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Biochemical markers of oxidative stress in zebrafish Danio rerio exposed to cadmium chloride

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ABSTRACT

Heavy metals are natural trace components of the aquatic environment, but their levels have increased due to domestic, industrial, mining and agricultural activities. Discharge of heavy metals into river or other aquatic environment can change both species diversity and ecosystems, due to their toxicity and bioaccumulation. These heavy metals are enters in the aquatic ecosystem as a result of direct input of atmospheric deposition, leaching of mineral and soil erosion due to rain water which causes the hazardous effects on aquatic biota especially fishes. These heavy metals when accumulated in the fish tissues, they damage and weaken the mechanisms concerned leading to physiological, pathological and biochemical changes.The Cadmium is non essential element for living organisms and its presence in fresh water in higher concentration are toxic to organisms, liver and ovary of the fish.The zebrafish were exposed to sublethal concentrations of Cd (20% and 80% of 96 h LC_{50} i.e. 1.05 and 4.18 mg/l) for 7, 14, 21 and 28 days period. The activity of antioxidant enzymes, catalase (CAT) and reduced glutathione (GSH) in zebrafish were decreased. There was increased lipid peroxidation (LPO) in liver and ovary. These observations clearly indicate the defensive nature and adaptive mechanism of cells against free radical induced toxicity.

Keywords: Zebrafish, cadmium**,** CAT, LPO, GSH.

INTRODUCTION

Heavy metals are metallic chemical elements with a specific gravity that is at least 5 times the specific gravity of water and having an atomic weight greater than Na, a density greater than 5 g/cm^3 and poisonous at low concentrations. Examples of heavy metals which are toxic to the environment including cadmium (Cd), copper (Cu), Arsenic (As), Chromium (Cr), Mercury (Hg) and Lead (Pb). They are natural components of the Earth's crust.Heavy metals cannot be degraded or destroyedto a small extent they enter our bodies via food, drinking water and air and also known as trace elements. Some heavy metals (e.g. copper,selenium, zinc, iron) are essential to maintain the metabolism of the human body.Heavy metals in the aquatic environment can affect biota and pose a risk to fish directly the consumers, such as humans and other wildlife. These metals may enter aquatic ecosystem from different natural and anthropogenic sources, including industrial or domestic sewage, storm runoff leaching from landfills/dumpsites and atmospheric deposits. Untreated community wastes use of fertilizers and pesticides as well as dumping of organic and inorganic wastes from industries is increasing environmental pollution to a great extent. Heavy metals have been recognized as strong biological poisons because of their persistent nature,tendency to accumulate in organisms and undergo food chain amplification[1]they also damage the aquatic fauna including. The contamination of freshwaters with a wide range of pollutants has become a matter of great concern over the last few

decades. Heavy metals are natural trace components of the aquatic environment, but their levels have increased due to domestic, industrial, mining and agricultural activities [2].Discharge of heavy metals into river or aquatic environment can change both aquatic species diversity and ecosystems, due to their toxicity and accumulative behaviors.Aquatic organisms such as fish and shell fish accumulate metals to concentration many times higher than present in water or sediment [3]. They can take up metals concentrated at different levels in their different body organs [4]. Certain environmental conditions such as salinity, pH, and water hardness can play an important factor in heavy metals accumulation in the living organisms up to toxic concentrations and cause ecological damage [5]. In This way heavy metals acquired through the food chain, which results pollution are potential chemical hazards, threatening consumers.At low levels, some heavy metals such as copper, cobalt, zinc, iron and manganese are essential for enzymatic activity and many biological processes. Some other metals like cadmium, mercury and lead have no essential role in living organisms and are toxic at even low concentrations. The essential metal also becomes toxic at higher concentrations. The highlighted anthropogenic sources of metals included industrial wastes from mining and run-off from roads, waste water, manufacturing and metal finishing plants they may also be leached from soils and rocks in contacts with water.

Heavy metals deplete glutathione, resulting enhanced production of Reactive Oxygen Species (ROS) such as catalase. ROS are considered as crucial mediators for the metal-triggered tissue injuries and apoptosis[6]. To prevent oxidation induced damage, there must be effective antioxidation systems in organisms. Some components of these systems involve reduced glutathione (GSH) and certain antioxidant enzymes including free redical scavenging enzymes, such as Superoxide Dismutase (SOD) and Catalse (CAT) Changes in the activityof enzymes and other biomarkers are the possible tools for aquatic toxicological research[7].Zebrafish can be used for biomonitoring of environmental contamination.

MATERIALS AND METHODS

Zebrafish, recommended by International Organization for Standardization(ISO, 1976)[8] and the Organization for Economic Co-operation and Development (OECD, 1992)[9]were collected, acclimatized for 15 days, stocked and bred under laboratory conditions. The aquaria were continuously aerated through stone diffusers connected to a mechanical air compressor. Water temperature was 25±2ºc and P^H was maintained between 6.6 and 8.5. The fish were fed twice daily alternately with egg, goat liver and raw brine shrimp pellets prepared in our laboratory. The experimental fishes were exposed to different concentrations (20% and 80% of 96 hour LC_{50}) of cadmium. 20 fishes for each concentration of metal were used. In the experimental aquaria water was replaced daily with fresh treatment of metal. The experiment was accompanied by the control. After the expiry of experiment periods (7, 14, 21 and 28 days) required number of treated fish was taken out from experiment and control groups. Six replicates for each concentration of cadmium were arranged.

Biochemical Assay

Lipid peroxidation (LPO)

LPO levels were estimated with thio-barbituric acid (TBARS) and color reaction for malandialdehyde (MDA) according to procedure by Placer et al., (1966)[10] . Tissues were homogenized in chilled 0.15 M KCl using a Teflon pestle to obtain 10% w/v homogenate. One ml of homogenate was incubated at 37ºC (± 0.5) for two hours. To each sample, 1 ml of 10% w/v tricholoro acetic acid (TCA) (s. d. fine chem. Ltd; Mumbai) was added. After through mixing, the reaction mixture was centrifuged at 2000 rpm for 10 minutes 1 ml of supernatant was taken with equal volume of 0.67% w/v TBA (thio-barbituric acid) and kept in boiling water bath for 10 minutes, cooled and diluted with 1 ml of distilled water.The absorbance pink color was observed, which measured at 535 nm against a blank. The concentration of MDA was read from a standard calibration curve plotted using 1, 1, 3, 3` tetra-methoxypropane (Sigma –Aldrich Co., St. Louis, USA) and the results were expressed as µmol of MDA formed 30 min^{-1} mg $protein^{-1}$.

Reduced glutathione (GSH)

The GSH levels was estimated according to procedures of Paglia et al., (1975)[11], with which it is determined by its reaction with 5,5`- dithiobis-2-nitrobenzoic acid (DTNB) to produce/yield a yellow chromophore that was measured spectrophotometrically at 412 nm. The results were expressed as GSH mg per mg $protein^{-1}$. The protein contents of tissues were assayed using the Lowery et al. (1951)[12] method with bovine serum albumin as the standard.

Catalase(CAT)

The CATactivity was estimated according to procedures by Sinha (1972) [13]. This method is based on the fact that in acetic acid dichromate is reduced to chromic acetate when heated in presence of H_2O_2 with the formation of perchromic acid as an unstable intermediate. The chromic acetate is measured colorimetrically at 620 nm. The catalase preparation is allowed to split H_2O_2 at different time intervals by the addition of a dichromatic acetic acid mixture and remaining H_2O_2 is determined colorimetrically. The results were expressed as µmol H_2O_2 utilized min^{-1} mg $protein^{-1}$.

Two way analysis of variance (ANOVA) was applied to test the significance of data. All the data are expressed as means (n=6) ± standard deviation (SD) and differences were considered significant at $P<0.05$.

RESULTS AND DISCUSSION

A significant elevation in LPO levels were observed in the test tissues exposed to cadmium for 7, 14, 21 and 28 days time intervals in response to concentration of 1.05and 4.18mg/l which is 20% and 80% of 96 hour LC_{50}. Liver tissues shows maximum elevation in LPO than 28 days Cd exposed zebrafish ovary. 7 days of exposure to 20% 96-h LC_{50} of Cd, to 8.79±0.24and 9.25±0.49 at 28 days of exposure to 12.03±0.69 12.78±0.59 in liver and ovary respectively. At 80% of 96 h LC_{50} at exposure period, 7 days concentrations increased to 9.49±0.46 to10.16±0.15 and 12.97±0.75 to13.05±0.58 in 28 days in liver and ovary respectively. (Table 1 and 2)

Catalase activity levels were significantly reduced in 7, 14, 21 and 28 days during exposure on zebrafish liver and ovary. In 7 days of exposure of 20% 96 hLC_{50} of Cd liver CAT activity reduced 135.00±0.39 to 142.41±0.23 comparing to controls in zebrafish tissues of liver and ovary respectively and after 28 days of exposure 20% 96 h LC_{50} CAT activity was observed 91.49±0.34 to 91.12±0.87 in both tissues respectively. In 7 days of exposure of 80% LC_{50} of Cd in liver and ovary CAT activity 126.40±0.20 to 110.03±0.54 observed, the ovary concentration greater then liver in CAT activity and after 80% 135.00±0.39 to 133.57±0.25 and at 28 days 83.02±0.19 to 77.53±0.85 observed liver and ovary respectively. (Table 3 and 4)

In GSH concentration in liver and ovary of zebrafish observed in 7 days of exposure4.00±0.15 to 2.59±0.49 and at 28 days of 96 h LC_{50} 1.51±0.94 to 1.59±0.373. The 80% of LC_{50} of Cd (4.18 mg/l),GSH level decreased in ovary in comparison to liver at 7 days exposure which are 3.15±0.13 to 2.00±0.46 and at 28 days exposure 1.65±0.65 to 1.25±0.28.(Table 5 and 6)

In aerobic organism, oxygen is an essential element for cells to maintain normal body function and metabolism. However, oxygen also can give rise to ROS (reactive oxygen species) such as superoxide radical (O_2), the hydroxyl radical (OH), the hydroperoxyl radical (OOH), and hydrogen peroxide (H_2O_2). In the body, the main source of ROS is cellular respiration, which involves mitochondrial electron transport [14-16]. Oxidative stress can occur when the generation of ROS exceeds the ability of antioxidant defense system to neutralize or eliminateROS [17]. Excessive production of ROS results in damage to various biological molecules such as nucleic acids, lipids, protein and carbohydrates. Reactive oxygen species can attack and damage cell membranes and the lipoproteins through a process called lipid peroxidation [18].Under aerobic conditions, all cells possess antioxidant defense mechanisms, which are divided into two groups, enzymatic and non-enzymatic antioxidants [19]. A variety of enzymes is involved in antioxidant protection inside cells. These endogenous antioxidants act through dismutation, decomposition and detoxification processes. They are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione S-transferase (GST). The main enzymes which help to detoxify ROS in all organisms are GPx and CAT. All these enzymes are found in fish tissues [20]. The levels of enzymes activity varies with species and muscle type [21].

In addition to antioxidants, which act as scavenger`s. These include glutathione (GSH) and lipid and water soluble antioxidants. In present study the zebrafish was exposed to $CdCl_2$ for a period of 7.14,21 and 28 days at suitable concentrations that is 20% of 96 hrs (1.05 mg/l) and 80% of 96 hrs (4.18 mg/l) and recorded a significant reduction in CAT (Catalase) and GSH (glutathione reduced) but in the LPO we observed significant induction in liver and ovary of zebrafish. Maximum reduction was recorded in GSH and CAT at the higher concentration 20% of 96 hrs LC_{50}as compared to the lower concentration of 80% of 96 hrs and maximum induction of LPO was recorded at the 80% of 96 hrs as compared to the higher concentration of 20% 96 hrs. These observations revealed that the decline in CAT, GSH and upgrade LPO level in different tissues was directly proportional to concentration of Cd. The

Cadmium can cause free radical mediated cellular damage which leads to metabolic alterations such as the enzymatic activities and membrane transport mechanism and injuries of biological systems at different levels[22]. The pattern of depletion in CAT and GSH and significant increase in LPO was observed in zebrafish, *danio rerio* in livers and gills exposed to pesticide dimethoate[23].

LPO is one of the main manifestations of oxidative damage, which plays an important role in the toxicity of many xenobiotics[24]. Toxic forms of activated oxygen react with cellular components resulting in protein oxidation, oxidative DNA damage as well as LPO. Our study revealed, enhanced LPO levels during Cd-exposure in liver and ovary of zebrafish *Danio rerio*. The enhanced LPO in our experiment also b due to inhibition in activity levels of antioxidants, which are more concerned with defense against free radical induction due to Cd intoxication. Some similar finding/ results were also reported in the liver of common carp (Cyprinuscarpio) [25]. Cd catalyses the production of toxic OH^- that causes LPO in different tissues of fish *Rhamadiaquelen*[26]. There was significant increase in the LPO level in liver tissue of rainbow trout exposed to Cd/Cr [27]. Enhanced LPO levels during Cd-exposure in tissues of *Oreochromismossambicus*[28].

Catalase is an inducible cytosolic enzyme which functions to protect the biological system against reactive oxgen species. The activity levels of CAT were significantly reduced in the test tissues of Cd exposed Zebrafish for 7, 14, 21 and 28 day's duration.Reduced CAT activity was observed in Cd exposed *rainbow trout*[29]. Catalase activity observed decreased exposed to cadmium on *Cyprinuscarpio*[30].Catalase reduced in hepatocytes of common carp (Cyprinus carp) to the toxicity of microcystin [31].Different tissues of fresh water fish Heteropneustes fossils (Bloch) exposed to Cd showed decreased CAT activity [32]. The decreasement in the CAT activity levels during Cd exposure might be due to enzyme protein oxidation as a result of accumulation of H_2O_2 and other cytotoxic radicals.An inhibition of catalase in liver of rainbow trout exposed to cd, possible mechanisms by which cadmium produces lower catalase activity, may include direct metal mediated structural alteration of the enzyme depression of catalase synthesis [33].

In addition some, investigators have suggested that severe oxidative stress also might suppress the activity of antioxidant enzymes due to oxidative damage and loss of the compensatory mechanisms [34-35]. The existence changes in GSH content[36], data may indicate a faster rate of GSH utilization or degradation, which could be responsible for the observed lower GSH content. GSH content was also reduced in the test tissues during all the exposure periods of cadmium in the ovary and liver of Zebrafish.The reports on GSH metabolism in tissues of freshwater fish *Oreochromisniloticus*subjected to Cd exposure [37]. Moreover, increase of GSH content may be related to prevention of oxidative challenge [38]. Aquatic organisms maintain high content of GSH in tissues and increased content has the function of protection. High content of GSH could be a consequence of its increased synthesis due to high cysteine accessibility which is necessary for GSH synthesis. GSH content increased after treatment with cadmium. This could provide the first line of defense against the influence of toxic heavy metal.

The findings revealed that heavy metal, Cd create harmful effects by generating reactive oxygen species that damage the cells by disturbing the fluidity balance.

Table 1: Effect of Cadmium on LPO in the Liver of *Danio rerio*.The values represent the means ± SD† of six individual observations and are significant at P<0.05 (two-way ANOVA)

	Treatment period (Days)			
Concentration(mg/l)*	7	14	21	98
Control	8.00±0.15 (100)	8.73±0.49 (100)	8.29±0.54 (100)	9.35±0.83 (100)
1.05	8.79±0.24 (110)	9.97±0.33 (115)	10.85±0.65 (124)	12.03±0.69 (129)
4.18	9.49±0.46 (116)	10.49±0.26 (121)	11.27±0.45 (131)	12.97±0.75 (138)

	Summary of computation for ANOVA				
Source of variation	d.f	S.S.	Variance	F-values	P<
Variation due to operation	3	11.19063	3.730208	11.40417	0.05
Variation due to concentration	2	13.04432	6.522158	19.93985	0.05
Total interaction	6	1.96255	0.327092		
Total	11	26.19749			

**The exposure concentrations used were 20 and 80% of the 96-h LC_{50} value, SD†=Standard deviation*

Table 2: Effect of Cadmium on LPO in the ovary of *Danio rerio*.The values represent the means ±SD† of six individual observations and are significant at P<0.05 (two-way ANOVA).

	Treatment period (Days)			
Concentration(mg/l)*	7	14	21	28
Control	9.15±0.25 (100)	9.25±0.39 (100)	10.15±0.63 (100)	10.23±0.26 (100)
1.05	9.25±0.49 (102)	10.16±0.82 (110)	11.67±0.29 (116)	12.78±0.59 (125)
4.18	10.16±0.15 (113)	10.55±0.28 (115)	12.08±0.57 (120)	13.05±0.58 (129)

	Summary of computation for ANOVA				
Source of variation	d.f	S.S.	Variance	F-values	p<
Variation due to operation	3	12.4414	4.147133	14.18268	0.05
Variation due to concentration	2	5.914217	2.957108	10.11294	0.05
Total interaction	6	1.75445	0.292408		
Total	11	20.11007			

**The exposure concentrations used were 20 and 80% of the 96-h LC_{50} value.*
SD†=Standard deviation

Table 3: Effect of Cadmium on CAT in the liver of *Danio rerio*.The values represent the means ± SD† of six individual observations and are significant at P<0.05 (two-way ANOVA).

	Treatment period (Days)			
Concentration(mg/l)*	7	14	21	28
Control	156.12±0.26 (100)	159.12±0.25 (100)	145.12±0.21 (100)	145.97±0.28 (100)
1.05	148.31±0.41 (95)	127.92±0.83 (82)	104.48±0.45 (72)	91.96±0.34 (63)
4.18	135.82±0.56 (87)	115.44±0.66 (74)	97.23±0.39 (67)	78.82±0.19 (54)

	Summary of computation for ANOVA				
Source of variation	d.f	S.S.	Variance	F-values	p<
Variation due to operation	3	2850.907	950.3023	8.062151	0.05
Variation due to concentration	2	4018.481	2009.241	17.04594	0.05
Total interaction	6	707.2323	117.8721		
Total	11	7576.62			

**The exposure concentrations used were 20 and 80% of the 96-h LC_{50} value.*
SD†=Standard deviation

Table 4: Effect of Cadmium on CAT in the Ovary of *Danio rerio*.The values represent the means ± SD† of six individual observations and are significant at P<0.05 (two-way ANOVA).

	Treatment period (Days)			
Concentration(mg/l)*	7	14	21	28
Control	148.45±0.67 (100)	152.00±0.67 (100)	149.56±0.98 (100)	150.00±0.64 (100)
1.05	136.57±0.45 (92)	120.08±0.78 (79)	103.19±0.87 (69)	88.59±0.87 (61)
4.18	120.24±0.54 (81)	106.40±0.69 (70)	94.22±0.95 (63)	75.51±0.85 (52)

	Summary of computation for ANOVA				
Source of variation	d.f	S.S.	Variance	F-values	p<
Variation due to operation	3	1709.069	569.6897	4.956146	0.05
Variation due to concentration	2	5317.725	2658.862	23.13138	0.05
Total interaction	6	689.6766	114.9461		
Total	11	7716.47			

**The exposure concentrations used were 20 and 80% of the 96-h LC_{50} value.*
SD†=Standard deviation

Table 5: Effect of Cadmium on GSH in the Liver of *Danio rerio*.The values represent the means ± SD† of six individual observations and are significant at P<0.05 (two-way ANOVA).

	Treatment period (Days)			
Concentration(mg/l)*	7	14	21	28
Control	5.00±0.09 (100)	5.12±0.56 (100)	4.81±0.29 (100)	4.85±0.84 (100)
1.05	4.25±0.15 (85)	3.99±0.29 (78)	3.41±0.43 (71)	3.05±0.94 (63)
4.18	3.40±0.13 (68)	3.17±0.43 (62)	2.69±0.23 (56)	2.27±0.65 (47)

	Summary of computation for ANOVA				
Source of variation	d.f	S.S.	Variance	F-values	p<
Variation due to operation	3	1.349292	0.449764	7.393717	0.05
Variation due to concentration	2	8.659817	4.329908	71.17983	0.05
Total interaction	6	0.364983	0.060831		
Total	11	10.37409			

**The exposure concentrations used were 20 and 80% of the 96-h LC_{50} value.*
SD†=Standard deviation

Table 6: Effect of Cadmium on GSH in the ovary of *Danio rerio*.The values represent the means ± SD† of six individual observations and are significant at P<0.05 (two-way ANOVA).

	Treatment period (Days)			
Concentration(mg/l)*	7	14	21	28
Control	3.19±0.58 (100)	3.00±0.45 (100)	3.13±0.32 (100)	3.25±0.39 (100)
1.05	2.76±0.49 (80)	2.19±0.56 (73)	2.09±0.26 (67)	1.88±0.33 (58)
4.18	2.34±0.46 (68)	1.77±0.55 (59)	1.59±0.29 (51)	1.25±0.28 (43)

	Summary of computation for ANOVA				
Source of variation	d.f	S.S.	Variance	F-values	p<
Variation due to operation	3	0.9007	0.300233	7.007975	0.05
Variation due to concentration	2	4.478617	2.239308	52.2694	0.05
Total interaction	6	0.25705	0.042842		
Total	11	5.636367			

**The exposure concentrations used were 20 and 80% of the 96-h LC_{50} value.*
SD†=Standard deviation

CONCLUSION

Results of the present study show biochemical effect of oxidative stress in zebrafish under sublethal exposure to cadmium chloride. These biochemical investigations can be used to study the mode of action of toxicants and cause for death by poisoning of aquatic organisms. Thus biochemical alterations in zebrafish are considered as biomarkers to access the health status of the fishes as well as aquatic bodies polluted by metals. Thus environmental protection is the major requirement of the society.

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