RESEARCH ARTICLE

Biochemical Markers of Oxidative Stress in Brain of Zebrafish *Danio rerio* Exposed to Different Heavy Metals Lead and Cobalt

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ABSTRACT- Aquatic organisms have been considered to concentrate metals several times greater than environmental levels. Fishes have been used for many decades to evaluate the pollution status of water and thus considered as excellent biological indicator of heavy metals in aquatic environments. Heavy metals are natural tress components of the aquatic environment, but their levels have increased due to domestic, industrial, mining and agricultural activities. These heavy metals when accumulated in the fish tissues, they damage and weaken the mechanisms concerned leading to physiological, pathological and biochemical changes. The lead is non essential element while cobalt is an essential element for living organisms but its presence in fresh water in higher concentration are toxic to organism's brain, liver, ovary, kidney and gills of the fish. The present study was aimed to investigate the changes due to two heavy metals (lead & cobalt) on the activity of the antioxidant enzyme, Catalase (CAT), Reduced glutathione (GSH), and Lipid peroxidation (LPO) in the brain of *Danio rerio* during 5, 10, 15 and 20 days of exposure period. For this study adult fishes were exposed to four different concentrations viz., 20, 30, 40 and 50 mg/l of cobalt and 5, 9, 13, and 17 mg/l of lead.

Key-words- Cobalt, Catalase, Glutathione, Heavy metals, Lead, LPO, Zebrafish

INTRODUCTION

Heavy metals are produced from a variety of natural and anthropogenic sources [1]. In aquatic environments, heavy metal pollution results from direct atmospheric deposition, geologic weathering or through the discharge of agricultural, municipal, residential or industrial waste products, also via wastewater treatment plants [2-4]. The contamination of heavy metals and metalloids in water and sediment, when occurring in higher concentrations, is a serious threat because of their toxicity, long persistence, and bioaccumulation and bio magnification in the food chain [5]. Generally, metals can be categorized as biologically essential and non-essential. The nonessential metals for example, aluminum (Al), cadmium (Cd), mercury (Hg), tin (Sn) and lead (Pb) have no proven biological function also called xenobiotics or foreign elements and their toxicity rises with increasing concentrations [6]. Essential metals for example, copper (Cu), zinc (Zn), chromium (Cr), nickel (Ni), cobalt (Co), Molybdenum (Mo) and iron (Fe) on the other hand, have known important biological roles [7] and toxicity occurs either at metabolic deficiencies or at high concentrations [8]. The deficiency of an essential metal can therefore cause an adverse health effect, whereas its high concentration can also result in negative impacts which are equivalent to or worse than those caused by non-essential metals [9].

The river system may be extremely contaminated with heavy metals released from domestic, industrial, mining and agricultural effluents. Heavy metal contamination may have disturbing effects on the ecological balance of the recipient environment and a diversity of aquatic organisms [10,11]. Among animal species, fishes are inhabitants that cannot escape from the detrimental effects of these pollutants [12]. The impact of metals, as well as other pollutants, on aquatic biota can be evaluated by toxicity test, which are used to detect and evaluate the potential toxicological effects of chemicals on aquatic organisms. However, little research has been done on the impact of contaminations on tropical ecosystems [13]. Fish are widely used to evaluate the health of aquatic ecosystems because pollutants build up in the food chain and are responsible for adverse effects and death in the aquatic systems. Fish can obtain their trace elements, either directly from the water through the gills or indirectly from food through the alimentary tract [14]. Heavy metals have been recognized as strong biological poisons because of their persistent nature, tendency to

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accumulate in organisms and undergo food chain amplification [15], they also damage the aquatic fauna. The contamination of freshwaters with a wide range of pollutants has become a matter of great concern over the last few decades.

Lead is a persistent metal which is commonly used in various industrial processes. It is toxic to living systems and may stay in the environment for a prolonged period of time, due to its persistency; it exists as a free metal in various compounds. Lead is a widespread environmental and occupational xenobiotic and is hazardous to humans and various ecosystems [16]. Its exposure to humans is mainly by ingestion through the mouth and inhalation from fumes and dust in the atmosphere [17]. Exposure to lead is mainly from anthropogenic sources due to its widespread usage. The form in which lead exists determines how toxic it is in the environment. Several studies link inorganic lead like lead acetate compounds to increased incidence of diseases in various organisms. Lead toxicity has been linked to incidence of neurological disorders, hypertension, cognitive impairments etc [18]. Chen *et al.* [19] reported that exposure of low doses of developmental lead to the embryo of zebrafish resulted in embryonic toxicity, behavioral alteration, and adult learning/memory deficit in zebrafish. It's accumulation in sediment is of significance for aquatic organisms. It is not a transition metal and cannot readily undergo valence changes, it can induce oxidative damage through direct effects on the cell membrane, interactions between lead and haemoglobin, which increase the auto-oxidation of hemoglobin, auto-oxidized δ-aminolevulinic acid, interactions with glutathione reductase, or through the formation of complexes with selenium, which decrease glutathione peroxidase activity [20]. Lead deposits in various fish organs like liver, brain, kidneys, spleen, digestive tract and gills [21].

Cobalt is an essential nutrient for man and is an integral part of vitamin B_{12}. It performs important biochemical function but its higher concentration in aquatic ecosystems becomes toxic to fish as it interferes with the enzyme systems [22]. It is reported to be a potential carcinogenic compound and has been included recently in group 2A carcinogens *i.e.,* probably carcinogenic to humans. Cobalt can be absorbed from the surrounding water through the gills as well as from the diet. The uptake of waterborne cobalt increased with a rise in temperature and decrease in waterborne calcium.

Also, heavy metals are known to induce oxidative stress and carcinogenesis by mediating free radicals e.g. reactive oxygen species [23]. They deplete glutathione, resulting enhanced production of Reactive Oxygen Species (ROS) such as catalase. ROS are considered as critical mediators for the metal-triggered tissue injuries and apoptosis. To prevent oxidation induced damage, there must be effective anti-oxidation system enzyme including free radical scavenging enzymes, such as Superoxide Dismutase (SOD) and Catalase (CAT) changes in the activity of enzymes and other biomarkers are the possible tool for aquatic toxicological research [24]. Zebrafish can be used for bio-indicator of environmental contamination.

MATERIALS AND METHODS

The present work was conducted in the Zebrafish laboratory, Department of Zoology, D.D.U. Gorakhpur University, Gorakhpur, India in the duration of April 2017. Zebrafish, recommended by International Organization for Standardization (IOS, 1976) [25] and the Organization for Economic Co-operation and Development (OECD) [26] were collected and acclimatized for a month, stocked and reared under laboratory conditions. The aquariums were aerated continuously through stone diffusers connected to a mechanical air compressor and the water temperature was maintained at 25±2°C. The fishes were fed twice daily alternately with raw and chopped goat liver and shrimp powder. Tubifex worm, Tetrabit and spirulina granules purchased from pets shop were also supplemented.

For the present study, mature adult zebrafish approximately 3.5 cm in length and 1 g in weight were procured from stock aquarium and exposed to four different concentrations viz., 20, 30, 40 and 50 mg/l of cobalt and 05.00, 09.00, 13.00 and 17.00 mg/l of lead calculated from our previous toxicity test. The concentrations of heavy metals were decided for exposures were below the range of 80% 96-h LC_{50} as calculated earlier Singh and Ansari [27]. Low concentrations were selected since fish can survive the stress of the toxicant. Twenty fishes were exposed to each concentration. The water in the aquarium was replaced daily with fresh treatment of metals. Each experiment was accompanied by their respective control. After exposure periods of 5, 10, 15 and 20 days, required number of treated fish were removed from the experimental and control groups. Their brain were removed and processed.

Biochemical assay- The activity of CAT (EC 1.11.1.6) was estimated according to procedures by Sinha [28]. This method is based on the fact that in acetic acid dichromate is reduced to chromic acetate when heated in the 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 dichromic acetic acid mixture and the remaining H_2O_2 is determined colorimetrically. The results were expressed as μM H_2O_2 utilized/min/mg protein.

Glutathione (GSH) content in the brain was estimated according to the method of Paglia *et al.* [29]. Tissue (brain) was lyses with 2.0 ml of 1g/l EDTA (ethylene diamine tetraacetic acid) solution and 1.5 ml of precipitating reagent (1.67 g glacial metaphosphoric acid, 0.2 g EDTA, 30 g sodium chloride, distilled water to 100 ml) was added. After mixing, the solution was allowed to stand for five minutes then centrifuged at 3000 rpm for 15 min. 0.50 ml of filtrate was added to 2 ml of disodium hydrogen phosphate (Na_2HPO_4) (0.1M, pH 7.4), and 0.25 ml of DTNB reagent (40 mg) was dissolved in 100 ml of

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10 g/l (1%) sodium citrate. A blank was prepared from 1.5 ml of precipitating reagent, 1 ml of distilled water, 2 ml of disodium hydrogen phosphate, and 0.25 ml of DTNB reagent. The absorbance of yellow color was read at 412 nm within a minute after adding DTNB. The results were expressed as GSH mg/mg protein.

LPO levels were estimated with thiobarbituric acid reacting substances (TBARS), and color reaction for malondialdehyde (MDA) according to procedures in Placer *et al.* [30]. 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 37 ±0.5°C for two hours. To each sample, 1 ml of 10% w/v trichloro acetic acid (TCA) was added. After thorough mixing, the reaction mixture was centrifuged at 2000 rpm for 10 minutes. 1 ml of supernatant was then taken with an equal volume of 0.67% w/v TBA (thio-barbituric acid) and kept in a boiling water bath for 10 minutes, cooled and diluted with 1 ml of distilled water. The absorption of the pink colour obtained 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 and the results were expressed as µmol of MDA formed/ min/ mg protein.

The protein contents of tissues were assayed using the method of Lowry *et al*. [31] with bovine serum albumin as the standard. Two way analysis of variance (ANOVA) was applied to test the significance of the data. All the data are expressed as means (n=6)±standard deviation (SD) and difference were considered significant at $P<0.05$.

RESULTS AND DISCUSSION

After the exposure of both heavy metals, the zebrafish was showed behavioral changes. They aggregated at one corner of the aquarium, resting at the bottom and frequently come to the surface followed by the heavy breathing with stronger opercular movement loss of equilibrium. Over secretion of mucus was also observed from the body surface. Their body color darkened, pectoral and pelvic fins got expanded and the fish rolled vertically prior to death.

In the brain CAT activity showed significant ($p<0.05$) decrease after 5, 10, 15 and 20 days of treatment period. Minimum changes in CAT activities were observed after 5 days of exposure period at each concentrations of lead which was 154.65±1.31 (95%), 148.32±1.63 (91%), 139.61±1.42 (86%) and 112.34±1.63 (69%) µM H_2O_2 utilized/min/mg protein as compared to control 162.35±1.86 (100%). But after 20 days of treatment period at all concentrations the maximum changes was 72.32±1.60 (45%) at 17 mg/l in CAT activity was observed, which showed a concentration and time-dependent action of lead (Table 1). While in the case of cobalt after 5, 10, 15 and 20 days of treatment period, minimum changes in CAT activities were observed after 5 days exposure period at each concentrations, which was 159.33±1.25 (97%), 151.65±1.32 (93%), 142.81±1.62 (87%) and 128.35±1.72 (79%) µM H_2O_2 utilized/min/mg protein as compared to control 163.45 µM H_2O_2 utilized/min/mg protein. But after 20 days of treatment period at all concentrations the maximum changes was 98.62±1.20 (60%) at 50 mg/l in CAT activity were observed which showed a concentration and time-dependent action of cobalt (Table 2). The results were shown that cobalt was less toxic as compared to lead.

Alteration in GSH level after 5, 10, 15 and 20 days treatment of both heavy metals are presented in table 3 and table 4. The reduction in GSH level was maximum after 20 days of treatment of 17 mg/l of lead and it was found to be only 1.61±0.35 (38%) GSH mg/mg protein as compared to control 4.25±0.32 (100%) (Table 3). While in case of cobalt, reduction was maximum after 20 days treatment period 1.80±0.12 (43%) GSH mg/mg protein as compared to control 4.18±0.13 (100%) at 50 mg/l concentration (Table 4).

Table 1: Effect of Lead on CAT activity (µM H_2O_2 utilised/min/mg protein) in the Brain of zebrafish

Concentrations (mg/l)	Treatment period (days)			
	5	**10**	**15**	**20**
Control	162.35±1.86 (100%)	163.23±1.27 (100%)	162.54±1.90 (100%)	161.75±1.23 (100%)
5	154.65±1.31 (95%)	147.35±1.38 (90%)	141.80±1.67 (87%)	137.62±1.25 (85%)
9	148.32±1.63 (91%)	139.66±1.75 (86%)	130.23±1.54 (80%)	118.35±1.66 (73%)
13	139.61±1.42 (86%)	121.32±1.62 (74%)	112.62±1.35 (69%)	102.82±1.23 (64%)
17	112.34±1.63 (69%)	108.45±1.32 (66%)	85.38±1.62 (53%)	72.32±1.60 (45%)

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Summary of computation for ANOVA

Source of variations	Degree of freedom	Sum of squares	Variance	F-values	Sign. level
Variation due to Operations	3	1772.9	590.96	10.02	P<0.05
Variation due to Concentrations	4	10684	2671.07	45.29	P<0.05
Total interaction	12	707.59	58.96		
Total	19				

***Dose was selected below 80% of 96-h LC_{50}.**
*** Values are mean ± SD of six individual observations and significant at p<0.05 (two-way ANOVA)**

Table 2: Effect of Cobalt on CAT activity (µM H_2O_2 utilised/min/mg protein) in the Brain of zebrafish

Concentrations (mg/l)	Treatment period (days)			
	5	**10**	**15**	**20**
Control	163.45±1.60 (100%)	161.65±1.43 (100%)	162.32±1.25 (100%)	163.21±1.54 (100%)
20	159.33±1.25 (97%)	152.80±1.74 (95%)	147.35±1.70 (91%)	141.66±1.32 (87%)
30	151.65±1.32 (93%)	143.63±1.20 (89%)	134.85±1.39 (83%)	129.81±1.25 (80%)
40	142.81±1.62 (87%)	135.25±1.38 (84%)	122.13±1.22 (75%)	110.28±1.74 (68%)
50	128.35±172 (79%)	117.90±1.63 (73%)	108.24±1.82 (67%)	98.62±1.20 (60%)

Summary of computation for ANOVA

Source of variations	Degree of freedom	Sum of squares	Variance	F-values	Sign. level
Variation due to Operations	3	1172.09	390.7	12.05	P<0.05
Variation due to Concentrations	4	5913.33	1478.3	45.62	P<0.05
Total interaction	12	388.82	32.40		
Total	19				

***Dose was selected below 80% of 96-h LC_{50}.**
*** Values are mean ± SD of six individual observations and significant at p<0.05 (two-way ANOVA)**

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Table 3: Effect of Lead on GSH activity (GSH mg/mg protein) in the Brain of zebrafish

Concentrations (mg/l)	Treatment period (days)			
	5	10	15	20
Control	4.33±0.45 (100%)	4.45±0.66 (100%)	4.35±0.15 (100%)	4.25±0.32 (100%)
5	3.90±0.35 (90%)	3.15±0.12 (71%)	2.85±0.13 (66%)	2.72±0.11 (64%)
9	3.10±0.23 (72%)	2.90±0.35 (65%)	2.61±0.13 (60%)	2.43±0.15 (57%)
13	2.92±0.35 (67%)	2.50±0.13 (56%)	2.15±0.25 (49%)	1.95±0.13 (49%)
17	2.80±0.26 (65%)	1.95±0.24 (44%)	1.80±0.62 (41%)	1.61±0.35 (38%)

Summary of computation for ANOVA

Source of variations	Degree of freedom	Sum of squares	Variance	F-values	Sign. level
Variation due to Operations	3	1.89	0.63	12.71	P<0.05
Variation due to Concentrations	4	12.70	3.17	63.81	P<0.05
Total interaction	12	0.59	0.04		
Total	19				

***Dose was selected below 80% of 96-h LC_{50}.**
*** Values are mean ± SD of six individual observations and significant at $p<0.05$ (two-way ANOVA)**

Table 4: Effect of Cobalt on GSH activity (GSH mg/mg protein) in the Brain of zebrafish

Concentrations (mg/l)	Treatment period (days)			
	5	10	15	20
Control	4.25±0.36 (100%)	4.50±0.24 (100%)	4.35±0.18 (100%)	4.18±0.13 (100%)
20	3.98±0.13 (94%)	3.58±0.17 (80%)	3.20±0.14 (74%)	3.14±0.16 (75%)
30	3.60±0.40 (85%)	3.10±0.12 (69%)	2.90±0.31 (67%)	2.81±0.20 (67%)
40	3.08±0.12 (72%)	2.83±0.17 (63%)	2.35±0.13 (54%)	2.08±0.10 (50%)
50	2.89±0.14 (68%)	2.10±0.13 (47%)	1.95±0.18 (45%)	1.80±0.12 (43%)

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Source of variations	Summary of computation for ANOVA				
	Degree of freedom	Sum of squares	Variance	F-values	Sign. level
Variation due to Operations	3	1.66	0.55	12.29	P<0.05
Variation due to Concentrations	4	10.90	2.72	60.32	P<0.05
Total interaction	12	0.54	0.04		
Total	19				

***Dose was selected below 80% of 96-h LC_{50}.**
*** Values are mean ± SD of six individual observations and significant at p<0.05 (two-way ANOVA).**

The effect of both metals on LPO also showed a significant change at different concentrations and exposure periods. At 17 mg/l of lead treatment for 20 days there was drastic increase in the MDA level 22.98±0.84 (158%), as compared to 14.58±0.65 (100%) (Table 5) and in the case of cobalt concentration at 50 mg/l for 20 days, increment in MDA level was 20.20±0.73 (138%), as compared to 14.69±0.58 (100%) (Table 6). However, it was observed that the changes were more profound with the lead exposure as compared to cobalt.

Table 5: Effect of lead on LPO activity (µM of MDA formed/30 min/mg protein) in the brain of zebrafish

Concentrations (mg/l)	Treatment period (days)			
	5	10	15	20
Control	13.65±0.89 (100%)	14.21±0.63 (100%)	13.35±0.80 (100%)	14.58±0.65 (100%)
5	14.50±0.38 (106%)	15.80±0.38 (111%)	16.01±0.19 (120%)	17.63±0.35 (121%)
9	15.44±0.63 (113%)	16.90±0.45 (119%)	17.65±0.34 (132%)	18.90±0.48 (130%)
13	16.85±0.12 (123%)	17.75±0.85 (125%)	18.90±0.28 (142%)	20.65±0.21 (142%)
17	17.01±0.30 (125%)	18.68±0.29 (131%)	20.35±0.65 (152%)	22.98±0.84 (158%)

Source of variations	Summary of computation for ANOVA				
	Degree of freedom	Sum of squares	Variance	F-values	Sign. level
Variation due to Operations	3	31.08	10.36	14.51	P<0.05
Variation due to Concentrations	4	81.24	20.31	28.46	P<0.05
Total interaction	12	8.56	0.71		
Total	19				

***Dose was selected below 80% of 96-h LC_{50}.**
*** Values are mean ± SD of six individual observations and significant at p<0.05 (two-way ANOVA)**

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Table 6: Effect of cobalt on LPO activity (µM of MDA formed/30 min/mg protein) in the brain of zebrafish

Concentrations (mg/l)	Treatment period (days)			
	5	10	15	20
Control	14.35±0.63 (100%)	13.96±0.35 (100%)	14.63±0.58 (100%)	14.69±0.58 (100%)
20	14.96±0.80 (104%)	14.80±0.80 (106%)	15.4±0.63 (109%)	16.40±0.35 (112%)
30	15.70±0.46 (109%)	15.60±0.46 (112%)	16.48±0.68 (113%)	17.50±0.64 (119%)
40	16.07±0.75 (112%)	16.21±0.75 (116%)	17.64±0.38 (121%)	18.86±0.48 (128%)
50	16.89±0.38 (118%)	17.10±0.38 (123%)	18.69±0.54 (128%)	20.20±0.73 (138%)

Summary of computation for ANOVA

Source of variations	Degree of freedom	Sum of squares	Variance	F-values	Sign. level
Variation due to Operations	3	13.67	4.55	17.46	P<0.05
Variation due to Concentrations	4	34.65	8.66	33.19	P<0.05
Total interaction	12	3.13	0.26		
Total	19				

***Dose was selected below 80% of 96-h LC_{50}.**
***Values are mean±SD of six individual observations and significant at p<0.05 (two-way ANOVA)**

Heavy metals are natural trace components of the aquatic environment, but their levels have increased due to domestic, industrial, mining and agricultural activities. In this way heavy metals acquired through the food chain, which results pollution and 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.

Aquatic organisms have developed several cellular defense paths, which under normal metabolic conditions regulate the level of ROS and protect against the deleterious effects of free radicals. This defense system includes both antioxidant enzymes, such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and condition due to over accumulation of ROS [32]. A similar response was found in fish exposed sub-chronically to industrial pollutants [33]. Reactive oxygen species have been reported to affect the physiology, growth, and survival of aquatic organisms [34,35]. Fish, like mammals, possess well-developed antioxidant defense systems for neutralizing the toxic effects of ROS [35]. Fish are largely being used for the assessment of the quality of aquatic environment and as such can serve as bio-indicators of environmental pollution [36,37]. Heavy metals accumulated in the tissues of fish may catalyze reactions that generated in the tissues of fish may catalyze reactions that generate ROS, which may lead to environmental oxidative stress. Defensive mechanisms to counteract the impact of reactive oxygen species are found in many mammalian species include aquatic animals such as fish. These systems include various antioxidant defense enzymes such as superoxide radical to H_2O_2, catalase acting on hydrogen peroxide, glutathione S-transferase family possessing detoxifying activities towards lipid hydroperoxides generated organic pollutants such as heavy metals.

The accumulation of heavy metals might have led to the production of superoxide anions which led to the induction of SOD to convert the superoxide radical to H_2O_2. SOD catalytically scavenges superoxide radical

which appears to be an important agent of toxicity of oxygen and this provides a defense against this aspect of oxygen toxicity [38]. GSH is known to be a substrate for the activity of glutathione S-transferase. The apparent increase in GSH levels with parallel elevation in the activity of glutathione S-transferase in the organs suggests an adaptive and protective role of this bio-molecule against oxidative stress induced by the heavy metals. Our results are in agreement with the findings of Pandey *et al.* [35] on *Wallgo attu* fish from the Panipat river in India. The decreased levels of antioxidant enzymes and reduced glutathione with lowered level of glutathione S-transferase in the gills could account for the marked elevation of lipid peroxidation observed. The gills are more exposed to contaminated water and as such metal can penetrate through their thin epithelial cells [39]. Furthermore, the apparent decrease in glutathione detoxification system in the gill, the first point of contact with environmental xenobiotics indicates that this system is a sensitive biochemical indicator of environmental pollution [40] in *Clarias gariepinus*. Increase in the activity of catalase and superoxide dismutase is usually observed in the face of environment pollutants [36,41] since superoxide dismutase-catalase system represents the first line of defense against oxidative stress [41]. Oxidative stress is believed to occur when the normal balance of the oxidant-to-antioxidant ratio is disturbed. Antioxidant defense systems are triggered as soon as organisms are exposed to oxidative stress. This acts as a compensatory mechanism that prevents damage induced by ROS, which attack nucleic acids, proteins, and membrane phospholipids [42,43]. The antioxidant defense system of organisms is composed of antioxidant enzymes such as catalase, glutathione peroxidase, superoxide dismutase, and non enzymatic molecules such as vitamins A and E [44]. For example, O_2^-, the parental form of intracellular reactive oxygen species, is a highly reactive molecule but can be converted to H_2O_2 by superoxide dismutase and then to oxygen and water by several enzymes including catalase, glutathione peroxidase, and lipid peroxiredoxin [42,45]. Therefore, examining the expression of antioxidant enzymes could be an effective method of measuring oxidative stress.

In present study, the zebrafish was exposed to lead acetate ($C_4H_6O_4Pb.3H_2O$) and cobalt chloride ($CoCl_2.6H_2O$) for a period of 5, 10, 15 and 20 days at suitable concentrations i.e. for the lead 5 mg/l, 9 mg/l, 13 mg/l and 17 mg/l and for the cobalt 20 mg/l, 30 mg/l, 40 mg/l and 50 mg/l and recorded a significant reduction in CAT (catalase) and GSH (reduced glutathion) but in the LPO we observed significant enhancement in the brain of zebrafish. Maximum reduction was recorded in GSH and CAT at the higher concentration for the lead at 17 mg/l and maximum increase in LPO was recorded at same concentration. Same pattern was recorded into cobalt a significant reduction in CAT (catalase) and GSH (reduced glutathion) but in the LPO, we were observed significant enhancement in the brain of zebrafish. Maximum reduction was recorded in GSH and CAT at the higher concentration was at 50 mg/l as compared to the lower concentration of 20 mg/l and maximum increase in LPO was recorded at the 50 mg/l as compared to 20 mg/l. These observations revealed that the decline in CAT, GSH and upgrade LPO levels in brain was directly proportional to concentration of lead and cobalt. In this investigation it is clear that lead is more toxic than cobalt. The heavy metals cause free radicals mediated cellular damage, which leads to metabolic alterations such as the enzymatic activities and membrane transport mechanism and injuries of biological system at different levels.

CAT is the primary enzyme responsible for eliminating the ROS formed during bio-activation of xenobiotics in hepatic tissues and the induction of CAT system provides the first-line of defense against ROS. CAT activity, however, gradually decreased after 5, 10, 15 and 20 days of exposure to heavy metals and the values obtained were significantly ($p<0.05$) lower than those of the control. Decreased CAT activity decreases in reaction rates resulting from the excess production of H_2O_2. This could have been because of the flux of superoxide radicals, which has been shown to inhibit CAT activity [46]. Tripathi and Singh [47], observed a decrease in CAT activity in the brains, gills, livers and skeletal muscles of *Channa punctatus* (Bloch). The increase or decrease of enzyme activity is related to the intensity of cellular damage. Thomas and Murthy [48], described the Monocrotophos treatment resulted in the decrease of CAT activity in the liver of Asian stinging catfish (*Heteropneustes fossilis*). A decrease in the activity of CAT has been previously reported in *Cyprinidae* fish living in Seyhan dam Lake of Turkey [40] and in starlet (*Acipenser ruthenus* L.) from the Danube river of Serbia.

The reduced glutathione (GSH) antioxidant system is the principal protective mechanism of cells and is a key factor in the development of immune response by immune cells. Reduced glutathione reduction might increase the risk of the oxidative stress [49]. However, oxidative stress can induce GSH rising by protective role in the organisms exposed to heavy metals. Reduced GSH and its metabolizing enzymes provide the major defense against ROS induced cellular damage [50]. Doyotte *et al.* [51] pointed out that a decreased enzyme activity response may accompany a first exposure to pollutants, which can be followed by an enhancement of antioxidant system. Thus, the existence of an inducible antioxidant system may reflect an adaptation of organisms. While Dimitrova *et al.* [52] suggested that the superoxide radicals by themselves or after their transformation to H_2O_2 (Hydrogen peroxide) cause an oxidation of the cysteine in the enzyme and decrease superoxide dismutase activity. Consequently, the decreased and increased superoxide dismutase activities might have reflected a cellular oxidative stress due to heavy metal exposure. On the other-hand, the enzymatic antioxidants such as superoxide dismutase, GSH have been shown to be sensitive indicators of increased oxidative stress in *Mugil sp.* obtained from polluted area containing high concentration of pollutants [53]. Cysteine is the limiting factor of GSH synthesis in cells and reduced glutathione has sulfide functional groups that can capture unpaired

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electrons and thus is capable of removing harmful free radicals [54]. Padmini *et al.* [55] observed a significant decrease in the level of GSH ($p<0.001$) in brain of *M. cephalus* inhabiting Ennore estuary (59%) when compared with brain of *M. cephalus* inhabiting Kovalam estuary. Joseph *et al.* [56] were found that GSH level was higher ($p<0.05$) in control with a value of 3.05±0.01, when compared to groups exposed to concentrations of $Pb(NO_3)_2$ (2.53±0.29, 0.94±0.14, and 0.82±0.10) at 28 days. For test organisms exposed to concentrations of $ZnCl_2$, GSH level was lower (0.10±0.05) though not significant at ($p<0.05$) in control at 28 days.

MDA is one of the LPO products deriving from oxidative attack on cell membrane phospholipids and circulating lipids, and its level directly reflects the degree of oxidative damage induced by contaminants [57]. The measurement of MDA content provides a relative measure of potential for pollutants to cause oxidative injury [58]. The elevated MDA level was considered as result of oxidative stress from xenobiotics. MDA, a major oxidation product of peroxidized polyunsaturated fatty acids, has been considered as an important indicator of lipid peroxidation.

The enhanced levels of LPO in the brains of *Danio rerio* in response to 20 days of exposures to lead and cobalt were observed during the present study suggest that production of ROS is increased which could be associated with the metabolism of the heavy metals leading to the peroxidation of membrane lipids in brain tissues. Lipid peroxidation is indicated by the presence of MDA in tissues.

CONCLUSIONS

The biochemical investigations can be used to study the mode of action of heavy metals and cause for death of aquatic organisms. Thus biochemical alterations in zebrafish may be considered as biomarkers to access the health status of the fishes as well as aquatic bodies polluted by heavy metals. Further research should be done in order to have a clear picture of heavy metal mediated oxidative stress and their effects on the environment and the risk they pose on it.

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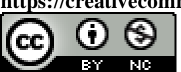

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