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Calcium Pre-Exposure Reducing Histopathological Alteration in Nile Tilapia (*Oreochromis niloticus*) After Lead Exposure

[1,2]P. Singhadach, [3]W. Jiraungkoorskul, [4]T. Tansatit, [3]P. Kosai and [5]C. Ariyasrijit
[1]Center for Environmental Health, Toxicology and Management of Chemicals,
CHE: 3328 Si Ayutthaya Road, Bangkok 10400, Thailand
[2]Toxicology Graduate Program,
[3]Department of Pathobiology, Faculty of Science, Mahidol University,
Bangkok 10400, Thailand
[4]Faculty of Veterinary Medicine,
[5]Mahidol University International College, Mahidol University,
Salaya Campus, Nakhonpathom 73170, Thailand

Abstract: This study was evaluated the influence of calcium to reduce the toxicity of sub-lethal lead concentration in Nile tilapia with emphasis on histopathological analysis. The values of 24, 48, 72 and 96 h LC_{50} of lead to tilapia were 247.51, 197.47, 183.74 and 182.38 mg L^{-1}, respectively. Fish were pre-exposed to vary dosages of calcium carbonate: 0 (G1 and G2); 20 (G3 and G4) and 60 (G5 and G6) mg L^{-1} for 4 days. After that, fish were post-exposed to 45 mg L^{-1} lead, which correspond to 25% of the 96 h LC_{50} (G2, G4 and G6) for 96 h. Histopathological changes were especially most evident in the group (G2) exposed to lead without calcium pre-exposure. The gills were observed edema, lamellar cell hyperplasia, epithelial lifting, lamellar fusion and aneurysm. There were blood congestion in sinusoids, vacuolation of hepatocytes and necrosis. Glomerulus's atrophy, tubular swelling and also necrosis were seen. However, the only observable lesion in the muscle was the infiltration of inflammatory cells and there were no histopathological changes observed in the brain and intestine of the lead treated fish. Fish with pre-exposed calcium (G4 and G6) showed slightly alteration when compare the only lead treatment groups. The results suggested that calcium pre-exposure may play an important role in the reduction of lead toxicity in fish.

Key words: *Oreochromis niloticus*, Nile tilapia, lead, calcium pre-exposure, histopathology

INTRODUCTION

Heavy metal toxicity has been an on going problem worldwide. The metal of interest for this study is lead (Pb), which is quite prevalent in the environment from batteries and lead-based paint as well as in water from industrial dumps. It thrives in the marine environment and is also accumulated into aquatic organisms such as fish, which are consumed by the masses of population (WHO, 1995). The problem of lead contaminated surface water and sediment in Klity Creek, Kanchanaburi Province, Thailand was reported in April 1998. The Pollution Control Department monitored the lead contamination along Klity Stream by sampling water, sediment and aquatic animals. These results showed that lead level in water before passing through mining area was low but increased after passing through mining (National Environment and Health Action Plans, 2008). Lead contamination of surface

Corresponding Author: Dr. Wannee Jiraungkoorskul, Department of Pathobiology, Faculty of Science, Mahidol University, Rama VI Road, Bangkok 10400, Thailand
Tel: (66) 02-201-5563 Fax: (66) 02-354-7158

water was in the range of 0.17-0.40 mg L^{-1}, much higher than the standard of 0.05 mg L^{-1}. For sediment, lead levels were in the range of 38,900-65,771 mg kg^{-1}, 20-100 times higher than the area without mining. Lead levels in fish were ten times higher than allowable standard in food of 1 mg kg^{-1}. Villagers, who relied on the water downstream or consumed fish from the contaminated water often experienced health impacts.

Waterborne lead causes the disruption of Na^{+}, Cl^{-} and Ca^{2+} regulation, disruption in hemoglobin synthesis and induction of spinal deformities. Generally, divalent metals such as lead, cadmium and zinc are considered Ca^{2+} antagonists. The earlier study in our laboratory, Nile tilapia (*Oreochromis niloticus*) were fed with 20 and 60 mg Ca^{2+} g^{-1} fish food and then exposed to 45 mg L^{-1} of waterborne Pb for 30 days, had reduced Pb tissue burdens (Lamchumchang *et al*., 2007). Similarly, several recent studies (Baldisserotto *et al*., 2005; Franklin *et al*., 2005) have shown that dietary Ca^{2+} is protective against the uptake of both waterborne and dietary cadmium, as well as against the uptake of waterborne zinc (Niyogi and Wood, 2006). Franklin *et al*. (2005) explained the interaction between calcium and cadmium (waterborne and dietary) in rainbow trout (*Oncorhynchus mykiss*). They concluded that the gut wall forms an important protective barrier reducing cadmium accumulation into internal tissues and suggested that Ca^{2+} and cadmium share common pathways/transport mechanisms in the gill and gut and that increased gastrointestinal Ca^{2+} uptake likely caused down regulation of branchial and gastrointestinal Ca^{2+} and therefore cadmium uptake pathways.

The objective of the present study was to evaluate the use of calcium pre-exposure as a protective agent against waterborne lead toxicity for Nile tilapia, *Oreochromis niloticus*, via histopathological analysis.

MATERIALS AND METHODS

Animals

This study was performed at the Department of Pathobiology, Faculty of Science, Mahidol University, Bangkok, Thailand, in 2008. Nile tilapia, *O. niloticus*, 10-20 g in body weight and 7-10 cm in total length, were purchased from a commercial hatchery in Thailand. The physicochemical characteristics of water were measured daily, according to the experimental procedures described in standard methods for the examination of water and wastewater (American Public Health Association, 2005). Acclimatization to laboratory conditions for 30 days was done using dechlorinated tap. Fish were fed twice a day with commercial fish food. The quantity of food was 2% of the initial body weight per day.

Experimental Design

Fish (n = 60) were randomly divided into six groups. Each fish was transferred to each aquarium as follows:

- **G1 and G2:** Without calcium pre-expose groups
- **G3 and G4:** With low calcium (20 mg L^{-1}) pre-expose groups
- **G5 and G6:** With high calcium (60 mg L^{-1}) pre-expose groups

After 4 days pre-exposure, fish in G2, G4 and G6 were post-expose with lead. The 96 h LC_{50} value of Nile tilapia exposed to Pb was determined in present laboratory as 182.38 mg L^{-1} (Lamchumchang *et al*., 2007). In this study, fish were exposed to 45 mg L^{-1}, which correspond to 25% of the 96 h LC_{50}.

After 96 h Pb post-exposure, fish from each group were anesthetized with 200 mg L^{-1} ethyl-3-aminobenzoate methanesulfonate salt, weighed and measured. The organs were removed and prepared for histopathological analysis.

Specimen Preparation for Light Microscopic Studies

Small pieces of tissues i.e., gills, liver, kidney, muscle, spleen, brain and intestine were fixed in the 10% buffered formaldehyde for 24 h, dehydrate through a graded series of ethanol and clear with xylene solutions. They were embedded in a block using melted paraffin at the embedding station. The paraffin blocks were sectioned at 4-5 μm thickness using a rotary microtome and stained with hematoxylin and eosin. The tissue glass slides were examined for abnormalities by a Nikon E600 light microscope and photographed by a Nikon DXM 1200 digital camera (Humason, 1972).

RESULTS

Gills (Control Group)

The gills consisted of a row of long thin filaments, the primary lamelae, which projected from the arch like the teeth of a comb. The surface area of each primary lamella was increased further by the formation of regular semilunar folds across its dorsal and ventral surface, the secondary lamellae. The primary lamellar epithelium was one or two cell layers thick. Chloride cells were identified as large epithelial cells with light cytoplasm, usually present at the base of secondary lamellae. Each secondary lamella was made up of two sheets of epithelium delimited by many pillar cells, which were contractile and separated the capillary channels. One to two erythrocytes were usually observed within each capillary lumen. No recognizable changes were observed in the gills of the control (G1) and Ca^{2+} pre-exposed (G3 and G5) throughout this experiment (Fig. 1A, C and E).

Treated Groups

A variety of histological studies revealed that lead affected gill tissue (G2). The lesions included hyperplasia, epithelial lifting or cell swelling and congestion. A typical chronology of damage from acute exposure to the tested substance started with the bending of the distal extremities of secondary lamellae, followed by a lifting of the outer layer of the lamellar epithelium, the formation of edematous

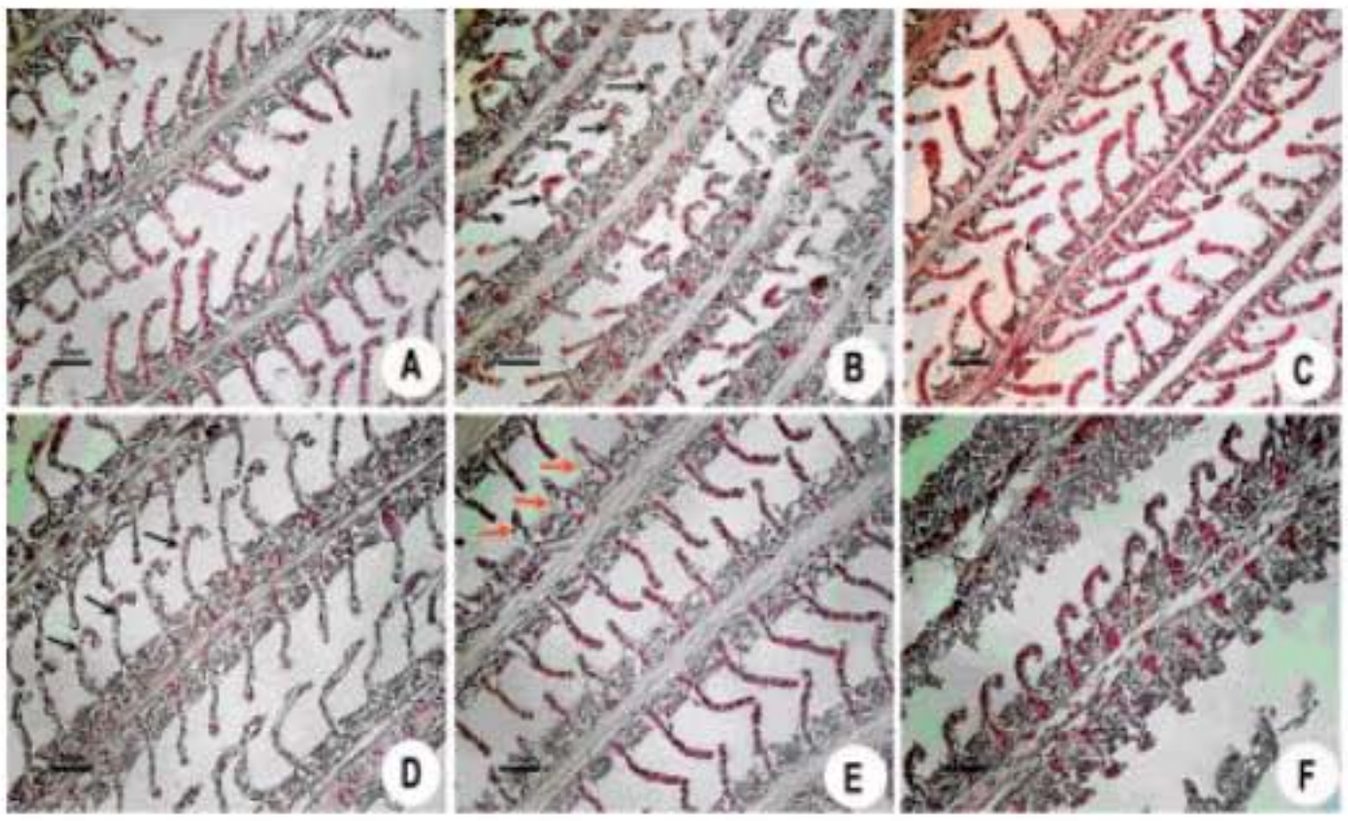

Fig. 1: Light micrographs of gill of *O. niloticus* in (A) control group showing normal arrangement of primary and secondary lamellae, (B-F) group 2 to 6, respectively. Black arrows in B and D for the example of the bending of the distal extremities of secondary lamellae. Red arrows in E for the example of the epithelial lifting

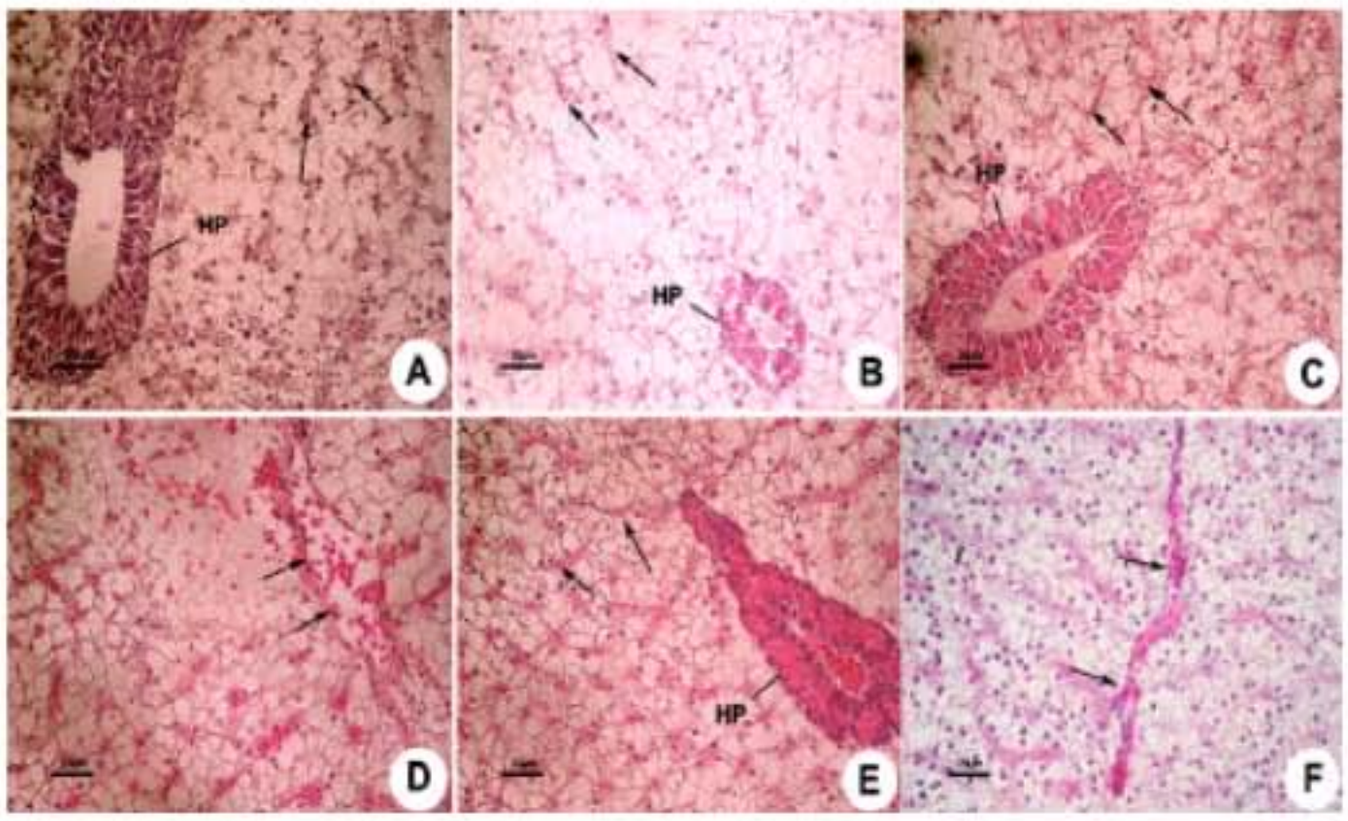

Fig. 2: Light micrographs of liver of *O. niloticus* in (A) control group showing normal hepatocytes and blood sinusoids, (B-F) group 2 to 6, respectively. HP: Hepatopancreas, v: Hepatic vein, Arrows: Sinusoids

spaces between the layers of epithelium which may become infiltrated with red blood cells and leukocytes. Finally, hyperplasic tissues were observed in the primary epithelial cells (Fig. 1B). Eventually, the whole epithelium sloughed off and the lamella lost its rigidity. Both G4 and G6 showed similar but less severe alterations than those of G2 (Fig. 1D, F).

Liver (Control)

The histology of fish liver differed from mammals in that there were far fewer tendencies for the deposition of hepatocytes in cords or lobules. Sinusoids, which were irregularly distributed between the polygonal hepatocytes, were fewer in number and were lined by endothelial cells with very prominent nuclei. Hepatocytes were polygonal and had a distinct central nucleus with densely staining chromatin margins and a prominent nucleolus (Fig. 2A).

Treated Groups

The hepatocytes in G2 began to swell and their vacuolization was observed. They showed congestion and exhibited increasing size and pyknotic nuclei in some area (Fig. 2B-F). The hepatocytes in G4 and G6 revealed similar alteration as G2, but the conditions were less severe.

Kidney (Control)

The kidney was located in a retroperitoneal position up against the ventral aspect of the vertebral column. It was a dark brown organ normally extending the length of the body cavity. It was divided into an anterior or head kidney largely composed of hemopoietic elements and a posterior or tail or excretory kidney. The nephron of the typical freshwater fish was composed of a well-vascularized glomerulus, proximal segments, distal segments and collecting duct system (Fig. 3A).

Treated Groups

There found to be some atrophy glomerulus and renal tubular necrosis in G2. Likewise, in G4 and G6 displayed similar alterations as those observed in G2, but they were less severe. Furthermore, there seemed to be a few hemosiderin accumulations in some area for a longer period of time (Fig. 3B-F).

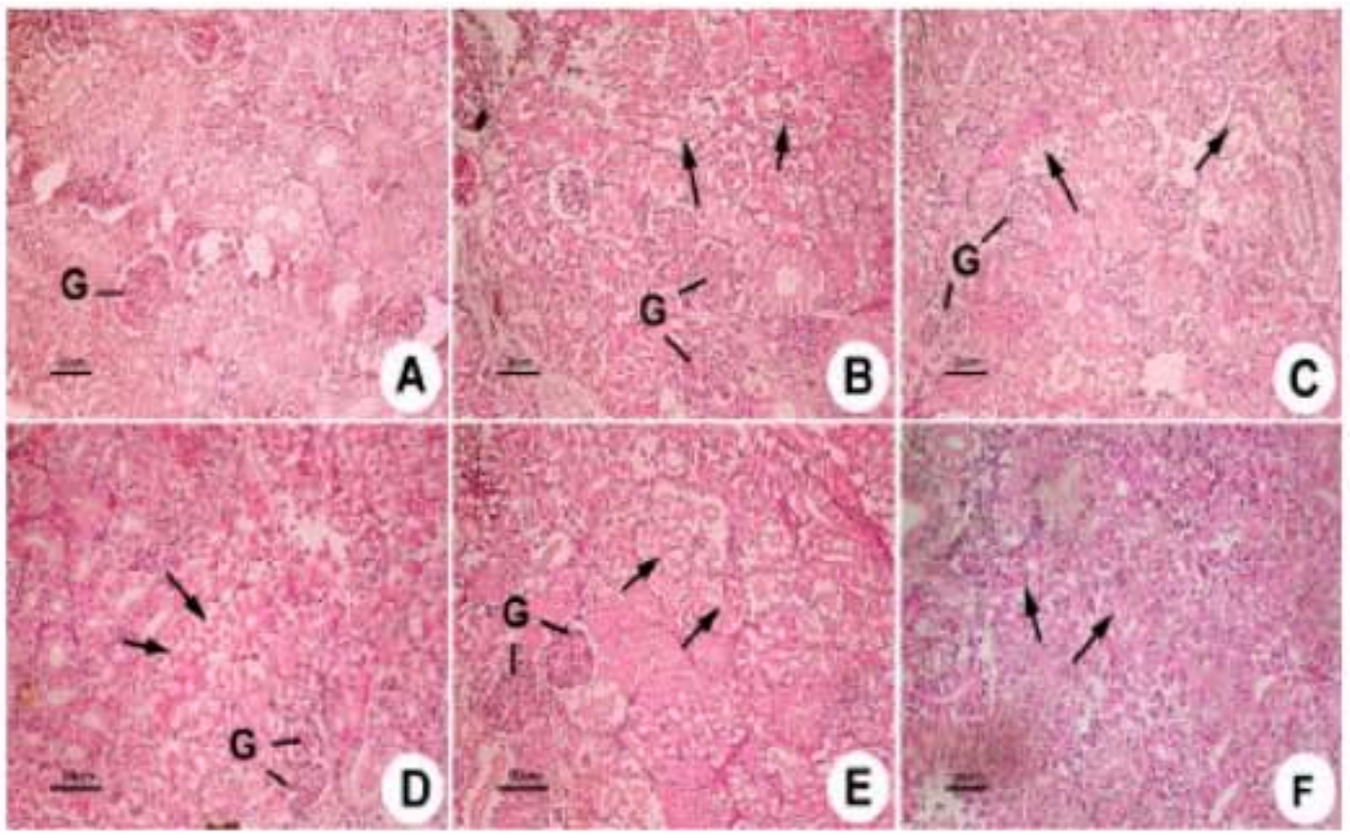

Fig. 3: Light micrographs of kidney of *O. niloticus* in (A) control group showing glomerulus (G) and renal tubules; (B-F) group 2 to 6, respectively, showing renal tubular necrosis (arrows)

Muscle (Control Group)

Histological examination of the myomeres had revealed a range of two main subdivisions of fiber types: the muscularis lateralis superficialis, consisting of the so-called red muscle fibers and the muscularis lateralis profundus, consisting of the white fibers. The red fibers were aerobic, slow-contractile fibers, similar to their counterparts in mammalian muscle and the white fibers were anaerobic, fast-contractile and fast-fatiguing fibers. Muscle cells had an elongated and cylindrical shape and were multinucleated. The nuclei of these muscles were located in the peripheral aspect of the cells (Fig. 4A).

Treated Groups

No recognizable changes were observed in the muscle of the experimental groups (Fig. 4B-F), except the infiltration of moderate inflammatory cells in G4 (Fig. 4D).

Spleen (Control Group)

The main elements of the spleen were the ellipsoids, the pulp and the melanomacrophage centers. Ellipsoids were the thick-walled filter capillaries which resulted from the division of the splenic arterioles. The splenic pulp consisted of sinusoid phagocytic tissue similar to that of the kidney, in which a large number of red blood cells might be held. Melanomacrophage centers, similar to those of the kidney, were usually located close to a vessel. Hemosiderin was sometimes present in the splenic corpuscle (Fig. 5A).

Treated Groups

No recognizable changes were observed in the spleen of the experimental groups, except a large number of megakaryocytes (Fig. 5B-F).

Brain (Control Group)

The brain was similar in its basic components to the brain of higher animals, but with many differences in form and complexity. For ease of description, the brain was usually divided into five

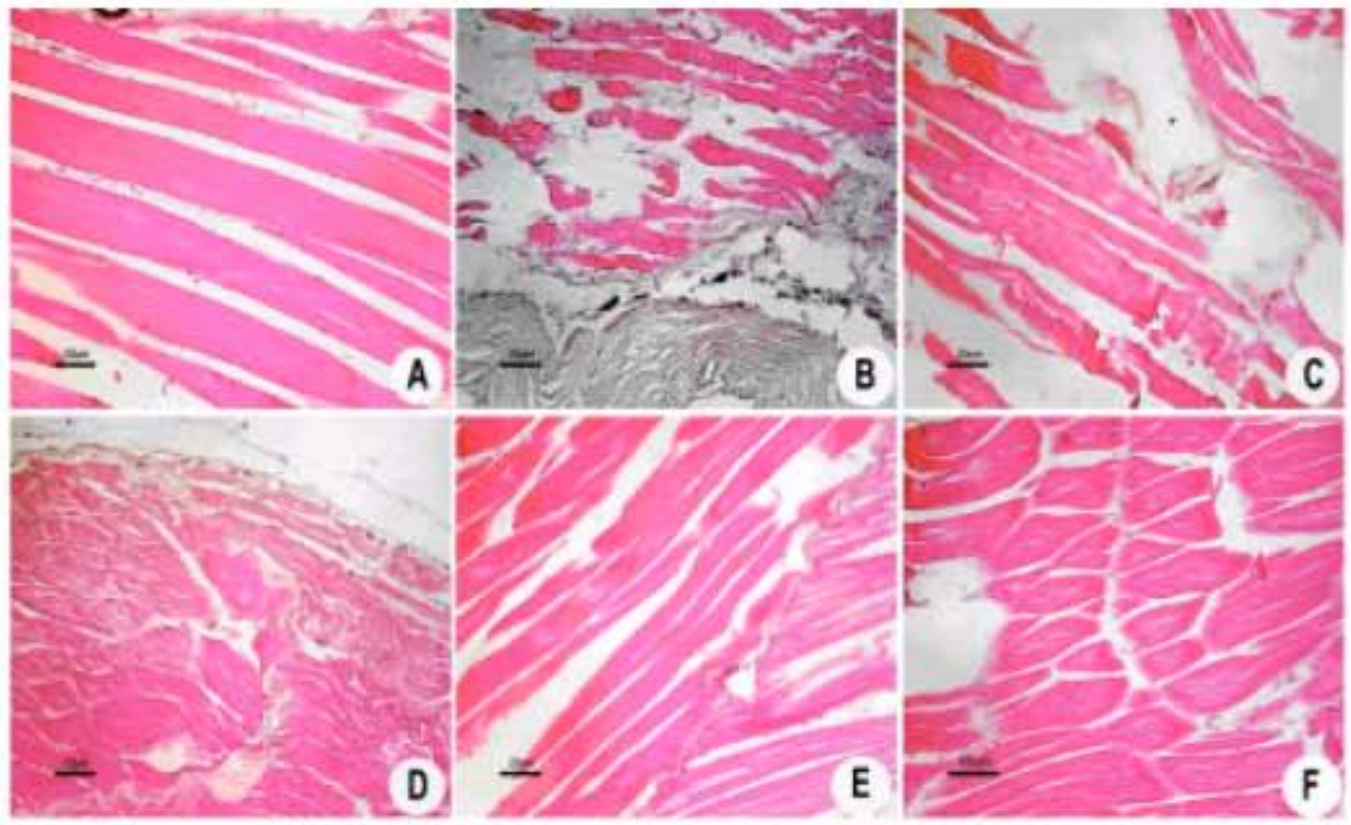

Fig. 4: Light micrographs of muscle of *O. niloticus* in (A) control group, (B-F) group 2 to 6, respectively

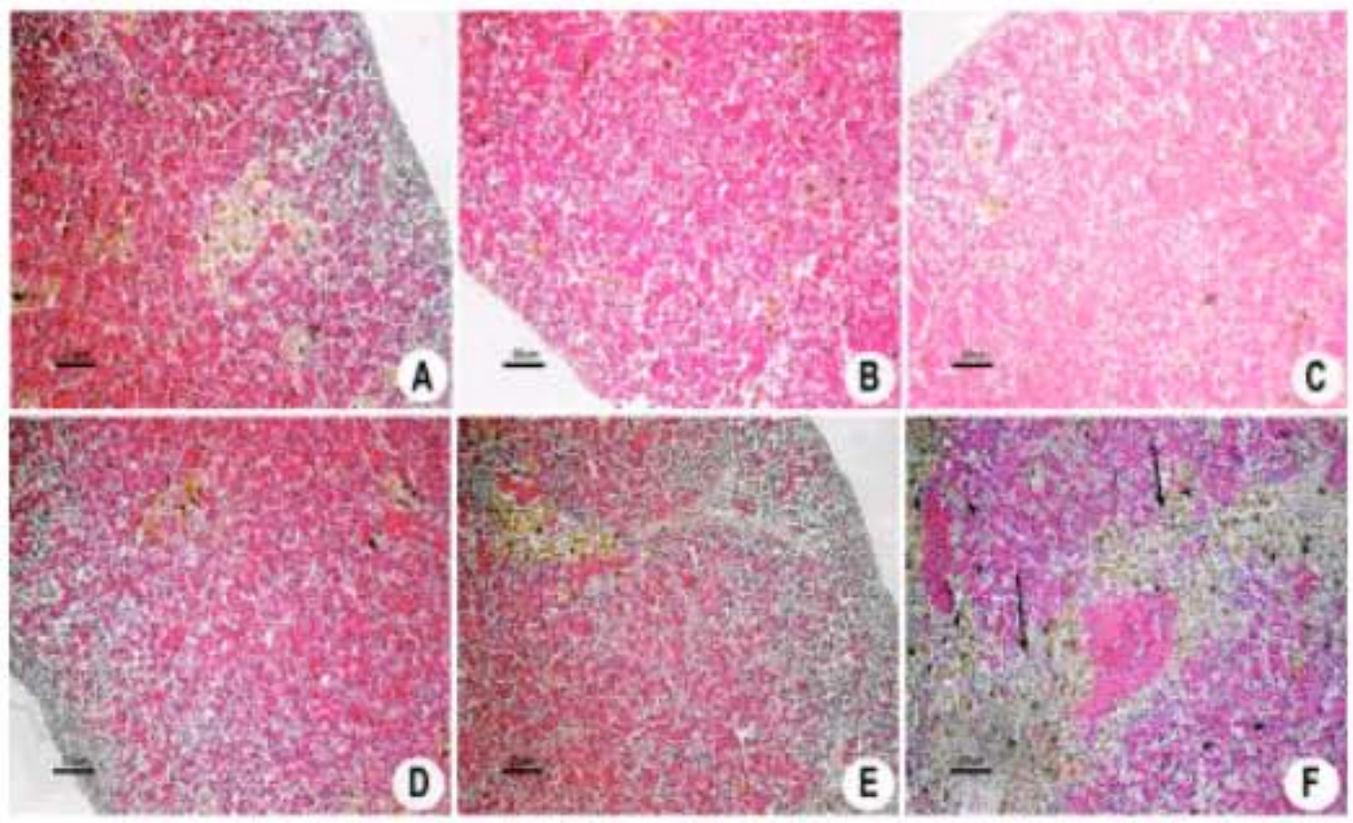

Fig. 5: Light micrographs of spleen of *O. niloticus* in (A) control group, (B-F) group 2 to 6, respectively. Arrows: A large number of megakaryocytes

divisions comprising of, from the anterior: telencephalon, diencephalon, mesencephalon, cerebellum and medulla oblongata. The cerebellum seemed to function in maintenance of muscle tone, postural reflexes and integration of stimuli from the eyes and acousticolateralis organs. It developed from the embryonic metencephalon and was composed of the corpus cerebelli and the valvula. The corpus cerebelli had a very small ventricle which was lined by ependymal cells surrounded by a granular (G) layer composed of small dark staining nuclei. The outer portion of the corpus cerebelli was composed of the molecular (M) layer consisting of fibers and a few nuclei (Fig. 6A).

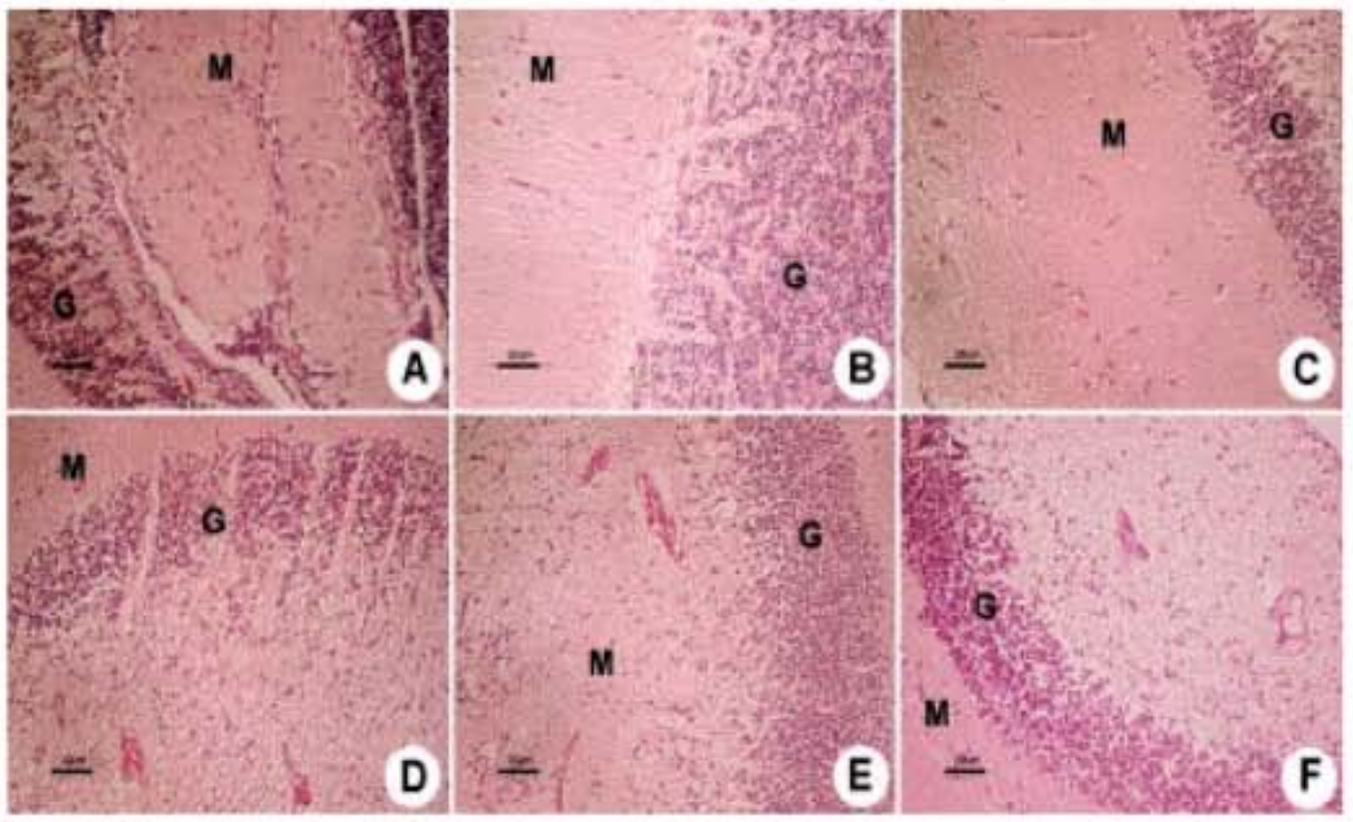

Fig. 6: Light micrographs of brain of *O. niloticus* in (A) control group, (B-F) group 2 to 6, respectively. Granular (G) layer composed of small dark staining nuclei, molecular (M) layer consisted of fibers and a few nuclei

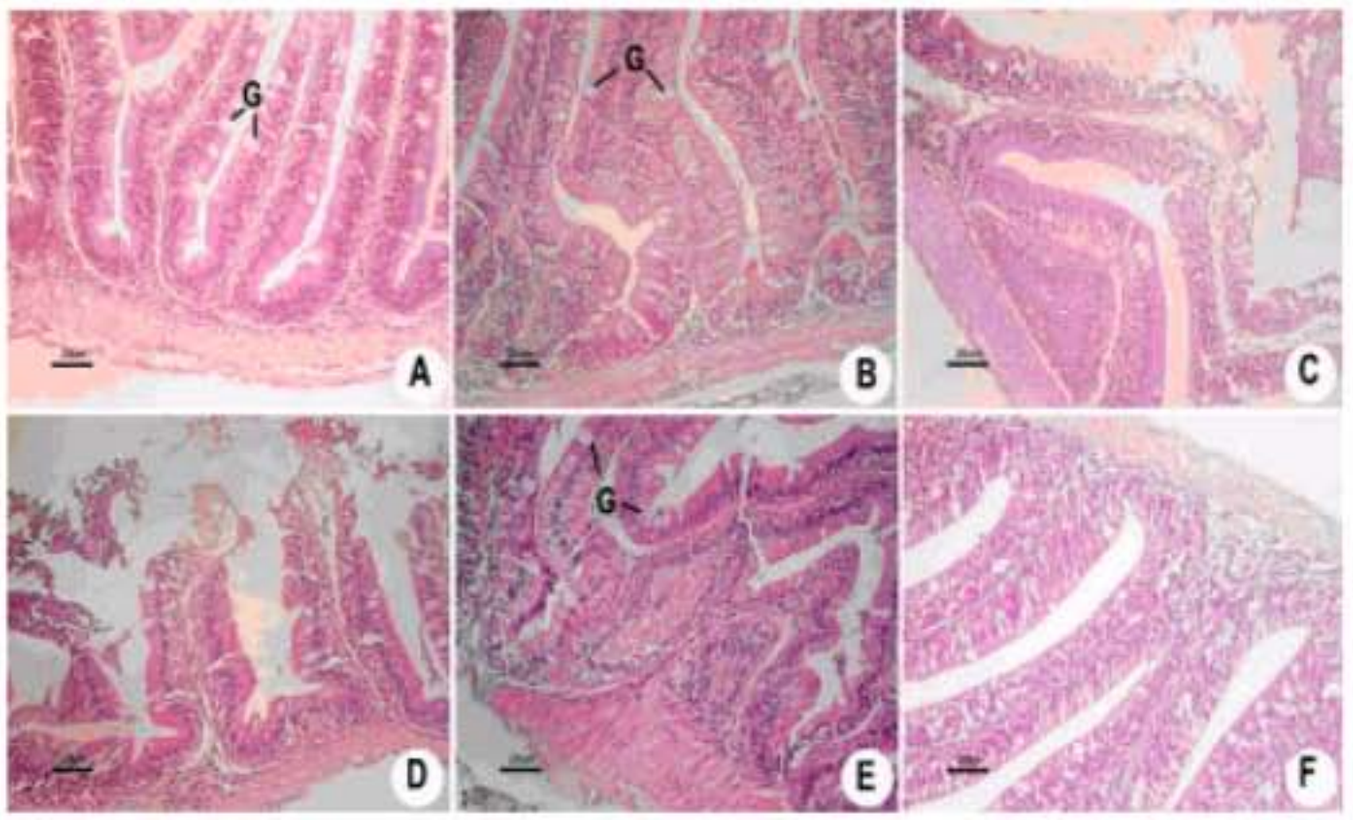

Fig. 7: Light micrographs of intestine of *O. niloticus* in (A) control group, (B-F) group 2 to 6, respectively. G: Goblet cells

Treated Groups

No recognizable changes were observed in the brain of the experimental groups (Fig. 6B-F).

Intestine (Control Group)

The intestine had simple columnar epithelium with scattered goblet cells. A thin lamina propria was part of the mucosa but was not clearly separated from the submucosa. Tall longitudinal folds, which often branched, projected into the lumen. The muscularis had inner circular and outer

longitudinal layers of smooth muscle. The serosa was composed of a very thin layer of connective tissue covered by mesothelium. The mucosa of the intestine had tall columnar epithelium with a striated border and nuclei near the center of the cell (Fig. 7A).

Treated Groups

No recognizable changes were observed in the intestine of the experimental groups (Fig. 7B-F).

DISCUSSION

In this study, histopathological alterations were observed in the gills, liver and kidney of the lead treated groups (G2, G4 and G6). These alterations were less severity in the calcium pre-exposure group (G4 and G6). It may suggest that calcium can play protect the lead toxicity in fish tissue. The gills were seen edema, lamellar cell hyperplasia, epithelial lifting, lamellar fusion and aneurysm. A number of studies have shown that significant gill lead burden occurs after both acute exposure (Palaniappan *et al*., 2008) and chronic exposure to sublethal concentrations of waterborne lead (Grosell *et al*., 2006). These obtained results agree with our earlier study in *Puntius altus* exposed to 10 mg L^{-1} cadmium for 96 h, *Poronotus triacanthus* exposed to 25 μg L^{-1} copper for 7 days were found filament cell proliferation, increase in intercellular spaces, epithelial lifting and thickening of the filament and lamellar epithelium (Jiraungkoorskul *et al*., 2006, 2007). However, fish with pre-exposed calcium (G4 and G6) showed slightly alteration when compare the only lead treatment group (G2). The outcomes of the present study came in agreement with those of Abdel-Tawwab *et al*. (2007), who reported that juvenile Nile tilapia which pre-exposed to 100 mg L^{-1} calcium oxide reducing the toxicity of 0.503 and 1.25 mg L^{-1} copper sulfate post-exposure for 6 weeks. The nature of the stimulus that triggers the morphological transformation of the gill during lead exposure is unknown. In general, lamellar epithelial lifting, hyperplasia and lamellar fusion are nonspecific responses that are known to be induced by many gill tissue irritants (Mallatt, 1985). However, focal points of cellular hypertrophy and necrosis followed by epithelial rupture reflect the direct deleterious effects of heavy metals in fish gills (Mallatt, 1985). All these lesions may impair respiratory function. Lifting or hyperplasia of epithelium results in an increase in the diffusion distance, thus affecting exchange of gases.

The histopathology showed that lead caused some alterations of the liver parenchyma, like blood congestion in sinusoids, vacuolation of hepatocytes and necrosis. Several studies had shown a variety of changes in the liver of *O. niloticus*, resulting from exposure to different toxic chemicals (Figueiredo-Fernandes *et al*., 2007; Jiraungkoorskul *et al*., 2002, 2008). A common morphologic response of the fish liver to toxicity is an accumulation of hepatic glycogen and/or lipid (Wolf and Wolfe, 2005). Lipid or glycogen vacuolization can cause an increase in the size of hepatocytes; however, Hinton *et al*. (2001) identified three additional potential causes of hepatocellylar enlargement: organelle proliferation (hypertrophy); the failure of sublethally-injured hepatocytes to mitotically divide (megalocytosis) and vacuolar swelling of the endoplasmic reticullum cisternae (hydropic degeneration). Moreover, a clearly pathologic response of the fish liver to anxious substance is hepatocyte necrosis (Myers *et al*., 1987; Wolf and Wolfe, 2005). In addition, these damages were less severity in the pre-exposed calcium groups suggesting that calcium did play a role in reducing the lead toxicity.

Glomerulus's atrophy, tubular swelling and also necrosis were seen in the lead alone treated group. In the earlier studies, creatinine and uric acid showed a significant increase in *O. niloticus* exposed to copper. These results may be due to the action of copper on glomeruli filtration rate and/or copper may cause pathological changes of the kidney resulting in dysfunction (Abbas *et al*., 2002; Abdel-Tawwab *et al*., 2007). Because, the excretion of divalent ions is a major function of the renal tubular epithelium, pollution with heavy metals would be highly likely to affect these cells. Fish with

pre-exposed calcium showed slightly alteration when compare the only lead treatment groups. The results suggested that calcium pre-exposure may play an important role in the reduction of lead toxicity in fish. However, the only observable lesion in the muscle was the infiltration of inflammatory cells and there were no histological changes observed in the brain and intestine of the lead treated fish.

In conclusion, these results show that the severity of the lesions are lesser in calcium pre-exposed fishes, however the mechanism is not know

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