ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES Knowledge to Innovation

Vol 8, Issue 5, 2015

ISSN - 0974-2441

Research Article

INDUCTION OF METALLOTHIONEIN WITH CADMIUM CHLORIDE IN A ECONOMICALLY IMPORTANT FRESHWATER FISH-GRASS CARP, *CTENOPHARYNGODON IDELLA* (VALENCIENNES, 1844)

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Received: 20 May 2015, Revised and Accepted: 11 June 2015

ABSTRACT

Objectives: Metallothioneins (MTs) have been widely considered for their potential use as a specific biomarkers to reflect the existence of heavy metal pollution, because their induction has been observed to be obviously elevated after heavy metal exposure in a large number aquatic organisms. However, relatively fewer efforts have been made to study the related effects of MT in fish species, such as *Ctenopharyngodon idella* (*C. idella*), a globally important aquaculture species. The objective of this study was to determine the cadmium (Cd) levels and MT induction in muscle, gill, kidney, and liver in *C. idella* during acute Cd exposure, to study 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).

Results: The MT induction levels were found in an increasing order of liver>kidney>gill >muscle. A positive correlation was shown between MT induction and Cd accumulation. The accumulation of Cd levels in all tissues is distinct by time-dependent and dose-dependent.

Conclusion: These results suggest that MT in the liver played an important role to detoxify high quantities of Cd. Cd accumulation showed a positive correlation with MT induction in all the tissues studied. Hence, the present investigation marks that MT levels can be considered as a biomarker for acute waterborne Cd.

Keywords: Heavy metals, Atomic absorption spectrophotometry, Biomarker protein, Metallothionein, *Ctenopharyngodon idella.*

INTRODUCTION

Heavy metals have been recognized as serious pollutants of the aquatic environment that causes serious impairment in metabolic, physiological and structural systems of both plants and animals. The accumulation of metals in an aquatic environment has a direct impact on the sustainability of the ecosystem. Interest in the heavy metals which are required for metabolic activities in organisms, lies in the narrow range between their essentiality and toxicity [1]. Fishes are considered as one of the most significant indicators in freshwater systems for the estimation of metal pollution [2].

Heavy metals are considered as a major source of environmental pollution. Cd which is one of the heavy metal has received considerable attention for its toxic effects on living individuals. Heavy metal contamination is typically derived from different sources such as mining, industrial waste discharges, sewage effluent, harbor activities, and agrochemicals. Cd is unique among metals because of its toxicity even at a very low dosage, long half-life (30 years in humans) and its low rate of excretion from the body [3]. The contamination of natural waters by heavy metals affects aquatic biota and poses considerable risks and concerns the environment [4,5] and human health.

The commercial and edible fish species, *C. idella* have been widely investigated in order to check heavy metal contamination in various tissues and their stress responses by way of secreting stress protein metallothioneins (MT). With the growth of fishes, the heavy metals accumulate in the internal organs such as muscles, liver, and intestine in a considerably higher concentration that make the fish unsuitable for human consumption [6].

Heavy metals become toxic when they are not metabolized by the body and accumulate in the soft tissues. The target organs for Cd toxicity have been identified as liver, kidney, gill and muscle [7]. Cd is one of non-essential heavy metals, known for its corrosive nature is widely used in paints and dyes, cement and phosphate fertilizers [8]. Cd occurs naturally in the environment, in significant amounts, but its release in the recent past ions steadily increasing due to anthropogenic activities causing pollution of soil and aquatic systems. The occurrence of cadmium toxicity in aquatic environments has been previously reported by earlier workers in various aquatic ecosystems [9-12]. Cd can be taken up from the environment into the body through pulmonary and enteric pathways. Cd like many other heavy metals competes with nutrient elements for binding sites as transport and storage proteins, metalloproteins and receptors. It acts as a stressor, leading to metabolic alterations similar to those observed in starvation conditions. It was reported that cadmium specifically inhibits some proteins such as phosphofructokinase, lactate dehydrogenase, etc. and retards glycolysis. The toxicity of cadmium is attributed to its ability to generate reactive oxygen species that may act as signaling molecules in the induction of gene expression and apoptosis [13]. Detoxification of heavy metals takes place by synthesis of stress proteins MT.

MTs are low-molecular-weight (approximately 6000-7000 Da) cysteine-rich, metal-binding proteins that are found in microorganisms, plants, and animals. MT was first isolated from horse kidney [14,15]. MTs are widely expressed in organisms; such as eukaryotes and are responsible for essential metal metabolism and heavy metal detoxification [16]. Cd 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].

METHODS

The freshwater fish, *C. idella* were collected from Poondi fish farm Tiruvallur district, Tamil Nadu, India. The fishes were acclimatized in the laboratory in a stone tank for 7 days at room temperature (30±20°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 control, 2.5 ppm and 5.0 ppm exposed for a period of 24, 48 and 72 hrs. Ten fishes with similar size, length, weight about (approximately 100±10 g) were selected and divided into four groups. Each group has 20 fishes. The fishes were fed every day with a 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 (10 L) containing 0, 2.5 ppm, 5.0 ppm 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, gill and muscle 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 of treatment. The tissues were handled with plastic forceps and kept in plastic homogenizing tubes. For each fish, approximately half of the sampling tissues 0.5 g of muscle, gill, kidney and liver were kept aside 0.25 g for MT measurements and the remaining tissue (0.25 g) for Cd measurement.

Cd determination

The Cd was determined according to the methods of Ma *et al*., [18]. The sampling tissues (0.25 g) were freshly weighed and cut into small pieces and dried in an oven at 80°C for about 48 hrs. Then tissues were digested in 10 ml; HNO_3 and 5 ml H_2O_2 over a hot plate at about 120°C. 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 wet weight tissue Cd levels were measured in the tissues of liver, kidney, gill and muscle.

MT quantification

MT was determined according to the methods of Ma *et al*., [18]. Sampling tissues were freshly weighed 0.25 g 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 phenylethylsulphonyl fluoride and 0.1 mM dithiothreitol. The homogenate was centrifuged at 16000 × g for 30 minutes at 4°C, and the supernatant was heated for 2 minutes in a boiling water bath (100°C). The heated sample was centrifuged at 10000 × g for 10 minutes to remove precipitated proteins. Volumes of 0.1 ml Cd solution (500 µg/L as $CdCl_2$) were mixed with 0.5 ml of sample (heat-denatured supernatant) and incubated at room temperature for 10 minutes 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 minutes. The hemoglobin was denatured in a water bath (100°C) for 2 minutes, cooled in ice for 3 minutes and centrifuged at 10000 × g for 15 minutes. 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.

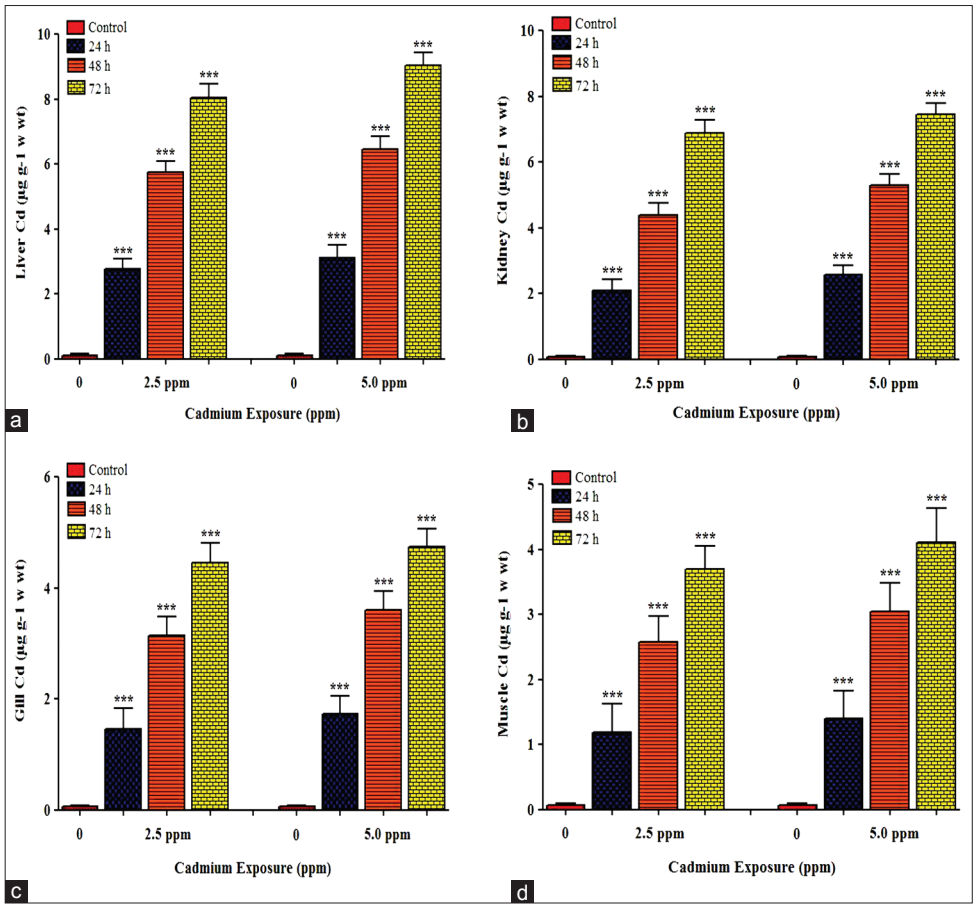

Fig. 1: Accumulation of cadmium in liver, kidney, gill, and muscle tissues of *Ctenopharyngodon idella* exposed to 0 (control), 2.5 and 5.0 ppm and the periods of 24, 48, and 72 hrs. The results were presented as mean ± standard deviation, (n=5). Statistical comparisons were made against control fish at each sampling day. (*The values are statistically significant at p<0.001), (a) Liver, (b) Kidney, (c) Gill, (d) Muscle**

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 w wt) = Cd conc. (µg/g 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 wet weight.

Statistical analysis

Statistical analysis of data was carried out using Graphpad Prism Version 5.0. The values are reported as mean ± standard deviation (SD). A 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, gill and muscle of fish tissues. The Cd levels increased significantly in all tissues in the following order: Liver>kidney>gill>muscle. The accumulation of Cd levels in all tissues is distinct and was time-dependent and dose-dependent. The highest Cd concentration was observed in liver 9.05±0.40 µg/g w wt, kidney Cd level were 7.45±0.35 µg/g w wt, the gill Cd level were 4.74±0.33 µg/g w wt and the Muscle Cd level were 4.11±0.52 µg/g w wt treated with 5.0 ppm of $CdCl_2$ during 72 hrs of Cd exposure. The Cd concentration in *C. idella* tissues such as liver, kidney, gill and muscle at concentration of 2.5 and 5.0 ppm of Cd for periods of 24, 48, 72 hrs were measured and illustrated in Fig. 1. The data were statistically tested and the values were found to be statistically significant at $p<0.05$.

MT induction

MT induction was measured in liver, kidney, gill, and muscle tissues. 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 to be in the following order in the tissues: Liver >kidney>gill>muscle. The highest MT concentrations were observed in the liver during 72 h of 5.0 ppm Cd exposure (33.65±0.89 µg/g w wt), the kidney (28.08±0.77 µg/g w wt), the gill (18.38±0.81 µg/g w wt) and the muscle (15.52±0.88 µg/g w wt), (n=5). The MT concentrations of control tissues were in the following order: Liver (0.21±0.10 µg/g w wt) > kidney (0.17±0.05 µg/g w wt) > gill (0.12±0.04 µg/g w wt) > muscle (0.14±0.05 µg/g w wt). After acute exposure to Cd, MT levels in all the tissues gradually increased in a similar pattern.

Correlation between MT levels and Cd accumulation

Fig. 3 shows the relationship between Cd accumulation and MT induction in liver, kidney, gill and muscle. 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: Liver of [MT] = 3.793

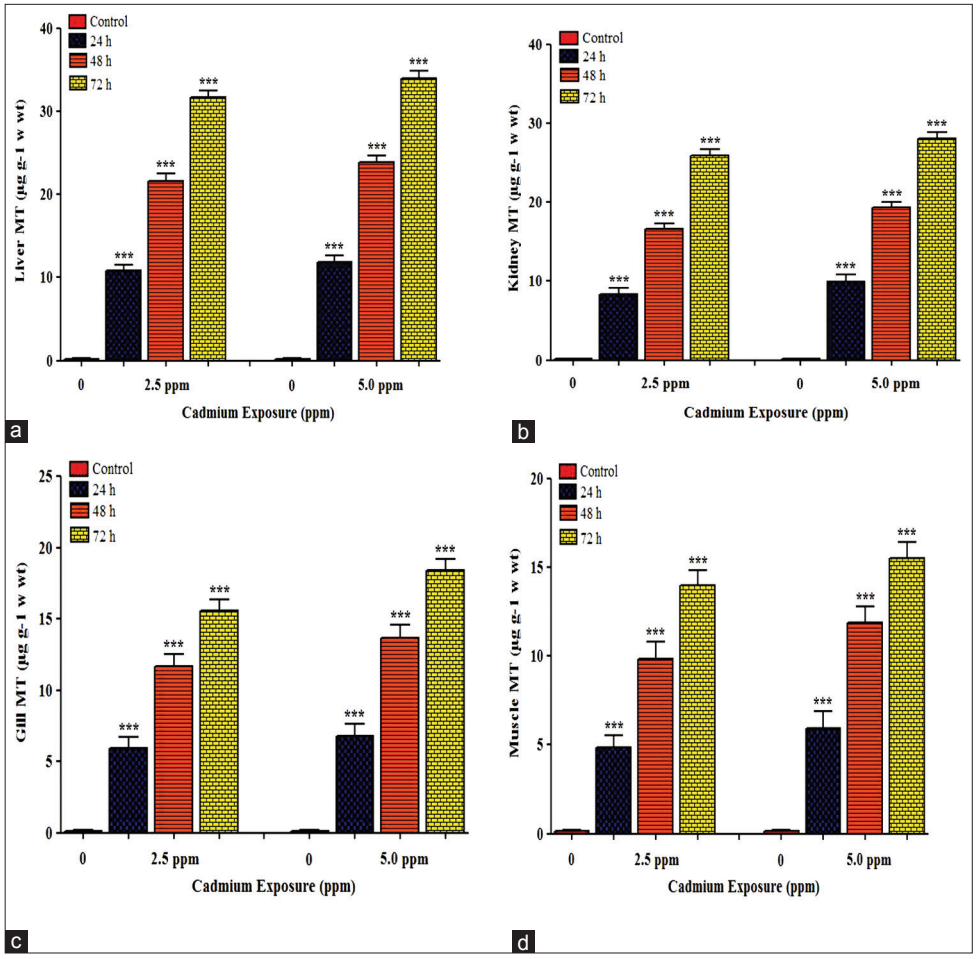

Fig. 2: Induction of metallothioneins concentrations in liver, gill and muscle of *Ctenopharyngodon idella* exposed to 0 (control), 2.5 and 5.0 ppm and the periods of 24, 48 and 72 h. The results were presented as mean ± standard deviation, (n=5). Statistical comparisons were made against control fish at each sampling day. (*The values are statistically significant at p<0.001), (a) Liver, (b) Kidney, (c) Gill, (d) Muscle**

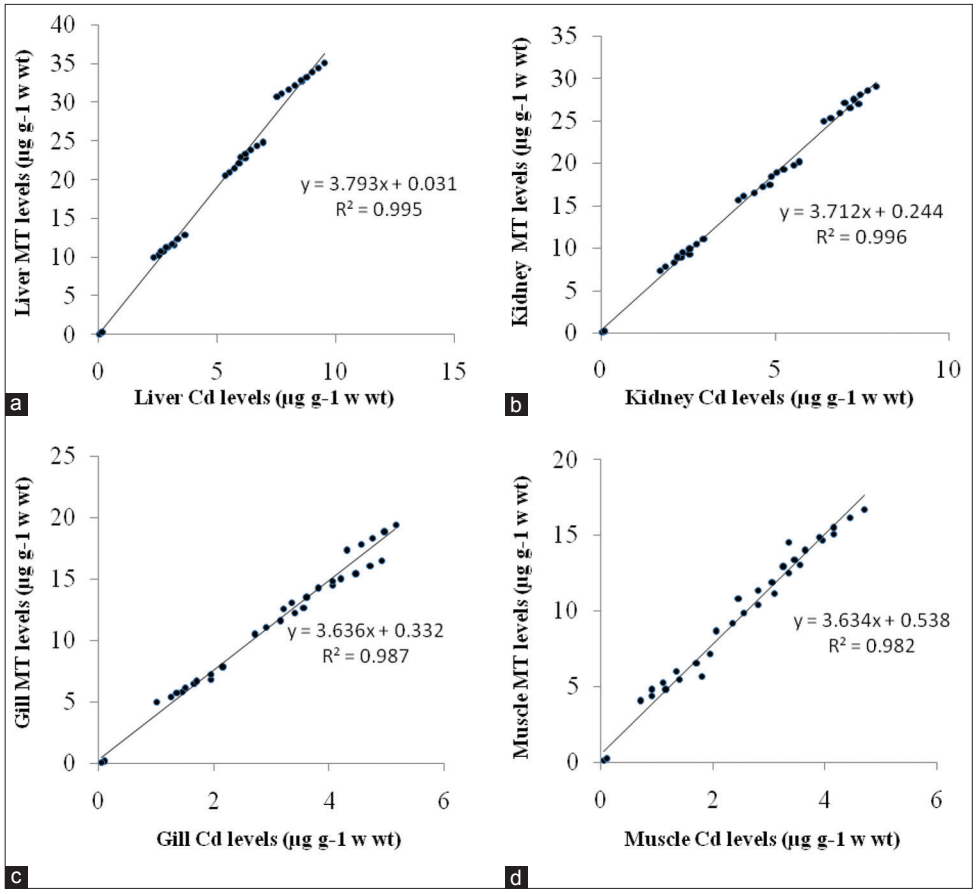

Fig. 3: The correlation between metallothioneins (MT) levels and cadmium (Cd) accumulation in the tissues of *Ctenopharyngodon idella* with a linear regression equation of liver of [MT] = 3.793 [Cd] + 0.031 (R^2 = 0.995, p<0.0001), and kidney of [MT]= 3.712 [Cd] + 0.244 (R^2 = 0.996, p<0.05) and gill [MT] = 3.636 [Cd] + 0.332 (R^2 = 0.987, p<0.001) and muscle [MT] = 3.634 [Cd] + −0.538 (R^2 = 0.982, p<0.001), (a) Liver, (b) Kidney, (c) Gill, (d) Muscle

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MT induction

Correlation between MT levels and Cd accumulation

DISCUSSION

Cd concentration in the muscle of the fish was low during 24 hrs of exposure and increased slightly during 48 hrs and 72 hrs of exposure during different Cd concentrations. Muscle is a type of tissue that is extrinsic to the target organ for Cd accumulation and MT synthesis [20]. But however the Cd concentrations were found to be higher in the liver followed by the kidney. This may be due to the fact that the first target organ for cd accumulation is the liver followed and the last target organs are the kidneys as reported by Jezierska, [20]; Kay *et al*., [21]; Giles, [22] and Glynn, [23].

Cd may bind to MT's; Cd that is present inside the cell, may induce synthesis of new MTs, and might bind to it. Cd-MT complex thus formed may be transported to the kidneys. Metal binding capacity depends on initial MT levels and on the intensity of MT synthesis and is thus, tissue specific, dependent on the concentration of Cd and is time - dependent.

The affinity of MT for heavy metals such as Zn, Cd, Hg, and Cu and their capacity to bioaccumulate in aquatic organisms, especially fishes has been demonstrated by Gill *et al*., [24]. The exposure of aquatic organisms to excess essential and non-essential metal induces MT expression in different fish species and tissues. MT are intrinsic to most fish species which makes them a possible candidate to serve as an indicator of environmental exposure to metals and also as a biomarker of metal pollution. In this study, the concentration of cellular stress proteins (i.e.,) metallothionein is a good indicator of water pollution.

MT concentrations in the tissues studied indicates that acute Cd exposure induces MT concentration in *C. idella* and was tissue - specific in all the tissues studied. MT concentrations were found to be in the following order liver>kidney>gill>muscle and falls in line with earlier reports [18] in freshwater crab, *Sinopotamon henanense.* Induction of MT shows dose and time - dependent increase as shown by Martinenz *et al*., [25] in cray fish, *P. clarkii* and Wu and Chen, [26] in shrimp, *L. vannamei.* After acute exposure to heavy metal Cd, the induction of MT increases the binding of Cd to protein (MT) which helps to sequester the Cd to decrease the Cd toxicity as advocated by Hogstrand and Haux, [27]. MT concentrations increase linearly with increasing Cd concentrations, indicating that MT can be used as an indication of Cd contamination in the tissues of *C. idella*.

In present study, MT concentrations were found to be at higher concentrations in the liver during 72 hrs of Cd exposure when compared to 24 hrs of Cd exposure followed by kidney, gills and muscle. Several reports have claimed that MT's play an important role in Cd detoxification in fish [28]. It is reported in this study that concentration of Cd-MT were in the following order: liver>kidney>gill>muscle and was positively correlated falls in the line with early reports [29]. In another study, the authors Haung *et al*., [30] showed that accumulation capacity of every single organ depends on other metals in water.

Table 1: Concentration of Cd in the control and treated tissues in *C. idella* exposed to $CdCl_2$ (2.5 and 5.0 ppm) for a period of 24, 48, 72 hrs (n=5)

Tissues	Control	2.5 ppm of Cd			5.0 ppm of Cd		
		24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs
Liver	0.10±0.05	2.76±0.33*	5.76±0.33*	8.05±0.42*	3.12±0.40*	6.46±0.38*	9.05±0.40*
Kidney	0.08±0.03	2.10±0.34*	4.39±0.37*	6.88±0.40*	2.56±0.30*	5.29±0.33*	7.45±0.35*
Gill	0.06±0.02	1.46±0.36*	3.14±0.35*	4.46±0.35*	1.73±0.33*	3.60±0.34*	4.74±0.33*
Muscle	0.07±0.03	1.19±0.43*	2.57±0.40*	3.69±0.36*	1.40±0.43*	3.04±0.44*	4.11±0.52*

Each values are indicates mean±SD, (n=5) in triplicates. Cd concentrations were expressed as µg/g wet weight tissue. *Indicates p<0.001 statistically significant between control and cadmium treated groups using Newman–Keul's test, SD: Standard deviation, Cd: Cadmium, *C. idella: Ctenopharyngodon idella*

Table 2: Concentration of MT in the control and treated tissues of *C. idella* exposed $CdCl_2$ (2.5 and 5.0 ppm) for a period of 24, 48, 72 h (n=5)

Tissues	Control	2.5 ppm			5.0 ppm		
		24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs
Liver	0.21±0.10	10.83±0.66*	21.65±0.89*	31.73±0.81*	11.83±0.84*	23.91±0.77*	33.65±0.89*
Kidney	0.17±0.05	8.32±0.77*	16.61±0.74*	25.94±0.83*	9.99±0.82*	19.32±0.67*	28.08±0.77*
Gill	0.12±0.04	5.93±0.74*	11.66±0.86*	15.54±0.81*	6.76±0.84*	13.69±0.89*	18.38±0.81*
Muscle	0.14±0.05	4.86±0.68*	9.82±0.96*	13.98±0.88*	5.93±0.94*	11.89±0.89*	15.52±0.88*

Each values are indicates mean±SD, (n=5) in triplicates. MT concentrations were expressed as µg/g wet weight tissue. *Indicates p<0.001 statistically significant between control and cadmium treated groups using Newman–Keul's test, SD: Standard deviation, *Cd: Cadmium, C. idella: Ctenopharyngodon idella*

CONCLUSION

The study showed significantly higher Cd concentrations in the liver and kidney and significantly higher MT concentration in the liver and kidney exposed to acute waterborne Cd; the organs being the sites of metal detoxification. The results showed that MT played an important role in detoxification of Cd in the liver and kidneys. Cd accumulation in the tissues showed a positive correlation with MT induction in all the tissues of *C. idella*. 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. Therefore, MT induction can be considered as a biomarker for acute waterborne Cd toxicity.

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