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Full Length Article

Alteration in Physiological and Histological Features of *Clarias gariepinus* Parenchyma Cell upon Exposure to Zinc Sulphate

Mohd Khalizan Sabullah[1,2], Nurul Aida Mohd Azri[1], Hassan Mohd Daud[3] and Siti Aqlima Ahmad[1*]
[1]*Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia*
[2]*Faculty of Science and Natural Resources, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia*
[3]*Department of Veterinary Clinical Studies, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia*
[*]For correspondence: aqlima@upm.edu.my
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Abstract

African catfish, *Clarias gariepinus*, specimens were subjected to separately expose to different concentration of zinc sulphate (25, 50, 75, 100, 150, 200 and 250 mg/L) for 96 h. By comparing to the unexposed specimens through physiological observation as the zinc sulphate concentration increases, the exposed specimens showed decreased food intake, lack of startle response, increased abnormalities in terms of swimming activity and pattern, surfacing activity, mucus secretion consequence to the increasing number of mortalities. Histological and ultrastructural alteration on exposed *C. gariepinus* gills was observed such as aneurysm, necrosis, rupture of capillaries, erythrocyte release and lamella fusion. Neurotic, hepatic, splenic and muscle cells demonstrated an increasing number of irregular polygonal shape, sinusoidal dilatation, vacuolation and parenchymatous degeneration associated with the toxic effect of zinc sulphate. *In situ* observation of *C. gariepinus* blood by scanning electron microscopy displayed huge differences in number of cells in exposed specimen compared to unexposed. The present study revealed that exposure to a toxic concentration of zinc sulphate significantly caused abnormalities in activity of the fish associated with histology changes of the parenchymal cell. Moreover, the baseline data is a useful reference for designing biomarker tool to assess the contamination level in the environment especially water bodies.

Keywords: *Clarias gariepinus*; Zinc sulphate; Physiological activity; Histology; Scanning electron microscope

Introduction

Soluble heavy metal has been documented to cause harmful effect on aquatic habitat. The major causes of heavy metal pollution in aquatic habitats come from the release of industrial effluents, waste disposal, sediment leaching and runoff from the agricultural area (Hayat *et al.* 2016; Sabullah *et al.* 2015a, 2020). Zinc (Zn) is an essential element and classified as trace metal, which is therefore present in picomolar range that plays role to regulate multiple functions of biological system especially cofactor of a number of enzymes that covers all of the six classes (McCall *et al.* 2000), but Zn is high potentially toxic to most living organisms. Zn in the form of inorganic sulphate salt is frequently used in agriculture as it has been proven effective to enhance the quality and quantity of crop production due to its dual function as fertilizer and herbicide (Faiz *et al.* 2015; Rasheed *et al.* 2019). Unfortunately, continuous and uncontrollable application may accidently contaminate the near water sources. For certain aquatic life, when the metal ion concentration reaches a certain amount, the metal toxicity may cause a broad range of effects and the organisms trigger reactions at all levels (Sabullah *et al.* 2015b; Ahmad *et al.* 2016a, b; Basirun *et al.* 2019a; Fadzil *et al.* 2019a).

The gill, liver, muscle, brain and blood have been given special importance in toxicological studies of organic and inorganic chemicals especially metal ion in different aquatic organisms due to its central role in interaction, metabolization and resistance to environmental contaminants. For instance, in gill epithelia of *Oncorhynchus mykiss*, *Oreochromis mossambicus* and *C. gariepinus*, exposure to heavy metals such copper and aluminum, respectively, is associated with structural damages such extreme curling and secondary lamellae inflammation with the epithelium extended away from the basement membrane (Heerden *et al.* 2004; Basirun *et al.* 2019b; Fadzil *et al.* 2019b;). Padrilah *et al.* (2017) mentioned about hepatoxicity of *C. gariepinus* upon exposure to copper such as the increasing number of dilated and congested blood vessels, development of melanomacrophage and necrotic area. Cadmium toxicity had caused swelling and coagulation necrosis of skeletal

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muscle fiber, as well as the deterioration of Purkinje cells and severe loss of granule cells in the brain of *Danio rerio* (Al-Sawafi *et al.* 2017). Liu *et al.* (2019) studied the accumulation of cadmium in *Carassius auratus gibelio*, and after 14 days' exposure, the gill showed the highest concentration followed by liver, gut and muscle. Erythrocyte morphology can be considered as an alternative parameter to evaluate the toxic effect of heavy metal (Gluhcheva *et al.* 2011).

The purpose of this study is to physiologically observe and assess the histological changes in gills, brain, liver, spleen and blood of African catfish, *C. gariepinus* where the specimens were acutely exposed to a different zinc sulphate concentration. The provided data could be useful for biomonitoring tools to assess the level of contamination in the environment especially river.

Material and Methods

Specimen preparation

Adult specimens of *C. gariepinus* (350–420 g; 42–45 cm) of both sexes with age around 2–3 month were obtained from Semenyih, Selangor. During the 20 days' acclimation period, one group of 10 sole specimens were maintained in a 400-L of free chlorinated tap water per tank with constant aeration and cleaning process was performed twice per week. Zinc contamination was carried out by adding zinc sulfate monohydrate; $ZnSO_4.H_2O$ (brand Merck) in each tank at different concentrations of 25, 50, 100, 150, 200 and 250 mg/L, while an untreated group was considered as a control of this study (Fig. 1). All the treated and control were let for 96-h exposure. Physical observation on the specimens was performed by semi quantifying several parameters such as the swimming activity, swimming pattern and position, startle response, food intake, mucus secretion and mortality (Basirun *et al.* 2019b; Pariza *et al.* 2019). Cellular abnormalities that occurred during exposure period performing histopathological (optical and ultrastructural) observations in gills, brain, liver, spleen and blood (Padrilah *et al.* 2017; Fadzil *et al.* 2019b).

Light microscopy

C. gariepinus from different treatments were collected and anesthetized in a box of ice for 20 min. Samples of in gills, brain, liver, spleen and blood were in the cassette followed by 48 h fixation in 10% formalin. After dehydration in an ascending concentration of alcohol (80, 95 and 100% for 2, 2.5 and 3 h, respectively), and chloroform for 3 h, the samples were embedded in paraffin wax. Sagittal sections of 5 mm thickness were stained with hematoxylin and eosin; H&E. The sections were visualized under light microscope (Leica DMRA II) and the selected areas were photographed for identifying the types of abnormalities on the parenchyma cells (Pariza *et al.* 2019).

Ultrastructural sample preparation

Around 1–2 mm^3 of each organ issues were excised in immersed in a fix solution (0.2 *M* sodium phosphate buffer pH 7.4 containing 2.5% glutaraldehyde solution) for 24 h at 4°C, followed by 2 h post fixed with 1% osmium tetroxide solution (prepared in 0.2 *M* sodium phosphate buffer 7.4) at 4°C. Next, tissues were dehydrated through soaked in a graded series of acetone; starting with 10 min of soaking in each acetone concentration of 35, 50, 75 and 95%, while at 100% for 10 min with 3 times changes. Resin infiltration was performed followed by polymerization process at 48 h. Ultrathin sectioning was performed in which the selected areas of interest were cut for ultrathin sections using a rotatory microtome. Finally, the ultrathin sections were examined under scanning electron microscope (SEM) (JOEL, Japan). the selected areas were photographed followed abnormalities determination.

Results

Observation of behavioral alterations

The evaluated changes in behavior and highlighted their significance as one of the crucial parameters for evaluating the systemic biomarker level of fish. The present study was conducted by observing and semi quantifying the behavioral alterations of the test specimen, *C. gariepinus*, during a 96-h treatment with $ZnSO_4$ concentration ranging from 25 to 250 mg/L (Table 1). During the test, the behavior of fish was found normal throughout the acclimatization phase, while at a lower concentration of $ZnSO_4$ (25, and 50 mg/L), the swimming pattern and food intake of fish were close to control, indicating no significant effect. Meanwhile, at the concentration of 50 and 75 mg/L, swimming activity and startle response were seen slightly affected with the increased secretion of mucus. Beyond 100 mg/L of $ZnSO_4$, significant behavior alteration was observed with 100% mortalities were recorded at the end of the exposure period at concentrations from 200 and 250 mg/L of ZnSO4. The changes of fish dorsal skin color from grey to pale associated to the increased formation of a white layer were noted as the concentration of $ZnSO_4$ increases.

For this study, *C. gariepinus* from the group of control, unaffected; 25 mg/L, initially affected or classified as slightly; 75 mg/L, moderately; 100 mg/L, and highly affected; 200 mg/L, by $ZnSO_4$ concentration were selected to visualize the changes in parenchymal cell of gill, liver, spleen, brain, muscle and blood.

Histopathological and ultrastructural changes in *C. gariepinus* gills

The untreated fish in this study showed a normal structure of gill filaments with no discovered changes in microscopic anatomy (Fig. 2). Similar pattern was observed in the treated fish with 25 mg/L of $ZnSO_4$, but a small number of

Table 1: Observation and semi-quantification of *C. gariepinus* abnormalities after 96 h exposure of $ZnSO_4$

Observation	96 h exposure of $ZnSO_4$ concentration (mg/L)							
	0	25	50	75	100	150	200	250
Swimming activity	Normal	Normal	Slower than normal	Slower than normal	Very slow	Very slow	Very slow	Very slow
Swimming Pattern and position	Normal	Normal	Normal	Normal	Vertical position	Vertical position	Vertical position and motionless	Vertical position and motionless
Startle response	Normal	normal	Under reactive	Under reactive	Under reactive	Under reactive	Under reactive/no response	Under reactive/no response
Food intake	+++	+++	+++	++	-	-	-	-
Mucus secretion	-	-	+	++	++	++	+++	+++
Mortality	-	-	-	+	++	++	+++	+++

- = none; ++ = moderate; + = little; +++ = high

Table 2: The histopathological abnormalities from the liver of *C. gariepinus* exposed to sub-lethal concentration of $ZnSO_4$ were quantitatively and semi-quantitatively recorded

Organ		Concentration ($mg.L^{-1}$)				
		Control	25	75	100	200
Gill						
•	Hyperplasia primary lamella	-	+	++	+++	+++
•	Secondary lamella fusion	-	+	+++	+++	+++
•	Lamellar aneurysms	-	+	++	+++	+++
Liver						
•	Dilated sinusoid	-	+	+	++	+++
•	Sinusoid congestion	-	+	++	+++	+++
•	Cytoplamic vacuolation	-	-	+	++	+++
•	Melanomacrophage formations	+	+	++	+++	+++
•	Necrotic area	-	-	-	+	+++
•	*Hepatic nuclei per mm^2	11281 ± 1084^{a}	10588 ± 1426^{a}	8842 ± 1074^{ab}	3204 ± 417^{c}	2762 ± 327^{c}
Spleen						
•	Melanomacrophage formations	+	+	++	+++	+++
•	Number of megakaryocyte	-	-	+	++	+++
•	Necrotic area	-	-	-	+	++
Muscle						
•	Parallel arrangement.					
•	deformation of the muscle fibers	-	-	+	++	+++
Brain						
•	Detachments around neurons	-	-	-	-	+
Blood						
•	Number of blood	Normal	N.O.	N.O.	lower	N.O.

Note: *The mean point with standard deviation was obtained from triplicate data

**Image of blood observed under SEM were qualitatively determined where control treatment considered as normal. Affected group show significantly lower in the number of blood cell compared to the control. N.O. = Not observed

-, +, ++, and +++ denoted as no, low, moderate and highly in the term of number and area that affecting the histology of parenchymal cell, respectively

abnormalities were observed such as the epithelial lifting of secondary lamellae and fusion between secondary lamellae (Fig. 2B). Severe damages were observed after treated with 75 and 100 mg/L of $ZnSO_4$ in which dysplasia, blebbing arrangement and multiple deformation of secondary lamellae were observed followed by congestion of blood and vacuolation at the primary lamellae. Besides, the highest concentration of $ZnSO_4$ at 200 mg/L displayed extreme changes with a total loss of structure associated with the absence of respiratory epithelium. At the ultrastructure level, by comparing to the untreated fish, treated fish with 150 mg/L of $ZnSO_4$ were seen to fully rupture with disorganization of secondary lamellae (Fig. 3).

C. gariepinus hepatological alteration

The liver is being the primary organ associated with mechanisms of detoxification and bioconversion besides being vital for multiple critical functions. The histological structure observed in exposed fish with 25 mg/L of $ZnSO_4$ was noted similar to that of unexposed or control fish, which consisted of disorganized hepatocytes into separate lobules but grouped into two-cell-thick branched laminae divided by sinusoids (Fig. 4A and 4B). Both showed a typical spherical structure of parenchymal cells with a densely central stain nucleolus. Histological abnormalities were initially observed at 75 mg/L of $ZnSO_4$ concentration treatment showing increasing number of congested sinusoids, vacuolation, and formation of melanomacrophage (Fig. 4C). Meanwhile, 100 and 250 mg/L showed a number of excessive lesions with blood retained in the dilated central vein, which gave a light pink color (Fig. 4D and 4E). A huge necrotic area and a number of melanomacrophage were also noted. Fig. 5 illustrates the comparison between

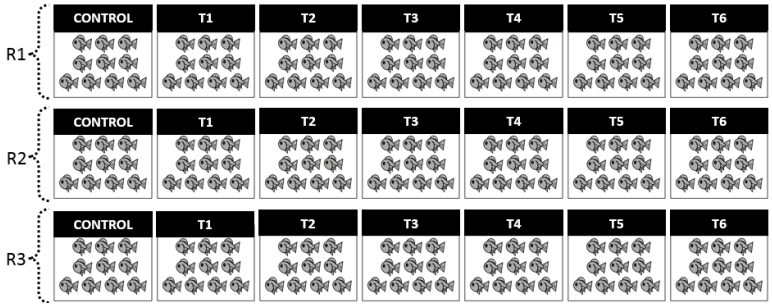

Fig. 1: *C. gariepinus* was exposed with different concentrations of $ZnSO_4$ for 96 h in a close system; 10 fishes per aquarium. Control served as untreated group while other aquarium marked as T1, T2, T2, T4, T5 and T6 denoted as 25, 50, 100, 150, 200 and 250 mg/L of $ZnSO_4$, respectively. R1 to R3 shows the study was run triplicate

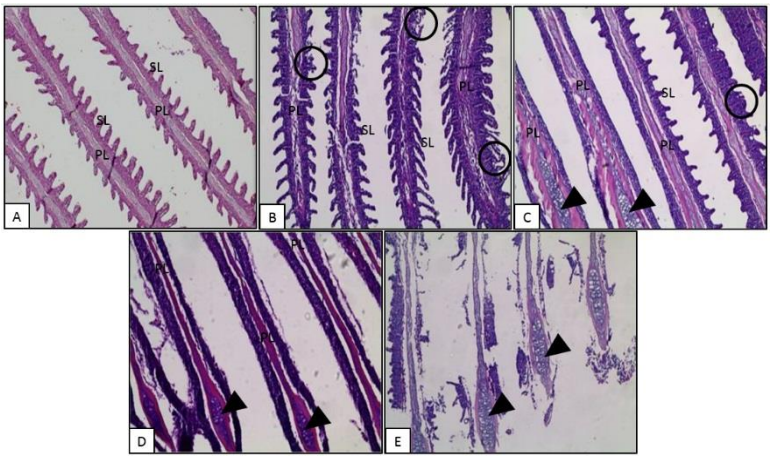

Fig. 2: Light microscopic observation on the gill sectioned of *C. gariepinus* stained by H&E. (**A**) control, (**B**) 25 mg/L, (**C**) 75 mg/L, (**D**) 100 mg/L, and (**E**) 200 mg/L. PL = Primary lamella, SL = Secondary lamella, Circle area = epithelial lifting of secondary lamellae, arrow head = blood congestion. 100x magnification

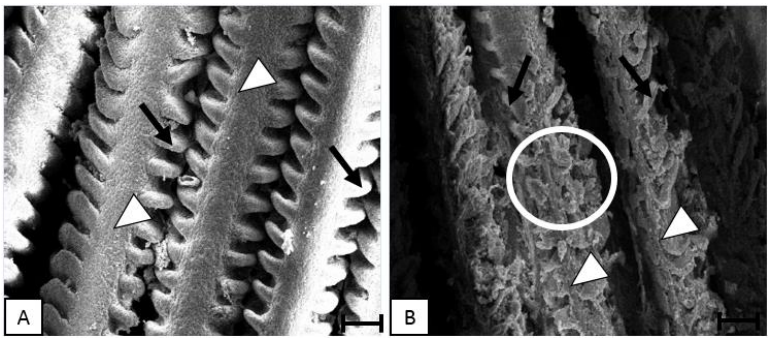

Fig. 3: Ultrathin section of *C. gariepinus* gill. (**A**) Control, and (**B**) 100 mg/L. White arrow head = Primary lamella, arrow = Secondary lamella, White circle = disorganization of secondary lamellae, and bar = 100 μm

control and severe effect of $ZnSO_4$ on hepatocyte where the formation of apoptotic bodies was highly developed, which was related to programmed cell death.

Histological change in splenic cells of *C. gariepinus*

Control and affected splenic cells at displayed normal and distinct spleen follicle with clear white and red pulps with marginal zone (Fig. 6). Abnormalities were found obviously after exposure to 75 mg/L of $ZnSO_4$ followed by 100 and 250 mg/L of $ZnSO_4$ where the increase hyperplasia in the melanomacrophage, scattered megakaryocyte numbers and widen of necrotic area were observed. Comparative observation was secondary validated under SEM. The ultrastructural observations in spleen tissue of control fish revealed typical tissue surface, while the surface of treated

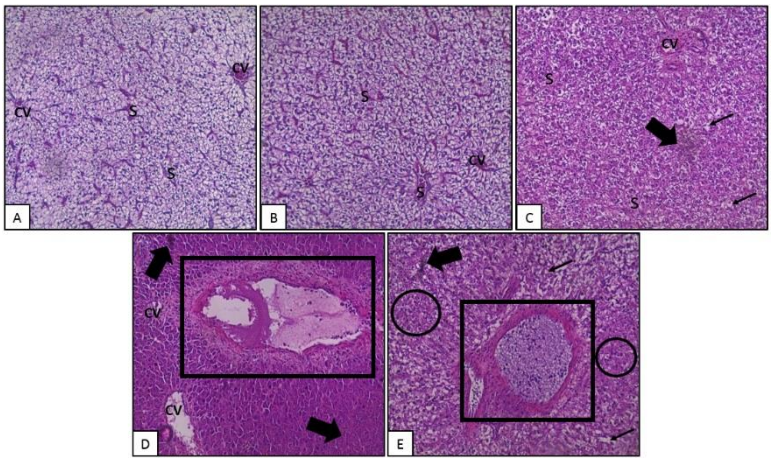

Fig. 4: Light microscopic observation on the liver sectioned of *C. gariepinus* stained by H&E. (**A**) control, (**B**) 25 mg/L, (**C**) 75 mg/L, (**D**) 100 mg/L, and (**E**) 200 mg/L. CV = central vein, S = Sinusoid, Circle area = necrotic area, thick arrow = melanomacrophage, thin arrow = vacuolation, box = dilation and congestion of central vein. 200x magnification

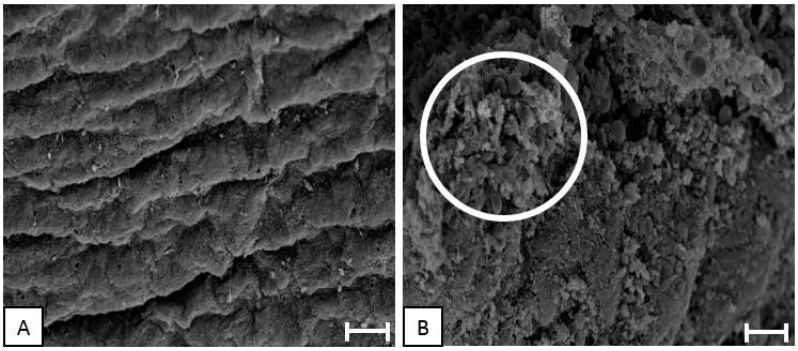

Fig. 5: Ultrathin section of *C. gariepinus* liver. (**A**) Control, and (**B**) 100 mg/L. White square = normal parenchymal arrangement, White circle = formation of apoptotic body, and bar = 10 μm

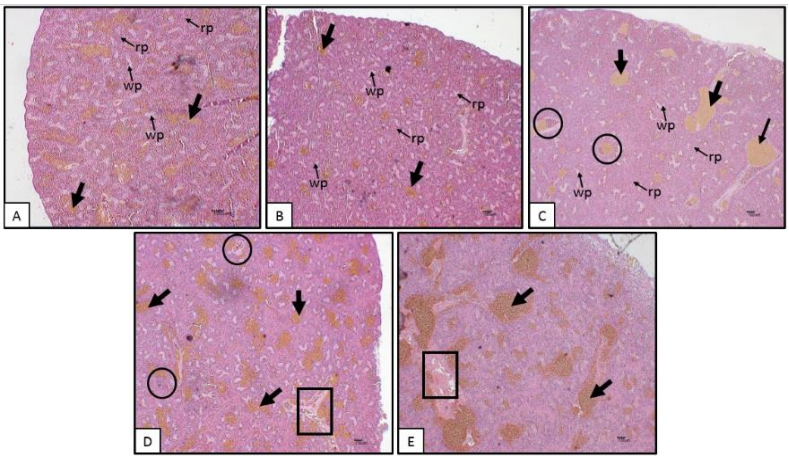

Fig. 6: Light microscopic observation on the liver sectioned of *C. gariepinus* stained by H&E. (**A**) control, (**B**) 25 mg/L, (**C**) 75 mg/L, (**D**) 100 mg/L, and (**E**) 200 mg/L. wp = white pulp, rp = red pulp, arrow = melanomacrophage, circle = megakaryocyte, and box = necrotic area. 40x magnification

sample showed an irregular structure on the development of apoptotic bodies and cell shrinkage. Besides, a number of crack and pit formations associated with cell deleterious processes were seen (Fig. 7).

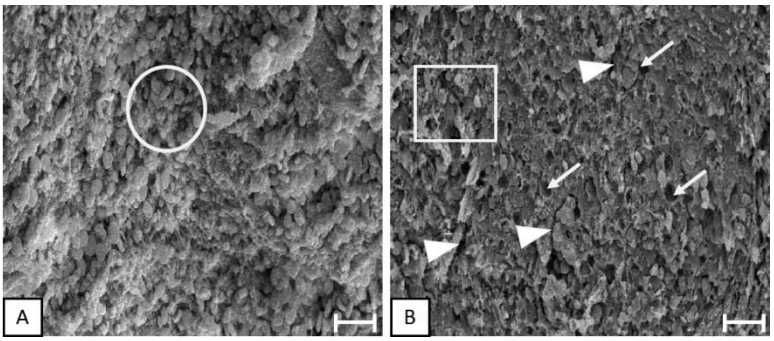

Fig. 7: Ultrathin section of *C. gariepinus* outer surface of spleen tissue. (**A**) Control, and (**B**) 100 mg/L. Circle = area of normal splenic cell, box = abnormal arrangement of splenic cell, arrow = formation of pit at the surface of spleen tissue, and arrow head = cracking at the surface of spleen tissue. Bar = 10 μm

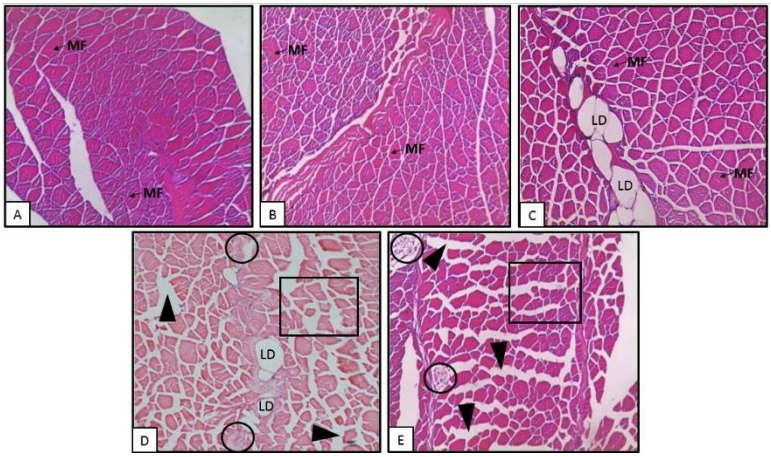

Fig. 8: Light microscopic observation on the skeletal muscle sectioned of *C. gariepinus* stained by H&E. (**A**) control, (**B**) 25 mg/L, (**C**) 75 mg/L, (**D**) 100 mg/L, and (**E**) 200 mg/L. MF = myofibril, LD = lipid deposition, box = degenerated myofibers, circle = a group of necrosis fiber, arrow head = inter myofibrillar space. 200x magnification

Muscle histopathology in the $ZnSO_4$ toxicity fish

Control study showed the typical architecture of muscle cross-sections and a similar result was determined in 25 mg/L and 75 mg/L of $ZnSO_4$ groups (Fig. 8). However, a significant effect was observed in 100 and 250 mg/L of $ZnSO_4$ concentration treatment with massive atrophy of muscle fiber including fragmented of myofibrils, wide necrotic area, degenerated myofibers and widened inter myofibrillar space. The three-dimensional structure of the muscular cell was visualized using SEM. Fig. 9A displays a typical vertical structure with several nodules in the control section while in Fig. 9B, the toxic effect of 100 mg/L of $ZnSO_4$ exposure was seen to result in disorientation and irregular sites of bending.

Brain histopathology in the $ZnSO_4$ toxicity fish

The toxic effect of $ZnSO_4$ of the fish brain was observed at the highest concentration treatment of 250 mg/L, while other concentrations showed no significant effect (Fig. 10). Affected brain displayed several detachments around neurons associated with cell death (Fig. 10E). The result (Fig. 11) was validated through observation using SEM where the ruptured of the cell surface with multiple blebbing were present (Fig. 11B).

Ultrastructural change in blood cell of *C. gariepinus*

A comparison of blood cells was observed at the ultrastructure level. Untreated fish showed an intact cellular spherical structure. However, 100 mg/L of $ZnSO_4$ treated fish showed a decreasing number of blood cells. The toxic effect of this compound was associated with the activity of white blood cells, some cells with oozed out cytoplasmic content and a lot of hemolysis (Fig. 12).

Discussion

Zinc residues can be indirectly poisonous to fish at higher waterborne levels and can be impacted either by Zn alone or more often together with other xenobiotic compounds.

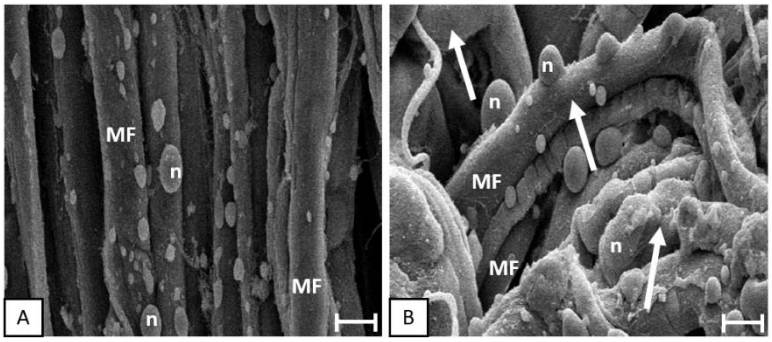

Fig. 9: Ultrathin section of *C. gariepinus* outer skeletal muscle area. (**A**) Control, and (**B**) 100 mg/L. MF = microfibril, n = node, and arrow = abnormal microfibril. Bar = 10 μm

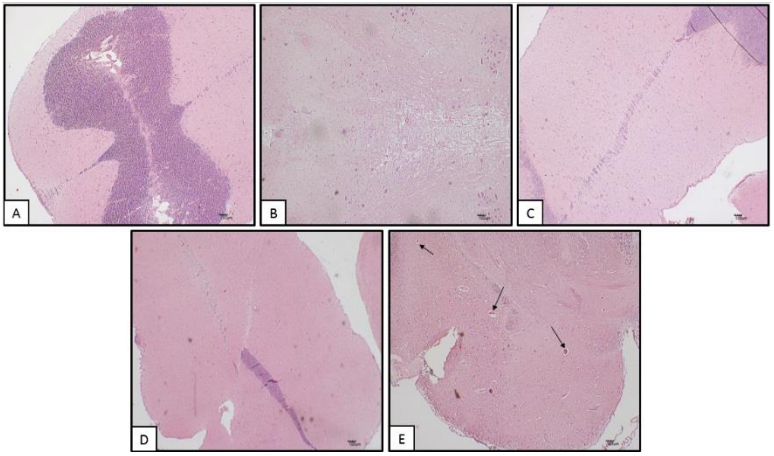

Fig. 10: Light microscopic observation on the brain sectioned of *C. gariepinus* stained by H&E. (**A**) control, (**B**) 25 mg/L, (**C**) 75 mg/L, (**D**) 100 mg/L, and (**E**) 200 mg/L. arrow = detachment of neuron. 40x magnification

The primary target of waterborne Zn exposure is the gills that block the Ca^{2+} uptake, leading to hypocalcaemia and eventual death. The other toxicity endpoints differ between freshwater and marine fish with survival, development, reproduction, and hatching being the most common. The present study showed that at the end of the exposure period, 100% mortality was noted beyond 200 mg/L of $ZnSO_4$ concentration exposure. At this concentration, all the parenchymal cell displayed histologically atypical associated with severe cell deleterious effect. Physiologically observation at 25 and 50 mg/L was considered as not much different from untreated fish; however, at 75 mg/L of $ZnSO_4$, the fish was seen slightly affected due to the presence of fish mortality, low food intake and mucus secretion as well as slow response, but swimming pattern was seen regular. This study showed that the physiological and behavior of the fish were adversely affected at ZnSO4 concentration of 100 mg/L, which was supported by observation and semi-quantitatively assessment based on the level of abnormalities by comparing all the treated and untreated fish. An acute inflammatory reaction and dysfunction of *C. gariepinus* gill were related to the fully ruptured of primary and secondary lamella at a concentration of 100 to 250 mg/L of ZnSO4 as the fish exhibited massive opercula motions, vertical position swimming position indicating breathing difficulty due to hypoxia and excessive mucus secretion associated with the toxic effect of ZnSO4.

Zinc is actively metabolized in the tissue of fish particularly in organs such as the liver. It has the potential to bioaccumulate as seen on different aquatic organisms such as *Oreochromis niloticus* (Taweel *et al.* 2012), *Carcinus maenas* (Chan *et al.* 1992) and *Channa punctatus* (Murugan *et al.* 2008). Hepatocyte arrangement in treated fish at 25 mg/L of $ZnSO_4$ was considered normal. However, 75 mg/L of $ZnSO_4$ showed a slightly effect on the fish in which the presence of melanomacrophage was associated with the response to foreign endogenous and exogenous substances through the process of detoxification and removal. Vacuolation in this sample was a mild effect of $ZnSO_4$ and would be recovered in a short period (Shubin *et al.* 2016). Beyond 100 mg/L of $ZnSO_4$ was considered as excessive exposure due to degeneration and widening of the necrosis area of hepatocytes associated with the cumulative effect of the metals that increased their accumulation in the liver. The cellular degeneration in the liver was also related to oxygen

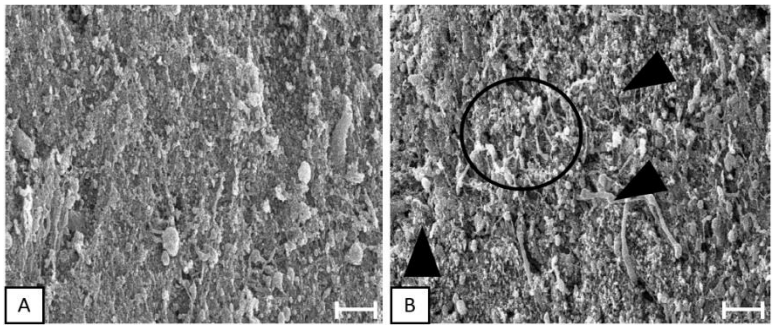

Fig. 11: Ultrathin section of *C. gariepinus* outer surface of fish brain. (**A**) Control, and (**B**) 100 mg/L. Circle = ruptured of cell surface, and arrow = blebbing related with apoptotic body. Bar = 10 μm

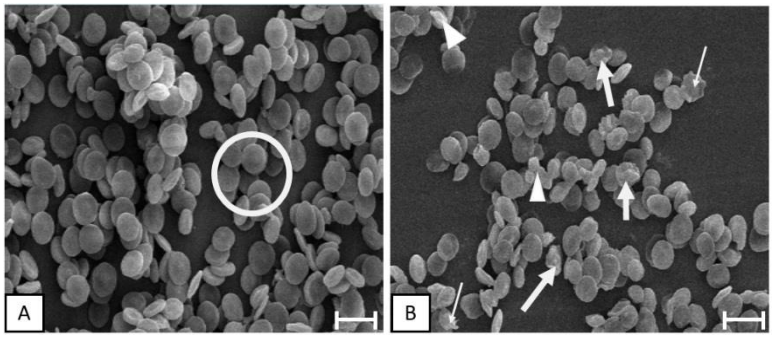

Fig. 12: Ultrathin section of *C. gariepinus* of fish blood. (**A**) Control, and (**B**) 100 mg/L of $ZnSO_4$. Circle = an example of the area of normal cell structure, arrow head = white blood cell, thick arrow = oozed out cytoplasmic content, and thin arrow = hemolysis. Bar = 10 μm
All the data were summarized in **Table 2**. Calculation on Hepatic nuclei per mm^2 was carried out based on the method developed by Figueiredo-Fernandes *et al.* (2007) and Sabullah *et al.* (2017) on the image of liver section under light microscope without calculating the cells present in sinusoids

deficiency as a result of gill dysfunction or vascular blood congestion and intravascular hemolysis noted in the blood vessels. When Zn accumulation exceeded the liver capacity, Zn may be transported *via* blood circulation to the next organ. The ultrastructural analysis showed the abnormal structure of the blood cell and hemolysis. In addition, splenic cell showed the same histological effect of $ZnSO_4$ with hepatocyte. 75 mg/L of $ZnSO_4$ treatment caused the abnormal size of melanomacrophage and induction of megakaryocytic proliferation, which were related to thrombocytosis due to excessive quantities of circulating platelets. Moreover, at a high concentration of 100 mg/L and 200 mg/L of $ZnSO_4$, the necrotic area was seen increased.

Only a few data related to histological abnormalities of fish skeletal muscle affected by Zn. Ciamarro *et al.* (2015) demonstrated a significant increase in size of distinctive fibers in the white pectoral muscle of fish *Astyanax altiparanae* after exposed to an urban lake water sample containing a high content of heavy metals including Zn. Tymoshenko *et al.* (2016) observed the alteration of skeletal muscle structure in the mature rat after drinking the water containing various heavy metals including $ZnSO_4$. Ismail *et al.* (2015) observed significant changes in the structure of skeletal muscle of fish in the water containing chromium, Zn, copper, lead, cadmium, mercury, and ferrum. All histological observations exhibited the fragmentation of the muscle fibers along with ruptured in muscle bundles associated with myolysis. Affected muscle fibers could be distinguished by wide extracellular spaces occupied by connective tissue. However, affected brain samples were only shown at 200 mg/L of $ZnSO_4$ with the presence of several neuron necroses. Toxic effect of 5 ppm of Zn was also noted in the brain of *Labeo rohita* fingerlings after 15-day exposure periods (Loganathan *et al.* 2006). Unlike the study by Saddick *et al.* (2017), the toxic effect of Zn nanoparticle was determined by analyzing the activity of oxidative stress-related genes and antioxidant enzyme activity in the brain of *Oreochromis niloticus* and *Tilapia zillii*. Overall, most affected cells were caused by the overreaction of oxidative stress and depletion of antioxidant activity under the influence of heavy metals. Reactive oxygen species occurs on account of two different pathways, 1) the generation of free radical; hydroperoxides (HO_2•), singlet oxygen, and 2) non-free radical; hydrogen peroxide.

Conclusion

Zn contamination in the aquatic habitat, even in low concentration, has been discovered to cause early signs of cellular and behavioral alterations in the adult *C. gariepinus*. Significant toxicological effects of Zn were determined

physiologically at the concentration of 100 mg/L of ZnSO4. Histological abnormalities of all the parenchymal cells were observed at this concentration. These data have strengthened the exploitation of *C. gariepinus* as a sentinel species for the non-point source of heavy metal pollution especially Zn.

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