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Research Article

METALLOTHIONEIN EXPRESSION IN MARINE CATFISH *ARIUS ARIUS* LIVER ON EXPOSURE TO CADMIUM USING IMMUNOHISTOCHEMISTRY AND WESTERN BLOT

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ABSTRACT

Objective: MT expression/induction has been identified using western blotting and immunohistochemical techniques have been proposed to specifically detect MT levels of liver. These immunohistochemical techniques are very sensitive and allow the detection of very low amount of proteins due to the use of antibodies. The objective of our study to identifying the MT expressions and localizations were analyzed in liver tissue by western blotting and immunohistochemical methods in marine catfish *Arius arius.*

Methods: MTs were localized by immunohistochemical method in liver treated with 20 mg/L of $CdCl_2$ for 72 hrs. MT expression (MT-I and MT-II) levels were analyzed by Western blotting method (using Mouse Monoclonal Anti-Metallothionein primary and Horseradish Peroxidase (HRP) secondary antibody). MT bands were detected using enhanced chemiluminescence system (West Femto Super Signal detection kit, Thermo Scientific Inc. USA) method.

Results: Antibodies showed a positive cross reactivity with MT proteins, MT immune reaction was high in the cytoplasm of hepatocytes and lesser in erythrocytes in Cd exposed fish liver. Western blotting showed increased MT expression levels in Cd treated tissues when compared with control tissues.

Conclusion: MT expression on exposure to Cd can be observed using immunohistochemistry mainly in the cytoplasm of hepatocytes of the liver tissues in *A. arius*. Western blotting showed that the MT expression levels were increased in fish on successive exposure to Cd. The differential characteristic localization/induction of MTs in different cell types described here suggests that the quantification of the specific expression of MT may be used in bio-monitoring programs as a biomarker of Cd exposure in aquatic environments.

Keywords: Metallothionein; Immunohistochemistry; Western blot; *Arius arius*; Cadmium; Liver

INTRODUCTION

Cd is a ubiquitous toxicant and most deleterious heavy metals [1]. Exposure of fish to low concentrations of this metal may lead to an increased body concentration that can results in several toxic effects including tissue damages, respiratory changes, vertebral alterations and ultimately death [2]. In metal detoxifications, MTs may reduce the toxic effects of metals by debasing the ratio of the uptake of heavy metal ions peroxides into cells [3, 4]. An important property assigned for MTs have been proposed to play a major role in metal storage and detoxification of heavy metals (especially Cd^{2+}). It may be noted that in higher organisms [5, 4].

MTs are a low molecular weight (6-14 kDa), cysteine-rich, non-enzymatic proteins ubiquitous in the animal kingdom [6], it was first described in the equine kidney by Margoshes and Vallee in 1957 [7]. The MT family is composed 4 isoforms: MT-I, MT-II, MT-III, and MT-IV [8]. MT-I and MT-II Co-exists in all tissues [6, 9]. Metallothionein-III is expressed mainly in brain only [10], and MT-IV is expressed in stratified squamous epithelium [11].

Heavy metals (especially Cd^{2+}) hormones, inflammation, acute stress and many chemicals can induce MT-I and MT-II activity [12]. The amino acid sequences of MTs from many mammalian sources reveal that all contain approximately 61 amino acids. It does not contain aromatic amino acids and histidine, one third of cysteine residues are present. All cysteines are known to participate in the coordination of 7 mol of Cd per mol of MT [13].

The immunochemical and western blot techniques are very sensitive and allow the detection of very low amount of proteins due to the use of specific antibodies. Immunochemical analyses might be of great importance allowing the detection of the specific cell types expressing MTs in organs of great complexity [14]. Cd accumulation and MT induction have been studied in major organ liver in different fish species [15, 16]. Hepatocyte cells are mainly found in liver, it makes up to 70-85% of liver cytoplasm mass in all organisms. Hepatocytes cells are involved in detoxification, excretion and modification of exogenous and endogenous substances [17].

In the present study, a marine catfish *A. arius* were exposed to a sublethal concentration of $CdCl_2$ 20 mg/L for 72 hrs. The main purpose of this study was to exhibit Cd-induced localization of MT expression in liver using immunohistochemistry technique and MT expression levels were determined by western blotting technique. The specific detection of MT expression may allow accurate identification of metal response that might usefully serve as subjects for biomarker assessments of water quality and MT can be used as a biomarker of Cd metal ion contamination.

MATERIALS AND METHODS

Experimental design

The Marine catfish *A. arius* were collected along the Coromandel Coast in the Bay of Bengal, Chennai, Tamil Nadu, Southern India. The fishes were acclimatized in the laboratory in a stone tank for 7 days at room temperature (30 $\pm 2^0$C). Aqueous solution of Cadmium chloride ($CdCl_2.6H_2O$) was used; twenty fishes were used in these experiments and weight approx. 100 to 150g. Two groups of 5 fish were exposed to 20ppm of $CdCl_2$ for 72 hrs, remaining 10 fishes were used as controls and were not exposed to Cd. Tanks were continuously aerated, water was changed every day. The content of Cd and other heavy metals were analyzed before the start of the study and found to be below detectable levels (BDL) to rule out their role or influence in the experiments.

Western Blots

Protein expression was assessed by western blotting method for MT and β-Actin in control and 24, 48 and 72 hrs of Cd treated (20 mg/L) marine catfish *A. arius* liver groups. MT protein (MT-I and MT-II) was identified and expressions were quantified, total proteins were extracted from fish liver tissues were lysed using RIPA buffer containing 1X protease inhibitor following which protein concentrations were measured using the Lowry's method (Lowry *et*

al., 1951). Proteins (20–50 µg) were electrophoresed in 15% SDS-PAGE and then transferred onto PVDF membranes. The membranes were incubated with primary antibody (Mouse monoclonal anti-MT antibody (Cat No. UC1MT (ab12228)), 1:1000 dilutions) against MT I and II in Tris-buffered saline. After being washed, the membranes were incubated with HRP conjugated secondary antibody (1:500). MT bands were detected using enhanced chemiluminescence system (West Femto Super Signal detection kit, Thermo Scientific Inc. USA) method.

Immunohistochemistry

Immunohistochemical evaluation of MT in fish was investigated at 48 hrs following MPTP administration. Five micrometer-thick paraffin sections through matched coronal levels of the MT were stained with Mouse monoclonal anti-MT primary antibody (1:50), using standard immuneperoxidase techniques. Briefly paraffin sections of fish liver were deparaffinized and hydrated with distilled water. Antigenic sites were exposed by incubating sections in Antigen Retrieval solution (Trisodium citrate pH= 6.2) for 20 min at 90°C using microwave oven. Following retrieval, slides were cooled in distilled water for 5 min. Phosphate buffered saline was used for washing between each step. Endogenous peroxidase activity was quenched by treating the sections with a 3.0% hydrogen peroxide. Nonspecific binding was blocked by 1hr incubation in 1.5% BSA. MT immunoreactivity was detected with an HRP-conjugated IgG secondary antibody at 1:100 dilutions and ImmunoCruz mouse ABC Staining kit. All slides were counterstaining with Mayer's hematoxylin and eosin visualized in light microscopy (Motic DMB1–2MP, China) (Stephenson et al., 2009).

Statistical analysis

The data obtained from the MT protein expression of *A. arius* liver treated with Cd and control groups were subjected to statistical analysis using one-way analysis of variance (ANNOVA). Newman-Keuls test was used to assess the significance of individual variations between the treated groups using GraphPad Prism, version 5.0. The values of $p<0.05$ were considered statistically significance.

RESULTS

Analysis of MT expression

MT protein (MT-I & MT-II) expression was assessed from liver tissue of marine catfish *A. arius* by western blotting method using Mouse monoclonal anti-MT primary and HRP conjugate

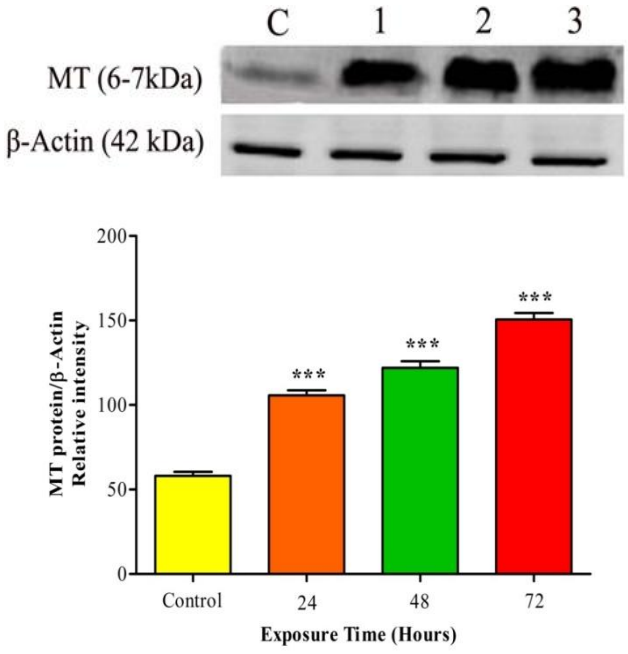

Fig. 1: Western Blot Analysis of MT expressions with β-Actin.

Lane C: Control, Lane 1: 24 hrs, Lane 2: 48 hrs and Lane 3: 72 hrs of MT protein expressions treated with Cd in *A. arius* liver. Bottom panel shows protein expression relative to that of β-Actin. '***' represents statistical significance between control Vs Cd treated groups at $p<0.0001$ level using Newman-Keul's test secondary antibody. Western blotting results showed increased MT expression levels in all Cd treated groups when compared with control liver group. MT protein bands were detected using chemiluminescence system (ECL Kit) method. MT bands were appeared with 6-7 kDa range. Antibody reacts with a specific MT protein after electrophoresis in 15 % SDS-PAGE, we should also be able to determine which proteins have elicited antibodies in a complex mixture of antigens. The expression of MT proteins of Cd treated and control liver tissues were analyzed. There was a significant increase in the protein levels of MT protein by 47.65 at 24 hrs, 63.91 at 48 hrs and 92.47 at 72 hrs when compared with control liver tissue in Cd treated liver samples.

Localization of MT

Localization of MTs in liver on *A. Arius* was reported by immunohistochemical methods using Mouse monoclonal anti-MT primary and HRP conjugate secondary antibody and stained with ImmunoCruz mouse ABC Staining kit. All slides were counterstaining with Mayer's haematoxylin and eosin visualized in light microscopy. The liver is characterized by polygonal shaped hepatocytes with a granular cytoplasm and centrally placed round nuclei.

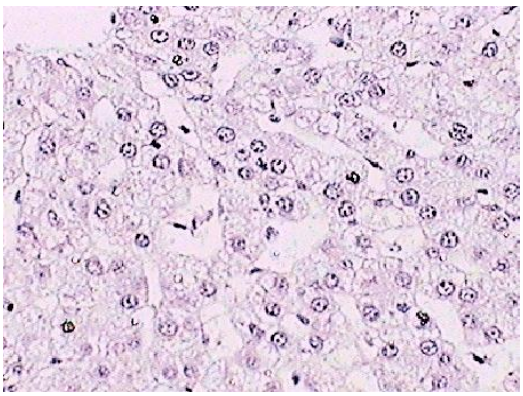

Fig. 2: Control liver section that has been stained with Mouse anti-MT and HRP conjugate antibody. There was no positively MT expression in the hepatocytes and other sections. (Magnification, 40X).

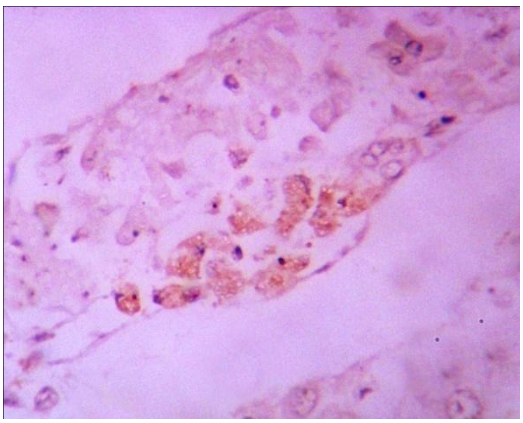

Fig. 3: Cd treated liver section that has been stained with Mouse anti-MT and HRP conjugate antibody. Showing immunolocalization of MT positively expression in the cytoplasm of hepatocytes.

Hepatocytes were arranged in well organized hepatic cords and separated by narrow blood sinusoids. In liver, *ir*MTs were mainly localized in hepatocytes and to a lesser extent in erythrocytes of Cd

exposed groups. *irMTs* were specifically localized in the lysosomes and in the cytoplasm of hepatocytes. MT positively expressions were showed cytoplasm of hepatocytes of Cd treated liver groups and negatively expressed in the control liver hepatocytes.

DISCUSSION

In Western blot the results indicate that MT expression levels were increased Cd treated liver groups in *A. arius* compared with the control. However, this method does not allow the determination as to which specific cell-types express significant induction of MT in the organs as a result of metal exposure during 72 hrs of Cd treatments. There was a significant increase in the protein levels of MT protein by 47.65 at 24 hrs, 63.91 at 48 hrs and 92.47 at 72 hrs when compared with control liver tissue in Cd treated liver samples. The use of antibodies against MT may be a good approach to detect the levels of proteins [20, 21]. However, antibodies against piscine MTs are scarce and include catfish (*Heteropneustes fossilis*), cod (*Gadus morhua*), rainbow trout (*Oncorhynchus mykiss*), dab (*Limanda limanda*) and perch (*Perca fluviatilis*) [21, 22]. Western blot analyses carried out here in confirmed those present of MT proteins and quantifying the MT expression levels only induced by Cadmium in treated liver tissues.

In liver, *ir*MTs were mainly localized in the cytoplasm of hepatocytes and lysosomes, to a lesser extent in erythrocytes. The immunolabelling produced in hepatocytes after Cd exposure was higher than in control liver on *A.arius.* The MT protein localization that has been detected in several fish tissues using specific antibodies by the method of immunohistochemistry techniques, these results are in occurrence with previous results; in turbot (Scophthalmus maximus) [23] exposed to Cd, in mammals [24]. Recent reports suggested the differential expression of MTs in selected cell-types of the gills of tilapia (*Oreochromis mossambicus*) after exposure to copper [25], in gills and kidney of salmon (*Salmo salar*) exposed to Cd [26, 27] in the liver and gills of turbot (*Scophthalmus maximus*) exposed to Cd, Cu and Zn [28, 29, 30] and in the gills of brown and rainbow trouts (*Oncorynchus mykiss*) environmentally exposed to sewage treatment plant effluents [20].

When the Cd enters into the blood after crossing the gills it is present in the plasma pool and it is gradually transferred to the erythrocytes until equilibrium is reached between the Cd pools in erythrocytes and plasma [31, 32]. Accordingly, immunolabelled erythrocytes were clearly observed under present exposure conditions confirming the presence of MTs and their function as Cd-binding proteins in these cells [32]. Cd is primarily distributed to the liver where, during chronic exposure to sublethal levels from 60 to 70% of the metal is sequestered by MTs, and to a lesser extent to the kidney and other organs [33, 34]. Cadmium when taken up by the liver, Cd can bind with glutathione (GSH) and be excreted into bile. More important, Cd can bind to MT and be stored. Some Cd bound to MT leaks into the plasma and then is taken up by the kidney. Circulating Cd-MT complex is a potent nephrotoxicant [35, 36]. The induction of fish MTs has been used as a biomarker of exposure to metals in both marine and freshwater environments [37, 38]. As a general rule, most of the metal stored in the liver is within the cytoplasam of hepatocytes [39] since the primary metal-binding protein, MT is cytosolic protein [40]. Immunohistochemistry revealed an increase in MT protein production in Cd-exposed hepatocytes, In addition, together with this cytosolic MT localization, the lysosomal population of hepatocytes also exhibited a strong MT labeling after Cd-exposure. The lysosomes constitute a major compartment for metal accumulation and sequestration [41] allowing a reduction of the toxic availability of Cd, at least transiently. Lysosomes can contain degradation products of MTs and serve as a final storage site of degraded MTs and possibly, of other metal-binding proteins [42, 43, 44].

CONCLUSIONS

MT expression after Cd exposure can be observed using MT-immunohistochemistry mainly in the hepatocytes of the liver tissues in *A. arius*. The MT levels were increased in liver on successive exposure to Cd. Western blotting helps in conforming the induction and expression of MT in cells and tissues which are exposed to Cd. The comparison of control and treated samples demonstrates the induction of MT on exposure to Cd and the characteristic tissue expression pattern of induction in observable**.**

The observation of the results clearly demonstrates that Cd is the inducting factor for MT protein (as all other heavy metals were BDL) and this is the cellular response to initial heavy metal exposure.

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