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METALLOTHIONEIN SYNTHESIS, MICRO-ARCHITECTURAL ALTERATIONS AND APOPTOSIS IN CADMIUM EXPOSED LIVER TISSUE OF MARINE CATFISH, *ARIUS ARIUS*

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ABSTRACT
Cadmium is one of the toxic heavy metal in the aquatic environment that affects aquatic organisms. They are also known to affect humans as they are carried through the food chain. In the present study, the effects of Cd were analyzed in the liver tissue of marine Catfish, *Arius arius*, when exposed to 20 ppm $CdCl_2$ for 24, 48 and 72 h. Cd accumulation and MT synthesis significantly increased in time dependent. A positive correlation was observed between Cd accumulation and MT synthesis. Typical morphological characteristic and physiological changes of apoptosis were observed following various methods such as; Histological alterations, Histomorphometric and Stereological analysis, DAPI nuclear staining and DNA fragmentation analysis. The activities of caspase-3 increased in time dependent after Cd exposure. The results showed significant alterations in the hepatocytes, hepatocytes nuclei and sinusoids in histology and histomorphometry with concomitant increase in apoptotic cell death in Cd exposed fish compared to control. From the results it can be concluded that Cd induced oxidative damage as well as apoptosis in the liver of marine catfish, *A. arius*. The induction of MT on Cd exposure shows its adaptive significance.

KEYWORDS: Cadmium toxicity; Histomorphometric and Stereological Analysis; Apoptosis; Caspase-3 activity; *Arius arius*; Metallothionein.

INTRODUCTION
Cd toxicity is an extremely devastating and a serious problem in aquatic organisms. Oceans, lakes and rivers are primarily contaminated by Cd and other heavy metals, mainly discharged from the industries (Swarup et al. 2006). Cd is an environmental pollutant ranked eighth in the Top 20 Hazardous Substances Priority List (ATSDR, 1999; Klaassen, 2009; Zakaria and Al-Busadah, 2015), and human activity has markedly increased the distribution of Cd in the global environment (Kalay and Canil, 2000; Swarup et al. 2006; Klaassen, 2009). Cd is one of the toxic and non-essential elements to living organisms with no biological functions (Burger, 2008). Cd is primarily used for electroplating with other metals and in nickeled batteries because of its relative resistance to corrosion and high electrical and thermal conductivity. These inputs may results in increased Cd levels in the aquatic ecosystems, which can be potentially toxic to aquatic organisms (Park, 2001; Sumit et al. 2014). Cd is a bivalent cation and is unable to generate free radicals directly; nevertheless there is increased production of Reactive Oxygen Species (ROS) after Cd exposure. The effect of Cd induced oxidative stress in the tissues and cells of animals and plants have been reported (Venod, 2009). Cd altered antioxidant defense systems and increased production of cellular ROS, such as singlet oxygen, hydrogen peroxide, and hydroxyl radicals. ROS can lead to oxidative stress within cells by reacting with macromolecules causing damages such as mutation, destruction of protein function and structure and apoptosis (Pathak and Khandelwas, 2006; Valko et al. 2006; Ognjanovic et al. 2010).

Fishes absorb heavy metals via the gills directly from the water in its ionic form Cd (II) (Ma et al. 2007), Cd ions from the water enters the chloride cells in the gills through calcium channels, binds to albumins and erythrocytes in the blood and then is transported to liver, kidney and other parts of the fish body (Wu and Chen, 2005; Mani et al. 2014; Perera et al. 2015). Excretion of Cd from the living organisms is a slow process (Thophon et al. 2003). In 1993, the FDA was reported; 50-70 % of Cd was deposited in liver tissues of fishes. The uptake of Cd through the food chain in aquatic organisms may lead to morphological alterations and pathological disorders

(Monteiro et al. 2005; Gabriel et al. 2006) led to apoptosis (cell death) and damage in the liver tissues has been reported by Kumar et al. (2005b), Farombi et al. (2007), Satarug and Moore, (2012). Cd heavy metal in aquatic organisms tends to accumulate in the human by way of food chain. One of the ways to regulate the level of metal in the cells is to immobilize the heavy metals by binding to biological molecules such as MT protein.

The liver is characterized by hexagonal shaped hepatocytes with granular cytoplasm and centrally placed round nuclei. Hepatocytes are arranged in a well-organized hepatic cords and separated by narrow blood sinusoids. The light microscopic examination of the control fish liver shows normal architecture (Mumford et al. 2007; Ahmad et al. 2011; Dar et al. 2011; Amin et al. 2013). Liver is an abdominal organ, hepatocytes constitute about 80% of the liver cell population, which played a vital role in detoxification and excretion of exogenous and endogenous substances (Van Dyk, 2003), it accomplished these detoxification action by means of three mechanisms as summarized by: A filtering system of large macrophages called kupffer cells lining the blood sinusoids, phase-I detoxification pathway and phase two detoxification pathways (Cabts, 2000). Detoxification systems for metals in fishes are derived from the specific feature of stress induced molecules to bind heavy metals. Particularly it was discovered that organisms protect themselves against the toxic effect of metals by synthesis of proteins called MTs, which are abundant throughout the whole animal kingdom (Fabrik et al. 2008).

MT was first isolated from horse kidney by Margoshes and Vallee in 1957. MT was found in higher plants, some prokaryotes and eukaryotic. MTs are widely expressed in eukaryotes that are responsible for heavy metal metabolism and detoxification (Kagi and Schaffer, 1988; Kagi, 1991; Mejarea and Leif, 2001; Duncan, 2009; Mani et al. 2014). MTs are low molecular weight (6,000–14,000 Da) proteins, containing about 25–35% cysteine, due to which they have high binding capacity for metals. All SH-groups may bind with metal ions; One MT molecule can sequester 7 Cd ions (Ma et al. 2007; Sumit et al. 2014). MT is known to be involved in the protection of organisms from the harmful effects produced by Cd and other heavy metals. In case of severe Cd stress, the amount of MT which is synthesized by the system may not be enough to bind and remove all the Cd, which leads to accumulation of Cd in the liver. Higher level of Cd induced apoptosis (cell death) and damage in the liver tissues. Accumulation of Cd acted as a inhibit apoptosis and mitogen leads to cancer in tissues (Satarug and Moore, 2012).

The objective of the present study was to analysis the impact of Cd in liver tissues of marine catfish, *A. arius* with reference to the effect of Cd on histo-architecture, apoptotic effect and MT synthesis.

MATERIALS AND METHODS

Animal Selection and Acclimatization

The marine catfish, *A. arius* (weight about 45 g ± 5 g and length about 16 cm ± 4 cm) were collected from the Coromandel Coast of Bay of Bengal (13°00.997 N, 080°16.687 E), Chennai, Tamil Nadu, India. The fishes were allowed to acclimatize in the laboratory in a stone tank (100 L) for 7 days at room temperature (30°C ± 2°C) with photoperiod of 12 h dark and 12 h light. The fishes were fed with commercial fish feed pellets (Hikari Marine a Pettets, Chennai). The feed was analyzed for Cd content which was found to be below detectable levels (BDL). Uneaten feed was removed daily along with dead fishes if any. Cd and other heavy metals concentrations in the experimental water were analyzed using an Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES (Perkin Elmer Optima-5300 DV)) at 228 nm before the start of the experiment and was found to be BDL.

Cadmium Chloride Treatment

$CdCl_2$ stock solution (100 ppm) was prepared by dissolving 100 mg of $CdCl_2$ in 1 L of DDH_2O. The fishes were divided into control and different time of Cd exposure groups. Group I – Control (without $CdCl_2$), Group II, III and IV at 20 ppm $CdCl_2$ for 24, 48, 72 h respectively. The tanks (100 L capacity) were continuously aerated and the water was cleaned at regular intervals. Each batch for the experiments contained 10% fishes. Three replicates were given for each experiment. As sub-lethal dose for short duration was selected for the experiments, no death fish was observed. The water samples analyzed were collected as per standard protocol (APHA, 1995) and were analyzed in triplicates. The LC_{50} values of Cadmium Chloride of the experimental fish were found 56.4 mg/L from our earlier studies (Mani et al. 2013).

Tissue Preparation

For each Cd concentrations, five fishes (n = 5) were randomly removed from the tanks during 0(Control), 24, 48 and 72 h of treatment respectively. At the end of Cd exposure, the fishes were sacrificed by an anesthesia with MS-222, and the liver tissues were removed. The tissues were handled with plastic forceps and kept in plastic homogenizing tubes to avoid contaminations. The fresh liver tissues were used for further analysis.

Quantification of Cd

Cd concentrations were quantified following the method of Ma et al. (2007). The liver tissue (0.25 g) were freshly weighed and dried in an oven at 80ºC for about 48 h. Then tissues were digested in 10 mL of HNO_3 and 5 mL H_2O_2 over a hot plate at about 120ºC. The metal (Cd) content of the fractions was measured by ICP-OES. Cd concentrations were expressed as µg g^{-1} wet weight tissue.

Quantification of MT

MT levels were quantified following the method of Ma et al. (2007). Sampling tissues (liver) 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 contained 0.1 mM Phenylmethylsulphonyl Fluoride (PMSF) and 0.1 mM Dithiothreitol (DTT). The homogenate was centrifuged at 16000 X g for 30 min at 4ºC, and the supernatant was heated for 2 min in a boiling water bath (100ºC). The heated sample was centrifuged at 10000 X g for 10 min 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 min to saturate the metal binding sites of MT. 0.5 mL of a 2 % (w/v) Bovine hemoglobin was then added and incubated at room temperature for 10 min. The hemoglobin was denatured in a water bath (100ºC) for 2 min, cooled in ice for 3 min, and centrifuged at 10000 X g for 15 min. 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. The concentration of Cd in the supernatant was determined using an ICP-OES.

The MT concentrations were calculated by the following equation:

MT Conc. (µg g^{-1} w wt) = Cd Conc. (µg g^{-1} w wt)/112.4/7 × 7000.

Based on previously reported data it was taken that 1 molecule of fish MT was bound to 7 molecules of Cd ions and the fish MT average molecular weight was assumed to be 7000 Daltons (Pedersen et al. 1994; Sumit et al. 2014). MT concentrations were expressed as µg g^{-1} w wt.

Western blot for Caspase- 3 Activity

Caspase-3 activity was performed following the method of Sumit et al. (2014). Caspase-3 activity was assessed by Western blot using specific antibodies (Caspase-3: Polyclonal Rabbit IgG primary (Cat.No. AF835, R&D Systems) and Anti-Rabbit IgG conjugated with HRP secondary antibody (Geni, Bangalore). β-Actin (housekeeping gene) was used as an internal control for quantify to Caspase-3. β-Actin primary antibody (sc-81178, Santa Cruz Biotech, USA) and HRP secondary antibody (Geni, Bangalore). Control and Cd treated (24, 48 and 72 h of 20 ppm of $CdCl_2$) liver tissues were weighted (50 g) and homogenized with 300 µL of ice-cold RIPA buffer with 1X protease inhibitor cocktail (Biobasic Inc., USA) was added. Homogenized tissues were centrifuged at 14,000 X g for 10 min at 4°C. Pellet was removed. The total proteins were present at supernatant. The protein concentrations were measured using the Lowry's method (Lowry et al. 1951). The supernatant was separated and added to a new 1.5 mL centrifuge tube and stored at -20ºC until further use. Equal amount of total protein (50 µg) was mixed with 2X sample buffer and boiled for 5 min. The proteins were separated on 15 % SDS-polyacrylamide gels and electrophoretically transferred into Polyvinylidene difluoride (PVDF) membranes (Millipore, USA). The blots/non-specific binding sites were blocked with 5 % blocking buffer for 2 h. After blocking, membranes were incubated with respective primary antibodies Polyclonal Rabbit IgG 1:1000 dilutions overnight at 4°C. Then the membranes were washed thrice with T-TBS, each for 10 min, followed incubation for 45 min at room temperature with the corresponding Anti-Rabbit IgG conjugated with HRP secondary antibody 1:500 dilutions. Finally, signals were visualized using super signal west femto maximum sensitivity substrate kit (Prod#34095, Thermo Scientific, USA). The signals were captured by Chemi Doc XRS system (Bio Rad, USA). The intensity of the bands was quantified using Quantity one software (Bio Rad, USA). The relative expression levels were expressed as the Caspase-3 activity/β-Actin relative intensity.

Histological Alterations Analysis

Histological alterations were carried out following the method of Banaee et al. (2012). At the end of the Cd treated, the liver tissue was dissected out and fixed in 10 % buffered formalin and post fixed in the same fixative. A small piece of liver was processed for paraffin technique and sections of 5 micron thickness were taken and stained with Haematoxylin and Eosin (H&E). The stained slides were subjected to histological analysis and documented under a light microscope (XSZ-801BN model, China) equipped with a 12.1 mega pixels camera (Casio, EX-Z450, Japan).

Histomorphometric and Stereological Analysis

The conventional principles and accepted morphometric procedures as outlined by Mori and Christensen, (1980) were used to obtain quantitative information. The diameters of the Hepatocytes, Sinusoid and Nucleus in hepatocytes were estimated using ocular micrometer scale. The diameter unit was expressed as micrometer (µm).

DAPI Nucleic Acid Staining

Nuclear morphology was performed following the method of Magesh et al. (2009). In the paraffin processed tissues, cross section was dewaxed with Xyline and dehydrated with serial concentrations of absolute alcohol and washed with deionised water. The sections were washed with PBS and incubated with 0.5 µg/mL of DAPI (4, 6-diamidino-2-phenylindole dihydrochloride) (Sigma Aldrich, USA) in the dark for 5 min. After five min the slide was washed thrice in PBS, drained excess buffer from the slide and mounted in the same buffer. The cells were viewed using an Olympus BX51 fluorescence microscope (Olympus, Tokyo, Japan) (magnification, 40x) with appropriate filters at 450 nm and photographed. Cells were condensed chromatin or

fragmented nuclei were considered as apoptotic. The incidence of apoptosis in each preparation was analyzed by counting around 300 cells and the percentage of apoptotic cells determined.

DNA Fragmentation Analysis

DNA fragmentation was performed following the method of Nagata et al. (2000). The control and Cd-treated liver tissues were washed with PBS. The tissues were homogenized and incubated with lysis buffer (10 mM Tris pH = 8.0, 20 mM EDTA, 200 mM NaCl, 0.2 % Triton X-100 and 100 µg/mL Proteinase K) for 90 min. The supernatant was collected after centrifugation and DNA was isolated using isopropanol and 4M NaCl (final concentration of 100 mM) at -20ºC kept overnight. The precipitated DNA was washed in 75 % ethanol and resuspended in TE buffer. The isolated DNA from control and Cd treated liver tissues were electrophoresed in 1 % agarose gel. 1 % agarose gel was prepared in Tris-Acetate-EDTA (TAE) buffer (1X) at pH = 8.0 and stained with 2 µL of ethidium bromide (0.5 µg/mL). The gel tray was removed from the casting unit and placed in the electrophoresis tank. 1X TBE buffer was poured into the tank until the agarose gel gets immersed. 1 µg of each DNA sample was loaded into the wells with gel loading buffer (2 µL of dye is used). Placed the lid on the tank and connected the electrodes to the power supply. The power was switched on; set at 100 V for 30 min. DNA fragments were visualized in the UV trans-illuminator and the details were documented.

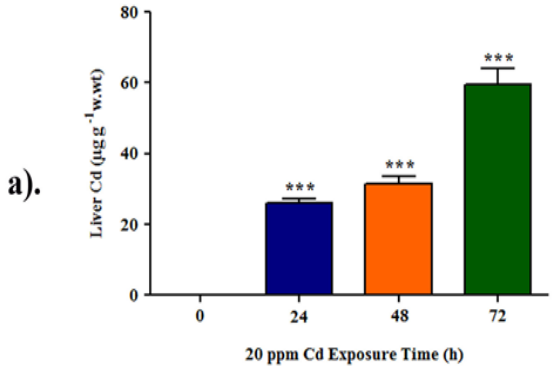
Statistical Analysis

The data were subjected to statistical analysis using one-way analysis of variance. The Newman - Keuls test was used to assess the significance of individual variations between the groups using GraphPad Prism 5.0. The values of $p<0.05$ were considered statistically significant.

RESULTS

Cadmium accumulation and MT synthesis levels

Fig.1a shows accumulated Cd concentrations, measured in the liver tissues of *A. arius* exposed to 20 ppm $CdCl_2$ for 24, 48 and 72 h respectively. The Cd levels significantly increased in a time dependent manner in the treated fish when compared to control.



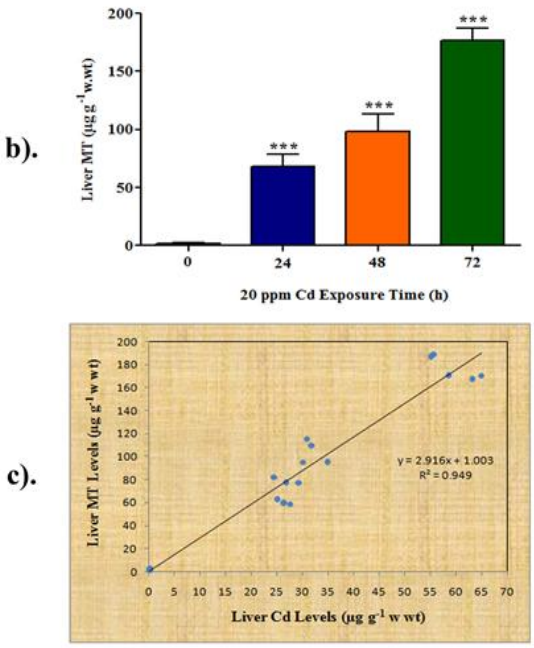


Fig. 1 Cd accumulation and MT synthesis in liver tissues of marine catfish, *A. arius*. The fishes were exposed to 20 ppm CdCl2 for 0(Control), 24, 48 and 72 h. The correlation between Cd accumulation and MT synthesis can be represented by a linear regression equation of *A. arius* liver tissues Y=2.916x + 1.003 (R2=0.949, $p < 0.001$). a)- Levels of Cd accumulation, b)- Levels of MT synthesis and c)- Correlation between Cd accumulation and MT synthesis. Each bar represents the Mean ± S.D, (n = 5). ***$p<0.001$ statistically significant between control and Cd treated liver.

The Cd levels in the liver of control fish was 0.096 ± 0.05 (µg g^{-1} w wt). During 24 h of exposure the Cd level was 26.03 ± 1.29 (µg g^{-1} w wt), during 48 h, Cd level was 31.36 ± 2.21 (µg g^{-1} w wt) and during 72 h, Cd level was 59.53 ± 4.42 (µg g^{-1} w wt). The data was statistically tested and the values were found to be statistically significant at $P < 0.001$. **Fig.1b** shows induced MT levels in control fish liver tissue, that was found to be 1.845 ± 0.53 (µg g^{-1} w wt), during 24 h of Cd exposure, the MT level was 68.199 ± 10.77 (µg g^{-1} w wt), during 48 h, the MT level was 98.413 ± 14.83 (µg g^{-1} w wt) and during 72 h, the MT level was 176.797 ± 10.18 (µg g^{-1} w wt). The data was statistically tested and the values were found to be statistically significant at $P < 0.001$. As shown in **Fig.1c**, MT levels increased linearly with increasing Cd concentrations and are described by the following regression equation: [MT] = 2.916 [Cd] + 1.003 (R^2 = 0.949, $p < 0.001$). A positive correlation was observed between Cd accumulation and MT synthesis of *A. arius* fish liver tissues, which clearly indicated that Cd accumulation in the liver and induced the synthesis of MT. In all control fishes Cd levels and MT levels were found to be low and hence their influence of Cd on MT synthesis is absent or negligible.

Caspase-3 Activity

In liver, Caspase-3 activities were found significant increase with respect to time of Cd exposure (**Fig.2a**). The caspase-3 levels of 0(control), 24, 48 and 72 h were 84.52 (Caspase-3 activity/β-Actin relative intensity), 111.58 (Caspase-3 activity/β-Actin relative intensity), 118.10 (Caspase-3 activity/β-Actin relative intensity) and 126.46 (Caspase-3 activity/β-Actin relative intensity) respectively (**Fig.2b**).

Liver Histological Alterations

Histological alterations were carried out control and Cd exposed liver tissues of *A. arius* on exposure to 20ppm of $CdCl_2$ for 24, 48 and 72 h. The light microscopy examination of the control fish liver tissue showed normal architecture with the distribution of the hepatocytes, hepatocytes nuclei and sinusoids. Whereas, Cd exposed liver tissues showed time dependent alterations like severe hypertrophy of hepatocytes and cytoplasmic vacuolation of the hepatocytes, focal necrosis of hepatic tissue, loosening of hepatic tissue; hepatic cells lose their original shape, got excessively distended and vacuolated. However, the alterations were more severe during 72 h of exposure when compared to other Cd exposed groups (**Fig.3**).

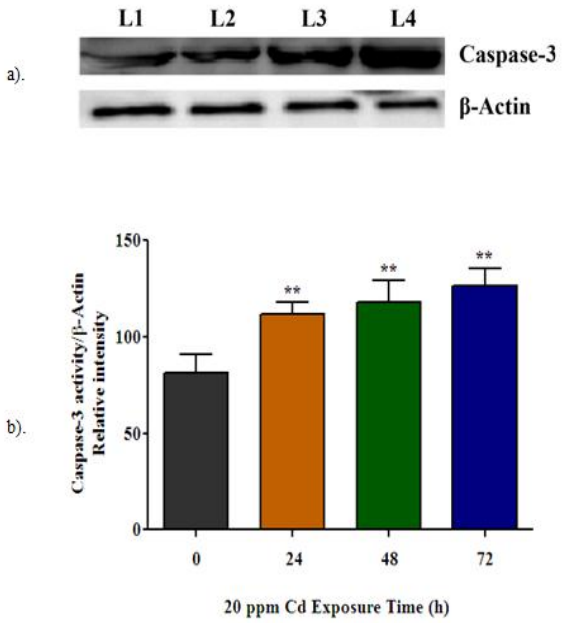


Fig. 2 Caspase-3 levels in liver tissues of *A. arius*. a). Western blot analysis of Caspase-3 with β-Actin as internal control, L1 – 0(Control), L2 - 24 h, L3 – 48 h, L4 – 72 h of 20 ppm CdCl2 exposure. b). Graphical representation of Caspase-3 levels. Each bar represents the Mean ± S.D, (n = 3).
**p < 0.01 statistically significant.

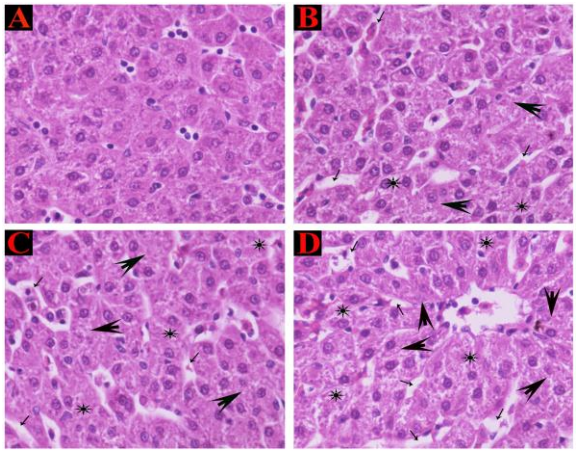


Fig. 3 T.S of liver tissues of *A. arius* fish as observed under Light microsope (40x). A.0(Control), B. 24 h, C. 48 h and D. 72 h of 20 ppm $CdCl_2$ exposure. Cd exposure resulted on altered cytoplasmic degeneration in hepatocytes **(✳),** sinusoids **(↙)**, hepatic cells lost their original shape and got excessively distended and mild vacuolated **(➹)** in liver tissues.

Histomorphometric Diametric Alterations Analysis in liver

The effects of Cd on the histological changes of liver tissues were quantified by Histomorphometric and Stereological analysis. The data illustrates that the Cd exposed liver showed time dependent alterations in the hepatocytes, hepatocytes nuclei and sinusoids structural diametric alterations were analyzed (**Fig.4**).

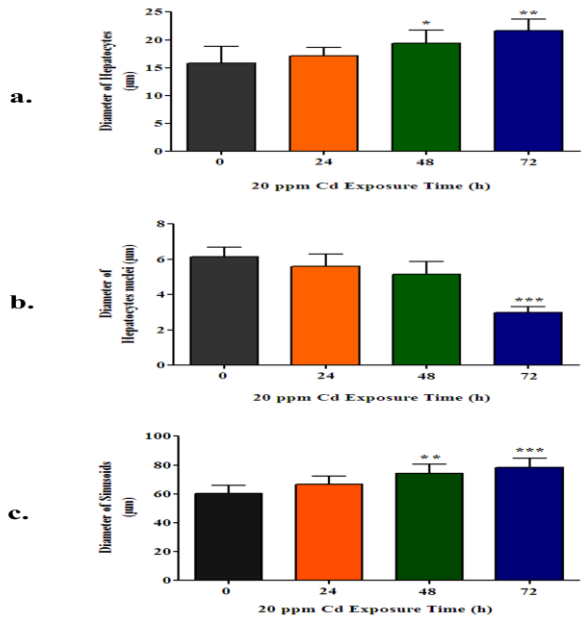
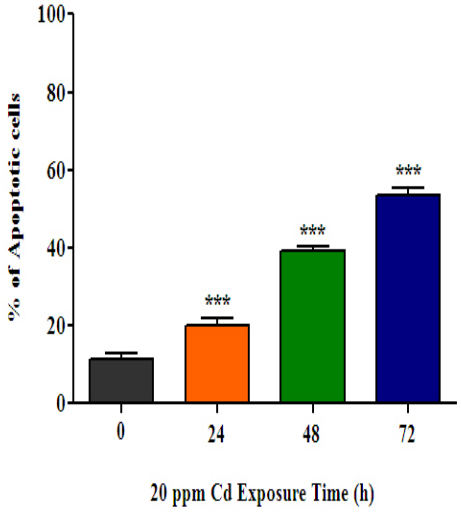


Fig. 4 Histomorphometric diametric alterations in liver tissues of *A. arius*. a). Diameter of hepatocytes, b). Diameter of Hepatocytes nuclei, c). Diameter of sinusoids on exposure to 20 ppm CdCl2 for a period of 0(Control), 24, 48 and 72 h. Each bar represents the Mean ± S.D of hundred independent observations in triplicates. *p < 0.05, **p < 0.01, ***p < 0.001 statistically significant..

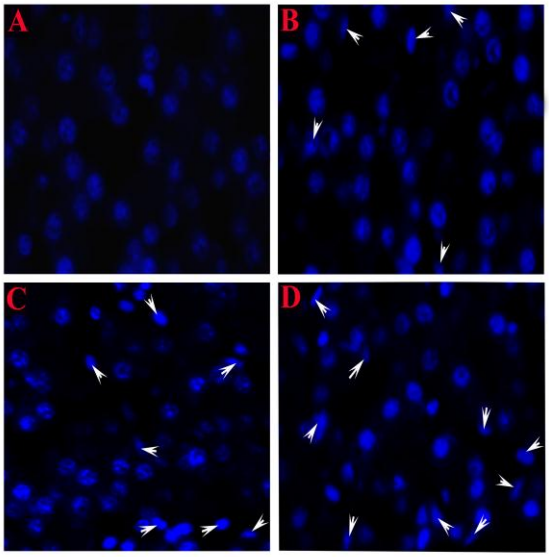
The diameter of hepatocytes in the liver tissues of control fish was 15.825 ± 2.98 µm. However it increased in all treated groups, which were time dependent. It increased to 17.158 ± 1.47 µm during 24 h, 19.404 ± 2.33 µm during 48 h and 21.679 ± 2.07 µm during 72 h of Cd exposure (**Fig.4a**). The diameter of hepatocytes nuclei in the liver tissues of control fish was 6.146 ± 0.53 µm. However it decreased in all treated groups, which were time dependent. It decreased to 5.596 ± 0.69 µm during 24 h, 5.171 ± 0.71 µm during 48 h and 2.996 ± 0.34 µm during 72 h of Cd exposure (**Fig.4b**). The diameter of sinusoids in the liver tissues of control fish was 60.508 ± 5.34 µm. However it increased in all treated groups, which were time dependent. It also increased to 66.575 ± 5.64 µm during 24 h, 74.408 ± 6.09 µm during 48 h and 78.342 ± 6.37 µm during 72 h of Cd exposure (**Fig.4c**). A significant increase in diameter of hepatocytes and sinusoids with concomitant reduction of diameter of hepatocytes nuclei when compared to control was observed.

Apoptotic studies by DAPI staining

Fig.5a shows the nuclear morphology in liver tissues of the control and Cd treated fishes. The control fish liver tissues showed normal nuclear morphology whereas Cd exposed fishes showed apoptotic morphology. Effect of Cd on nuclear morphology on fish liver was studied by the DAPI staining. DAPI is known to form fluorescent complexes with normal double-stranded DNA and used to find apoptotic nuclei. Apoptotic nuclei can be identified by the reduced nuclear size and lobulated nucleus (Apoptotic bodies).



a.



b.

Fig. 5 Analysis of nuclear morphology in *A. arius* liver tissues by DAPI using florescent microscope. a). Cells nuclear morphology, A. 0(Control), B. 24 h, C. 48 h and D. 72 h of 20 ppm CdCl2 exposed liver tissues. A white arrow indicates apoptotic bodies. b). Quantification of apoptotic nuclear morphology in control and Cd exposed liver tissues. Each bar represents the Mean ± S.D, (n = 5) (300 cells in each replicates).
***$p < 0.001$ statistically significant

The control liver tissues showed normal nuclear morphology with a round clear nucleus in hepatocytes (**Fig.5a.A**). Cd exposed liver tissues showed apoptotic nuclear morphology (**Fig.5a.B-D**). **Fig.5b** shows quantitative study of the Cd exposed fish liver. Apoptotic morphology was statistically significant ($p < 0.001$) and an increase in number of nuclei showed apoptotic morphology when compared to control. In control apoptotic cells were 11.47 ± 1.39 %, it increased 20.05 ± 2.07 % during 24 h, 39.18 ± 1.06 % during 48 h and 53.49 ± 2.97 % during 72 h of Cd exposure. The effects were more prominent during 72 h of Cd exposed liver than other study periods.

DNA Fragmentation Analysis

Fig.6 shows the Cd induced apoptotic DNA fragmentation in the liver tissues which was clearly seen in 1 % agarose gel and stained using Ethidium bromide. In the absence of Cd (Control) no ladder was observed (**Fig.6.L1**), and a genomic DNA ladder formation was observed in treated liver tissues **(Fig.6.L2–L4)**. Electrophoresis of DNA isolated from control and Cd treated tissues showed a significant increase in the DNA ladder pattern in time dependent manner. The degradation of DNA into oligonucleotide fragments was maximum during 72 h of Cd exposure, confirming the induction of apoptosis by Cd (**Fig.6.L4**).

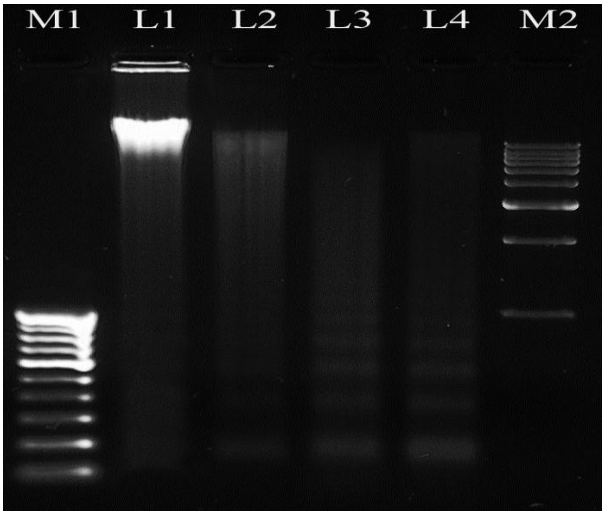


Fig. 6 DNA fragmentation analysis in liver tissue of *A. arius*. 1% Agarose gel showing DNA fragmentation of treated with 20 ppm CdCl2. M1– Marker (1 kb DNA ladder), L1– Control, L2 – 24 h, L3 – 48 h, L4 – 72 h of Cd exposed liver, M2– Marker (100 bp DNA ladder)**.**

DISCUSSION

Aquatic ecosystem is exposed to a variety of pollutants due to anthropogenic activities by way of industrial effluents. The heavy metals in the water tend to accumulate in aquatic organism; fish being the target organism. The heavy metals in the fish pose a serious threat to human being as these metals gain entry into the human system by way of aquatic food chain (Park, 2001; Ma et al. 2007; Barkhordar et al. 2013). The marine catfish, *A. arius*, is an edible fish and is eaten by various organisms including man. So the marine catfish *A. arius* was used as the experimental animal in the present study. In the present study an attempt has been made to study the effects of Cd histological alterations, histomorphometric diametric alterations, nuclear morphological changes and DNA fragmentation on the liver tissue of *A. arius*. The fishes respond to the Cd toxicity by synthesizing stress protein, one of which is MT protein. In the present study, the effects of Cd on oxidative stress and apoptosis of liver tissues in marine fishes were investigated.

Most aquatic animals absorb heavy metals via gill and transfer the metals to the blood and other parts of the body (Ma et al. 2007). In 1993, the FDA was reported; 50-70 % of Cd was deposited in liver tissues of fishes. According to Kent, (1998) the liver are involved in the detoxification and removal of toxic substances circulating in the blood stream (Klavercamp et al. 1984). Cd and MT levels indicate that the highest Cd concentrations were observed during 72 h of Cd exposure in *A. arius* liver tissues. The results clearly showed time dependent and similar to many studies in aquatic organisms such as Rainbow trout (Chowdhury et al. 2005), *Sinopotamon henanense* (Ma et al. 2007), *Litopenaeus vannamei* (Wu and Chen 2005) and *Clarias gariepinus* (Sumit et al. 2014). Cd accumulated mainly in liver tissue that store metals to detoxify/excrete it by synthesizing MTs (Rao and Padmaja 2000). Previous reports have shown that after metal exposure, a high level of metal accumulation occurs in organs like liver (Ma et al. 2007; Sumit et al. 2014). The highest MT levels were observed in liver tissues during 72 h of Cd exposure in *A. arius*, indicating the possible role of liver in storage of Cd for detoxification and excretion that is initiated by the MT synthesis. The results of the present study correlate with earlier work (Sumit et al. 2014) in fresh water catfish, C*larias gariepinus.* MT concentrations in *A. arius* were in complete agreement with those of many other investigations in that the synthesis of MT increased in a time dependent manner and these results were supported with earlier works of Martinez et al. (1993, 1996) in crayfish, *Procambarus clarkia*, Wu and Chen (2005) in shrimp, *Litopenaeus vannamei*, Sumit et al. (2014) in catfish, *Clarias gariepinus*, Suresh et al. (2015) in grass carp, *Ctenopharyngodon idella.*

The correlation analysis of Cd accumulation and MT synthesis in marine catfish, *A. arius* liver tissues were investigated. The relationship between Cd accumulation and MT synthesis after exposure to Cd was found to be significantly increased. A positive correlation was observed between Cd accumulation and MT synthesis of *A. arius* liver tissues, which clearly indicated that Cd significantly accumulated in the liver and stimulated the synthesis of MT. This might be due to self-adaptation mechanism to remove the Cd from the system by forming Cd-MT complex (Jana et al. 2009). Similar studies Sumit et al. (2014) also showed an increased MT on exposure Cd. The earlier reports also confirmed our present study findings that the Cd exposure enhanced MT in various fishes, *Rainbow trout* (Chowdhury et al. 2005) and *Litopenaeus vannamei* (Wu and Chen, 2005).
Cd induced histological alterations in the liver tissues of *A. arius* was time dependent manner. Under the present investigation, it has been observed that the liver tissues Cd exposed for 0(Control), 24, 48 and 72 h exhibited several histological alterations like deshaping and hypertrophy of hepatocytes and cytoplasmic vacuolation in the hepatocytes and focal necrosis of hepatic tissue, loosening of hepatic tissue and the hepatic cells losing their original shape. Similar results have been reported in other fishes (Van-Dyk, 2003; Giari et al. 2007; Van-Dyk et al. 2007). These findings lend support to the observations of the present study. In case of Cd stress, the amount of MT which is synthesized by the system may not be enough to bind and remove all the Cd, which thus lead to accumulation of Cd in the liver. Excess Cd induced mitochondrial membrane lipid peroxidation, which can cause damage to organelles. Cd induced oxidative stress in cells, the consequence of which is primarily peroxidation damage to cell membranes (Arroyo et al. 2012). Furthermore, the interaction of Cd and MT leads to production of highly reactive OH^- free radicals via Fenton reaction. These high reactive OH^- radicals interact with plasma membrane, macromolecules and proteins (Valko et al. 2006).

Apoptosis shows chromatin condensation and DNA fragmentation, and is mediated by Caspases (Hengartner, 2000; Elmore, 2007). The family of Caspases regulates apoptosis. Caspases are normally present in the cell as proenzymes that require limited proteolysis to activate enzymatic activity (Nunez et al. 1998). Once activated, Caspases cleave a variety of intracellular polypeptides, including major structural elements of the cytoplasm and nucleus. Increased Caspase-3 activity during 72 h of Cd exposed fish tissues might be due to increased Cd (Vaculova and Zhivotovsky, 2008). Among this Caspase-3 is the most commonly activated during the apoptotic process Janicke et al. (1998). Caspase-3 is a key executioner of apoptosis, its activation is mediated by initiator Caspases such as Caspase-9 that cleave a number of substrates which act in response to DNA strand breaks leading to apoptosis (Nicholson and Thornberry, 1997; Mancini et al. 1998; Soldani and Scovassi, 2002).

Cd stimulates free-radical production, resulting in oxidative deterioration of lipids, proteins and DNA, as well as initiating various pathological conditions in aquatic organisms and animals. The effect of Cd on the liver tissues of *A. arius* was studied culminating in apoptosis. Cd exposed liver of fish showed time dependent increase in severity of alterations resulting in apoptosis. The Cd induced liver toxicity is mediated by the up-regulation of ROS, which cause oxidative damage to lipid contents of membranes (Shaikh et al. 1999; Packer and Cadenas, 2002). Overproduction of ROS normally induces oxidative stress unless it was scavenged with endogenous antioxidants. Thus, overproduction of ROS could be attributed to the depletion of antioxidants or to the direct action of Cd on peroxidation reaction and iron-mediated peroxidation (Casalino et al. 2002; Pillai and Gupta, 2005). Primary injury of cells resulting from the binding of Cd to sulfhydryl groups in mitochondria and secondary injury initiated by the activation of kupffer cells have also been mentioned as a possible mechanism of toxic effect of Cd in the liver (Rikans and Yamano, 2000). Inactivation of sulfhydryl groups causes oxidative stress, mitochondrial permeability transition and dysfunction (Jurczuk et al. 2004). It is also suggested that kupffer cells released proinflammatory cytokines and chemokines, which stimulated the migration and accumulation of neutrophils and monocytes in the liver (Bilzer et al. 2006). Dudley also suggested that hepatocytes injury may be caused by ischemia due to sinusoidal endothelial cell dysfunction. Cd has been found to accumulate in endothelial cells, leading to necrosis and denudation of hepatic sinusoids. The hepatotoxicity of Cd has also been attributed to the formation of toxic metabolites when it is activated by hepatic cytochrome p450 (Wong et al. 1981) to a highly active metabolite xenobiotics (Savides and Oehne, 1983). This could be the possible explanation to the altered histology of liver in the present study.

To examine whether the apoptotic pathway was involved, DAPI staining was done on the tissues treated with 20 ppm $CdCl_2$. During 24, 48 and 72 h of treatment of Cd the various stages precluding apoptosis such as chromatin condensation, membrane blebbing, cell shrinkage, increased number of nuclear body fragments and irregular edges around the nucleus were observed in Cd treated tissues. While the control tissues exhibited round clear edged, stained cell nuclei with uniformly. The induction of apoptotic cell death by Cd has also been demonstrated by various studies on fishes (Pulido and Parrish 2003; Gonzalez et al. 2006; Risso-de Faverney et al. 2004). DAPI assay also showed an increase in apoptotic cells that was in time dependent manner in Cd treated tissues. Thus it is evident that Cd induces apoptosis (Filipic and Hei 2004; Bertin and Averbeck, 2006). The DNA fragmentation technique is well studied of apoptotic cell death (Wyllie, 1980). DNA fragments were absent in the control tissue. Since the Cd treated tissues showed clear fragmentation it provides further support for its apoptotic activity. The highest fragments were observed during 72 h of Cd exposure compared to other Cd treated groups. The fragments increased in a time dependent manner. These results correlate with earlier works of McMurray and Tainer, (2003); Filipic and Hei, (2004); Bertin and Averbeck, (2006); Martelli et al. (2006); Badisa et al. (2007).

CONCLUSION

The present study on the liver tissues of *A. arius* clearly demonstrates that sub-lethal concentration of Cd stimulates oxidative stress, alters structure of hepatocytes and Cd accumulation in the liver, leading to alteration in the physiology and metabolic regulation of liver and fish as a whole. Fishes on exposure to heavy metals protect themselves by synthesizing metal binding protein like MT. The synthesis of MT is a significant adaptation which enables organisms to detoxify/excrete and reduce the harmful effects of heavy metals in habitants. However, in long run or over dose, the accumulation of Cd even in sub-lethal concentration can lead to a serious problems to the fish and affect their population.

CONFLICT OF INTEREST STATEMENT

There is no conflict of interests.

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