

THE ROLE OF ASCORBIC ACID AS AN ANTIOXIDANT AGAINST GENOTOXICITY OF ZINC OXIDE NANO-PARTICLES ON TISSUES OF *HETEROBRANCHUS LONGIFILIS*

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Received February 19, 2025; Revised April 01, 2025; Accepted April 03, 2025

ABSTRACT

*The indiscriminate discharge of effluents from Zinc oxide nano-particles (ZnO-NPs) industries into water bodies can cause genetic disorders in fish. The genotoxicity of ZnO-NPs was investigated on Juveniles of Heterobranchus longifilis through exposure to sub-lethal concentrations (6.00, 8.00, 10.00, 12.00 mg/L) of ZnO-NPs for 60 days, and fish were fed on ascorbic acid (50.00, 250.00, 500.00, 1000.00 mg AA/kg diet) for 30 days. After each experiment, the micronuclei (MN) abnormalities and deoxyribonucleic acid (DNA) quality were evaluated. The commonest abnormalities recorded are micronucleated, binucleated, multinucleated, altered, echinocyte cells, kidney-shaped and deformed nuclei. However, groups ameliorated with a 500 mg AA/kg diet recorded little or no abnormalities. A dose and time-dependent increase in MN induction and frequency occurred compared to control but decreased ($p < 0.05$) as the concentration of AA increased. There were highest MN induction and frequencies in the liver (38.23%) than in gills (26.32%) and blood (24.61%) of ZnO-NPs-exposed fish, with the lowest MN induction and frequency in the liver (10.21%), gills (1.76%) and blood (1.61%) of 500 mg AA/kg diet-group. DNA quality reduced with increasing concentrations and exposure periods but improved in the 500 mg AA/kg diet group. This indicated that ZnO-NPs are toxic, but 500 mg AA/kg-diet was able to minimize MN induction and DNA damage in *H. longifilis*.*

Keywords: Amelioration, Ascorbic acid, DNA, Micronucleus, Zinc oxide nano-particles, *Heterobranchus longifilis*

INTRODUCTION

Micronuclei (MN) are chromatin masses appearing as small nuclei outside the nucleus, which could result from the breakage of chromosomes. MN of chromosome fragments or dysfunction of the mitotic spindle apparatus leads to entire chromosomes lagging in the anaphase stage and failing to become incorporated into daughter cell nuclei during cell division (Okonkwo *et al.*, 2011; Luzhna *et al.*, 2013). Genetic damage such as fragmentation of chromosomes or changes in their number may lead to alteration of gene number and their orders (Okonkwo *et al.*, 2011;

Luzhna *et al.*, 2013) and consequently may cause disturbance in gene expressions and overall activities of cells and daughter cells (Harabawy and Mosley, 2014). Formation of nuclear abnormalities may, therefore, represent a process by which the cell eliminates any amplified genetic material from the nucleus (Shimizu *et al.*, 1998). The binuclei and blebbed-nucleated cells exhibit a similar origin to MN and are considered genotoxic MN analogues (Serrano-García and Montero-Montoya, 2001). MN is reported to be a sensitive biomarker for the detection of genotoxic damage induced by various pollutants (Moraes *et al.*, 2004). MN frequency also allows the detection

of pollutant concentration gradients. MN formations are damages that are irreversible and continue to manifest in future generations through heredity. This may tend to reduce the population of the affected species, and the abnormalities may be biomagnified along the food chain, thereby affecting humans in the higher trophic levels (Moraes *et al.*, 2004). Several nuclear abnormalities and micronucleus (MN) induction in different fish species exposed to metal oxides have been reported by several researchers. Çavaş *et al.* (2005) and Kumar *et al.* (2012) reported elevated frequency in *Oreochromis niloticus* exposed to copper, cadmium and chromium. Bagdonas and Vosyliene (2006) recorded a significant increase in MN of rainbow trout exposed to varying concentrations of zinc sulphate and copper. Obiakor *et al.* (2010) also reported MN induction in *Synodontis clarias* exposed to a binary mixture of copper and zinc. Amongst different investigations reviewed on the effects of metal oxides on fish, data on the genotoxicity of their nanoparticles on fish are less available, most especially on *Heterobranchius longifilis* Valenciennes, 1840 (Siluriformes: Clariidae). Because the lack of information on *H. longifilis*, makes it difficult to know the potential ecotoxicity of ZnO-NPs on this fish, investigation of its effect on the fish will enable us to know the biology, survival and conservation strategy of the fish and also the potential risk ZnO-NPs may cause to *H. longifilis* and preventive measures that will help to neutralize the toxic effect of ZnO-NPs which may enable us to conserve and prevent the genetic composition of the fish. The genotoxicological implication of ZnO-NPs on *H. longifilis* and ameliorative potentials of ascorbic acid (AA) were therefore investigated through the evaluation of MN induction and frequency in erythrocytes of the blood, gills and liver and the determination of DNA quality of *H. longifilis* exposed to ZnO-NPs and ameliorated with ascorbic acid.

MATERIALS AND METHODS

Ethics: The study was approved (UERC/ASN/2016/648) by the Animal Care and Use Committee of the University of Ilorin, Ilorin, Nigeria. The methods of all experiments were carried out

following the guidelines for the care and use of fish in research (DeTolla *et al.*, 1995).

Experimental Setup: Five hundred (500) juveniles of *H. longifilis* (average weight 19.40 ± 3.65 g and average length 12.45 ± 0.13 cm) were obtained from a commercial fish hatchery in Lagos, Nigeria and transported in well-aerated plastic aquaria containing water from the hatchery to the laboratory in the Department of Zoology, University of Ilorin. The fish were not fed throughout the day due to stress, which may prevent easy digestion and cause mortality. Feeding commenced the following day and was fed twice daily at 9.00 am and 5.00 pm with commercial feeds (Coppens floating fish feed, 2 mm size, 40% crude protein and 3000 kcal/kg) at 4% of initial body weight (Ekanem *et al.*, 2012). The hatchery water was replaced by chlorine-free bore-hole water, and the fish were kept in a large tank of 1000-litre capacity at 24° C and acclimatized to laboratory conditions for 14 days before experiments. The water was kept oxygen-saturated with aerators. Unconsumed feed and faecal wastes were removed and renewed every 24 hours to maintain the toxicant's concentration (Brouwer and Heibloem, 1986) and to reduce the pollution risk of disease outbreak and mortality. Feeding was stopped 24 hours before the commencement of the experiment (USEPA, 2016).

During the tests, water quality parameters were continuously monitored for temperature, pH, dissolved oxygen (DO), biochemical oxygen demand (BOD), chemical oxygen demand (COD), and electrical conductivity (EC) according to the procedure of APHA (1999).

Commercial ZnO-NPs with an average particle size of about 100 nm were purchased from Sigma Aldrich, USA. A stock solution of ZnO-NPs was prepared by dispersing zinc oxide powder into distilled water, and the suspending solution of ZnO-NPs was stirred using a magnetic stirrer for about two hours to break aggregates or precipitates. Each aquarium containing the experimental fish was renewed with freshly prepared ZnO-NPs every 24 hours.

Chronic Toxicity: Based on the result of the acute tests (Abdulkareem and Owolabi, 2019),

the test fishes were exposed to varying concentrations (6.0, 8.0, 10.0 and 12.0 mg/L) measured from the stock solution of ZnO-NPs for 60 days. 450 fish were divided into five groups of 30 fish each. One group served as the control without toxicant, while four groups were exposed to four measured concentrations (6.0, 8.0, 10.0 and 12.0 mg/L) of ZnO-NPs. Positive control groups were injected with colchicine. The experiment was also set up in triplicate (Pathiratne and Athauda, 1998). The fish were fed twice a day at 4% of their body weight, according to Ekanem *et al.* (2012). The aquaria containing the fish were cleaned, and the toxicant was renewed every day to keep the toxicant concentration constant (Brouwer and Heibloem, 1986). At the end of every fifteen days of the experimental period, blood was collected from two fish per replicate from each of the experimental and control groups and dissected to remove the gills and liver for MN and DNA quality assay.

Ameliorative Experiment: To evaluate the ameliorative effect of ascorbic acid (Vitamin C) on the catfish exposed to ZnO-NPs for 30 days, 320 catfish with an average weight of 19.40 ± 3.65 g were distributed randomly into eight groups in 32 plastic aquaria (50 litres capacity) of 10 fish each, containing 10 litres of chlorine-free bore-hole water. Among the eight groups, three of the groups (A, B and C) were the control without ZnO-NPs. Group A was fed on basal feed, while Group B was fed on 50 mg of ascorbic acid/kg (lowest concentration) and Group C with 1000 mg of ascorbic acid/kg (highest concentration). The remaining five groups (D, E, F, G and H) were exposed to 1/10 of the LC_{50} of ZnO-NPs (Narra *et al.*, 2012) for 30 days. Group D was fed on a basal diet without ascorbic acids, and groups E, F, G, and H were fed on diets formulated with different concentrations of ascorbic acids (50, 250, 500 and 1000 mg AA/kg diet), respectively. The fish were fed twice daily (8.00 am and 5.00 pm) for 30 days. The test solutions (water containing 10 mg/L of ZnO- NPs of groups D, E, F, G and H were renewed every 24 hours (Vutukuru *et al.*, 2007) with freshly prepared ZnO-NPs to maintain the ZnO-NPs concentration. Two catfish from each

replicate per group were randomly sacrificed for MN and DNA quality assay.

Feed Formulation: Basal diet with 0 mg of ascorbic acids 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. Furthermore, the basal diets were supplemented with graded levels (50, 250, 500 and 1000 mg) of ascorbic acids to constitute the experimental diets (Table 1). These ingredients were ground, milled, weighed, mixed and pelleted with a meat mincer through a 3 mm die. The pelleted ingredients were air-dried and kept in an air-tight container. Both the dietary ingredients and the formulated diets were assayed for their proximate compositions (AOAC, 2005).

Micronucleus and DNA Quality Assay: Blood samples were collected with a syringe through the caudal fin. One drop of blood was used to make smears. Three slides of tissues were prepared from each fish by smearing blood, gill, and liver homogenates on each slide for the MN assay. The slides were air-dried overnight at room temperature and then fixed in absolute methanol for 20 minutes, after which they were allowed to dry overnight. Fixed smears were then stained in 10% May-Grunwald and 5% Giemsa stains. Stained slides were rinsed with distilled water to remove excess stain and allowed to dry. The blood smears were examined with a light microscope using oil immersion at x100 magnification. The number of MN in the erythrocytes, gills and liver homogenate were expressed per 1000 erythrocytes for each fish. Chromatin bodies with small refractive, circular or ovoid shapes with the same staining and focusing pattern as the main nucleus were scored as MN (Al-Sabti and Metcalfe, 1995). The MN frequency (%) was calculated as: $MN (\%) = \frac{\text{Number of cells containing MN} \times 100}{\text{Total number of cells scored}}$.

Blood was also collected into EDTA bottles, while gills and liver were kept in different sample bottles containing sucrose solution for DNA damage analysis.

Table 1: Basal diet and its variations with graded levels of ascorbic acid supplementation fed to *Heterobranchus longifilis* exposed to zinc oxide nano-particles

Ingredient (%)	Basal diet (0 mg ascorbic acid)	50 mg ascorbic acid	250 mg ascorbic acid	500 mg ascorbic acid	1000 mg ascorbic acid
Maize corn	30.0	30.0	30.0	30.0	30.0
Soya bean	20.0	20.0	20.0	20.0	20.0
Groundnut cake	18.0	18.0	18.0	18.0	18.0
Fish meal	30.0	30.0	30.0	30.0	30.0
Bone meal	0.4	0.4	0.4	0.4	0.4
Vitamin C (mg)	0.2	50.2	250.2	500.2	1000.2
Methionine	0.4	0.4	0.4	0.4	0.4
Lysine	0.4	0.4	0.4	0.4	0.4
Salt	0.2	0.2	0.2	0.2	0.2
Premix	0.2	0.2	0.2	0.2	0.2
Total (%)	100.0	100.0	100.0	100.0	100.0
Calculated composition					
Dry matter	90	90	90	90	90
Moisture content	10	10	10	10	10
Crude protein	40	40	40	40	40
Crude fibre	5	5	5	5	5
Ether extract	6.5	6.5	6.5	6.5	6.5
Ash	10.5	10.5	10.5	10.5	10.5
Carbohydrate	20	20	20	20	20
Nitrogen-free extract (NFE)	28	28	28	28	28
Metabolizable energy	68.9	68.9	68.9	68.9	68.9

DNA Extraction using Gill Homogenate: DNA extraction was performed using the Zymo Research Quick-DNA Miniprep Kit, purchased from Inqaba Biotech, South Africa. 200 μ L of gill homogenate was measured using a micropipette. Simultaneously, 260 μ L of Proteinase K Storage Buffer was added to 5 mg of Proteinase K (final concentration: 20 mg/mL) and mixed thoroughly by vortexing. To each 200 μ L sample, 95 μ L of distilled water and 95 μ L of 2X Digestion Buffer were added, followed by 10 μ L of Proteinase K. The mixture was incubated at 55°C for 1 – 3 hours to facilitate digestion (Green and Sambrook, 2012).

Following digestion, 700 μ L of Genomic Lysis Buffer was added to each tube and mixed thoroughly by vortexing. The samples were centrifuged at 10,000 rpm for 1 minute to remove insoluble debris. The supernatant was carefully transferred to a Zymo-Spin IIC column placed in a collection tube, followed by centrifugation at 10,000 rpm for 1 minute.

Next, 200 μ L of DNA Pre-Wash Buffer was added to the spin column in a fresh collection tube and centrifuged at 10,000 rpm for 1 minute. This was followed by the addition of 400 μ L of DNA Wash Buffer, with centrifugation at 10,000 rpm for 1 minute (Zymo Research, 2021). The

spin column was then transferred to a clean microcentrifuge tube, and ≥ 50 μ L of DNA Elution Buffer or distilled water was added. The sample was incubated at room temperature for 2 – 5 minutes and centrifuged at maximum speed for 30 seconds to elute the DNA. The eluted DNA was stored at $\leq -20^{\circ}\text{C}$, and DNA quality was assessed using a spectrophotometer.

DNA Extraction (Blood and Liver Homogenate

Method): Cells were pelleted in Eppendorf tubes by centrifugation at 13,200 rpm for 30 seconds. Subsequently, 200 μ L of Lysis Buffer was added to the cells, followed by incubation in a 37°C water bath for 2 hours (Green and Sambrook, 2019). Next, 100 μ L of 5M potassium acetate was added, mixed by inversion several times, and centrifuged at 13,200 rpm for 10 minutes. The supernatant from each sample was carefully transferred to new Eppendorf tubes.

To precipitate DNA, 200 μ L of isopropanol was added to the supernatant, gently inverted, and centrifuged at 13,200 rpm for 2 minutes. After centrifugation, the supernatant was removed, leaving behind small pellets. 300 μ L of distilled water was then added to each pellet, followed by gentle tapping to aid dissolution. Subsequently, 30 μ L of 3M sodium

acetate was added and mixed thoroughly by inversion (Ausubel, 2002). 300 μ L of isopropanol was added, and the samples were centrifuged again at 13,200 rpm for 2 minutes. The supernatant was carefully removed, leaving only the pellets behind (Green and Sambrook, 2012).

For further purification, 300 μ L of 70% ethanol was added to each sample and centrifuged at 13,200 rpm for 2 minutes. The supernatant was discarded, and the Eppendorf tubes were inverted onto sterile tissue paper to drain any remaining liquid (Sambrook and Russell, 2001). Finally, the pellet was resuspended in 50 μ L of TE Buffer (distilled water) and stored at -20°C until further use for DNA quantification.

Spectrophotometric Analysis of DNA: After the extraction of DNA from the tissues of both the control and the ZnO-NPs-exposed groups, the DNA quantification was estimated by ultraviolet spectrophotometric measurement of the absorption at absorbance 260 and absorbance 280 nm on a sample of the DNA in solution (Ogunkanmi *et al.*, 2008). The fraction of light passing through the solution was measured. The samples were held in quartz cuvettes, this is because they do not absorb in the UV range the way most glasses and plastics do. TE (Tris-EDTA) buffer was used to zero the instrument, and the DNA samples themselves were diluted in TE buffer (10%) such that the absorbance 260 readings obtained were above 0.02 but below 0.8 (Beer's Law). The absorption at a 260/280 ratio of 1.8 suggests pure DNA, while a 260/280 ratio of >1.8 suggests RNA contamination, and a 260/280 ratio of <1.8 suggests protein and/or phenol contamination (Johnson, 1981).

Data Analysis: The data obtained from the experiment was subjected to a one-way analysis of variance (ANOVA) followed by significant mean separation using Duncan's Multiple Range Test (DMRT) (Duncan, 1955). All data were analysed using IBM Version 20.0 and Microsoft Excel 2013. The test of significance was at 95% ($p < 0.05$). The results of data analysis were expressed as mean $\pm$ SE.

RESULTS AND DISCUSSION

Water Quality Parameters: Throughout the study, key water quality parameters, including temperature, pH, dissolved oxygen (DO), biochemical oxygen demand (BOD), chemical oxygen demand (COD), and electrical conductivity (EC), were monitored. The temperature remained stable at 24°C, ensuring optimal metabolic function in *H. longifilis*. The pH was maintained between 7.2 – 7.4, within the suitable range for freshwater fish. The DO levels remained above 5 mg/L, supporting adequate respiration, while BOD and COD levels were controlled to minimize organic load and prevent hypoxia. These parameters ensured a stable aquatic environment, reducing potential confounding stressors on the fish.

Water quality plays a critical role in genotoxicity studies as pollutants and poor water conditions can contribute to oxidative stress and DNA damage. The study demonstrated a dose- and time-dependent increase in MN frequency in the blood, liver, and gills of *H. longifilis* exposed to ZnO nanoparticles (ZnO-NPs). The highest MN frequency (38.23%) was observed in the liver, followed by the gills (26.32%) and blood (24.61%). The liver's higher susceptibility may be due to its role in detoxification and bioaccumulation of pollutants. The increased MN frequency in ZnO-NP-exposed fish suggests that ZnO-NPs cause chromosomal aberrations due to oxidative stress-induced DNA damage. Elevated reactive oxygen species (ROS) production can lead to lipid peroxidation, affecting cell integrity and nuclear stability (Ahmad *et al.*, 2006).

Dietary Parameters: Diet significantly influenced MN formation and DNA integrity. Fish supplemented with ascorbic acid (AA) exhibited a dose-dependent reduction in MN frequency. The 500 mg AA/kg diet showed the most significant protective effect, reducing MN frequency to 10.21% (liver), 1.76% (gills), and 1.61% (blood). This suggests that ascorbic acid acts as an antioxidant, neutralizing ROS and mitigating ZnO-NP-induced genotoxicity (Hao and Chen, 2012). The highest AA dose (1000 mg/kg) led to slightly increased MN frequency, possibly due to pro-oxidant effects at excessive concentrations.

These findings highlight the protective role of dietary antioxidants against nanoparticle-induced genotoxicity in aquatic organisms (Alkaladi *et al.*, 2015).

Micronucleus: Normal erythrocytes were revealed in the blood of *H. longifilis* in the control group, while different abnormalities, such as MN, binucleated cells, echinocytes, altered cells, kidney-shaped nuclei, and so on, were evident in the exposed group. The commonest abnormalities recorded in almost all concentrations are micronucleated cells. However, the erythrocytes in the fish exposed to ZnO-NPs and ameliorated with varying concentrations of ascorbic acid recorded mild abnormalities, such as micronucleated cells.

The abnormalities of erythrocytes may be due to the liberation of the reaction of reactive oxygen species (ROS), which causes lipid peroxidation for unsaturated fatty acids in the cell membrane and subsequently damages the cell membrane (Ahmad *et al.*, 2006). Parveen and Shadab (2012) reported that fish that inhabit polluted water or are exposed to toxicants recorded nuclear abnormalities. The binuclei and blebbed-nucleated cells exhibit a similar origin as MN and are considered genotoxic analogues of MN (Serrano-García and Montero-Montoya, 2001). The formation of binuclear cells is an indicator of abnormal cell division due to the blocking of cytokinesis (Mahboob *et al.*, 2014). Crott and Fenech (2001) attributed the occurrence of blebbed nuclei as a precursor of MN. The occurrence of kidney-shaped nuclei may represent precursors of MN or binuclei phenomena (Harabawy and Mosley, 2014).

The occurrence of MN in a cell is an indication of structural or numerical chromosomal aberrations during mitotic division (Fenech, 2000). Çavaş *et al.* (2005) reported MN in erythrocytes as a sensitive biomarker of genotoxicity in fish because of its easy induction even on short-term exposure of 24 – 96 hours. Many types of nuclear aberrations, such as blebbed, lobbed and notched, were reported by Carrasco *et al.* (1990), Ferraro *et al.* (2004), and Anbumani and Mohankumar (2012).

These abnormal nuclear morphologies in different fish species were used as signals of cytogenetic damage, which were reported to probably be due to failure in cell division, genotoxicity, mutagenicity or cell death (Bombail *et al.*, 2001; Pacheco and Santos, 2002). The nuclear abnormalities reported in this study may be attributed to a failure in chromosome segregation. The blockage of cytokinesis could also lead to abnormal cell division, which may eventually cause binucleated cells, thereby leading to genetic imbalance and carcinogenesis in cells (Potapova and Gorbysky, 2017). Nuclear lesions can also result from the amplification of genes through the breakage-fusion-bridge cycle (Shimizu *et al.*, 1998; Lo *et al.*, 2002). From the result of this study, ZnO-NP-exposed fish recorded higher MN than the control groups, except the positive control groups (injected with colchicine), and the frequency of MN increased as the concentration of ZnO-NPs and exposure period increased. This, therefore, indicated that MN and nuclear lesion assays are sensitive tools for evaluating the mutagenic and genotoxic effect of ZnO-NPs.

Micronucleus Frequency: A time-dependent gradual increase in the MN frequency was observed in the blood, gills and liver as the exposure periods increased to 60 (Figures 1 – 3). The induction in the MN also increased as the concentration increased compared to the values and frequency of MN in that of the control (Table 2). A significant ($p < 0.05$) higher MN induction and frequency were observed in the erythrocytes of fish exposed to higher concentrations of ZnO-NPs and maximum frequency of MN in fish exposed for 60 days (Table 2). MN induction was significant ($p < 0.05$) in the erythrocytes of fish exposed to varying concentrations of ZnO-NPs compared to that of the control group (Figure 1a) with a significant ($p < 0.05$) highest MN frequency in the highest concentration (Figure 1e) and significant ($p < 0.05$) lowest MN frequency in lowest concentration (Figure 1b) after 60 d exposure to ZnO-NPs. The highest percentage frequency of MN was revealed in the liver (38.23%) compared to the blood (24.61%) and gills (26.32%).

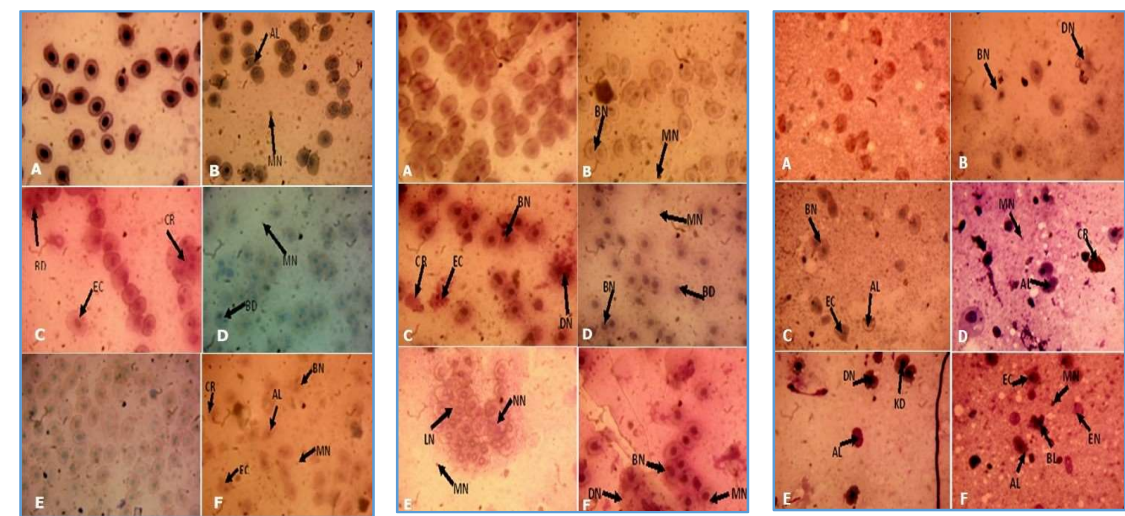


Figure 1: Micronucleus (MN) formation in the erythrocytes of *H. longifilis* after exposure to zinc oxide nano-particles for 60 days. **Key:** (a) Normal erythrocytes in the blood of *H. longifilis* in the control group without ZnO-NPs; (b) Altered (AL) and Micronucleus (MN) in the erythrocytes of blood of *H. longifilis* exposed to 6 mg/L of ZnO-NPs; (c) Bud (BD), Crenated (CR), Echinocytes (EC) and Enucleated (EN) in the erythrocytes of the blood of *H. longifilis* exposed to 8 mg/L of ZnO-NPs; (d) Bud (BD) and Micronucleus (MN) in the erythrocytes of blood of *H. longifilis* exposed to 10 mg/L of ZnO-NPs; (e) Altered (AL), Crenated (CR), Echinocytes (EC) and Micronucleus (MN) in the erythrocytes of the blood of *H. longifilis* exposed to 12 mg/L of ZnO-NPs; (f) Altered (AL), Binucleated (BN), Crenated (CR), Echinocytes (EC) and Multinucleated (MT) in the erythrocytes of blood of *H. longifilis* treated with 0.05% colchicine

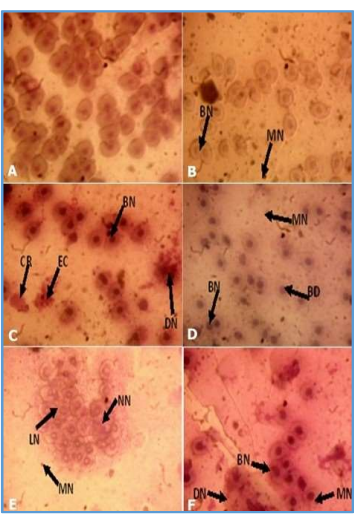


Figure 2: Micronucleus (MN) formation in the gills of *H. longifilis* after exposure to zinc oxide nano-particles for 60 days. **Key:** (a) Normal erythrocytes in the gills of *H. longifilis* in the control group without ZnO-NPs; (b) Micronucleated (MN) and Binucleated (BN) in the erythrocytes of gills of *H. longifilis* exposed to 6 mg/L of ZnO-NPs; (c) Binucleated (BN), Crenated (CR), Deformed nucleus (DN) and Echinocytes (EC) in the erythrocytes of gills of *H. longifilis* exposed to 8 mg/L of ZnO-NPs; (d) Binucleated (BN), Bud (BD) and Micronucleated (MN) in the erythrocytes of gills of *H. longifilis* exposed to 10 mg/L of ZnO-NPs; (e) Micronucleated (MN), Notched nuclei (NN) and Lobed nuclei (LN) in the erythrocytes of gills of *H. longifilis* exposed to 12 mg/L of ZnO-NPs; (f) Altered (AL), Binucleated (BN), Crenated (CR), Echinocytes (EC) and Multinucleated (MT) in the erythrocytes of blood of *H. longifilis* treated with 0.05% colchicine

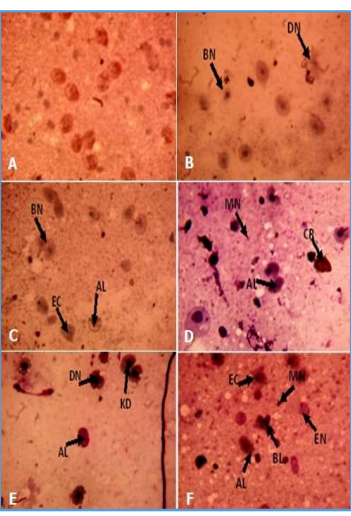


Figure 3: Micronucleus (MN) formation in the liver of *H. longifilis* after exposure to zinc oxide nano-particles for 60 days. **Key:** Normal erythrocytes in the liver of *H. longifilis* in the control group without ZnO-NPs; (b) Binucleated (BN) and Deformed nucleus (DN) in the erythrocytes of liver *H. longifilis* exposed to 6 mg/L of ZnO-NPs; (c) Binucleated (BN), Echinocytes (EC) and Altered (AL) in the erythrocytes of liver of *H. longifilis* exposed to 8 mg/L of ZnO-NPs; (d) Altered (AL), Micronucleated (MN) and Crenated (CR) in the erythrocytes of liver *H. longifilis* exposed to 10 mg/L of ZnO-NPs; (e) Altered (AL), Deformed nucleus (DN) and Kidney shaped (KD) in the erythrocytes of liver *H. longifilis* exposed to 12 mg/L of ZnO-NPs; (f) Altered (AL), Blebbed (BL), Echinocytes (EC), Enucleated (EN) and Micronucleated (MN) in the erythrocytes of liver of *H. longifilis* treated with 0.05% colchicine

Table 2: Micronucleus frequencies in erythrocytes of the blood, gills and liver cells of *Heterobranchus longifilis* exposed to zinc oxide nano-particles for 60 days

Tissue	Concentration (mg/L)					Colchicine (+ve)
	0.00 (-ve)	6.00	8.00	10.00	12.00	
Blood	5.64 ± 1.12 ^a	8.27 ± 0.53 ^b	17.53 ± 3.14 ^c	19.52 ± 2.18 ^d	24.61 ± 2.13 ^d	13.35 ± 1.12 ^e
Gill	5.93 ± 1.15 ^a	7.59 ± 1.16 ^b	16.95 ± 0.58 ^c	20.45 ± 2.15 ^d	26.32 ± 2.12 ^d	12.76 ± 2.13 ^e
Liver	12.56 ± 0.58 ^a	16.43 ± 1.25 ^b	29.18 ± 1.13 ^c	34.27 ± 1.16 ^d	38.23 ± 3.14 ^d	26.34 ± 1.15 ^e

Key: Means on the same row with different letter superscripts are significantly different ($p < 0.05$)

The MN frequency increases as the ZnO-NPs concentration and exposure period increase (Table 2). Diet supplementation with varying concentrations of ascorbic acid in groups E, F, G and H significantly ($p < 0.05$) led to a decrease in the frequencies of MN in these cells compared to

those recorded in Group D that was exposed to ZnO-NPs only (Table 3). However, the frequency level of MN decreased as the concentration of ascorbic acid supplementation in the diet increased, but MN frequencies increased in the highest concentration of ascorbic acid (Group H)

Table 3: Micronucleus frequencies in erythrocytes of the blood, gill and liver of *Heterobranchus longifilis* exposed to zinc oxide nano-particles and ameliorated with ascorbic acid for 30 days

Tissue	Group								Colchicine (+ve)
	A (-ve)	B	C	D	E	F	G	H	
Blood	1.25 ± 1.30 ^a	1.93 ± 1.45 ^a	3.12 ± 1.21 ^d	9.38 ± 1.28 ^f	3.13 ± 1.16 ^d	2.17 ± 0.80 ^b	1.61 ± 1.80 ^b	3.78 ± 0.62 ^{cd}	4.15 ± 2.58 ^e
Gill	1.42 ± 0.15 ^a	1.91 ± 1.12 ^a	2.87 ± 0.65 ^b	9.48 ± 1.60 ^e	5.61 ± 0.38 ^d	2.31 ± 3.17 ^b	1.76 ± 0.45 ^a	3.82 ± 1.25 ^c	5.34 ± 2.45 ^d
Liver	0.81 ± 2.00 ^a	1.83 ± 1.12 ^b	2.34 ± 0.42 ^c	23.42 ± 2.35 ⁱ	20.57 ± 1.18 ^h	15.62 ± 2.73 ^e	10.21 ± 3.08 ^d	16.24 ± 1.64 ^f	18.16 ± 1.23 ^g

Key: Normal feed (A); 50 mg ascorbic acid/kg diet (B); 1000 g ascorbic acid/kg diet (C); ZnO-NPs (10 mg) + Normal feed only (D); ZnO-NPs (10 mg) + 50 mg ascorbic acid/kg diet (E); ZnO-NPs (10 mg) + 250 mg ascorbic acid/kg diet (F); ZnO-NPs (10 mg) + 500 mg ascorbic acid/kg diet (G); ZnO-NPs (10 mg) + 1000 mg ascorbic acid/kg diet (H). Means on the same row with different letter superscripts are significantly different ($p < 0.05$)

compared to control groups (groups A, B and C). Group G, which was fed on diets with 500 mg/kg ascorbic acid, recorded the lowest MN frequencies in the liver (10.21%), gills (1.76%) and blood (1.61%) throughout the ameliorative period of 30 days (Table 3). A dosage-dependent gradual increase in the MN formation after exposure to ZnO-NPs and ameliorated with ascorbic acid (AA) for the period of 30 days was observed in the blood gills and liver of *H. longifilis* (Figures 4 – 6).

The high percentage frequency of MN recorded in the liver compared to blood and gill could be that the liver provides earlier and more sensitive detection of MN frequencies than blood and gill. The liver is, therefore, sensitive to the damage induced by ZnO-NPs compared to blood and gills. This is in contrast with the findings of Ali *et al.* (2008), who reported blood as the more sensitive tissue to MN induction.

However, *H. longifilis* treated with colchicine, which serves as a positive control, revealed a higher frequency of MN. Some fish species are less sensitive than others in MN frequency analysis (Al-Sabti and Metcalfe, 1995; Arias-Estévez *et al.*, 2008). This may be a result of interspecies sensitivity, such as metabolic capacity, DNA repair efficiency and defence mechanisms (Rodriguez-Cea *et al.*, 2003). These differences may also be related to the development of adaptive mechanisms of tolerance to chemical stress which help in the promotion of an increase in the replacement rate of dead or damaged cells to maintain normal physiological conditions or inhibition of nuclear division which leads to the expression of MN (Çavaş and Ergene-Gözükar, 2005; Bolognesi and Hayashi, 2011). It may also be due to

different biotic factors such as age, sex, genetic makeup, and conditions of treatment or different scoring criteria.

The increase in the frequencies of nuclear abnormalities (MN, binuclei, etc.) in the group exposed to ZnO-NPs is in accord with reports of Alkaladi *et al.* (2014), who exposed *O. niloticus* to ZnO-NPs.

DNA Quality: There was a significant ($p < 0.05$) reduction in the quality of DNA in the blood, gills and liver of *H. longifilis* exposed to ZnO-NPs compared to the control group (Figure 7). The absorbance ratio varied between 1.17 – 1.2 in blood, 1.04 – 1.05 in gills and 1.02 – 1.04 in the liver of control groups, while 0.06 – 0.37 in blood, 0.06 – 0.28 in gills and 0.05 – 0.27 in the liver of the exposed groups. The erythrocytes in the blood, gills and liver of fish exposed to different concentrations of ZnO-NPs exhibited significantly low DNA quality (0.05 – 0.37) compared to the control with 1.02 – 1.17 (Figure 7). The DNA quality decreased as the concentration of ZnO-NPs and exposure period increased to 60 days (Figure 7).

There was an increase in the quality of DNA in the blood, gills and liver of *H. longifilis* fed on varying concentrations of ascorbic acid compared to the groups that were exposed to ZnO-NPs and fed on normal basal feed (Figure 8). The absorbance ratio varied between 1.31 – 1.39 in blood, 1.15 – 1.25 in gills and 1.17 – 1.27 in the liver of control groups, while 0.08 – 0.12 in blood, 0.10 – 0.13 in gills and 0.07 – 0.08 in the liver of the ZnO-NPs-exposed groups without amelioration (Figure 8).

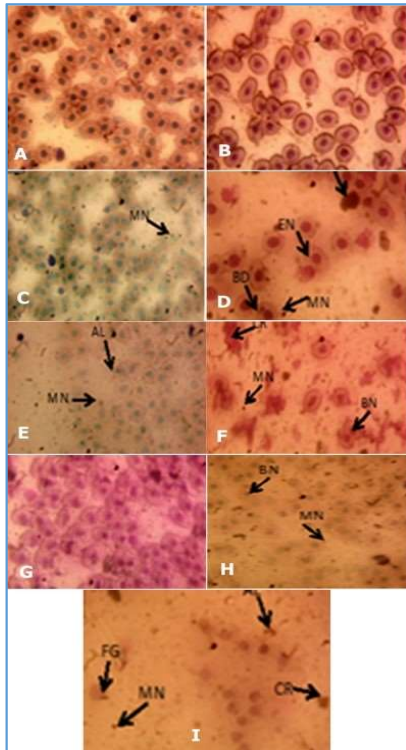


Figure 4: Micronucleus (MN) formation in erythrocytes of *H. longifilis* after exposure to zinc oxide nano-particles and ameliorated with varying concentrations of ascorbic acid (AA) for the period of 30 days. Key: (a) Erythrocytes of blood of *H. longifilis* in the group fed on normal diet only showed normal architecture; (b) Normal erythrocytes in the blood of fish fed on diet with 50 mg AA/kg diet without toxicant; (c) Normal erythrocytes with Micronucleated (MN) in the blood of fish fed on diet with 1000 mg AA/kg diet only; (d) Bud (BD), Enucleated (EN), Kidney shaped (KD) and Micronucleated (MN) in the erythrocytes of blood of fish exposed to 10 mg/L of ZnO-NPs with normal diet; (e) Altered (AL) and Micronucleated (MN) in the erythrocytes of blood of fish exposed to 10 mg/L of ZnO-NPs with diet containing 50 mg AA/kg diet; (f) Binucleated (BN) Crenated (CR) and Micronucleated (MN) in the blood of fish exposed to 10 mg/L of ZnO-NPs with diet containing 250 mg AA/kg diet; (g) normal architecture of erythrocytes in the blood of fish exposed to 10 mg/L of ZnO-NPs with diet containing 500 mg AA/kg diet; (h) Binucleated (BN) and Micronucleated (MN) in the erythrocytes of blood of fish exposed to 10 mg/L of ZnO-NPs with diet containing 1000 mg AA/kg diet. (i) Altered (AL), Crenated (CR), Fragmented (FG) and Micronucleated (MN) in the erythrocytes of blood of fish treated with 0.05% colchicine

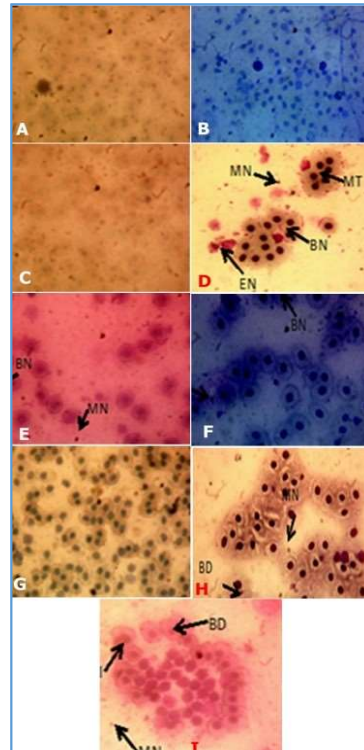


Figure 5: Micronucleus (MN) formation in gills of *H. longifilis* after exposure to zinc oxide nano-particles and ameliorated with ascorbic acid (AA) for the period of 30 days. Key: (a) Erythrocytes of gills of *H. longifilis* in the group fed on normal diet only without toxicant showed normal architecture; (b) Normal erythrocytes in the gills of fish fed on diet with 50 mg AA/kg diet without toxicant; (c) Normal erythrocytes of the gills of fish fed on diet with 1000 mg AA/kg diet without toxicant; (d) Binucleated (BN), Enucleated (EN), Micronucleated (MN) and Multinucleated (MT) in the erythrocytes of gills of fish exposed to 10 mg/L of ZnO-NPs with normal diet; (e) Binucleated (BN) and Micronucleated (MN) in the erythrocytes of gills of fish exposed to 10 mg/L of ZnO-NPs with diet containing 50 mg AA/kg diet; (f) Binucleated (BN) and Micronucleated (MN) in the gills of fish exposed to 10 mg/L of ZnO-NPs with diet containing 250 mg AA/kg diet; (g) normal architecture of erythrocytes with Enucleated (EN) in the gills of fish exposed to 10 mg/L of ZnO-NPs with diet containing 500 mg AA/kg diet; (h) Bud (BD) and Micronucleated (MN) in the erythrocytes of gills of fish exposed to 10 mg/L of ZnO-NPs with diet containing 1000 mg AA/kg diet. (i) Bud (BD), Binucleated (BN) and Micronucleated (MN) in the erythrocytes of gills of fish treated with 0.05% colchicine

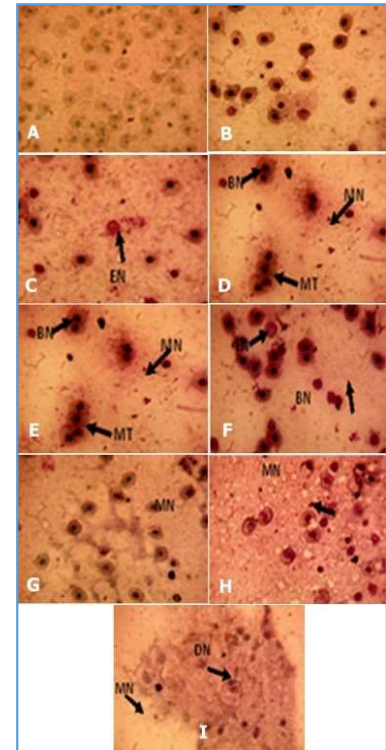


Figure 6: Micronucleus (MN) formation in the liver of *H. longifilis* after exposure to zinc oxide nano-particles and ameliorated with ascorbic acid (AA) for 30 days. Key: (a) Erythrocytes of liver of *H. longifilis* in the group fed on normal diet only showed normal architecture; (b) Normal erythrocytes in the liver of fish fed on 50 mg AA/kg diet only; (c) Normal erythrocytes with Enucleated (EN) in the liver of fish fed on 1000 mg AA/kg diet only; (d) Binucleated (BN), Micronucleated (MN) and Multinucleated (MT) in the erythrocytes of liver of fish exposed to 10 mg/L of ZnO-NPs with normal diet; (e) Altered (AL) and Micronucleated (MN) in the erythrocytes of liver of fish exposed to 10 mg/L of ZnO-NPs fed on 50 mg AA/kg diet; (f) Binucleated (BN) and Enucleated (EN) in the gill of fish exposed to 10 mg/L of ZnO-NPs fed on 250 mg AA/kg diet; (g) normal architecture of erythrocytes with Micronucleated (MN) in the liver of fish exposed to 10 mg/L of ZnO-NPs fed on 500 mg AA/kg diet; (h) Micronucleated (MN) in the erythrocytes of liver of fish exposed to 10 mg/L of ZnO-NPs fed on 1000 mg AA/kg diet. (i) Deformed nucleus (DN) and Micronucleated (MN) in the erythrocytes of liver of fish treated with 0.05% colchicine

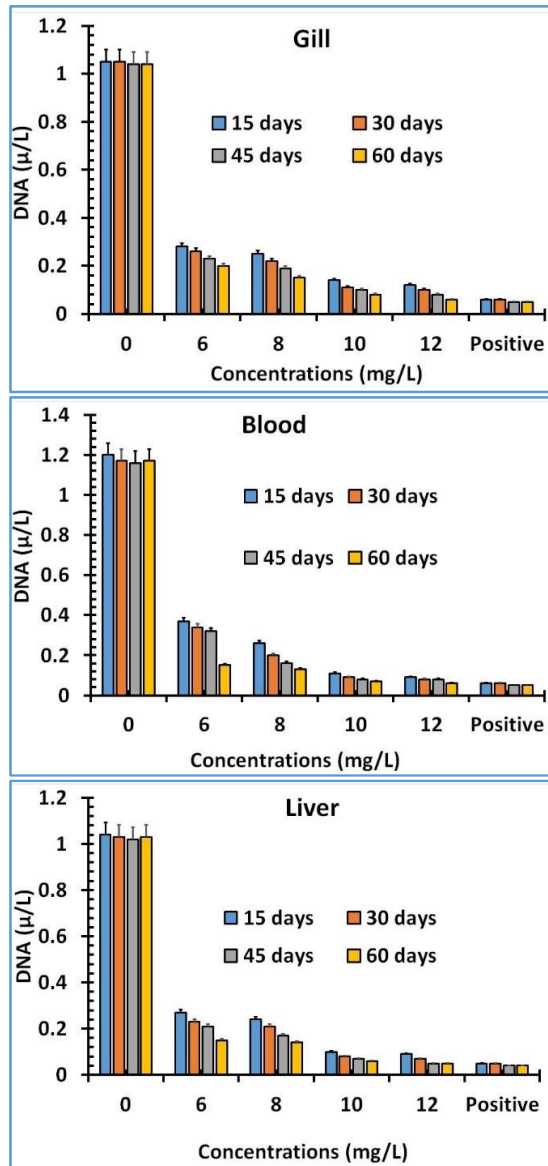


Figure 7: Level of DNA quality in the blood, gills and liver of *Heterobranchus longifilis* exposed to ZnO-NPs for 60 days

However, the absorbance ratio in ameliorated groups E to G varied between 0.15 – 1.28 in blood, 0.22 – 1.17 in gills and 0.18 – 1.17 in liver, while Group H, with the highest concentration of ascorbic acid, recorded between 0.61 – 0.91. The quality of DNA in Group D exposed to ZnO-NPs only is significantly lower ($p < 0.05$), recording 0.07 – 0.12 compared to the control groups A, B and C that were fed on normal and ascorbic acid formulated feed without toxicant, respectively. However, the quantity of DNA in the groups exposed to toxicant and ameliorated with varying concentrations of ascorbic acid recorded high

significant quality of DNA compared to Group D that was exposed to toxicant only. The DNA quality significantly increased as the concentration of ascorbic acid inclusion in the diet increased, but the quality reduced in Group H with the highest inclusion of ascorbic acid in the diet (Figure 8).

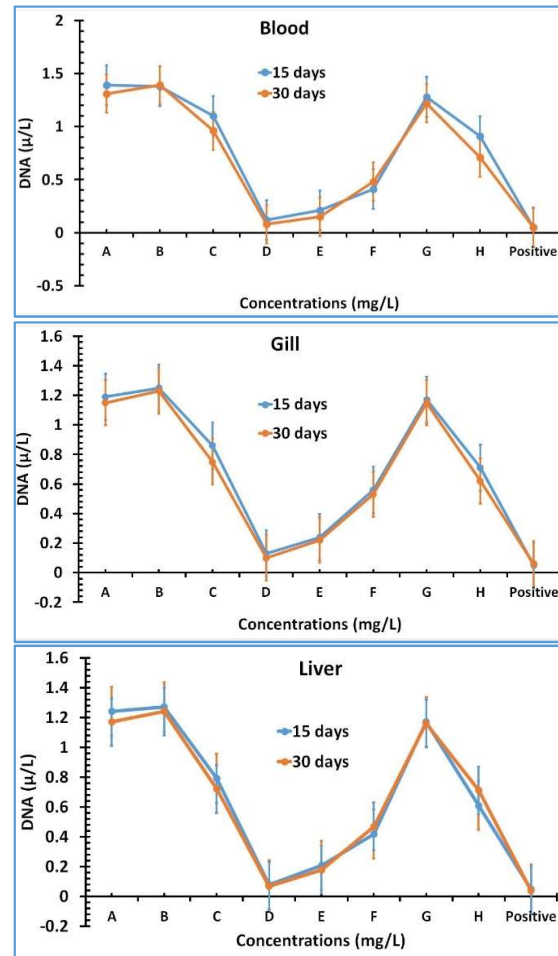


Figure 8: Level of DNA quality in the blood, gills and liver of *Heterobranchus longifilis* exposed to ZnO-NPs and ameliorated with ascorbic acid for 30 days

The ability of ZnO-NPs to accumulate in tissues as a result of their large surface area and small size enables it to interact easily with biomolecules that generate a high reactivity which triggers the formation of reactive oxygen species (ROS) such as superoxide anion ($O_2^{\cdot -}$), hydroxyl radicals and hydrogen peroxides (Yang *et al.*, 2009). An increase in ROS production induced by the accumulation of ZnO-NPs could cause DNA damage, thereby reducing the quality of DNA

(Singh and Walker, 2009; Yang *et al.*, 2009). Hydroxyl radicals cause severe damage to cells and tissues (Frei, 1994). Hydrogen peroxides formed from the hydroxyl radicals caused more damage to DNA (Yang *et al.*, 2009). Modification of protein, lipids and nucleic acids as a result of high oxidative stress could cause damage to DNA, which may affect the quality. The small size, large surface area and surface charge of ZnO-NPs allow its easy penetration into cells, easy dissolution in solvent and entry into blood capillaries and lymphatic tissues, where they are carried to other organs and accumulate and could cause DNA damage.

Conclusion: The commonest aberrations recorded in the tissues of ZnO-NPs-exposed fish are micronucleated, binucleated, multinucleated, altered, echinocyte cells, kidney-shaped and deformed nuclei. However, the groups ameliorated with a 500 mg/kg diet of ascorbic acids recorded little or no aberration. A dose and time-dependent increase in MN induction and frequency occurred compared to control but decreased significantly ($p < 0.05$) as the concentration of ascorbic acids increased with the lowest MN induction and frequency in the group fed on a diet with 500 mg/kg diet. The liver recorded the highest percentage frequency of MN compared to the blood and gills. A gradual linear decrease in the DNA quality was recorded as the concentration of ZnO-NPs and exposure period increased. The quality of DNA improved as the concentration of ascorbic acids increased from 50 – 500 mg/kg diet but reduced in the groups fed on a diet with the highest concentration of ascorbic acids (1000 mg/kg). Despite the benefits derived from the advent of nanotechnology by the industrial, agricultural and medicinal sectors, the release of wastes from the industries into the aquatic environment causes serious pollution and damage to the entire ecosystem, which may lead to the extinction of the aquatic animals, especially fish.

ACKNOWLEDGEMENTS

The University of Ilorin in Nigeria supported the research work through the Staff Development Award (SDA).

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