EFFECTS OF ZINC IN REDUCING CADMIUM TOXICITY WITH SPECIAL REFERENCE TO OXIDATIVE ENZYME LEVELS AND HEMATOLOGICAL PARAMETERS IN ZEBRAFISH (*DANIO RERIO*)

Short title: Zinc role in reducing cadmium toxicity

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Abstract: The protective effects of zinc on cadmium toxicity were investigated in this study, with a focus on oxidative enzyme levels in the zebrafish liver and blood biochemical parameters of cadmium, zinc, and cadmium + zinc treated zebrafishes. We observed synergetic effects of zinc on reducing cadmium toxicity in all of the parameters we studied. The effectiveness of zinc treatment in lowering cadmium toxicity was demonstrated by the growth and survival rate of zebrafish subjected to cadmium. Results have proved that the zinc treatment has increased the survival percentage along with body weight and length. When zebrafish were administered cadmium, the oxidative enzyme succinate dehydrogenase (SDH) in the liver homogenate reduced drastically, from 6.86 mU/mg (control) to 2.73 mU/mg (cadmium treated). However, when cadmium and zinc were given simultaneously, the SDH incidence increased (4.97 mU/mg). Similar pattern was observed in other tested enzymes. Hematological properties of treated fish blood clearly indicate that zinc has the capability in reducing cadmium toxicity. To summarize, the findings show that even modest doses of cadmium are hazardous to zebrafish and cause changes in hematological and oxidative enzyme levels. Lower doses of zinc, on the other hand, can significantly diminish cadmium's detrimental effects.

Index Terms **- Cadmium, Zinc, Toxicity, Oxidative enzymes, Blood Biochemistry**

I. INTRODUCTION

Cadmium is a naturally occurring pollutant in water, and excessive levels of cadmium in surface water pose a serious hazard to the aquatic ecology, which includes fish [1]. Because their feeding and metabolic activities concentrate metals in specific body parts, high quantities of metals can accumulate in the tissues of most aquatic organisms [2]. According to certain investigations on this mechanism in fish, cadmium may have harmful effects that cause changes in physiological processes in the blood and tissue of the fish [3]. Although the mechanisms of cadmium toxicity are unknown, it is thought that cadmium damages cells mostly by producing reactive oxygen species (ROS) [4], which causes single-strand DNA damage and impairs nucleic acid and protein synthesis. As a result, it is essential to monitor heavy metal concentrations in aquatic habitats, and cadmium toxicity has thus become a local as well as a worldwide concern. When it comes to chemistry of Cadmium (Cd), it is a transition metal with an atomic number of 48 and an atomic mass of 112.411g, belongs to the d-block and the 12 group of the periodic table.

In vitro studies show that cadmium causes cytotoxicity and free radical-dependent DNA damage at doses ranging from 0.1 to 10 mM [5]. Cadmium influences male reproductive in mice models at a dose of 1 mg/kg body weight, according to in vivo research [6]. Long-term cadmium exposure has been linked to autoimmune diseases, cancer, cardiovascular disease, and hepatic dysfunction in several studies [7-9]. When cadmium exposure is done via oral or respiratory route it gets accumulated in the tissue and causes oxidative stress and metabolic disorder of essential elements which leads to tissue damage [10]. Cadmium can accumulate in many organs, including the liver, kidney, heart it can adversely affect organ function and overall health [11]. Zinc is an essential metal that is involved in protein structure, catalysis, and function regulation. Zinc has been proven in numerous studies to minimise cadmium toxicity; however, the underlying mechanisms have not been well investigated [12-14]. Because zinc is an essential component of Cu/Zn–SOD, it can also act as an antioxidant indirectly by inducing the synthesis of metallothionein (MT), a thiol-rich protein that can bind metals with pro-oxidant activity, such as cadmium, and provide thiol groups that can scavenge hydroxyl radicals and singlet oxygen [15]. The available data indicate that zinc is one of the most important nutritional factors influencing the metabolism and toxicity of heavy metals, including cadmium [16]. Mutual interactions between cadmium and zinc may occur at various stages of both metals' metabolism, i.e. absorption, distribution in the organism and excretion, as well as at the stage of zinc biological functions [17]. Enhanced consumption of zinc may decrease cadmium absorption from the digestive tract and its accumulation in the organism, and as a result it may ameliorate the toxic effects of cadmium, including mainly the liver damage [18].

Moreover, zinc is a crucial metal that plays key roles in protein structure, catalysis, and regulation of their function [19]. Zinc is readily available as a nutritional ingredient, affordable as a dietary supplement, and has not been described to have adverse side effects [20]. On the other hand, dietary zinc deficiency increases both cadmium absorption and body burden, thus enhancing its toxicity [21]. It has also been suggested that zinc may have a protective influence on cadmium retention [22]. However, the effects of Zn have not been comprehensively studied in living organisms until

now. It is known that many effects of the toxic action of cadmium result from its interactions with essential elements. Zinc as a micronutrient can impact toxicity of metals notably cadmium by interacting with the metal at its primary site of action. As stated previously, zinc boosts synthesis of MT, a thiol-rich protein that sequesters cadmium and prevents acute hepatotoxicity, leading instead to chronic kidney toxicity as cadmium-MT is excreted from the liver and absorbed by the kidney [23]. Therefore, the main objective of this study was to evaluate the role of zinc in lowering cadmium toxicity in zebrafish, with a focus on oxidative enzyme levels and blood biochemical analysis.

In this study, zebrafish were selected for exposure experiments and exposed to test solutions (cadmium, zinc and cadmium +zinc) under static conditions for 90 days. Zebrafish was chosen for this study as it has been established as a model of choice due to number of advantages like easy maintenance and short size so that they are user friendly and easy to handle. Furthermore, its genome has a significant level of homology with that of humans.

II. METHODOLOGY

***Animals and experimental protocol*:**

Adult zebrafish were purchased from a local commercial dealer and acclimated to soft water in a laboratory condition for two weeks prior to the experiments. Fish were maintained in aquaria at 26+10C with well oxygenated water (pH 7.0) under a photoperiod of 14:10 h light: dark cycle. Fish were fed daily with a commercial food (TetraBits, Melle, Germany) and live blood worms. The uniform conditions of water quality were maintained as follows: water temperature, 22–24^{0}C; pH, 6.8–7.2; dissolved oxygen, 5–7 mg /L; conductivity, 650 lS/cm. The concentrations of heavy metals were almost zero or could not been detected, composition of water meets the water quality standard of fisheries. Water for each group was completely renewed every day and the aquaria were thoroughly cleaned.

Rules of the "Institutional Animal Ethics Committee of Sri Venkateshwara University" were strictly followed during the experiment and steps were taken to protect the welfare of experimental animals.

Experimental groups and sample preparation: Treated fish were randomly divided into four groups **(Figure 1)** as given below with 07 + 07 (2 batches) fishes in each group and with 14 fish livers were used to compose one sample (n=14 pools).

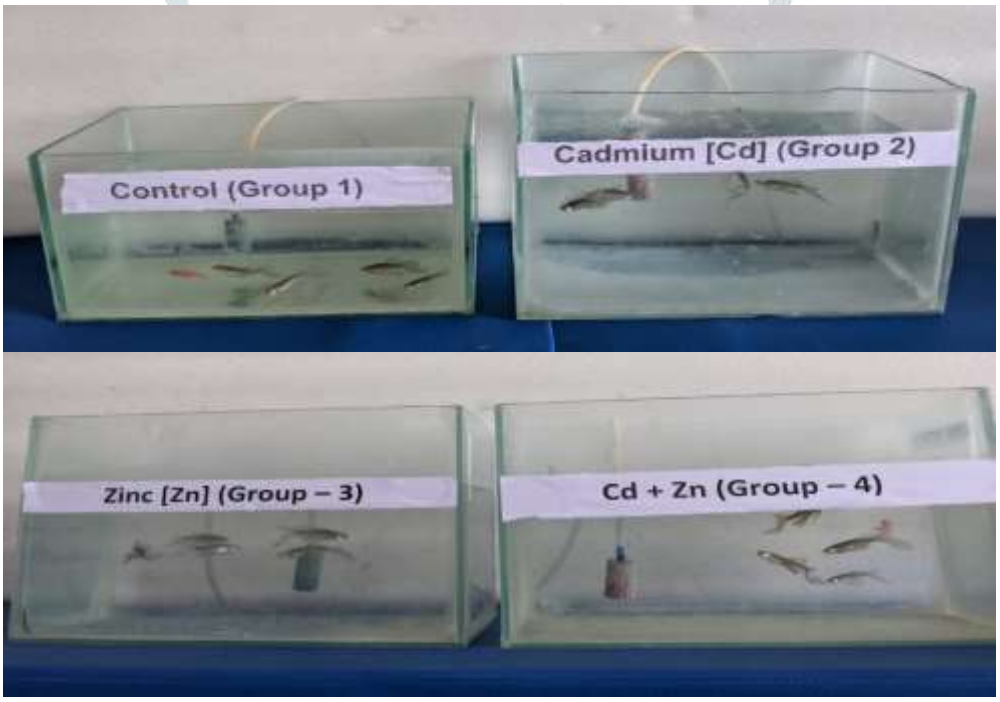

Figure 1 Experimental set up used in this study

Control Group 1: (Untreated): not exposed to Cadmium and Zinc

Treatment Group 2: Cadmium (Cd) exposed (100 ppm/per litre daily for 90 days – [Dissolved 0.100 g of cadmium metal in 4 mL concentrated nitric acid. Add 8.0 mL concentrated nitric acid and dilute to 1000 mL with water (1.00 mL = 100 µg Cd)].

Treatment Group 3: Zinc (Zn) (100 ppm ppm/per litre daily for 90 days – [Dissolved 0.440 g of zinc sulfate in water containing 1 ml of 5M acetic acid and add sufficient water to produce 100.0 ml. Diluted 1 volume of this solution to 10 volumes with water immediately before use).

Treatment Group 4: Cd (100 ppm daily for 90 days) + Zn (100 ppm daily for 90 days) exposed.

Zebrafish lengths (mouth to caudal peduncle), weight, and survival rate were analyzed quantitatively after 90 days of treatment. Figure 1 represents the experimental set up of the present study.

Enzyme assays:

***Succinate dehydrogenase (SDH) Assay*:** This enzyme is responsible for converting succinate to fumarate as part of the Krebs cycle. SDH activity was estimated by Colorimetric Assay Kit (BioVision) as per the protocol provided with the kit.

***Lactate Dehydrogenase (LDH) Assay*:** LDH is an oxidoreductase enzyme that catalyzes the interconversion of pyruvate and lactate. Cells release LDH into the bloodstream after tissue damage. LDH activity was estimated by Kit method (Sigma Aldrich) as per the protocol provided with the kit.

***Glucose 6 Phosphate Dehydrogenase* (G-6-PD):** G-6-PD activity was estimated by Kit method (Sigma Aldrich) as per the protocol provided with the kit

***Blood Biochemical assays*:** Blood was collected using a micropipette tip through a lateral incision in the dorsal aorta region that was about 0.2 cm long. Without adding anticoagulant, the blood was collected from treated zebrafish into separate tubes and subject for centrifugation and serum collected was used far biochemical analysis. All the blood parameters were estimated by using diagnostic kits supplied by SD fine, Kanbaxy, span diagnostics Ltd., India, as per the procedures mentioned on the kit.

***Statistical analysis*:** All the experimental data given in the results were means of triplicates and followed Duncan's new Multiple range (DMR) test to find significant difference ($P<0.05$) between values of each sampling [24].

III. RESULTS AND DISCUSSIO.

As summarized in **Table 1**, cadmium had negative impact on physiological properties of zebrafish when compared to control and zinc treated fishes as the drastic decrease in the body weight and length was observed in cadmium treated. Cadmium exposure had an obvious negative effect on the rate of overall growth of the test animals. Mortality rate also observed in the cadmium treated fishes. However, zinc has helped to regain the physiological properties of zebrafish that were treated with cadmium. Survival percentage was 70 percent in case of cadmium treated zebrafish. However, in case of cadmium + zinc treated, survival rate has been increased with 90 %.

Table 1 Zebrafish lengths (mouth to caudal peduncle), weight, and survival rate after and before treatment

Parameter	Control	Cadmium (Cd)	Zinc (Zn)	Cd + Zn
BODY WEIGHT (g)	Before treatment			
	0.23 ± 0.02^{a}	0.21 ± 0.01^{a}	0.21 ± 0.02^{a}	$0.23^{a} \pm 0.01$
	After treatment			
	1.85 ± 0.05^{c}	0.75 ± 0.03^{a}	1.80 ± 0.05^{c}	1.55 ± 0.03^{b}
BODY LENGTH (cm)	Before treatment			
	1.09 ± 0.05^{b}	0.92 ± 0.03^{a}	1.12 ± 0.04^{b}	1.02 ± 0.07^{b}
	After treatment			
	2.63 ± 0.05^{c}	1.53 ± 0.05^{a}	2.54 ± 0.05^{c}	2.1 ± 0.05^{b}
SURVIVAL (%) AFTER TREATMENT	100	70	100	90

Means ± S.E. in each row, followed by the same letter are not significantly different ($P \leq 0.05$) from each other according to DMR test.

Cadmium is a powerful uncoupling agent and inhibits the succinate- and malate/pyruvate-stimulated respiration [25]. As a result, in this study, the liver homogenate of zebrafish treated with cadmium showed a significant decrease in succinate dehydrogenase (2.73 mU/mg) compared to control (6.86 mU/mg) and zinc treatment (8.89 mU/mg). The succinate dehydrogenase activity was nearly doubled with 4.97mU/mg when cadmium and zinc were mixed, confirming the synergistic effects of zinc (Table 2). Cadmium has been observed to cause oxidative stress and because of its prooxidative properties, causes oxidative damage to various macromolecules [26]. Zinc, on the other hand, was found in a study by Amara et al [27] to reduce oxidative stress and oxidative damage produced by cadmium. However, in case of lactate dehydrogenase (LDH), the activity was low (3.97 milliunits/mL) compared to control (5.24 milliunits/mL), zinc treated (6.80 milliunits/mL) and zinc + cadmium treated (4.89 milliunits/mL). Reduced lactate dehydrogenase activity could be caused by cadmium's direct increase in permeability and decrease in mitochondrial membrane potential, which results in cytochrome C release and caspase pathway activation [28].

However, in cadmium-treated zebrafish liver homogenate, glucose-6-phosphate dehydrogenase activity was high (8.67 mU/ml), followed by zinc + cadmium treated (7.53 mU/ml), untreated, control (6.87 mU/ml), and zinc treated

(6.71 mU/ml). Under oxidative stress, glucose-6-phosphate dehydrogenase levels have been found to rise in general, particularly in liver cells [29]. According to prior research, cellular membranes have been identified as cadmium's target areas, and hazardous metal-induced lipid peroxidation has been suggested as a key process in the destruction to several tissues and organs, particularly the liver [30,31]. The findings strongly indicate that increased dietary zinc consumption during cadmium exposure can, at least in part, protect against a number of cadmium toxicity consequences, such as oxidative damage to cellular membranes, by preventing changes in the structural frame of lipids.

Table 2 Alternations in the levels of oxidative enzymes of liver homogenate of zebrafish after treatment

Liver Homogenate	Group-I	Group -II	Group-III	Group-IV
Succinate Dehydrogenase [SDH] (mU/mg)	6.86 ±0.39^{c}	2.73 ± 0.16^{a}	8.89 ± 0.62^{d}	4.97 ± 0.63^{b}
Lactate dehydrogenase [LDH] (milliunits/mL)	5.24 ± 0.14^{a}	3.97 ± 0.26^{c}	6.80 ± 0.52^{b}	4.89 ± 0.8^{a}
Glucose 6 Phosphate Dehydrogenase [G-6-PDH] (mU/ml)	6.87 ± 0.32^{c}	8.67 ± 0.5^{a}	6.71 ±0.032^{c}	7.53 ± 0.58^{b}

Means ± S.E. in each row, followed by the same letter are not significantly different ($P \leq 0.05$) from each other according to DMR test

Hematological parameters have shown great promise in measuring the acute and chronic toxicity of heavy metals in mammals and other animals. These parameters include the measurement of blood glucose, potassium, phosphorous and a variety of enzymes. The current study additionally looked at the use of a few hematological measures in zebrafish that had been exposed to cadmium and zinc. Hematological parameters have long been thought to be a good predictor of physiological changes and health condition in fish, and they can be influenced by metal exposure [32]. When all hematological indicators were compared to the normal range, cadmium was shown to be more hazardous than the control and zinc-treated groups in lowering the values significantly (Table 3). When it comes to white blood cell differential counts, such as lymphocytes, monocytes, neutrophils, eosinophils, and basophils, cadmium-treated zebrafish have a significantly lower percentage than control and zinc-treated zebrafish. When cadmium was used, blood glucose dropped dramatically, but when zinc was used, it stayed almost the same. These findings showed that zinc supplementation in the diet can help to mitigate the negative effects of cadmium on the characters studied. The way cadmium is absorbed by cells is yet unknown, and further research is needed to determine the predicted amount in the circulation and the pathway of cadmium uptake in cells. Several cellular transporters and ion channels, including calcium channels, have been discovered to transport cadmium across the cell membrane. Higher blood cadmium levels are related with increased coronary heart disease (CHD) mortality.

Zinc supplement use seemed to be inversely associated with recent exposure, with greater doses of zinc supplements associated with lower blood cadmium concentrations [33]. The duration of zinc supplement use was inversely associated with serum zinc, but there was no evidence of an association between duration of zinc supplement use and cadmium exposure [34]. Despite the fact that zinc is required for regular cellular function and can be harmful in excess, homeostatic mechanisms have evolved to keep the intracellular zinc content in check. The activity of zinc transporters, zinc-permeable ion channels, and cysteine-rich metallothionein metal-binding proteins regulates the quantity of intracellular free zinc. Overall, the findings of this investigation demonstrated that zinc protects organisms from cadmium-induced toxicity.

Table 3 Effect of cinc in reducing Cadmium toxicity with reference to blood biochemical analysis in Zebrafish

Blood parameter	Control	Cadmium (Cd)	Zinc (Zn)	Cd + Zn	Range (%)
White blood cell differential counts					
Lymphocytes (%)	85.42 ± 4.36^{c}	64.21 ± 3.41^{a}	81.45 ± 4.71^{b}	79.64 ± 3.84^{b}	71-92
Monocytes (%)	12.25 ± 1.95^{d}	4.25 ± 2.37^{a}	10.42 ± 3.21^{c}	9.25 ± 2.42^{b}	5-15
Neutrophils (%)	14.42 ± 0.45^{d}	8.45 ± 1.23^{a}	12.48 ± 2.15^{c}	10.23 ± 1.48^{b}	2-18
Eosinophils	1.41 ± 0.04^{c}	0.78 ± 0.01^{a}	1.14 ± 0.07^{b}	1.15 ± 0.0^{b}	0-2
Basophils	0.98 ± 0.07^{c}	0.14 ± 0.08^{a}	0.69 ± 0.12^{b}	0.72 ± 0.12^{b}	0-2
Serum Biochemical analytes					
Albumin	2.7 ± 0.32^{c}	1.12 ± 0.48^{a}	2.1 ± 0.74^{b}	1.57 ± 0.25^{b}	3.3 g/dl
ALP	7.0 ± 0.85^{d}	2.45 ± 0.45^{a}	4.21 ± 1.12^{c}	3.42 ± 0.77^{b}	2.0 – 10.0 U/L^{3}
ALT	370 ± 14.14^{d}	165 ± 12.5^{a}	340 ± 14.26^{c}	285 ± 9.45^{b}	343.0-410.0 U/L

Total bilirubin	0.51 ± 0.07^{b}	0.12 ± 0.05^{a}	0.37 ± 0.04^{b}	0.28 ± 0.03^{b}	0.2 – 0.6 mg/dl
BUN	3.5 ± 0.74^{d}	1.48 ± 0.07^{a}	2.18 ± 0.7^{b}	2.75 ± 0.48^{c}	3.0 – 4.0 mg/dl
Calcium	15.2 ± 1.72^{c}	10.45 ± 1.24^{a}	13.24 ± 2.32^{b}	12.75 ± 3.12^{b}	10.3-18.6 mg/dl
Phosphorus	23.1 ± 2.14^{b}	20.5 ± 2.32^{a}	22.75 ± 1.75^{b}	22.89 ± 2.15^{b}	20.3-24.3 mg/dl
Glucose	79.45 ± 7.32^{d}	68.23 ± 1.48^{a}	74.81 ± 2.41^{c}	71.25 ± 1.46^{b}	91.0 g/dl
Potassium	6.4 ± 0.85^{d}	5.12 ± 0.75^{a}	6.21 ± 1.21^{c}	5.91 ± 1.2^{b}	5.2 – 7.7 mEq/L
Total Protein	5.1 ± 0.17^{d}	4.15 ± 0.42^{a}	4.58 ± 1.14^{c}	4.38 ± 2.14^{b}	4.0 – 5.8 g/dl

ALP = Alkaline phosphatase; ALT – alanine transaminase; BUN – Blood Urea Nitrogen

Means ± S.E. in each row, followed by the same letter are not significantly different ($P \leq 0.05$) from each other according to DMR test

IV. SUMMARY AND CONCLUSION

In connection to the development of cadmium toxicity, the body's zinc status is critical. As stated earlier discussion, numerous studies have found that increased zinc supply can minimize cadmium absorption and accumulation, as well as avoid or mitigate cadmium's negative effects, whereas zinc deficiency can enhance cadmium accumulation and toxicity. These findings address a gap in our knowledge of the significance of zinc supplementation in aquatic animal health; dietary zinc may help zebrafish and other aquatic species avoid cadmium accumulation and toxicity.

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