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Metallothionein mRNA expression in freshwater teleost, *Channa punctata* (Bloch) under the influence of heavy metal, cadmium – a dose kinetic study

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Abstract

Increased Metallothionein (MT) synthesis is associated with increased capacity for binding metals such as copper, zinc and cadmium, as well as protection against metal toxicity. A study was carried out using quantitative real-time reverse-transcriptase polymerase chain reaction (q-RT-PCR) in freshwater teleost, *Channa punctata* (Bloch), after exposure to sub-lethal concentrations of cadmium. Post 14 days exposure, the heightened MT mRNA expression was seen only in kidney, whereas in contaminated liver and gills, the MT gene expression collapsed significantly below the basal level. The difference in the effect of various concentrations of cadmium on MT mRNA transcript level in the tissues under study was found significant. The study indicates that the expression of MT mRNA in *C. punctata* occurs in tissue specific and dose dependent manner.

Keywords: Cadmium, *Channa punctata*, Dose response, Metallothionein, Tissue specific

Introduction

Metals differ from other toxic substances in that they are neither created nor destroyed by humans. Nevertheless, their utilization by humans influences the potential for health effects in at least two major ways. First, by environmental transport that is, by human or anthropogenic contributions to air, water, soil and food, and second, by altering the speciation or biochemical form of the element.

Protein binding of metals has been the subject of a major review on the molecular biology of metal toxicology (Zalups and Koropatnick 2000). Several kinds of metal-protein interactions may be considered. Among the metalloproteins are the metallothioneins (MTs), a class of low-molecular-weight proteins (6-7 kDa) with high cysteine content (~30% of the amino acids are cysteines), lack of aromatic amino acids, a selective capacity to bind heavy-metal-ions via mercaptide linkages, and ubiquitous in distribution (Bae et al. 2005). MT was first discovered as a cadmium and zinc binding protein in the equine renal cortex (Margoshes and Vallee 1957). Subsequently a role for MTs or MT-like proteins (MTLPs) in protecting against the toxic effect of cadmium (Cd) was suggested. MT

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genes have now not only been implicated in detoxification of toxic heavy metals but also in the homeostasis of the essential metals such as (Zn) zinc and (Cu) copper (Olsson and Kille 1997).

Two types of reference patterns of expression enable the estimation of contaminant effects on the biomarker levels in bio indicator organism. Contaminant-independent patterns define the physiological range of expression. Natural fluctuations of biomarker levels may be due to biological parameters such as age, sex, reproductive activity and inter-individual variations, as well as to seasonal changes in the environmental conditions. Contaminant-dependent patterns caused by chronic exposure to contaminants have to be obtained through characterized dose response to model compounds administered via realistic environmental routes (Tom and Auslander 2005).

Despite the fact that laboratory studies suggest, MT mRNA expression pattern may be studied to assess the effects of metal exposure, most of the studies evaluating the use of MT mRNA expression as a bio-monitoring tool have been restricted to exposure of specimens under laboratory conditions to metals through intraperitoneal or intramuscular injections (Baksi et al. 1988; George et al. 1996). Only a very limited number of studies effectively determined MT mRNA expression levels due to model compounds administered via realistic environmental routes (Knapen et al. 2007; Tiwari et al. 2010). Thus, present study was undertaken to assess the MT mRNA expression in a dose response manner due to waterborne exposure of metal, cadmium in freshwater fish, *Channa punctata*.

Materials and methods

Experimental animals

Healthy specimens of freshwater murrel, *Channa punctata* (Bloch, Family Channidae and Order Perciformes) were procured from local fish market of Lucknow, U.P., India (45 specimens). The specimens had an average wet weight and length of 21.34 ± 2.79 g and 12.05 ± 0.56 cm (mean±SEM) respectively. The specimens were given prophylactic treatment by bathing them in 0.05% (w/v) potassium permanganate ($KMnO_4$) solution for 2 min to avoid any dermal infections. The fishes were then acclimatized for 15 days under laboratory conditions prior to metal exposure. The fishes were fed, *ad libitum,* with boiled chicken eggs, goat liver and poultry waste material.

Test chemical

For the present study, analytical-grade cadmium chloride ($CdCl_2.H_2O$) (98%), manufactured by Himedia Lab. Ltd., Mumbai, India was used as the test compound (as a source of metal, cadmium).

In vivo exposure experiment

The acute toxicity bioassay to determine the 96 h median lethal concentration ($^{96}LC_{50}$) of cadmium was conducted in the semi-static system following standard methods (APHA et al. 2005). On the basis of determined $^{96}LC_{50}$ value (14.95 mg/l), three concentrations of cadmium viz., d_1 (= 0 mg/l), d_2 (= 3.74 mg/l), and d_3 (= 7.48 mg/l) were selected for the waterborne exposure of Cd in the semi-static system. The exposure was continued up to 14 days and tissue sampling was done on day 14^{th} at the rate of five fish per dose (n = 5).

The fish maintained in tap water without the test chemical (control group) were considered as calibrator (d_1 = 0 mg/l Cd) for the dose kinetic study. During experiment, the test solution was changed after every 24 h to maintain the appropriate concentration of the metal in the test aquaria. The physico-chemical properties of test water were determined according to the standard procedures (APHA et al. 2005). From each dose group, the liver, kidney and gill tissues were taken from each individual. The tissue sample were placed in sterile tubes and immediately stored in RNA *later* RNA Stabilization Reagent (Qiagen GmbH, Germany) following the manufacturer's instructions, for further RNA extraction.

Primers for the real time amplification of MT cDNA

The quantitative real-time reverse-transcriptase polymerase chain reaction (q-RT-PCR) primers (MT-2 F', forward; MT-2 R', reverse) for the real-time PCR amplifications of MT were designed on the basis of the sequence information of *C. punctata* MT gene (GenBank Accession No. FJ869867).

Beta-actin (β-actin) was used as an internal control to normalize mRNA levels in the real-time PCR amplification study. The sequence information of β-actin mRNA in *C. orientalis* (GenBank Accession No. GQ219743) was used for the designing of β-actin F', forward; β -actin R', reverse primers (Table 1). For the primer production sequence information was obtained from GenBank (http://www.ncbi.nlm.nih.gov/GenBank/) and entered into Primer3, a web-based software package which designed optimal primers according to given

specifications. Melt curve and primer efficiency analyses were conducted for each primer for quality control purposes.

Table 1. Primer sequences used in RT-PCR for the amplification of Metallothionein and Beta-actin mRNA

Primer	Sequence (5'->3')	Length (bp)	Tm (°C)	GC%	Reference (GenBank Accession No.)
MT-2 F' Forward	CTGCAACTGCGGAGGA	16	57.92	62.50	FJ869867
MT-2 R' Reverse	GGTGTCGCATGTCTTTCCTT	20	60.12	50.00	
β-actin F' Forward	GTGCCCATCTACGAGGGTTA	20	59.96	55.00	GQ219743
β-actin R' Reverse	AAGGAAGGAAGGCTGGAAGA	20	60.32	50.00	

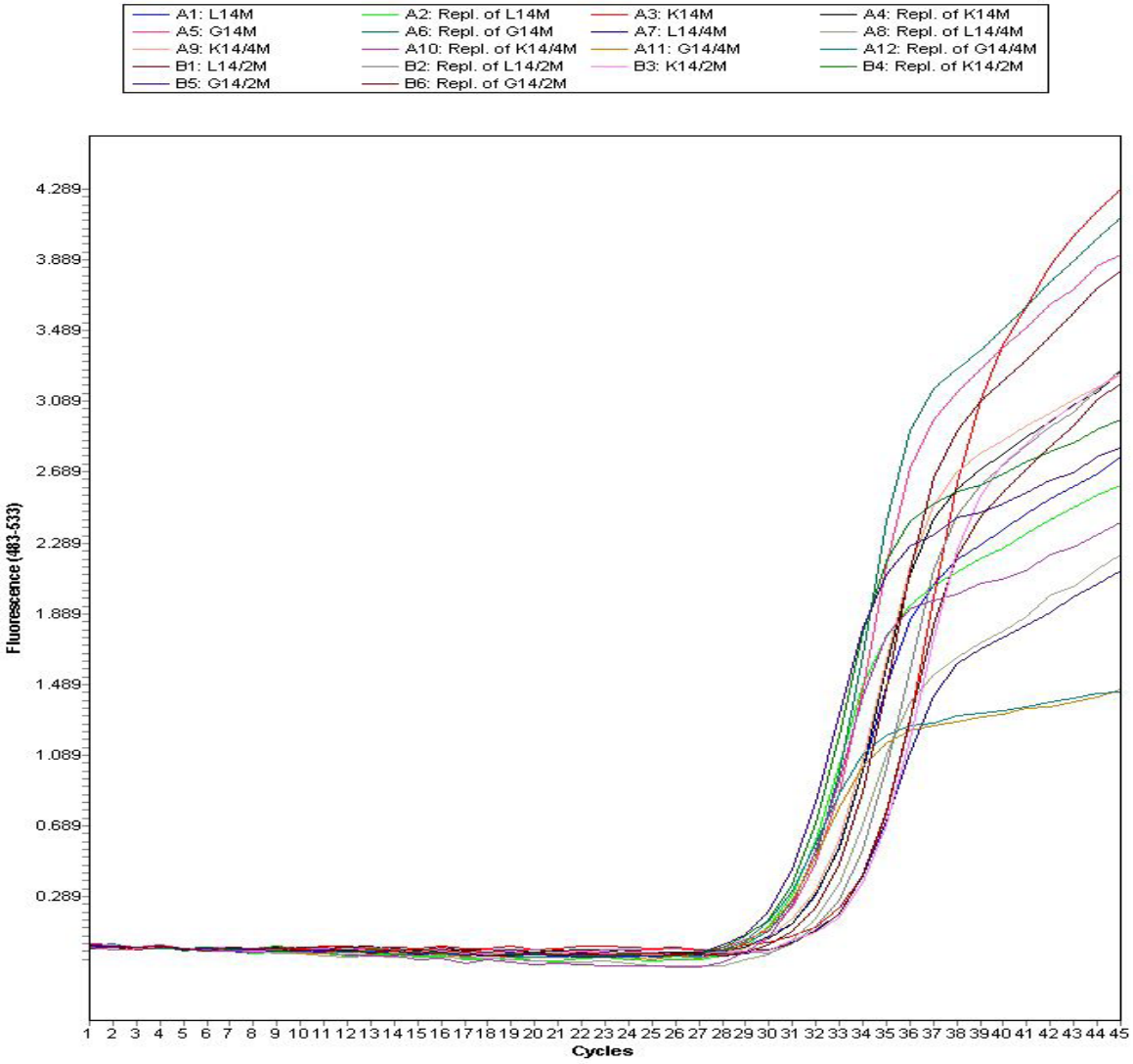

Fig. 1. Amplification curves of metallothionein mRNA (target). Figure shows fluorescence of metallothionein mRNA amplification in L- liver, K- kidney, and G- gill tissues of *C. punctata* after exposure to 0, 3.74 ($^{96}LC_{50}/4$) and 7.48 ($^{96}LC_{50}/2$) mg/l Cd for 14 days. A1 - B6 represent corresponding wells.

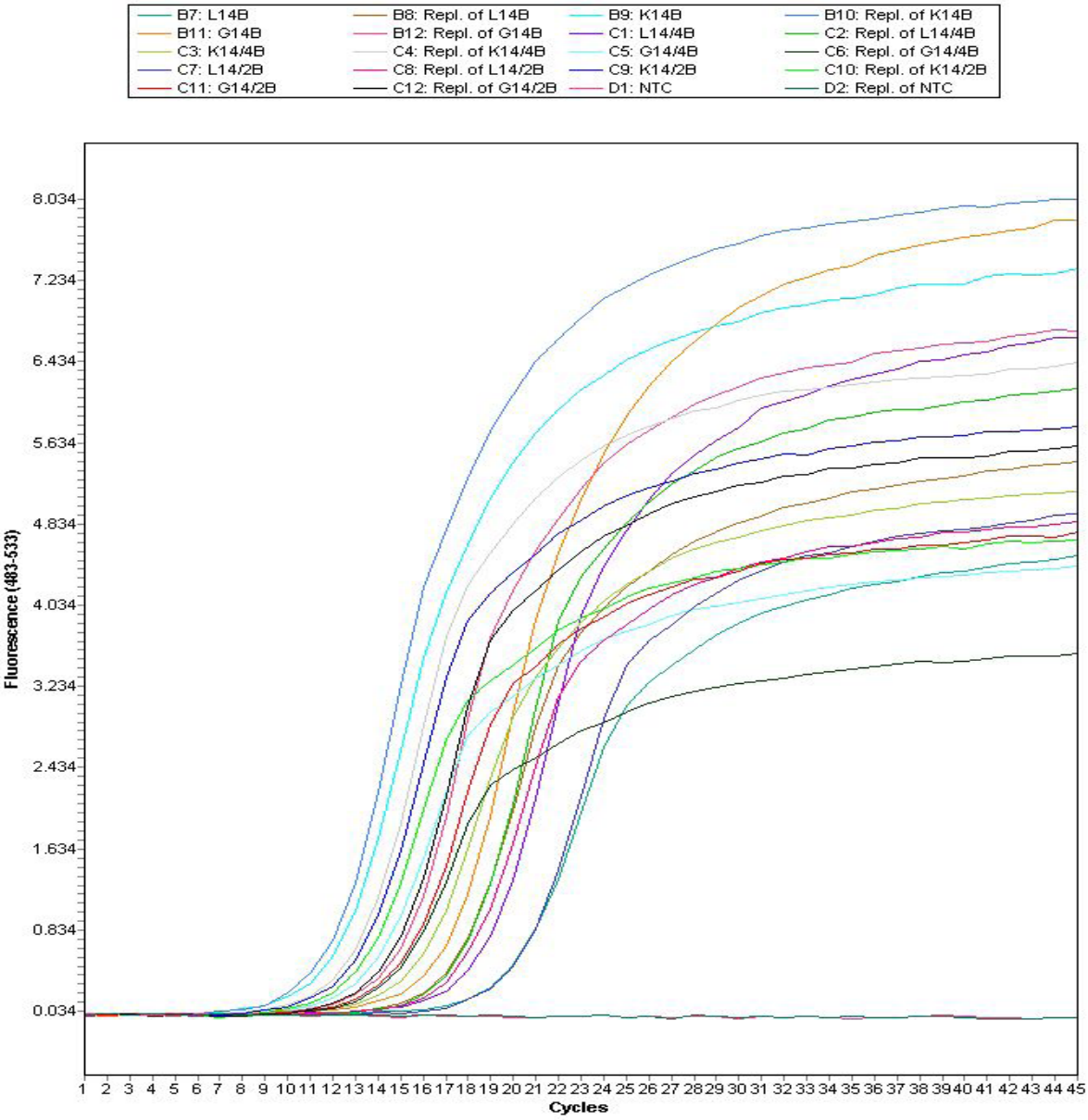

Fig. 2. Amplification curves of β-actin mRNA (reference). Figure shows fluorescence of β-actin mRNA amplification in L- liver, K- kidney, and G- gill tissues of *C. punctata* after exposure to 0, 3.74 ($^{96}LC_{50}/4$) and 7.48 ($^{96}LC_{50}/2$) mg/l Cd for 14 days. B7 – D2 represent corresponding wells.

Total RNA extraction

Total RNA was isolated from each sample using acid guanidinium isothiocyanate-phenol-chloroform extraction following the TRI Reagent extraction protocol (Molecular Research Center, Inc. Cincinnati, OH) (Chomczynski and Sacchi 1987). RNA pellets were finally resuspended in RNAse/DNAse-free water to a concentration of approximately 100 ng/µl.

For quality control purposes, samples were quantified spectrophotometrically at 260 and 280 nm (PowerWave XS Microplate Spectrophotometer, Bio Tek Instruments, Inc., USA). Only samples with absorbance 260 to absorbance 280 ratios (A_{260}/A_{280}) greater than 1.7 were used in subsequent analyses. RNA integrity was checked by denaturating formaldehyde agarose (FA) gel electrophoresis with MOPS (3[N-morpholino] propanesulfonic acid, 200 mM; sodium acetate, 20 mM; ethylenediaminetetraacetic acid, 10 mM) buffer (Sambrook and Russell 2001). Visual inspection of the gels showed no signs of RNA degradation in any of the samples.

Expression of MT mRNA

q-RT-PCR amplifications were carried out using the QuantiTect SYBR Green RT-PCR kit according to the manufacturer's instructions (Qiagen). Reaction mixture included 25 µl of kit Master-Mix (contains nucleotides, buffer, *Taq* enzyme, $MgCl_2$ and SYBR Green I dye), 1 µl of 50 µM gene specific primers and 50 ng total RNA. The mixture was placed in a Light-Cycler real-time PCR (Light-Cycler 480 System, Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) and incubated at 50 °C (30 min), 95 °C (15 min), 94 °C (15 s), 56 °C (30 s) and 72 °C (30 s) to reverse transcription, PCR initial activation, denature the cDNA, anneal the primers and extend the product, respectively. Reactions were carried out for a total of 45 cycles. All amplifications were performed in triplicates.

For the q-RT-PCR data (Figs. 1, 2) analysis a method of comparison of C_T (threshold fluorescence) values, the $2^{\wedge} (-\Delta\Delta C_T)$ method, was used (Livak and Schmittgen 2001).

Cadmium analysis

On day 14th in all dose groups, cadmium level was analyzed using an atomic absorption spectrophotometer (AAnalyst 300 Spectrometer, Perkin Elmer, USA). Three replicate samples were analyzed to obtain an average cadmium concentration from each sampling. The sample digestion and analysis was done following the standard methods (APHA et al. 2005).

Data analysis

Statistical analyses MT mRNA expression data were performed using SPSS computer software (SPSS 16.0.2 2008). To achieve homogeneity of variance, all gene expression data [$2^{\wedge} (-\Delta\Delta C_T)$ values] were log transformed. Comparisons between the effects of exposure concentrations on mean relative tissue specific mRNA expression was done using multiple comparison technique, Tukey's HSD post hoc test ($\alpha = 0.01$).

Results

Physico-chemical properties of the test water

The physico-chemical characteristics of the test water are presented in Table 2. During the experiment, temperature of the test water ranged from 19.3 to 22.5 (21.1 ± 0.40) °C, dissolved oxygen from 6.72 to 8.13 (7.51 ± 0.16) mg/l, and pH from 7.14 to 7.95 (7.45 ± 0.08) respectively. The total hardness of the test water varied from 169 to 198 (182.8 ± 2.96) mg/l as $CaCO_3$ during experimental period.

Table 2. Physico-chemical properties of the test water

Parameters	Unit	Mean ± SEM	Range
Ambient temperature	°C	22.9 ± 0.42	20.5-24.7
Water temperature	°C	21.1 ± 0.40	19.3-22.5
pH	-	7.45 ± 0.08	7.14-7.95
Conductivity	µS/cm	280.3 ± 7.18	239-303
Dissolved oxygen	mg/l	7.51 ± 0.16	6.72-8.13
Total alkalinity	mg/l as $CaCO_3$	261.1 ± 4.04	242-278
Total hardness	mg/l as $CaCO_3$	182.8 ± 2.96	169-198

Table 3. Metal concentration in the test water in various dose groups

Cd Concentration (mg/l)		Decrease in Conc. (%)
Dissolved	Measured (Mean)*	
0	N.D.	-
3.74	2.91	22.19
7.48	6.56	12.30

Cadmium concentration in test water

The measured concentrations of cadmium in the test water using AAS in each dose group (d_1 = 0 mg/l, d_2 = 3.74 mg/l, and d_3 = 7.48 mg/l Cd) are shown in Table 3. The measured concentration of waterborne Cd was found to be slightly lesser in comparison to that of the dissolved in all samples, and showed decrease in level up to 22.19% in the 0-14 day time period.

Dose kinetics of MT mRNA expression

On day 14th post Cd exposure at doses 0, 3.74 and 7.48 mg/l, MT mRNA expression was monitored by q-RT-PCR (Fig. 3). Liver, kidney and gill tissues were analyzed of which, heightened expression was seen only in kidney, whereas liver and gill MT mRNA expression were below the basal level. Dose dependent up regulation was observed in kidney MT mRNA level in an inversely proportional manner. It was found to be significantly up regulated with 1.24 and 0.58 (logarithm values) or around 18-, and 4-fold induction level, at 3.74, and 7.48 mg/l Cd exposure (P < 0.01). The difference in the effect of various concentrations on MT mRNA transcript level in the tissues under study was found significant (P < 0.01). For pair wise comparison, all differences were found significant (P < 0.01).

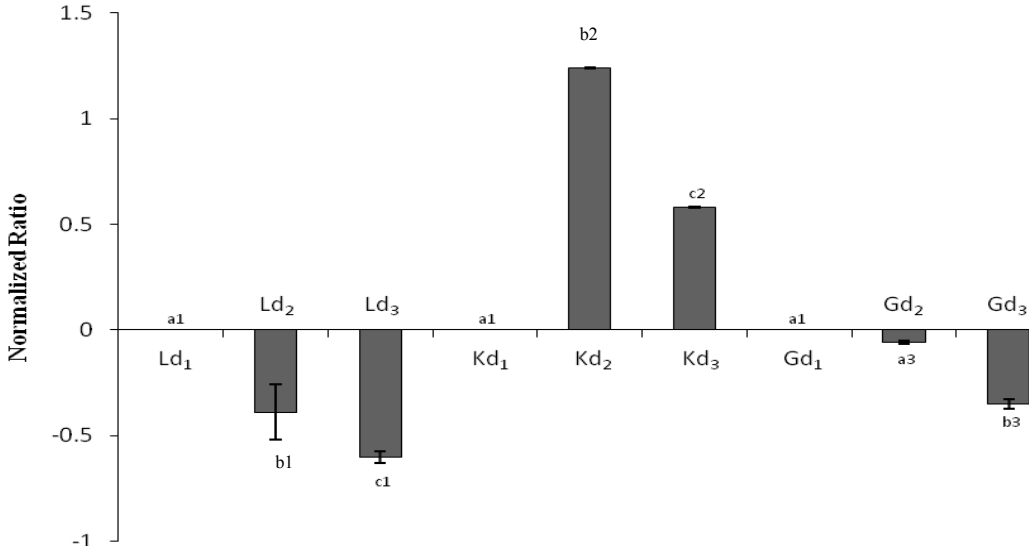

Fig. 3. Mean relative [log 2^ ($-\Delta\Delta C_T$)] metallothionein mRNA expression (± SD) in liver (L), kidney (K), and gill (G) tissues of *C. punctata* (n = 5) after exposure to (d_1= 0, d_2= 3.74, d_3= 7.48 mg/l) Cd for 14 days. Relative expression (ΔC_T) in control sample (d_1= 0 mg/l Cd) used as calibrator. Different alphabet superscript letters denote significant difference (P < 0.01) in mRNA expression between doses within tissues. Different numeric superscript values denote significant difference in mRNA expression (P < 0.01) between tissues within dose.

Discussion

Present study was conducted for the assessment of MT mRNA expression pattern in freshwater teleost, *C. punctata* on various sub lethal concentrations of cadmium as the survival of fish was observed on these concentrations up to 14 days in our study, irrespective of environmentally relevant concentration. So that information can be obtained on expression pattern of metallothionein for further biomarker studies using the model test organism.

In the present study on day 14th post 3.74 and 7.48 mg/l waterborne Cd exposure, the MT mRNA level was found in the following order: kidney > gill > liver (Fig. 3). On this time point only kidney shows significant up regulation in MT mRNA level, whereas in liver and gill the same was below basal level, and seems to be the prominent site for MT synthesis and metal uptake in *C. punctata* after a prolong Cd exposure. The results clearly indicate an inversely proportional dose response in the tissue.

Freshwater fish can take up waterborne Cd via the gills and dietary Cd via the gut. The mechanism of uptake for both routes is considered to be the non-selective uptake of Cd through calcium uptake systems. During

waterborne exposure after uptake across gill, Cd is probably bound to transport proteins in the blood plasma as in higher vertebrates (Zalups and Ahmad 2003) and distributed via the circulatory system to various internal organs where toxicological responses may be elicited (Roesijadi and Robinson 1994). Cadmium accumulates in organs such as the gills, liver, kidney and gastrointestinal tract of fish in an unregulated manner (Chowdhury et al. 2003).

Studies revealed that MT induction is dependent on cell type and occurs primarily in the chloride cells of the gills and much less in other cell types (Dang et al. 2000). Cd ions (Cd^{2+}) that are in direct contact with gills could bind in a non-specific manner to mucopolysaccharides (constituents of mucoproteins, glycoproteins) present on the outside of the gills and this mucopolysaccharides barrier can depress Cd-uptake in the gills (Isani et al. 2009). Piscine MT mRNA level seems to be vary in a tissue specific manner according to species (Dang et al. 2009; Oliveira et al. 2010).

Decrease in metal concentration level was found to be inversely proportional to the dose. The decrease in the concentration after metal analysis may be attributed to the precipitation of the metal in test water as well as the hardness and relative higher pH of test solution causing lesser solubility of Cd (Playle et al. 1993).

The study indicates nonlinear response with the metal concentration, as ~ 2 fold decrease in MT mRNA level was reported with the increasing metal concentration (Fig. 3). For significant decrease in MT mRNA level in the tissues at higher concentration, it may be remarked that the acutely lethal concentrations affect the capacity of the cells to synthesise proteins so that MT synthesis cannot keep up with cadmium accumulation.

Due to which MT production becomes too slow to sequester all the Cd that enters the cells. This indicates that the limited capacity of the cells to bind Cd in MT is not the result of a direct toxic effect of Cd on the capacity of the cells to synthesize proteins.

Cadmium is initially taken up by the liver through circulatory system, where it can bind to MT and be stored. Some amount of Cd bound to MT leaks into plasma and subsequently is taken up by the kidney. This circulating Cd-MT complex is a potent nephrotoxicant (Klaassen and Liu 1997). In lysosomes of the kidney, cadmium is released from Cd-MT complex. When a critical concentration of Cd is reached in the kidney, renal injury occurs (Klaassen et al. 1999). It has also been suggested, Cd not bound to MT induces free radicals and lipid peroxidation, which may in turn depress renal functions (Thevenod and Friedmann 1999). Although tubular cells are able to synthesize MT themselves, the free cadmium ion will still exert adverse effects when it exceeds the synthetic capacity of tubular cells (Lu et al. 2001).

It has been shown that under acute and highly toxic circumstances, Cd can be directly transported via the blood to the kidneys, where it can induce MT-synthesis (Hollis et al. 2001). Moreover, it was observed that in the kidney the binding of Cd to the MT occurred at lower cytosolic Cd concentrations. At higher renal Cd concentrations the rate of MT induction in the kidneys did not appear to be high enough to keep track with the high Cd accumulation rates in order to bind all newly incoming Cd.

Clearly, the toxicity of Cd and the role of MT in the detoxification process both depend on the duration and concentration of the exposure. As such, a fixed critical cadmium tissue concentration above which toxic effects occur cannot be defined. Further, binding of Cd to MT will also have its effect on the regulation of zinc (Dallinger et al. 1997). For instance in the kidney, where the incoming Cd led to a total saturation of the Cd-MT pool, it is likely that the disturbance of the zinc homeostasis causes several indirect toxic effects.

It may be proposed that the initial effect of nonspecific Cd-binding to intracellular ligands (proteins) can be regarded as a toxic interaction and that detoxification of Cd by MT was by transfer of Cd to newly synthesised MT, that is, it was a “rescue” phenomenon (Huang 1993). Therefore, there will be a progressive inhibition of cellular processes including MT mRNA transcription with increasing concentration of Cd. Indeed these data where increasing concentration of Cd result in a progressively greater inhibition of MT mRNA synthesis (i.e., cytotoxicity) support such a proposal.

To interpret the results in terms of the role of MTs in the detoxification of cadmium in fish, two major factors should be considered. First, Cd competes with other elements for the available binding sites on the MT molecule. Since one of the functions of MT consists in the homeostatic regulation of zinc and copper, the concentration of these essential elements in organs will decrease the ability of cadmium to be bound to this protein. Secondly, Cd can also be bound to other molecules. Currently, no evidence exists for the presence of metal-binding proteins with a higher affinity for Cd than MT in teleosts. However, proteins with a lower affinity for the metal may also bind Cd, because of their relative high concentrations in the cell compared to MT. The influence of other elements and other cadmium-binding proteins will be the highest when low concentrations of Cd are present in the cell and MT concentrations are at their lowest level.

Conclusions

It may be concluded by the results of present study that dose and duration dependent MT mRNA expression occurs in a tissue specific manner in *C. punctata* after Cd exposure. In addition renal MT mRNA level also indicates the prolong presence of high concentration of metal in water bodies and thus, provides a possible index of susceptibility to the adverse effect of cadmium.

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References

APHA, AWWA, WPCF. 2005. Standard Methods for Examination of Water and Wastewater. 21st ed. American Public Health Association, Washington, DC, USA.

Bae H, Nam SS, Park HS, Park K. 2005. Metallothionein mRNA sequencing and induction by cadmium in gills of the Crucian Carp, *Carassius auratus.* J Health Sci 51(3): 284-290.

Baksi SM, Libbus N, Frazier JM. 1988. Induction of metal binding proteins in striped bass, *Morone saxatilis*, following cadmium treatment. Comp Biochem Physiol 91C(2): 355-363.

Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156-159.

Chowdhury MJ, Grosell M, McDonald DG, Wood CM. 2003. Plasma clearance of cadmium and zinc in non-acclimated and metal-acclimated trout. Aquat Toxicol 64: 259-275.

Dallinger R, Egg M, Kock G, Hofer R. 1997. The role of metallothionein in cadmium accumulation of Artic char (*Salvalinus alpinus*) from high alpine lakes. Aquat Toxicol 38: 47-66.

Dang F, Zhong H, Wang WX. 2009. Copper uptake kinetics and regulation in a marine fish after waterborne copper acclimation. Aquat Toxicol 94: 238-244.

Dang ZC, Flik G, Ducouret B, Hogstrand C, Bonga SEW, Lock RAC. 2000. Effects of copper on cortisol recepror and metallothionein expression in gills of *Oncorhynchus mykiss.* Aquat Toxicol 51: 45-54.

George SG, Todd K, Wright J. 1996. Regulation of metallothionein in teleosts: Induction of MTmRNA and protein by cadmium in hepatic and extrahepatic tissues of a marine flatfish, the Turbot (*Scophthalmus maximus*). Comp Biochem Physiol 113C(2): 109-115.

Hollis L, Hogstrand C, Wood CM. 2001. Tissue-specific cadmium accumulation, metallothionein induction, and tissue zinc and copper levels during chronic sublethal cadmium exposure in juvenile rainbow trout. Arch Environ Contam Toxicol 41: 468-474.

Huang PC. 1993. Metallothionein structure/function interface. In: Metallothionein III: Biological roles and medical implications (ed. By KT Suzuki, N Imura and M Kimura), pp. 407-426. Birkhauser Verlag, Basel.

Isani G, Andreani G, Cocchioni F, Fedeli D, Carpene E, Falcioni G. 2009. Cadmium accumulation and biochemical responses in *Sparus aurata* following sub-lethal Cd exposure. Ecotoxicol Environ Saf 72(1): 224-230.

Klaassen CD, Liu J. 1997. Role of metallothionein in cadmium induced hepatotoxicity and nephrotoxicity. Drug Metab Rev 29: 79-102.

Klaassen CD, Liu J, Choudhry S. 1999. Metallothionein: an intracellular protein to protect against cadmium toxicity. Annu Rev Pharmacol Toxicol 39: 267-294.

Knapen D, Reynders H, Bervoets L, Verheyen E, Blust R. 2007. Metallothionein gene and protein expression as a biomarker for metal pollution in natural gudgeon populations. Aquat Toxicol 82: 163-172.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. Methods 25: 402-408.

Lu J, Jin T, Nordberg G, Nordberg M. 2001. Metallothionein gene expression in peripheral lymphocytes from cadmium-exposed workers. Cell Stress Chaperones 6(2): 97-104.

Margoshes M, Vallee BL. 1957. A Cadmium protein from renal cortex. J Am Chem Soc 79: 4813-4814.

Oliveira M, Ahmad I, Maria VL, Serafim A, Bebianno MJ, Pacheco M, Santos MA. 2010. Hepatic metallothionein concentrations in the golden grey mullet (*Liza aurata*) - relationship with environmental metal concentrations in a metal-contaminated coastal system in Portugal. Mar Environ Res 69(4): 227-233.

Olsson PE, Kille P. 1997. Functional comparison of the metal-regulated transcriptional control regions of metallothionein genes from cadmium-sensitive and tolerant fish species. Biochim Biophys Acta 1350: 325-334.

Playle RC, Dixon DG, Burnison K, 1993. Copper and cadmium binding to fish gills: estimates of metal-gill stability constants and modelling of metal accumulation. Canadian J Fish Aquat Sci 50: 2678-2687.

Roesijadi G, Robinson WE, 1994. Metal regulation in aquatic animals: mechanisms of uptake, accumulation, and release. In: Aquatic Toxicology- Molecular, Biochemical and Cellular Perspectives (ed. By DC Mullins and GK Ostrander), pp. 387-419. Lewis Publishers, Florida.

Sambrook JD, Russell DW. 2001. Molecular Cloning- A Laboratory Manual, 3rd edn. CSHL Press, Cold Spring Harbor, New York.

SPSS 16.0.2. 2008. Command Syntax Reference. SPSS Inc, Chicago, Illinois.

Thevenod F, Friedmann JM. 1999. Cadmium-mediated oxidative stress in kidney proximal tubule cells induces degradation of Na^+/K^+-ATPase through proteasomal and endo-/lysosomal proteolytic pathways. FASEB J 13: 1751-1761.

Tiwari M, Nagpure NS, Saksena DN, Lakra WS. 2010. Time kinetic study of metallothionein mRNA expression due to heavy metal, cadmium exposure in freshwater murrel, *Channa punctata* (Bloch). J Ecophysiol Occup Hlth 10: 85-96.

Tom M, Auslander M. 2005. Transcript and protein environmental biomarkers in fish- a review. Chemosphere 59: 155-162.

Zalups RK, Ahmad S. 2003. Molecular handling of cadmium in transporting epithelia. Toxicol Appl Pharmacol 186: 163-188.

Zalups RK, Koropatnick J. 2000. Molecular Biology and Toxicology of Metals. Taylor and Francis, New York.