## The Effect of Copper on the Ultrastructure of *Puntius javanicus* Hepatocyte

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Histology assessment is necessary to verify the presence of liver toxicity. The present study was carried out to compare ultrastructure alterations affected by copper (Cu) in the liver of *Puntius javanicus* exposed in vivo for 96 hours to sublethal copper sulfate (CuSO₄) concentration of 0.5, 1.0 and 5.0 mg/L. Our results indicated that Cu have significant effects on *Puntius javanicus* hepatocyte ultrastructure. The toxicity of copper was visualized using transmission electron microscope (TEM) where the affected cells show abnormalities of the shape of the nucleus, ripped nuclear membrane, swollen cells and lipid droplet deposition. However, at higher CuSO₄ exposure, other abnormalities were observed which are the development of pyknotic nucleus along with damaged organelles such as mitochondria, Golgi apparatus and endoplasmic reticulum disorientation. Irreversible cell injury was also observed where the hepatic nuclei was undergoing karyorrhexis with the formation of apoptotic body consisted of free scattered damaged organelle. This comparative study provides additional knowledge about the elimination effects of copper for the evaluation of the health status of fish species such as *Puntius javanicus* toward exposure by this contaminant and as an alternative source for biomarker of metal toxicity.

### INTRODUCTION

Small amounts of copper may regulate multiple process in body metabolism especially in fish. Fish need this essential micronutrient to activate several functions or as the cofactor of enzymes involved in cell biosynthesis, protection from free radical attack, respiration and maintaining 3D protein structure (Linder, 1991; WHO, 1998; Daniel et al., 2004). Copper deficiencies may decrease several optimum biochemical activities but excessive level will also lead to the increasing toxicity level and inhibition of several biological functions.

Commercial uses of copper includes pest control (de Oliveira-Filho et al., 2004), electronic components (Gan et al., 2013) and drug development (Szymanski et al., 2012). Uncontrolled and poor management of copper will lead to environmental contamination by run-off and leaching into the water bodies. Previous studies have proved that copper had negative effects to aquatic organisms (Van Heerden et al., 2004; Campagna et al., 2008). At toxic concentrations, several behaviours in fish were affected such as the decreasing of swimming performance, less sensitivity of avoidance behavior and the decreasing of feed intake (Plaut, 2001; Ali et al., 2003; Vieira et al., 2009; Ezeonyejiaku et al., 2011).

The toxicity of this compound to fish can be evaluated from the molecular to structural level. The gills are the first organ that comes into contact with waterborne contaminants. However, the liver is the primary target organ for contaminant accumulation and storage especially copper (Perkins et al., 1997). Elevated levels of copper concentrations may lead to the increasing toxicity which disrupts other biological functions (Figueiredo-Fernandes et al., 2007). At toxic levels, copper may cause the generation of reactive oxygen species (ROS) which disrupts biochemical functions and cellular morphology (Paris-Palacios et al., 2000; Monteiro et al., 2005). Production of ROS is related to Fenton and Haber Weiss reaction (Kehrer, 2000; Letelier et al., 2005; Do...
Lago et al., 2011) which then interacts with DNA, protein, lipid or other possible pathways to damage or produce other free radicals that damage the mitochondria and lead the occurrence of programmed cell death such as apoptosis and necrosis (Cooke et al., 2003; Elmore, 2007; Giorgio et al., 2007; Rodrigues et al., 2010; Kang et al., 2012). Copper can also affect glucose metabolism as mentioned by Martinez et al., (2004), who reported the decrease of glucose concentration in Prochilodus lineatus in response to copper exposure. In the duration of copper exposure, the increasing of total plasma ammonia affects muscle metabolic status and interfere with the nervous system functions which are exhibited as the decrease of swimming performance associated with low contraction force in skeletal muscle (Sjøgaard, 1991; Beaumonth et al., 1995; Grosell et al., 2002). However, copper toxicity depends on the concentration level, fish species, exposure duration and other factors (Paris-Palacios et al., 2000).

Fish are good candidates as an environmental tool for monitoring the existence of contaminant in the aquatic medium because fish organs, especially the liver, is highly sensitive to the presence of xenobiotics (Paris-Palacios et al., 2000). Thus, in this study, the in vivo stress effects caused by the chemical actions of CuSO4 on Puntius javanicus liver was studied using TEM.

MATERIALS AND METHODS

Specimen Treatment and Sample Preparation:

Puntius javanicus weighing 400-600 g was obtained from the Agriculture Development Centre, Pahang, Malaysia and brought alive to the laboratory. Fish were distributed randomly in three groups of nine animals in each aquarium. The fish was acclimatise at laboratory condition for 15 days (12d:12n) using fully aerated and chlorine-free tap water (75 L). Water was renewed once a week to maintain the hygiene and cleanliness of the water. Each group was separately treated with the final CuSO4 concentration of 0.5, 1.0 and 5.0 mg/L in the aquarium. After 96 hours treatment, the fish were killed and had the livers removed. The livers were cut into approximately 1 mm3 sections and then subsequently fixed for 20 hours using 4% of glutarydehyde. The samples were then washed and immersed three times in 0.1 M sodium cacodylate buffer for 10 minutes each. Post fixation was performed by soaking in 1% cold osmium tetroxide for 2 hours and then washing three times with 0.1 M sodium cacodylate buffer for 10 minutes each. The test sample was dehydrated by being immersed three times in a series of increasing acetone concentrations (35% for 10 minutes; 50% for 10 minutes, 75% for 10 minutes, 95% for 10 minutes and finally 100% for 10 minutes). The infiltration process was performed using a resin mixture and acetone with the ratio of 1:1, 3:1, and 100% resin for one, two and 12 hours incubation, respectively. Another two hours incubation in 100% resin was carried out to ensure a complete resin infiltration. The sample was embedded in the beam capsule filled with resin prior the polymerisation process for 48 hours at 60 °C.

Sample Sectioning:

The sample was cut using a rotatory microtome to ultrathin sections. Silver sections were picked up using a grid and then dried using filter paper. Uranyl acetate was used to stain the section samples before being washed with distilled water. Staining process was continued using lead for 10 minutes and washed again using double distilled water. Prepared sections were visualised using TEM. Each of the treated samples was observed for changes in the affected tissue such as abnormalities and histological alterations (Velcheva et al., 2010) and was then photographed.

RESULTS

Ultrastructure alteration was observed for each section of copper-treated concentration on P. javanicus liver tissues for 96 hours using the TEM where all samples showed alterations with different appearance associated with CuSO4 concentrations. There was no fish mortality except signs of swimming disorder at 1 and 5 mg/L of CuSO4 concentration. The present study shows the first copper treatment (0.5 mg/L) displayed by toxicant-related changes in ultrastructure. Figure 1A shows normal cells including the regular form of a nucleus with double nuclear membrane and pore, numerous natural organelles such as mitochondria, endoplasmic reticulum and distributed ribosome and single lipid droplet. However, cellular abnormalities were observed in Figure 1B which displayed the swollen cell, vacualisation and the existence of lipid droplet (lipidosis) in the hepatocyte which were also seen by Abdel-Moneim and Abdel-Mohsen (2010). The hepatocyte of P. javanicus exposed to 1 mg/L copper displayed the beginning of nucleus deformation (Figure 2). Ripped nuclear membrane was also visualised which is associated with the development of pyknotic nucleus by the accumulation of heterochromatin (Figure 2B and 2C). Several copper-treated samples at 1 and 5 mg/L exhibited the budding formation of the cells with the scattered organelle, shrinkage, dense and karyorrhexis nucleus (Figure 2D and 2C). Numerous vacuole formation was also visualised in Figure 3. This may be due to the deleterious effects of either cellular degradation or phagocytosis by nearby macrophage.
Fig. 1: Ultrastructure of *P. javanicus* hepatocyte exposed with CuSO4 concentration of 0.5 mg/L. (A) Normal cell with normal shape of nucleus, mitochondria and Golgi apparatus with single appearance of lipid droplet, X2000. (B) Affected cell showing normal nucleus and the presence of pleomorphic mitochondria with dilate cristae and dense matrices, cytoplasm degeneration marked with X and deposition of numerous lipid droplet, X1800. (C) The nucleus was undergoing pyknosis with dilated ER fractionation, X1600. (D) The portion of three neighboring hepatocyte shows the existence of normal cells and affected cell which is nuclear dense to form pyknotic cell and the appearance of mitochondria damage, X1500.

Note: N=Nucleus, m=Mitochondria, ER= Endoplasmic reticulum, ga= Golgi apparatus, d= Lipid droplet, V= Autophagy vacuole

Fig. 2: Ultrastructure of *P. javanicus* hepatocyte exposed with CuSO4 concentration of 1 mg/L. (A). Note pyknotic cell was surrounded by mitochondria consisted of electron-dense matrical precipitation (arrow), X2500 (B) Pyknotic cells shows the ruptured of the nuclear envelope (arrow head). V mark shows mitochondria inside the autophagic vacuole. X2000 (C) Nucleus was undergoing karyorrhexis. Note the RER fragmentation, swollen and damage mitochondria, X2700 (D) Nuclear and mitochondria damaged, and ER fragmentation along with the formation with apoptotic body (*), X2000.
Fig. 3: Ultrastructure of *P. javanicus* hepatocyte exposed with CuSO₄ concentration of 0.5 mg/L. (A) Pyknotic nucleus shrinks from the normal shape surrounded by dilated ER, X2500 (B) Nucleus karyorrhexis shows abnormal shape. Also note ER fragmentation and damage mitochondria (m) and numerous vacuole containing granular material (V), X2000 (C) Damage of karyorrhexis nucleus, dilated and matric dense mitochondria were visualised. Note also damage mitochondria into the autophagy vacuole, X2000. (D) Damage of karyorrhexis nucleus and surrounded by an abundance of dilated mitochondria. Also note vacuole contained with damage organelle, X2000.

Note: N=Nucleus, m=Mitochondria, ER= Endoplasmic reticulum, ga= Golgi apparatus, d= Lipid droplet, V= Autophagy vacuole.

**DISCUSSION**

In Figure 1, the presence of lipidosis which is the abnormal accumulation of triglycerides within parenchymal cells shows the development of fatty liver syndrome induced by copper exposure. This observation was also reported by Shaw and Handy (2006), and Liu *et al.*, (2010) using waterborne copper exposure of *Oreochromis niloticus* and *Synechogobius hasta*. Elevated number of lipid droplets may block the metabolism of lipid-protein conjugate and decrease the synthesis of protein in the hepatocyte (Cheville, 1994). The present study shows that increasing CuSO₄ exposure is associated with the increase in number of irregularities of nuclei shape. It is normal that the toxic effects on hepatocytes produced numerous amounts of vacuoles to remove the damaged organelles such as degenerated membrane organelle and pyknotic nucleus out of the system (Chishti and Roikiewicz, 1992). Nucleus damage was detected and this situation is normal due to the unsustainability towards stressor toxicity. This is related to the overproduction of ROS caused by the toxicant-imbalanced defence mechanism of antioxidants needed to scavenge the ROS.

ROS production and reaction was the cause of the decreasing permeability of the nuclear envelope and the losing of main barrier function which leads to the membrane bilayer destruction (Kodiba *et al.*, 2004; Sánchez *et al.*, 2005). This situation allowed other ROS such as hydrogen peroxide (H₂O₂) to gain access and cause damage through oxidation of purines and pyrimidines, especially guanine bases leading to the breakage of double and single DNA strands (Duthie and Dobson, 1999; Linder *et al.*, 2012). The synthesis of the intracellular enzyme caspase also damages the nucleus through the release of cytochrome c from affected mitochondria by the stressor, which then activates these apoptotic compounds causing the breakage of cytoplasmic and nucleus protein including DNA (Van Cruchten and Broeck, 2002; Mufti *et al.*, 2007). Mitochondria destruction was visualised from Figures 2 and 3. Previous studies mentioned that mitochondria play the centrals role in the activation of cell apoptosis (Ott *et al.*, 2002; Hong *et al.*, 2009). The pore formation of the outer mitochondria membrane by BID and BAX allows the ion influx which leads to the swelling of mitochondria and rupture of the outer membrane, followed by the releasing of cytochrome c into the cytoplasm (Van Cruchten and Broeck, 2002). The interaction of cytochrome c with Apaf-1 resulting in the activation of procaspase-9, followed by activation of caspase-3 by caspase-9 which is the biochemical characteristic of apoptosis by breaking up cytoplasmic and nuclear envelope including DNA strand (Ott *et al.*, 2002; Van Cruchten and Broeck, 2002).
Through the observation obtained, the irreversible cell injury was caused by karyorrhexis of the hepatocyte nucleus and formation of the apoptotic body consisting cytoplasm with abundant of organelle. Apoptotic and necrotic cell will be eliminated through the prosess of phagocytosis by nearby phagocytic cell (Van Cruchten and Broeck, 2002; Elmore, 2007).

CONCLUSION

In conclusion, the TEM visualisation showed that the highest exposure concentration of CuSO4 is associated with the increasing numbers of hepatic ultrastructure abnormalities. This deleterious effect was related to the production and the reaction of ROS towards hepatocyte biochemical. *P. javanicus* liver ultrastructure could be an alternative and valuable sensitive indicator of metal toxicity especially copper. Thus, further study is needed to quantify the regulation and the remaining activity of the anti-oxidant enzymes after in vivo exposure with different CuSO4 concentrations.

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