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METALLOTHIONEINS ROLE AGAINST CADMIUM TOXICITY IN ZINC AND CALCIUM SUPPLEMENTED *OREOCHROMIS MOSSAMBICUS (TILAPIA)*

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ABSTRACT

Present study is carried out to know the role of Metallothioneins (MT) in detoxification of cadmium (Cd) toxicity in fresh water teleost, *Oreochromis mossambicus* before and after supplementation with trace elements such as zinc (Zn) and calcium (Ca). After specific time periods, the fish were sacrificed and tissues like liver, kidney, gill, brain and muscle were isolated and used for metallothionein purification and quantification. Purified MT protein containing samples was subjected to SDS-PAGE. Clear visible bands were observed in the test tissues approximately at 6.5 KDa against a standard low range molecular weight protein marker. Further, MT protein levels were significantly elevated in the test tissues during Cd exposure and also after supplementation with Zn and / or Ca. Maximum MT protein synthesis was observed in 30d fish kidney under combined supplementation of both Zn and Ca. Thus, tissues that contain an excess amount of MT are resistant to Cd toxicity.

KEY WORDS: Cadmium, Trace elements, Metallothionein, *Tilapia*

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INTRODUCTION

Cells react to stressful environments with a broad range of diverse homeostatic responses. Among an array of responses, stress response proteins including metallothioneins (MTs) play an important role against metal induced stress. From a biochemical stand point, MT is a novel protein and has low molecular weight (6-7 kDa), Cysteine (Cys) – rich and stress response protein with a high affinity for divalent heavy metals such as Cd[1,2,3,4]. When MT binds to essential divalent metals (e.g. zinc and copper) it may serve as a metal reservoir for apoenzymes and zinc-finger transcription regulators[5,6]. MT protein that is induced by other divalent metal cations (e.g. mercury, cadmium etc.,) protects essential cellular functions[7] and enhances the survival of both cells and whole organisms that are exposed to toxic heavy metals. These MTs are ubiquitously present in a large variety of prokaryotic and eukaryotic species as well as in all mammalian organs and tissues examined so far both in animal and plant kingdom and are increasingly being demonstrated to play a vital role in metal homeostasis[8,9]. In mammals, four isoforms of MT have been reported (MT-I, II, III and IV). MT-I and II are found in the liver and kidney tissues. MT – III has been detected in mouse and human brain, and MT – IV has been found in certain stratified epithelia[10,11,12,13]. The biological function of MT is likely related to the physiologically relevant metals (Zn, Cu and Fe) that binds this protein. In mammals, among MT isoforms, MT-I is found to bind Zn and Fe under normal physiological conditions. Both Zn and Fe are essential trace elements in biology as components of a wide variety of metalloproteins and wealth of enzyme families[14,15]. Zn is known to be the most effective MT inducer and Fe is categorized as indirect MT inducer[16]. Fe binds significantly less tightly to MT than Zn[17]. The relative affinities of these metal ions to sulfur ligands are well established from inorganic thiolate complexes[18]. MT is primarily synthesized on free polysomes[19]. Based on their synthesis, MT protein has been often considered to function exclusively as an intracellular protein. While MT lacks the signal peptide sequences or other protein trafficking signals that would result in proteins entering the traditional secretory pathway, it is nevertheless detected in serum, urine, pancreatic secretions and other biological fluids as well as in branchoalveolar spaces, liver sinusoids and other extra cellular spaces. This pool of extracellular MT may originate from necrotic cell death that may accompany some forms of stress but it is also possible that MT may be selectively released from stressed cells by non-traditional secretory pathway[20]. Though, few studies were oriented towards MT and Cd[21,22,23], studies related to the interactions between Zn, Ca and Cd with respect to MT is poorly understood. Indeed, there is no sufficient work on MT with regard to supplementation of trace elements Zn and Ca in order to reduce the Cd induced toxicity in fish. Hence, the present work was carried out to determine whether the MT synthesis is helpful in reducing the Cd induced stress in fish under Cd exposure and also with supplementation of essential trace elements Zn and / or Ca.

MATERIALS AND METHODS

I. Chemicals

Cadmium as cadmium chloride ($CdCl_2$), zinc as zinc chloride ($ZnCl_2$) and calcium as calcium chloride ($CaCl_2$) were purchased from Merck (Dormstadt, Germany). The 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 for this study were of the highest purity.

II. Maintenance of animals (fish)

Fish *O. mossambicus* (*Tilapia*) weighing 10 ± 2 gm was collected from the local fresh water ponds and acclimatized to laboratory conditions for a week in separate troughs. The laboratory temperature was maintained at $28^0C \pm 2^0C$, relative humidity was 50 ± 20% and 12-h light-dark cycle. The fish were fed *ad libitum* with ground nut cake and water was renewed for every 24 hrs with routine changing of troughs leaving no fecal matter.

III. Experimental design

Fish were divided into two groups. First group served as control and other group as experimental. The experimental group was exposed to sub lethal concentration of $CdCl_2$ i.e., 5 ppm ($1/10^{th}$ of LC_{50} / 48 hrs) daily for 7, 15 and 30 days (d) time periods[24] (). Then 15d Cd exposed animals were subjected to Zn and Ca supplementation (i.e., 1 ppm) for again 7, 15 and 30d long sojourn[25]. After specific time periods fish were sacrificed and tissues like liver, kidney, gill, brain and muscle were isolated and used immediately for the MT protein purification and quantification studies.

IV.Metallothionein purification

The initial isolation of the MT protein from liver, kidney, gill, brain and muscle homogenates were carried out by following Fowler *et al.,*[26]. The clear supernatants thus obtained from test tissue homogenates were again subjected to purification process. Supernatant fractions of each tissue was applied to a column of Sephadex, G-75 (5 x 50 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.4). Further purification of MT protein was carried out by Ion exchange chromatography using DEAE-32CELLULOSE by following the method of Overnell and Coombs[27]. Purified MT protein samples were subjected to SDS–PAGE.

V. Quantification of MT

Purified MT protein quantification was performed by using Lowry *et al.,*[28] in the test tissue samples of control, Cd exposed as well as Zn and / or Ca supplementation to 15d Cd exposed fish.

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

Tissues such as liver, kidney, gill, brain and muscle were isolated from control and Cd exposed fish. The test tissues were subjected to purification and quantification of MT protein as described by Overnell and Coombs[27]. The supernatants thus obtained after centrifugation, were subjected to gel filtration by using Sephadex G-75 column and DEAE - Ion Exchange chromatography. The fractions were collected from the samples after performing Ion Exchange chromatography and were characterized by using SDS–PAGE. The characterization of MT was based on purification and quantification of MT protein from the tissue samples which were already standardized in our laboratory[29]. After staining and de-staining, the Gel electrograms were examined under Alpha Image Analyzer (Alpha Innotech) in liver, kidney, gill, brain and muscle of control and Cd exposed fish. The bands were visible and approximately with a molecular weight of 6.5 KDa in liver (Plate: 1,6,11 and 16), kidney (Plate: 2,7,12 and 17), gill (Plate: 3,8,13 and 18), brain (Plate: 4,9,14 and 19) and muscle (Plate: 5,10,15 and 20) of control, Cd exposed and Zn and / or Ca supplemented fish against a standard low range molecular weight protein marker (Cat. No. M 3546) obtained from Sigma Chemical Co. (St Louis, Mo, USA). The visibility of clear bands in all gel electrograms of test tissues at 6 - 7 KDa indicates the presence of MT, as the MT protein molecular weight was estimated approximately to be 6 -7 KDa by Overnell and Coombs[27] and Kagi *et al.*,[30] .

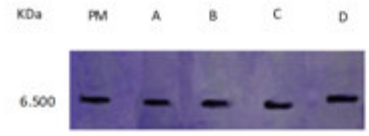


Plate – 1 Liver

PM: Protein Marker
A: Control liver
B: 7d Cd exposed liver
C: 15d Cd exposed liver
D: 30d Cd exposed liver

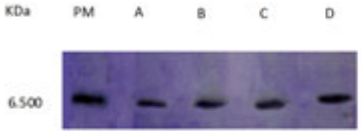


Plate – 2 Kidney

PM: Protein Marker
A: Control Kidney
B: 7d Cd exposed Kidney
C: 15d Cd exposed Kidney
D: 30d Cd exposed Kidney

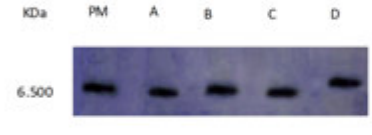


Plate – 3 Gill

PM: Protein Marker
A: Control Gill
B: 7d Cd exposed Gill
C: 15d Cd exposed Gill
D: 30d Cd exposed Gill

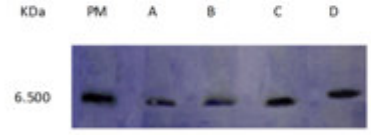


Plate – 4 Brain

PM: Protein Marker
A: Control Brain
B: 7d Cd exposed Brain
C: 15d Cd exposed Brain
D: 30d Cd exposed Brain

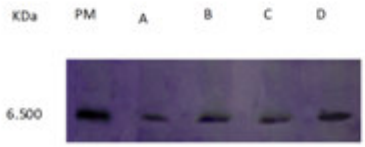
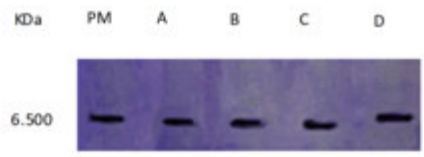


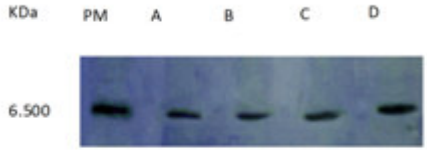
Plate – 5 Muscle

PM: Protein Marker
A: Control Muscle
B: 7d Cd exposed Muscle
C: 15d Cd exposed Muscle
D: 30d Cd exposed Muscle



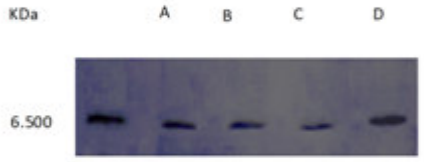
PM: Protein Marker
A: 15d Cd exposed liver
B: 7d Ca supplemented liver
C: 15d Ca supplemented liver
D: 30d Ca supplemented liver

Plate – 6 Liver



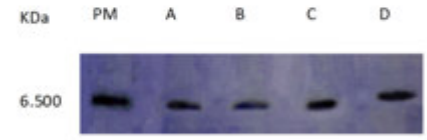
PM: Protein Marker
A: 15d Cd exposed Kidney
B: 7d Ca supplemented Kidney
C: 15d Ca supplemented Kidney
D: 30d Ca supplemented Kidney

Plate – 7 Kidney



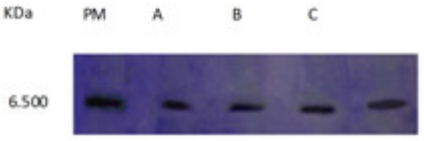
PM: Protein Marker
A: 15d Cd exposed Gill
B: 7d Ca supplemented Gill
C: 15d Ca supplemented Gill
D: 30d Ca supplemented Gill

Plate – 8 Gill



PM: Protein Marker
A: 15d Cd exposed Brain
B: 7d Ca supplemented Brain
C: 15d Ca supplemented Brain
D: 30d Ca supplemented Brain

Plate – 9 Brain



PM: Protein Marker
A: 15d Cd exposed Muscle
B: 7d Ca supplemented Muscle
C: 15d Ca supplemented Muscle
D: 30d Ca supplemented Muscle

Plate – 10 Muscle

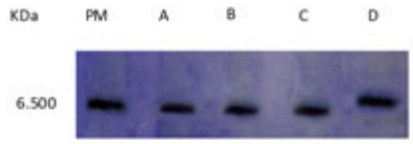


Plate – 11 Liver

PM: Protein Marker
A: 15d Cd exposed Liver
B: 7d Zn supplemented Liver
C: 15d Zn supplemented Liver
D: 30d Zn supplemented Liver

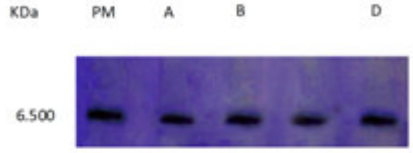


Plate – 12 Kidney

PM: Protein Marker
A: 15d Cd exposed Kidney
B: 7d Zn supplemented Kidney
C: 15d Zn supplemented Kidney
D: 30d Zn supplemented Kidney

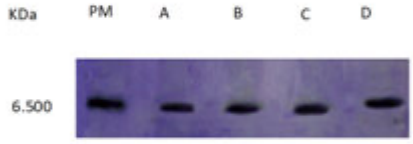


Plate – 13 Gill

PM: Protein Marker
A: 15d Cd exposed Gill
B: 7d Zn supplemented Gill
C: 15d Zn supplemented Gill
D: 30d Zn supplemented Gill

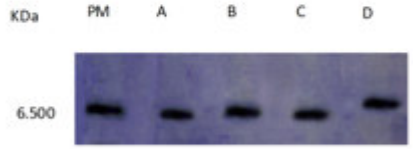


Plate – 14 Brain

PM: Protein Marker
A: 15d Cd exposed Brain
B: 7d Zn supplemented Brain
C: 15d Zn supplemented Brain
D: 30d Zn supplemented Brain

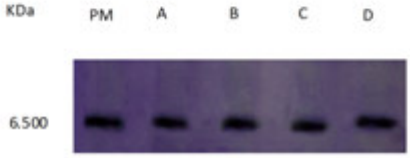
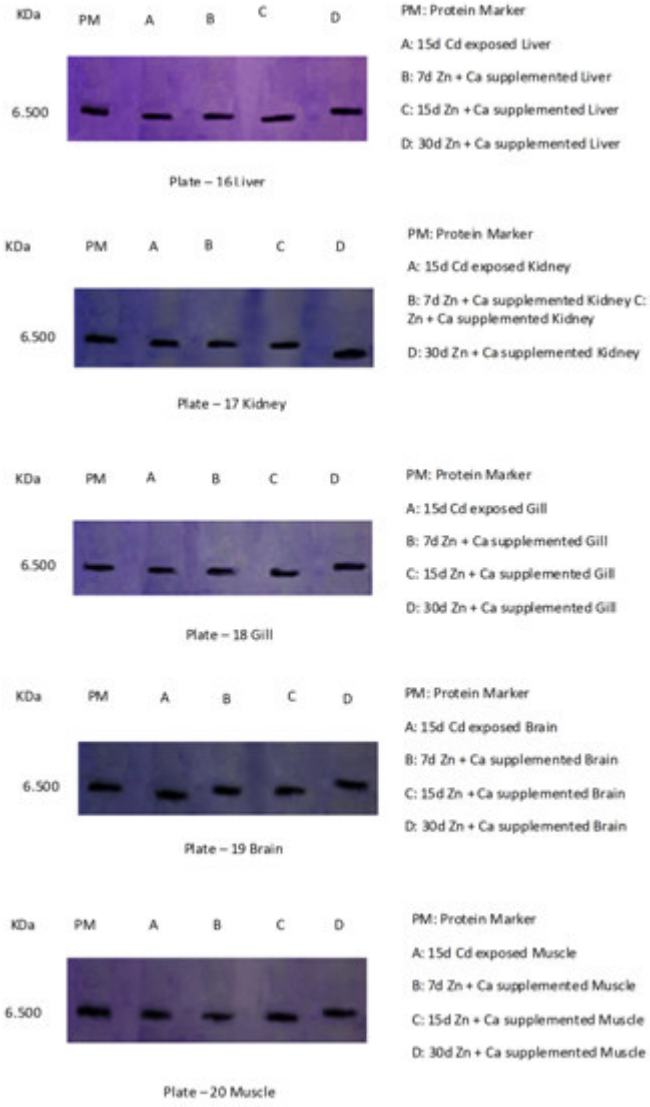


Plate – 15 Muscle

PM: Protein Marker
A: 15d Cd exposed Muscle
B: 7d Zn supplemented Muscle
C: 15d Zn supplemented Muscle
D: 30d Zn supplemented Muscle



The presence of bands in control and experimental animals around 6-7 KDa clearly indicates the expression of MT protein in the test tissues. After purification and characterization, the quantification of MT protein content was carried out in test tissues of fish. The synthesis of the metallo protein, MT in Cd exposed fish liver, kidney, gill, brain and muscle both before and after supplementation with Zn and / or Ca were tabulated (Table: 1-4). The significant increase in the synthesis of MT protein levels in Zn and / or Ca supplemented fish over Cd exposed fish as well as control are statistically significant at $p<0.05$.

Table 1

MT concentration (µ g / g wet weight of the tissue) levels in different tissues of O. mossambicus.

S.No.	Tissue	control	Cd exposure		
			7d	15d	30d
1.	Liver	1.147±0.151	2.514 ± 0.252	3.968 ± 0.113	6.553 ± 0.153
2.	Kidney	0.835±0.123	1.643 ± 0.106	3.365 ± 0.219	6.986 ± 0.184
3.	Gill	0.835±0.213	1.531 ± 0.309	3.812 ± 0.210	4.976 ± 0.125
4.	Brain	0.428±0.218	0.654 ± 0.218	1.646 ± 0.328	3.463 ± 0.126
5.	Muscle	0.572±0.122	0.973 ± 0.304	1.823 ± 0.162	3.957 ± 0.104

All values are expressed as Mean ± SD of 6 individual samples.
All values are significant at P<0.05 level.

Table 2
MT concentration (µ g / g wet weight of the tissue) levels in different tissues of O. mossambicus after Ca supplementation.

S.No.	Tissue	15d Cd	Ca supplementation		
			7d	15d	30d
1.	Liver	3.968 ± 0.113	4.156 ± 0.406	6.236 ± 0.389	9.896 ± 0.284
2.	Kidney	3.365 ± 0.219	4.854 ± 0.451	6.961 ± 0.213	8.428 ± 0.415
3.	Gill	3.812 ± 0.210	4.786 ± 0.399	6.616 ± 0.270	8.576 ± 0.155
4.	Brain	1.646 ± 0.328	2.361 ± 0.404	3.050 ± 0.136	4.464 ± 0.120
5.	Muscle	1.823 ± 0.162	2.612 ± 0.308	3.437 ± 0.238	5.684 ± 0.304

All values are expressed as Mean ± SD of 6 individual samples.
All values are significant at P<0.05 level.

Table 3
MT concentration (µ g / g wet weight of the tissue) levels in different tissues of O. mossambicus after Zn supplementation.

S.No.	Tissue	15d Cd	Zn supplementation		
			7d	15d	30d
1.	Liver	3.968 ± 0.113	4.756 ± 0.326	6.854 ± 0.390	9.185 ± 0.356
2.	Kidney	3.365 ± 0.219	5.155 ± 0.531	7.187 ± 0.345	10.396 ±0.468
3.	Gill	3.812 ± 0.210	5.154 ± 0.400	6.680 ± 0.480	8.992 ± 0.500
4.	Brain	1.646 ± 0.328	2.471 ± 0.605	3.323 ± 0.346	4.986 ± 0.432
5.	Muscle	1.823 ± 0.162	2.945 ± 0.336	3.864 ± 0.543	5.995 ± 0.653

All values are expressed as Mean ± SD of 6 individual samples.
All values are significant at P<0.05 level.

Table 4
MT concentration (µ g / g wet weight of the tissue) levels in different tissues of O. mossambicus after Zn + Ca supplementation.

S.No.	Tissue	15d Cd	Zn + Ca supplementation		
			7d	15d	30d
1.	Liver	3.968 ± 0.113	5.050 ± 0.561	7.100 ± 0.516	10.155 ±0.532
2.	Kidney	3.365 ± 0.219	5.756 ± 0.606	7.736 ± 0.494	10.680 ±0.492
3.	Gill	3.812 ± 0.210	5.453 ± 0.619	6.924 ± 0.540	9.576 ± 0.506
4.	Brain	1.646 ± 0.328	2.771 ± 0.534	3.876 ± 0.602	5.326 ± 0.448
5.	Muscle	1.823 ± 0.162	3.154 ± 0.488	4.260 ± 0.490	6.362 ± 0.636

All values are expressed as Mean ± SD of 6 individual samples.
All values are significant at P<0.05 level.

Results revealed a profound increase in MT levels in all the test tissues of Cd exposed fish during the time periods when compared to the controls. 30d Cd exposed fish kidney showed maximum synthesis of MT protein (6.986 ± 0.184µg / g wet weight of the tissue) than the other tissues (Fig-1). After supplementation with Zn and / or Ca to 15d exposed fish, the MT levels were highly elevated in all the test tissues (Fig. 2 - 4). Maximum MT protein synthesis was found in 30d fish kidney under the combined supplementation of Zn and Ca (10.680 ±0.492 µg / g wet weight of the tissue). Moderate increment in the synthesis of MT protein was found in 30d Zn supplemented fish kidney and liver (17.141 ± 0.363 µg / g wet weight of the tissue and 14.827 ± 0.313 µg / g wet weight of the tissue respectively). From the present investigation, it is clear that the MT protein synthesis was high in the fish tissues that received combined supplementation of Zn and Ca than the individual supplementation of either Zn or Ca to the Cd induced *O. mossambicus*.

Figure 1
MT concentration (µ g / g wet weight of the tissue) levels in different tissues of O. mossambicus.

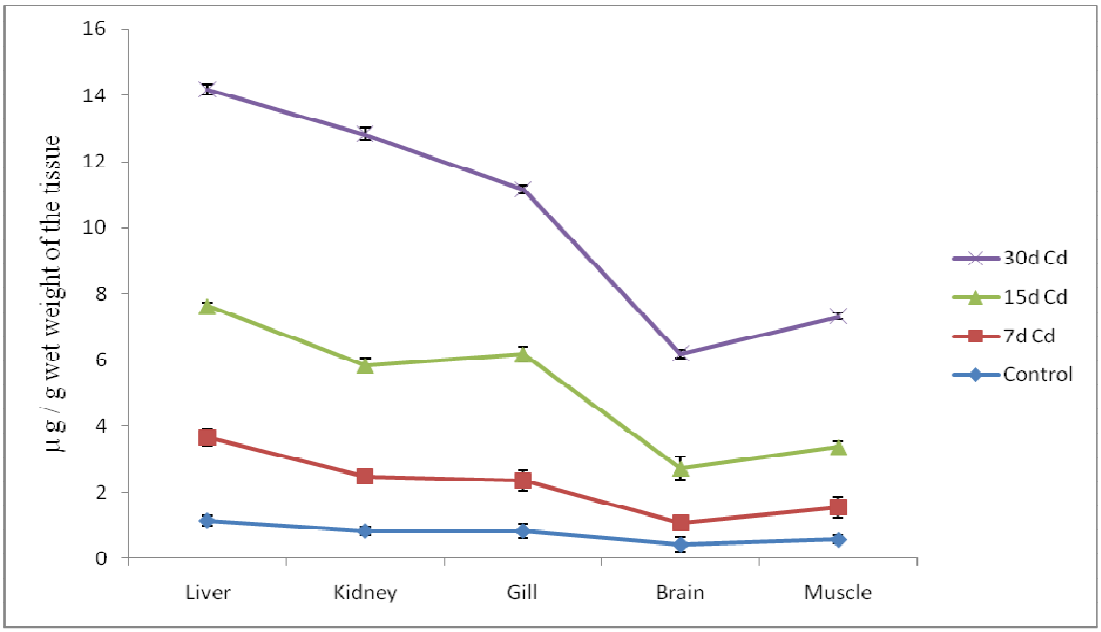


Figure 2
MT concentration (µ g / g wet weight of the tissue) levels in different tissues of O. mossambicus after Ca supplementation.

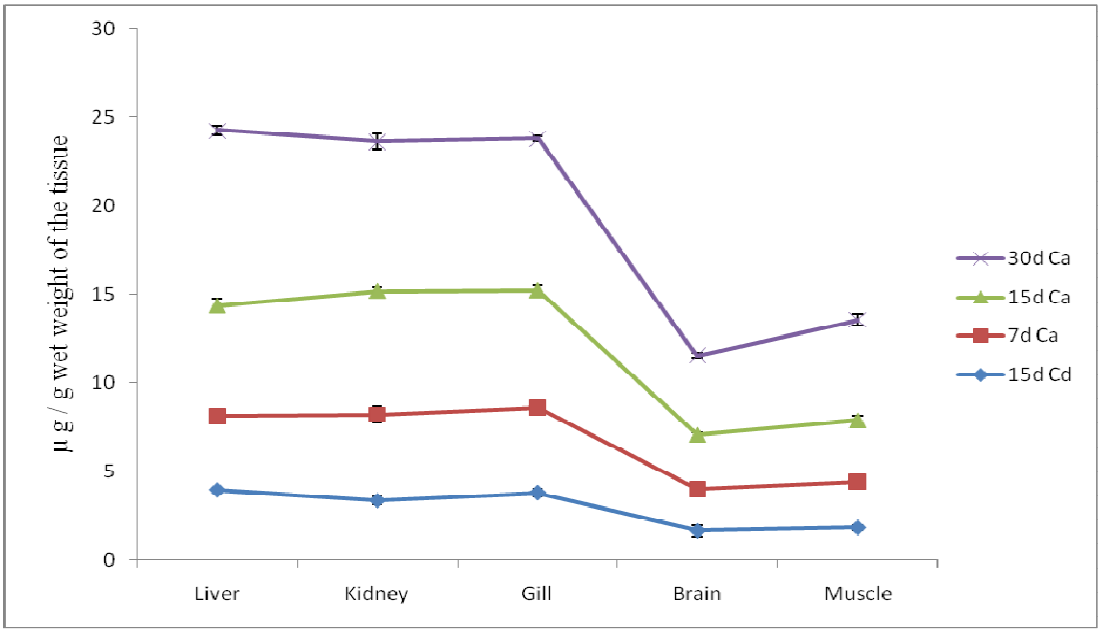


Figure 3
MT concentration (µ g / g wet weight of the tissue) levels in different tissues of O. mossambicus after Zn supplementation.

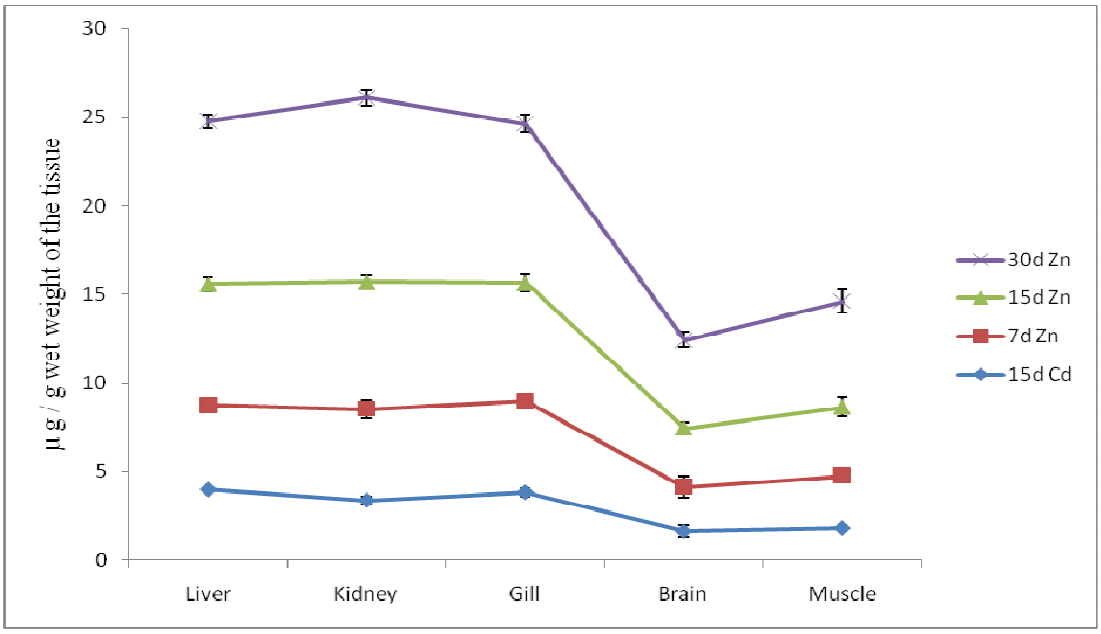
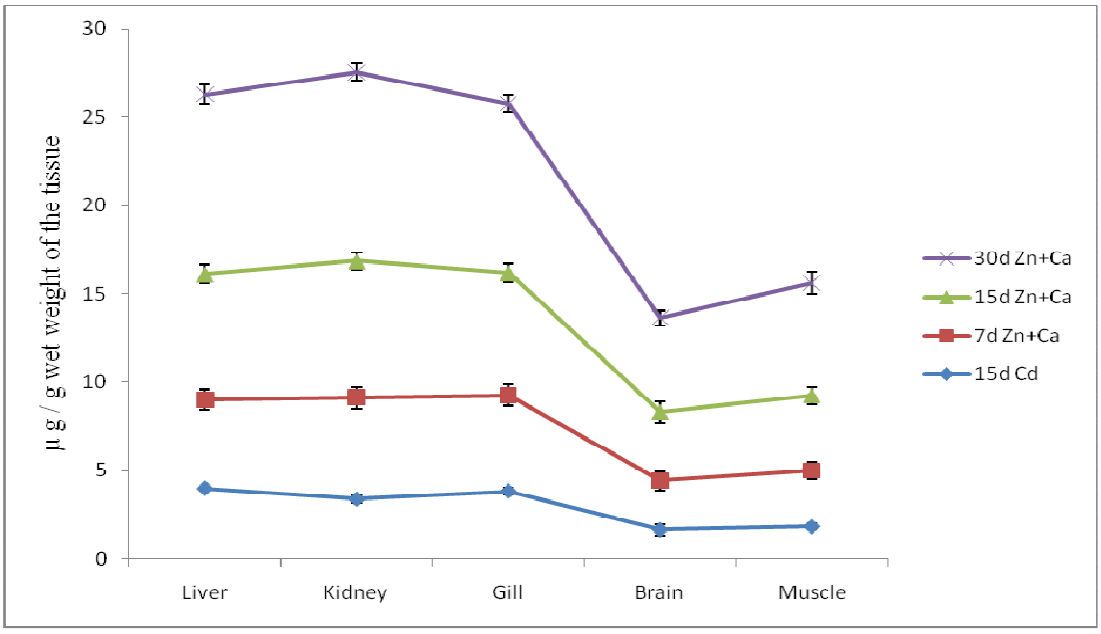


Figure 4
MT concentration (µ g / g wet weight of the tissue) levels in different tissues of O. mossambicus after Zn + Ca supplementation.



DISCUSSION

In recent years, Cd has been recognized as one of the most toxic environmental and industrial pollutant due to its ability to induce severe alterations in various organs and tissues. One of the tissue protection mechanisms against these toxic effects of Cd is MT synthesis[31,3]. MTs are cytoplasmic proteins that sequester certain divalent metal cations and are considered as primary cellular defense against the toxic transition metal Cd. MT protein levels were significantly increased in Cd exposed fish over controls in the present study which indicates that Cd exposure induces MT synthesis. Our results are in consonance with earlier reports of Haki *et al.*,[23] and Kukner *et al.*,[31]. Previous studies

of Lu *et al*., [32,33], Chaumont *et al*., [34], Chen *et al*., [13] and Kukner *et al*., [31] revealed that the MT synthesis was high in Cd exposed workers. To protect from heavy metal toxicity, organisms synthesizes more MT protein as it involves in the homeostasis of essential metal ions (Zn, Ca, Cu and Fe), detoxification of heavy metals (Hg and Cd), protection against oxidative damage through scavenging of ROS, cell proliferation and apoptosis[35,36,37,7,38,39,40]. High levels of MT protein synthesis were found in the test tissues of fish during supplementation with essential trace elements like Zn and Ca over Cd exposed as well as control fish. Increased synthesis of MT has been thought to produce antioxidant effect against ROS in Cd and other heavy metal intoxications[41,42]. More synthesis of MT protein was found in kidney tissue than other tissues in all the modes of supplementation with Zn and / or Ca as well as Cd exposure. A notable induction of MT protein under Cd over load in the kidney of the present study suggests that kidney might serve as a "Critical Organ" to Cd toxicity. The present data show low level of hepatic MT concentration than renal MT concentrations. Many reports suggested that ingestion of Cd is absorbed and transported to plasma where it binds with albumin to form Cd-albumin complex[43] via pulmonary or gastro intestinal route. Cd-albumin is absorbed predominantly by liver and Cd is released from the albumin in the liver tissue. The released Cd induces synthesis of MT in the liver and most of the Cd is bound to MT[44,45]. As liver is the first site of Cd bioaccumulation, where Cd binds with MT[46], the Cd-MT complex transported to kidney tissue might have caused MT increase in the renal tissue. It is believed that MT plays an important role in Zn metabolism and is popularly known as a reservoir of Zn. Zn-MT rescues the function of Cd - substituted tramtrack, a zinc finger transcription factor[47,48,49,50]. When Cd displaces Zn in tramtrack, this protein loses DNA binding activity. Incubating Zn - MT with Cd tramtrack *in vitro* allows the exchange of Cd and Zn, with the transcription factor regaining its DNA binding activity. Hence, Zn-MT may rescue zinc finger proteins from inactivation by other metals. Ca is another essential trace element that plays an important role in MT synthesis. It is an essential nutrient to almost all organisms[51,52,53] and plays an essential role in biological processes. Ca induces MT either indirectly or by way of antioxidant response elements[54,55]. In the present study, the supplemented Ca influenced the expression of MT in all the test tissues of fish under experimentation. The mechanism of MT protein induction by Ca is not elucidated. Quantification of MT protein revealed that the kidney tissue showed more expression of MT than the liver tissue under Zn and / or Ca supplementation at all the time periods of experimentation. Yasutake and Hirayama[56] reported that the supplementary Zn and / or Ca enhances MT turnover in the kidney than other tissues, although the mechanisms of such processes are not understood at present. However our studies suggest that the increase of MT protein level in all the test tissues is probably mediated by the differential expression of MT gene. Interestingly, supplemented Zn and / or Ca was found to elevate the MT protein expression in the test tissues of Cd exposed fish, providing further evidence of the ameliorative effects of Zn and / or Ca supplementation against Cd induced stress response in *O. mossambicus*. It is well known that Zn and / or Ca provides protection against Cd induced alterations through the induction of MT either directly or indirectly[57,58,2], activation of antioxidant defense system and decreases the ROS generation[59,60,61,62,63].

CONCLUSION

From the MT studies, it is clear that when Cd exposed fish were subjected to Zn and / or Ca supplementation, MT protein provided protection against Cd induced toxicity in the test tissues. Based on the overall discussion it may be concluded that the combined supplementation of Zn and Ca together was more effective in the MT protein synthesis as well as in reducing the Cd body burden in the tissues of *O. mossambicus*.

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