CD4 cell activation with the CD8 marker and metallothionein expression in the gills of cadmium-exposed mosquito fish (Gambusia affinis Baird and Girard 1853) juveniles

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ABSTRACT

This experiment aimed to determine how Cd exposure impacts CD4 cell activation, macrophage cells, pinocytosis activity, metallothioneine expression, and Cd levels in juvenile Gambusia affinis gills. Four treatment groups were used, with one control and treatments A, B, C, and D at dosages of 0.03 mg/L, 0.023 mg/L, 0.015 mg/L, and 0.008 mg/L, respectively. The results showed that the number of CD4 with CD8 cell markers differed significantly from each treatment compared to the control (0.33%). The value in A, namely 0.54%, was the highest, followed by B, C, and D of 0.46%, 0.44%, and 0.42%, respectively. The number of macrophages increased as the Cd level of the medium increased, as did the activity of pinocytosis. Furthermore, the immunofluorescence test on the gills with the Anti-MT Mouse and goat IgG fluorescein in Rhodamine on the gills showed that luminescence increased with increasing Cd levels in the gills. Similarly, the MT intensity increased at Cd-exposed gills as compared to the control. As the Cd level in the gills increased, the MT levels ascended significantly.

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1. Introduction

Heavy metals occur naturally at trace amounts in water bodies; nevertheless, their background levels are increasing, particularly in areas with significant industrial, agricultural, and mining activity [1–4]. The cadmium (Cd) level in unpolluted water was 0.003 mg/L, while it reached 2 mg/L in polluted water [5–7]. Fish may be exposed to high amounts of heavy metals since most of the heavy metals released into the environment are mainly end up in the aquatic phase. Heavy metal accumulating in the tissues of aquatic species can be harmful if it reaches a threshold level [3,8,9]. Cd may have a variety of detrimental effects on fish, such as alterations in respiration [10–12], disruption of osmoregulation [7,13,14], histological disturbances [14–16], and enzymatic activity impairment [17–19].

Cd is easily bound to protein in fish tissues. It interferes with physiological functions, weakens the immune system, and ultimately leads to death [20–22]. Fish health depends on the effectiveness of both cellular and humoral immunity. Lymphocytes mediate cellular immunity as the host’s immune response to intracellular pathogens that infect the body [23–25], which indicates the body’s immune response to an infection by the presence of lymphocyte activity.

Antibodies are protein structures that function to prevent foreign cells and substances like Cd from entering and damaging the body [1,22,24,26]. The configuration of the antigen-antibody

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molecule is structured in such a manner that only the antibody appears in response to a particular antigen that matches the surface [27–29]. The cluster of differentiation (CD) is the surface molecule of epoite leukocytes and is identified by monoclonal antibodies. Monoclonal antibody cells recognize just a specific single epitope of the antigen [25,30,31]. The CD4 cells act as adhesion molecules linked with the major histocompatibility complex (MHC) II by signal transduction. Furthermore, CD4 cells are divided into two large subsets that perform different functions in the body’s defense system. They are of white blood cell or lymphocyte [32–35], and constitute an essential part of the immune system and activate Th1 cells, which further stimulate macrophages to destroy microbes [21,36,37]. CD4 cells are differentiated from CD8 based on the particular protein on the surface [38–40]. CD4 cells are most affected by infection or exposure to foreign bodies such as cadmium. CD4 expression using CD8 marker of cell activation has been widely studied. The use of CD8 markers is closely related to its ability to predict the number of CD4 cells [26,41,42].

The ability to accumulate Cd concentration in the organism’s body is determined by the metallothionein (MT) level [16,19,22]. MT is used as an indicator of heavy metal contamination due to its high sensitivity and accuracy [43–45]. The level of MT in an organism can be assessed through its density and intensity using immunohistochemistry. This technique detects the presence of antigens in tissues by using stained antibodies [26,46,47]. One of the examples is the immunohistochemical technique using histochemical immunofluorescent observations with Confocal Laser Scanning Microscopy (CLSM) [48–51]. Given the significant effect of Cd in the waters, this study aims to examine the activation of Cd4 cells in young Gambusia gills and the expression of the MT due to Cd exposure.

2. Materials and methods

2.1. Adaptation

Mosquito fish (Gambusia affinis) with a length of 3–4 cm and a weight of 2–3 g were obtained from the Freshwater Aquaculture Installation in Punten, Batu, East Java and kept in a 120-liter tank with continuous aeration in 12 h:12 h light:dark cycles. During the 14-day acclimatization period in the laboratory, the pelletized feed was given twice daily. Around 20% of the water exchanges were performed every day, and feces and other waste particles were siphoned off. The following water parameters were measured during the acclimation and experimental phase: temperature ranged from 28 to 30 °C, dissolved oxygen concentrations were between 7.0 and 7.4 mg/L, and pH values were 7.5–8.0.

2.2. Cd exposure experiment

Cd (1000 mg/L) stock solutions were prepared by dissolving 2,744 g Cd(NO3)2.4H2O (Merck, Darmstadt, Germany) in 1000 ml deionized water. Based on the obtained acute toxicity test LC50 values (0.030 mg/L) [52], fish were subjected to various Cd concentrations for 96 h, with the following concentrations: Group (A) 0.030 mg/L (100% of LC50), Group (B) 0.023 mg/L (75% of LC50), Group (C) 0.015 mg/L (50% LC50), Group (D) 0.008 mg/L (25% LC50), and Group (E) without Cd as a control, with two replications. One hundred fish were divided into 10 tanks, with 10 fish in each tank containing 4 L of media. The experiment was conducted in 12 h:12 h light:dark cycles, applied to all tanks with continuous aeration.

During the in vivo experiment, 30% of the experiment media was changed every day to maintain a consistent Cd concentration [53]. The fish were fed pelleted feed every day, and the feces and detritus were sucked from the tanks. This study’s experiment was carried out in conformity with the University’s Institutional Animal Care guidelines and procedures.

2.3. Isolation of samples for flow cytometry

Fish from each treatment was sedated by gradual cooling [54]. Following that, the gills were dissected, cut into small pieces (about 2 mm) and placed in a petri-dish, in which they were crushed with 5 ml of phosphate buffered saline PBS solution, then placed to a 15 ml propylene tube. The suspension is then crushed again with 10% detergent ammonium sulfate salt. The propylene tube was centrifuged at a speed of 2,500 rpm, at a temperature of 10 °C, for 15 min. The supernatant was carefully removed and centrifuged again at 2,500 rpm, at 10 °C, for 25 min. The pellet and supernatant were separated, then the pellet was re-suspended with 1 ml of PBS solution. The suspension was transferred to a 1.5 ml microtube for cell antibody staining and flow cytometry analysis [55].

2.4. Antibody staining on cells and flow cytometry analysis

The gill cell isolation suspension [56] was divided into 5 microtubes of 50 µL each, and each microtube was filled with 0.5 ml of PBS. All microtubes were centrifuged for 5 min at 2,500 rpm and 10 °C. The pellet and supernatant were separated. Subsequently, the pellet was suspended in 50 µL of the particular antibody solution (Anti-Metallothionein antibody [UC1MT]-ab1228) for extra-cellular staining. The suspension was incubated at 4 °C for 20 min in the darkroom. The incubated cell suspension was then mixed with 400 µL of PBS solution before being transferred to a cuvette for flow cytometry (Biosciences-BD FACSCalibur-USA) examination. Meanwhile, the pellet was suspended in 50 µL of fixation buffer (cat. #420801 [Biolegend]) for the intracellular staining. The suspension was incubated at 4 °C for 20 min in the darkroom. The incubated cell suspension was then mixed with 400 µL of PBS solution before being transferred to a cuvette for flow cytometry examination using cytometer flow (Biosciences-BD FACSCalibur-USA). Flow cytometry analysis was also performed on the identified and analyzed species using BD CellQuest ProTM software for acquisition and gating. The percentage of the total cell population (CP) is calculated using the following formula:

\[ CP = LR/(UR + LR) \]

where LR is the low right of the quadrant, UR is the upright of the quadrant.

2.5. Sampling of histological preparations

First, the gills were immediately placed in 10% phosphate-buffered formalin (NBF) fixative and allowed to stand for 24 h, then washed with 70% alcohol [58]. Following fixation, commence paraffin tissue embedding, followed by tissue cutting using a microtome (Leica RM2135, Wetzlar, Germany) with an incision thickness of 5 µm. The sliced tape was stretched by placing it in a water bath at a constant temperature of 40 °C. The tape was taken with the item glass and air-dried for 1 h. Afterward, the specimen can be used for histochemical analysis.
2.6. Immunofluorescent analysis for MT

Specimens were deparaffinated overnight in a 40 °C oven, then immersed in xylol twice for 10 min each, followed by a rehydration process for 5 min in ethanol concentrations of 90% and 70%, respectively [59]. Subsequently, the specimens were washed three times in PBS for 5 min each. Specimens were soaked with Triton-X with FCS at room temperature, then washed three times with PBS for 5 min each. Next, the specimens were put into 2% Bovine Serum Albumin (BSA) in PBS, left at room temperature for ±60 min, and washed with PBS 3 times, 8 min each. Then they were labeled with primary antibody (mouse-anti MT) in 2% BSA (1:1000) for ±60 min, then washed with PBS 3 times, each for 8 min. They were then labeled with secondary antibody (goat IgG fluoresce in Rhodamine).

Fig. 1. Flow cytometry results. Percentage of CD4 cells expressing CD8 cell markers in gills of Gambusia fish exposed to different Cd levels (A = 0.030 mg/L, B = 0.023 mg/L, C = 0.015 mg/L, D = 0.008 mg/L and control (without Cd)).
in 2% BSA (1:1500) for 60 min before being rinsed with PBS three times for 8 min each. This stage is performed in a dark room. The slides were then drained, cleaned with paper towels, and placed in 10% glycerol in a dark room. The specimens were then examined in the dark with a Confocal Laser Scanning Microscope (CLSM) at a magnification of 400 times. The intensity of the MT is recorded.

2.7. Concentration of Cd in gills

To obtain a consistent weight, the gills of each fish were homogenized and dried in an oven at 65 °C for 48 h [60]. In 3 mL nitric acid (Merck, Darmstadt, Germany), 0.2 g of homogenized tissue was digested for 4 h at 90 °C. After cooling, samples were filtered using filter paper (Whatman, 0.45 μm) and deionized water was added until a volume of 50 mL was reached. Cd concentrations were measured using an atomic absorption spectrophotometer (Shimadzu AA-7000, Tokyo, Japan), and the results were expressed as mg/kg dry weight. Analytical blanks were processed in the same method as the samples, and concentrations were measured using standard solutions produced in the same acid matrix. The accuracy of the Cd measurement was confirmed using dogfish muscle reference material (DORM-4) given by the National Research Council of Canada (Ottawa, Canada), with a Cd recovery of 106% and a detection limit of 0.01 mg/kg. The reagents used in this study were all quality-analytical grades.

2.8. Data analysis

Before being utilized for statistical analysis, data were validated for normality and homogeneity and presented as mean ± standard error. It was log converted if the data did not fulfill the normality and homogeneity of variance requirements. The data were analyzed statistically using one-way ANOVA followed by Tukey’s post hoc comparison test to determine the effect of different Cd concentrations on the percentage of Cd4 cell activation, the number of macrophages and pinocytosis of cells in the gills, the intensity of MT and the concentration of Cd in gills. The differences were considered to be statistically significant at p < 0.05. Furthermore, the fraction of Cd4 cells seen through flowcytometry, gill histochemistry visualization, and immunofluorescence were evaluated descriptively.

3. Results

3.1. Activation of Cd4 cells with the Cd8 marker

Fig. 1 shows the flowcytometry findings of Gambusia gills and the percentage of Cd4 cells expressing Cd8 cell markers as follows, which recorded: control 0.33%, treatment A 0.54%, B 0.46%, C 0.44%, and D 0.42%. The Cd4 cell percentages in all Cd treatments were higher than in control, with treatment A having the highest proportion. Fig. 2 depicts the number of Cd4 cells that have been activated with Cd8 cell markers. The higher the cadmium exposure concentration, the greater the number of Cd4 cells activated.

An increase in the number of activated Cd4 cells is important for the body’s immunity. These cells differentiate depending on the type of stimulant. For example, the cytokines produced on antigen recognition are the effector phase of interferon (IFN). Cd4 cell effectors function as a defense for infection in the phagocytosis process, as shown in Fig. 3. They also secrete interleukin-2 (IL-2) which acts as an autocrine growth factor and stimulates cell proliferation, including the processes of B cells and T cells. In this experiment, the percentage value of Cd4 cell counts decreased with increasing Cd in the media. The number of macrophages rose as the Cd content in the media increased, as did the pinocytosis activity of macrophages (Fig. 3). The number of macrophages in this study correlated positively with the intensity of pinocytosis activity.

3.2. Metallothionein expression

Fig. 4 shows the Anti-MT Mouse immunofluorescent technique on the gills, while Fig. 5 shows the average MT intensity in the gills. With decreasing levels of Cd in the media, gill luminosity (Fig. 4) and MT intensity in gills (Fig. 5) decreased. The intensity of absorption (measured by wavelength) in gills increases significantly as the level of Cd in the medium rises (Fig. 6).

Fig. 6 shows the difference in cadmium absorption in each treatment. Treatment A showed the highest absorption intensity, while treatments B, C, and D had similar absorption intensities, whereas the control had the lowest absorption intensity.

The Cd concentrations in gills of Cd-exposed fish were significantly higher than those in the control groups (p < 0.05). There were no significant differences in the Cd concentrations in gills of fish exposed to 0.023, 0.015, and 0.008 mg/L (Fig. 7).

4. Discussion

The findings discovered an increase in the percentage of Cd4 cell activation in Cd-exposed gills but not in control gills. Treatment A produced the most Cd4 cells, whereas treatments B, C, and D produced similar amounts (Figs. 1 and 2). Increased antigen levels in the fish body can activate the immune system’s Cd4 cells. According to Refs. [61–63], the presence of antigens in the body of mice causes activation of T cells and B cells via T Cell Receptors (TCR), with Th cells inducing proinflammatory cytokines and displaying Cd8 markers on Th cells, indicating a movement response to T cell in inflamed tissue due to the presence of antigens. In this study, the number of macrophages correlated positively with the amount of pinocytosis activity. The Cd-exposed fish had more macrophages and more pinocytosis than the control fish (Fig. 3). The phagocytic activity decreased in fish with lower cadmium exposure. The findings of this study are consistent with previous research that found lower phagocytic activity in fish caught relatively from uncontaminated water [64–66] and higher phagocytic in fish captured from contaminated water [67,68]. Phagocytosis is an important component of both non-specific and specific immune responses, and it serves as the first line of defense against invading agents [33,47,69]. The number of macrophages in fish from polluted and non-polluted locations can be utilized as a biomarker of environmental pollution and to compare macrophage activity.

MTs are proteins that can be used as a heavy metal-binding biomarker. MT can be found in gill tissue [2,12,70]. Changes in Cd
accumulation in the gills are due to Cd exposure were reflected in MT levels. The MT concentrations increased significantly as the Cd content in the media increased, with fish exposed to 0.030 mg/L Cd showing the highest MT concentration. The MT response is considered to be connected to the cell growth system that occurs in gill cells via the involvement of cell growth factors in the form of insulin-like growth factors (IGF-1). Cell death can stimulate IGF-1 excretion from gill cells. This growth factor will regulate cytokine responses, promote regeneration, and play a role in T cell proliferation both inside and outside the thymus [38,70,71]. As a result, a large number of T lymphocytes will enter the circulatory system in search of cytokines [39,47,61]. Similarly, the amount of MT in circulation has the ability to increase. When the thymus produces MT, it enters the bloodstream and plays an important role in numerous organs. Metallothionein (MT) is a protective factor against toxic metals like cadmium and mercury. It decreases metal toxicity by binding to it [16,52,72].

Gambusia affinis has shown a high ability to survive in a polluted environment despite being exposed to heavy metals. The fish can multiply pinocytotic cells and macrophages as part of the innate immune system. Therefore, activating the cellular immune system through the intermediary of cytotoxic or CD8 T cells is important. Cell activation also occurs through the infection by a helper or CD4 T cells, as proven by an increase in the number of macrophages in pinocytosis activity and the elevated function of the metallothionein enzyme that is demonstrated in the primary lamellae of the Gambusia fish gills. This study contributes to the advances of the immunology field by providing scientific data on fish defenses in response to heavy metals.

5. Conclusions

In conclusion, Cd-exposed fish showed higher CD4 and CD8 cell markers than control fish. According to an immunofluorescence test, the luminescence of the gills increased as Cd levels in the gills increased. Cd-exposed gills had greater MT intensity than the

![Fig. 3. The number of macrophage and pinocytosis of cells in gambusia gills exposed to various levels of Cd and control for 96 h. There is a significant difference between different letters (p < 0.05). Lowercase letters indicate a significant difference in macrophage; capital letters indicate a significant difference in pinocytosis.](image)

![Fig. 4. Luminescence level in gills exposed to various Cd concentration (A exposed to 0.03 mg/L, B 0.023 mg/L, C 0.015 mg/L, D 0.008 mg/L, and Control) observed by CLSM.](image)
control. The MT levels in the gills increased as the Cd level in the gills increased.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare no conflict of interest.

Fig. 5. MT intensity in gills exposed to different level of Cd (A exposed to 0.03 mg/L, B 0.023 mg/L, C 0.015 mg/L, D 0.008 mg/L, and Control) observed by CLSM.

Fig. 6. Absorption intensity in gills exposed to various levels of Cd and control for 96 h. There is a significant difference between different letters ($p < 0.05$).

Fig. 7. Cd concentrations in gills of fish exposed to different Cd levels. There is a significant difference between different letters ($p < 0.05$).

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