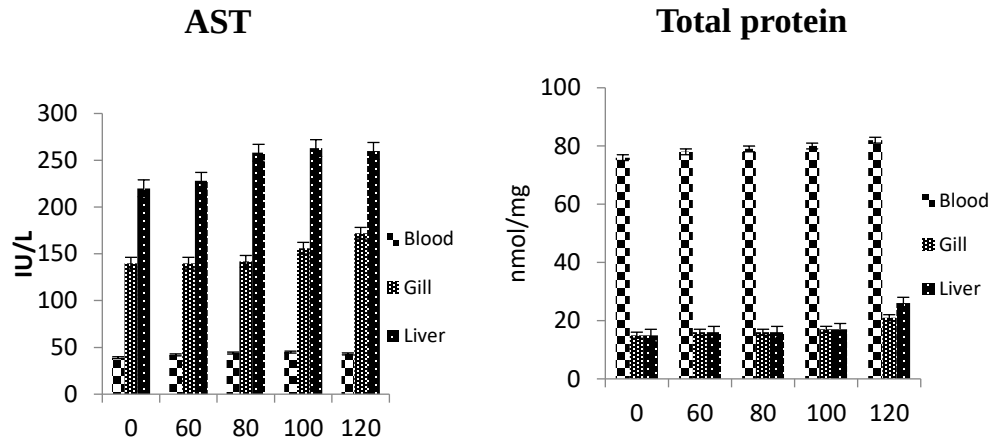


Fig.1c: Aspartate amino transferase (AST)

Fig. 1d: Total Protein

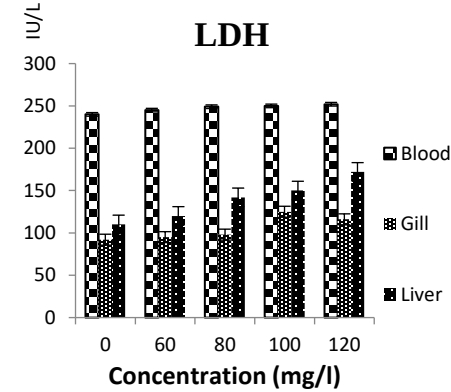


Fig. 1e: Lactate dehydrogenase (LDH)

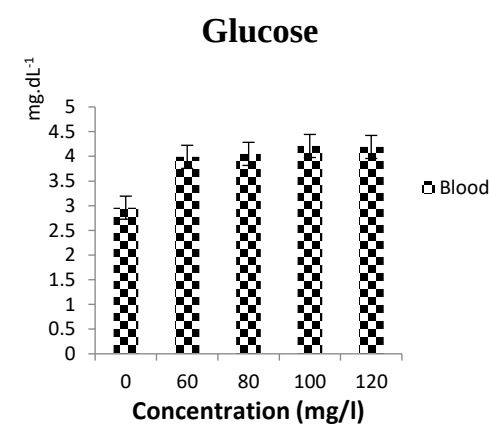


Fig. 1f: Glucose levels

Fig.1a-f: Alanine amino transferase (ALT), Aspartate amino transferase (AST) and Lactate dehydrogenase (LDH), Acetylcholine esterase (ACHE), Total Protein and Glucose levels in the tissues of *Heterobranchus longifilis* exposed to varying concentrations of zinc oxide nano-particles for 96 h. Values are means $\pm$ SE (n=3). *Significantly different from the control ($P < 0.05$).

The activities of ALT and AST in the blood, gills and liver of *H. longifilis* after exposure to varying concentrations of ZnO-NPs for 45 days are illustrated in Figure 2. At the end of 15 days of exposure, a significant ($P < 0.05$) increase in the activities of ALT was examined in the blood and gills, with a slight increase in the liver compared to control. After 30 days of exposure, activities of ALT also increased greatly in the blood and gills as the concentration and exposure period increased compared to control.

The value of ALT was significantly higher at the highest concentration and 45 days of exposure. Activities of ALT in the blood and gills therefore showed both concentration and time dependent increase, while concentration dependent increase alone was demonstrated in the liver of exposed group compared to control.

Activities of AST in the blood illustrated an insignificant ($P > 0.05$) increase between 30th and 45th day of exposure, but significantly ($P < 0.05$) higher than the 15th day. The value of AST in those exposed to the highest concentration (12.00 mg/l) recorded a significant ($P < 0.05$) increase compared to control. Activities of AST in the gills of ZnO-NPs exposed fish showed significant increase ($P < 0.05$) as the concentrations and exposure group increased compared with the control group. The enzyme activities of AST in the liver of the fish exposed to ZnO-NPs was also found to increased significantly ($P < 0.05$) as the ZnO-NPs concentrations and exposure period increased.

The activities of LDH and AChE in the blood, gills and liver of *H. longifilis* exposed to ZnO-NPs for the period of 45 days are shown in Figure 3. After 15 days of exposure, there was significant ($P < 0.05$) increase in the activities of LDH in the blood, gills and liver as the concentration increased, but revealed both concentration and time dependent increase after 30 and 45 days of exposure to ZnO-NPs compared to the control group. However, activities of LDH showed insignificant ($P > 0.05$) increase in the gills of *H. longifilis* exposed to ZnO-NPs, while a significant ($P < 0.05$) increase in the liver was demonstrated. The values of AChE in both the blood and liver increased significantly ($P < 0.05$) as the concentrations and exposure period increased from 15 to 45 days of exposure compared to control, except in the gills where an insignificant increase was recorded.

The activities of total protein in the blood, gill and liver of *H. longifilis* after exposure to ZnO-NPs for 45 days are shown in Figure 4. There was a significant ($P < 0.05$) increase in the values of total protein in the gill and liver of fish exposed to ZnO-NPs as the exposure period increased from 15 to 45 days compared to control. The total protein level increased as the concentration and the exposure period increased, except in the blood of the exposed fish which showed an insignificant ($P > 0.05$) difference after exposure for 15 and 30 days to ZnO-NPs compared to the control group. However, a significant ($P < 0.05$) increase was observed in the blood of exposed fish after 45 days of exposure to ZnO-NPs compared to control. The activities of glucose in the blood of *H. longifilis* exposed to ZnO-NPs 45 days are shown in Figure 4. The level of glucose in the blood of *H. longifilis* exposed to ZnO-NPs was significantly ($P < 0.05$) higher than those in the control group. However, there was insignificant ($P > 0.05$) difference in the level of glucose between 15th and 30th day of exposure to ZnO-NPs. The glucose level increased significantly ($P < 0.05$) after 45th day of exposure compared to control and it was concentration and time dependent.

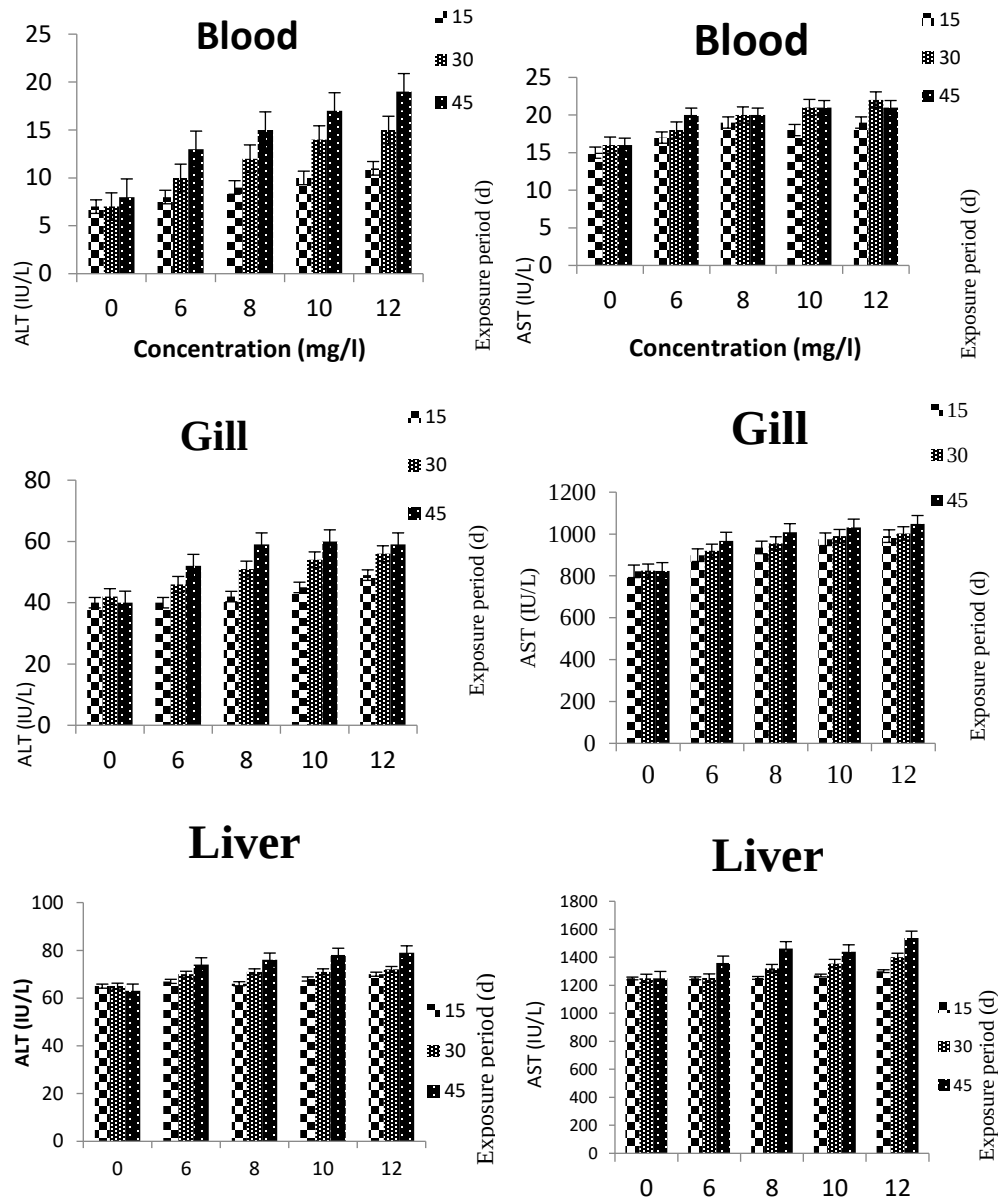


Fig. 2: Activities of alanine amino-transferase (ALT) and aspartate amino-transferase (AST) in blood, gill and liver of *Heterobranchus longifilis* exposed to varying concentrations of zinc oxide nano-particles for 45 days. Values are means $\pm$ SE (n=3). *Significantly different from the control ($P < 0.05$).

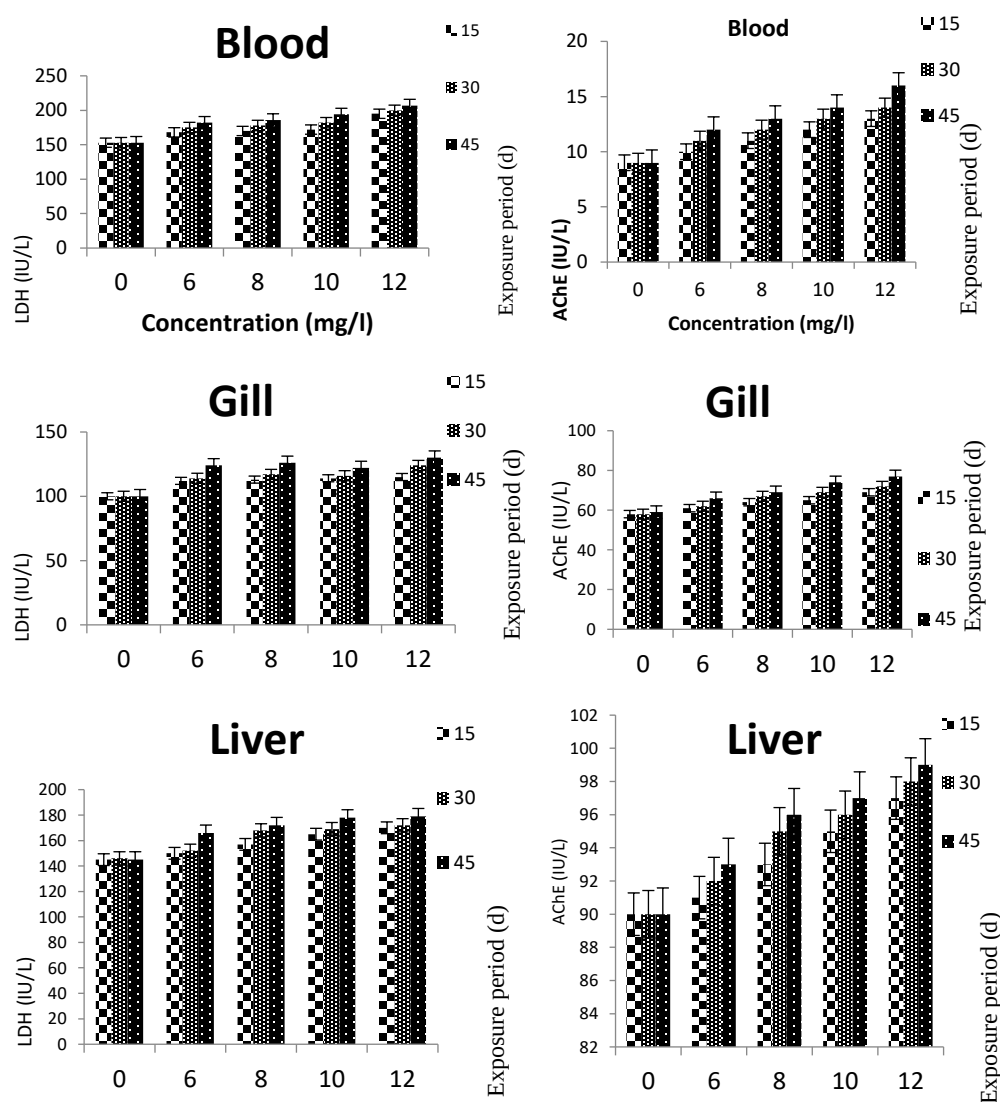


Fig. 3: Activities of lactate dehydrogenase (LDH) and acetylcholine esterase (AChE) in blood, gill, and liver of *Heterobranchus longifilis* exposed to varying concentrations of zinc oxide nano-particles for 45 days. Values are means $\pm$ SE (n=3). *Significantly different from the control ($P < 0.05$).

The activities of total protein in the blood, gill and liver of *Heterobranchus longifilis* after exposure to ZnO-NPs for 45 days are shown in Figure 4. There was a significant ($P < 0.05$) increase in the values of total protein in the gill and liver of fish exposed to ZnO-NPs as the exposure period increased from 15 to 45 days compared to

control. The total protein level increased as the concentration and the exposure period increased, except in the blood of the exposed fish which showed an insignificant ($P > 0.05$) difference after exposure for 15 and 30 days to ZnO-NPs compared to the control group. However, a significant ($P < 0.05$) increase was observed in the blood of exposed fish after 45 days of exposure to ZnO-NPs compared to control.

The activities of glucose in the blood of *Heterobranchus longifilis* exposed to ZnO-NPs for 45 days are shown in Figure 4. The level of glucose in the blood of *Heterobranchus longifilis* exposed to ZnO-NPs was significantly ($P < 0.05$) higher than those in the control group. However, there was insignificant ($P > 0.05$) difference in the level of glucose between 15th and 30th day of exposure to ZnO-NPs the glucose level increased significantly ($P < 0.05$) after 45th day of exposure compared to control and it was concentration and time dependent.

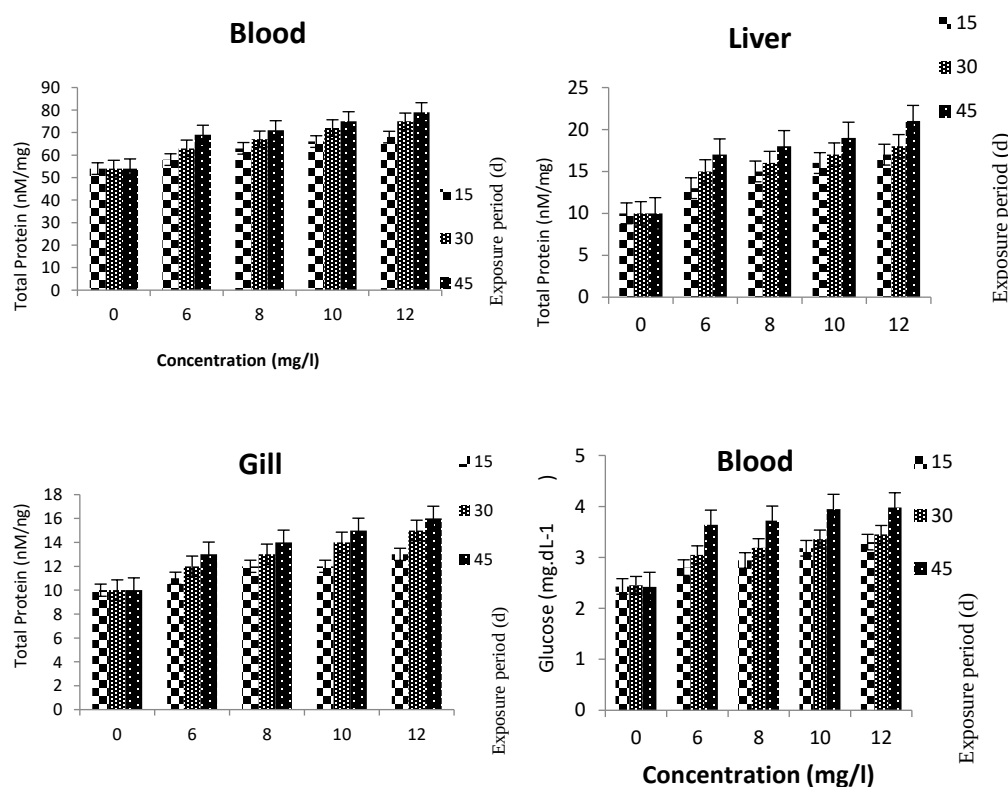


Fig. 4: Total protein in the in blood, gill, liver and glucose level in the blood of *Heterobranchus longifilis* exposed to varying concentrations of zinc oxide nano-particles for 45 days. Values are means $\pm$ SE ($n=3$). *Significantly different from the control ($P < 0.05$).

DISCUSSION

The biochemical oxygen demand (BOD) revealed the concentration of organic matter. Addition of ZnO-NPs to the test water could lead to rise in BOD due to the accumulation of zinc which block dissolution of oxygen. Increased BOD and COD concentrations as the concentration of ZnO-NPs increased could lead to blockage of dissolution of oxygen which may lead to an increase in oxygen demand. The recorded BOD and COD in the exposed groups were above the WHO (2004) limits, which probably led to the low rate of growth in the fish exposed to ZnO-NPs. The levels of TDS, turbidity and electric conductivity were highly above the recommended limits for fish habitat. This could prevent the fish from extracting the dissolved oxygen from the environment, thereby inducing oxidative stress. Dissolved oxygen (DO) measures the concentration of free molecular oxygen dissolved in water. This served as one of the important elements for water quality. Recorded DO during the exposure period is below the maximum limit of 5.0 mg/l of DO (WHO, 2004). This could be due to organic waste and the utilization of oxygen by the fish in the aquatic environment. However, during the chronic exposure, the DO level was still tolerable because it was still above 2mg/l. An increase in the concentration of BOD and COD could probably be as a result of decrease in the concentration level of DO which resulted from high metabolic activity and unconsumed feed due to toxicity induced by ZnO-NPs. The value of BOD and COD of the water in the control group is within the NESREA (2011) limits of 4 mg/l and 30 mg/l respectively. The values of temperature recorded during the study is within the recommended limits by NESREA (2011) for fish. The values of the mean hydrogen ion concentration (pH) obtained during the experiment revealed that pH in the exposure period was within the NESREA (2011) recommended limits of 6.5-8.5.

The results of the present study provided evidences to show that ZnO-NPs elicited toxicity by affecting biochemical enzymes parameters, causing damage to the tissues and leakage of the tissue enzymes into blood and production of free radicals which then caused oxidative stress and alteration in the activities of the biochemical enzymes. Increase in the activities of alanine amino transferase (ALT) and aspartate amino transferase (AST) in tissues is an indication of severity of liver and muscle damage. The increment in ALT and AST observed in this study was similar to the reports of Amutha and Subramanian (2009), Subramanian *et al.* (2011) and Alkaladi *et al.*, (2014) who exposed *Oreochromis mosambicus* to ZnO-NPs. Higher significant values of ALT and AST in the blood of ZnO-NPs-exposed fish could be an indication that the liver undergone chronic effect of tissues damage which may eventually lead to leakage of the liver cell thereby releasing the enzymes into the blood stream (Subramanian *et al.*, 2011). Lactate dehydrogenase catalyzes the conversion of lactate pyruvate; it is therefore important in energy formation by inducing the anaerobic oxidation when fish is in stress and need oxygen. Activities of LDH indicate stress in organism (Ishikawa *et al.*, 2007). Elevation of these enzymes in the gills, liver and blood therefore shows the level of tissue damage (Svoboda *et al.*, 2001) which could be as a

result of the release of the enzyme from the destroyed tissues (Mishra and Shukla, 2003). The elevated LDH activity in this study was in agreement with the reports of Li *et al.*, 2011.

Decrease in the value of protein could be due to production of reactive oxygen species which could directly attack protein to produce irreversible carbonyl protein, thereby resulting to decrease catalytic activity of enzyme proteases (Zhang *et al.*, 2008). This could have negative effect on the structure, function and integrity of proteins (Madhusudhanan *et al.*, 2004). Increase in the value of protein could be due to mobilization of more protein released into the blood stream which are used as an alternative source of energy demand as a result of stress induced by ZnO-NPs intoxication (Juliana *et al.*, 2008). Mobilization of glycogen may lead to increase in the blood glucose level between 15 and 45 days of exposure when the stressed fish is in need of more energy to cope with the stress situation. Similar reports were given by Seth and Sexana (2003).

CONCLUSION

The catfish, *Heterobranchus longifilis* exposed to ZnO-NPs recorded a significant increase in the activities of enzymes ALT, AST, AChE and total protein with a reduction in glucose level after 45 days of exposure. Thus, it is concluded that ZnO-NPs may be toxic to this fish and the toxicity is both concentration and exposure period dependent.

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