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Histopathological Changes in the Gills of *Oreochromis mossambicus* Exposed to Mercury Chloride ($HgCl_2$)

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ABSTRACT
Most of the fish deaths caused by pollutants demonstrate gills damage, since it located outside the body that directly exposed to water as a medium of life, thus this organ is the first to be affected if pollutants contaminated water environment. The objective of this research was to find out histopathological changes of tilapia fish (*Oreochromis mossambicus*) gills exposed to mercury chloride ($HgCl_2$). This study used 36 male tilapia fish weight of 200-300 grams collected from farmer's ponds in Cadek, Aceh Besar, which grouped into 4 treatment groups with 3 replications. The fish in PO group was considered as negative control group, while fish in PI, P2, and P3 were exposed to 0.25, 0.50, and 0.75 ppm of $HgCl_2$, respectively, for 10, 20, and 30 consecutive days. Fish were sacrificied to collect the gills then followed by histopathological examination. The results showed that fish in control group (P0) demonstrated normal histological structure of gills containing primary lamella, secondary lamella, interlamellar cell, erythrocyte, and pillar cell. Meanwhile the fish gill exposed to HgCl2 showed several alterations such as edema, congestion, primary and secondary lamella hyperplasia, and secondary lamella fusion in all treatment groups. To conclude, $HgCl_2$ exposure caused gills damage histopathologically, which more severe along with the increasing of $HgCl_2$ concentration and the longer time of exposure.

Keywords: *Mercury chloride, Gill, Histopathology, Lamella fusion, Edema*

1. INTRODUCTION

Mercury (Hg) is a water pollutant harmful to the environment and may accumulate in aquatic organisms [1]. Mercury waste in public waters is converted by the activity of microorganisms into methyl-mercury (Me-Hg) components, which have toxic properties, strong binding capabilities and high solubilities, especially in aquatic animals bodies [2,3]. Mercury not only pollutes water bodies but also accumulates in sediments and in fish bodies and other aquatic biota. As in the case of mercury pollution in Minamata Bay, Japan, mercury content in shells found in contaminated area reaches 1.7-6 mg /L, while in the fish collected in uncontaminated area ranges from 0.01-1.7 mg /L, and aproximately 10-55 mg/L in polluted areas [4].

The Food and Drug Administration (FDA) limits a maximum threshold for mercury content of 0.0005 ppm for water and 0.5 ppm for food and the World Health Organization (WHO) sets a lower maximum limit of 0.0001 ppm for water. Japan, Switzerland, Sweden determine a threshold for 1 ppm of mercury in marine products that may be consumed, while Germany and United States governments allow 0.5 ppm (mg/kg) [2, 5].

Mercury is a neurotoxin that enters the aquatic ecosystem through atmospheric deposition or comes from externalization of industrial wastes [2]. This compound cannot be degraded by aquatic organisms, but it is fat-soluble (lipophilic). Fat-soluble metals are able to penetrate the cell membrane, and eventually mercury metal ions will accumulate in cells and other

organs such as liver, kidneys, meat, and gills [5]. Metal accumulation in fish begins with the process of entering through the gills, absorbed into all body tissues, and accumulated. Various factors influence the process of Hg uptake and accumulation; include metabolic rate, size and type, alkalinity and pH. In addition, the demethylation process, temperature, contamination level, time, source and form of Hg, as well as life level of organisms significantly affect the process of 70% Hg intake through food and absorption into fish body tissue [6].

Most of the fish deaths caused by pollutants occur due to damage to the gills and gill associated-organs, since the thin epithelium of the gills directly contacts to dissolved and suspended polluted water as a medium of life [7, 8]. The minor damage to the gills can disrupt the function of the gills as a regulator of osmosis, leading to breathing disorder. For instance, blood flow congestion due to physical trauma, contaminants, or disturbance of the circulatory system in the lamellae result in edema or swelling of the cells around the blood vessels. Congestion and edema will reduce the efficiency of gas diffusion because the absorption surface area of the secondary gill lamellae will be narrowed [9].

Previous research have reported several histopathological changes of gills exposed to heat and pollutant (acids, ammonia, heavy metals, and pesticides). This lead to changes in the structure of chloride cells [10], lamellar hyperplasia which occurs inline with an increase in the number of mucosal cells at the base of the lamellae and results in lamellae fusion [11], congestion and edema [12], and necrosis [13]. Fish that are exposed to heavy metals, detergents, and pesticides undergo hyperplasia with the separation between epithelial cells and the pillar cells which lead to secondary lamella structure damage and increase in the amount of chloride cells [14, 15].

Mercury exposure in fish for a long time period causes structural and functional abnormalities such as hyperactivity and skin irritation, respiratory disorders, balance disorders, gasping, blackened skin discoloration, making sudden and rapid movements, circling movement, swimming backwards, excess mucus and ending in death [3]. Furthermore, it can also cause changes in the histological conditions of several organs such as liver, kidneys, and gills [16].

2. MATERIALS AND METHODS

2.1. Experimental Animal

The experimental animals used in this research were 36 male tilapia fish (*Oreochromis mossambicus*) weighed 200-300 grams collected from Cadek village, Aceh Besar

2.2. Research Design

This research implemented a completely randomized design with a 3x4 factorial experimental arrangement with 3 replications. P0 was control group without exposed to $HgCl_{2;}$ while P1, P2, and P3 were exposed to $HgCl_2$ at concentration of 0.25 ppm, 0.5 ppm, and 0.75 ppm, respectively. The treatments were given for 30 consecutive days and the gills sampling was performed on 10^{th}, 20^{th}, and 30^{th} day. All fish were eutanized using clove oil at dose of 0.15 ml/liter, then subsequently necropsied to collect the gills for histopathological preparations.

2.3. Histological Preparations

Gills samples were fixed in Davidson's solution for 48 hours, dehydrated in rising concentration of ethanol (70%, 80%, 90%, and 96%), cleared using xylene, infiltrated and embedded in paraffin, sliced with a thickness of 5 um using a rotary microtom. The tissues were stretched out in water at temperature of 50 ºC, mounted on an object glass, incubated for 24 hours at 37ºC, and stained with hematoxyline-eosin (H&E) [17]. The slides were observed using a light microscope, and the histopathological changes found were documented using a photomicrograph. The parameters observed in this study were edema, secondary lamellar hyperplasia, congestion, secondary lamella necrosis, and secondary lamella fusion.

3. RESULTS AND DISCUSSION

The gill of tilapia fish in control group (P0) demonstrated normal histological structure with intact primary lamella, secondary lamella, interlamellar cel, erytrhocyte and pillar cell as shown in Figure 1. Fath El-Bab [18] stated that the gills of a typical teleost comprise two sets of four holobranches consisting of two hemibranches each. Hemibranches comprise primary and secondary lamellae with vascularization, epithelial, interlamellar, pillar, and chloride cells. The chloride cells were not observed in this research since the fish used were fresh water fish. The chloride cells were usually found in marine fishes.

Figure 1 revealed the primary lamellae consist of epithelial tissue, cartilage, and a vascular system. The secondary lamellae protrude along the entire length of the primary lamellae, consisting of blood arterioles through which oxygen exchange with the surrounding water takes place. The capillary lumens separated by darkly stained pillar cells, which served as a structural support for the secondary lamellae. The lamellae are enclosed by an epithelial cell layer that supported by a basal membrane. Large spherical mucous cells are present and occur randomly throughout the gill epithelium. The mucous cells are cloudy and grey in coulour after hematoxilin and eosin staining [19].

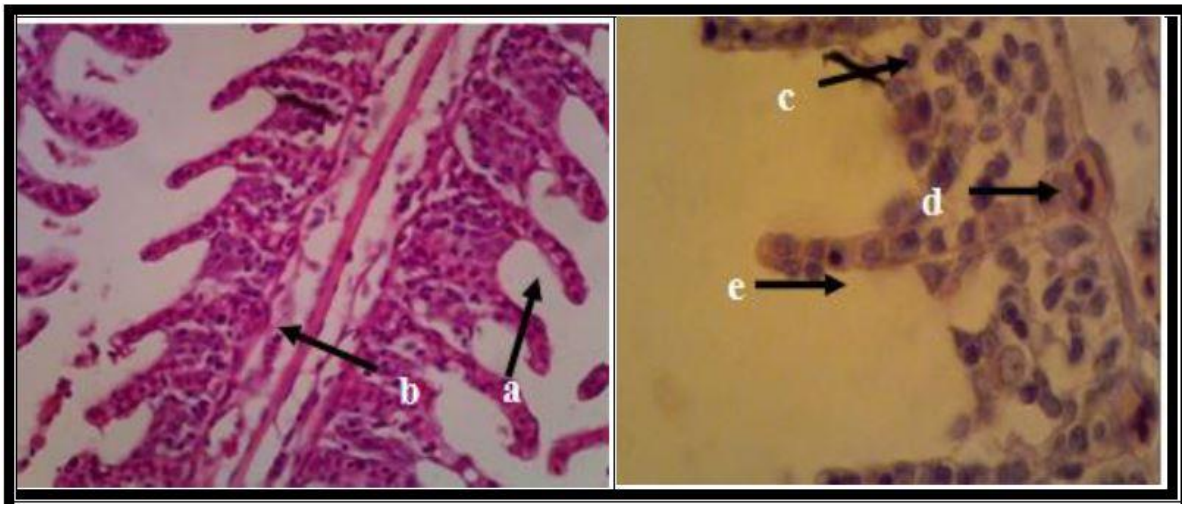

Figure 1. Histological figure of tilapia fish gills in control group shows a. secondary lamellae, b. primary lamellae, c. interlamellar cell, d. erythrocyte, e. pillar cell (HE 400x; 1000x).

The histopathological examination demonstrated that exposure of 0.25 ppm, 0.50 ppm, and 0.75 ppm $HgCl_2$ changed the cellular structure and morphology of tilapia gill. After 10 days, minimum changes observed in fish exposed to 0.25 ppm $HgCl_2$ was edema that may lead to erosion of epithelial cells. On day 20 and 30, necrotic cells, congestion, rupture of epithelial cells, and hyperplasia were also observed. Similarly, the gill of fish in group P1 did not show any alteration. The gill of fish exposed to 0.50 ppm $HgCl_2$ showed congestion and erosion of epithelium cells (day 10), congestion and fusion of secondary lamellae (day 20), and hyperplasia, congestion, and necrosis (day 30). The fish exposed to 0.75 ppm $HgCl_2$ showed histopathological changes in the form of congestion and hyperplasia of epithelial surface (day 10), detachment of epithelium from underlying pillar system, hyperplasia of secondary lamellae, congestion, and fusion of secondary lamellae (day 20), severe erosion of epithelial cells, congestion and fusion of lamellae (day 30). All histopathological changes of tilapia fish gills exposed to various concentration of $HgCl_2$ were shown in Figure 2. This results proved that the length of exposure time of $HgCl_2$ affected histopathological changes of tilapia gill, the higher the dose the higher the level of histopathological changes.

Edema of the respiratory lamellae is the early symptom resulting from exposure to substances such as heavy metals, pesticides, and therapeutics (e.g. hydrogen peroxide, formalin), as well as the effect of water acidification after acid rainfall or irritation by various suspensions [28]. Edema is a swelling of cell occurs due to an excessive accumulation of fluid in the tissues and may cause the separation of epithelial layer from the underlying systems mast cells. This could lead to the destruction of gill secondary lamellae structure (epithelial lifting). This alteration is characterized by the detachment of epithelial cells of the secondary gill lamellae due to the outflow of serous fluids into the spaces of the gill tissue [19]. It is fish physiological adaptation form when experience interference from the environment.

Edema is often associated with the cell infiltrates and is a symptom of osmoregulation disorders that represents a kind of defense mechanism. At the terminal stage of edema, the respiratory epithelium separates entirely from both primary and secondary lamellae followed by necrosis of the epithelial cells, leading to respiratory and osmotic regulation disturbances and even death [20]. Elevation of the epithelium of gill lamella is an immediate effect of different toxic substances and reflects an acute inflammatory process in the gills. Separation of the epithelium from the supporting cells of the respiratory lamellae is a process that initiates necrosis of the gills. Edema of the gill lamellae with concurrent dilatation of the blood vessels often results from an accumulation of suspensions in the gills [21].

The edema observed in this study probably due to infiltration of toxic mercuric chloride into the gills which then irritated the cells and resulted in swollen cells. Mercuric chloride entering the gills together with other metal ions forms fat soluble-ions, which will penetrate the gill cell membrane and lead to the accumulation of metal ions in the gills. Otherwise, the loss of liquid regulation in the cells inhibit the rate sodium ion exchange, which in turn increase the sodium concentration and water deposition in the cells [20].

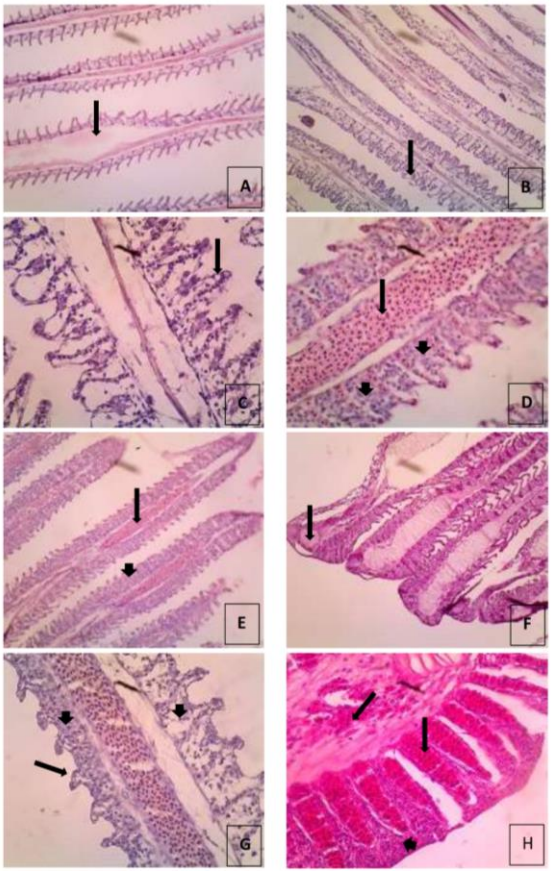

Figure 2. Histopathological changes observed in the gills of tilapia exposed to $HgCl_2$: A. edema in the primary lamellae (arrows), B & C epithelial detachment (arrows), D & E. congestion (arrow) and epithelial hyperplasia leading to complete lamellar fusion (arrow head), F. rupture of blood vessel forming hematoma (arrow), G. shortening of secondary lamellae (arrows) and necrosis, H. hemorrhage (arrow head) and cell hyperplasia in secondary lamellae (arrows) (H&E, 100x, 400x).

This result is in line with previous research reported by Dogan and Canli [9], that the occurrence of edema is caused by increased intra vascular hydrostatic pressure which causes blood plasma fluid to leak out and flow into the interstitial space. This might be caused by various pathologic conditions related to vascular permeability changes. Edema also occurred in a study reported by Mohammed *et al*. [8] that was caused by $HgCl_2$ in *Oreochromis niloticus* at the lowest concentration (0.16 ppm).

Congestion were observed in all treatments except for control group. The higher the concentration of mercury given and the longer the exposure time, the more severe the congestion that occurred in the gills. Congestion is an increase in the volume of blood in the blood vessels which lead to swelling of blood capillaries. The capilaries looks bulging and full of erythrocyte block the lumen [20].

Congestion occurs due to physical trauma or circulatory system disorders caused by the onset of inflammatory reaction after changes in the biochemical structure of cells by heavy metals. This damage results in disruption of the gills function in the process of respiration since blood flow which carries oxygen is obstructed. This condition leads to asphyxia, which eventually resulted in death if the exposure happened for a long time of period [22]. Raissy and Ansari [23] stated that congestion at the most severe level will cause blood vessels to burst or blood exit the cardiovascular circulation (arteries, veins and capillaries), which in turn will cause cell death or necrosis caused by trauma; biological agents such as viruses, bacteria, fungi, and parasites; chemical agents; and blood supply disruption in a particular area.

Congestion or blockage of blood vessels in gills can be triggered by the breakdown of cell structure pillars resulting in increasing blood flow in the lamellae and form a uniform space filled with blood lead to dilatation of the lamellae [20]. Congestion obtained in this study can be cause by metal contents in $HgCl_2$. Metal affect the permeability of cell membranes and lead to the occurrence of resistance in the ion exchange system, which in turn lead to there disruptions in the fluid transport into and out of cells.

Hyperplasia occurs in all fish gills in treatment groups except control. Hyperplasia is the formation of excess tissue due to an increase in the number of cells. Thus, part of the secondary lamellae will be covered due to the increase in the number of cells, lead to the disruption of oxygen exchange process in the gills [20]. Heavy metals that enter the body through the gills will bond with erythrocytes and circulate in the blood plasma to all organs in the body of the fish including the gills. Furthermore, the amount of oxygen bound by red blood cells in the gills is decrease, which causes the gills to adapt by actively moving to increase the amount of oxygen in the blood which plays a role in the metabolic process in the fish body [24].

Furthermore, Maftuch et al. [25] also states that hyperplasia is damage to fish with prolonged exposure to toxic chemicals in the form of heavy metals and also due to disruption of ion transport in cells. The observed hyperplasia in lamellar epithelium could increase the thickness of the filament epithelium which prevent or retard the access of toxic metals into the blood circulation. Such hyperplasia could provoke partial or complete fusion of the secondary lamellae, which could result in great disturbance of ionic regulation and gas exchange leading to the disturbance of fish health [26]. The observed lamellar vasodilation might be caused by the increased permeability due to the prolonged exposure to the metals, which could lead to

degeneration and necrosis. This change could be considered as general defense response to increase the space across which waterborne toxicants must diffuse to reach the blood vessels [27].

In this study, secondary lamella fusion was observed in treatment P2 (day 20) and P3 (day 20 and 30). These results are consistent with the research conducted by Selvanathan et al [14] which found lamella fusion on *Clarias batrachus* Linn exposed to mercury and cadmium. Lamella fusion is the attachment of two secondary lamellae due to excess mucus on the gills which cover the secondary lamellae. This excess mucus is secreted by mucus gland as one of the immune body responses to protect the gills from mercuric chloride, however this mechanisme also lead to oxygen uptake inhibition from water. Maftuch et al. [25] added that lamellar fusion is a condition of gas diffusion efficiency reduced due to damage of hyperplasia on the epithelial tissue of fish gills and fused secondary lamellae.

Lamella fusion occurs due to the continuous increase of hyperplasia and causes infiltration of new cells into the secondary lamellae spaces which then triggers adhesions on both sides of the lamella [20]. Lamellar fusion is a serious level of damage since it is an advanced stage of damage of hyperplasia. The effects of heavy metals, ammonia intoxication, excessively high or low water pH, and parasitic infestations that destroy the gill cells are assumed due to excessive proliferation. Poleksic et al [28] reported that chronic exposure to even diluted toxic substances causes proliferation of the cells between the respiratory lamellae. Adhesions of the respiratory lamellae are common and results from a change in the electrical charge of glycoproteins in the gill cells, which consequently attracts neighbouring lamellae, and causes hyperplasia and hypertrophy. Adhesions of the gill lamellae over a large area and destruction of the supporting cells are a sign of poor health status in fish [29]. In differential diagnostic, it is important that in comparison with other factors, the adhesions of the lamellae caused by external parasites, such as freshwater ick, are limited only to the locations of parasites on the gill surface [23].

Necrosis is the death of cells or tissue, characterized histopathologically by the unclear to disappear boundaries of cells and cell nuclei [20]. Necrosis occurs due to blood clots or severe congestion. In addition, necrosis also caused by the blood flowing into the veins has lost nutrients thus the cell loses its membrane integrity which results in the release of cell material and eventually cell death (necrosis) [11]. Necrosis occurred in all treatment groups, this result is in line with research conducted by Khosnood et al [13], which observed necrosis in Persian Sturgeon (Acipenser persicus) fish exposed to 15 ppb mercuric chloride for 15 days. Bose et al [30] stated that necrosis occurs due to hyperplasia and excessive secondary lamella fusion resulted in not intact of gill tissue due to the high concentration of mercury in the water which absorb and accumulate continuously in gill tissue. In this study, the lamellae was severely damaged which indicated severe water pollution.

Necrosis of the gills develops as a result of prolonged exposure to irritants include suspensions found in water. Choudary et al [31] reported that necrosis of the gill epithelial cells from lead toxicity. At the initial stage of necrosis, pyknosis or karyorrhexis are observed microscopically; these phenomena are typical of degenerative processes [20]. Necrosis is the final stage in the most advanced cases, necrosis may result in complete atrophy of the soft tissue covering the gill filaments and, consequently, in uncovering of the cartilaginous elements [19].

4. CONCLUSION

Histopathological changes of tilapia fish gills caused by mercury exposure are edema, congestion, hyperplasia, secondary lamellae fusion, and necrosis. The higer concentration and the longer time of $HgCl_2$ exposure the more severe the histopathological damage of tilapia fish gills.

AUTHORS' CONTRIBUTIONS

All authors read and approved the final manuscript.

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