

Detoxification mechanisms in oxidative stress and Reactive Oxygen Species (ROS) in gills of gambusia fish (*Gambusia affinis*) exposed to Cadmium

Moh. Awaludin Adam^{1,2*}, Ach. Khumaidi¹, Ramli¹, Ira Mei Widiastuti³, Ernawati⁴, Era Insivitawati⁵, and Agoes Soegianto⁶

¹Faculty of Science and Technology, University of Ibrahimy, Situbondo, Indonesia

²Research Center for Limnology, The National Research and Innovation Agency (BRIN), Cibinong, Bogor, Indonesia

³Aquaculture, Faculty of Animal Husbandry and Fisheries, University of Tadulako, Palu, Central Sulawesi, Indonesia

⁴Technology of Fisheries Product, Faculty of Agriculture, University of Yudharta, Pasuruan, East Java, Indonesia

⁵Polytechnics of Marine and Fisheries, KKP, Sidoarjo, East Java, Indonesia

⁶Faculty of Science and Technology, University of Airlangga, Surabaya, Indonesia

Abstract. This research aims to measure and analyze oxidative stress and ROS, aside from analyzing the detoxification mechanism in the gill of gambusia fish exposed to Cd. During maintenance and acclimatization, fish were fed pellets with smooth water circulation. After seven days of acclimation, the test fish were then transferred to a 4-liter aquarium. Before treatment, fish are fasted for 24 hours to ensure the stomach is empty. The study used 5 treatment groups with 1 control (C), treatment A: 0.03; B: 0.015; C: 0.011; D: 0.007; E: 0.003 (mg/L). The results showed a significant difference between the treatment and control groups ($p < 0.05$). Treatments A and B were showed the highest reactive levels. Stress caused by Cd can increase ROS activity in the gill of gambusia fish. The production of ROS and the exposure to Cd stressors trigger the production of antioxidant enzymes such as catalase (CAT), hydrogen peroxide (H_2O_2), and superoxide dismutase (SOD). This study concludes that exposure to Cd in juvenile gambusia fish causes oxidative stress through increased antioxidant enzymes and ROS. Analysis related to the mechanism of detoxification of heavy metals in the body of juvenile gambusia fish.

1 Introduction

Cadmium (Cd) is a heavy metal widely used in industry, including semiconductors, manufacturing, welding, soldering, ceramics, and painting. Around 13,000 tons of Cadmium are produced worldwide each year, including nickel-cadmium batteries, pigments, chemical

* Corresponding author: moha@lipi.go.id

stabilizers, metal coatings, and alloys [1, 2]. The Agency for Toxic Substances and Disease Registry (ATSDR) has listed Cd as seven of the 275 most dangerous substances in the environment. Cd remains a source of attention for populations living in polluted areas, especially in developed and developing countries [3]. Cd is dangerous both by inhalation and by swallowing and can cause acute and chronic toxicity. The Cd intake route involves the respirator, intestine, and skin [4].

Cadmium in the body is predominantly bound to metallothioneins. Cadmium-metallothionein (MTs-Cd) complex is distributed to various tissues [5], organs and finally reabsorbed in the kidney tubules. There is no mechanism of cadmium excretion in organisms [6]. Cd can be accumulated for life-long inside the body. The half-time Cadmium in the renal cortex is 20-35 years [7]. Acute exposure to Cd causes dysuria, polyuria, chest pain, fatigue, and headaches. Chronic poisoning is usually found in cases after several years of exposure and is clinically characterized by variable features [4]. Nasorespiratory involvement includes emphysema, rhinitis, changes in the nasal mucosa, and anosmia [6]. In fish, an immense amount of Cd is stored in gills [8], kidneys, liver, and meat [2].

Clinical damage to the gills has been demonstrated, especially those closely related to exposure [9]. However, in the past few decades, exposure to Cadmium found in populations has produced subtle effects on the kidneys, such as tubular bones. Cadmium in gills has been measured both autopsies and in vivo [10]. In comparison, some other damage to gills is discussed in the previous article [8, 11, 12]. The destructive mechanism of Cd also occurs in the kidneys through the formation of Reactive Oxygen Species (ROS) and oxidative stress with nonenzymatic reactions. Besides, the ferritin/lysine system can cause oxidative DNA damage through the generation of ROS. Fenton's similar response to free iron ions is released by ferritin, which undergoes oxidation [9].

In the respiratory process, there is a rapid absorption of oxygen molecules and transformation into reactive oxygen compounds, representing the gills' defense mechanism. Reactive oxygen compounds at physiological concentrations can modulate redox-sensitive signals and improve cellular immunological function [13]. Our research aims to measure and analyze oxidative stress and ROS in the gills of juvenile gambusia fish exposed to Cd. Analysis related to the mechanism of detoxification of heavy metals in the body of juvenile gambusia fish.

2 Materials and methods

2.1 Test fish

This research was experimental to analyze the impact of Cd exposure on gambusia (*Gambusia affinis*) fish that have been cultivated in Freshwater Aquaculture Ponds (IBAT) - Punten, Batu City, East Java, Indonesia. Fish used with an average weight of 1.5 grams and a body length of 5 cm. Then, the fish are taken to the Fish Reproduction Laboratory of the Faculty of Science and Technology (FSAINTEK), Ibrahimy University, for adaptation. During maintenance and acclimation, fish are fed pellets with smooth water circulation. After seven days of acclimation, the test fish were then transferred to a 4-liter aquarium. Before treatment, fish are fasted for 24 hours to ensure the stomach is empty. The study used 5 treatment groups with 1 control (C), treatment A: 0.03 mg / L; B: 0.015 mg / L; C: 0.011 mg / L; D: 0.007 mg / L; E: 0.003 mg / L.

2.2 Cadmium exposure

Fish prepared beforehand are then exposed with standard Cadmium (Cd^{2+}) for 28 days [14] with an interval of 4 days. After the exposure, the water changes once (according to the results of acute toxic tests). Fish that have been exposed during the trial period were directly dissected to take fresh gill for protein isolation. After isolating the protein, it was then tested as needed in conducting an antioxidant/oxidative stress test.

2.2.1. Hydrogen peroxide test

The process of determining the H_2O_2 concentration was using a modified method based on [15]. The solution was measured spectrophotometrically at $\lambda = 505 \text{ nm}$. Standard and solution tests consist of 1 M H_2O 2200 μL and 200 μL serum with the addition of 160 μL PBS pH 7.4, 160 μL FeCl_3 (251.5 mg FeCl_3 dissolved in 250 ml distilled water), and 160 μL o-phenanthroline (120 mg o-phenanthroline dissolved in 100 ml distilled water) for both solutions. The composition of the empty solution is identical to the test solution, except for the absence of FeCl_3 in the open place. After preparation, all resolutions were incubated for 30 minutes at room temperature, then centrifuged at 12,000 rpm for 10 minutes, and the absorbance of standard tests (As), (Au) and empty solutions (Ab) measured at $\lambda = 505 \text{ nm}$, using the supernatant of every solution.

2.2.2 Malondialdehyde test

MDA was accepted by method [16]. Spectrophotometry at 532 nm. Serum in homogenization of 100 mg. Then add 1 mL of distilled water and then discard it in the Eppendorf tube. After that add 100% TCA 100 μL , 1% Na-Thio 100 μL and 250 μL 1 N HCl. The solution is heated at 100°C for 20 minutes. Then centrifuged to 3,500 rpm for 10 minutes. The supernatant is taken. After that, add distilled water to 3500 μL . It can be read with a spectrophotometer with a maximum wavelength of 500-600 nm.

2.2.3 Superoxide dismutase assay

SOD activity in the supernatant was measured by the method [17]. The supernatant (500 μL) was added to 0,8 ml of carbonate buffer (100 mM, pH 10.2) and 100 μL epinephrine three mM). Changes in the sample's absorbance were recorded at 480 nm on a spectrophotometer for 2 minutes with 15 seconds of interval

2.2.4 Catalase assay

Catalase activity of fish gill samples started with the preparation of lysate solution by the method [18]. Taking 20 μL samples added 800 μL of 0.5% Triton X-100 solution, then the preparation of a standard solution and the mother liquor by dissolving 10 μL of catalase in 50 mL phosphate buffer. Ten μL lysate and 12.5 mL phosphate buffer were mixed. The reaction starts after adding 1 mL H_2O_2 . The whole solution was vortex slowly, then the decrease in absorbance was read by spectrophotometer at a wavelength of 240 nm, A240 values range from 0.02-0.10.

2.2.5 GSH assay

The method to measure the GSH levels was following [19] by mixing 50 μL with 1.78 mL in 0.1 M phosphate pH 8 and 0.2 mL TCA 5%. The mixture was centrifuged at 1500 g for 5 minutes at 4°C. The supernatant was then added with 0.01 mL DNTB and left for 60 minutes. The combination was then examined using a spectrophotometer at a wavelength of 412 nm to determine GSH levels.

2.2.6 GPx test

Glutathione peroxidase (GPx) enzyme activity in fish gill samples was carried out by the method [20] utilizing a 100 μL sample diluted with 200 μL physiologic NaCl (0.85% NaCl solution). 0.1 mL of the solution is taken and then added with 0.4 mL of Triton-X 0.5%. This mixing is called hemolysate and put in a 100 μL test tube of hemolysate. 100 mL of Drabkin solution was added and shaken, then added with 2.6 mL of phosphate buffer and beaten slowly. Add 0.1 mL NADPH, 0.01 mL GSSG-R, 0.01 mL NaNO_3 , 0.1 mL GSH and then shake. Before reading the absorbance rate with a spectrophotometer at 340 nm wavelength, insert the silica cuvette, add 1 mL H_2O_2 with an interval of 1-2 minutes, and blank 100 μL .

2.2.7 Protease test

Protease activity was carried out by the method [21]. A total of 1 mL from 2% casein mixed with 1 mL of borate buffer (0.01 M pH 8), 0.2 mL of 0.05 M hydrochloric acid, and 0.2 mL of sample to be determined by its activity then incubated at 37°C for 10 minutes and added 2 mL of 0.1 M trichloroacetic acid, distributed at room temperature 37°C for 10 minutes, then centrifuged. The 1.5 mL portion of the mixture was mixed with 5 mL of 0.5 M disodium carbonate and 1 mL of the Folin Ciocalteus reagent, left for 20 minutes at 37°C room temperature. Then read the absorbance at a wavelength of 578 nm. Protease activity was calculated in units of U (units) per mL of sample extract.

2.2.8. ROS assay

The ROS test was carried out according to the instructions in the ELISA ROS kit on a UV-1601 spectrophotometer by collecting 40 μL samples, then adding 400 μL TCA, centrifuged 1000 rpm for 10 minutes, adding reagents (Na thiosulphate, 400 μL TBA, 1 N HCl, and one distilled water (mL), then incubated at 100°C for 15 minutes, the temperature was reduced (cooling) for several minutes then read on the spectrophotometer.

2.3 Statistical analysis

Data were presented as mean \pm SD. The difference was tested with one-way ANOVA. For all results, the average level of MDA, H_2O_2 , GSH, GPx, CAT, SOD, and ROS activity be presented in Fig. 1.

3 Results

3.1 Oxidative stress and ROS

Some of the results of oxidative stress testing, antioxidants, and ROS on Cd exposed fish are intended to analyze the extent to which gambusia fish can survive if observed from oxidative

stress markers. ANOVA test results showed significant differences between cases and control groups ($p<0.05$). Fig. 1 shows a substantial difference between the treatment and control groups ($p<0.05$). Treatments A and B were the treatments that showed the highest reactive levels.

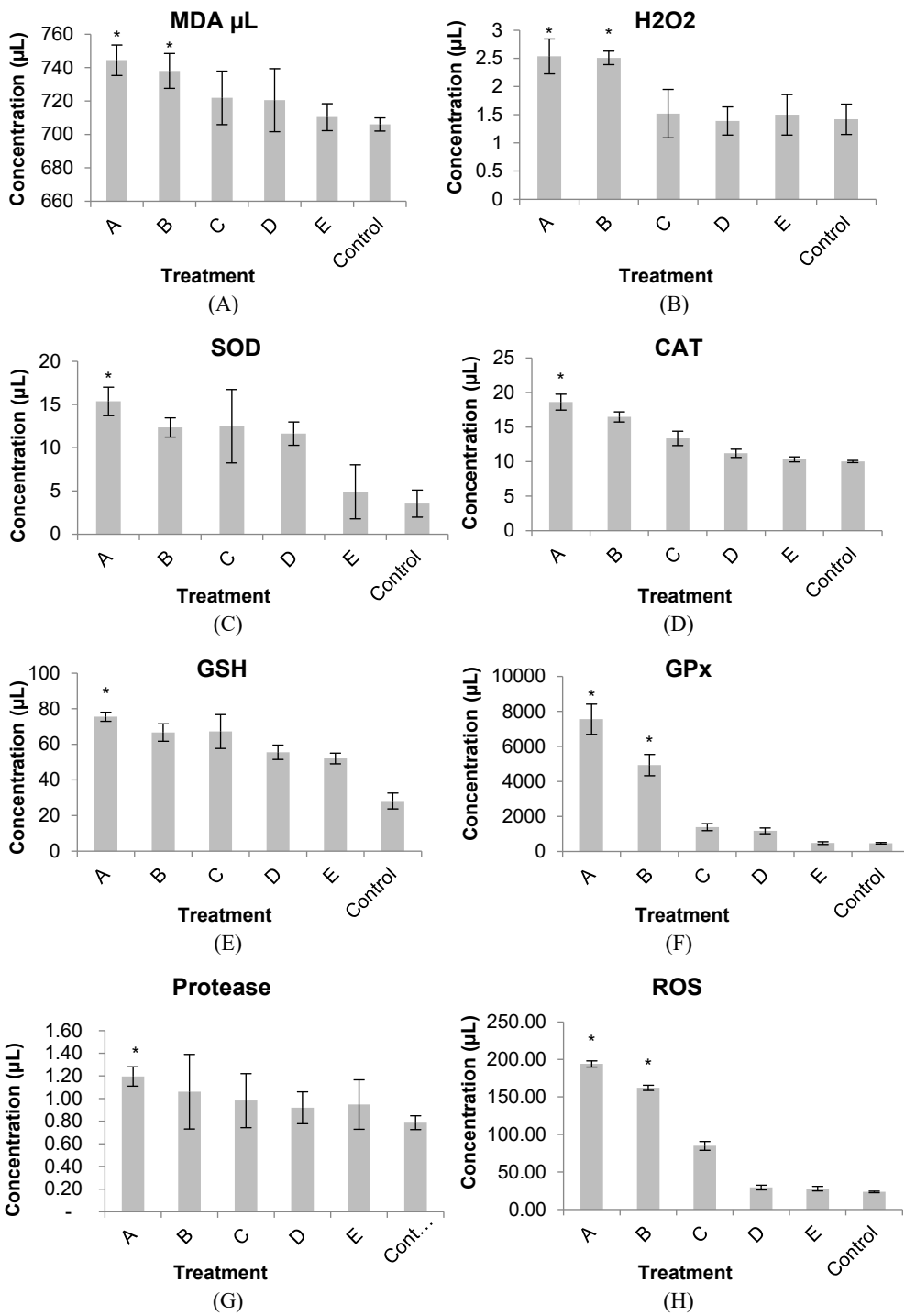


Fig. 1. Comparison of the results of oxidative stress testing in gambusia fish exposed to Cd in (A) MDA activity, (B) Hydrogen Peroxide (H_2O_2), (C) Superoxide (SOD), (D) Catalase (CAT), (E) Glutathione S-hydrogen (GSH), (F) Glutathione peroxidase (GPx), (G) Protease and (H) Reactive Oxygen Species (ROS) compared to treatment and control. On the average \pm SD at the average treatment (* $p < 0.05$), (** $p < 0.01$).

4 Discussion

4.1 Oxidative stress and ROS

Oxidative stress was a dangerous condition when there was an imbalance between ROS, including superoxide, hydrogen peroxide, hydroxyl radicals, and non-defense antioxidants, including SOD, catalase, and peroxidase. Stress caused by Cd can increase ROS activity in the gambusia fish gill. The process of diffusion of greater environmental concentrations in fish cells results in the uptake of ecological material. An exciting mechanism that explains the indirect role of Cd as free radicals can cause stress and disruption of homeostasis cells or stimulation of the immune system.

Triggering ROS production and exposure to the Cd stressor can trigger the production of antioxidant enzymes. Those are catalase (CAT), hydrogen peroxide (H_2O_2), superoxide dismutase (SOD) (Fig. 1). An average increase in all antioxidant levels indicates that oxidative stress has occurred in gambusia fish exposed to Cd. The disorder is mediated by reactive oxygen species (ROS) produced by cells in response to stressors. The greater the concentration of the stressor, the greater the concentration of ROS caused. The data shows the same thing as the analysis of ROS data. The higher the attention of the Cd stressor, the more the production of antioxidant enzymes increased. This means that the gambusia fish body (especially the gill) fights by boosting the immune response (antibodies) in the presence of foreign material that enters.

SOD is considered a key enzyme in the regulation of intracellular ROS concentrations. SOD acts as the first line of defense against ROS, breaking down superoxide into H_2O_2 . SOD acts as the first line of defense against ROS, dismutating superoxide to H_2O_2 . Thus, increased SOD activity indicates that it plays a positive role in controlling cellular levels of this ROS and repairing oxidative damage to free radicals by stressed Cd. Permeability of the mitochondrial transition pore (PTPM) in the outer membrane of the mitochondria allows SOD to leak into the cytoplasm, which is then converted to H_2O_2 in the cytoplasm by SOD. H_2O_2 was another enzyme produced as less reactive, which was not charged and can spread through the aquaporin membrane such as aquaporin-8.

Aquaporin-8 has been detected in the membrane in the mitochondria and shows its function as waterways and hydrogen peroxide. Despite their low reactivity load, some proteins contain specific cysteine residues that are susceptible to oxidation by hydrogen peroxide, essential for hydrogen peroxide-based signaling systems. H_2O_2 can be converted into hydroxyl radicals, a very reactive species. H_2O_2 and oxygen production of other toxic species in cellular compartments and results in acceleration of lipid peroxidation and further oxidative damage. An increase in MDA levels in the gill of fish exposed to Cd shows an increase in lipid peroxidation. It is known that the result of oxygen radicals due to metals results in an attack not only of DNA but also of other cellular components involving residues of double phospholipid unsaturated fatty acids, which are very sensitive to oxidation.

The whole process of lipid peroxidation consists of three stages: initiation, propagation, and cessation of initiation. The first stage involves a ROS attack that randomizes a hydrogen atom from the methylene group in the lipids. A double bond adjacent to the methylene group weakens the bond between carbon and hydrogen so that hydrogen can more easily be removed from fatty acid molecules. Fatty acids without double bonds or one bond may

undergo oxidation but not lipid chain peroxidation, leading to lipo-peroxyl radicals ($\text{ROO}\bullet$). Once formed, lipo-peroxyl radicals ($\text{ROO}\bullet$) can be rearranged through a cyclization reaction to endoperoxide (a precursor of malondialdehyde). The end product of this peroxidation process becomes MDA. MDA is mutagenic in bacteria and mammalian and carcinogenic cells in mice.

An increase in lipid peroxidation with increased MDA levels has been observed in an experimental group of Cadmium arbitrate rats. It has been reported in other studies that the administration of PT Cd via different routes can cause an increase in fat peroxidation of erythrocyte membranes and tissues of the liver, kidney, brain, and testes. So that, the MDA can be used as an indicator of oxidative damage. MDA is one of the main manifestations of oxidative damage and has been found to play an essential role in Cd toxicity. MDA is a well-known lipid peroxidation indicator and is elevated in the liver and kidney after exposure to Cd.

Superoxide (O_2^-), free radicals produced accidentally or by reactions catalyzed by various enzymes, will be neutralized to H_2O_2 by the SOD enzyme. H_2O_2 will be converted to H_2O and O_2 by catalase. Therefore, most enzymes that produce and need O_2 are peroxisome with the SOD, CAT, and GPx enzymes. Peroxides formed by the reaction of OH radicals with unsaturated fatty acids will be reduced to fat origin by glutathione peroxidase (GPx), which is dependent on selenium (Se) as a cofactor on the membrane and plasma phospholipids. Therefore, Se becomes an optimizer of good antioxidant activity. Glutathione which is oxidized will be reduced by glutathione reductase, which depends on NADPH.

4.2 Detoxification mechanism of exposure to Cd

Free radicals are molecules or atoms that do not have electron pairs in external orbitals, so they are unstable and very reactive to cells in the body to get electron pairs. Abnormalities in protein, lipids, and carbohydrates can occur due to damage of cell constituents caused by free radical activity in the body [22]. Reactive oxygen species (ROS) are free radicals that result from biochemical processes in the body [23], in this case, exposure to heavy metals Cd (Figure 2). One source of free radicals is chemical pollution in toxins that will drastically increase free radicals' levels. Some drugs such as anesthetics, pesticides, and solvents used in industry are exogenous sources of free radicals. Free radicals are by-products of metabolism and are produced continuously in living things. Reactive oxygen species (ROS) in excess amounts can lead to oxidative stress resulting in oxidative damage to proteins, DNA, and fats [24–26].

Lipid peroxidation reactions that produce free radicals occur through three stages: initiation, propagation, and termination [27]. The Initiation Stage is the initial formation stage of free radicals, which causes many free radicals to form. The propagation stage is a reaction to the formation of free radicals by involving the same number of free radicals, and it is very reactive. The termination stage is when radical compounds change into non-radicals when two radical molecules react [28].

High levels of malondialdehyde (MDA) in plasma in the picture above can indicate free radicals and oxidative damage to cell membranes as free radical compounds attack lipid membranes that contain polyunsaturated fatty acids will form MDA, which is one of the end products of peroxidation lipids. Free radicals and oxidative damage can be inhibited and prevented by antioxidant compounds by adding hydrogen atom groups to electrons that do not have a pair to be stable [29]. Exposure to Cd^{2+} given to gambusia fish, both acute and subchronic doses, demonstrates the existence of different results. Acute dose treatment describes direction with high quantities and momentarily causes an increase in antioxidant enzymes getting higher as well, especially in the GSH enzyme, which provides detection

ability. In contrast, the treatment of subchronic doses with low doses and more prolonged exposure causes a slower increase in antioxidant enzymes.

Essential antioxidants produced in gambusia fish bodies play an important role because they can neutralize free radicals by providing one electron to form stable molecules and ending free radical reactions. Antioxidants are not only essential to prevent oxidative stress and tissue damage. Still, its are also crucial in preventing the increase in pro-inflammatory production of cytokines, which are the result of activation of the body’s defense response that occurs continuously through the antioxidants that function is catalyzing the molecular oxidation process carried out by catalase and glutathione peroxidase. In addition, the enzyme also functions to repair tissue or cells that have been damaged by free radicals, which are carried out by the protease enzyme. Cell damage caused by free radicals is thought to cause heavy metal exposure [30].

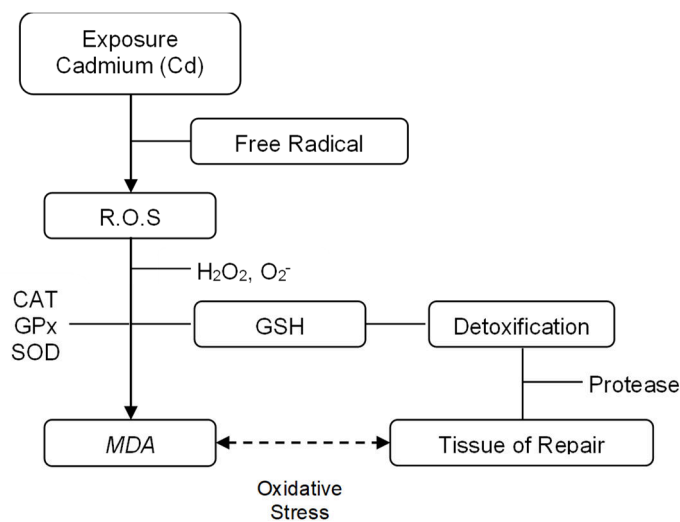
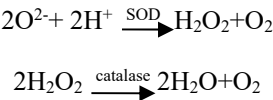


Fig. 2. Detoxification mechanism of exposure to Cd.

Antioxidants are the body’s first defines that will prevent the free radical attack on the periodontium tissue from maintaining a healthy periodontium tissue condition. [31], to avoid oxidative damage from the production of superoxide (O²⁻), then antioxidants such as superoxide dismutase (SOD) are stimulated to convert superoxide (O²⁻) to hydrogen peroxide (H₂O₂) so that SOD acts as a catalyst to convert superoxide into oxygen and hydrogen peroxide. Hydrogen peroxide will eventually be removed by the second enzyme involved, called catalase, more present in intracellular cells than extracellular cells. This catalase acts as a destroyer of hydrogen peroxides and superoxide. In summary, it can be concluded as below:



The mechanism to prevent hydroxyl radicals (OH⁻) that will damage cells is binding free radicals with antioxidants, which can also be done by glutathione (GSH), so the body can still depend on antioxidant defenses from ROS attacks that are still under normal conditions. Although the mechanism of action of each antioxidant is different, its role is still to protect cells and tissues from staying healthy. To prevent oxidation, the Cd metabolite is then rapidly

detoxified by active cell glutathione (GSH) reserves so that Cd will bind to the sulfinyl groups contained in GSH. Formation of mercapturic acid and cysteine conjugates due to the reaction between GSH and Cd, which is then excreted in the urine. The peroxidation of lipid will occur if the form of metabolite Cd is inversely proportional to the rate of detoxification of GSH [32].

5 Conclusion

This study concluded that exposure to Cadmium caused oxidative stress in the gill of gambusia fish through an increase in SOD, CAT, MDA, GPx, GSH, and H₂O₂ enzymes. The analysis related to the mechanism of detoxification of heavy metals in the body of juvenile gambusia fish.

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