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Research Article

PROTECTIVE ROLE OF TRACE ELEMENTS AGAINST CADMIUM INDUCED ALTERATIONS IN THE SELECTED OXIDATIVE STRESS ENZYMES IN LIVER AND KIDNEY OF FRESH WATER TELEOST, *OREOCHROMIS MOSSAMBICUS* (TILAPIA)

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ABSTRACT

Cadmium (Cd) is a non essential heavy metal that enters human and animal bodies via different industrial products, environmental pollution and different contaminated foods. The present study is carried out to investigate Cd induced alterations in the lipid peroxidation (LPO) and antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) in liver and kidney of fresh water teleost, *Oreochromis mossambicus* (Tilapia) exposed to Cd before and after supplementation with trace elements such as calcium (Ca) and zinc (Zn) either individually or in combination. The fish were exposed to cadmium chloride ($CdCl_2$) at a dose of $1/10^{th}$ LC_{50} i.e. 5ppm for 7, 15 and 30 days (d) time intervals. After 15d Cd exposure, the fish were then supplemented with trace elements like Ca (1ppm) and Zn (1ppm) either individually or in combination for again 7, 15 and 30d time intervals. After the specified time intervals, liver and kidney tissues were isolated and used for assay of antioxidant enzymes as well as LPO. Results revealed significant elevation of LPO during Cd exposure for all the time durations. Maximum LPO level was found in 30d Cd exposed fish liver tissue (26.415 ± 0.286 µ moles of MDA formed / gm wet wt. of the tissue) than kidney (21.692 ± 0.193 µ moles of MDA formed / gm wet wt. of the tissue). Both SOD and CAT activity levels were markedly inhibited at all the time intervals of Cd exposure. After supplementation with trace elements Ca and / or Zn, the LPO levels were significantly decreased. Maximum decrease in LPO levels were found in kidney and liver under Ca alone supplementation followed by combination of Ca + Zn and Zn alone supplementation. Maximum SOD activity levels were found in 30d Zn supplemented kidney tissue (0.628 ± 0.212 superoxide anion reduced / mg protein / min) followed by 30d combined supplementation of Ca and Zn (0.621 ± 0.201 superoxide anion reduced / mg protein / min). Whereas Maximum CAT activity levels were found in 30d Ca supplemented kidney tissue (0.605 ± 0.261 µ moles of H_2O_2 / mg protein / min) followed by 30d combined supplementation of Ca and Zn (0.583 ± 0.219 µ moles of H_2O_2 / mg protein / min). Our findings clearly evidenced that the Ca alone supplementation is very effective in reducing the Cd toxicity when compared to other modes of supplementation in the teleostean fish *Oreochromis mossambicus.*

Keywords: Cadmium, Trace elements supplementation, Oxidative stress enzymes, Tilapia

INTRODUCTION

Heavy metals like Cd, Pb and Hg have no known biological functions and consequently detrimental to essential life processes. These metals in the form of inorganic compounds from natural and anthropogenic sources continuously enter the aquatic ecosystem where they pose a serious threat because of their toxicity.

Among the heavy metals, Cd is one of the most toxic, non-essential heavy metal; known for its corrosive nature and is widely used in paints and dyes, cement and phosphate fertilizers[1]. Cd occurs naturally in the environment in significant amounts but its release in the recent past is steadily increasing due to human activities causing pollution at considerably toxic amounts was reported by earlier workers in various aquatic ecosystems[2]. Bioenhancement of Cd transfer along a food chain was studied by Seebaugh[3] and fish were reported to be used as biological indicators to assess water pollution[4]. In aquatic systems, as fish occupy the upper trophic level, there are greater chances of transferring Cd to higher organisms particularly to man.

Cd has an extremely long half-life (20-30 Years) in the human body[5] and is highly cumulative, especially in the liver and kidney[6,7,8,9]. It is a ubiquitous toxic metal and induce oxidative damage by disturbing the prooxidant – antioxidant balance in the tissues[10]. Cd inhibits oxidative stress enzymes which protect tissues by either binding to sulfhydryl groups essential for the enzymes, replace the bivalent metals like zinc (Zn), copper (Cu), iron (Fe) and manganese (Mn) required for the enzymes or decreases the bioavailability of selenium (Se) required for the enzymes[11]. Cd like many other heavy metals is antagonistic to essential trace elements like Zn, Fe, Cu, Ca etc.,[12] and competes with these trace elements for binding sites as transport and storage proteins, metalloenzymes and receptors.

Zn is a ubiquitous essential trace element with numerous functions in biological systems. It occurs in all living cells as a constituent of metallo enzymes involved in major metabolic pathways. It plays a catalytic, inhibitory or accessory role in the regulatory enzymes such as kinases or phosphatases. Zn controls several enzymes of intermediary metabolism, DNA and RNA synthesis, gene expression, immunocompetence and plays a significant role in homeostasis of hormones[13]. It has been noted that Zn is a constituent of several enzymes (more than 300 enzymes) in the body and can prevent cell damage through activation of the antioxidant defense system[14,15].

Ca plays diverse role in the living organisms. In most of the vertebrates it is a major component of the skeleton but it also has vital functions in the body fluids and soft tissues. It acts as a cofactor in various enzymatic processes and couples stimulus excitation reactions, as in muscle contraction or the secretion of exocrine and endocrine glands. Both Ca and Cd are divalents and they use the same transitional channel interacting with each other antagonistically. Zohouri[16] have been reported that high concentrations of Ca either in water or diet clearly envisages ameliorating effects on water borne Cd toxicity in fish *Oreochromis mossambicus.*

Hence, an attempt is made in the present investigation on the interactions of Ca and / or Zn against Cd induced toxicity in liver and kidney of teleostean fish *Oreochromis mossambicus.*

MATERIALS AND METHODS

Chemicals: Cadmium as cadmium chloride ($CdCl_2$), calcium as calcium chloride ($CaCl_2$) and zinc as zinc chloride ($ZnCl_2$) were purchased from Merck (Dormstadt, Germany). All other chemicals which were used in the present study were obtained from the standard chemical companies like Sigma Chemical Co. (St Louis, Mo, USA) and SD Fine Chemicals. The chemicals used in this study were of the highest purity.

Animals: Fish *Oreochromis mossambicus* (Tilapia) weighing 10 ± 2 gm were collected from the local fresh water ponds and acclimatized to laboratory conditions for a week in separate troughs. The laboratory temperature was maintained at $28^0C \pm 2^0C$. The fish were

feed *ad libitum* with ground nut cake and water was renewed for every 24 hrs with routine changing of troughs leaving no faecal matter. The protocol and animal use has been approved by the Institutional Animal Ethics Committee (Resol. No. 10(ii)/a/CPCSCA/IAEC/SVU/AUR-JO dt 22-12-2008), Sri Venkateswara University, Tirupati, Andhra Pradesh, India.

After acclimatization the fish were divided into two groups, namely control and experimental. Control fish received only deionised water without Cd. The experimental fish were exposed sub lethal concentration i.e., 5 ppm of $Cdcl_2$ ($1/10^{th}$ of Lc_{50} / 48 hrs) daily for 7, 15 and 30 days (d) time intervals. The LC_{50} / 48hr value for $Cdcl_2$ to the test animals were already evaluated from our laboratory[17].

Then the 15d Cd exposed fish were divided into three groups. Group- I received supplementation of Ca (1 ppm) for 7, 15 and 30d. Group-II received Zn supplementation (1 ppm) and Group-III animals were supplemented with both Ca and Zn at the above said doses for 7, 15 and 30d long sojourn.

Isolation of tissues: After specific time intervals, the control and experimental fish were decapitated and tissues such as liver and kidney were quickly isolated under ice cold conditions and weighed to their nearest mg using Shimadzu electronic balance (Schimadzu, AUX 220). After weighing, tissues were immediately used for the assay of oxidative stress enzymes like SOD, CAT and the levels of LPO or were stored at -80 ^{0}C for future use.

Lipid peroxidation (LPO)

The LPO was determined by the TBA method of Ohkawa[18]. The tissues were homogenized in 1.5% KCl (20% W/V). To 1ml of tissue homogenate 2.5 ml of 20% TCA was added and the contents were centrifuged at 3,500g for 10 minutes (min) and the precipitate was dissolved in 2.5ml of 0.05M sulphuric acid. To this, 3ml of thiobarbituric acid (TBA) was added and the samples were kept in a hot water bath for 30 min. The samples were cooled and malonaldehyde (MDA) was extracted with 4ml of n-butanol and the colour was read at 530nm in a UV spectrophotometer (Hitachi U-2000) against the reagent blank. Trimethoxy pentane (TMP) was used as external standard. Values are expressed in µ moles of MDA formed / g tissue / hr.

Superoxide dismutase (SOD) (E.C. 1.15.1.1)

SOD activity was determined according to the method of Misra and Fridovich[19] at room temperature. The tissue was homogenized in ice cold 50 mM phosphate buffer (PH 7.0) containing 0.1 mM EDTA to give 5% homogenate (W/V). The homogenates were centrifuged at 10,000 rpm for 10 min at 4^{0}C in cold centrifuge. The supernatant was separated and used for enzyme assay. 100 µl of tissue extract was added to 880 µl (0.05 M, pH 10.2, containing 0.1 mM EDTA) carbonate buffer; and 20 µl of 30 mM epinephrine (in 0.05% acetic acid) was added to the mixture and measured the optical density values at 480 nm for 4 min using UV-Spectrophotometer (Hitachi U-2000). Values are expressed in superoxide anion reduced / mg protein / min.

Catalase (CAT) (E.C. 1.11.1.6)

CAT activity was measured by a slightly modified method of Aebi[20] at room temperature. The tissue was homogenized in ice cold 50 mM phosphate buffer (pH 7.0) containing 0.1 mM EDTA to give 5% homogenate (W/V). The homogenates were centrifuged at 10,000 rpm for 10 min at 4^{0}C in cold centrifuge. The resulting supernatant was used as enzyme source. 10 µl of 100% ethylalcohol (EtOH) was added to 100 µl of tissue extract and then placed in an ice bath for 30 min. After 30 min the tubes were kept at room temperature followed by the addition of 10 µl of Triton X-100 RS. In a cuvette containing 200 µl of phosphate buffer and 50 µl of tissue extract was added 250 µl of 0.006 M H_2O_2 (in phosphate buffer) and decreases in optical density measured at 240 nm for 60 seconds (s) in a UV spectrophotometer (Hitachi U-2000). The molar extinction coefficient of 43.6 M cm^{-1} was used to determine CAT activity. One unit of activity is equal to the moles of H_2O_2 degraded / mg protein / min.

Estimation of protein content

Protein content of the tissues was estimated by the method of Lowry[21]. 1 % (W/V) homogenates of the tissues were prepared in 0.25 M ice cold sucrose solution. To 0.5ml of homogenate, 1ml 10% TCA was added and the samples were centrifuged at 1000g for 15 min. Supernatant was discarded and the residues were dissolved in 1ml of 1N sodium hydroxide. To this 4ml of alkaline copper reagent was added followed by 0.4ml of folin-phenol reagent (1:1folin:H_2O). The color was measured at 600nm in a UV spectrophotometer (Hitachi U-2000) against reagent blank. The protein content of the tissues were calculated using a protein (BSA) standard graph.

DATA ANALYSIS

The data was subjected to statistical analysis such as mean, standard deviation and analysis of variance (ANOVA) using standard statistical software, SPSS (version 16) software. All values are expressed as Mean ± SD of 6 individual samples. Significant differences were indicated at $p < 0.05$ level.

RESULTS

The data on the alterations in the oxidative stress enzymes such as SOD, CAT as well as LPO in Cd exposed fish liver and kidney both before and after supplementation with Ca and / or Zn were tabulated in Table 1 – 4.

Table 1: Shows alterations in the activity levels of selected oxidative stress enzymes in the liver of Cd exposed *Oreochromis mossambicus*.

S. No.	Name of the enzyme	Control	7 days	15 days	30 days
1.	LPO	12.223 ± 0.217	14.523 ± 0.181	19.223 ± 0.179	26.415 ± 0.286
2.	SOD	0.949 ± 0.028	0.799 ± 0.018	0.699 ± 0.012	0.524 ± 0.023
3.	CAT	0.754 ± 0.021	0.618 ± 0.030	0.538 ± 0.023	0.394 ± 0.019

All values are expressed as Mean ± SD of 6 individual samples.

All values are significant at $p < 0.05$ level.

Table 2: Shows alterations in the activity levels of selected oxidative stress enzymes in the liver of Cd exposed *Oreochromis mossambicus* after supplemented with Ca and / or Zn.

Enzyme	Calcium (Ca)			Zinc (Zn)			Calcium and Zinc		
	7d	15d	30d	7d	15d	30d	7d	15d	30d
LPO	18.171 ± 0.158	16.536 ± 0.172	13.082 ± 0.170	18.238 ±1.065	17.125 ±0.684	15.075 ±1.115	18.000 ±1.080	16.725 ±1.060	13.700 ±0.620
SOD	0.726 ± 0.216	0.797 ± 0.119	0.916 ± 0.127	0.738 ±0.266	0.824 ±0.232	0.929 ±0.246	0.731 ±0.265	0.805 ±0.232	0.920 ±0.188
CAT	0.601 ± 0.105	0.643 ± 0.214	0.727 ± 0.250	0.581 ±0.016	0.627 ±0.017	0.696 ±0.024	0.599 ±0.015	0.632 ±0.022	0.711 ±0.017

All values are expressed as Mean ± SD of 6 individual samples.

All values are significant at $p < 0.05$ level.

Table 3: Shows alterations in the activity levels of selected oxidative stress enzymes in the kidney of Cd exposed *Oreochromis mossambicus*.

S. No.	Name of the enzyme	Control	7 days	15 days	30 days
1.	LPO	9.515 ± 0.387	11.570 ± 0.266	15.068 ± 0.138	21.692 ± 0.193
2.	SOD	0.695 ± 0.011	0.567 ± 0.025	0.398 ± 0.011	0.215 ± 0.021
3.	CAT	0.660 ± 0.112	0.557 ± 0.206	0.410 ± 0.117	0.295 ± 0.300

All values are expressed as Mean ± SD of 6 individual samples.

All values are significant at P < 0.05 level.

Table 4: Shows alterations in the activity levels of selected oxidative stress enzymes in the kidney of Cd exposed *Oreochromis mossambicus* after supplemented with Ca and / or Zn.

Enzyme	Calcium (Ca)			Zinc (Zn)			Calcium and Zinc		
	7d	15d	30d	7d	15d	30d	7d	15d	30d
LPO	13.947 ± 0.103	13.091 ± 0.182	10.085 ± 0.287	14.083 ±1.044	13.315 ±1.357	10.433 ±0.995	14.000 ±1.036	13.201 ±1.088	10.251 ±1.278
SOD	0.423 ± 0.118	0.506 ± 0.217	0.601 ± 0.158	0.435 ±0.265	0.532 ±0.319	0.628 ±0.212	0.430 ±0.350	0.526 ±0.347	0.621 ±0.201
CAT	0.420 ± 0.314	0.467 ± 0.230	0.605 ± 0.261	0.415 ±0.021	0.438 ±0.029	0.466 ±0.021	0.418 ±0.019	0.442 ±0.015	0.583 ±0.021

All values are expressed as Mean ± SD of 6 individual samples.

All values are significant at P < 0.05 level.

A significant increase in LPO was observed in both the test tissues exposed to Cd. Liver tissue showed profound increment in LPO levels (26.415 ± 0.286 µ moles of MDA formed / gm wet wt. of the tissue) than kidney in 30d Cd exposure (Fig-1).

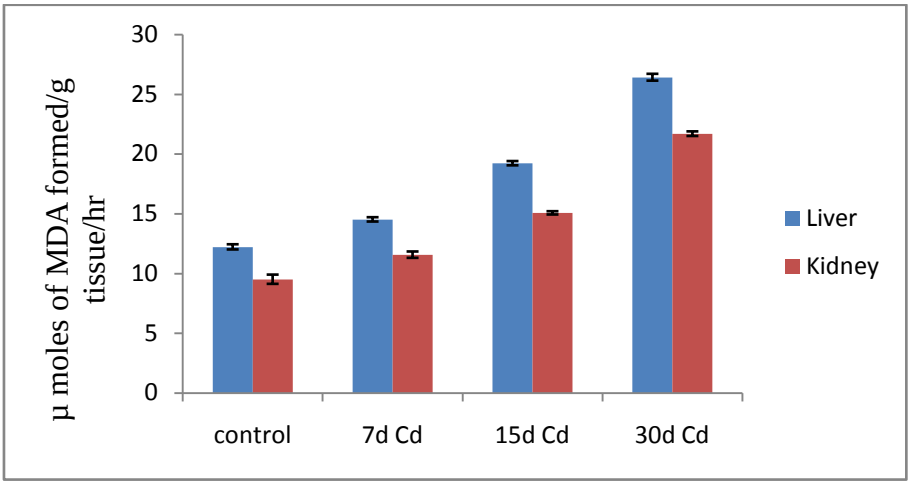

Fig. 1: The levels of LPO (µ moles of MDA formed / g tissue / hr) in liver and kidney of Cd exposed *Oreochromis mossambicus*.

LPO levels were gradually decreased after supplementation with Ca and /or Zn. Maximum reduction was found in liver (13.082 ± 0.170 µ moles of MDA formed / gm wet wt. of the tissue) under 30d Ca supplementation followed by combined supplementation of Ca and Zinc and Zn alone supplementation(Fig-2,3,4).

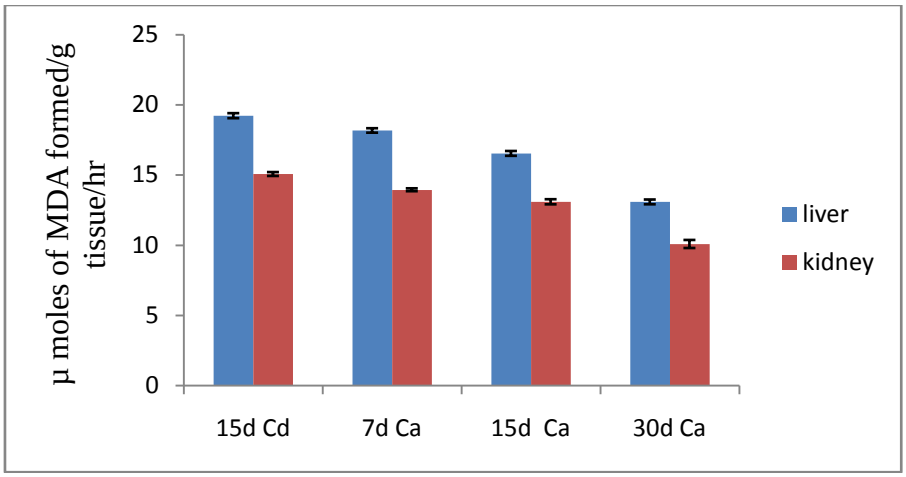

Fig. 2: The levels of LPO (µ moles of MDA formed / g tissue / hr) in liver and kidney of Cd exposed *Oreochromis mossambicus* after Ca supplementation.

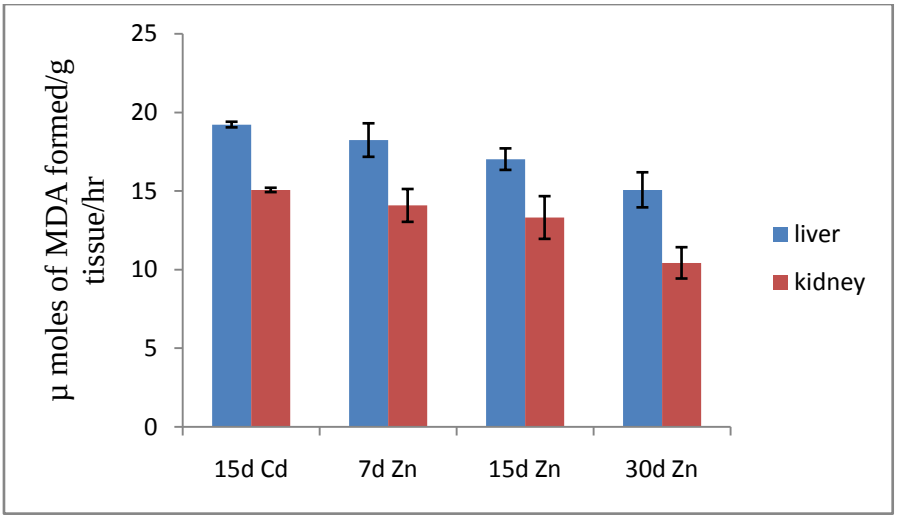

Fig. 3: The levels of LPO (µ moles of MDA formed / g tissue / hr) in liver and kidney of Cd exposed *Oreochromis mossambicus* after Zn supplementation.

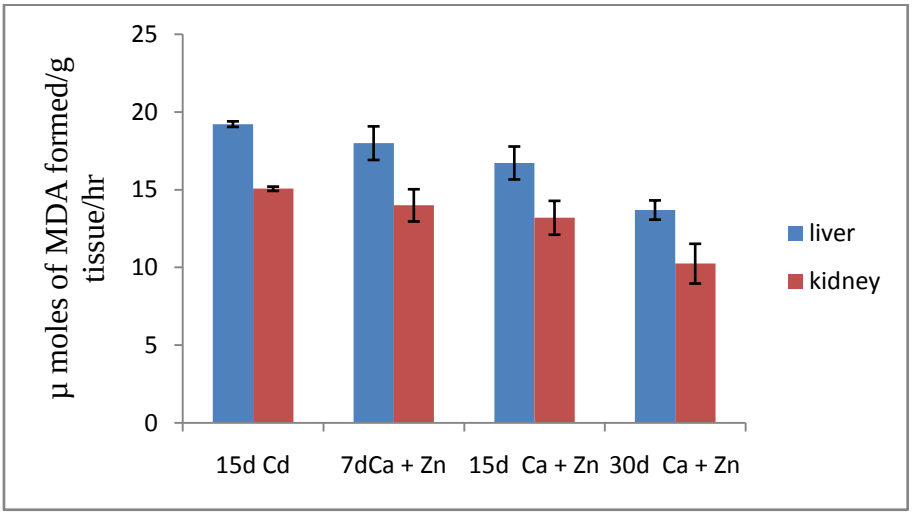

Fig. 4: The levels of LPO (µ moles of MDA formed / g tissue / hr) in liver and kidney of Cd exposed *Oreochromis mossambicus* after Ca and Zn supplementation.

SOD activity levels were assayed in the selected tissues like liver and kidney of fish exposed to sub lethal concentration of Cd. The activity levels were significantly decreased in both the test tissues of fish during 7, 15 and 30d Cd exposure. Maximum depletion of SOD activity was observed in 30d Cd exposed fish kidney (0.215 ± 0.215 superoxide anion reduced / mg protein / min) than liver (Fig-5).

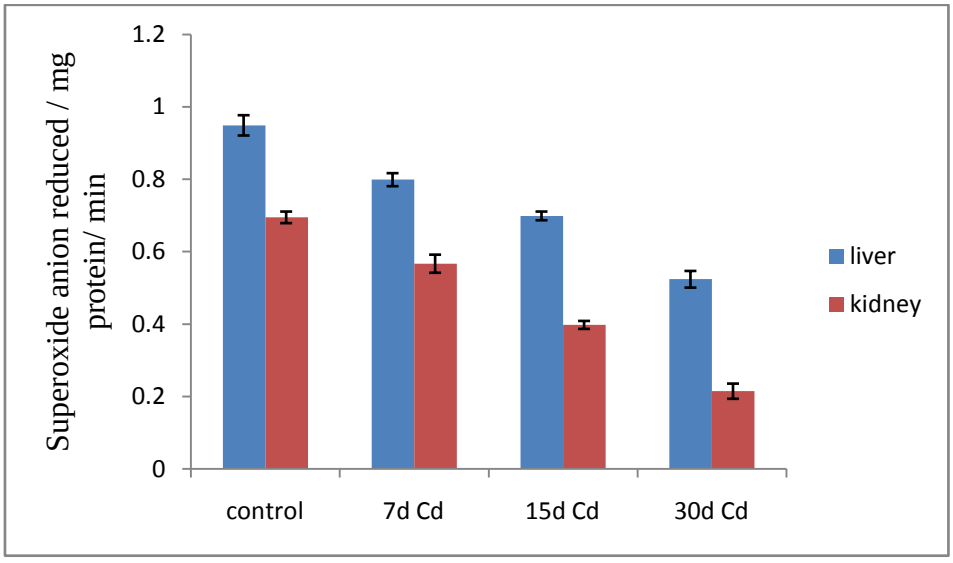

Fig. 5: SOD activity levels (superoxide anion reduced / mg protein / min) in liver and kidney of Cd exposed *Oreochromis mossambicus*.

When 15 d Cd exposed fish were supplemented with Ca and /or Zn as above said time intervals, the SOD activity levels were increased in both the test tissues (Table-2, 4). Maximum increase in SOD activity was observed in kidney tissue (0.628 ± 0.212superoxide anion reduced / mg protein / min) of fish supplemented with Zn for 30d followed by combined supplementation of Ca and Zn and Ca individual supplementation (Fig-6,7,8).

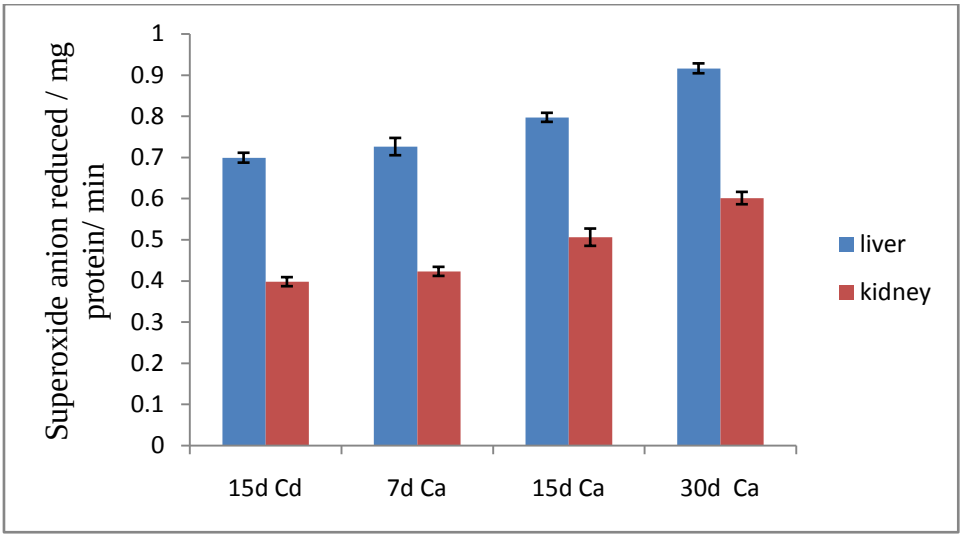

Fig. 6: SOD activity levels (superoxide anion reduced / mg protein / min) in liver and kidney of Cd exposed *Oreochromis mossambicus* after Ca supplementation.

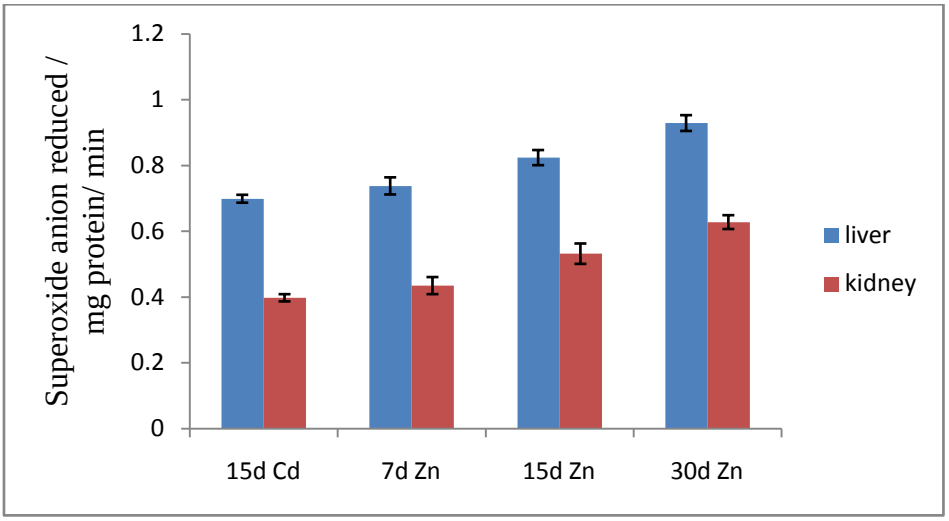

Fig. 7: SOD activity levels (superoxide anion reduced / mg protein / min) in liver and kidney of Cd exposed *Oreochromis mossambicus* after Zn supplementation.

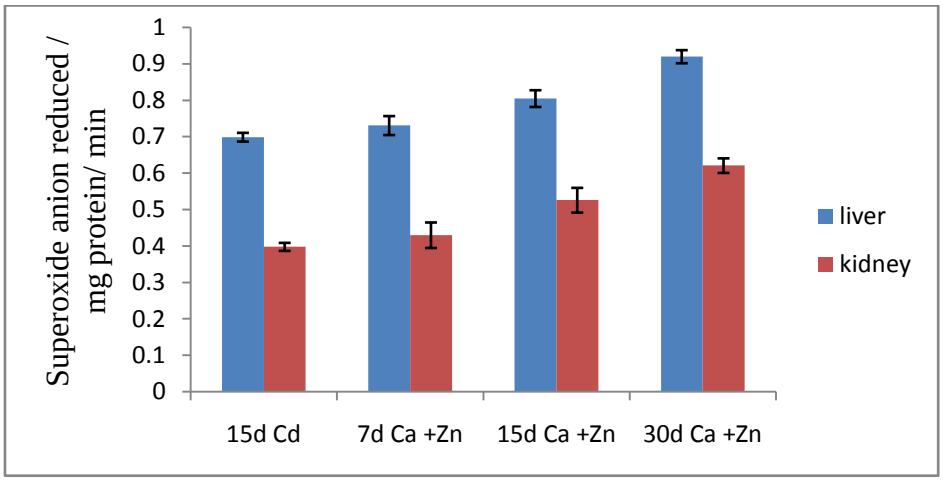

Fig. 8: SOD activity levels (superoxide anion reduced / mg protein / min) in liver and kidney of Cd exposed *Oreochromis mossambicus* after supplementation with Ca and Zn.

The specific activity levels of CAT were determined in the selected tissues of Cd exposed fish and as well as control (Table- 1, 3). CAT activity levels were significantly reduced in kidney (0.295 ± 0.300µ moles of H_2O_2 / mg protein / min) of 30d exposure fish (Fig-9).

CAT activity levels were significantly elevated in both the experimental tissues after supplementation with Ca and / or Zn. Maximum elevation in CAT activity was found in kidney (0.605 ± 0.261 µ moles of H_2O_2 / mg protein / min) subjected to 30d Ca supplementation than other modes of supplementation (Fig-10, 11,12).

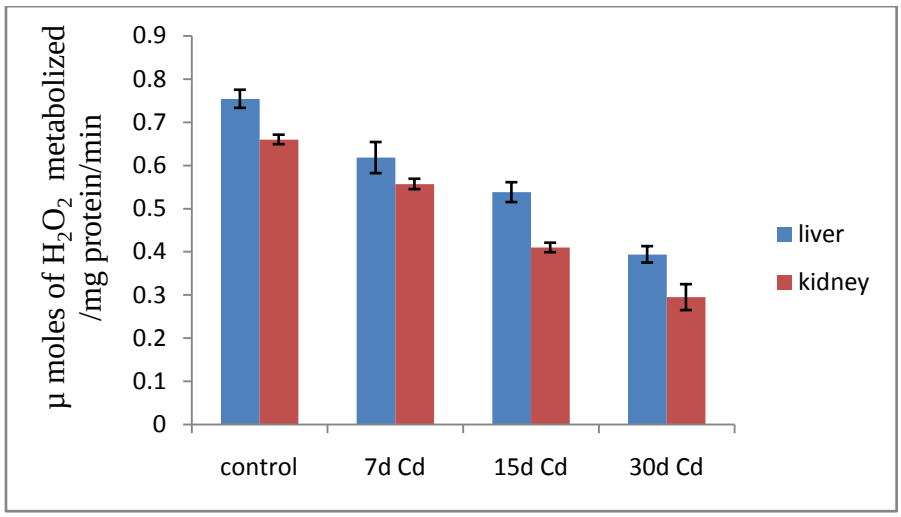

Fig. 9: CAT activity levels (µ moles of H_2O_2 metabolized/ mg protein / min) in liver and kidney of Cd exposed *Oreochromis mossambicus*.

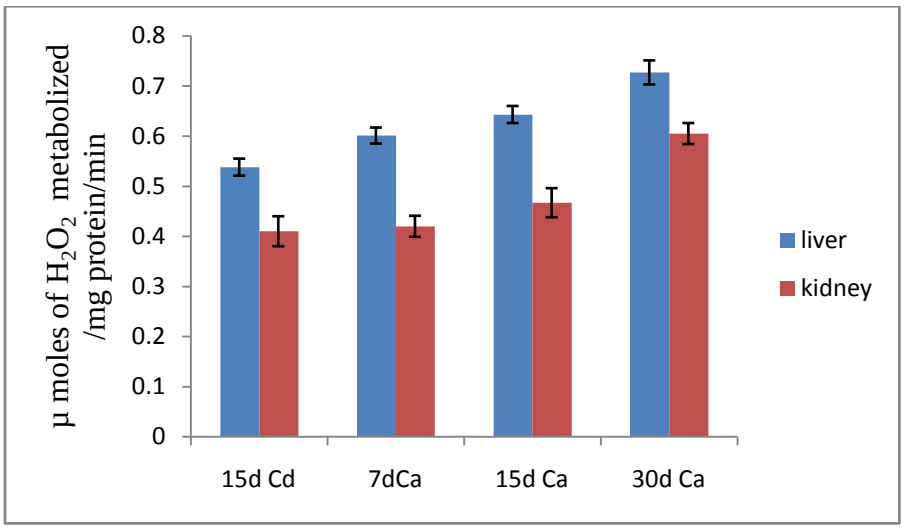

Fig. 10: CAT activity levels (µ moles of H_2O_2 metabolized/ mg protein / min) in liver and kidney of Cd exposed *Oreochromis mossambicus* after supplementation with Ca.

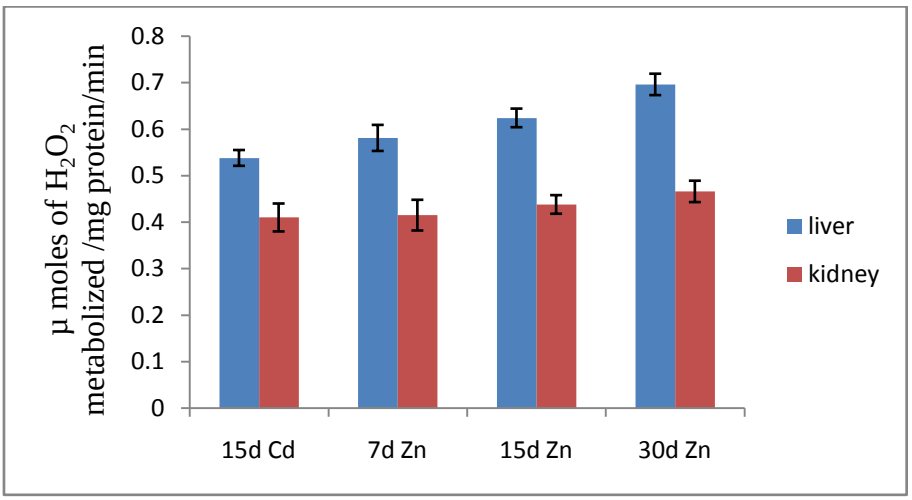

Fig. 11: CAT activity levels (µ moles of H_2O_2 metabolized/ mg protein / min) in liver and kidney of Cd exposed *Oreochromis mossambicus* after supplementation with Zn.

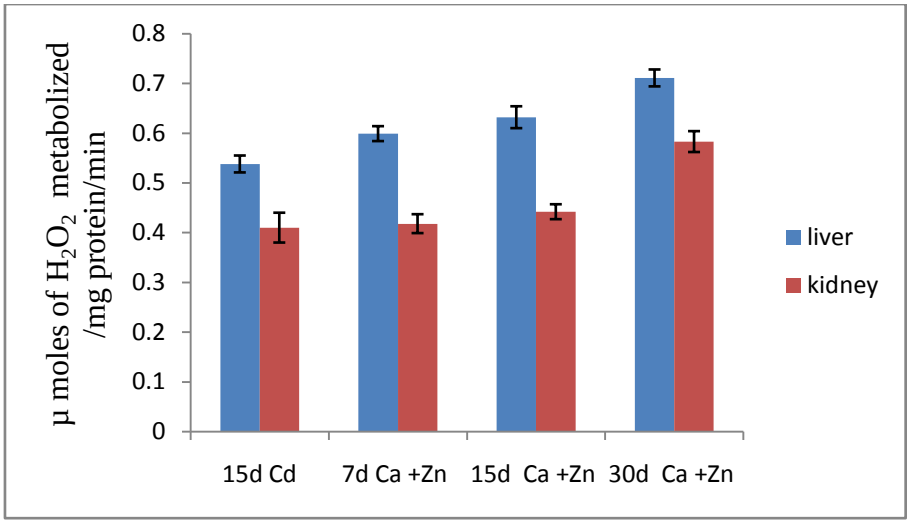

Fig. 12: CAT activity levels (μ moles of H_2O_2 metabolized/ mg protein / min) in liver and kidney of Cd exposed *Oreochromis mossambicus* after supplementation with both Ca and Zn.

DISCUSSION

The results of the present investigation revealed that Cd induces significant alterations in the levels of LPO and certain enzymatic antioxidant enzymes status in liver and kidney of teleostean fish *Oreochromis mossambicus* at all specific time intervals. These activities were progressively reversed after using trace element supplements like Ca and / or Zn. Cd may induce oxidative damage in different tissues by enhancing peroxidation of membrane lipids in tissues and altering the antioxidant systems of the cells. The peroxidative damage to the cell membrane may cause injury to cellular components due to the interaction of metal ions with the cell organelles.

The present study findings revealed that LPO was enhanced during Cd exposure, which may be due to interaction of Cd with membrane phospholipids and thus causing membrane disorganization and further fragility. The enhanced LPO in this study could also be due to inhibition on activity levels of antioxidants, which were more concern with defense against free radical induction due to Cd intoxication. Similar findings were observed in the liver of common carp (*Cyprinus carpio*) [22]. MDA elevation induced by Cd was also reported for different fish tissues[23].

Similar results were observed by several workers in different animals exposed to heavy metals. Wang and Wang[24] reported that Cd significantly increased the LPO level in the contaminated copepods after 12d of exposure, so the treated animals had encountered oxidative injury. Company[25] also demonstrated that Cd exposure (101.2 mg/ l) notably increase the LPO level in the vent mussel. Talas[26] reported that there was significant increase in the LPO level in liver tissue of rainbow trout exposed to Cd / Cr at the dose of 2ppm. In one study, Cd exposed fish showed increased LPO in gills of the calm *Ruditapes decussates* [27].

Ca and / or Zn supplementation significantly counteracted the enhancement of LPO caused by Cd. This finding is in harmony with the findings of Ng[28] who reported that elevated Ca protects against Cd induced toxicity in rainbow trout. However in our study supplementation of Ca and / or Zn reduced LPO significantly in all the test tissues. A lower level of LPO means a lower degree of membrane damage. So Ca and Zn might have alleviated the Cd-induced membrane damage and aids protect the cell.

The decrease in SOD activity could be due to its inhibition by the excess production of ROS as evidenced by LPO in the present study. Decreased SOD levels indicate the product of O_2 radicals increased by the lowered ability of the tissues that can scavenge free radicals. Therefore, the enhanced LPO in the liver, kidney and other tissues might result from the reduction of their SOD activity. These findings are in accordance with Han[29] on growing pigs. Casalino[30] proposed that Cd binds to the imidazole group of the His – 74 in SOD, which is vital for the breakdown of H_2O_2, thus causing its toxic effects and Cd probably interacting with metal moieties of SOD (Cu, Zn or Mn) and thus reducing its activity. Alternatively, Cd may alter the protein conformation by interacting with the enzyme, there by altering its functional activity[31]. The alterations in SOD activity may depend on several factors such as Cd dose, Cd exposure time, type of Cd administration and the state of the animal[32]. In this study, a significant reduction in SOD activity with the exposure period of both the test tissues was observed in Cd subjected animals. Similar observations have been reported in rainbow trout[26]. Oost[33] observed that a significant decrease in SOD activity in the liver tissue of rainbow trout exposed to 2 ppm heavy metal (Cd, Cr). When the test fish were supplemented with Ca and / or Zn there was a significant increase in SOD activity in both the experimental tissues. Similar findings were reported in Cd exposed rainbow trout[34] and rats[35] subjected to Ca and Zn supplementation.

In the present study the activity levels of CAT were significantly reduced in both the tissues of Cd exposed fish for 7, 15 and 30d (Table-1, 3). The decrease in CAT activity could be due to its inactivation by superoxide radical or due to decrease in the rate of the reaction as a result of the excess production of H_2O_2. Similar findings were observed in Cd exposed rainbow trout[36]. Vaglio and Landriscina[37] also reported that CAT activity levels were decrease in the fish *Sparus aurata* following *in vivo* exposure to Cd. According to Radhakrishnan[37], different tissues of fresh water fish, *Heteropneustes fossilis (Bloch)* exposed to Cd showed decrement CAT activity. Pratama yoga[39] suggests that Cd cause LPO through inhibition of the CAT activity. CAT is a manganese or heme containing enzyme, functions to rapidy dismutate H_2O_2 to water and oxygen. By inhibiting this enzyme activity, H_2O_2 production within the cell is increased and leads to the production of hydroxyl radical and subsequently results in the cellular damage via the metal catalyzed Haber-Weiss reaction. After supplementation with Ca and / or Zn, CAT activity levels were significantly increased in both the test tissues. Cd is known to decrease the trace element absorption in the body[5,40]. Hence the CAT activity levels were decreased during all the Cd exposure periods. However with trace element supplementation like Ca and / or Zn in our study may have a significant role in protecting the cells from Cd induced injury.

In the present study it is clear that LPO, SOD and CAT showed a trend towards normalcy in their activity levels in both the test tissues after supplementation with Ca and / or Zn to the Cd treated fish thereby indirectly suggesting certain therapeutic measures to Cd induced toxicity in vertebrates.

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