Effect of mercury chloride to number of melano-macrophage centers on the kidney of carp fish (Cyprinus carpio)

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Abstract. Mercury chloride can cause immunotoxic effects on fish. The accumulation or aggregate of melano-macrophages centers (MMCs) in the kidney is a feature of cellular immune response, so it can be used as a bioindicator of heavy metal toxicity in waters. This research aims to determine an effect of heavy metal exposure concentration of mercury chloride (HgCl₂) on a number of MMCs from common carp kidney. This research using four treatments of mercury chloride: 0 ppm, 0.01 ppm, 0.05 ppm and 0.1 ppm. The main parameters were the number of MMCs in kidney common carp. Supporting parameter was behavior change, water quality and concentration of mercury in the water and kidney. The results of the research showed that the concentration the heavy metal exposure concentration of mercury chloride (HgCl₂) affected the number of MMCs from common carp (Cyprinus carpio) kidney. The number of MMCs in the kidney of common carp exposed to mercury chloride 0.01 ppm, 0.05 ppm and 0.1 ppm increased compared to carp was not exposed to mercury chloride (0 ppm).

1. Introduction
Heavy metals are part of industrial waste as a result of the pace of development in the industrial sector [1, 2]. Heavy metals that are toxic include mercury (Hg), cadmium (Cd) and lead (Pb) [3]. The heavy metal mercury can cause damage to the liver and kidneys of exposed organisms, especially the type of inorganic mercury that is mercury chloride (HgCl₂) which is also corrosive to the intestine [4]. Mercury chloride (HgCl₂) can cause changes in fish behavior and can give an immunotoxic effect [5]. The accumulation of metals in fish begins with the process of uptake through the gills and then is absorbed into the entire body tissue and stored in the organs [6]. The accumulation of metals is present in fish tissues, therefore the monitoring of heavy metals in fish tissues can help to assess the health of aquatic ecosystems [7]. Al-Madani et al. [8] state that one of the organs that can accumulate mercury chloride (HgCl₂) is the kidney, which functions for excreting waste products and plays an important role in maintaining homeostasis by regulating body fluids.

The research results of El-Boshy and Taha [9] showed that mercury chloride causes impaired cellular or humoral immune response in tilapia. The cellular immune response can be an indicator of exposure to heavy metals through the changes and appearance of pigments in MMCs in the liver and kidneys of the anterior part [10]. Visible changes in MMCs resulting from pollution response include the quantities, sizes, and pigments seen. [11]. Melano-macrophages centers are the accumulation or aggregate of macrophages [12]. Observation of the structure of MMCs can be seen from changes in
the number and appearance of pigments [13]. The MMC structure is easily visualized histologically by
the presence of three pigments namely hemosiderin, melanin and lipofuscin. [11, 14]. Hemosiderin is a
storage form of iron that is derived from food sources and from the red blood cell hemolysis [13].

The results of the study from Reddy [10] concluded that an increase in the number and size of
MMCs in the liver and kidney of *Cyprinus carpio* exposed to cadmium for 30 days is considered a
histopathologic biomarker due to stress caused by the metabolic toxicity of the aquatic environment.
Based on this background, a study was conducted to determine the change in the number of MMCs in
the goldfish kidney exposed to mercury chloride. The anterior part of the kidney organ of where most
of the complex hematopoietic tissue is located. Histopathological features of kidneys’ anterior showed
the cellular structure melano-macrophage centers [15, 16].

The use of carp as a test fish in this study was based on the Environmental Protection Agency [17]
which mentions that carp can be used as bioindicators. Coban [18] state that carp (*Cyprinus carpio*) is
a water biota that has met the requirements to be used as bioindicators because it is easily adapts to
contaminated water conditions. According to Osswald [18], the extensive distribution of carp makes
these fish as ecologically compatible aquatic organisms for the study of the impact of water toxicity.

2. Methodology

2.1. Materials

The test fish used were carp fish obtained from the fish market of Gunung Sari with lengths of 18-20
cm and weight of 75-80 gr. Other materials used in the research were mercury chloride (HgCl$_2$), pellet
feed, aquadest, formalin buffer, liquid paraffin, 70 %, 80 %, 90 %, 96 % alcohol, absolute alcohols I,
absolute alcohols II, xylol I, xylol II and substances color hematoxylin-eosin (HE).

2.2. Methods

2.2.1. Preparation

An aquarium measuring 50 cm x 35 cm x 30 cm was first washed with liquid soap, followed by
disinfecting chlorine with a dose of 100 ppm, according to Wahjuningrum [19] and dried in the sun.
The aquarium was filled with 25 liters of water and given the aerator. Each treatment used five fish
with five replications. Before being treated, the carp fish were acclimated beforehand.

Measurements of mercury in water were carried out before the water medium was treated with
mercury chloride, while the mercury content in the kidney organ was measured after the carp was
exposed to mercury chloride for 21 days. The measurement of mercury content was carried out using
the Atomic Absorption Spectrophotometer (AAS) at the Environmental Health Engineering and
Disease Control (Balai Besar Teknik Kesehatan Lingkungan dan Pengendalian Penyakit), Surabaya.

2.2.2. Procedures

The study was conducted by exposing the carp to mercury chloride concentrations of 0.01 ppm, 0.05
ppm, and 0.1 ppm for 21 days [9]. Pellet feed was given ad libitum in the morning and evening.
Substitution of water was done every three days by adding water with the same mercury chloride
(HgCl$_2$) concentration. Water quality measurements were performed in the morning at the beginning
and end of the study.

After 21 days of exposure to mercury chloride, the carp were autopsied to retrieve the kidney organs
as histology preparation material. The steps for making histologic preparations consist of fixation,
dehydration and clearing, infiltration, paraffin blocking, microtome cutting and staining. Organ
fixation is done by soaking the organs with formalin buffer solution for at least 24 hours. The stages of
dehydration were done using 70 % alcohol for one hour, 80 % alcohol for two hours, 90 % alcohol for
two hours, 96 % alcohol for two hours, absolute alcohol for two hours. The clearing process aimed to
replace the alcohol solution from the tissue by inserting a piece of kidney organ into the xylol for two
hours. The next stage was infiltration which was entering pieces of kidney organ into liquid paraffin
with a temperature of 56 - 60°C for two hours. To facilitate the cutting of the tissue to 4-7 microns
thick, paraffin blocks with iron molds were made. In order for the tissue to expand properly, it was
dipped in a warm water temperature of 42-45°C [20]. Next it was put on an object glass and dried over the hot plate. According to Hibiya [21] and Suresh [14], to facilitate the observation of the hemosiderin pigment of the anterior renal tissue, the hematoxylin-eosin staining method was used to show a brownish yellow color.

2.2.3. Parameters
The main parameter in this study is the number of the MMCs of kidney organs exposed to mercury chloride for 21 days. The observation of the number of MMCs in the anterior kidney from histopathology was conducted using a 400x microscope connected to the computer to determine the bar size of the drawn image.

Supporting parameters include the change of carp behavior during the study, the mercury content in carp kidney organs and water media prior to treatment. Water quality parameters measured include temperature, pH and dissolved oxygen (DO) at the beginning and end of the study.

2.2.4. Analysis of data
Data on the number of MMCs were analyzed using Analysis of Varians (ANOVA) with SPSS version 17, followed by the Duncan's test to determine the differences between treatments.

3. Result
The results of the measurements of mercury in water before treatment were 0 ppm (treatment A), 0.0164 ppm (treatment B), 0.0546 ppm (treatment C) and 0.0700 ppm (treatment D). The results of the measurements of mercury content in the renal carp organ after exposure to mercury chloride for 21 days were 0 ppm (treatment A), 3.4032 ppm (treatment B), 30.422 ppm (treatment C) and 26.7605 ppm (treatment D).

The histopathological observation data is the number and size of the visible melano-macrophage centers. The observations showed differences in the number and size of melano-macrophages in the carp kidney exposed to mercury chloride (HgCl₂) characterized by the hemosiderin pigment (Figure 1). The average data on the number of MMCs of carp kidney exposed to mercury chloride (HgCl₂) concentrations of 0 ppm, 0.01 ppm, 0.05 ppm, and 0.1 ppm are presented in table 1. The result of Analysis of Varians (ANOVA) with 95% confidence interval showed that mercury chloride concentration (HgCl₂) had a significant effect (p <0.05) on the number of MMCs of the carp kidney. Duncan Multiple Range Test results showed significant differences (p <0.05) of MMCs at 0.05 ppm treatment with 0 ppm, 0.01 ppm, and 0.1 ppm.
**Figure 1.** Kidney histopathology of carp (400x magnification). A: HgCl$_2$ concentration 0 ppm, B: HgCl$_2$ concentration 0.01 ppm, C: HgCl$_2$ concentration 0.05 ppm and D: HgCl$_2$ concentration 0.1 ppm. Melano-macrophage centers (1), tubules (2), glomeruli(3).

**Table 1.** The average number of melano-macrophage in the carp kidney (*Cyprinus carpio*).

| Treatment   | Number (mm$^{-2}$) | Description          |
|-------------|--------------------|----------------------|
| A (0 ppm)   | 21.45 ±2.39        | no significant difference at significance level ($\alpha = <0.05$) |
| B (0.01 ppm)| 50.35 ±3.88        |                      |
| C (0.05 ppm)| 78.95 ±5.47        |                      |
| D (0.1 ppm) | 65.25 ±2.61        |                      |

Data on the behavior of carp fish exposed to mercury chloride are presented in table 2.

**Table 2.** Data changes in behavior of carp.

| Treatment   | Change of Behavior                                                                 |
|-------------|-------------------------------------------------------------------------------------|
| A (0 ppm)   | No change in behavior, normal swimming fish                                         |
| B (0.01 ppm)| Fish tend to swim faster, the accumulation of mucus                                  |
| C (0.05 ppm)| Fish tend to be hyperactive, lots of mucus accumulation and fish try to jump out of the aquarium |
| D (0.1 ppm) | Fish tend to be hyperactive, very much mucus accumulation, loss of balance and try the fish jump out of the aquarium |

Water quality measurements aim to monitor water quality conditions for carp during maintenance. Water quality measurements during the study ranged from 25.8 - 29.04°C, pH 7.1 – 7.8 and DO 3.95 – 4.14 mg / L. The value was still in the optimum range for the life of the carp, namely the temperature of 23 – 30°C, pH 6.5 - 9 [22, 23] and dissolved oxygen 3-5 mg / L [24]. This suggests that the change...
in the number of melano-macrophages of the carp of each treatment group is caused by exposure to mercury chloride, rather than influenced by water quality conditions.

4. Discussion

Mercury can enter through food and then into the digestive tract after experiencing absorption in the intestine, mercury compounds will be brought into the liver. Mercury entering into the heart will be divided into two; some will accumulate in the liver and some will be sent to the bile. Mercury in the bile will be carried to the excretory organs of the kidney, where some will accumulate in the kidney and some will be excreted through urine [25]. Subanri [25] states that mercury is a systemic poison and usually accumulates in the liver, kidneys, and spleen. The result of the highest measurement of mercury content in the renal organ is at treatment with a mercury concentration 0.05 ppm that was 30.422 mg/kg, but at treatment with a mercury concentration of 0.1 ppm it is seen that there is a decrease of mercury content in the kidney which was 26.7605 mg/kg.

The result of the Javed and Usmani [26] study proved that the concentration of heavy metals in the kidney organs are higher than in the gills, liver, muscles, and integument. Thophon [27] also proved that renal tubules and gills lamellae were the primary target organs for the effects of acute toxicity from cadmium while in subchronic exposure, but toxic effects on the gills were less than those of the kidneys and liver. Sadeghi [28] also proved that there was a tendency of chromium accumulation in kidney organs of Epaulet grouper (Epinephelus stålīcakē) exposed to chromium pollution.

Mercury chloride (HgCl$_2$) can cause cellular or humoral immune response [9]. Cellular immune responses are indicators of changes in the number of melano-macrophages in the liver and kidneys, especially the anterior kidney [10]. Melano-macrophages plays an important role in the response of fish to foreign materials, including infectious agents [29,30]. Melano-macrophages are an aggregation of macrophages [15]. Macrophages function as the main phagocytic cells that play a role in the process of phagocytosis [11, 31, 32, 33].

The highest number of MMCs in the kidney was found in the 0.05 ppm treatment which significantly differed (p <0.05) to the treatment of 0 ppm, 0.01 ppm, and 0.1 ppm. The presence of heavy metals in organs will cause cell or tissue damage [34]. The damaged cells or tissue will be digested by phagocyte cells and will be concentrated in macrophages [15]. This mechanism will stimulate the formation and increase the number of MMCs [30]. According to Bols [11], the visible changes in melano-macrophages are resulted from pollutant responses include the amount, size and pigment seen. The results of Sayed and Younes [34] prove the immune role of MMCs as indicated by an increase in the number and size of MMCs in the spleen, kidney and liver organs of Clarias gaprīepīnus exposed to silver nanoparticles. Tjahjaningsih [35] also proved that there is accumulated macrophage activity in the kidney, spleen, and liver of carp due to the exposure to mercury chloride. The macrophage activity illustrates the role of melano-macrophage centers (MMCs) in capturing non-antigenic materials that were damaged cells by exposure to mercury chloride. Reddy [24] also proves that macrophage activity in the kidneys and liver of carp can play a role in the clearance of the macromolecular circulation and foreign particles due to cadmium exposure.

In the treatment of 0.1 ppm concentration of mercury chloride (HgCl$_2$), a visible decrease in the number of MMCs was observed. Excessive mercury chloride exposure can be very toxic, thus causing a decreased immune response expression due to an immunosuppression mechanism. According to Middleton [36], a substance that toxic can inhibit macrophage phagocytosis activity at a certain concentration limit. This is also evident from the changes in the behavior of carp fish in the treatment of 0.1 ppm mercury concentration that appeared hyperactive, tried to get out of the aquarium, irregular swimming and high mucus accumulation. Nirmala [37] state that mercury can disrupt the taste sensors and receptors of the sight of fish so that there are changes in behavior. Changes in the behavior of carp in the mercury concentration treatment of 0.1 ppm are very visible i.e hyperactive fish, trying to get out of the aquarium, irregular swimming, and accumulation of high mucus. This is in accordance with the statement of Guedenon [38] which stated that changes in fish behavior in response to mercury chloride toxicity (HgCl$_2$) include fish becoming hyperactive by attempting to jump out due to skin
irritation, respiratory distress, loss of balance, swimming backwards, swirling, excessive mucus accumulation and ending with death.

5. Conclusion
Based on this research, it can be concluded that the levels of exposure to the heavy metal mercury chloride (HgCl₂) has an effect on the change of the number of melano-macrophage of the kidney of carp fish (Cyprinus carpio). The melano-macrophage centers of carp that were exposed to 0.01 ppm, 0.05 ppm and 0.1 ppm mercury chloride had increased MMCs compared to carp fish that were not exposed it (0 ppm).

7. References
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