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Research Article

METALLOTHIONEIN INDUCTION IN FRESH WATER CATFISH *CLARIAS GARIEPINUS* ON EXPOSURE TO CADMIUM

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ABSTRACT

Objective: Cadmium (Cd) is a ubiquitous trace metal, biochemically classified as a nonessential element. It occurs naturally in the aquatic environment and is released as a result of anthropogenic activities and natural processes. Metallothionein (MT) has been proposed as a specific biochemical probe for heavy metals exposure in aquatic organisms. The objectives of this study were to determine the Cd levels and MT induction in liver, kidney, brain and gill of *Clarias gariepinus* during acute Cd exposure, to study of the relationship between tissue-specific Cd accumulation and MT induction.

Methods: Cd accumulation and MT induction levels was determined according to the methods of Ma *et al*., 2007. Cd concentration was determined using an atomic absorption spectrophotometer (Perkin Elmer Optima-5300 DV).). Cd concentration was expressed as μg g^{-1} wet weight tissue Cd levels were measured in the tissues of liver, kidney, gill and brain in *C. gariepinus*.

Results: The Cd levels increased significantly in all tissues in the following order: Liver > Kidney > Gill > Brain. The accumulation of Cd levels in all tissues is distinct by time-dependent and dose-dependent. In gills, Cd accumulation levels gradually increased rapidly to reach a peak during a period of 48 hrs and then declined gradually. The MT induction levels were found in the following order in the tissues: liver > kidney >gill> brain. A positive correlation was shown between MT induction and Cd accumulation.

Conclusion: In conclusion, the results indicated that Cd exposure clearly resulted in MT induction and hence MT levels can be considered as a biomarker for acute waterborne Cd pollution. The liver and kidney Cd accumulation and MT induction levels was found to be higher than gill and brain. These results suggest that MT played an important role in liver to deal with high quantities of Cd. Cd accumulation in the tissues showed a positive correlation with MT induction in all the tissues studied.

Keywords: Cadmium; Metallothionein; *Clarias gariepinus*; Liver; Kidney; Gill; Brain; AAS

INTRODUCTION

Cadmium is a ubiquitous trace metal, biochemically classified as a nonessential element. It occurs naturally in the aquatic environment and is released as a result of anthropogenic activities and natural processes [1]. In recent years, Cd has become a problem of higher magnitude because of the toxic nature of the pollutant in seas, rivers and estuaries waters [2]. Cd is considered as a hazardous environment pollutant widely distributed in nature that has a broad spectrum of toxic effects in mammalian tissues causing nephro-toxicity, hypertension and osteomalacia [3, 4]. In India, with the rapid industrialization, industrial waste discharge, and mining, has contributed to widespread Cd contamination, Aquatic organisms are exposed to elevated levels of heavy metals [5]. Cd does not break down in the environment, and can bio-accumulate for many years after exposure to low levels of this metal. Fish may absorb metal directly from contaminated water or indirectly from feeding on living organisms in the contaminated water [6]. Several studies show Cd bioaccumulation in different fish tissues viz., skin, gill, muscle, brain, liver, kidney and intestine [7]. The toxic pollutant affects water quality, feeding, and swimming behavior of fish, delays the hatching and maturation period [8].

Gills are the major entry site of metals and act as a transient store for accumulated metals [9]. Therefore, heavy metals can be bio-accumulated and biomagnified via the food chain and finally assimilated by human consumers resulting in health risks [10]. Fishes are major part of the human diet due to high protein content, low saturated fat and sufficient omega fatty acids which are known to support good health therefore, various studies have been underway worldwide on the contamination of different fish species by heavy metals Fishes have been widely used as bio- Indicators for heavy metals [11]. MT's are low-molecular-weight (approximately 6000 to 7,000 Da) cysteine-rich, metal-binding proteins that are found in microorganisms, plants, and animals [12, 13]. MT was first isolated from horse kidney [12]. MT's are widely expressed in organisms; such as eukaryotes and are responsible for essential metal metabolism and heavy metal detoxification [14]. Most of MT-related studies have focused on the potential role of MT's as a specific biomarker for heavy metal exposure [15]; Cd is an ubiquitous toxicant which has been recognized as one of the most deleterious heavy metal [16]. Exposure of fish to very low concentrations of this metal may lead to an increased body concentration that can result in several toxic effects including tissue damages, vertebral alterations, and respiratory changes and ultimately death [17].

The objectives of this study were (1). to determine the Cd levels in liver, kidney, brain and gill of *C. gariepinus* during acute Cd exposure, (2) to investigate the induction of MT in sample tissues, (3) to study of the relationship between tissue-specific Cd accumulation and MT induction.

MATERIALS AND METHODS

The Fresh water fish *C. gariepinus* were collected from Poondi fish farm in Thiruvallur district, Tamil Nadu, India. The fishes were acclimatized in the laboratory in a stone tank for 7 days at room temperature (30 ±2^{0}C). For the experiment, analytical grades, aqueous solution $CdCl_2$ were used. The solution was prepared in distilled water for acute toxicity studies. The various concentration of $CdCl_2$ such as 0 (control), 5.0 ppm, 10.0 ppm and 20.0 ppm exposed to a period of 24, 48 and 72 hrs. Eighty fishes with similar size, length, weight about (approx. 20 ± 30 g) were selected and divided into four groups. Each group has 20 fishes. The fishes were fed every day with commercial fish pellet. Uneaten foods were removed at the end of the day. One group was kept as a control, the other three groups were transferred to aquaria (100L) containing 0, 5.0 mg L^{-1}, 10.0 mg L^{-1} and 20.0 mg L^{-1} of $CdCl_2$, respectively. Aquaria were checked every day and dead fishes were removed. The test solutions were renewed daily to maintain the waterborne Cd concentration. In our biological experiments, the influence of cadmium chloride in water on levels of Cd in liver, kidney, gill and brain and the levels of MT in the tissues were studied. The content of

other heavy metals were also analyzed in test water and found to be below detectable limits (BDL) to rule out their role.

Sample Preparation

For each acute Cd concentration, five fishes were randomly removed from the tanks after 24, 48 and 72 hrs. The tissues were handled with plastic forceps and kept in plastic homogenizing tubes. The hepatosomatic index (HIS) for each fish was estimated (HIS =liver weight / body weight X 100). For each fish, approximately half of the sampling tissues 0.5g of liver, kidney, gill and brain were kept aside 0.25 g for MT measurements and the remaining tissue (0.25 g) for Cd measurement.

Table 1: Morphological characteristics of *C. gariepinus* after exposure to Cd for a period of 24, 48 and 72 hrs (n = 5), (Mean ± SD)

		Concentrations of Cd mg / L		
Items	**Control**	**5.0 mg L^{-1} Cd**	**10.0 mg L^{-1} Cd**	**20.0 mg L^{-1} Cd**
Body weight (g)	24.46 ±1.74	25.53±1.27	22.71 ±1.01	23.71±1.86
Liver weight (g)	0.58 ±0.29	0.61±0.51	0.55±0.41	0.57±0.55
Kidney weight (g)	0.16 ±0.42	0.18±0.32	0.13±0.64	0.14±0.39
Brain weight (g)	0.25±0.53	0.29±0.38	0.24 ±0.31	0.25±0.36
Gill weight (g)	0.47 ±0.27	0.49±0.22	0.43 ±0.33	0.46±0.28
HIS 100% (Liver)	2.37 ± 0.21	2.39±0.36	2.42 ±0.18	2.40±0.27

Cd Determination

Cd was determined according to the methods of Ma *et al*., 2007 [18]. The sampling tissues (0.25g) were freshly weighed and cut into small pieces, and dried in an oven at 80ºC for about 48 h. Then tissues were digested in 10 ml; HNO_3 and 5 ml H_2O_2 over a hot plate at about 120ºC. The metal (Cd) content of the fractions was measured by atomic absorption spectrometer (Perkin Elmer optima 5300 DV). Cd concentration was expressed as µg g^{-1} wet weight tissue Cd levels were measured in the tissues of liver, kidney, gill and brain.

MT Quantification

MT was determined according to the methods of Ma *et al*., 2007 [18]. Sampling tissues were freshly weighed 0.25g and placed in a homogenizing tube kept on ice, then gently homogenized in 4:1 (v/w) 0.01 M Tris-HCl (pH 8.0) buffer with a glass homogenizer and teflon pestle. The homogenization buffer also contained 0.1 mM phenylmethylsulphonyl fluoride (PMSF) and 0.1 mM dithiothreitol (DTT). The homogenate was centrifuged at 16 000 X g for 30 min at 4ºC, and the supernatant was heated for 2 min in a boiling water bath (100ºC). The heated sample was centrifuged at 10 000 X g for 10 min to remove precipitated proteins. Volumes of 0.1 ml Cd solution (500 µg L^{-1} as $CdCl_2$) were mixed with 0.5 ml of sample (heat-denatured supernatant) and incubated at room temperature for 10 min to saturate the metal binding sites of MT. 0.5 ml of a 2% (w/v) Bovine hemoglobin (Sigma Chemical) was then added and incubated at room temperature for 10 min. The hemoglobin was denatured in a water bath (100 ºC) for 2 min, cooled in ice for 3 min, and centrifuged at 10 000 x g for 15 min. The denatured proteins, except for MT which is heat stable, were removed by centrifugation. Steps from the addition of the bovine hemoglobin until centrifugation were repeated three times. The amount of Cd ions in the final supernatant was proportional to the amount of MT present. The concentration of Cd in the supernatant was determined using an atomic absorption spectrophotometer (Perkin Elmer Optima-5300 DV).

The MT concentration was calculated by the following equation:
MT Conc. (µg g^{-1} w wt) = Cd Conc. (µg g^{-1} w wt)/112.4/6 × 6000

According to Pedersen *et al*., 1994 [19], 1 mol fish MT was bound to 6 mol metal ions and the fish MT average molecular weight was assumed to be 6000 Da. MT concentration was expressed as µg g^{-1} wet weight.

Ratio of Actual Cd to Theoretical Maximum Cd-MT

Calculations for the Cd-binding potential of Liver, Kidney, Brain and Gill of the fish were done. This is the ratio of actual tissue Cd concentration to theoretical maximum metal bound by MT. The ratio was calculated using the following equation.

The ratio = Cd Conc. (µmol g^{-1}w wt) / MT Conc. (µmol g^{-1}w wt) / 6

A ratio of 6 mol Cd/mol MT was taken for the fishes MT. If the ratio exceeds 1, some Cd is not bound to the MT.

Statistical Analysis

Statistical analysis of data was carried out using Graphpad Prism Version 5.0. The values are reported as mean ± SD. One-way analysis of variance was utilized to test the differences between the control and Cd-exposed of each sampling. The data of different hrs of sampling were compared by ANOVA unifactorial analysis was used to test the differences between the control and Cd-exposed groups. Regression analysis was used to studies the correlation between MT levels and Cd accumulation in different tissues.

RESULTS

Cadmium Accumulation

Cd concentrations were measured in Liver, kidney, gills and brain of fish tissues. The Cd levels increased significantly in all tissues in the following order: Liver > Kidney > Gill > Brain. The accumulation of Cd levels in all tissues is distinct by time-dependent and dose-dependent. The highest Cd concentration of 54.11 ± 6.21 µg g^{-1} w wt were observed in the liver during 72 hrs of 20.0 mg L^{-1} , after during 72 hrs of Cd exposure, the kidney Cd level were showed 29.52 ± 0.19 µg g^{-1} w wt treated with 20.0 mg L^{-1} of $CdCl_2$. During 48 hrs of Cd exposure, the gill Cd level were showed 14.33 ± 0.25 µg g^{-1} w wt treated with 20.0 mg L^{-1} of $CdCl_2$. During 72 hrs of Cd exposure, the brain Cd level were showed 9.76 ± 0.58 µg g^{-1} w wt treated with 20.0 mg L^{-1} of $CdCl_2$. In gills, Cd accumulation levels gradually increased rapidly to reach a peak during a period of 48 hrs and then declined gradually. The brain showed the lowest Cd concentration than all other tissues. The Cd concentration in *C. gariepinus* tissues such as liver, kidney, gill and brain at concentration of 0, 5.0, 10.0, 20.0 ppm of Cd for periods of 24, 48, 72 hrs were measured and illustrated in **(fig.1)** The data was statistically tested and the values were found to be statistically significant at $P < 0.05$.

MT Induction

MT induction was measured in fish liver, kidney, gills and brain. The concentrations of MT are illustrated in **(fig.2)**. MT levels in all tissues of the fishes after acute exposure to $CdCl_2$ were measured. The MT levels were found in the following order in the tissues: liver > kidney >gill> brain. The highest MT concentrations were observed in the liver during 72 hrs of 20.0 mg L^{-1} Cd exposure (137.13 ± 8.56 µg g^{-1} w wt), followed by the kidney during 72 hrs of 20.0 mg L^{-1} Cd exposure (85.48 ± 2.38 µg g^{-1} w wt), the gill during 48 hrs of 20.0 mg L^{-1} Cd exposure (59.28 ± 4.23 µg g^{-1} w wt), and the brain during 72 hrs of 20.0 mg L^{-1} Cd exposure (33.94 ± 1.24 µg g^{-1} w wt) , (n = 5). The MT concentrations of control tissues were in the following order: Liver (6.76 ± 1.59 µg g^{-1} w wt) > kidney (4.39 ± 1.35 µg g^{-1} w wt) > gill (3.71 ± 1.01 µg g^{-1} w wt) > brain (1.72 ± 0.37 µg g^{-1} w wt). After acute exposure to Cd, MT levels in all the tissues gradually increased with a similar pattern. But the gills MT induction levels gradually increased rapidly to reach the highest peak during in the period of 48 hrs and then declined gradually.

In all controls Cd levels were found to be extremely low and hence their influence of MT induction can be considered absent or negligible.

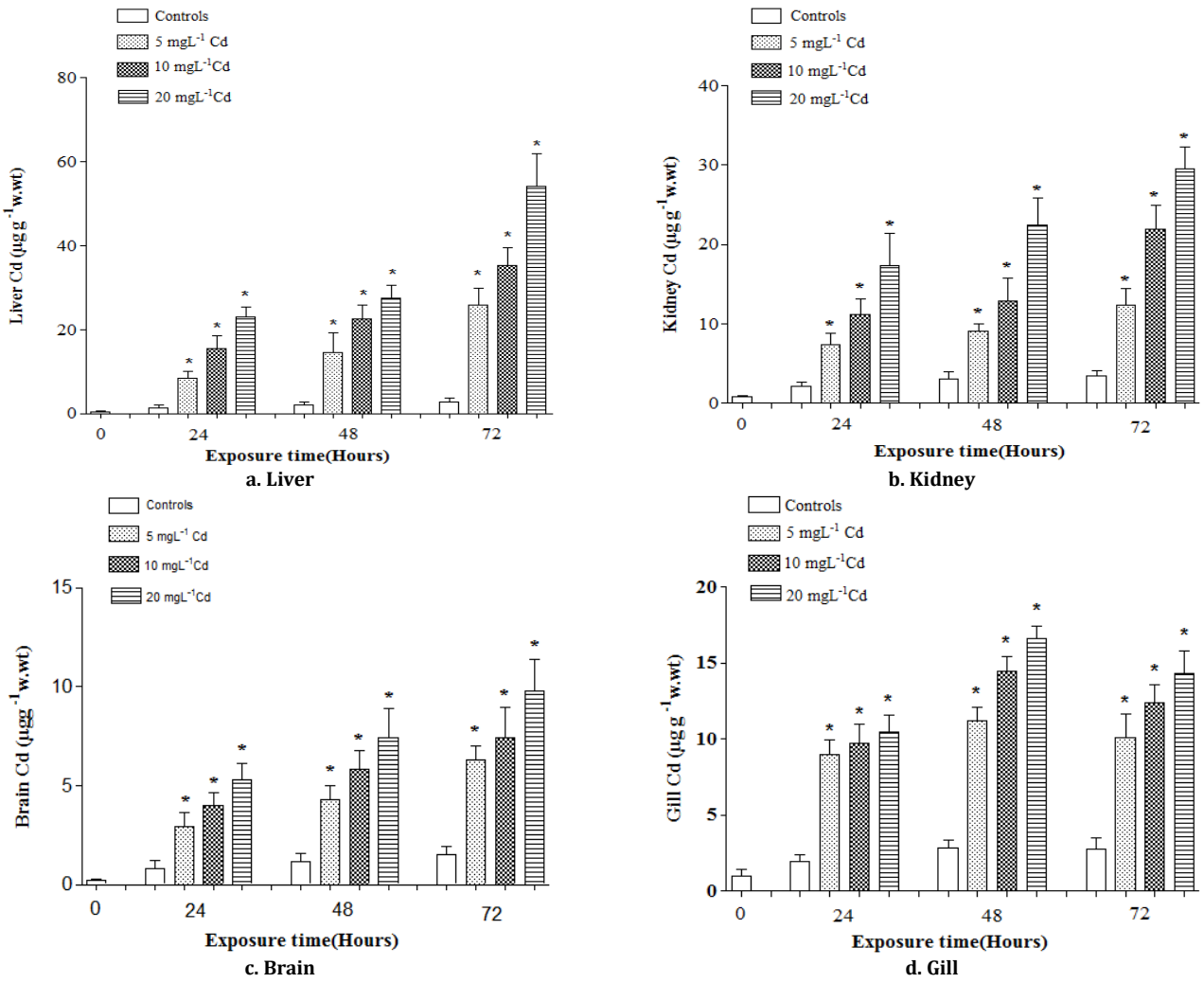
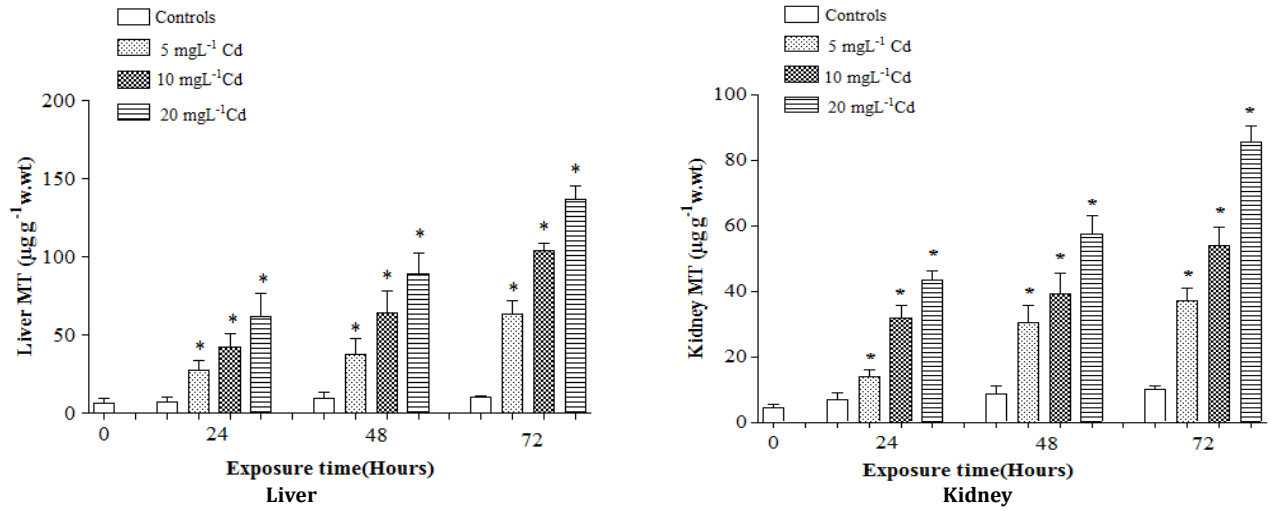
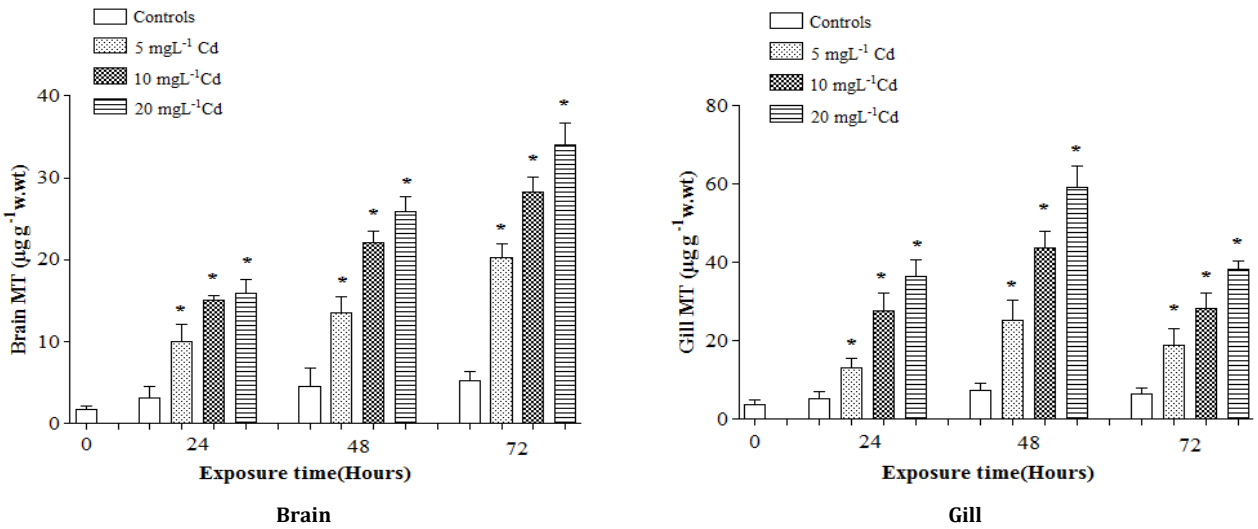


Fig. 1: Accumulation of Cd in liver, kidney, gill and brain of *C. gariepinus* exposed to 0 (Control), 5.0, 10.0 and 20.0 ppm and the periods of 24, 48 and 72 hrs. The results were presented as Mean ± SD, (n=5). Statistical comparisons were made against Control fish at each sampling day. (* The values are statistically significant at P < 0.05)

Correlation between MT induction and Cd accumulation

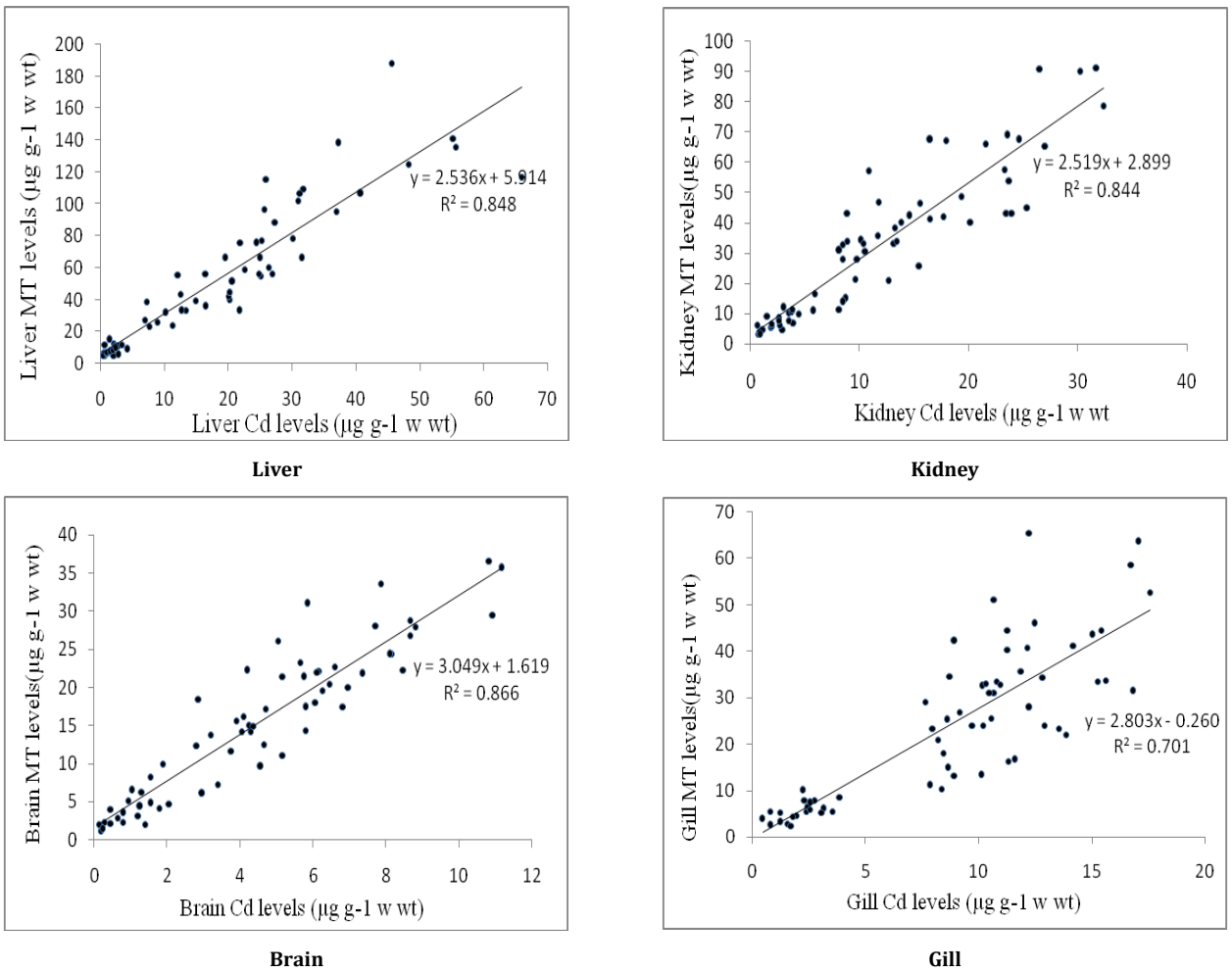
Fig. 3: Shows the relationship between Cd accumulation and MT induction in liver, kidney, brain and gill. A positive correlation was shown between MT induction and Cd accumulation. MT concentrations increased linearly with increasing Cd concentrations and may be described by the following regression equations: [MT] = 2.536 [Cd] + 5.014 (R^2 = 0.848, p<0.05) for liver and [MT] = 2.519 [Cd] + 2.899 (R^2 = 0.844, p<0.05) for kidney and [MT] = 3.049 [Cd] + 1.619 (R^2 = 0.866, p<0.05) for brain and [MT] = 2.803 [Cd] + -0.260 (R^2 = 0.701, p<0.05) for gill.





Brain **Gill**

Fig. 2: Induction of MT concentrations in liver, kidney, gill and brain of *C. gariepinus* exposed to 0 (control), 5.0, 10.0, 20.0 ppm and the periods of 24, 48 and 72 hrs. The results were presented as Mean ± SD, (n=5). Statistical comparisons were made against Control fish at each sampling day. (*The values are statistically significant at P < 0.05)



Liver **Kidney**

Brain **Gill**

Fig. 3: The Correlation between MT levels and Cd accumulation in the tissues of *C. gariepinus* with a linear regression equation of liver of Y= 2.536 [Cd] + 5.014 (R^2 = 0.848, p<0.05), and kidney of Y= 2.519 [Cd] + 2.899 (R^2 = 0.844, p<0.05) and brain Y= 3.049 [Cd] + 1.619 (R^2 = 0.866, p<0.05) and gill Y=2.803 [Cd] + -0.260 (R^2 = 0.701, p<0.05).

Ratio of Actual Cd to Theoretical Maximum Cd-MT

The ratio of actual Cd to theoretical maximum Cd-MT for the liver, kidney, brain and gill are presented in **(fig.4)**. The ratios in all sampling tissues of controls were below 1.0, indicating that there was an adequate amount of MT to bind Cd in all studied tissues of controls. After acute exposure to waterborne Cd , the ratios increased significantly in the liver, kidney, and brain ($p<0.05$) at each sampling day, but a small rise in the brain was not significant at each sampling day, indicating that liver had greater Cd-binding potentials of MT than gill, kidney and brain.

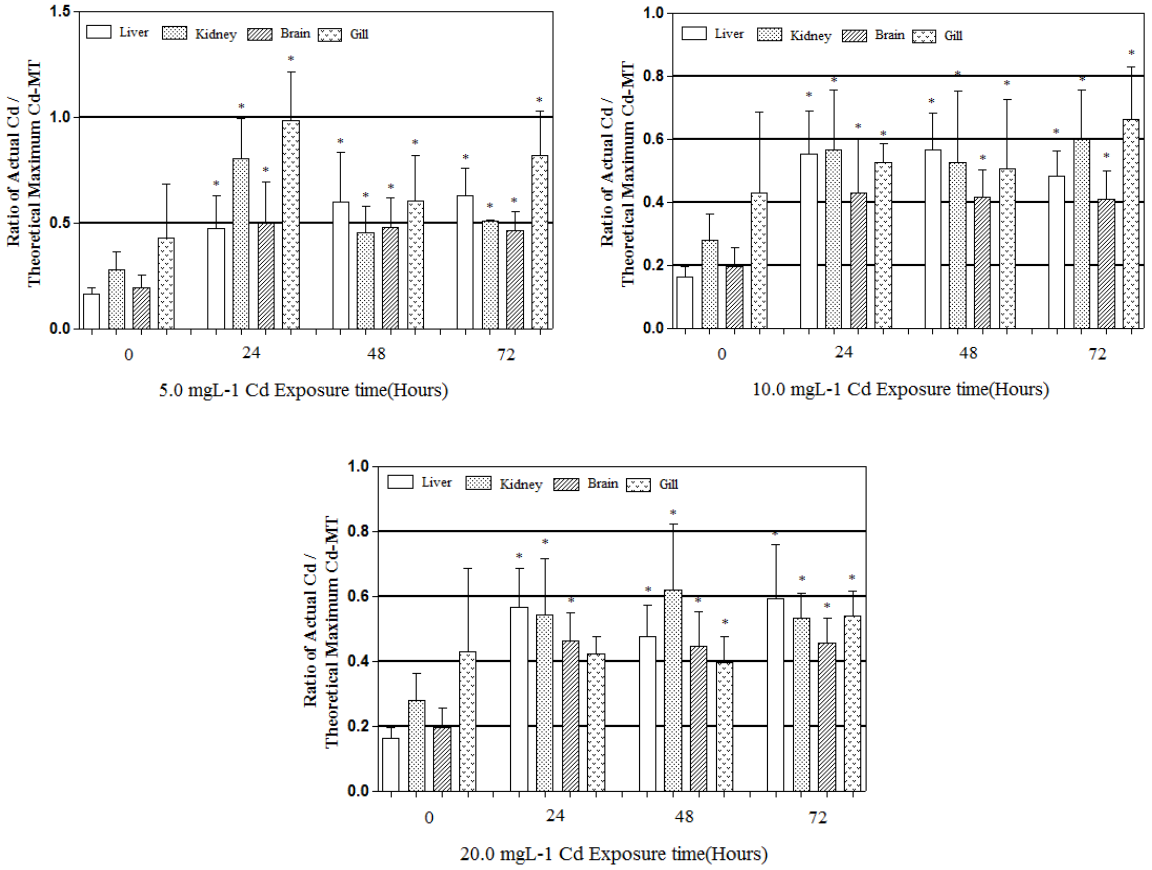


Fig. 4: Ratio of actual Cd to theoretical maximum Cd-MT in liver, kidney , brain and gill of *C. gariepinus* exposed to 0 (controls), 5.0, 10.0, and 20.0 mg L^{-1} Cd. Results were presented as mean ± SD, (n = 5). Statistical comparisons were made against background ratios (controls) at day 0 (*p<0.05).

DISCUSSIONS

Cadmium Accumulation

The results indicate that the accumulation of Cd levels in *C. gariepinus* was clearly tissue-specific and similar to many aquatic organisms such as *Rainbow trout* [20]; *Litopenaeus vannamei* [21] and *Sinopotamon henanense* [18]. The heavy metal usually accumulates mainly in organs such as liver that store metals to detoxify it by producing metallothioneins [22, 23, 24]. The liver is the target tissue while studying metal concentrations in aquatic environments [25]. The concentration of Cd levels in liver and kidney is higher than gill and brain in fishes because the liver and kidney are the major targets for Cd distribution to detoxify them by binding with MT. The gills are the major entry site of heavy metals and act as a transient store for accumulation of metals [9].

Most aquatic animals absorb heavy metals via the gills and transfer the metals to the blood and other parts of the body, So Cd existed within gill with a quite complicated and fluctuating pattern. At the initial stage of exposure, the accumulation of Cd elevated rapidly [18]. Cd accumulation in brain is much lower, when compared to liver, kidney and gill and showed dose and time dependence. After absorption of Cd by gill, liver and kidney, the metals detoxified and then transferred to interior tissues the brain. In this study, metal concentrations were studied in major osmoregulatory tissues, and revealed tissue specific Cd uptake in fishes. The present study shows distribution of accumulated Cd in fish differs among organs as evidenced by the works of De Conto Cinier *et al*., 1999 [26]; Asagba *et al*., 2008 [27]. Previous reports have shown that during waterborne metal exposure, a high level of metal accumulation occurs in organs like liver, kidney and gills [28, 26].

MT Induction

In fresh water fish, Cd uptake occurs mainly through the gills [29], and Cd entering the fish's body, is distributed and then deposited to some other organs [21]. It is opined that waterborne Cd ultimately accumulates in the liver to detoxify and accumulates in the kidney [26, 27] and intestine also detoxify and also induces the synthesis of MT. MT's are a class of low molecular weight, sulfur-rich metal-binding proteins with a high affinity for heavy metal ions such as Cd [30, 31] which controls both the kinetics of bioaccumulation and the manifestation of toxic effects, and ultimately determine metal tolerance [32]. MT was identified as a Cd -binding protein and, for this reason, has been considered as an important factor involved in the protection of organisms from the harmful effects of toxic heavy metals such as Cd. The improvement of analytical techniques and the increased understanding of the mechanisms of MT induction have strongly encouraged the use of MT as an environmental biomarker for metal pollution [33, 34]. MT has no known catalytic function hence measurements of the concentrations are based upon the quantification assay of protein itself. In the present study the liver

and kidney are the main organs of Cd accumulation followed by gill and brain up to 72 hrs of the study which is similar to earlier works of Chawry *et al*., 2005 [20] in rainbow trout and Wu and Chun, 2005 [21] instance. Higher concentration of Cd was found in the liver tissue that induces synthesis of new MT'S and forms Cd-MT complex, so the complex formed may be sequested [35] to other detoxifying organs. Cd contents and MT's concentration differ significantly in fishes exposed to various concentrations of Cd for 0, 24, 48 and 72 hrs. The low Cd content in the liver at 0 day of exposure indicates low level of Cd in the fresh water but however the levels of Cd increases significantly up to 72 hrs of exposure.

MT's are abundant cytosolic proteins in parenchymatous tissues. These proteins are found in plants and animals (vertebrates and invertebrates), eukaryotic microorganisms and in several prokaryotes [36, 37]. Major concentrations of MT are found in the liver and the kidneys of vertebrates [38, 39]. The variation in the level of heavy metals among different species depends upon its feeding habits, age, size and length of the fish and their habitats [40, 23, 41]. Metallothioneins (MT's), considered as metal biomarkers by many authors, are a highly conserved protein that provides great interests in phylogenetical studies.

Correlation between MT Level and Cd Accumulation

The results indicate a correlation between MT level and Cd accumulation of *C. gariepinus* tissues such as liver, kidney, gill and brain. The relationship between MT induction and Cd accumulation in the liver, kidney, gill and brain after acute exposure to Cd was found to be significant. Similar to many other studies on chronic Cd exposure on *Cyprinus carpio* [42] and white shrimp *Litopenaeus vannamei* [21] a positive correlation was observed between MT induction and Cd accumulation both in the liver, kidney, gill and brain of *C. gariepinus* in the present study. MT concentrations increase linearly with increasing Cd concentrations, indicating that MT can be used as an indicator of Cd concentration in the tissues of fishes. This fish being a commercially important fish is consumed locally by the people of Chennai and has an impact on the health of the people. Hence further characterization and the mechanism of MT functioning is mandatory.

Tissue Cd to MT Ratios

The calculated ratios of actual Cd to theoretical maximum Cd-MT for all tissues tested were<1.0 in controls at day 0, suggesting that the background MT was theoretically enough to bind all of the Cd in the tissues in the non exposed groups. The same pattern was observed for trout exposed to waterborne Cd [43, 20], in a similar analysis for trout exposed to waterborne Cd.

CONCLUSION

In conclusion, MT induction can be considered as a biomarker for acute waterborne Cd pollution. In the study a higher concentration of Cd accumulation levels was seen in the liver and the higher MT induction levels in the liver of *C. gariepinus* was observed in the present study. The liver and kidney Cd accumulation and MT induction levels was found to be higher than gill and brain. In gills acute exposure to Cd accumulation and MT induction levels gradually increased rapidly to reach the highest peak and then declined gradually after 48 hrs in *C. gariepinus*. The brain Cd accumulation and MT levels much lower than liver, kidney and gill in *C. gariepinus* during the acute Cd exposure and were tissue-specific. Moreover, the calculated ratios of actual Cd to theoretical maximum Cd-MT in liver were <1.0 under acute waterborne Cd at all sampling days, indicating that the liver had higher Cd-binding potentials than kidney, gill and brain. These results suggest that MT played an important role in liver to deal with high quantities of Cd. Cd accumulation in the tissues showed a positive correlation with MT induction in all the tissues studied. The control Cd levels and MT levels were minimum and as other metals were also BDL, it is inferred that MT induction is dependent on Cd in the study.

Conflict of interest statement

There is no conflict of interests.

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