CHAPTER 4

MATERIALS AND METHODS

4.1 EXPERIMENTAL ANIMAL – *C. gariepinus* (BURCHELL, 1822)

Distribution

Freshwater catfish *C. gariepinus* (Burchell, 1822) North African catfish are found throughout Africa and the Middle East, and live in freshwater lakes, rivers, and swamps, as well as human-made habitats, such as oxidation ponds or even urban sewage systems.

It was introduced all over the world in the early 1980s for aquaculture purposes, so is found in countries far outside its natural habitat, such as Brazil, Vietnam, Indonesia, and India.

![Freshwater catfish, C. gariepinus](image)

**Figure 4.1: Freshwater catfish, C. gariepinus**

Identification of the Experimental Fish

*C. gariepinus* (Burchell, 1822) commonly called as the North African catfish (figure 4.1), the experimental fish *C. gariepinus* for this research work was identified by the Zoological Survey of India (ZSI), Chennai, Tamil Nadu, India.
Classification

Kingdom : Animalia
Phylum : Chordata
Class : Actinopterygii
Order : Siluriformes
Family : Clariidae
Genus : Clarias
Species : C. gariepinus

Description

Dorsal spines: 0; Dorsal soft rays: 61-80; Anal spines: 0; Anal soft rays: 45 - 65; Vertebrae: 56 - 63. Body depth is 6-8 times in standard length, the head 3-3.5 times. The head is somewhat between rectangular and pointed in dorsal outline; the snout is broadly rounded. The eyes have a supero-lateral position and are relatively small. Teeth on the premaxilla and lower jaw are small, fine and arranged in several rows. Nasal barbels from 1/5 to 1/2 times as long as the head in fishes longer than 12 cm, and from 1/2 to 4/5 of the head length in smaller individuals; maxillary barbels rarely shorter than the head, usually somewhat longer and reaching to a point midway between the origin of the dorsal fin and the insertion of the pelvic fins; outer mandibular barbel longer than the inner pair.

Max length : 170 cm, average length : 90.0 cm,
max. published weight: 60.0 kg
Justification

Freshwater catfish, *C. gariepinus* is commonly available in and around Chennai, Tamil Nadu. It’s an edible fish, suitable for the experimental and laboratory work. It is predatory and has been used in bioaccumulation and biomagnification studies (Olumuji and Mustapha et al., 2012). Literature search has revealed that not much work has been done on effects of Cadmium on *C. gariepinus*. Also the MT of this species has not yet been characterized. Hence the selection of this fish for this study.

4.2 ANIMAL SELECTION AND ACCLIMATIZATION

- Experimental Animal - Freshwater catfish - *C. gariepinus*.
- Specimen Collection site - Poondi Fish Farm, Thiruvallur Dist, Tamil Nadu, South India.
- Average wt of Specimens - 75 g ± 5 g
- Average Length - 13 cm ± 2 cm
- Acclimatization Process - 7 days in a 100 L stone tank at RT (30°C ± 2°C) with photoperiod of 12 h dark and 12 h light.
- Feed - Commercial fish feed. It was tested for heavy metals and found to be BDL.
- Fishes Per Experiment - 5

Procedure

The freshwater catfish, *C. gariepinus* (weight: 75 g ± 5 g and length: 12 cm ± 2 cm) was collected from Poondi fish Farm, Thiruvallur Dist, Tamil Nadu, South India. The fishes were allowed to acclimatize in the laboratory in
a 100L stone tank 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. The water and the feed was analyzed for Cd and other heavy metal content and was found to be BDL. Uneaten feed was removed daily along with dead fishes if any. The physico-chemical parameters of the water samples were analyzed and found to be within the normal ranges. Cd and other heavy metals concentrations in the experimental water were analyzed using an Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) at 228 nm (Perkin Elmer Optima-5300 DV) before the start of the experiment and was found to be below detectable levels (BDL) to rule out their influence in the experiments. After acclimatization, each batch was labeled and maintained in separate 50L plastics tubs.

4.3 BIOLOGICAL STUDY

4.3.1 Quantification of Cadmium

Cadmium concentrations were estimated by following the method of Ma et al., (2007) with slight modifications.

- Tissues: Liver, Kidney and Gill.
- Concentrations of Cadmium used: 0.5, 10 and 20 ppm
- Duration of exposure: 0(control), 24, 48 and 72 h.
- Reagents:
  - Cadmium Chloride (Merck),
  - Conc. HNO₃ (AG)
  - H₂O₂ (AG).
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Procedure

Tissue preparation

For each of the Cd concentrations, five fishes (n=5) were randomly removed from the tanks during 24, 48 and 72 h of treatment respectively. The fishes were sacrificed after anesthetizing with MS-222. Then the liver, kidney and gill tissues were removed. The tissues were handled with plastic forceps and kept in plastic homogenizing tubes to avoid contamination. Fresh tissues were used for quantification of Cadmium.

Quantification of Cadmium in tissues

The fresh sample tissues (0.25 g each of liver, kidney and gill) were weighed, cut into small pieces and dried in an oven at 80°C for about 48 h. The tissues were digested in 10 mL concentrated HNO₃ and 5 mL H₂O₂ over a hot plate at about 100°C. The metal (Cd) content of the fractions was measured by inductively coupled plasma optical emission spectrometry (ICP-OES) (Perkin Elmer optima 5300 DV) at IIT, Chennai. Cd concentrations were expressed as µg g⁻¹ w/wt tissue.

4.3.2 Histological Alterations Study

Histological alterations studies were carried out following the method of Banaee et al., (2012).

- Tissues: Liver and Kidney
- Concentration of Cadmium used: 20 ppm
- Duration of exposure: 0(Control), 24, 48 and 72 h.
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➢ **Reagents:** Cadmium Chloride (Merck), 10% Formalin (AG), Alcohol (AG), Xylene (AG), Hematoxylin and Eosin (Sigma).

**Principle**

Histology is the study of the microscopic anatomy of cells and tissues of plant and animals. It is commonly performed by examining cells and tissues by fixing, sectioning and staining, followed by examination under suitable microscope. The ability to visualize or differentially identify microscopic structures is enhanced through the use of suitable histological stains. Histology is an essential tool of biology and medicine.

**Procedure**

After exposing the fishes to various Cd concentrations, the liver and kidney tissues were dissected out and fixed in 10% buffered formalin and post fixed in the same fixative. A small piece of liver and kidney tissues were processed for paraffin technique and sections of 5µ thickness were taken and stained with haematoxylin and eosin (H&E). The stained slides were subjected to histological analysis, observed and documented under a light microscope (XSZ-801BN model, China) equipped with a 12.1 mega pixels camera (Casio, EX-Z450, Japan).

4.3.3 **Histomorphometric and Stereological Analysis**

Histomorphometric and Stereological analysis were carried out by the method of Elias and Hyde, (1980) and Mori and Christensen, (1980).

**Procedure**

The conventional stereological principles and accepted morphometric procedures as outlined by Elias and Hyde, (1980) were used to obtain
quantitative information. The procedure followed was as described by Prakash et al., (2008). Stereological analysis was performed by the Point Count method, superimposing lattice of 121 intersections. In liver the diameter of Hepatocytes, Hepatocyte nuclei and Sinusoids were calculated using ocular micrometer scale (Mori and Christensen, 1980). The diameters were expressed in micrometer (µm).

In kidney, the volume density of the Glomerulus, Bowman’s capsule and Tubules per unit area (723 µm²; an area in the section observed with the 10 x objective) were quantified and the values were given in relative terms. The diameter of the Glomerulus, Bowman’s capsule and Tubules were estimated using ocular micrometer scale (Mori and Christensen, 1980) for the specified concentrations and duration of exposure.

4.3.4 DNA Fragmentation Study

DNA fragmentation was performed using the method of Nagata et al., (2000).

- **Tissues** - Liver and Kidney
- **Concentration of Cadmium used** - 20 ppm
- **Duration of exposure** - 0(control), 24, 48 and 72 h.
- **Reagents**: Lysis buffer (Sigma), Proteinase K (Sigma), Isopropanol (AG).

**Principle**

DNA fragmentation is a key feature of programmed cell death and also occurs in certain stages of necrosis. DNA cleavage during apoptosis occurs at sites between nucleosomes, the protein containing structures that occur in chromatin as ~180 bp fragments or multiples thereof. DNA fragmentation is
analyzed using agarose gel electrophoresis to observe the “ladder” pattern at ~180 bp intervals.

Necrosis on the other hand, is characterized by random DNA fragmentation which forms a “smear” on agarose gels. Thus, when DNA undergoing apoptosis is isolated from liver and kidney tissues and run on an agarose gel, it shows a characteristic DNA ladder pattern.

**Procedure**

**DNA isolation**

1. The tissues were washed with PBS.
2. 0.5 mL of 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) were added to the tissues (50 mg) and incubated for 90 minutes at 37°C. The tissues were then homogenized manually or by using a sonicator.
3. Then centrifuged at 14,000 rpm at RT for 5 minutes.
4. The supernatant was transferred to a fresh eppendorf tube.
5. Equal volume of isopropanol and 25 µL of 4M NaCl (100 mM final concentration) were added.
6. Tubes were incubated overnight at -20°C.
7. They were then centrifuged at 14,000 rpm at room temperature for 20-25 minutes to obtain DNA pellet.
8. DNA pellet was dissolved in 30 µL of Milli-Q water.
9. Incubate for an hour at 37°C.
10. The isolated DNA from Control and Cadmium exposed fish liver and kidney tissues were electrophoresed in 1% agarose gel.
Agarose Gel Electrophoresis for DNA

Agarose Gel Electrophoresis was performed as described by Maniatis and Fritsch, (1982).

Principle

Electrophoretic separation is carried out in agarose gel for DNA samples. DNA molecules and their fragments are considerably larger than protein, therefore larger pore sized agarose gel is required. The mobility of DNA molecules during gel electrophoresis is dependant on the pore size. The gel concentration is chosen based on the type of molecules to be separated.

Reagents

TAE buffer (stock solution 50X), Tris base -242 g, Acetic acid glacial – 57.1 mL, EDTA 0.5M, Working concentration 1X, Gel loading buffer (6X), Bromophenol blue (Sigma), Sucrose (Sigma), Agarose (Sigma), Ethidium Bromide (Sigma).

Procedure

Gel electrophoresis

1. 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).
2. The gel tray was removed from the casting unit and placed in the electrophoresis tank.
3. 1x TBE buffer was poured into the tank until the agarose gel (1%) got immersed.
4. 1 µg of each of the DNA sample was loaded into the wells with gel loading buffer (2 µL of dye is used).

5. Place the lid on the tank and connect the electrodes to the power supply. The power was switched on; set at 100 V for 30 minutes.

6. DNA fragments were visualized in the UV trans-illuminator and the observations were documented.

4.3.5 Dapi Nucleic Acid Staining

Nuclear morphology was performed by following method of Magesh et al., (2009).

- Tissues - Liver and Kidney
- Concentration of Cadmium used - 20 ppm
- Duration of exposure - 0 (control), 24, 48 and 72 h.
- Reagents: 4-6-diamidino-2-phenylindole (DAPI) (Sigma), 1X Phosphate buffered saline (AG), Xylene (AG).

Principle

The fluorescent dye 4, 6-diamidino-2-phenylindole (DAPI) binds selectively to DNA and forms strongly fluorescent DNA-DAPI complexes with high specificity. On adding DAPI to tissue cells it is rapidly taken up into cellular DNA yielding highly fluorescent nuclei and no detectable cytoplasmic fluorescence. DAPI shows fluorescence specifically for AT rich regions.

Procedure

1. In the paraffin processed tissues, cross section was dewaxed with Xylene and dehydrated with serial concentrations of absolute alcohol and washed with distilled water.
2. The sections were washed with PBS and incubated with 0.5 μg/ml of DAPI in the dark for 5 minutes.

3. After five minutes the slide was washed thrice in PBS, drained excess buffer from the slide and mounted in the same buffer.

4. The cells were viewed using an Olympus BX51 fluorescence microscope (Olympus, Tokyo, Japan) (magnification, 40x) with appropriate filters at 450 nm and photographed.

5. Cells were condensed chromatin or fragmented nuclei were considered as apoptotic.

6. The incidence of apoptosis in each preparation was analyzed by counting around 300 cells and the percentage of apoptotic cells determined.

4.3.6 Caspase-3 Estimation

Caspase-3 activity was performed by following the method of Fiddo et al., (1995).

- Tissues - Liver and Kidney
- Cadmium Concentration used - 20 ppm
- Duration of exposure - 0 (control), 24, 48 and 72 h.

- Antibody: Polyclonal Rabbit IgG (Cat.No. AF 835, R&D Systems) Primary antibody and Anti-Rabbit IgG conjugated with HRP (Geni, Bangalore) secondary antibody.

Procedure

Caspase-3 expression was assessed by Western blot using β-Actin (internal control), Polyclonal Rabbit IgG primary and Anti-Rabbit IgG conjugated with HRP secondary antibody.
4.3.7 Catalase Enzyme Assay

Catalase levels in response to Cd treatments were evaluated by the method of Sinha et al., (1972). 

- Tissues: Liver and Kidney
- Cadmium Concentrations used: 5, 10 and 20 ppm
- Duration of exposure: 0 (control), 24, 48 and 72 h.

Procedure

1. The tissues (50mg) were homogenized in 50 mM phosphate buffer, pH 7.0, and centrifuged at 16,000g for 45 min.
2. The supernatant was used as the enzyme source.
3. The reaction mixture contained 2 mL of phosphate buffer (pH 7.0) 0.45 mL H₂O₂, and 0.025 mL of enzyme source.
4. The absorbance was read at 570 nm using spectrophotometrically and the enzyme activity was expressed as micromoles of H₂O₂ consumed/min/ mg protein.

4.3.8 GST Enzyme Assay

The GST levels in response to Cadmium treatment were analyzed in the tissues using the method of Habig et al., (1974).

- Tissues: Liver and Kidney
- Concentrations of Cadmium used: 5, 10 and 20 ppm
- Duration of exposure: 0 (control), 24, 48 and 72 h.
- Reagents: CdCl₂ (AG), 50 mM Tris–HCl buffer (pH 7.4), 0.2 M sucrose, M potassium phosphate buffer (pH 6.9), 30 mM CDNB and 30 mM GSH.
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Procedure

1. Enzymatic assay was performed on *C. gariepinus* liver and kidney.

2. The tissues (50 mg) were homogenized in 50 mM Tris–HCl buffer, pH 7.4, and containing 0.2 M sucrose and centrifuged at 16,000g for 45 min at 4°C.

3. The obtained pellet was discarded and the supernatant was used as the enzyme source.

4. The reaction mixture which is about 3 mL contained 2.4 mL of 0.3 M potassium phosphate buffer (pH 6.9), 0.1 mL of 30 mM CDNB and 0.1 mL of 30 mM GSH as enzyme source.

5. The reaction was initiated by glutathione. The GST levels were measured using spectrophotometrically.

6. The absorbance was read at 340 nm against a reagent blank.

7. The results were expressed as μM/min/mg protein.

4.3.9 SOD Enzyme Assay

SOD activity was assayed by the method of Misra and Fridovich *et al.*, (1972).

- **Tissues** - Liver and Kidney

- **Concentrations of Cadmium used** - 5, 10 and 20 ppm

- **Duration of exposure** - 0(control), 24, 48 and 72 h.

- **Reagents**: CdCl₂, 50 mM phosphate buffer (PH 7.0), 0.1 mM EDTA, 30 mM epinephrine, Carbonate buffer
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Procedure

1. The tissue was homogenized in ice cold 50 mM phosphate buffer (PH 7.0) containing 0.1 mM EDTA to give 5% homogenate (W/V).

2. The homogenates were centrifuged at 10,000 rpm for 10 min at 4°C in cold centrifuge.

3. The supernatant was separated and used for enzyme assay.

4. 100 µl of tissue extract was added to 880 µl (0.05 M, pH 10.2, containing 0.1 mM EDTA) carbonate buffer; and 20 µl of 30 mM epinephrine (in 0.05% acetic acid) was added to the mixture and optical density was measured at 480 nm for 4 min using UV-Spectrophotometer (Hitachi U-2000).

5. Values are expressed as superoxide anion reduced / mg protein / min.

4.4 BIOCHEMICAL STUDY

4.4.1 Estimation of Total Protein

Total protein concentrations of Control and Cd treated liver and kidney tissues were determined by the method of Lowry et al., (1951).

- Tissues - Liver and Kidney
- Concentrations of Cadmium used - 20 ppm.
- Duration of exposure - 0(Control), 24, 48 and 72 h.

- Reagents: CdCl₂, Lowry’s reagent, Folin-Ciocalteau reagent (1N), BSA.
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Principle

In alkaline solutions, protein molecules form a complex with copper ions and the amino acids containing phenolic hydroxyl group *viz.* tyrosine and tryptophan present in the copper-protein complex reacts with Folin-Ciocalteau reagent to give blue colour due to the reduction of phosphomolybdate and phosphotungstate anions respectively. The intensity of the colour developed is directly proportional to the concentration of proteins available in the test samples.

Procedure

1. Total proteins were quantified in control and Cd treated tissues using Lowry’s method.
2. 1 µL of the protein was made up to 10 µL with distilled water and 100 µL of Lowry’s reagent was added, shaken well and allowed to stand for 10 minutes.
3. 10 µL of Folin-Ciocalteau reagent was added, shaken well, and kept at room temperature for 20 min.
4. Standard solutions containing BSA at concentrations of 2.5 - 12.5 µg and blank were treated in a similar manner.
5. The colour developed was measured at 720 nm.
6. The protein concentrations were calculated from the standard graph.

4.4.2 Quantification of MT

MT was quantified by following the method of Ma *et al.*, (2007) with slight modifications.
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- Tissues: Liver, Kidney and Gill.
- Concentrations of Cadmium used: 5, 10 and 20 ppm
- Duration of exposure: 0 (Control), 24, 48 and 72 h.
- Reagents: Cadmium Chloride (Merck), 0.01 M Tris-HCl (pH 8.0) (Thermo Fisher Scientific), 0.1 mM Phenylmethylsulphonyl Fluoride (PMSF) (Sigma), 0.1 mM Dithiothreitol (DTT) (Sigma), 2% (w/v) Bovine Hemoglobin (Sigma).

Procedure

1. Sample tissues 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.

2. The homogenization buffer also contained 0.1 mM phenylmethylsulphonyl fluoride (PMSF) and 0.1 mM dithiothreitol (DTT).

3. The homogenate was centrifuged at 16000 X g for 30 min at 4°C, and the supernatant was heated for 2 min in a boiling water bath (100°C).

4. The heated sample was centrifuged at 10000 X g for 10 min to remove precipitated proteins.

5. Volumes of 0.1 mL Cd solution (500 µg/L as CdCl₂) 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.
6. 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 (100 ºC) for 2 min, cooled in ice for 3 min, and centrifuged at 10000 x g for 15 min.

7. 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.

8. The amount of Cd ions in the final supernatant was now proportional to the amount of MT present.

9. The concentration of Cd in the supernatant was determined using an inductively coupled plasma optical emission spectrometry (ICP-OES) (Perkin Elmer Optima-5300 DV).

The MT concentrations were calculated by the following equation:

\[
\text{MT Conc. (µg g}^{-1}\text{ w wt) = Cd Conc. (µg g}^{-1}\text{ w wt)}/112.4/7 \times 7000.}
\]

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)

MT concentrations were expressed as µg g\(^{-1}\) w wt.

### 4.4.3 MT Expression Confirmation by Western Blot

Protein expressions for MT and β-Actin (internal control) in liver and kidney tissues of freshwater catfish, C. gariepinus was analysed by the method of Fiddo et al., (1995).

- Tissues - Liver and Kidney
- Concentration of Cadmium used - 20 ppm
Duration of exposure - 0 (Control) and 72 h.

**ANTIBODY:**

**Metallothionein:**

*Primary Antibody* : Monoclonal mouse Anti-Metallothionein IgG (Cat No. UC1MT (ab12228), Abcam.

*Secondary Antibody* : Anti-Mouse IgG conjugated with HRP from Bangalore, Geni.

**Beta Actin:**

*Primary Antibody* : Monoclonal Mouse Anti-β-Actin IgG (Cat No. MA 1-91399, Thermo Scientific Inc, USA)

*Secondary Antibody* : Anti-Mouse IgG conjugated with HRP from Bangalore, Geni.

Developing Solution : Enhanced chemiluminescence system (ECL) (West Femto Super Signal detection kit, Thermo Scientific Inc, USA) and quantified using Chemi Doc XRS Imaging System, Bio-Rad (USA).

**Principle**

An overall examination and confirmation of the expression of proteins in tissues and cells is possible by the technique called Western blot. Proteins were extracted from tissues and separated on polyacrylamide gel (PAGE) on the basis of size and transferred on to PVDF or Nitrocellulose membranes. Following transfer, the proteins of interest were detected by incubation of the membrane with specific antibodies, followed by detection with an enzymatically labelled (HRP-conjugated) secondary antibody. The protein expressions were visualized by chemiluminescent method using enhanced chemiluminence (ECL) reagent.
Procedure

1. Control and Cd treated liver and kidney tissues were weighed (50 g) and homogenized in 300 μL of ice-cold RIPA buffer with protease inhibitor cocktail (Biobasic Inc., USA).

2. Homogenized tissues were centrifuged at 14,000 rpm for 10 min at 4°C. Pellet was removed. The proteins were present at supernatant.

3. The supernatant was separated and added to a new 1.5 mL centrifuge tube and stored at -20°C until further use.

4. Equal amount of total protein (50 μg) was mixed with 2X sample buffer and boiled for 5 min.

5. The proteins were separated on 12% SDS-polyacrylamide gels and electrophoretically transferred (SDS-PAGE) into Polyvinylidene difluoride (PVDF) membranes (Millipore, USA).

6. The blots/non-specific binding sites were blocked with 5% blocking buffer for 2 h.

7. After blocking, membranes were incubated with respective primary antibodies Anti-MT antibody (ab12228-UC1MT, Abcam) and β-Actin (sc-81178, Santa Cruz Biotech, USA) 1:1000 dilution overnight at 4°C.

8. Then the membranes were washed thrice with T-TBS, each for 10 minutes, followed incubation for 45 min at room temperature with the corresponding horseradish peroxidase-conjugated secondary antibody (Merck, India) (1:500 dilutions).

9. 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).
10. The intensity of the bands was quantified by Quantity one software (Bio Rad, USA).

### 4.4.4 Immunohistochemical Analysis

Immunohistochemical Analysis was performed by the method of Stephenson et al., (2009).

- **Tissues** - Liver and Kidney
- **Concentration of Cadmium used** - 20 ppm
- **Duration of exposure** - Control and 72 h.

- **Reagents**: MT primary and Secondary antibody, 0.1 M Trisodium citrate (pH= 6.2) (Fisher Scientific), 3.0% Hydrogen peroxide (Fisher Scientific), 1.5% BSA (Thermo Fisher Scientific), 1X Phosphate buffered saline (Fisher Scientific), ABC staining kit (Thermo Fisher Scientific).

**Principle**

Immunohistochemistry is a widely used biological technique that can be used for studies in anatomy, physiology, immunology and biochemistry. Developed from the antigen-antibody binding reaction, it can be considered as methods that visualize distribution and localization of specific antigen or cellular components in separated tissues, or tissue sections.

**Procedure**

1. Immunohistochemical evaluation of MT in fish was investigated at 48 h following MPTP administration.
2. Five micrometer-thick paraffin sections through matched coronal levels of the MTs were stained with monoclonal mouse Anti-
Metallothionein Ig G primary antibody (1:50), using standard immune-peroxidase techniques.

3. Briefly paraffin sections of fish liver were deparaffinised and hydrated with distilled water.

4. Antigenic sites were exposed by incubating sections in Antigen Retrieval solution (0.1M 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.

5. 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 1h incubation in 1.5% BSA.

6. MT immuno-reactivity was detected with an Anti-Mouse IgG conjugated with HRP 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).

4.4.5 Metallothionein Isolation and Purification

MT isolation and purification was performed by following method of Honda et al., (2005) with slight modifications.

- Tissues - Liver.
- Concentrations of Cadmium used - 20 ppm.
- Affinity Column - 1-mL Hi Trap™ Chelating HP columns were purchased from Amersham Pharmacia Biotech (Sweden).
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- Sephadex G-25 (Sigma).

➤ Reagents

1. Cadmium chloride, Bovine Serum Albumin, β-Mercaptoethanol and Protease inhibitors these all chemicals were purchased from Sigma chemicals.

2. Nickel Sulfate (0.1M NiSO$_4$.6H$_2$O).

For 10 mL, 0.26 g NiSO$_4$.6H$_2$O was dissolved in 10 mL Milli-Q water.

3. Equilibration/Washing Buffer

For 100 mL, 0.788 g Tris HCl and 0.876 g NaCl were dissolved in 80 mL Milli-Q water and pH was adjusted to 7.4 with HCl and made up to 100 mL. Stored at 4°C.

4. Elution/Blank Buffer

For 100 mL, 0.788 g Tris HCl and 0.876 g NaCl and 3.404 g imidazol was dissolved in 80 mL Milli-Q water and pH was adjusted to 7.4 with HCl and made up to 100 mL. Stored at 4°C.

5. 0.1 M EDTA Buffer

For 10 mL, 0.3724 g EDTA was dissolved in 10 mL Milli-Q water. Stored at 4°C.

6. Molecular Markers: Apparent molecular weights were determined by measurement of relative motilities using the broad range SDS-PAGE molecular weight standards from ovalbumin 43 kDa, carbonic anhydrase 29 kDa, Soyabean trypsin inhibitor 20.1 kDa, lysozyme 14.3 kDa, aprotinin 6.5 kDa (Geni, Bangalore).

Principle
The principle of Affinity Chromatography is that the stationary phase consists of a support medium (e.g. cellulose beads) on which the substrate (or sometimes a coenzyme) has been bound covalently, in such a way that the reactive groups that are essential for enzyme binding are exposed. As the mixture of proteins is passed through the chromatography column, those proteins that have a binding site for the immobilized substrate will bind to the stationary phase, while all other proteins will be eluted in the void volume of the column.

**Procedure**

1. The total proteins were extracted from the fish liver tissue.
2. 1g of liver tissue was homogenized with 3 mL of RIFA buffer and 2 mL of 0.1 mM protease inhibitors in a homogenizer.
3. The homogenate was centrifuged at 18,000 × g for 60 min at 4°C.
4. The supernatant was immediately applied to a HiTrap™ Chelating HP column previously loaded with NiSO₄.6H₂O, as described below.

**AFFINITY CHROMATOGRAPHY**

1. Affinity chromatography was performed using a 1 mL HiTrap™ Chelating HP column.
2. 800 µL of the total proteins from the supernatant obtained from the liver tissues were loaded to the affinity column previously loaded with NiSO₄.6H₂O.
3. After metal loading the column was equilibrated with 10 volumes equilibrating buffer.
4. After applying the supernatants, 10 volumes of equilibrating buffer were passed through the column, this is washing step, non-specific binding proteins removed from the column resins.

5. The elution was performed with elution buffer containing 500 mM imidazol (for reduced of protein denaturing condition) were collected at a flow rate of 1 mL/min through the entire procedure. The purification was performed at room temperature.

6. Once finished the elution procedure, the column was washed with 10 volumes of 0.1 mM EDTA (this step is called washing step) followed by 10 volumes of distilled fresh water to remove the column, according to the protocol enclosed to the manufacturers technical data sheet.

7. Aliquots (20 µL) of each fraction obtained from the elution step were applied to 12% SDS-PAGE. MT positive fractions were deduced on the gel by the molecular mass.

**Desalting column (Sephadex G-25)**

1. Imidazol was removed from purified MT fractions using desalting column (Sephadex G-25).

2. MT was obtained by the incubation of MT positive fractions, obtained as described above and deduced from SDS-PAGE, with 50 mM DTT and 10 mM HCl solution according to Dallinger et al. (2001) and Honda et al., (2005).

3. The elution preparation was applied to a Sephadex G-25 (Sigma) column. Pre-equilibrated with 10 mM HCl and 10 mM NaCl. The fractions were collected.

4. The entire procedure to obtain MT was performed under 4°C.
5. A collected fraction was applied to 12% SDS-PAGE and stained with CBB.
4.4.6 Maldi-TOF MS

MT molecular weight was performed the following method of Lazarev et al., (2009).

- **Reagents:** 50 mM Ammonium bi-carbonate (Fisher Scientific), 40% ethanol, Acetonitrile (Fisher scientific), Trypsin (Sigma), 1% Trifluoroacetic acid (Fisher scientific), A-cyano-4-hydroxycinnamic acid (Sigma).

**Procedure**

1. MALDI-TOF MS was used for the analysis of peptide mass.

2. Purified protein (20 µg) was resolved on a 12% SDS-PAGE and visualized by CBB R-250.

3. The protein bands were excised from the gel, sliced into 1 mm$^3$ cubes and incubated overnight at room temperature with 200 µL of 50 mM NH$_4$HCO$_3$ in 40% ethanol.

4. After the stain removal, the liquid was removed and replaced with 50 µL of 50 mM NH$_4$HCO$_3$. Subsequently, NH$_4$HCO$_3$ buffer was removed and replaced with 150 µL of acetonitrile and incubated for 10 min.

5. After this step was repeated two times, the gel pieces were dried for a few minutes. Then replace with 100 µL of 50 mM NH$_4$HCO$_3$.

6. The samples were homogenated, then centrifuged at 12,000 rpm for 15 min at 4°C. The MT present supernatant was used for MALDI-TOF MS analyzes.
4.4.7 *Maldi-TOF MS (PMF)*

MT identification and amino acid sequence was performed by the method of Lazarev *et al.*, (2009) with slight modification. MALDI-TOF mass spectra were used for the analysis of peptide mass fingerprinting and MS/MS ion search.

**Procedure**

**In-gel trypsin digestion**

1. Purified protein (20 µg) was resolved on a 10% SDS-PAGE and visualized by CBB R-250.

2. The protein bands were excised from the gel, sliced into 1 mm$^3$ cubes and incubated overnight at room temperature with 200 µL of 50 mM NH$_4$HCO$_3$ in 40% ethanol.

3. After the stain removal, the liquid was removed and replaced with 50 µL of 50 mM NH$_4$HCO$_3$. Subsequently, NH$_4$HCO$_3$ buffer was removed and replaced with 150 µL of acetonitrile and incubated for 10 min.

4. After this step was repeated two times, the gel pieces were dried for a few minutes.

5. Twenty microlitres of fresh trypsin solution (12.5 ng/µL) in 50 mM NH$_4$HCO$_3$ buffer was added to the tube on ice.

6. Excess enzyme solution was discarded using the gel loading tips after 10 min of rehydration, and the solution was replaced with an equivalent volume of fresh 25 mM NH$_4$HCO$_3$ buffer, followed by incubation for 12 h at 36°C.

7. The digestion was terminated by addition of 10 µL of 1% trifluoroacetic acid (TFA) for MALDI-TOF MS analysis.
Sample preparation for MALDI-TOF MS

1. The 20 µL of trypsin digested purified protein solution was used for the MALDI-TOF MS analysis, recorded on an AB SCIEX Voyager DE Pro MALDI-TOF (Applied Biosystems, Foster City, CA) Time-of-Flight spectrometer, with a Pulsed Nitrogen Laser (337 nm; 3-ns pulse width).

2. The spectra were recorded in the linear, positive high-mass mode. A saturated solution of α-cyano-4-hydroxycinnamic acid in a 1:1 mixture of acetone and water along with 0.1% trifluoroacetic acid was used for obtaining the mass spectra.

Peptide Mass Fingerprinting and Ms/Ms Ion search

For identification of the peptide fragments obtained from MALDI-ToF, selected peptide masses were submitted to Mascot (http://www.matrixscience.com) for NCBI PDB search to get the closest match possible. Suitable filters and selection criteria for the obtained peptide fragment mass enabled proper matching of available protein sequences and the query sequence.

From the obtain sequence match, apart from identification, phylogenetic trees and homology can be drawn or evolutionary inferences made.

4.5 STATISTICAL ANALYSIS

Statistical analysis was carried out by Graph pad prism software. Where ever appropriate, comparison of controls and treated groups were carried out using suitable tests such as one-way ANOVA. In certain experiments co-relation and regression analysis were done. For each comparison suitable levels of significance (p<0.001, p<0.05) testing was used.