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Abstract. The aim of this study was to assess the impact of cadmium nitrat ($CdNO_3$) in *Gambusia affinis* on growth and the development of histopathological changes in gill organs and on the activity of some oxidative stress markers during a 96-hours acute toxicity test. Tests were performed on *G. affinis*. The average initial weight of fish used in the experiment was 3±1.2 g and the average initial total length of fish was 5.21±3.48 mm. The fish were exposed to a range of $CdNO_3$ concentrations (1 mg/L, environmental concentration, 0.1, 0.01, 0.001 nad 0.0001 mg/L) for 96 hours. Each test on a $CdNO_3$-treated group was performed in duplicate. There were no significant differences between the specific growth rates of fish from the test groups and from the control group. Histopathological examination revealed no pathological changes in organs of fish exposed to any $CdNO_3$ concentration. Significantly lower CAT and SOD activity ($p<0.05$, $p<0.01$) was found in fish exposed to $CdNO_3$ concentrations of 1, 0.1 and 0.01 mg/L compared to the control group; the control group was observed. H2O2, GSH and GPx exhibited significantly higher activity ($p<0.01$) in the 1, 0.1, 0.01 and 0.001 mg/L groups compared to the control group. But none of these differences were significant ($p>0.05$). No significance differences ($p>0.05$) between the control group and any experimental group were found with respect to Protease activity. The MDA level was significantly higher ($p<0.01$) at a concentrations of 1, 0.1, 0.01 and 0.001 mg/L compared to the control, while in the other experimental groups no significant differences compared to the control group ($p>0.05$) were observed. According to our results, all tested concentrations of $CdNO_3$, including the environmental concentration, had an inhibit on oxidative stress markers and detoxifying enzymes in exposed fish, but did not affect fish growth or cause the development of histopathological changes in the fish organism.

1. Introduction

Heavy metals are a mixed group of elements with metallic properties that include those important. Poisoning due to heavy metals has traditionally been thought to occur only in the setting of industrial or occupational exposure, and to present with severe and obvious clinical signs and symptoms; however, this is now uncommon in modern industrialized economies [1].

Cadmium is regularly found in ores together with zinc, copper and lead. Therefore volcanic activity is one natural reason for a temporary increase in environmental cadmium concentrations [2]. Cd (cadmium) is a non-essential, ubiquitous and toxic transition metal which can be found in the aquatic

environment from anthropogenic sources such as metal mining and processing, agricultural use of pesticides. It can accumulate through water and diet into aquatic organisms, which can have harmful effect via food chains [3].

Due to toxic potencies and persistence in the environment, metals are contaminants of global concern to aquatic wildlife, including fishes. Occurrence of metals in freshwater ecosystems results from activities including discharge of municipal effluents, industrial discharges and mining activities. Cadmium (Cd) are of particular concern as an ubiquitous in the environment and can lead to a variety of adverse effects on in aquatic wildlife, particularly fish [4]. The greater than additive toxicity of Cd observed these metals not only have common, but also independent binding sites and mechanisms of action, which could exacerbate the pathophysiological effects caused by each metal alone [5, 6].

Many studies have dealt with the acute effects of cadmium and heavy metals on organisms, especially on aquatic organisms [7, 8, 9]. One aspect of cadmium toxicities is the generation of cytotoxic reactive oxygen species (ROS) that cause oxidative deterioration of biological macromolecules [10]. In the cells, there is a balance between ROS production and antioxidant defense which is an important removal mechanism of reactive oxygen species [11]. Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) constitute a part of the antioxidant system that protects cells against ROS [12]. When the rate of ROS generation exceeds the antioxidant capacity of cells, severe oxidative stress will result in oxidative damage. In addition to the enzyme index, a central measure of oxidative stress is lipid peroxidation (LPO), as indicated by malondialdehyde (MDA) levels, which can accumu-late as a consequence of cellular damage [9].

It is necessary to learn more about the acute effects of this and other heavy metal;as cadmium on fish organisms and on early developmental stages in order to evaluate the range of the problem accurately. In many studies, biomarkers of oxidative stress have been used to evaluate toxic effects, because the aquatic environment have the potencial to induce oxidative stress in fish organisms (i.e. through the production of free radicals and reactive oxygen species [4, 5, 6, 13, 14, 15, 16].

The aim of this study was to assess the impact of cadmium nitrat (CdNO3) in surface waters on fish under experimental conditions. In detail, the aim was to assess the effects of acute exposure to CdNO3 on fish growth and the development of histopathological changes in gill organs and on the activity of some oxidative stress markers. Thus, the activities of the enzymes catalse (CAT), superoxide dissmutase (SOD), hydrogen peroxidase (H2O2), glutathione S-peroxiadase (GSH), glutathione peroxidase (GPx), Protease and and the products of the lipid peroxidation (MDA) were determined.

2. Material and methods

2.1. Experimental fish

Cadmium nitrat ($CdNO_3$) toxicity tests were performed on *G. affinis*, which is one of the model organisms most commonly used in toxicity tests to determine the effects of chemicals and pharmaceuticals on fish in the aquatic environment [17, 18, 19, 20, 21]. Experimental procedures were in compliance with europan legislation [22, 23].

2.2. The acute toxicity test

Tests were performed on *G. affinis*. The average initial weight of fish used in the experiment was 3±1.2 g and the average initial total length of fish was 5.21±3.48 mm, according to EPA test and OECD test modifided [8, 24]. Aqueous testing solutions of CdNO3 (cadmium standart solution) were prepared from the active compound produced by EMD-Millipore, made in German (100 ml, 1000 ppm/ml). The fish were randomly distributed into 4 liter glass aquaria, 10 specimens per each. The experiment was conducted in a flow-through system and the volume of each test solution was replaced twice a day. The fish were exposed to a range of CdNO3 concentrations (1 mg/L, environmental concentration, 0.1, 0.01, 0.001 nad 0.0001 mg/L) for 96 hours.

During the tests, living conditions were checked at 24-hour intervals (aclimatisations period) and the number of dead fish was recorded in each concentration. The mean values for water quality were: temperature, 26±1 °C; oxygen saturation above 70% (ranging from 87% to 92%); and pH from 8.3 to 8.5.

2.3. Histopathological examination

The fish (10 specimens from each concentration) were prepared for histopathological examination modifeded (of selected organs – gills), fixed in buffered 10% neutral formalin, dehydrated, embedded in paraffin wax, sectioned on a microtome at a thickness of 4 μm, and stained with haematoxylin and eosin. Five sections from each fish were examined at different levels.

2.4. Fish sampling and homogenization

At the end of the test, the fish were immediately frozen, and stored at –85 °C until analyses modifeded [8]. Whole body samples were weighed and homogenised (1:10 w/v) using phosphate buffer (pH=7.2). The homogenate was divided into two portions, to obtain a supernatant fraction for the determination of CAT, SOD, H_2O_2, GSH, GPx, Protease and MDA activities.

2.5. Measurement of oxidative stress parameters and detoxifying enzyme

The activity of CAT was determined spectrophotometrically by measuring the breakdown of H2O2 at 240 nm. The specific activity was expressed as the μmol of decomposed H2O2 per min per mg of protein [25]. The SOD activity in supernatant was measured by the method [26]. The supernatant (500 μl) was added to 0.800 ml of carbonate buffer (100 mM, pH 10.2) and 100 μl of epinephrine 3 mM). The change in absorbance of each sample was then recorded at 480 nm in spectrophotometer for 2 min at an interval of 15 sec. Determination of H2O2 concentration by the modified FOX2 method [27].

Solutions measured spectrophotometrically at λ = 505 nm. Standard and test solutions consisted of 1 M H2O2 200 μL and 200 μL serum, respectively, with the addition of 160 μL PBS pH 7.4, 160 μL FeCl3 (251.5 mg FeCl3 dissolved in 250 ml distilled water) and 160 μL o-fenantroline (120 mg o-phenantroline dissolved in 100 ml distilled water) for both solutions. The tissue of GSH activity were prepared [28] in 0.1 M sodium phosphate-0.005 M EDT A buffer (pH 8.0) and kept on ice until used. o-Phthalaldehyde (OPT) solution was prepared daily in reagent-grade absolute methanol just prior to use. All other reagents employed were reagent grade quality. The tissue hydrogen peroxide level was determined according to the methods that provided by Philip J. [29] using HRP and phenol red. The values were expressed as nanomole of hydrogen peroxide per milligram protein. The catalytic concentration of GPx was calculated from the amount of NADPH oxidation to NADP+ produced by the reaction at 340 nm. Protease activities in the tissues were estimated by the ninhydrin method as described by [30]. Homogenate (4 %) was prepared in cold phosphate extraction buffer (50 mmol, pH 7) and centrifuged at 3,000 rpm for 15 min. To 2 mlof the supernatant, 0.5 ml of 1 % casein and 2 mlof 0.1 mol phosphate buffer (pH 5) were added. MDA, the secondary product of lipid peroxidation, was estimated in the tissue homogenates utilizing the colorimetric reaction of TBA [31]. It gives an index of the extent of progress of lipid peroxidation, and since the assay estimates the amount of TBA reactive substance, e.g., MDA, it is also known as TBARS test. Tissue homogenates (16 %) were preparedin cold 50 mmol Tris–hydrochloric acid (HCl) (pH 6.8) extraction buffer. To 0.8 ml of the homogenate, 2 ml of 15 % trichloroacetic acid (TCA) was added and centrifuged at 5,000 rpm for 15 min.

2.6. Statistical analysis

All data were tested for normal distribution using the Shapiro-Wilk test. After testing for homogeneity of variance across groups (the Levene test), an analysis of variance (one-way ANOVA) was used. The differences between test groups with different concentrations and the control group were assessed using Dunnett`s test with $p<0.05$ chosen as the level of significance.

3. Result

3.1. Mortality

In all CdNO3-exposed groups and in the control group, mortality did exceed experimental period 96 hours (mortality was observed in the control group in Table 1).

Table 1. Mortality exceed experimental period 96 hours.

Consentration (mg/L)	Mortality (hours)				Totals
	24	48	72	96	
1.0000	3	4	2	1	10
0.1000	1	3	2	2	8
0.0100	0	1	1	3	5
0.0010	0	1	1	2	4
0.0001	0	0	0	1	1
Control	0	0	0	0	0

NB: at the research was analys of LC_{50}-96h at concentration of 0.03 mg/L

3.2. Histopathological examination

Histopathological examination did not reveal any path-ological lesions in any CdNO3-exposed fish or in fish from the control group (Figure 1).

3.3. Oxidative stress markers and detoxifying enzyme

The effects of acute exposure to CdNO3 on selected oxidative stress markers and the activity of detoxifying enzyme are presented in Table 2.

Significantly lower CAT and SOD activity ($p<0.05$, $p<0.01$) was found in fish exposed to CdNO3 concentrations of 1, 0.1 and 0.01 mg/L compared to the control group; however, no significant difference in CAT and SOD activity between the 0.001 and 0.0001 mg/L group and the control group was observed. H2O2, GSH and GPx exhibited significantly higher activity ($p<0.01$) in the 1, 0.1, 0.01 and 0.001 mg/L groups compared to the control group. In the other experimental groups, the H2O2, GSH and GPx activity was lower than in the control group, but none of these differences were significant ($p>0.05$).
No significance differences ($p>0.05$) between the control group and any experimental group were found with respect to Protease activity.

The MDA level was significantly lower ($p<0.01$) in fish exposed to CdNO3 at a concentration of 0.0001 mg/L and significantly higher ($p<0.01$) at a concentrations of 1, 0.1, 0.01 and 0.001 mg/L compared to the control, while in the other experimental groups no significant differences compared to the control group ($p>0.05$) were observed.

Table 2. Comparison of the activities of Catalase (CAT, μmol.min–1.mg protein–1), (SOD, nmol.min–1.mg protein–1), hydrogen peroxidase (H2O2, nmol.min–1.mg protein–1), glutathione S-hydrogen (GSH, nmol.min–1.mg protein–1), glutathione peroxidase (GPx, nmol.min–1.mg protein–1), protease activity (PA, nmol.min–1.mg protein–1) and the level of lipid peroxidation (MDA, nmol.min–1.mg protein–1) after CdNO3 exposure between different treatment groups and control.

Indicatio ns	Concentration of $CdNO_3$					
	Control	1 mg/L	0,1 mg/L	0,01 mg/L	0,001 mg/L	0,0001 mg/L
CAT	0.55±0.27	3.41±0.47[b]	2.45±0.22[b]	2.28±0.42[b]	1.63±0.50	0.73±0.34
SOD	0.36±0.16	3.04±0.18[b]	2.53±0.26[b]	2.42±0.37[b]	1.58±0.24[a]	1.51±0.04[a]
H_2O_2	8.82±1.92	92.57±9.54[b]	69.19±8.94[b]	29.62±3.89[b]	24.13±3.03[b]	15.84±3.08[a]
GSH	25.73±3.03	110.53±22.29[b]	97.95±11.67[b]	89.88±10.02[b]	64.85±10.85[b]	54.56±4.32
GP_X	1165.33±42.78	3531.19±488.01[b]	3153.92±292.75[b]	2709.59±147.71[b]	2387.66±171.12[b]	1909.79±101.65
Protease	0.79±0.06	1.20±0.09[b]	1.06±0.33[a]	0.98±0.24	0.92±0.14	0.95±0.22
MDA	2.70±1.14	31.53±7.11[b]	27.21±5.17[b]	21.53±2.04[b]	12.49±2.93[b]	8.91±1.42[a]

[a] mean ± standard error of the mean ($p<0.05$)
[b] mean ± standard error of the mean ($p<0.01$)

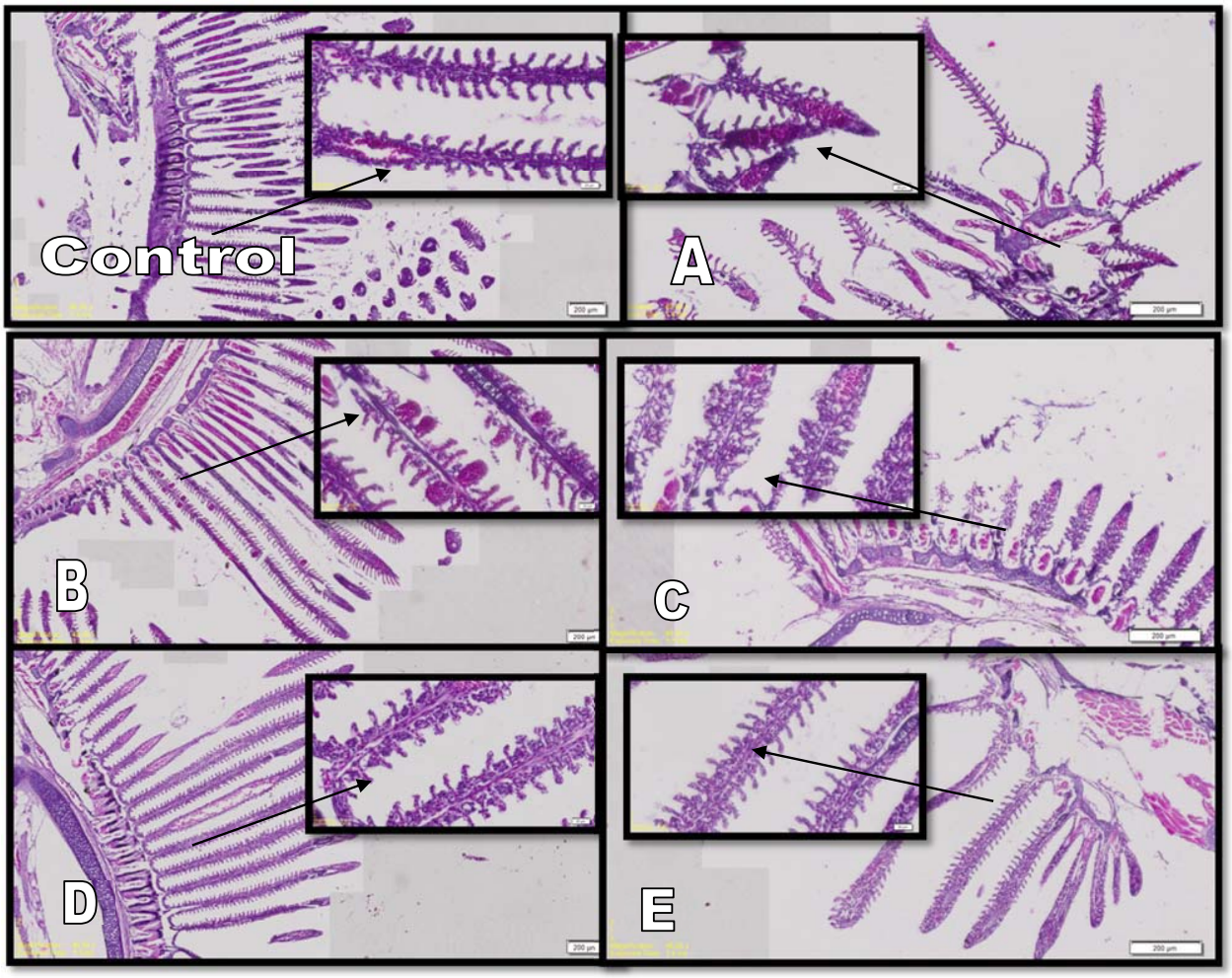

Figure 1. Gills histology of fish effects of cadmium exposure ($CdNO_3$) (A) 1 mg/L; (B) 0,1 mg/L; (C) 0,01 mg/L; (D) 0,001 mg/L; and (E 0,0001 mg/L; with control)

3.4. Validity of the tests

Our tests met all conditions required by the OECD –mortality in the control groups was below 10%, the dissolved oxygen concentrations were at least 60%, the water temperature did not differ by more than ± 1 °C among test aquariums, and test substance concentrations were above 80% of the initial measured concentration.

4. Discussions

The result of histopathological analysis shows that the damage caused by exposure to treatments A, B and C undergoes many cell changes (Figure 1). The treatment gives us an idea how big the effect of the cell level in the gill organs of gambusia fish that has the function as a respirator and homeostasis. So that will be the main and most important part in the process affected by foreign body exposure. It is

assumed to occur because of the release of cells from the support tissue (basement membrane) caused by necrosis cells due to reduced oxygen levels in the environment that stimulate the occurrence of stress due to hypoxia. Hypoxia occurs when blood cells that carry oxygen into the tissue cannot meet the metabolic processes in the body for various purposes. Hypoxia can lead to pathological conditions such as necrosis, hyperplasia, hyperemia and hypertrophy in various organ tissues namely gills, liver, spleen, and kidney.

In addition, the effect of other exposures is that secondary lamella hyperplasia of the gill occurs due to uncontrolled epithelial division, whereas in primary lamella is caused by excessive chloride cell division. Hyperplasia of lamella gill cells begins with several occasions such as edema, cell death and the release of epithelial cells in lamella gill. The fusion of lamella is due to the continuous increase of pathology of hyperplasia and causes the intermediate space between the secondary lamella by the new cell-cell which then triggers attachment on both sides of the lamella. This incident states that the incidence of lamella fusion is a severe level of damage because the fusion of lamella is an advanced stage damage from damage to hyperplasia.

Radicals are naturally formed by the system in the body and have a beneficial effect that is not realized. The immune system is the main system of the body that uses free radicals. Foreign body attack or tissue damage is characterized by free radicals by the immune system. Antioxidants work by protecting lipids from the peroxidation process by free radicals. When free radicals get electrons from antioxidants, the free radicals no longer need to attack the cells and the oxidation chain reaction will be cut off. After giving electrons, antioxidants become free radicals by definition. Antioxidants in this state are dangerous because they have the ability to make electron changes without being reactive. The human body has an antioxidant defense system. Antioxidants are formed in the body and also obtained from foods such as fruits, vegetables, grains, beans, meat and oil. There are two antioxidant defense lines in the cell. The first line of defense, contained in fat soluble cell membranes containing vitamin A (beta-carotene) E, and coenzyme Q. The body under normal circumstances will produce free radicals associated with physiological cell metabolism. For example, the synthesis of some hormones will produce free radicals, as well as polymorphonucleic leukocytes will form free radicals to kill bacteria that help the body fight infection.

The entry of heavy metals in living bodies can occur through the air, water, and food consumed by living things or it can be said that accumulates heavy metals in the body of living things through the food chain. Heavy metals need living things as essential metals in metabolic processes and also as co-enzyme factors but in very small quantities. If the absorption of metals beyond the safe limits will be harmful to the body of these living things, because it will poison that can disrupt the metabolic process. Western metal is a harmful pollution substance, the metal that enters our body through our digestive system will react with the element of sulfur and enzyme in our body so that enzyme will not work well besides also heavy metal entering our body also will react with Cluster carboxylate (-COOH) is also amino (- NH_2) in amino acids.

Free radicals are basically unpaired molecules in their chemical structures so that these free radicals will look for couples to bond. Basically all biomolecules pair up to achieve stability, so to achieve this stability free radicals will look for other free electrons to bind to achieve stability. The nature of oxygen (O_2) is essentially an electron acceptor so that it will receive free electrons even when it reaches stability, thus forming superoxide (O_2.-), these oxygen-free free radicals are called Reactive Oxygen Species (ROS). X + + Y X. + Y: (The process of occurring oxygen species) The heavy metals in our bodies will also trigger ROS due to the disabling of antioxidant enzymes such as Superoxidedismutase (SOD), Catalase (CAT), and Glutation Peroxidase (GPOD), which function as antioxidants.

The formation of ROS in our body is also caused by oxidative stress, ROS will easily damage peroxide fat from the pleated membrane, cell membrane from phospolipid, and lipoprotein by spreading in chain reaction. ROS itself can attack all types of biomolecules such as nucleic acids, proteins and amino acids that interfere with metabolism. DNA damage is a consequence of the modification of genetic material resulting in cell death, mutagenesis, carcinogenic and aging by our body ROS is generated by our body's metabolic processes which will be used programmatically to disable cells. ROS

consists of Superoxidaradical (O_2-), HydrogenPeroxide (H_2O_2), Hydroxyl Radical (OH) and Singlet Oxigen (O_2), and will increase if exposed to UV rays, Ionic Radiation and if exposed to pollution.

5. Conclusions

According to our results, the acute exposure of *Gambusia affinis* $CdNO_3$ did not affect fish growth and did not cause the development of histopathological changes in the fish organism. However, we can conclude that $CdNO_3$ (even at the environmental concentration) may have a negative impact on some biochemical processes which are connected with the production of reactive oxygen species and free radicals in the fish organism.

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