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RESEARCH ARTICLE

Sequencing and Expression of Metallothionein mRNA in Freshwater Murrel after Cadmium Exposure

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Abstract Metallothionein (MT) is a cysteine-rich, low molecular weight metal sequestering protein that has been shown to be involved in essential metal homeostasis and in detoxification of heavy metals. In the study sequences of MT genomic DNA and cDNA of freshwater murrel, *Channa punctata* (Bloch) were reported. cDNA was verified as the *C. punctata* MT gene by the characteristics of its predicted translation product, namely a high cysteine content, conserved Cys-Cys, Cys-X-Y-Cys, and Cys-X-Cys motifs, and a molecular weight of around 6 kDa. The quantitative real-time reverse-transcriptase polymerase chain reaction (q-RT-PCR) study was carried out in liver, kidney and gill tissues after exposure of the organism to waterborne cadmium for 8 h. At all the time points (viz. 1, 2, 4 & 8 h), a significant up regulation of MT transcript level was observed only in the liver. The results of time kinetics study indicated that expression pattern of *C. punctata* MT mRNA was found to be in tissue specific manner and the transcript level in the liver could be used as a biomarker of heavy metal contamination in water bodies.

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Introduction

Metal's utilization by humans influences its potential health effects in at least two major ways: first, by environmental transport, that is, by anthropogenic contributions to air, water, soil and food, and second, by altering the speciation or biochemical form of elements [1].

Cadmium (atomic weight, 112.41; periodic table group, IIB; valence, +2) is a toxic metal. Because of its non-corrosive properties, its main use is in electroplating or galvanizing. It is also used as a colour pigment for paints and plastics and as a cathode material for nickel–cadmium batteries. Cadmium (Cd) is a by-product of zinc and lead mining and smelting, which are important sources of environmental pollution.

Living organisms respond at the cellular level to a variety of stimuli as diverse as heat, heavy metals, anoxia, amino acid analogues, infection and inflammatory agents by synthesizing different groups of highly conserved polypeptides [2]. Of these a group of induced proteins is represented by low-molecular-mass, cysteine-rich, heavy-metal-binding proteins known as metallothioneins (MTs). They lack aromatic amino acids, have a selective capacity to bind heavy-metal-ions via mercaptide linkages, and are ubiquitous in distribution [3, 4].

The characterization of MTs from different species has revealed that the proteins are uniform in length, consisting of 60–61 amino acids. The most unique feature is the high content of cysteine, generally 20 out of 61 amino acids. The alignment of Cys-Cys, Cys-X-Cys and Cys-X-Y-Cys sequences, where X and Y are amino acids other than

cysteine, is the criterion that allows the distinction between different structural MT classes and that leads to many isoforms of the same protein [5].

In the present, it is paid more attention to that MTs in the aquatic animal can work as a bio-indicator responding to environmental pollution, especially MT gene expression in fishes exposed in the field may be assessed to evaluate the contamination in waters. In the present study sequences of MT genomic (g)DNA and complementary (c)DNA of *Channa punctata* (Bloch), a fish species, widely distributed in Indian freshwater system, were analyzed. Furthermore, expression pattern of MT mRNA in candidate tissues was also investigated using quantitative real-time reverse-transcriptase polymerase chain reaction after heavy metal Cd, exposure.

Materials and Methods

Experimental Animals

Healthy specimens of freshwater murrel, *Channa punctata* (Bloch) were procured from local sources. The specimens had an average wet weight and length of 21.34 ± 2.79 g and 12.05 ± 0.56 cm (mean ± SE), 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 heavy metal exposure. The fishes were fed, ad libitum, with boiled chicken eggs. No mortality was occurred during the acclimation period.

Test Chemical

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

In Vivo Exposure Experiment

The specimens were exposed to the sub lethal concentration of waterborne cadmium i.e., 1/4th of 96 h $LC_{50} = \sim 3.74$ mg L^{-1} [6]. The exposure was continued up to 8 h and tissue sampling was done at five time points—$t_1 = 0$, $t_2 = 1$, $t_3 = 2$, $t_4 = 4$, and $t_5 = 8$ h at the rate of five specimens per duration ($n = 5$). The specimens maintained in tap water without the test chemical were considered as calibrator ($t_1 = 0$ h) for the time-course study. The physico-chemical properties of test water were determined according to the standard procedures [7].

For DNA extraction, the blood was collected from the fish by caudal vein puncture technique. At each sampling time point, the liver, kidney and gill tissues were taken from each individual. The tissue samples 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 Amplification of MT gDNA and cDNA

The primers MT-1 F′ (CCTGCAACTGCGGAGGAT) and MT-1 R′ (TGGTGTCGCATGTCTTTCCT) for the amplification of MT gDNA of *C. punctata* were designed on the basis of those for *Oreochromis mossambicus* (GenBank accession no. AY257202). The reverse transcription-polymerase chain reaction (RT-PCR) primers MT-2 F′ (CTG CAACTGCGGAGGA) and MT-2 R′ (GGTGTCGCATGT CTTTCCTT) for MT cDNA synthesis as well as real-time PCR amplifications 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 quantitative real-time reverse-transcriptase polymerase chain reaction (q-RT-PCR). The sequence information of β-actin mRNA in *C. gachua* (GenBank accession no. GQ219743) was used for designing of the β-actin Forward (GTGCC CATCTACGAGGGTTA) and β-actin Reverse (AAGGAA GGAAGGCTGGAAGA) primers.

Genomic DNA Extraction and MT gDNA Amplification

Genomic DNA was carefully extracted from 10 μL anticoagulated blood using DNeasy Blood & Tissue DNA extraction kit (Qiagen) according to the manufacturer's instructions.

PCR amplification of MT gDNA using gene specific primers was performed in a 20 μL total reaction mixture volume for 30 cycles of 95 °C for 1 min, 95 °C for 1 s, 57 °C for 5 s, 72 °C for 25 s and 72 °C for 10 s. The PCR reaction was set in a thermal cycler (PTC-0200 DNA Engine Cycler, Bio-Rad Laboratories, Inc., USA) using PyroStart Fast PCR Master Mix (Fermentas International Inc., Canada) following manufacturer's instructions.

RNA Extraction and Reverse-Transcriptase Polymerase Chain Reaction

Total RNA was isolated from each tissue samples using acid guanidinium isothiocyanate–phenol–chloroform extraction following the TRI Reagent (Molecular Research Center, Inc. Cincinnati, OH) extraction protocol [8]. For quality control purposes, samples were quantified spectrophotometrically. Only samples with 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; EDTA, 10 mM) buffer [9]. Visual inspection of the gels showed no signs of RNA degradation in any of the samples.

Gene specific reverse transcription was performed using the RevertAid H Minus First Strand cDNA Synthesis Kit as per the manufacturer's instructions (Fermentas). The RT-PCR was carried in a thermal cycler (PTC-0200 DNA Engine Cycler, Bio-Rad) for 30 cycles of 94 °C for 4 min, 94 °C for 45 s, 57 °C for 45 s, 72 °C for 60 s, and 72 °C for 2 min.

Nucleotide Sequencing

For the sequence analysis of MT gDNA, MT and β-actin cDNA, the amplified PCR products were gel purified using the QIAquick PCR purification kit according to the manufacturer's instructions (Qiagen), and applied to an automatic 96 capillary DNA sequencer (ABI 3730xl DNA Analyzer, Applied Biosystems, Foster City, CA, USA).

Expression of MT mRNA

The q-RT-PCR amplifications were carried out in LightCycler real-time PCR (LightCycler 480 System, Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) using QuantiTect SYBR Green RT-PCR kit according to the manufacturer's instructions (Qiagen). For the q-RT-PCR data analysis, a method of comparison of C_T values, the $2^{\wedge}(-\Delta\Delta C_T)$ method, was used [10].

Data Analysis

Statistical analyses were performed using SPSS (version 16.0.2, 2008) computer software [11]. The gene expression data [$2^{\wedge}(-\Delta\Delta C_T)$ values] were of exponential in nature and log transformed before applying data analysis. Comparisons between the effects of exposure duration on mean relative tissue specific gene expression was done using multiple comparison technique i.e., Tukey's HSD post hoc test ($\alpha = 0.01$).

Results

Physico-Chemical Properties of the Test Water

During the experiment, temperature of the test water varied from 19.3 to 22.5 °C, and pH value ranged from 7.14 to 7.95. The dissolved oxygen concentration was normal, varying from 6.72 to 8.13 mg L^{-1} during the experiment. The conductivity of the test water ranged from 239 to 303 µS cm^{-1}. The total hardness and total alkalinity of the test water varied from 169 to 198 and 242 to 278 mg L^{-1} as $CaCO_3$, respectively during experimental period.

Sequence of MT gDNA and cDNA

Using PCR protocol, a discrete band of approximately 500 bp in length was examined in 1.5 % TAE agarose gel (Fig. 1). It was sequenced and a MT gDNA fragment of 559 bp in length was detected. Figure 2 shows the MT gDNA sequence (GenBank accession no. FJ869867). The sequence analysis of gDNA revealed 87 bp coding sequence (CDS) region along with a 472 bp long 5′-untranslated region (5′-UTR) (Fig. 2a). The 87 bp long CDS encodes a polypeptide of 28 amino acids (partial N-terminal and C-terminal) (GenBank protein i.d. ACR25278) for MT in *C. punctata* (Fig. 2b). Deduced amino acid sequence shows four characteristic Cys-Cys, Cys-X-Y-Cys, and Cys-X-Cys motifs.

The hypothetical deduced amino acid sequence was compared with those of the published sequences using NCBI blastp algorithm (Table 1). The alignment of putative MT protein sequence (GenBank protein i.d. ACR25278) with other sequences available in the GenBank database using CLUSTAL W (1.81) multiple sequence alignment program confirmed that the sequenced genomic DNA fragment was indeed a MT gDNA fragment, and the deduced 28 amino acids long sequence from the MT gDNA corresponds to *C. punctata* MT (Fig. 3).

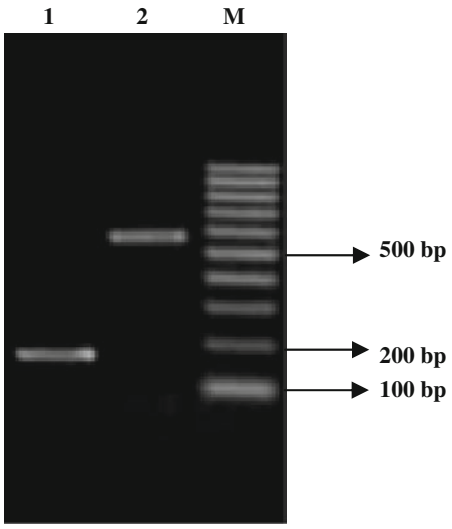

Fig. 1 RT-PCR and PCR Product of Metallothionein of *C. punctata*. Amplified cDNA (from kidney), and gDNA (from blood) product with gene specific primers electrophoresed on 1.5 % agarose gel. *Lane 1*, cDNA product (165 bp) amplified by primers of MT-2 (F′, R′); *lane 2*, gDNA product (559 bp) amplified by primers of MT-1 (F′, R′); *lane M*, 100 bp molecular marker

(a)

```
   1 gcacgtgcac aaagtgctca ttatttgcca tgtggtatca gcctacaact ggactcaggt
  61 gaggtgtaca actgactcac actgggtata agagctgttg ccatgacatt ggccttctgt
 121 ttgagagttg agcctgtgac catctacatc agaccgcaga cttcagaaaa agatcaggtg
 181 agacgctaaa ctgctcctgc gcttcaggtt cgaataacca tttattttct aatttattgc
 241 ctcactggtg tagaacacca cattattaga ttgagtctaa acatgcagaa gctgtaataa
 301 caccaggctg aaagaggagc aggacatttg cttctgggag aaacactgtg tgatcgggat
 361 agaacatatg aaataaataa tgttttagtt ttgctcattt tgttccttct acttgcagca
 421 atggcgttcc agggaaatgt gatcagctcg cagcctcagg tcacagtcat agAAGCTGCT
 481 GCGACTGCTG CCCATCCGGC TGCAGCAAAT GCGCCTCTGG CTGCGTGTGC AAAGGAAAGA
 541 CATGCGACAC CAGCTGCTG
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(b)

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474     AGC TGC TGC GAC TGC TGC CCA TCC GGC TGC AGC AAA TGC GCC TCT   518
1        S   C   C   D   C   C   P   S   G   C   S   K   C   A   S    15

519     GGC TGC GTG TGC AAA GGA AAG ACA TGC GAC ACC AGC TGC          557
16       G   C   V   C   K   G   K   T   C   D   T   S   C          28
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Fig. 2 Sequence of MT gDNA (559 bp), and CDS region <473..> 559 (**a**), and deduced amino acid sequence (**b**), GenBank accession no. FJ869867 in freshwater murrel, *C. punctata*. **a** *Underlined* and *uppercase letters* depict CDS (Coding Sequence); **b** Below the sequence line—*bold* and *uppercase letters* shows amino acid sequence, 28 aa (protein i.d. -ACR25278). The *bold* and *highlighted letters* show characteristic Cys-Cys, Cys-X-Y-Cys, and Cys-X-Cys motifs

Table 1 Sequences showing significant alignments with *C. punctata* MT (GenBank protein i.d. ACR25278)

Fish species	Score (bits)	*E* value
Channa punctata MT[a]	96.5	3e−19
Graying (*Thymallus thymallus* MT-A)	96.5	3e−19
Chinook salmon (*Oncorhynchus tshawytscha* MT-A)	96.5	3e−19
Arctic char (*Salvelinus alpinus* MT-A)	96.5	3e−19
Oreochromis aureus MT	96.5	3e−19
Oreochromis mossambicus MT	96.5	3e−19
Atlantic salmon (*Salmo salar* MT-A)	96.5	3e−19
Chum salmon (*Oncorhynchus keta* MT-A)	93.5	2e−18
Coho salmon (*Oncorhynchus kisutch* MT-A)	93.1	3e−18

[a] Deduced amino acid sequence from CDS of *C. punctata* MT gDNA (GenBank accession no. FJ869867) was compared with the published sequences at NCBI using blastp (protein–protein BLAST) algorithm

After RT-PCR protocol a discrete band of approximately 150 bp in length was obtained (Fig. 1). Using sequencing reaction it revealed a 165 bp long open reading frame (GenBank accession no. FJ869865) (Fig. 4). The CDS (<1..>165) encodes a hypothetical polypeptide of 55 amino acids (GenBank protein i.d. ACR25276). Deduced amino acid sequence of MT protein (partial N-terminal and C-terminal) shows five characteristic Cys-Cys, Cys-X-Y-Cys, and Cys-X-Cys motifs, typically found in this protein family.

Using NCBI blastp algorithm the putative MT protein sequence from MT cDNA (GenBank protein i.d. ACR25276) was compared with those of the published sequences in the databases (Table 2). The CLUSTAL W (1.81) multiple sequence alignment program conformed that the sequenced mRNA fragment was indeed a MT cDNA and corresponds to a *C. punctata* MT protein sequence, after aligning deduced 55 amino acids long polypeptide with other sequences available in the GenBank database (Fig. 5). The predicted protein shows characteristic richness of cysteine (21.82 %), and is characterized by a molecular weight of 5.7 kDa (using BioEdit Sequence Alignment Editor Computer program) which in the same order as the MT molecular weight.

Time Kinetics of MT mRNA Expression

The varied MT mRNA expression pattern was observed according to the tissues viz., liver, kidney and gill in the time course study for 8 h after waterborne exposure to 3.74 mg L^{-1} Cd (Fig. 6). Duration dependent up regulation in MT mRNA expression was found in the liver only. The weakest MT mRNA expression was found in gill tissues, where at all the time points it was below basal level, and the strongest in the liver, indicating that this tissue is a major site of MT transcription. In kidney at all the time points, MT mRNA expression was found to be below basal level.

Liver MT mRNA was found to be significantly heightened with 0.67, 0.37, 0.52, and 0.58 (logarithm values) or around 5-, 2-, 3-, and 4-fold induction level, after 1, 2, 4, and 8 h cadmium exposure, respectively ($P < 0.01$). It diminishes significantly after a 1 h exposure ($P < 0.01$).

```
Oreochromis_mossambicus      MDPCECAKTGTCNCGGSCSCTKCSCKSCKKS-CCDCCPSGCSKCASGCVC
Oncorhynchus_keta            MDPCECSKTGSCNCGGSCKCSNCACTSCKKASCCDCCPSGCSKCASGCVC
Salmo_salar                  MDPCECSKTGSCNCGGSCKCSNCACTSCKKASCCDCCPSGCSKCASGCVC
Oreochromis_aureus           MDPCECAKTGTCNCGGSCTCTKCSCKSCKKS-CCDCCPSGCSKCASGCVC
Oncorhynchus_kisutch         MDPCECSKTGSCNCGGSCKCSNCACTSCKKASCCDCCPSGCSKCASGCVC
Thymallus_thymallus          -DPCECSKTGSCKCGGSCKCSNCACTSCKKPSCCDCCPSGCSKCASGCVC
Salvelinus_alpinus           MDPCECSKTGSCNSGGSCKCSNCACTSCKKASCCDCCPSGCSKCASGCVC
Oncorhynchus_tshawytscha     MXPCECSKTGSCNCGGSCKCSNCACTSCKKASCCDCCPSGCSKCASGCVC
C._punctata                  ------------------------------SCCDCCPSGCSKCASGCVC
                                                            **********************

Oreochromis_mossambicus      KGKTCDTSCCQ
Oncorhynchus_keta            KGRTCDTSCC-
Salmo_salar                  KGKTCDTSCCQ
Oreochromis_aureus           KGKTCDTSCCQ
Oncorhynchus_kisutch         KGXTCDTSC--
Thymallus_thymallus          KGKTCDTSCCQ
Salvelinus_alpinus           KGKTCDTSCCQ
Oncorhynchus_tshawytscha     KGKTCDTSC--
C._punctata                  KGKTCDTSC--
                             ** ******
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Fig. 3 *C. punctata* MT deduced protein sequence aligned with MT from other fish species using CLUSTAL W (1.81) multiple sequence alignment. The GenBank accession numbers of the proteins are as follows: *O. mossambicus* MT (P52726); Chum salmon, *O. keta* MT-A (ABA03254); Atlantic salmon, *S. salar* MT-A (NP_001117149); *O. aureus* MT (Q7ZZP9); Coho salmon, *O. kisutch* MT-A (ABA03252); graying, *T. thymallus* MT-A (ABA03250); Arctic char, *S. alpinus* MT-A (AAP31403); Chinook salmon, *O. tshawytscha* MT-A (ABA03255); and Snakehead murrel, *C. punctata* MT (ACR25278). *Represents conservation of amino acid among all sequences. The characteristic Cys-Cys, Cys-X-Y-Cys, and Cys-X-Cys motifs are represented by *bold letters*

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gca cgt gca caa agt gct cat tat ttg cca TGT ggt atc agc  42
 A   R   A   Q   S   A   H   Y   L   P   C   G   I   S   14

cta caa ctg gac tca ggt gag gtg tac aac ctc agc TGC TGC  84
 L   Q   L   D   S   G   E   V   Y   N   L   S   C   C   28

gac TGC TGC cca tcc ggc TGC agc aaa TGC gcc tct ggc TGC 126
 D   C   C   P   S   G   C   S   K   C   A   S   G   C   42

gtg TGC aaa gga aag aca tgc gac acc agc TGC TGC cag     165
 V   C   K   G   K   T   C   D   T   S   C   C   Q      55
```

Fig. 4 Sequence of MT cDNA (165 bp), GenBank accession no. FJ869865 in freshwater murrel, *C. punctata.* (Below the sequence line)—*uppercase letters* shows deduced amino acid sequence of MT protein, 55 aa (protein i.d. -ACR25276) from CDS (coding sequence); *bold, uppercase*, *underlined letters* shows Cysteine. The *bold* and *highlighted letters* show characteristic Cys-Cys, Cys-X-Y-Cys, and Cys-X-Cys motifs

Table 2 Sequences showing significant alignments with *C. punctata* MT (GenBank protein i.d. ACR25276)

Fish species	Score (bits)	E value
Channa punctata MT[a]	107	3e−22
Arctic char (*Salvelinus alpinus* MT-A)	56.2	1e−06
Atlantic salmon (*Salmo salar* MT-A)	55.8	1e−06
Graying (*Thymallus thymallus* MT-A)	55.5	2e−06
Chum salmon (*Oncorhynchus keta* MT-A)	52.8	1e−05
Oreochromis aureus MT	46.6	7e−04

[a] Deduced amino acid sequence from CDS of *C. punctata* MT cDNA (GenBank accession no. FJ869865) was compared with the published sequences at NCBI using blastp (protein–protein BLAST) algorithm

Post 2 h Cd exposure, liver showed significant ($P < 0.01$) up regulation of MT transcript level. Non significant up regulation in transcript level was found on 8 h in comparison to 4 h post Cd exposure. The difference in the effect of metal exposure on MT mRNA level in the tissues under study was found significant. For pair wise comparison, all differences were found significant.

Discussion

The primary structure of MT is evolutionarily conserved, especially with respect to the position of cysteine residues

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Oncorhynchus_keta          MDPCECSKTGSCNCGGSCKCSNCACTSCKKASCCDCCPSGCSKCASGCVC
Salmo_salar                MDPCECSKTGSCNCGGSCKCSNCACTSCKKASCCDCCPSGCSKCASGCVC
Oreochromis_aureus         MDPCECAKTGTCNCGGSCTCTKCSCKSCKKS-CCDCCPSGCSKCASGCVC
Salvelinus_alpinus         MDPCECSKTGSCNSGGSCKCSNCACTSCKKASCCDCCPSGCSKCASGCVC
Thymallus_thymallus        -DPCECSKTGSCKCGGSCKCSNCACTSCKKPSCCDCCPSGCSKCASGCVC
C._punctata                ---ARAQSAHYLPCGISLQLD---SGEVYNLSCCDCCPSGCSKCASGCVC
                                                   **********************

Oncorhynchus_keta          KGRTCDTSCC-
Salmo_salar                KGKTCDTSCCQ
Oreochromis_aureus         KGKTCDTSCCQ
Salvelinus_alpinus         KGKTCDTSCCQ
Thymallus_thymallus        KGKTCDTSCCQ
C._punctata                KGKTCDTSCCQ
                           ** ********
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Fig. 5 *C. punctata* MT deduced protein sequence aligned with MT from other fish species using CLUSTAL W (1.81) multiple sequence alignment. The GenBank accession numbers of the proteins are as follows: Chum salmon, *O. keta* MT-A (ABA03254); Atlantic salmon, *S. salar* MT-A (NP_001117149); *O. aureus* MT (Q7ZZP9); Arctic char, *S. alpinus* MT-A (AAP31403); Graying, *T. thymallus* MT-A (ABA03250); and Snakehead murrel, *C. punctata* MT (ACR25276). *Represents conservation of amino acid among all sequences. The characteristic Cys-Cys, Cys-X-Y-Cys, and Cys-X-Cys motifs are represented by *bold letters*

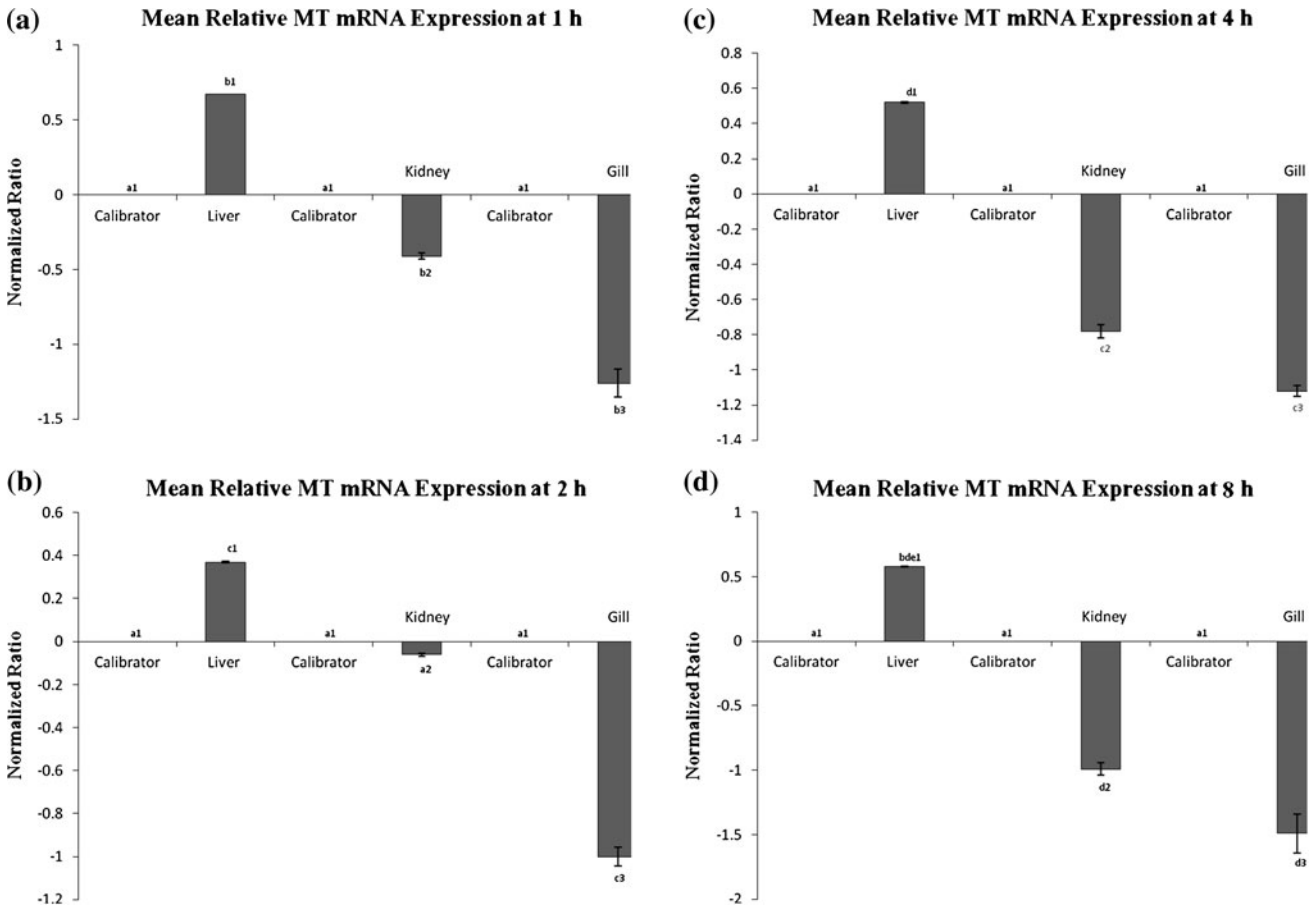

Fig. 6 Mean relative [log 2^(−ΔΔC_T)] metallothionein gene expression (±SD) in liver, kidney, and gill tissues of *C. punctata* ($n = 5$) after exposure to Cd for 1 h (**a**), 2 h (**b**), 4 h (**c**), 8 h (**d**). Calibrator refers to the relative expression (ΔC_T) in control sample ($t_1 = 0$ h). Different *alphabet superscript letters* denote significant difference ($P < 0.01$) in gene expression between time points within tissues. Different *numeric superscript* values denote significant difference in gene expression ($P < 0.01$) between tissues within time point

[12]; hence, physicochemical characteristics of fish and mammalian MTs are similar. In the present study, we identified the MT gene, a cadmium-binding protein in an Indian freshwater murrel, *C. punctata*. From the result of sequence analysis, the amplified PCR products were identified as a part of MT cDNA and gDNA, respectively. All the 165 bp sequenced are included in the translated sequence of *C. punctata* MT, and no un-translated region (UTR) is present. Comparing the MT cDNA of *C. punctata* with metallothioneins from other fish species allowed us to conclude that there are important similarities between the MTs in the teleosts (Fig. 5).

However, irrespective of considerable structural similarity, fish MTs differ from those of mammals and birds, as they lack the asparagine residue at position 4 in the N-terminal part of the molecule [12]. The murrel MT protein shows typical MT pattern, such as lack of aromatic residues and presence of five glycine residues. In the majority of vertebrates, two major isoforms of MT, (designated MT-I and MT-II) that can be resolved by ion exchange chromatography, are found. These isoforms are encoded by two co-ordinately regulated genes lying about 6 kb apart on the same chromosome [13]. In the murrel, only one isoform appears to be present.

In the 8 h time kinetic study, the MT transcript level was found in the following order: liver>kidney>gill due to waterborne Cd-uptake. Only liver showed up regulation in MT transcript level, whereas the remaining two have the same below basal level, and seems to be the major site for metal uptake.

Although induction of MT in gill and kidney has been reported in fishes, liver seems to be more actively involved in the formation and storage of MT as compared to other organs. It may be co-related to the fact that in comparison to the liver and kidney, relatively little Cd accumulated in the gills. This transcript level order may be attributed to the lower metal-binding capacity of the gills as a consequence of the low gill MT concentrations present [14].

Studies revealed that MT induction is dependent on cell type and occurs primarily in the chloride cells of the gills and much less in the other cell types [15]. Cd transport through the epithelial gills is not well understood. Cd ions that are in direct contact with gills could bind in a non-specific manner to the mucopolysaccharides present on the outside of the gills and this mucopolysaccharides barrier can depress Cd-uptake in the gills [16]. In other words, gills could be the first target for Cd accumulation before its distribution to other organs [17]. After Cd absorption by the gills, the metal is probably transferred to the liver where it induces the synthesis of MT, which in turn is delivered to the blood and finally accumulated in the kidney [18, 19]. The up regulation in liver MT mRNA level attests liver as a potent organ for Cd detoxification in *C. punctata*.

The results clearly indicate that heightened MT mRNA level was observed in *C. punctata* after exposure to waterborne Cd, only in the liver. The metal sequestering function of MT protein that has been shown to be involved in essential metal homeostasis and in the detoxification of heavy metals is performed by the formation of a metal-metallothionein complex. Metals induce MT by displacing zinc from the intracellular binding sites, making additional Zn available for interacting with the inhibitor. Increased metal influx result in induction of thionein, mediated by displaced Zn, and increased metal flux to MTs.

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Conflict of interest The authors declare that there are no conflicts of interest.

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