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Detection of Metallothionein Protein Biomarkers (MTs) and Pinocytosis Activity in Gambusia Fish (*Gambusia affinis*) Exposed to Cadmium

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ABSTRACT

This study aims at detecting the metallothionein protein that binds cadmium in Gambusia fish using MT ELISA kit and Western Blotting testing. The continuation of the detection is used as a basis for analysing the macrophage and pinocytosis activity on Gambusia fish exposed to cadmium. The methods used to detect metallothionein protein are the MT ELISA kit and Western Blotting test. Meanwhile, to check the fish immunity, macrophage cells and pinocytosis activity were tested. The results indicated that the total proteins detected by the method of MT ELISA kits showed an increasing number with concentrations of exposure to Cd good at acute doses and sub-chronic higher doses (treatment A: 0.066 ± 0.019 ng/mL; B: 0.053 ± 0.022 ng/mL; C: 0,045 ± 0,014 ng/mL; D : 0,021 ± 0,012 ng/mL; E : 0,032 ± 0,019 ng/mL; and control k : 0,018 ± 0,018 ng/mL). Similarly, in the pinocytosis activity, the number of macrophage cells between treatment and control was significantly different (Cd ≥ $3x10^6$ treatment; Cd ≥ $1x10^6$ no treatment) and the number of pinocytosis activity was significantly different (Cd ≥ 24 PA treatment; Cd ≥ 10 no PA treatment). Increasing pollutants from cadmium exposure affect the fish health. Pollution causes changes in macrophage's immune cells and phagocytosis *Gambusia affinis,* which can change the overall innate immunity system and fish health. The change of immune cells associated with the species and biological conditions is not present. Analysis of the number of immune cells revealed that Gambusia has decreased its macrophage pinocytosis activity, thereby indicating that phagocyte activity is a sensitive biomarker and a good bio-indicator for cadmium pollution.

INTRODUCTION

Exposure to cadmium as a biomarker is defined as a measurable effect of toxic and harmful substances (heavy metals) to living organisms at the level of sub-cellular or physiological, regarded as a sensitive indicator to environmental pollution. Heavy metal is the most dangerous toxic substance because even at low concentrations it is very toxic to biota (Hertika et al. 2018). The metals discharged into the environment will pitch the couch and accumulate in the environment, which then enter into the cycle of life (Adam et al. 2018). Some biomarkers that are often used in detecting heavy metals include metallothionein and protein stress (Siddiqui et al. 2012).

Metallothionein (MT) detection by the Western blotting technique has been widely discussed instead of the MT ELISA Kit method (Hertika et al. 2018). MTs immobilized on membranes are detected by two methods; (1) binding of radioactive cadmium and (2) immuno-chemical staining using protein-A or colloidal gold conjugates, the detection of which were 0.4 and 0.06-0.16 microgram (Kumar 2012). Whereas, in EL it is used by MTs detected by proteins that have bind metals to certain spectrophotometer waves.

The fish's body defence system against cadmium exposure is observed through macrophages in T cells (Galvez et al. 2006) and a pinocytosis activity (Moulis 2010), for fluid (liquid) materials. The magnitude of the activity shows the presence of proteins that play a role in the fish's body defence. Pinocytosis activity takes place in lysosomes endocytosis. Endocytosis is the entry of macromolecules from outside the cell into the cell and then will be taken to small and irregular vesicles. These mechanisms take place on the cell membrane with the help of hydrolytic enzymes (Esteban et al. 2015). The immune defence mechanism in the fish appears to be related and equally competent compared to mammals (Enane et al. 1993). As in mammals, monocytes migrate to tissues and become macrophages (Hodgkinson et al. 2015). The calculation of macrophages can be used to compare the activity of infused macrophages from the control fish and those given treatments. In general, fish has an innate immune system that develops both a non-specific function and phagocytes that play an important role in de-

fence (Secombes & Fletcher 1992, Esteban et al. 2015). The phagocytic function has been used as immunological parameters to evaluate the status of Mohatan and the immune function in fish species (Secombes & Fletcher 1992). With being differently associated, there are a variety of biotic and biotic factors such as pollutants (Hooper et al. 2007) and pathogens (Neumann et al. 2001).

Many experimental and epidemiological reports have confirmed the immunotoxicity of pollutants in various marine biota, in both marine and freshwaters. However, the immune response measured in the number of matological cells varies among species, especially in freshwater fish. Studies conducted on sites contaminated with waste or exposed to other types of stresses show that haematological cell numbers can change dramatically, affecting the fish immunity (Seeley et al. 1990, Esteban et al. 2015).

This study aims to detect a metallothionein protein that binds cadmium as the heavy metal using the MT ELISA kit and Western Blotting tests. Furthermore, the detection is used as a basis for an analysis of the macrophage cell number and the pinocytosis activity of the gambusia fish exposed to cadmium.

MATERIALS AND METHODS

Sample of fish and cadmium exposure: Gambusia fish were obtained from the results of own cultivation in the Freshwater Aquaculture Installation (IBAT) - Punten, Batu City, East Java. Ten fish were prepared for each aquarium with a total of 18 aquarium treatments and replications. After being acclimatized for 7 days, the fish were given $CdNO_3$ exposure according to the treatments (0.03 mg/L, 0.015 mg/L, 0. 0115 mg/L, 0.0075 mg/L and 0. 0035 mg/L compared to the control group). Exposure to Cd was carried out once a day for 28 days and every 4 days there was a water change process.

MT ELISA Kit test: The ELISA Kit procedure was carried out according to the instructions in the product package. Previously, preparation of reagent, the sample and standard solutions was conducted. Following that, the standard solution and the ELISA solution were incubated for 60 min at 37°C. The plate was washed for as many as 5 times. After that, the substrate solution A and B was added and then incubated for 10 min at 37°C during the formation of colour. The solution process ended and then the OD value was read for 10 minutes before it was calculated.

Test western blotting: The protein isolation sample was prepared. Then the semi-dry transfer cell was cleaned by using 70% ethanol solution and the 9 layer Whatman paper that was previously soaked in the buffer was attached. Following this, it was coated with the 6 Whatman paper. The dry transfer cell device was installed and operated at 0.16 volts for 60 minutes. Then staining was conducted by soaking the 5% blot to solution for 30 minutes. After that, the solution was washed with 3 PBST levels for 5 minutes each. It was then sloughed with secondary antibodies and washed with PBST 4 times. Furthermore, colourizing was conducted by using NBT-BCIP. The resultant was cleaned with distilled water and put into the scanner.

Macrophage and pinocytosis activities: Macrophages were taken from the gills aseptically, weighed, crushed using a network grinder and diluted in RPMI 1640+ media (Roswell Park Memorial Institute, SIGMA) with a ratio of 1:10. RPMI 1640+ medium containing 1 mg per 100 mL pen/strep (Sigma), 0.2 mg per 100 mL heparin and 0.1% (v/v) bovine fetal serum (FBS, Sigma). It was then sterilized using millipore 0.22 mm filter. Suspension of macrophages was applied to the groove of the haemocytometer and the total cell count was obtained in four small rooms under the light microscope as follows:

$$\Sigma \text{ cell} = \text{number of cells} \times 4 \times 10^6 \times 1/\text{fp} \quad ...(1)$$

Where: fp is the dilution factor (Risjani et al. 2014).

To measure the pinocytosis activity, the rest of the cell suspension was dropped into a slide and incubated at a room temperature for 60 minutes. Object glasses were washed with RPMI 1640+ and then added with 1 mL of yeast suspension (10^9 cells/mL). Macrophages and yeast were incubated at 26° C for 45 minutes and then the object glasses were washed three times with RPMI 1640+, fixed with methanol 96% (v /v) and placed at room temperature for 5 minutes. The dried object was added with a drop of Giemsa, incubated for 20-30 minutes, and then washed under running water. The glass object was observed under a microscope, and the macrophage pinocytosis activity (PA) was determined as follows:

$$PA = (\text{number of pinocytosis macrophages by yeast/100 cells}) \times 100\% \quad ...(2)$$

RESULTS

MT ELISA test: The results of testing on the detection of MT proteins using the ELISA Kit illustrate that there is a significant effect of exposure to cadmium (Fig. 1).

Protein detection results in Fig. 1 show that there is a significant difference between the Gambusia fish with the treatment dose and no treatment. There was an increase in the amount of MT protein with an increased concentration of Cd exposure both at acute and sub-chronic doses (treatment A: 0.066 ± 0.019 ng/mL; B: 0.053 ± 0.022 ng/mL; C: 0.045 ± 0.014 ng/mL; D: 0.021 ± 0.012 ng/mL; and E: 0.032 ± 0.019 ng/mL; and control: 0.018 ± 0.018 ng/mL)

Western blotting test: As seen in Fig. 2, Western Blot analysis shows protein induction with a molecular weight

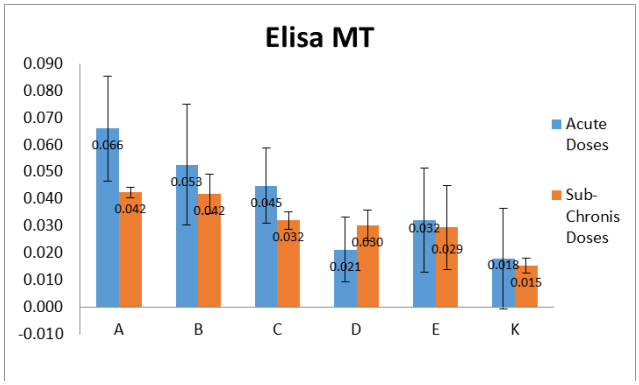

Fig. 1: Measurement of MT protein using the MT ELISA Kit method. Comparison between treatments at acute doses and sub-chronic doses of Cd exposure in Gambusia gills. MT protein production is greater in acute doses than that in subchronic doses.

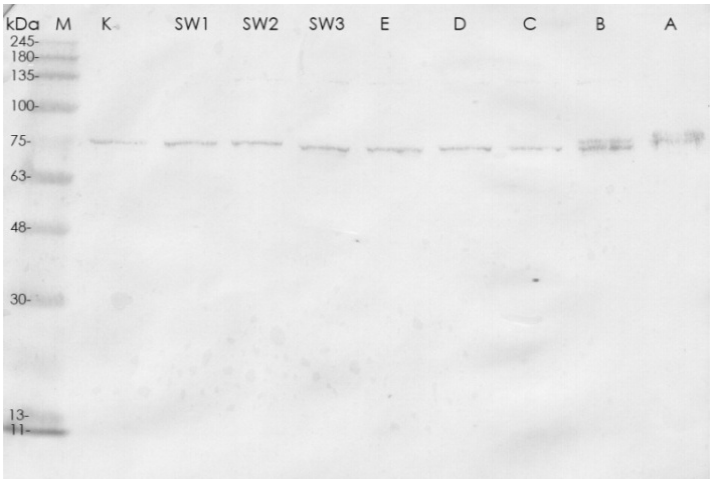

Fig. 2: Western blooting test results of Cd uptake on the sample absorption at a molecular weight of 75 kDa. Absorption in K0, K1, K2 and K3 as control areas still had MT-Cd absorption but was thin. Likewise, C, D and E were almost the same as K. Absorption on A and B showed the thickest band.

of about 75 kDa. This induction was detected most clearly in the highest cadmium concentration (in A and B treatments at a concentration of 0.03 mg/L and 0.015 mg/L). The new protein is immunologically cross-reactive with 70 kDa MT protein from Gambusia fish (Fig. 2C, 2D, 2E, 2K).

Macrophage test and phagocytic activity: The body's defence resulted from Cd exposure seen in Fig. 3 showed a significant difference from the number of macrophage cells. The picture shows the indicator of a cellular level activity of fish gills in the defence from Cd. In this case there is an event of endocytosis.

Pinocytosis activity: The pinocytosis activity shown in Fig. 4 shows the magnitude of the activity, which also depends on the amount of exposure concentration. The higher the exposure concentration, the greater is the amount of the pinocytosis activity. When the results of the number of macrophages and pinocytosis activity are compared, it is shown that there is a positive correlation between the increase in the number of macrophages and the increase of the amount of the pinocytosis activity.

DISCUSSION

In this study, it is clearly shown that the proteins detected were MTs proteins as biomarkers of heavy metal binding. The test results estimated that the total cadmium uptake was higher in samples with Cd exposure treatment than in the control group. The gills that are sampled for ELISA and WB testing are the first organ in the process of respiration and osmoregulation. This means that the homoeostasis process first occurs in the gills. Several studies have shown that the effect of Cd exposure on the gill organs is the accumula-

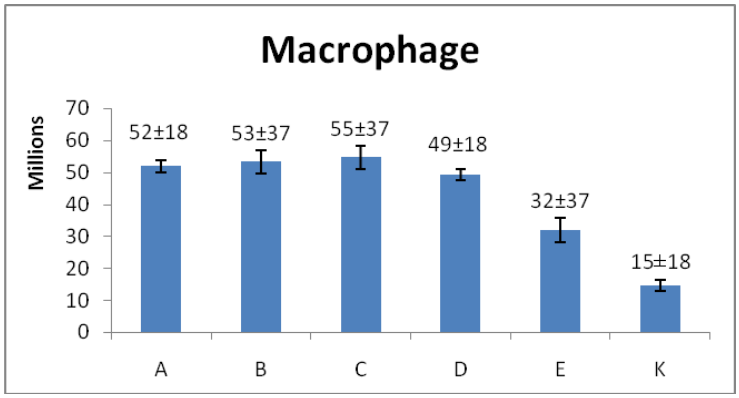

Fig. 3: The number of produced macrophage cells caused by Cd exposure to the gills of Gambusia fish.

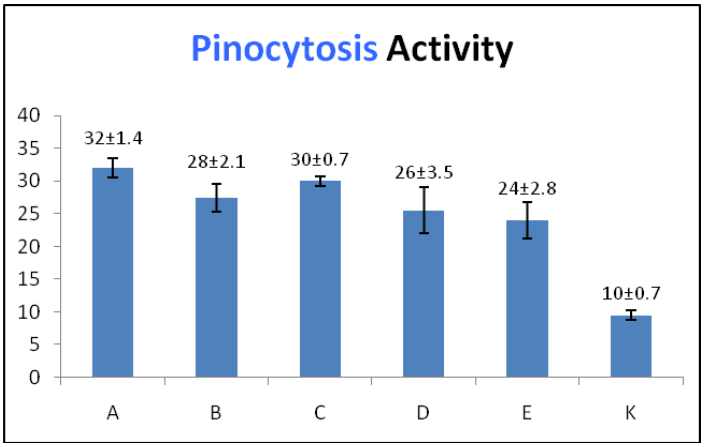

Fig. 4: Number of pinocytosis activities in the Gambusia fish gills after Cd exposure.

tion of cells in the filaments of the secondary lamellae on the gills due to the absorption process of Cd.

It is known that toxic metals can interfere with the immune system (Cooper 1991), but it should be noted that metals such as mercury, lead, and cadmium can display immunostimulatory effects on the development of autoimmune diseases (Ohsawa et al. 1997). Some research results reported that there is an increase in the circulating autoantibodies levels found in animals and humans exposed to toxic metals, including cadmium (Duruibe & Ogwuegbu 2007, Hart et al. 2001, Johri et al. 2010). Individuals who are exposed to cadmium and nickel demonstrated high titers in basic DNA antioxidant autoantibodies (Desamehta et al. 1995). Lacroix-Desmazes et al. (1998) found that anti-laminin autoantibodies are more prevalent in humans exposed to cadmium showing a urine cadmium level of 20 g/g Cr. Jin et al. (2003) first reported that a high level of MT-Ab exists in healthy subjects in patients suffering from atopic dermatitis, either with or without metal allergies. Patients who experience metal allergies have a very high prevalence of MT-Cd. However, the MT mechanism of present autoantibodies in the serum of healthy individuals and atopic dermatitis patients who have allergies to metals remains unclear. Yet, it may be caused by the release of MT intracellular into circulation or to receipt Tural changes under excessive exposure to metals (Jin et al. 2003, Desaimehta et al. 1995). In fact, some autoantibodies called natural autoantibodies (AHA) can be present in the serum of healthy individuals with the absence of deliberate immunization by any antigen (Korte et al. 1994). In humans and mice, NAA is known to bind to various surface cells that are evolutionarily conserved, intracellular and circulating antigens, such as glaramic acid decarboxylase, ointment interfering, factor VIII, and glomerular basement membranes (Poletaev & Osipenko 2003, Lacroix-Desmazes et al. 1998, Korte et al. 1994). Natural autoantibodies can be encoded by germ line genes and produced in the early stages of life, not dependent on the stimulation by foreign antigens

(Lacroix-Desmazes et al. 1998). According to research that has been done, there is no subject in this case that has used metallothionein products and no one has a history of a metal allergy or a hereditary disease. Thus, the small variance at the MT-Cd level in this subject cannot be associated with the exposure to xenogenic antigens.

In addition, the result of this study does not show any significant relationship between cadmium exposure and the MT- CD level although it was known that cadmium exposure can increase metallothionein release into blood plasma of humans who are exposed to cadmium (Nordberg 2009). It appears that an increase in the metallothionein plasma levels does not stimulate an autoimmune reaction in this case. It is believed that the presence of this phenomenon may occur. The level of cadmium exposure in this study is not as high as that reported in the study of Jin et al. (2003) on anti-laminin autoantibodies; thus, it may not be high enough to generate an immune response to metallothionein (Desaimehta et al. 1995).

In this study, the number of macrophages showed an equal value to the number of pinocytosis activities both in the number of macrophages in fish with increased treatment and the number in the control fish (Fig. 3). On the contrary, pinocytosis activities of macrophages were significantly different between fish with treatments and the control fish (Fig. 4). In particular, the macrophages of all treated fish show the same capacity for phagocytosis. Nonetheless, in some cases, the phagocytic activity decreases in blood with lower cadmium exposure. The similar findings of other studies show that the phagocytic activity was higher in fish from the relatively uncontaminated water (Seeley et al. 1990), and lower in fish swept from the contaminated water (Moulis 2010). Phagocytosis plays a key role in both non-specific and specific immune responses and represents an immune line of defence against invading agents (Yamamoto et al. 2014, Hodgkinson et al. 2015, Risjani et al. 2014). Based on our findings, MT-Cd uptake and the number of macrophages could be used as a biomarker of environmental pollution and can be used to compare the activity of macrophages in fish from contaminated and uncontaminated areas.

The lower phagocytic activity in the treated fish correlates with the critical levels of water dissolved oxygen and other high levels of pollutants. In the previous discussion, we showed that water quality with cadmium exposure has a critical level of dissolved oxygen. Dissolved oxygen is one of the most important parameters of water quality because oxygen is very important for all organisms that live in water. Furthermore, oxygen levels in the environment can modulate the immune response; hypoxia suppresses the activity of macrophages and reduces the level of circulating antibodies, which in turn increase hyperoxia (Watts et al. 2001, Uribe et al. 2011). The discharge of pollutants into the anthropogenic aquatic environment affects aquatic organisms, as indicated by changes in the immune cells (Risjani et al. 2014). This pollution impacts several physiological responses of aquatic organisms, including physiological changes in circulating phagocytic tissues and phagocytic activities, similar to observations in studies that have been carried out.

CONCLUSION

Increasing pollutants from cadmium exposure affect fish health. Pollution causes changes in the immune and phagocytic cells of the *Gambusia affinis* macrophages, which can alter the innate immune system and overall health of the fish. Furthermore, changes in the immune cells are associated with lost species and biological conditions. Thus, an analysis on the number of immune cells revealed that Gambusia experienced a decrease in the macrophage pinocytosis activity, indicating that the phagocytic activity is a sensitive biomarker and a good bioindicator of cadmium pollution.

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