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IMMUNOLOCALIZATION OF METALLOTHIONEIN IN DIFFERENT TISSUES OF FRESH WATER CATFISH, *CLARIAS GARIEPINUS* ON EXPOSURE TO CADMIUM

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ABSTRACT: Metallothionein (MT) is a cysteine-rich protein that binds to heavy metals, and it is induced by them. The objective of this study to identify the MT protein induction/expression and localization in liver and kidney tissues (treated with 20 mg/L of CdCl₂ for 72 hours) by immunohistochemical techniques and Western Blotting methods in the fresh water catfish, *Clarias gariepinus*. MT expression (MT-I and MT-II) levels were analyzed by Western blot 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. Antibodies showed a positive cross reactivity with MT proteins, MT immune activity was high in the cytoplasm of hepatocytes in Cd exposed fish liver and also detected in the nephrocytes present in the proximal tubules of the nephron and more precisely in their basal labyrinth in kidney. Western blotting showed increased MT expression levels in Cd treated tissues when compared with control tissue. The differential induction/localization of MTs in different cell types described in this study suggests that the presence and quantification of MT can be used in bio-monitoring programs as a biomarker of Cd exposure in aquatic environments.

INTRODUCTION Cadmium is a toxic heavy metal, which is a common pollutant of aquatic medium. Organisms like fish living in contaminated aquatic systems can absorb this heavy metal, show bioaccumulation, damaged tissues, vertebral alterations, necrosis and ultimately apoptosis and death¹. The release of discharge of large number of pollutants, especially heavy metals and pesticides, pose a threat to human life². The water pollution is no longer considered to be an aesthetic problem, but a serious economic and public health problem as well.

Unfortunately, raw or inadequately treated sewage of millions of people still flow into lakes, rivers and seas, creating several kinds of disorders³. In metal detoxifications, MTs may reduce the toxic effects of metals by debasing the ratio of the uptake of heavy metal ions peroxides into cells⁴. An important property observed and conformed for MTs is that they play a major role in metal storage and detoxification of heavy metals⁵.

MTs are low molecular weight (6-14 kDa), cysteine-rich, non-enzymatic proteins in the animal kingdom, it was first described in the horse kidney by Margoshes and Vallee in 1957⁶. MT family is composed 4 isoforms: MT-I, MT-II, MT-III, and MT-IV⁷. Metallothionein-I and MT-II exists in all tissues^{8,9}. Metallothionein-III is expressed mainly in the brain only¹⁰, and MT-IV is expressed in

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stratified squamous epithelium¹¹. Heavy metals, hormones, inflammation, acute stress, and many chemicals can induce MT-I and MT-II activity¹². The amino acid sequences of MTs from many mammalian sources reveal that all contain approximately 61 amino acids of remarkably similar composition. It does not contain aromatic amino acids and histidine. All cysteines are known to participate in the coordination of 7 mol of Cd or zinc (Zn) per mol of MT¹³. Immunochemical techniques are very sensitive and allow the detection of very low amounts of MT 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 complexity¹⁴. Hepatocyte is a cell of the main tissue of the liver; it makes up to 70-85% of the liver cytoplasmic mass. Hepatocytes are involved in detoxification, modification and excretion of endogenous and exogenous substances¹⁵.

In the present study, freshwater catfish *C. gariepinus* were exposed to a sub-lethal concentration of CdCl₂ for 3 days. The main purpose of this study was to exhibit Cd-induced localization of MT expression in liver and kidney using immunohistochemical techniques and confirming MT protein by Western Blotting. MT can be used as a biomarker of Cd metal ion contamination.

MATERIALS AND METHODS:

Animals and Cadmium chloride exposure

The Fresh water catfish *C. gariepinus* (75 to 100g) were collected from fish farm, Poondi, Tamil Nadu, Southern India and they were used in the present study. The fishes were allowed to acclimatized in the laboratory in a stone tank for the period of 10 days at room temperature (30 ± 2°C) and 12 hrs dark and 12 hrs night. The fishes were divided into two groups based on the presence and absence and time cadmium exposure.

Group I – Control (without Cadmium), group II Cadmium exposed (20ppm CdCl₂) for 72 hrs. Tanks were continuously aerated and the water was changed every 24 hrs. The physico-chemical property, presence of Cd and other heavy metals in the tank water were checked before the experiment

and it was found to be below detectable levels (BDL) to ensure that the MTs were induced due to the addition of sub lethal dose of CdCl₂ only. At the end of Cadmium exposure the fishes were sacrificed by lethal dose of anesthesia (tricaine) and the liver and kidney tissues were dissected out and it was subjected to various analyses.

Western Blot

MT protein (MT-I and MT-II) expression was confirmed by Western Blot method. Total proteins were extracted from fish liver and kidney tissues were lysed using RIPA buffer containing 1X protease inhibitor following which protein concentrations were measured using the Lowry's method¹⁶. 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) and quantified using Chemi Doc XRS Imaging System, Bio-Rad (USA).

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 immunoperoxidase techniques. Briefly paraffin sections of fish liver and kidney were deparaffinised 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 immune-reactivity was detected

with an HRP-conjugated IgG secondary antibody at 1:100 dilutions and ImmunoCruz mouse ABC Staining kit. All slides were counterstained with Mayer's hematoxylin and eosin and visualized in light microscopy (Motic DMB1-2MP, China)¹⁷.

RESULTS

Western blot analysis of MT

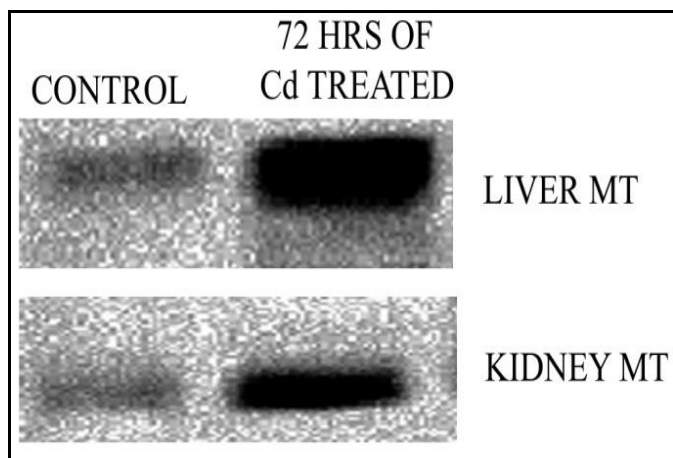


FIG. 1: RESULTS OF WESTERN BLOT ANALYSIS OF *C. GARIEPINUS* FISH LIVER AND KIDNEY THAT WERE TREATED WITH 20 MG/L OF CADMIUM CHLORIDE FOR 72 HOURS AT 15% SDS-POLYACRYLAMIDE GEL.

MT protein (MT-I & MT-II) expression was assessed from liver and kidney tissues of fresh water catfish *C. gariepinus* by western blot method using Mouse monoclonal Anti-MT primary and HRP secondary antibody. Western blot analysis of MT protein showing an increase in MT protein levels at 72 hours of Cd treated tissues compared with control. Antibody reacts with a specific protein after electrophoresis in 15 % SDS-PAGE; we should also be able to determine which proteins have elicited antibodies in a complex mixture of immunogens.

Detection of Antigen by Antibody Binding on Blots *in situ*, showed that the proteins transferred to PVDF membrane remained without being exchanged. Because a blot could be saturated with bovine serum albumin to block the residual binding capacity of the sheet, it can be treated as a solid-phase immunoassay. In the following immunological applications, an indirect technique was used throughout. Thus, antibody bound by the immobilized antigen was detected by a secondary, labeled antibody directed against the primary antibody, and in each case excess unbound

antibody was washed out. Western blot analysis (**Fig. 1**) revealed that the selected mouse anti-MT antibody cross-reacted specifically with *C. gariepinus* MT-I & MT-II. MT from fish liver and kidney samples exhibited bands at 6-14 kDa range. The intensity of the bands (optical density) was higher in Cd-exposed tissues than in controls of liver and kidney.

Immunolocalization of MT

In light-microscopic examination, the presence of immunoreactive MTs (*irMTs*) was visualized (**Fig. 2**). *irMTs* were easily observed in liver and up to a lesser extent in the kidney of Cd-exposed fishes. Localization of MTs in liver and kidney on *C. gariepinus* fish was observed by immunohistochemical methods. The liver is characterized by polygonal shaped hepatocytes with a granular cytoplasm and centrally placed round nuclei. Hepatocytes were arranged in well organized hepatic cords and separated by narrow blood sinusoids. The kidney is characterized by well built haemopoietic tissue, uriniferous tubules and glomerulus with clear Bowman's capsule.

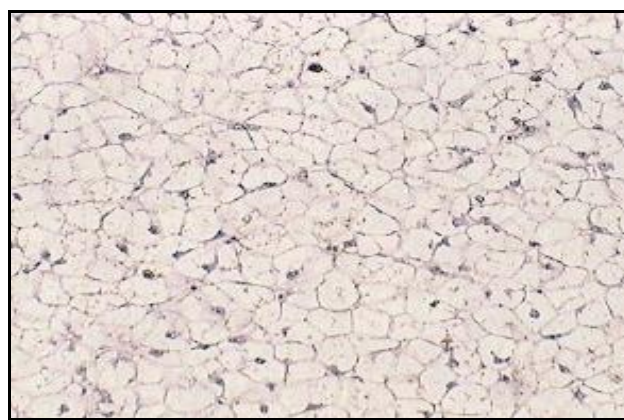


FIG. 2: CONTROL LIVER SECTION THAT HAS BEEN STAINED WITH SPECIFIC ANTIBODIES. THERE WAS NEGATIVELY MT EXPRESSION IN THE HEPATOCYTES AND OTHER SECTIONS. (MAGNIFICATION, 40X)

In liver, *irMTs* were mainly localized in hepatocytes and to a lesser extent in erythrocytes of Cd exposed groups. As observed under the light microscope, *irMTs* were specifically localized in the lysosomes and in the cytoplasm of hepatocytes. The immunolabelling produced in hepatocytes after Cd exposure was higher than in control liver. *irMTs* have also been localized in macrophages and blood cells present in the liver sinusoids. When the liver tissues were stained with mouse anti-MT

and HRP, the hepatocytes nuclei appeared purple colour. The arrows pointed out in the cytoplasm of hepatocytes on Cd treated liver (Fig. 3) that are stained are identified as MT expressed hepatocytes and negative expression of MT proteins in hepatocytes of control liver (Fig. 2).

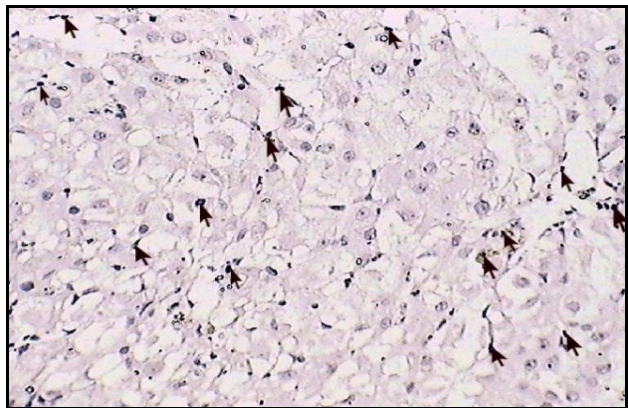


FIG. 3: Cd TREATED LIVER SECTION THAT HAS BEEN STAINED WITH SPECIFIC ANTIBODIES. SHOWING POSITIVELY MT EXPRESSIONS IN CD EXPOSED LIVER TISSUES AT 72 HOURS. IMMUNOLABELLING IN THE CYTOPLASM OF HEPATOCYTES TO NUCLEI. (MAGNIFICATION, 40X)

Low levels of *irMT* were localized in the kidney of Cd- exposed fishes. The immunolabelling was mainly detected in the nephrocytes placed in the proximal tubules of the nephron and more precisely in their basal labyrinth. Immunohistochemistry of the kidney (Fig.4 and Fig.5) belongs to control and Cd treated groups incubated with primary monoclonal antibody to MT protein. The arrows pointed out in the kidney, MT protein was checked in the cytoplasm of kidney tubule cells, especially in the proximal tubules and some distal tubules of the renal cortex.

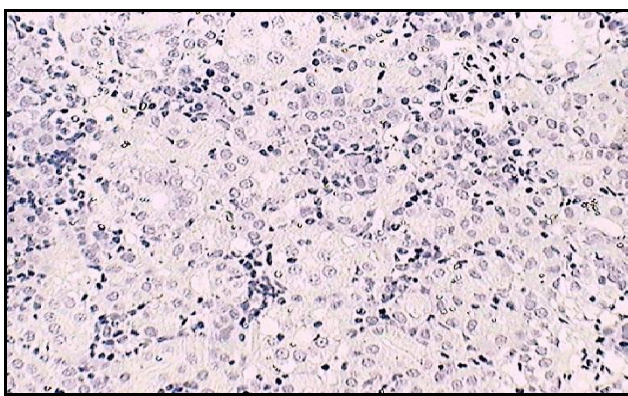


FIG. 4: CONTROL KIDNEY SECTION THAT HAS BEEN STAINED WITH SPECIFIC ANTIBODIES. THERE WAS NEGATIVELY MT EXPRESSION IN THE NEPHROCYTES. (MAGNIFICATION, 40X)

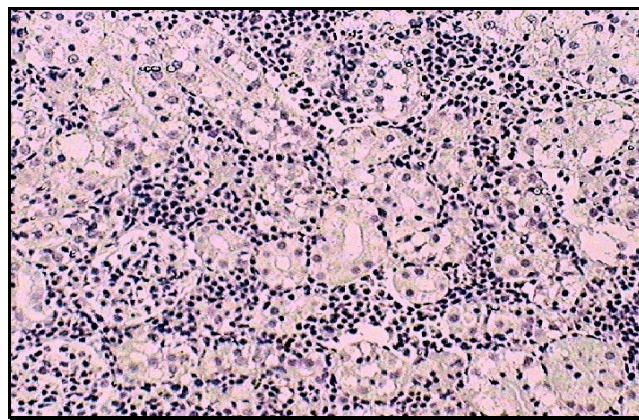


FIG. 5: Cd TREATED KIDNEY SECTION THAT HAS BEEN STAINED WITH SPECIFIC ANTIBODIES. SHOWING POSITIVE MT EXPRESSION IN FISH KIDNEY AT 72 HOURS OF CD EXPOSURE PROXIMAL TUBULES AND MORE PRECISELY IN THEIR BASAL LABYRINTH IN NEPHROCYTES. (MAGNIFICATION, 40X)

Immunohistochemical staining of the liver and kidney of freshwater catfish revealed MT-I and MT-II immunoreactivity. In liver MT was stained in the hepatocytes and in kidney MT was stained in the nephrocytes, Where MT distribution was not uniform and diffused. In the kidney, positively MT expression was detected in the cytoplasm of the collecting duct epithelium and the proximal and distal tubular epithelium. The glomeruli and vascular endothelial cells stained positively for MT expression in Cd treated. The controls did not show a positive expression.

DISCUSSIONS: Western blot results indicate induction of MT levels in liver and kidney of Cd exposed fish. 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 hours of Cd treatments. The use of antibodies against MT may be a good approach to detect the levels of MT proteins^{18, 19}. 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*)²⁰. Western blot analyses conformed with previous results.

In the liver, *irMTs* 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 *C. gariepinus*. The

MT protein localization that has been detected in several fish tissues using specific antibodies by the method of immunohistochemical techniques. MT was localized in the liver of hepatocytes. These results are in concurrence with previous results: in liver of turbot (*Scophthalmus maximus*) exposed to Cd, Cu and Zn^{21, 22, 23}, in kidney of salmon (*Salmo salar*) exposed to Cd^{24, 25}) and in the gills of brown and rainbow trouts (*Oncorhynchus mykiss*) environmentally exposed to sewage treatment plant effluents²³.

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^{26, 27}. So kidney MT protein levels were low compare than liver tissues. The induction of fish MTs has been used as a biomarker of exposure to metals in both freshwater and marine environments^{28, 29}. As a general rule, most of the metal stored in the liver is within the cytosol of hepatocytes³⁰ since the primary metal-binding protein; MT is cytoplasmic protein and is mainly localized in the cytoplasm of hepatocytes³¹.

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 sequestration³² 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^{33, 34}. Metals cannot be degraded metabolically so they have to be excreted via gills, skin, intestine liver or kidney³⁵. The main cell-type of the kidney is the nephrocytes which contains numerous, invaginations of the plasma membrane, often in the form of a well developed basal labyrinth. The presence of *ir*MT was mainly restricted to the basal part of the nephrocytes that form the proximal tubules. In the present study, MT expression in the kidney after Cd-exposure was lower when compared with Cd treated tissues.

CONCLUSIONS: In conclusion, MT induction and expression on Cd exposure can be observed

and confirmed using western blot and MT-immunohistochemical techniques mainly in the hepatocytes of the liver and nephrocytes of the kidney tissues in *C. gariepinus*. The comparison of control and treated samples demonstrates the induction of MT on exposure to Cd and the characteristic tissue expression pattern of induction is observable. The observation of the results clearly demonstrates that Cd is the inducing factor for MT protein and this is the cellular response on initial exposure to heavy metals and subsequences and initiation of detoxification process especially in the liver. This could be the reason for elevated MTs in liver when compared to kidneys.

CONFLICT OF INTEREST: There is no conflict of interests.

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