

**Cadmium Induces the Activation and Expression of MTF-1 Protein
As a Biomolecular Response of Sea Urchins *Diadema setosum* (Leske, 1778) at Ambon Bay,
Maluku Province**

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ABSTRACT

It is known that cadmium has toxic effect on living organisms. However, the bodies of living organisms have the ability to regulate a wide range of toxic entering by using the biomolecular response that aims at maintaining cell homeostasis. In this research, an induction of cadmium in sea urchins *Diadema setosum* was carried out. Cadmium was induced in 6 levels of concentration in six treatment groups of *D. setosum* from Ambon Bay, Maluku Province. Each group consisted of 7 individuals. The examination of MTF-1 protein activation was carried out by immunohistochemical method, the measurement of MTF-1 protein concentration was carried out by the indirect ELISA method (Enzyme-Linked Immunosorbent Assay) and the examination of MTF-1 protein expression was carried out by western blotting method. The data were analyzed using descriptive analysis and One Way ANOVA followed by Duncan test (0.05 significantly) to determine the mean difference of MTF-1 protein concentration of *D. setosum*. The research results showed that cadmium induction activated MTF-1 characterized by the condition of liver cells of *D. setosum* which was brown. The higher the exposure of cadmium concentration on *D. setosum*, the higher the concentration of MTF-1 expressed.

Keywords: *Heavy metals cadmium, MTF-1, Diadema setosum*

1. Introduction

Cadmium is nonessential heavy metal (Olive et al., 2001; Martin and Prognonec, 2010), and has a highly reactive nature and can inactivate a wide range of enzyme activities which are required by cell (Jonak et al., 2004). Cadmium is accumulated in organs, mainly the kidneys, and can cause toxicity in many animals (Garcia-Rico et al., 2002). The research conducted by Ohta et al. (2000) found that the accumulation of cadmium in male rats (Wistar Male Rats) caused osteoporosis, and livers and kidneys diseases.

Cadmium in biomolecular level is absorbed by organisms and is accumulated in the cytosol through the formation of metal-ligand complex (Dailanis and Kaloyianni, 2004). Moreover, cadmium can induce damages to the membrane function (Smiri et al., 2010) and damaging the lipid composition of cell membrane (Dailanis and Kaloyianni, 2004). According to Gzyl et al. (2009), cadmium cannot participate in a redox reaction, but it has the ability to produce free radicals. However,

Smiri et al. (2010) explained that cadmium induced toxicity or caused toxic effects by its ability to induce oxidative stress mediated by H_2O_2 , which would trigger an increase in the reduction of the activity of antioxidant enzymes such as catalase and peroxidase along with the increase of lipid peroxidation, the production of radical superoxide (Ali et al., 2009). According to Chowdhary et al. (2006), the induction of cadmium will trigger the release of Zn^{2+} ions from the protein structure.

The research conducted by Almeida et al. (2009) found that the cadmium increased lipid peroxidation in Nile tilapia (*Oreochromis niloticus*) liver. Through an Cd's attachment into the cysteine residue or triggering the formation of ROS, then cadmium shows an effect on the genomic and post genomic process on the liver (hepar), kidneys (ren), lungs (pulmo), and brain (cerebrum). Additionally, Rumahlatu et al. (2012) report that *D. setosum* accumulated Cd showed in the expression of the MT-1 protein (metallothionine) on gonad, intestine, and liver cells. MT (metallothionines) protein is a plasma

membrane protein (Jiang et al., 2004) with non-enzymatic nature and functions as a metal trap (Nielsen et al., 2006) which is regulated by the MTF-1 (Metal Transcription Factor 1). MTF-1 is a conserved protein that regulates gene expression in response to heavy metals, oxidative stress (Formigari et al., 2008) and hypoxia (Jiang et al., 2004). Gunther et al. (2012) explained that the MTF-1 protein is a regulator of transcriptional pluripotent which is involved in cellular adaptation under various stress conditions, particularly the stress due to the exposure to heavy metals. Thus, MTF-1 is a major generator of metallothioneine protein. Therefore, the research aimed to determine the activation, concentration, and expression of the MTF-1 protein in response to biomolecular of sea urchins *D. setosum* was conducted while exposing by cadmium.

2. Materials and Methods

All This research was a non-factorial experiment in a completely randomized design (CRD) 6 levels of heavy metals Cd concentration with 7 replications for 4 weeks. The 42 samples of *D. setosum* collected from the breeding at Aquaculture Centres Indonesian Institute of Sciences-LIPI Ambon, Indonesia. The research was conducted in three stages, namely: preparing the test animals, determining the test animals, and giving treatments.

The preparation of the test animals began with the stage of cultivation step. *D. setosum* cultivation used flowing water system, the spawning stage, and the larval stage, preserving stage and raising stage. The spawning stage was done in a pond starting from the selection, spawning, fertilization, and hatching. The larval stage was done in a controlled container (fiberglass tank). The phase was done since the eggs hatched until the age was 12-15 days. The preserving stage, seed maintenance of *D. setosum* was done in a fiberglass tank for 1 month. The raising stage is the phase of raising the seed which was carried out for 3-8 months in fiberglass tank until the *D. setosum* gained the body circumference of 10-25 cm and a body weight of 40-160 g.

The determination and sortation of *D. setosum* for treatment individual was passed the preserving stage for 8 months until reach 90 g (body weight) and 15 cm (body diameter). As many as 42 individuals were divided into six groups depending on Cd treatment exposure. Subsequently, the 7 individuals of *D. setosum* were placed into the aquarium basin/tank (100 x 60 x 70 cm). An adaptation phase was done for 1 week in laboratory. Cadmium exposure

treatment of *D. setosum* individuals described as follows. *D. setosum* (42 individuals) which have undergone the capacitation process divided into six groups according to the levels of heavy metal (Cd) concentrations, namely 0, 1, 3, 6, 9, and 12 µg/L Cd. It dissolved for 4 weeks in aquarium tank (200 L sea water) and supported by air circulation using the electrical aerator. All the treatments were repeated 7 times. During the treatments, chemical and physics factor measurements were performed in the treatment tank, such as the change of temperature, pH, salinity, and dissolved oxygen in the morning, afternoon and evening. Water was changed once a week in the aquarium. The feeding in the form of seagrass was given every morning by binding the seagrass on the boulder and placed in the treatment tank and spreading the seagrass on the surface of the water of the treatment tank. After treatment, a surgery for 42 individuals of *D. setosum* was performed. The liver organs which had been dissected were put into a sample pot for analysis. The analysis of activation, concentration, and expression of the MTF-1 protein was conducted in the Laboratory of Physiology and Histology, Faculty of Medicine, Brawijaya University, Malang Indonesia.

Examination of the activation of MTF-1 protein

The analysis of the MTF-1 protein activation in the *D. setosum* liver was done by immunohistochemical methods. It started with the making of preparations of liver specimens by tissue fixation. Tissue was embedded in the paraffin block, cut into thin slice, and put in object glass to become a slide. After finishing a slide, the examination of MTF -1 protein activation was performed by soaking the tissue slides in xylene twice, each for 15 minutes. After that the slide was incubated in ethanol series 100 %I, 100 %II, 95 %, 90 %, 80 %, 70 % for 5 minutes in each solution. Then it was incubated again in water for 5 minutes.

The slides that had been prepared were soaked into H₂O₂ 0.3% for 30 minutes at room temperature. For the frozen sections, the incubation time was 10-30 minutes, and for the frozen tissue arrays, the incubation time was 5-10 minutes. Then, the slides were rinsed with water followed by 1 x PBS (Sigma) (pH 7.4), and then the circle of tissue dissect with Pap Pen. Slides were incubated with 1% normal serum / PBS [Mix 1x 3.5 ml of PBS, pH 7.4 for 1 drop (about 35 µl/drop) normal serum in a tube for 30 minutes at room temperature. The slides were incubated with PBS which was diluted with the antibody in a humid room for 1 hour at a room temperature. Then the slide was rinsed with 1 x PBS for 3 times for 5 minutes.

Then, the slides were incubated by diluting PBS Biotin-labeled secondary antibody for 30 minutes at a room temperature. The slides were rinsed using 1 × PBS for 3 times for 5 minutes.

Preparing the detection solution: Mix 1 × PBS 1.33 ml, 1 drop (about 35 µl/drop) from the solution A, and 1 drop (about 35 µl/drop) from the solution B in the tube and incubating the mixture at a room temperature for 30 minutes. Then, adding the detection solution to the tissue sections and incubating it at a room temperature for 30 minutes. The slides were rinsed with 1 × PBS for 3 times, each for 5 minutes. The slides were dripped with DAB liquid (Diamino-benzidine tetrahydrochloride). After that, the cells were dripped with counter stains with hematoxylin for 10 minutes. Cells were washed with flowing water and then with distilled water for 10 minutes. Cells were left at a room temperature. The tissue was placed on an object glass and dripped with entelan. After that, the liver cells undergoing activation of MTF-1 protein were observed using photonic microscope (Olympus) with slide blot shooting at 400x magnification field of view.

Measurement of MTF-1 protein concentration

The measurement of MTF-1 protein concentration was carried out by using the indirect ELISA method (Enzyme-Linked Immunosorbent Assay) Following Lequin method (Lequin, 2005). Sample preparation was carried out by softening the liver organ of *D. setosum* through thawing step. The ELISA reader was performed by making ELISA plate plan and coating buffer based on the sample code and location of the sample. After that, performing Coating Antigen with the levels 1:40 diluted with coating buffer and incubated at a temperature of 4 °C overnight. The next day the plate was washed with a solution of 0.2 % PBS Tween as much as 100 µl and repeated for 6 times. After that, 100 µl of primary antibody anti MTF-1 (1: 400) was added into assay buffer. After that, the ELISA plate was incubated at room temperature for 2 hours while being shaken with ELISA plate shaker. In the next stage, it was washed with a solution of PBS Tween 0.2 % as much as 200 µl for 6 times, and then 100 µl secondary antibody IgG biotin anti-rabbit (1: 800) was added into assay buffer and incubated at room temperature for 1 hour while being shaken. After that, the plate was washed again with PBS Tween 0.2 % for 6 times.

After that, 100 µl SAHRP solutions (1: 800) were added to the assay buffer and

incubated at room temperature for 1 hour while being shaken. After that, the solution was washed with PBS Tween 0.2% 200 µl for 6 times. And then, each was added 100 µl of good substrate sure blue TMB microwell, incubated for 20-30 minutes in a dark room. At this stage, if there reaction occurs between the antigen and the antibody, the solution would turn blue. After that, 100 µl of HCl 1N was added to stop the reaction. At this stage, the solution which was previously blue would turn yellow. After that, the sample was read by using an ELISA reader at a wavelength of 450 nm. The results of absorbance were then converted into a standard curve, and the levels of MTF-1 protein of each sample would be observed eventually.

Examination of MTF-1 protein expression

The examination of MTF-1 protein expression was carried out using western blotting following the method of Young and Hongbao (2010). The examination of MTF-1 protein expression was preceded by the examination of SDS-PAGE, namely by performing electrophoresis on the samples of protein standard broad range (Biolab). The gel, the results of SDS PAGE was soaked in 100 mL deionized water (pH 7.0) for 5 minutes. And then the gel, NC membrane, and sponge were soaked in transfer buffer for 5 minutes. Furthermore, it was arranged sequentially in each well: (1) black able, (2) sponge, (3) two sheets of filter paper, (4) gel, (5) NC membrane, (6) 3 pieces of filter paper, (7) sponge, (8) whiteable. Then it was put into the chamber, electrified from the negative to the positive pole (100 volts, 120 minutes). Furthermore, the NC membrane was rinsed with deionizing three times, and immersed in blocking buffer (5 % BSA), and incubated at 4 °C overnight. NC membrane was washed with TBS 0.2 % Tween 3 times for 5 minutes and added antibodies in TBS BSA 1 %. Incubated for 2 hours and shook. After that, the gel was washed with TBS Tween 0.2 % 3 times for 5 minutes. Then IgG biotin anti-rabbit was added in TBS, incubated for 1 hour and shaken. Washed again with TBS Tween 0.2 % for 3 times for 5 minutes, and SAHRP was added in TBS, incubated for 1 hour and shaken. After that, it was washed with TBS Tween 0.2 % for 3 times for 5 minutes. After that, TMB substrate was added to the membrane for 15-30 minutes until the ribbon on the membrane emerged. Stop the reaction with distilled water.

Data analysis

The data were analyzed with descriptive statistics to describe the activation, concentration, and expression of the MTF-1 protein. To determine the effect of the concentration of Cd on the concentration of MTF-1 protein, inferential statistical analysis One Way ANOVA was used, and then followed by Duncan test ($p < 0.05$) to determine if there is statistical differences between the mean concentrations of MTF-1 protein in *D. setosum*.

3. Results and Discussion

Activation of MTF-1 protein *Diadema setosum* from Ambon Bay, Maluku Province due to exposure cadmium

The results showed that there were The results showed that there were smearing in immunohistochemical of the *D. setosum* liver tissue by using rabbit antibodies anti MTF-1. Figure 1 showed that the cells expressing MTF-1 had the brown color, whereas the cells that did not express MTF-1 had the blue color. This phenomenon is consistent with the findings of Lichten and Schaffner (2001) on rats that state cells of the normal liver are purplish blue and look close to each other between the adjacent cells, whereas the cells of a liver expressing MTF-1 look dark brown or stained and form patches cell between the adjacent cells. In a

culture medium, the majority of MTF-1 is located in the cytosol and plays a role in detecting changes in levels of heavy metals such as Cd (Li et al., 2006).

In general, the cells undergoing the expression of MTF-1 protein seem to spread and form clusters of cells. The morphology of brown cell reflects the expression of MTF-1 protein that occupies the cytoplasm and the nucleus of liver cells of *D. setosum*. It seems that the higher the exposure to the concentration of Cd, the higher the number of the brown liver cells indicating the expression of the MTF-1 protein. This means that the concentration of Cd tends to increase the expression of the MTF-1 protein. Gunes et al. (1998), states that in mice expressing the MTF-1 protein, the liver cells of the mice were stained brown indicating the disruption of hepatocytes cell development leading to liver damage and subsequently liver enlargement, sinusoid happened and dissociation of epithelium compartment, as well as losing the selective power of the expression of cytokeratin in the liver illustrating the possibility of necrosis incidence rate more than apoptosis. The results are related to the role of MTF-1, namely as the primary regulator at the cellular level that regulates the expression of target genes in responding to various stress conditions, especially due to the presence Cd through the enlargement of the liver (Wimmer et al., 2005; Balamurugan et al., 2009).

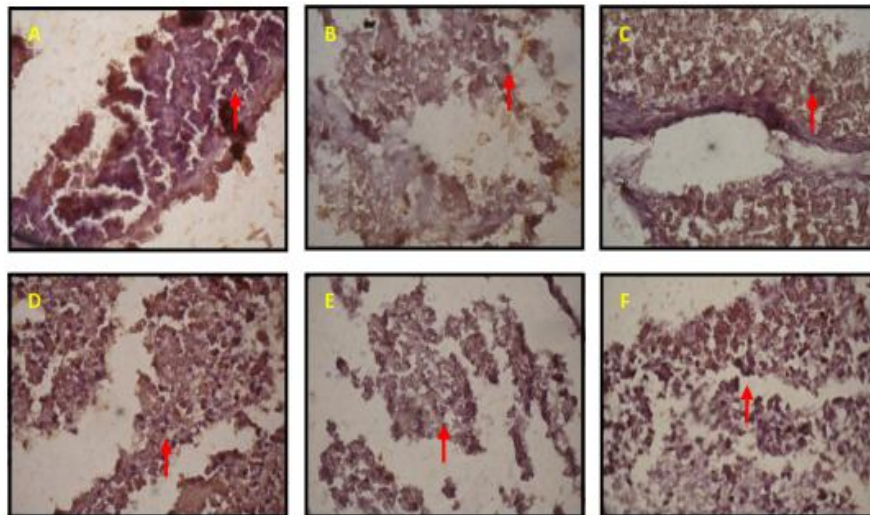


Figure 1. Results of immunohistochemical smear using antibody rabbit anti MTF-1 liver tissue *D. setosum*. observation used olympus microscope for shooting a regular slide with 1000x magnification zoom. Image with Notation: A) control; B) concentration of 1 µg/L Cd; C) concentration of 3 µg/L Cd; D) concentration of 6 µg/L Cd; E) concentration of 9 µg/L Cd; and F) concentration of 12 µg/L Cd. Arrows indicate liver cells that express the MTF-1 protein, in which the cells are brown.

Concentration and expression of MTF-1 *Diadema setosum* due to the exposure of heavy metals cadmium

The determination result of the concentration of MTF-1 protein by ELISA reader (Table 1) shows that the concentration increases along with the increasing exposure to Cd. It can be seen that the concentration levels of MTF-1 protein increased in sequence from low to high, that is, in tank 1 <2 <3 <4 <5 <6. In addition, a semi quantitative test using western blotting (Figure 2) shows that MTF-1 protein was stained with rabbit antibody anti MTF-1. This shows that the expression of MTF-1 protein increases along with the increasing exposure to the concentrations of Cd. It is characterized by the thick ribbon in the results of western blotting test. It can be assumed that higher the levels of accumulated heavy metals in the liver tissue of *D. setosum* are the MTF-1 protein. It is a transcriptional regulator protein, performing a control function by capturing Cd that accumulated in the liver tissue of *D. setosum*. Janssens et al. (2009) state that in the case of oxidative stress due to the Cd, MTF-1 is rapidly translocated to the nucleus to overcome the damage of cells by stimulating the expression of metallothionein in response

to cell exposure to heavy metals, because chemically MTF-1 contains six attachment in binding Cd. This means that MTF-1 acts as a positive mediator to initiate the expression of MT (metallothionein) gene (Radtke et al., 1995; Klassen et al., 1999; Sakulsak, 2012). Therefore, it can be concluded that the activation, concentration, and expression of MTF-1 are in accordance with the activation, concentration, and expression of MT. When stimulated, it will undergo translocation into the nucleus, in which, according to Kimura and Itoh (2008) the activation of MTF-1 depends on the presence of free zinc in the cell. Furthermore, the free Zn accumulated in the cells will trigger the activation of MTF-1 protein (Kimura et al., 2009). Saydam et al. (2002) also explain that free Zn will be bound to finger Zn in MTF-1 and causes a conformational change in the protein and triggers the phosphorylation process. MTF-1 which is phosphorylated will then undergo translocation into the nucleus and binds to Metal Response Element (MRE) on the promoter domain of MT protein (Kimura et al., 2009). The research results by Adams et al. (2002) show that the MTF-1 which is phosphorylated will accumulate in the nucleus after the phosphorylation.

Table 1. Concentration of MTF-1 protein at 6 treatment Cd tanks

Concentration of Cd ($\mu\text{g/L Cd}$)	Mean concentration of MTF-1 (ng/ml)
0.0 (Control)	4371.429
1.0	6957.143
3.0	8985.714
6.0	13628.571
9.0	14757.143
12.0	20757.143

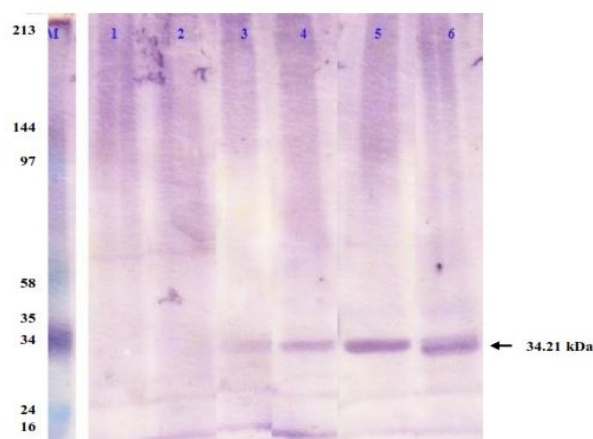


Figure 2. Expression of MTF-1 protein by western blotting test on the liver *D. setosum* at 6 tanks of Cd concentration treatments. It can be seen that at the top row of the concentration levels at 6 treatment tanks, the purplish brown ribbon is the MTF-1 protein which is marked with arrows. M: marker, 1 to 6: Cd concentration at 6 treatment tanks.

Table 2. Results of analysis of variance, the effect of the concentration of Cd on the concentration of MTF-1 protein in *D. setosum*

Source of Variance		Sum of Squares	df	Mean Square	F-Value	Sig. (p)
Concentra-tion of MTF-1 Protein	Between Groups	1.250E9	5	2.500E8	11.625	.000
	Within Groups	7.742E8	36	2.151E7		
	Total	2.024E9	41			

Table 3. Results of duncan test of analysis of variances how that the concentration of Cd has a significant effect on the concentration of MTF-1 protein in liver organ of *D. setosum*

Concentration of Heavy Metals Cd	N	The mean concentration of MTF-1 Protein in Liver Organ	Concentration of Heavy Metals Cd
Control	7	4.3714E3	a
Concentration Cd 0.02	7	6.9571E3	a
Concentration Cd 0.06	7	8.9857E3	ab
Concentration Cd 0.12	7	1.3629E4	bc
Concentration Cd 0.18	7	1.4757E4	c
Concentration Cd 0.24	7	2.0757E4	d

In addition, the results of Duncan test (Table 3) show that there is a difference in groups of the concentration levels of Cd. This shows that. It's concentration significantly increases the concentration of MTF-1 protein. The higher the concentration of Cd exposed, the higher the concentration of MTF-1 protein activated in *D. setosum*. The high concentration of MTF-1 protein is presumably related to the increased accumulation of heavy metals Cd in the cytoplasm, where Cd which binds to the MT protein caused the Zn to become free and activates the MTF-1 to be activated into the nucleus and binds with Metal Response Element (MRE). According to Langmade (2000), the MTF-1 which is activated will bind with MRE, so that it will increase the transcription of MT. Furthermore, if the concentration of MT has sufficed the needs of the cells, MTF-1 will release its attachments with the MRE and stop the metal-binding protein transcription (Saydam et al., 2002).

The research results as concentration and expression of MTF-1 protein are a biomolecular response due to exposure to Cd. The response is a control mechanism that is performed by MTF-1 to process MT protein transcription, where the cell detoxifies the Cd by the MT-1 protein for the recovery process, in which the MT protein binds with the Cd, thus the Zn becomes free, and it triggers the MTF-1 protein to be activated into the nucleus and attach with MRE. Waelput et al. (2001) explain that MT protein generally binds to Zn metal, but it will be easily detached by the induction of Cd.

Furthermore, the free Zn accumulated in the cells will trigger the activation of MTF-1 protein (Kimura et al., 2009).

4. Conclusion

The results of the examination of Cadmium inducing the activation, concentration and expression of MTF-1 protein show that the induction of Cd in *D. setosum* activates MTF-1 protein, and the greater the concentration of cadmium in the body of *D. setosum*, the greater the concentration of MTF-1 expression on the liver organ. The results of this research indicate that the activation, concentration, and expression of MTF-1 protein in the liver organ of *D. setosum* are a form biomolecular response as a mechanism to maintain homeostasis in *D. setosum* cells.

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