CHAPTER 5

RESULTS

5.1 CADMIUM ACCUMULATION

The fishes that were exposed to Cd concentrations of 5, 10 and 20 ppm for a period of 24, 48 and 72 h showed accumulation of Cd in the liver, kidney and gill tissues of freshwater catfish, C. gariepinus in a dose and time dependent manner. The accumulated Cd levels were observed to increase significantly in the following order: Liver > Kidney > Gill. A control batch was maintained separately against which the accumulation of Cd was compared and results obtained. The Cadmium concentrations were expressed as ‘µg g-1 w wt’.

5.1.1 Cadmium Accumulation in Liver Tissue (in µg g-1 w wt.)

After 24 h of 5 ppm Cd exposure, Cd accumulation level was calculated to be $8.39 \pm 1.78$. For 10 ppm Cd exposure, accumulation level was calculated to be $15.47 \pm 3.01$. For 20 ppm Cd exposure, Cd accumulation level was calculated to be $23.03 \pm 2.40$. (Table 5.1)

After 48 h of 5 ppm Cd exposure, Cd accumulation level was calculated to be $14.01 \pm 3.45$. For 10 ppm Cd exposure, Cd accumulation level was calculated to be $22.52 \pm 3.44$. For 20 ppm Cd exposure, Cd accumulation level was calculated to be $27.56 \pm 3.11$.

After 72 h of 5 ppm Cd exposure, Cd accumulation level was calculated to be $25.01 \pm 2.87$. For 10 ppm Cd exposure, Cd accumulation level was calculated to be $35.38 \pm 4.21$. For 20 ppm Cd exposure, Cd accumulation level was calculated to be $54.11 \pm 7.90$. 
In the control batch pre experimental Cd present in the liver was calculated to be 0.62 ± 0.21. For 24 h, Cd present was calculated to be 0.66 ± 0.13. After 48 h, Cd present was calculated to be 0.7 ± 0.14. After 72 h, Cd calculated to be 0.64 ± 0.05.

The highest Cd accumulation of 54.11 ±7.90 µg g$^{-1}$ w wt was observed in the liver after 72 h of 20 ppm Cd exposure.
The data were statistically tested and the values were found to be statistically significant at $P < 0.05$. Figure 5.1 represents the data of Table 5.1 graphically.

### 5.1.2 Cd Accumulation in the Kidney (in µg g⁻¹ w wt.)

After 24 h of 5 ppm Cd exposure, Cd accumulation level was calculated to be 7.36 ± 1.44. For 10 ppm Cd exposure, Cd accumulation level was calculated to be 11.17 ± 1.38. For 20 ppm Cd exposure, Cd accumulation level was calculated to be 17.71 ± 1.42 (Table 5.2).

After 48 h of 5 ppm Cd exposure, Cd accumulation level was calculated to be 10.31 ± 1.43. For 10 ppm Cd exposure, Cd accumulation level was calculated to be 13.16 ± 1.51. For 20 ppm Cd exposure, Cd accumulation level was calculated to be 22.39 ± 2.37.

After 72 h of 5 ppm Cd exposure, Cd accumulation level was calculated to be 13.32 ± 1.70. For 10 ppm Cd exposure, Cd accumulation level was calculated to be 21.88 ± 2.34. For 20 ppm Cd exposure, Cd accumulation level was calculated to be 29.52 ± 2.69.

In the control batches, pre-experimental Cd accumulation level was calculated to be 0.58 ± 0.18. After 24 h, Cd level was calculated to be 0.62 ± 0.15. After 48 h Cd level was calculated to be 0.63 ± 0.15. After 72 h Cd level was calculated to be 0.61 ± 0.09.

The highest Cd accumulation of 29.52 ± 2.69 µg g⁻¹ w wt was observed in the kidney after 72 h of 20 ppm Cd exposure.
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Figure 5.2 Cadmium accumulation in kidney of *C. gariepinus*

(Each bar indicated Mean ± S.D, (n=5) in triplicates. ***p<0.001 statistically significant between control and cadmium treated kidney using Newman-Keul’s test. The control groups were maintained separated).

The data was statistically tested and the values were found to be statistically significant at P < 0.05. Figure 5.2 represents data of Table 5.2 graphically.

5.1.3 Cd Accumulation in the Gill (in µg g-1 w wt)

After 24 h of 5 ppm Cd exposure, Cd level was calculated to be 7.18 ± 0.66. For 10 ppm Cd exposure, Cd level was calculated to be 9.15 ± 0.97. For 20 ppm Cd exposure, Cd accumulation level was calculated to be 11.06 ± 0.23. (Table 5.3)
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After 48 h of 5 ppm Cd exposure, Cd accumulation level was calculated to be $8.6 \pm 0.79$. For 10 ppm Cd exposure, Cd accumulation level was calculated to be $12.08 \pm 1.82$. For 20 ppm Cd exposure, Cd accumulation level was calculated to be $15.39 \pm 1.61$.

After 72 h of 5 ppm Cd exposure, Cd accumulation level was calculated to be $10.06 \pm 1.54$. For 10 ppm Cd exposure, Cd accumulation level was calculated to be $13.95 \pm 1.77$. For 20 ppm Cd exposure, Cd accumulation level was calculated to be $17.73 \pm 1.55$.

In the control batches, pre-experimental Cd level in gill was calculated to be $0.58 \pm 0.16$. After 24 h, Cd level was calculated to be $0.59 \pm 0.12$. After 48 h, Cd level was calculated to be $0.60 \pm 0.12$. After 72 h, Cd level was calculated to be $0.58 \pm 0.14$.

![Figure 5.3 Cadmium accumulation in the gill of C. gariepinus](image)

*Figure 5.3 Cadmium accumulation in the gill of C. gariepinus (Each bar indicated Mean ± S.D, \(n=5\) in triplicates. ***p<0.001 statistically significant between control and cadmium treated gill using Newman-Keul’s test. The control groups were maintained separated).*
The highest Cd accumulation of $17.73 \pm 1.55 \mu g \; g^{-1} \; w \; wt$ was observed in the gill after 72 h of 20 ppm Cd exposure.

The data was statistically tested and the values were found to be statistically significant at $P < 0.05$. Figure 5.3 represents data of Table 5.3 graphically.

### 5.2 HISTOLOGICAL ALTERATIONS STUDIES

#### 5.2.1 Liver

The Cd exposed liver tissues of *C. gariepinus* were analyzed for histological alteration from the normal after exposing to 20 ppm for different time periods and compared.

The light microscopic examination of the control fish liver shows normal architecture with respect to the distribution of hepatocytes, hepatocytes nuclei and sinusoids. Whereas treated fishes exposed to 20 ppm Cd a period of 24, 48 and 72 h, show time dependent alterations like degrees of hypertrophy of hepatocytes, cytoplasmic vacuolation of the hepatocytes, focal necrosis of hepatic tissue, loosening of hepatic tissue. Hepatic cells lost their original shape, got excessively distended and vacuolated. The alterations were more severe during 72 h of exposure when compared to 24, 48 h exposed fishes (Figure 5.4).

#### 5.2.2 Histological Alterations in Kidney Tissue

Histological studies were carried out on exposed *C. gariepinus* kidney tissues.

The light microscopy examination of the control fish kidney show normal architecture with normal distribution of Glomerulus and Tubules.
Whereas, Cd exposed groups showed time dependent alterations like vacuolar degeneration, pycnotic nucleic in the glomerulus, neutrophilic infiltration, tubules nucleus showed pycnotic nature with vacuolation, degeneration and necrosis (Figure 5.5).

5.3 HISTOMORPHOMETRIC AND STEREEOLOGICAL ANALYSIS

5.3.1 Liver Tissues

The effect of Cd on the histology of liver tissue was quantified by Histomorphometric and stereological analysis. The data illustrates that the Cd exposed fishes showed dose and time dependent alterations in the hepatocytes, hepatocytes nuclei and sinusoids structural diameter (Figure 5.6).

5.3.1.1 Hepatocyte Diameter (µm)

The hepatocyte diameter in the control was 15.75 ± 1.11. It increased to 18.54 ± 0.58 µm after 24 h, 20.54 ± 2.44 µm after 48 h and 24.19 ± 2.86 µm after 72 h of Cd exposure (Figure 5.6a).

5.3.1.2 Hepatocytes Nuclei Diameter (µm)

The hepatocyte nuclei diameter in control were 4.19 ± 0.42 µm. It increased to 3.65 ± 0.25 µm after 24 h, 3.38 ± 0.11 µm after 48 h and 2.95 ± 0.35 µm after 72 h of Cd exposure (Figure 5.6b).

5.3.1.3 Sinusoids Diameter (µm)

The diameter of sinusoids in control was 46.71 ± 2.09 µm. It increased to 65.75 ± 2.90 µm after 24 h, 72.99 ± 3.62 µm after 48 h and 108.61 ± 4.92 µm after 72 h of Cd exposure (Figure 5.6c).
A significant increase in hepatocyte and sinusoid diameter with concomitant reduction of hepatocytes nuclei diameter when compared to control was observed.

5.6 a.

![Graph showing hepatocyte diameter vs Cd exposure time]

5.6 b.

![Graph showing nucleus diameter in hepatocytes vs Cd exposure time]
5.6 c.

Figure 5.6 Histomorphometric diametric alterations in liver tissue

- Diameter of hepatocytes
- Diameter of hepatocyte nucleus
- Diameter of sinusoids on exposure to sub-lethal dose (20 ppm) of Cd for a period of 0 (control), 24, 48 and 72 h.

*Each bar indicated Mean ± S.D, (n=100) in triplicates. *p<0.05, **p<0.01, ***p<0.001 statistically significant between control and Cd treated liver tissues using Newman-Keul’s test.*

5.3.2 Kidney Tissues

The histological alterations of kidney tissues due to Cd were quantified by Histomorphometric and Stereological analysis. The data obtained showed that the Cd exposed fishes showed dose and time dependent alterations (Diameter, Volume and Numerical Density) in the Bowman’s capsule, glomerulus and tubules.
5.3.2.1 Histomorphometric Diameter Alterations in Nephrons

Histomorphometric diameter alterations were measured in Bowman’s capsules, Glomerulus and Tubules of kidney (Figure 5.7).

5.3.2.1.1 Bowman’s capsules

The Bowman’s capsule diameter in the control was 34.75 ± 1.60 µm. It increased to 36.89 ± 2.01 µm after 24 h, 38.86 ± 1.97 µm after 48 h and 45.99 ± 2.31 µm after 72 h of Cd exposure.

5.3.2.1.2 Glomerulus

The glomerular diameter in control was 32.08 ± 1.58 µm. It decreased to 31.70 ± 0.81 µm after 24 h, 30.31 ± 1.45 µm after 48 h and 28.87 ± 1.92 µm after 72 h of Cd exposure.

5.3.2.1.3 Tubules

The tubular diameter in control was 36.10 ± 2.76 µm. It increased to 40.09 ± 1.64 µm after 24 h, 42.12 ± 2.68 µm after 48 h and 46.42 ± 3.03 µm after 72 h of Cd exposure.

A significant increase in Bowman’s capsule and tubule diameter with concomitant reduction of glomerulus diameter when compared to control were observed.
5.3.2.2 Histomorphometric Volume Alterations in Nephrons

The volume of the glomerulus and tubules structure showed time dependent decrease on exposure to Cd when compared to control fish (Figure 5.8).

5.3.2.2.1 Glomerulus

The volume of glomerulus in control was 0.0712 ± 0.0082 mm$^3$. It reduced to 0.0692 ± 0.0038 mm$^3$ after 24 h, 0.0564 ± 0.0133 mm$^3$ after 48 h and 0.0542 ± 0.0053 mm$^3$ after 72 h of Cd exposure.
5.3.2.2 Tubules

The volume of tubules in control was 0.5012 ± 0.0534 mm$^3$. It also reduced 0.4404 ± 0.0280 mm$^3$ after 24 h, 0.374 ± 0.0343 mm$^3$ after 48 h and 0.343 ± 0.0393 mm$^3$ after 72 h of Cd exposure.

A significant decrease in glomerular and tubular volume when compared to control was observed over the different exposure times.

![Histomorphometric volume alterations in nephrons](image)

**Figure 5.8 Histomorphometric volume alterations in nephrons**

*Each bar indicated Mean ± S.D, (n=100) in triplicates. ***p<0.001.***

***p<0.001 statistically significant between control and Cd treated kidney using Newman-Keul’s test.*

5.3.2.3 Numerical Density Alterations in Nephrons

The numerical density of the glomerulus and tubules was found to decrease in the Cd exposed groups when compared to that of control fish kidney (Figure 5.9).
5.3.2.3.1 Numerical Density of Glomerulus

The numerical density of glomerulus in control was 14263.07 ± 679.88. It reduced to 11582.29 ± 718.12 after 24 h, 10263.54 ± 737.43 after 48 h and 7892.05 ± 923.91 after 72 h of Cd exposure.

![Graph showing numerical density alterations of glomerulus and tubules](image)

Figure 5.9 Histomorphometric numerical density alterations in nephrons
Each bar indicated Mean ± S.D, (n=100) in triplicates. *p<0.05, **p<0.01, ***p<0.001 statistically significant between control and Cd treated kidney using Newman-Keul’s test.

5.3.2.3.2 Numerical Density of Tubules

The numerical density of tubules in control was 63042.52 ± 2605.14. It reduced to 54846.68 ± 2169.39 after 24 h, 47793 ± 2071 after 48 h and 40742.26 ± 2650.15 after 72 h of Cd exposure.

A significant decrease in glomerulus and tubules numerical density when compared to control was observed (Figure 5.9).
5.4 DNA FRAGMENTATION STUDIES

5.4.1 Liver tissue

The Cd induced DNA fragmentation in liver tissue was observed in 1% agarose gel and detected by Ethidium Bromide (Figure 5.10). In the control tissue not exposed to Cd no ladder was observed (Figure 5.10 L1). While a genomic DNA ladder formation was observed in the liver tissue treated with 20 ppm of Cd (Figure 5.10 L2 – L4).

These results show an oligonucleosomal DNA fragmentation pattern comprising of ~180 base pairs fragments or multiples, the so-called “DNA ladder” which is a marker of apoptotic cell death. Electrophoresis of DNA isolated from control and Cd treated tissues showed a significant increase in the DNA ladder pattern in a dose and time dependent manner. The degradation of DNA into oligonucleotide fragments was maximal during 72 h of Cd exposure, confirming the induction of apoptosis (Figure 5.10 L4).

5.4.2 KIDNEY TISSUE

The Cd induced DNA fragmentation in kidney tissue was observed in 1% agarose gel and detected by Ethidium Bromide (Figure 5.11). In the control tissue not exposed to Cd no ladder was observed (Figure 5.11 L1). While a genomic DNA ladder formation was observed in the kidney tissue treated with 20 ppm of Cd (Figure 5.11 L2 – L4).

These results show an oligonucleosomal DNA fragmentation pattern comprising of ~180 base pairs fragments or multiples, the so-called “DNA ladder” which is a marker of apoptotic cell death. Electrophoresis of DNA isolated from control and Cd treated tissues showed a significant increase in the DNA ladder pattern in a dose and time dependent manner. The degradation of
DNA into oligonucleotide fragments was maximal during 72 h of Cd exposure, confirming the induction of apoptosis (Figure 5.11 L4).

5.5 APOPTOSIS

Apoptotic studies by DAPI staining on Cd exposed liver and kidney tissues were performed in Cd treated liver and kidney tissues of, *C. gariepinus* for a period of 24, 48 and 72 h of 20 ppm of Cd exposure to confirm the mode of cell death. Features such as chromatin condensation, nuclear shrinkage and apoptotic bodies are seen typically in cells undergoing apoptosis were observed during all Cd exposure periods. The results indicate that the cytotoxic effect of Cd in tissues was due to apoptosis.

5.5.1 Apoptosis of Hepatocytes

Figure 5.12 show the nuclear morphology of the liver tissues of the control and Cd treated fishes. The control fishes showed normal nuclear whereas Cd exposed fishes showed apoptotic morphology. Effect of Cd on nuclear morphology on fish liver was studied by 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).

Figure (5.12A) showed normal liver tissue with polygonal structure of hepatocytes and round clear hepatocytes nuclei and normal sinusoids. Cd exposed liver shows apoptosis Figure (5.12 B - D).

5.5.1.1 Quantification of Apoptotic Cells in Liver

Figure 5.13 shows the results of the quantitative study of cadmium exposed fish liver tissues’ apoptotic morphology. They were statistically
significant (p<0.001) and showed increased number of nucleii showing apoptotic morphology when compared to control.

![Graph showing quantification of apoptotic cells in Cd exposed liver tissue](image)

**Figure 5.13 Quantification of apoptotic cells in Cd exposed liver tissue**

Each bar represents the Mean ± S.D, (n=100) in triplicates. ***p<0.001 statistically significant between control and Cd treated liver tissue using Newman-Keul’s test. Percentage of apoptotic cells in liver control was 9.14 ± 1.39 %. It increased to 12.33 ± 2.07 % after 24 h, 25.52 ± 1.06 % after 48 h and 39.58 ± 2.97% after 72 h of cadmium exposure. The effect was more prominent after 72 h of cadmium exposed liver tissue than the other study periods.

5.5.2 Nuclear Morphology of Kidney Tissues

Figure 5.14 shows the nuclear morphology of the kidney tissues of the control and Cd treated fishes. The control fishes showed normal nuclear whereas Cd exposed fishes showed apoptotic morphology. Effect of Cd on nuclear morphology on fish kidney 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). The data (Figure 5.14 A) shows normal kidney with a round clear nucleus in both glomerulus and tubules. Cd exposed groups (Figure 5.14 B - D) shows apoptotic like morphology.

5.5.2.1 Quantification of Apoptotic Cells in Kidney

Figure 5.15 shows the results of Cd exposed kidney tissues which showed significantly (p < 0.001) higher apoptotic nuclear morphology when compared to control.

Percentage of apoptotic cells in kidney control was 7.84 ± 1.38 %. It increased to 11.81 ± 2.71 % after 24 h, 21.64 ± 2.39 % after 48 h and 25.65 ± 2.99 % after 72 h of Cd exposure. The effect was more prominent during 72 h Cd exposed kidney than the other study periods.

![Chart showing quantification of apoptotic cells in Cd exposed kidney tissue](image)

**Figure 5.15 Quantification of apoptotic cells in Cd exposed kidney tissue**

*Each bar represents the Mean ± S.D, (n=100) in triplicates.

***p<0.001 statistically significant between control and Cd treated kidney tissues using Newman-Keul’s test.*
5.6 ANALYSIS OF CASPASE-3 ACTIVITY

Caspase-3 activity was assessed from liver and kidney tissues of freshwater catfish, *C. gariepinus* by Western blot using Polyclonal Rabbit IgG primary antibody and Anti-Rabbit IgG conjugated with HRP secondary antibody. Western blot results showed elevated caspase-3 levels in Cd treated liver and kidney tissues after 72 h of exposure, when compared to the control tissues. The Antibody is made to react with caspase-3 after electrophoresis in 12 % SDS-PAGE and is useful to determine which caspase have elicited antibodies in a complex mixture of immunogens. The intensity of the bands (optical density) was elevated in Cd exposed tissues than in control of liver and kidney. The β-Actin housekeeping genes were incubated with monoclonal mouse anti-β-Actin IgG primary antibody and anti-mouse IgG conjugated with HRP secondary antibody. The Caspase-3 and β-Actin bands were detected using chemiluminescence system (ECL Kit) method. The Caspase-3 levels were quantified using β-Actin expression.

5.6.1 Caspase-3 Activity in Liver

In liver tissue, Caspase-3 expressions were found to significantly increase with respect to duration and concentration of Cd exposure (Figure 5.16). Caspase-3 expressions levels were quantified. Control tissue caspase-3 levels was 80.42 (Caspase-3 activity/β-Actin relative intensity). After 24 h of Cd treated liver tissues Caspase-3 level was 109.71 (Caspase-3 activity /β-Actin relative intensity). For 48 h Cd treated liver tissues Caspase-3 expression level was 115.08 (Caspase-3 activity /β-Actin relative intensity) and after 72 h, Cd treated liver tissues Caspase-3 level was significantly increased to 127.14 (Caspase-3 activity /β-Actin relative intensity) (Figure 5.17).
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Figure 5.16 Western blot of Caspase-3 in liver tissue of *C. gariepinus*

*L1* = control; *L2* = 24 h Cd exposure; *L3* = 48 h Cd exposure; *L4* = 72 h Cd exposure

Figure 5.17 Caspase-3 expression in liver tissue of *C. gariepinus*

Each bar represents the Mean ± S.D. (*n* = 3) in triplicates.

***p < 0.001** statistically significant between control and Cd treated liver using Newman-Keul’s test.
5.6.2 Caspase-3 Activity in Kidney

In kidney tissue, Caspase-3 expressions were found to significantly increase with duration and time of Cd exposure (Figure 5.18). Caspase-3 expressions levels were quantified. Control tissue caspase-3 levels were 75.58 (Caspase-3 activity/β-Actin relative intensity), after 24 h of Cd treated kidney tissues Caspase-3 levels was 98.89 (Caspase-3 activity /β-Actin relative intensity), after 48 h of Cd treated kidney tissues Caspase-3 levels was 105.38 (Caspase-3 activity /β-Actin relative intensity) and after 72 h of Cd treated kidney tissues Caspase-3 expression levels significantly increased to 111.13 (Caspase-3 activity /β-Actin relative intensity) (Figure 5.19).

![Figure 5.18 Caspase-3 activities in kidney tissue of C. gariepinus](image)

![Figure 5.19 Caspase-3 expression levels in kidney tissue of C. gariepinus](image)
Each bar represents the Mean ± S.D, (n=3) in triplicates. ***p<0.001 statistically significant between control and Cd treated kidney using Newman-Keul’s test.

5.7 DETOXIFICATION ENZYMES

5.7.1 GST Enzyme Activity

GST enzymes activity in response to Cd treatments was analyzed in the liver and kidney tissues of *C. gariepinus* for a period of 72 h. The data were graphically represented in Figure 4.20 and 4.21. The highest level of GST enzyme 6.09 ± 1.33 μM min/mg proteins in the liver after 72 h treated with 20 ppm of Cd. After 72 h of Cd exposure in the kidney, GST levels showed 5.83 ± 1.03 μM min/mg protein with 20 ppm of Cd. (Figure 5.20 and 5.21)

The GST enzyme levels in control liver and kidney tissues shows the 0.17 ± 0.02 μM min/mg protein and 0.16 ± 0.02 μM min/mg protein respectively. In the liver and kidney, GST enzyme levels show significant increase for the 72 h of Cd exposure study. The data were subjected to statistical analysis of one way ANOVA and the values were found to be statistically significant at P < 0.05.

![Figure 5.20 GST enzyme activity in liver tissue of *C. gariepinus* on exposure to Cd](image)

Figure 5.20 GST enzyme activity in liver tissue of *C. gariepinus* on exposure to Cd
The results were represented as mean ± SD. Statistical comparisons were made against Control fish on each sampling day. (***Statistically significant at p < 0.001)

Figure 5.21  GST enzyme activity in kidney tissue of *C. gariepinus* on exposure to Cd

The results were represented as mean ± SD. Statistical comparisons were made against Control fish on each sampling day. (***Statistically significant at p < 0.001)

5.7.2 Catalase Enzyme Activity

Catalase is an important enzyme in antioxidant defense system protecting animals from oxidative stress. The effect of 5, 10 and 20 ppm of Cd on liver and kidney is graphically represented in Figure 4.22 and 4.23. The
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The highest level of CAT enzyme levels was 457.6 ± 16.07 μmol H₂O₂ consumed/min/mg protein in liver after 24 h of Cd exposure with 5ppm (Figure 5.22). After 24 h of Cd exposure, the kidney CAT level is 386.6 ± 10.64 μmol H₂O₂ consumed/min/mg proteins after treated with 5ppm of Cd (Figure 5.23).

The CAT levels of control tissues value were in the following order, liver 497.2 ± 7.98 μmol H₂O₂ consumed/min/mg proteins, in kidney 409.5± 12.11 μmol H₂O₂ consumed/min/mg proteins, CAT activities decreased on exposure to increased Cd concentrations when compared to the control CAT activity. The data were subjected to statistical analysis of one way ANOVA and the values were found to be statistically significant at P < 0.05.

![Catalase enzyme activity in liver tissue of C. gariepinus exposed to Cd](image)

The results were represented as mean ± SD. Statistical comparisons were made against Control fish on each sampling day. (***Statistically significant at p < 0.001, *p<0.05).
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Figure 5.23 Catalase enzyme activity in kidney tissue of *C. gariepinus* exposed to Cd

The results were represented as mean ± SD. Statistical comparisons were made against Control fish on each sampling day. (***Statistically significant at \( P < 0.001 \)).

5.7.3 SOD Enzyme Assay

SOD is an important enzyme in antioxidant defense system protecting animals from oxidative stress. The effect of 5, 10 and 20 ppm of Cd for tissues such as liver and kidney is graphically represented in Figure 4.24 and 4.25. The highest level of SOD enzyme levels was shows in the values of 0.946 ± 0.19 in superoxide anion reduced / mg protein / min in the liver after 24 h of Cd exposure with 5ppm, after 24 h of Cd exposure (Figure 5.24), in the kidney SOD level is 0.822 ± 0.21 in superoxide anion reduced / mg protein / min when treated with 5ppm of Cd (Figure 5.25).
Figure 5.24 SOD enzyme activity in liver tissue of *C. gariepinus* exposed to Cd
The results were represented as mean ± SD. Statistical comparisons were made against Control fish on each sampling day. (**Statistically significant at p < 0.01, ***p<0.01).

Figure 5.25 SOD enzyme activities in kidney tissue of *C. gariepinus* exposed to Cd
The results were represented as mean ± SD. Statistical comparisons were made against Control fish on each sampling day. (**Statistically significant at p < 0.01, ***p<0.01).
The SOD levels of control liver tissue were 1.042 ± 0.36 superoxide anion reduced / mg protein / min. In kidney 0.87 ± 0.11 superoxide anion reduced / mg protein / min. CAT activity decreased on exposure to increasing Cd concentrations and time when compared to the control SOD activity. The data were subjected to statistical analysis of one way ANOVA and the values were found to be statistically significant at P < 0.05.

5.8 MT INDUCTION

Induced MT levels were measured in Liver, Kidney and Gill tissues of freshwater catfish *C. gariepinus* after exposure to different concentrations of Cd over different durations and in the control groups. The MT levels were observed to increase significantly in the following order, Liver > Kidney > Gill. The induction of MT in the tissues is clearly time dependent and dose dependent.

5.8.1 MT Levels in Liver Tissue (in µg g⁻¹ w wt)

After 24 h of 5 ppm Cd exposure, MT level was 27.62 ± 4.55. For 10 ppm Cd exposure, MT level was 42.36 ± 2.79. For 20 ppm Cd exposure, MT level was 62.32 ± 5.76. (Table 5.4)

After 48 h of 5 ppm Cd exposure, MT level was 38.13 ± 9.76, For 10 ppm Cd exposure, MT level was 61.11 ± 12.28. For 20 ppm Cd exposure, MT level was 89.23 ± 14.63 µg g⁻¹ w wt.

After 72 h of 5 ppm Cd exposure, MT level was 63.88 ± 8.40. For 10 ppm Cd exposure, MT level was 111.81 ± 10.84. For 20 ppm Cd exposure, MT induction level was estimated to be 141.13 ± 11.58 µg g⁻¹ w wt.

The liver tissues MT level in the pre experiment control batches was 7.168 ± 0.73. For 24 h controls, the MT level was 7.308 ± 0.68. For 48 h
control liver tissue, MT level was 7.78 ± 1.20 and after 72 h MT level was 7.92 ± 2.11 µg g⁻¹ wet wt.

The induced MT levels were expressed as µg g⁻¹ wet wt.

The highest MT induction of 141.13 ± 11.58 µg g⁻¹ w wt was observed in the liver after 72 h of 20 ppm Cd exposure (Figure 5.26).

![Figure 5.26 MT induction levels in liver of C. gariepinus](image)

*Figure 5.26 MT induction levels in liver of C. gariepinus*

Each bar indicated Mean ± S.D. (n=5) in triplicates. **p<0.001 statistically significant between control and cadmium treated liver using Newman-Keul’s test.**

The data was statistically tested and the values were found to be statistically significant at P < 0.05. Figure 5.26 represents data of Table 5.4 graphically.
5.8.2 MT Levels in Kidney Tissue (in µg g⁻¹ w wt)

After 24 h of 5 ppm Cd exposure, MT level was 13.79 ± 2.31. For 10 ppm Cd exposure, MT level was 30.53 ± 5.99. For 20 ppm Cd exposure, MT level was 49.32 ± 11.62 µg g⁻¹ w wt. (Table 5.5).

After 48 h of 5 ppm Cd exposure, MT level was 30.49 ± 5.20. For 10 ppm Cd exposure, MT level was 39.24 ± 9.06 and for 20 ppm Cd exposure, MT level was 56.45 ± 12.18 µg g⁻¹ w wt.

After 72 h of 5 ppm Cd exposure, MT level was 35.91 ± 6.30. For 10 ppm Cd exposure, MT induction level was 56.07 ± 10.99 and for 20 ppm Cd exposure, MT level was 83.08 ± 11.18 µg g⁻¹ w wt.

In the control batches, the kidney tissue MT level was 6.59 ± 1.33 in the pre treatment batch. For 24 h controls, MT level was 6.87 ± 1.96 µg g⁻¹ w wt, for 48 h MT level was 6.79 ± 1.33 and for 72 h controls, MT level was 6.86 ± 1.78.

The MT induction levels were expressed as µg g⁻¹ w. wt.

The highest MT induction of 83.08 ± 11.18 µg g⁻¹ w wt was observed in the kidney after 72 h of 20 ppm Cd exposure (Figure 5.27).
Figure 5.27 MT induction levels in kidney of C. gariepinus
Each bar indicated Mean ± S.D. (n=5) in triplicates. ***p<0.001 statistically significant between control and cadmium treated kidney using Newman-Keul’s test.

The data was statistically tested and the values were found to be statistically significant at P < 0.05. Figure 5.27 represents data of Table 5.5 graphically.

5.8.3 MT Levels in Gill Tissue (in µg g-1 w wt)

After 24 h of 5 ppm Cd exposure, MT level was 14.49 ± 5.48. For 10 ppm Cd exposure, MT level was 27.71 ± 4.43 and for 20 ppm Cd exposure, MT level was 36.96 ± 5.21 µg g-1 w wt. (Figure 5.28, Table 5.6)

After 48 h of 5 ppm Cd exposure, MT level was 18.93 ± 4.28. For 10 ppm Cd exposure, MT level was 28.52 ± 5.02 µg g-1 w wt and for 20 ppm Cd exposure, MT level was 39.67 ± 4.85 µg g-1 w wt.
After 72 h of 5 ppm Cd exposure, MT induction level was 25.11 ± 5.26. For 10 ppm Cd exposure, MT induction level was 40.48 ± 6.98 and 20 ppm Cd exposure, MT induction level was 57.28 ± 7.97.

In the control batches, prior to treatment, the gill tissues MT level was 6.11 ± 2.83. For 24 h controls, MT level was 6.36 ± 1.78, for 48 h control gill tissue, MT level was 6.20 ± 1.80 and for 72 h control gill tissue, MT level was 6.32 ± 2.15. The MT induction levels were expressed as µg g⁻¹ wet wt.

![Figure 5.28 MT induction levels in gill of C. gariepinus](image)

Each bar indicated Mean ± S.D. (n=5) in triplicates. ***p<0.001 statistically significance between control and cadmium treated gill using Newman-Keul’s test.

The highest MT induction of 57.28 ± 7.97µg g⁻¹ wet wt was observed in the gill after 72 h of 20 ppm Cd exposure (Figure 5.28). The data was statistically tested and the values were found to be statistically significant at P < 0.05. Figure 5.27 represents data of Table 5.6 graphically.
5.9.1 Correlation between Cd Accumulation and MT Induction

The relationship between Cd accumulation and MT induction in liver, kidney and gill tissues of *C. gariepinus*. Figure 5.29 shows a positive correlation between Cd accumulation and MT induction in liver tissue. MT concentrations increased linearly with increasing Cd concentrations for the limited time-dose study, and is be described by the following regression equations: $[\text{MT}] = 2.536 \times [\text{Cd level}] + 5.014 \ (R^2 = 0.848, p<0.05)$ for liver.

Figure 5.30 shows the positive correlation between Cd accumulation and MT induction in kidney tissue. MT concentrations increased linearly with increasing Cd concentrations and may be described by the following regression equations: $[\text{MT}] = 2.519 \times [\text{Cd level}] + 2.899 \ (R^2 = 0.844, p<0.05)$ for kidney.

![Figure 5.29](image)

**Figure 5.29** Correlation between Cd accumulation and MT induction in liver tissues of *C. gariepinus*. 
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Figure 5.30 Correlation between Cd accumulation and MT induction in kidney tissues of *C. gariepinus*.

Figure 5.31 shows a positive correlation between Cd accumulation and MT induction. MT concentrations increased linearly with increasing Cd concentrations and are represented by the following regression equations: $[\text{MT}] = 2.803 \times [\text{Cd}] - 0.260$ ($R^2 = 0.701$, p<0.05) for gill.

Figure 5.31 Correlation between Cd accumulation and MT induction in gill tissues of *C. gariepinus*. 
5.10 IMMUNOLOCALIZATION OF MT

Under light-microscopic examination, the presence of immunoreactive MTs (irMTs) was visualized. irMTs were easily observed in liver and to a lesser extent in the kidney of Cd exposed fishes. Localization of MTs in liver and kidney tissues in freshwater catfish, *C. gariepinus* were observed by immunohistochemical method. 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 defined haemopoietic tissue, uriniferous tubules and glomerulus with clear Bowman’s capsule.

5.10.1 MT Localization in Liver

In liver, *ir*MTs were mainly localized in hepatocytes and to a lesser extent in erythrocytes of Cd exposed liver (Figure 5.32).

Under the light microscope, *irMTs* were seen to be 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 monoclonal mouse IgG primary and anti-mouse IgG conjugate with HRP secondary antibody, the cytoplasm of hepatocytes in Cd treated liver (Figure 5.32b) that were stained are identified as MT expressed hepatocytes brownish blue colour. The control liver tissue shows negative or poor expression of MT proteins in hepatocytes (Figure 5.32a).
5.10.2 MT Localization in the Kidney Tissues

Figure 5.33 shows enhanced levels of irMT localized in the kidney of Cd exposed fishes when compared to control. The immunolabel was mainly detected in the nephrocytes present in the proximal tubules of the nephron and more precisely in their basal labyrinth. Immunohistochemistry for control and Cd treated groups of the kidney tissue is carried out by incubation of 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.

Immunohistochemical staining of the liver and kidney of freshwater catfish revealed MT immunoreactivity. In liver, MT was stained in the hepatocytes and in kidney; MT was stained in the nephrocytes. It is clear that MT distribution was not uniform and is diffused. In the kidney, positively MT expression was detected in the cytoplasm of the collecting duct epithelium and the proximal and distal tubular epithelium (Figure 5.33b). The glomerulus and vascular endothelial cells stained positively for MT expression in Cd treated. The controls did not show a positive expression (Figure 5.33a).

5.11 MT – WESTERN BLOT

MT protein expression was assessed for confirmation from liver and kidney tissues of freshwater catfish, C. gariepinus by Western blot method using monoclonal mouse IgG primary and anti-mouse IgG conjugate with HRP secondary antibody. Western blot results shows elevated MT expression levels in Cd treated liver and kidney tissues respectively after 72 h, for 20ppm concentration when compared to the control fish tissues. The anti-MT Antibody reacts specifically with MT proteins and after electrophoresis in 15 %
SDS-PAGE it was useful to determine which proteins had elicited Ag-Ab complexes in a mixture of immunogens.

Detection of antigen by antibody binding on blots in situ, shows that the proteins transferred to PVDF membrane remained without being exchanged. A blot is saturated with bovine serum albumin to block the residual binding capacity of the sheet, and it can be treated as a solid-phase immunoassay. In the following immunological application 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. Figure 5.34 and Figure 5.35 show Western blot result that revealed the selected mouse anti-MT antibody cross-reacting specifically with MT.

MT from fish liver and kidney tissues exhibited bands at 6 - 7 kDa.

The intensity of the bands was elevated in Cd exposed liver and kidney tissues than in control. The β-Actin housekeeping genes were incubated with monoclonal mouse anti-β-Actin IgG primary and anti-mouse IgG conjugated with HRP secondary antibody. The MT proteins and β-Actin bands were detected using chemiluminescence system (ECL Kit) method. The MT expression levels were quantified, using β-Actin expressions. The highest MT expression was seen after 72 h of Cd exposure in liver and kidney tissues (Figures 5.34 and 5.35).

In liver tissue, MT protein was found to significantly increase with respect to duration of Cd exposure and concentration. MT expression levels were quantified. Control levels shows 29.08 (MT protein/β-Actin relative intensity) and 72 h Cd treated liver tissues shows MT levels of 133.75 (MT protein/β-Actin relative intensity) (Figure 5.34), which shows a significant increased.
In kidney tissue, MT protein was found to significantly increase with respect to duration of Cd exposure. MT expression levels were quantified. Control levels show 26.89 (MT protein/β-Actin relative intensity) and 72 h Cd treated liver tissues show MT levels of 114.97 (MT protein/β-Actin relative intensity) (Figure 5.35). They were significantly increased when compared to the controls.

Figure 5.34 MT expression in liver tissue of *C. gariepinus*

a. Western blot analysis of MT expressions with β-Actin for Control and 72 h sub-lethal dose (20 ppm) Cd exposed liver tissue.
b. Graphical representation of MT expression levels. Each bar represents the Mean ± S.D of three independent observations in triplicates.

a. Western blot analysis of MT expression with β-Actin for Control and 72 h sub-lethal dose (20 ppm) Cd exposed kidney tissue.

Figure 5.35 MT expression in kidney tissue of *C. gariepinus*.

b.
b. Graphical representation of MT expression levels. Each bar represents the Mean ± S.D of three independent observations in triplicates.

5.12 MT ISOLATION AND PURIFICATION

5.12.1 Affinity Chromatography

The total protein concentration was determined by the Lowry method using bovine serum albumin as a standard.

Metallothionein was isolated and purified from liver tissue of *C. gariepinus* exposed to sub-lethal (20 ppm) cadmium for 72 h. Purification was carried out by affinity chromatography (figure 5.36) using 1 mL HiTrap™ metal chelating column (figure 5.37). MTs were purified by Ni²⁺ loaded resins and eluted with 500mM imidazol. The elution was performed with 1 mL/min and the fractions were collected. Seven eluted fractions were obtained. The highest MT concentration was obtained in third fraction (figure 5.38).
Figure 5.37 The affinity chromatography column

Figure 5.38 Eluted fractions of affinity chromatography from freshwater catfish, *C. gariepinsu* liver homogenate for MT

Elution fractions (500mM imidazol; flow rate 1 mL/min) of MT from liver extracts started to be eluted in fractions 2, 3, 4 and 5. The purest form of MT were then visualized using 12% SDS-PAGE stained with CBB. Lane 5 shows pure MT as it had no non-specific bands. The molecular markers were helpful to identify the MT protein by comparing its approximate molecular mass. (Figure 5.39).

To eliminate or reduce the possibility of MT remaining bound to the column and not be completely eluted by imidazol, EDTA solution was passed throughout the column after the washing procedure. The wash aliquots were then run in SDS-PAGE. No protein band from aliquots were obtained after EDTA washing step. Thus, it was concluded that this method had made highly pure MT isolation possible without significant loss of protein.
5.12.3 Western Blot

Purified MT protein was confirmed using Western blot with specific anti-MT antibodies. The antibody shows a positive cross reactivity with MT proteins (Figure 5.40).

![Western Blot Image]

**Figure 5.40** Purified MT confirmation by Western blot.

Purified MT protein was detected using chemiluminescence system (ECL Kit) method. MT bands appeared in the PVDF membrane, proving the Antibody reacted with specific MT protein after electrophoresis in 12 % SDS-PAGE.

5.12.4 Evaluation of Ni\(^{2+}\) Leakage from the Column

There is a possibility that metal sequestration with proteins (MT) would take place while pass through the column. The results obtained shows that (figure 5.41) nickel was seen only after EDTA washing and after sample loading and the MT fractions (fractions 8-10) are very low concentration. The nickel concentrations were measured using ICP-OES. It was seen that no
significant nickel leakage was observed in the MT positive fractions (fraction 1-7).

![Figure 5.41 Evaluation of Ni\(^{2+}\) leakage from the column.](image)

Figure 5.41 Evaluation of Ni\(^{2+}\) leakage from the column.

Higher nickel concentrations were detected just after loading the column with NiSO\(_4\)-6H\(_2\)O as well as by passing EDTA through the column in order to wash it out fractions 8-10. The results indicate the nickel was completely removed from the column by EDTA washing. Hence the possibility loss of MT due to leak of Ni chelated to column was eliminated or reduced.

5.13 MALDI-TOF MS

5.13.1 Molecular Weight of MT

MT protein molecular weight determination was performed using MALDI-TOF MS and is depicted in Figure (5.42). The result shows that the purified MT protein molecular weight was found to be 6125 Daltons in the liver tissue of freshwater catfish, *C. gariepinus* in the liver tissue.
Figure 5.42 Molecular weight of MT protein in freshwater catfish *C. gariepinus* using MALDI-TOF MS

5.13.2 Peptide Mass Fingerprinting

MALDI-TOF MS analysis of MT protein by peptide mass fingerprinting and MS/MS ion, m/z charge ratio successfully shows mass fragments with minimal background, enabling a search for matches in protein mass databases. The search parameters for peptide mass was carried out with Mascot software on NCBI database. NCBI database search was conducted in comparison with taxonomy of Eukaryota (53438708 sequences; 19231914498 residues). Proximal or similarity based major hits were observed, with gi|443254575, metallothionein [*Clarias macrocephalus*] with protein score of 87 [Protein score is -10 x Log (P), where P is the probability that the observed match is a random event (Figure 5.43). If the protein scores greater than 65 are significant (p < 0.05)] showing highest or maximum peptide match or similarity. The freshwater catfish *C. gariepinus*’ MT protein mass was
deducted to be around 6125 Daltons after analyzes and matching observed mass of *C. macrocephalus* (6125.1 Daltons). *C. gariepinus* MT shows 93% amino acid sequence similarity with *C. macrocephalus* fish MT sequence. There is a mismatch of only 4 sites out of 60 amino acids in the MT peptide. The search however failed to find any other convincing similarities in the database searches. (Figure 5.44). Hence it can reasonably be concluded that the liver tissue peptide sequence analysed by MALDI-TOF is Metallothiothionein. And the 93% match indicated that we have got partial sequences with very high level of similarity and homology with the MT of *C.macrocephalus*. The phylogenetic tree shows the similarity and homology of the MT with other related fish MT (Figure 5.45).

![Figure 5.43](image)

**Figure 5.43** Mascot score histogram protein score of *C. gariepinus* matched with *C. macrocephalus*
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**MASCOT Search Results**

**Protein View: gi|443254575**

**metallothionein** [Clarias macrocephalus]

- **Database:** NCBI
- **Score:** 87
- **Expect:** 0.033
- **Nominal mass (M̄):** 6120
- **Calculated pI:** 8.05
- **Taxonomy:** Clarias macrocephalus

This protein sequence matches the following other entries:
- gi|443254577 from Clarias macrocephalus

Sequence similarity is available as an NCBI BLAST search of gi|443254575 against nr.

**Search parameters**

- **Enzyme:** Trypsin: cuts C-term side of KR unless next residue is P.
- **Mass values searched:** 18
- **Mass values matched:** 6

**Protein sequence coverage:** 93%

**Matched peptides shown in bold red.**

1. MDPCRCRGKT TCTC6FSCKK SNQCSTYCKK SCSCCPSTC SSCASGCVCK

51 GSDCDSKCCQ

Unformatted sequence string: **60 residues** (for pasting into other applications).

Figure 5.44 Mascot search results (continued in next page)
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Figure 5.44 Mascot search results

Figure 5.45 Phylogenetic Tree for MT from *C. gariepinus* showing relationship with other fish MT