CHAPTER 5

RESULTS

5.1. Effects of mercuric chloride and cadmium chloride exposure singly on oxidative stress, antioxidant defenses and immune functions in macrophages from freshwater fish *Channa punctatus Bloch.*

5.1.1. Study of effects of mercuric chloride and cadmium chloride exposure singly on ultra-structural changes bioaccumulation, morphological alteration in macrophages from freshwater fish *Channa punctatus*

5.1.1.1. Heavy metal analysis in different organs of untreated and treated fish

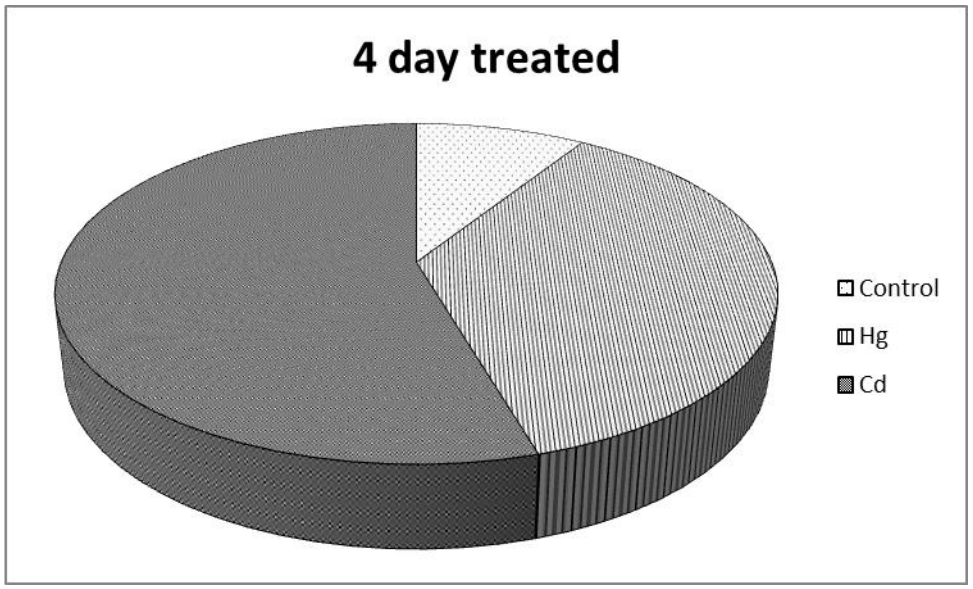
Table. 5.1. Heavy metal analysis in different organs of untreated and treated fish after 7 days of exposure (ppm/g tissue)

UNTREATED GROUP	**INTESTINE**	**LIVER**	**GILLS**	**MUSCLES**
MERCURY ACCUMULATION	**0.072±0.001**	**0.039± 0.02**	**0.044±0.023**	**0.025±0.017**
CADMIUM ACCUMULATION	**0.041 ± 0.01**	**0.046±0.016**	**0.0388±0.014**	**0.027±0.07**
TREATED GROUP	**INTESTINE**	**LIVER**	**GILLS**	**MUSCLES**
MERCURY ACCUMULATION	**1.065±0.06**	**1.05 ± 0.024**	**0.921±0.025**	**0.42 ± 0.018**
CADMIUM ACCUMULATION	**0.93 ± 0.021**	**0.92±0.015**	**0.87 ± 0.011**	**0.58 ± 0.022**

Note: The values were statistically significant at $P < 0.05$.

5.1.1.2. Effect of mercury and cadmium on morphological alteration of macrophages

a)



b)

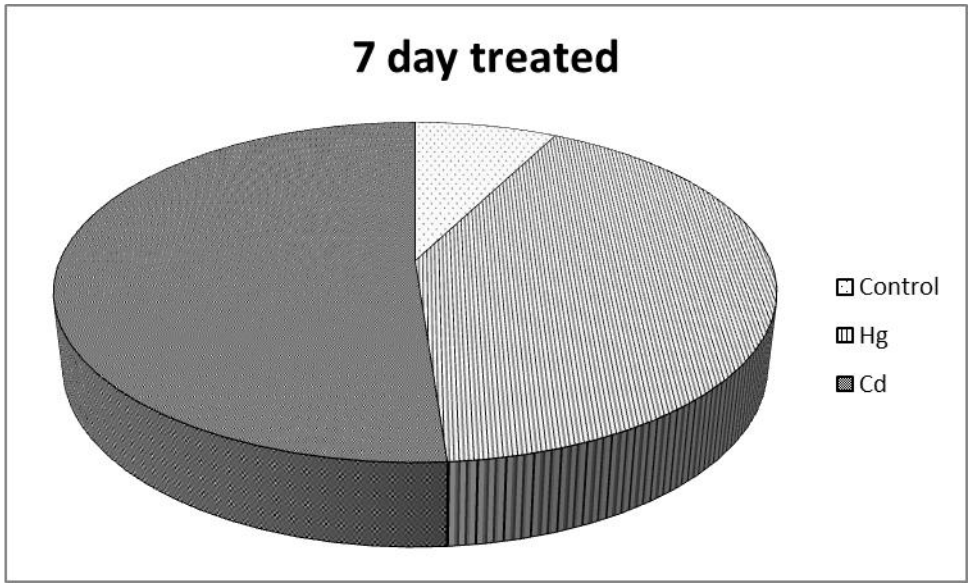
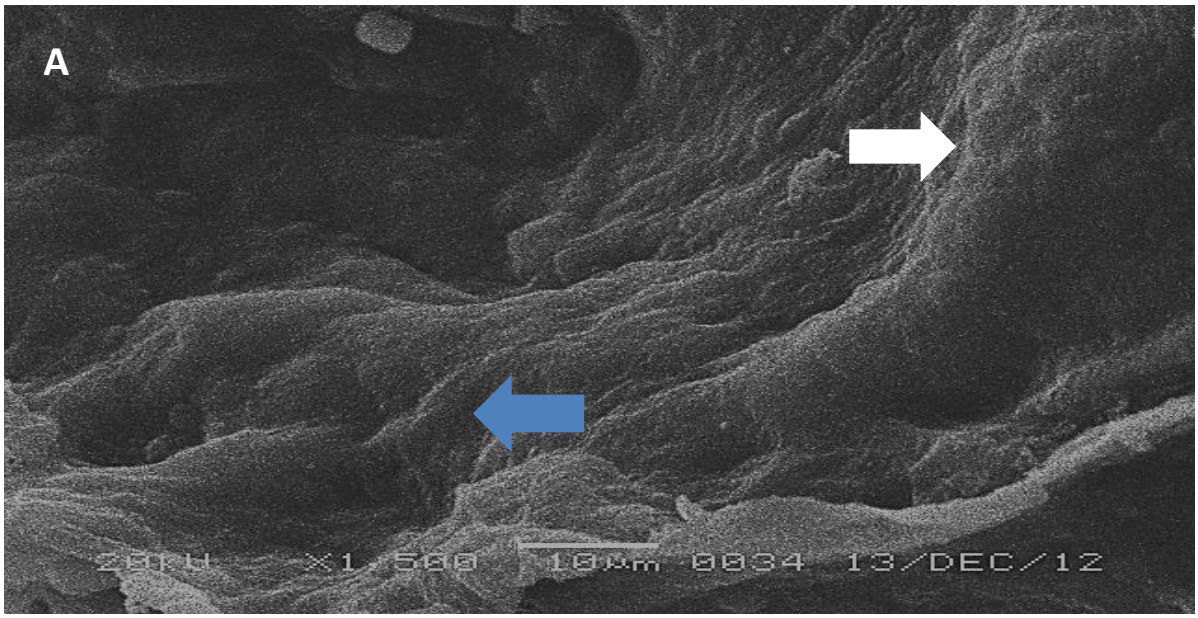


Fig. 5.1: Morphological alteration of intestinal macrophages when treated singly with mercury and cadmium after 4 days (a) and 7 days (b) of exposure. Hg, Cd (4 days) $P< 0.05$; Hg, Cd (7 days) $P< 0.05$.

5.1.1.3. Ultra structural analysis of tissue by scanning electron microscope



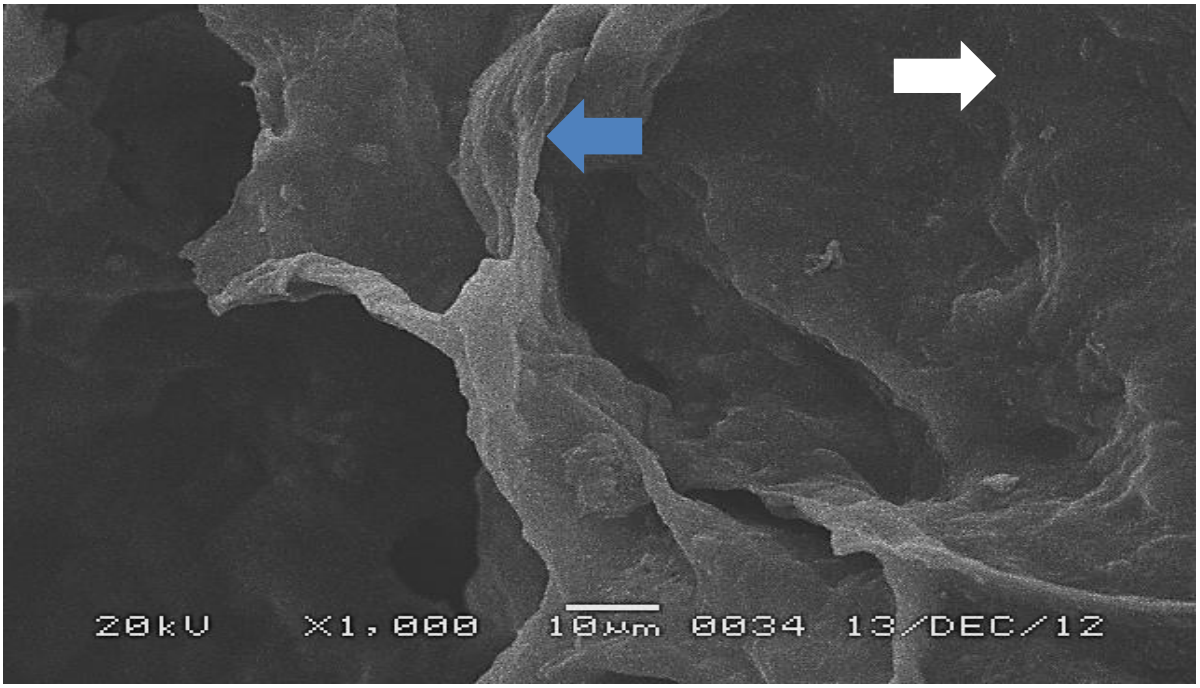


Fig. 5.2: Ultramicroscopic photographs of untreated fish showing prominent mucosal foldings (blue arrow) and normal epithelium (white arrow) after 4 days (A) and 7 days (B).

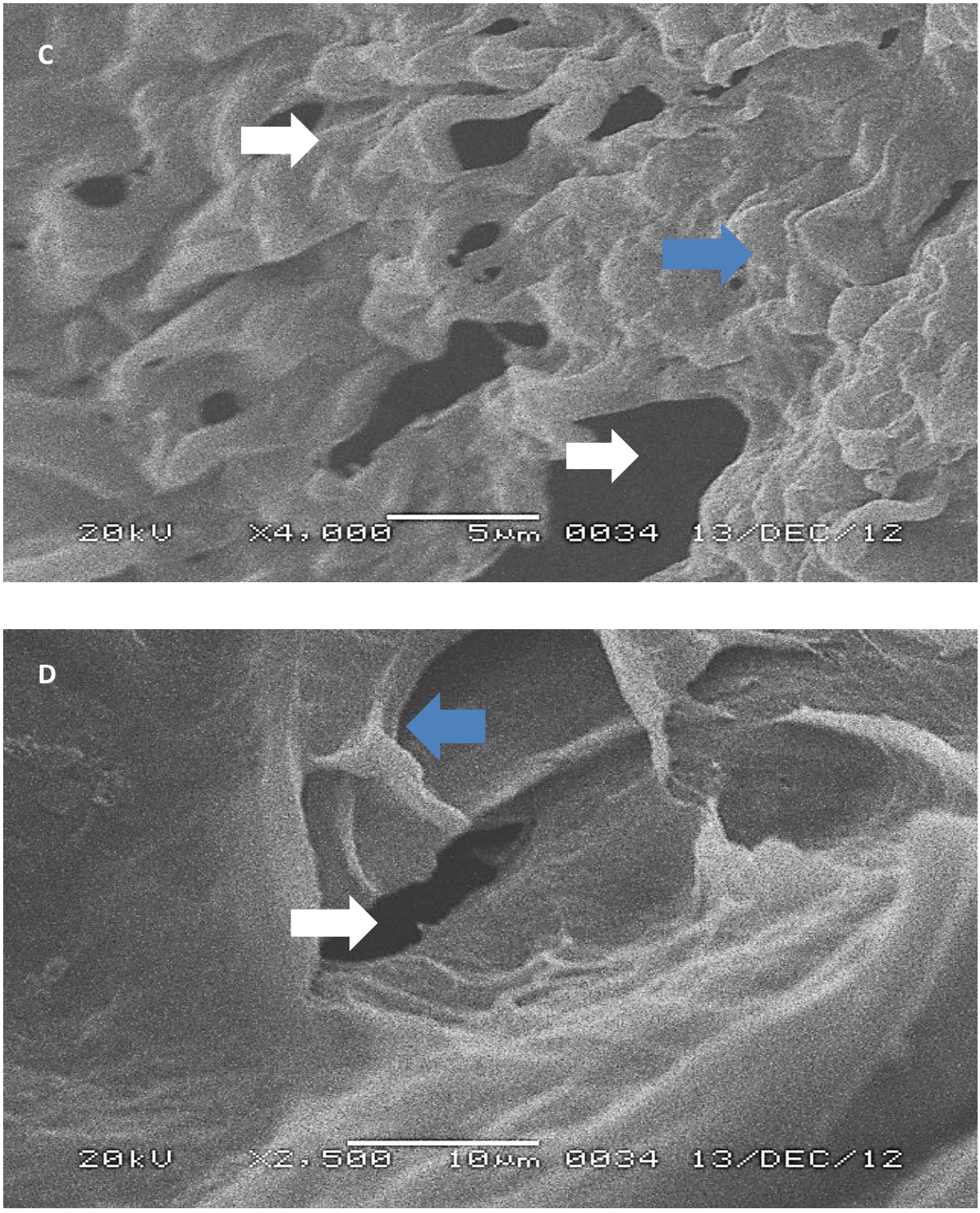


Fig. 5.3: Ultramicroscopic photographs of mercury treated fish showing debris of mucosal folds (blue arrow), damaged and degenerated epithelium (white arrow) after 4 days (C) and 7 days (D) of exposure.

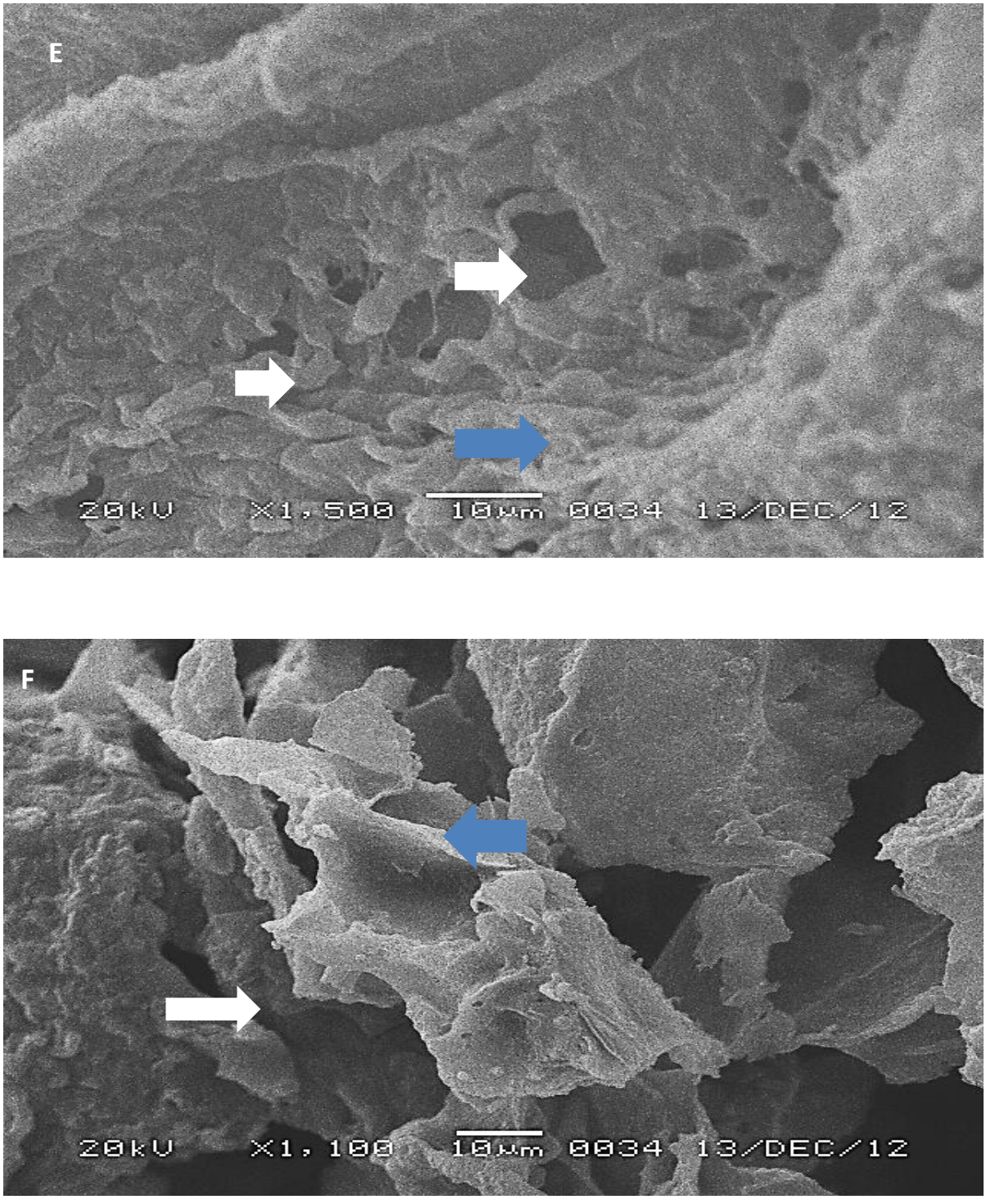
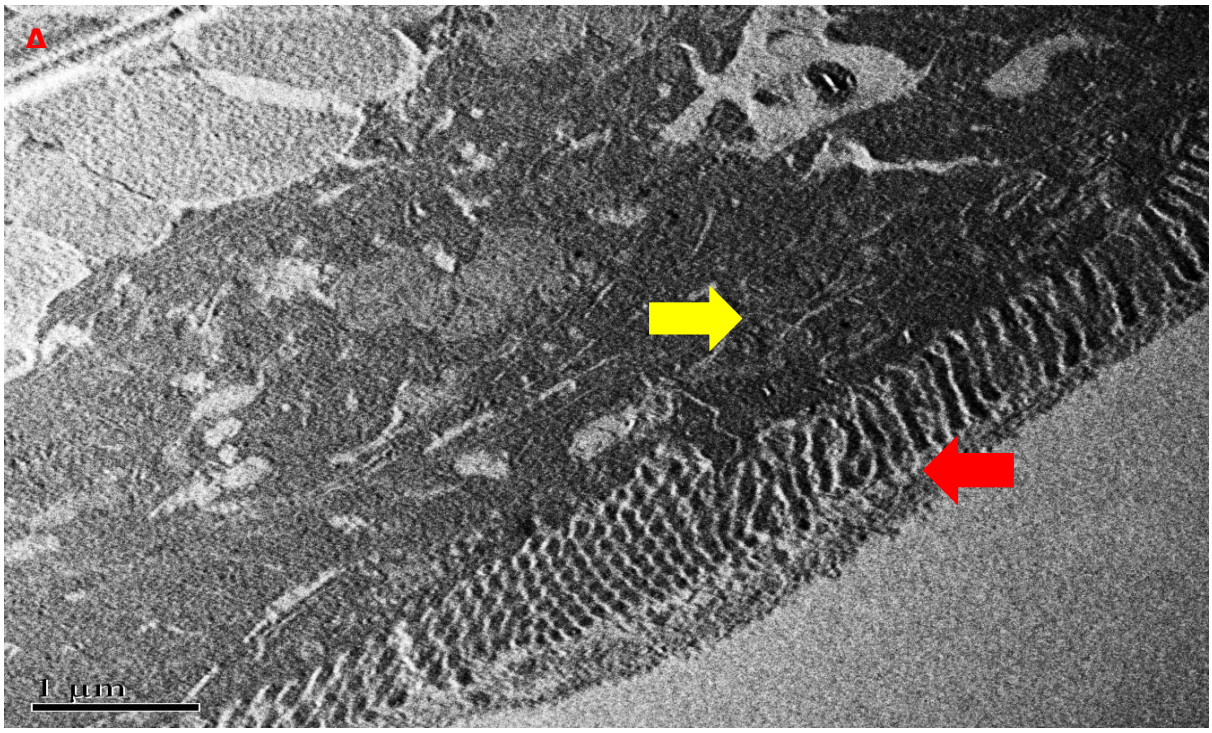
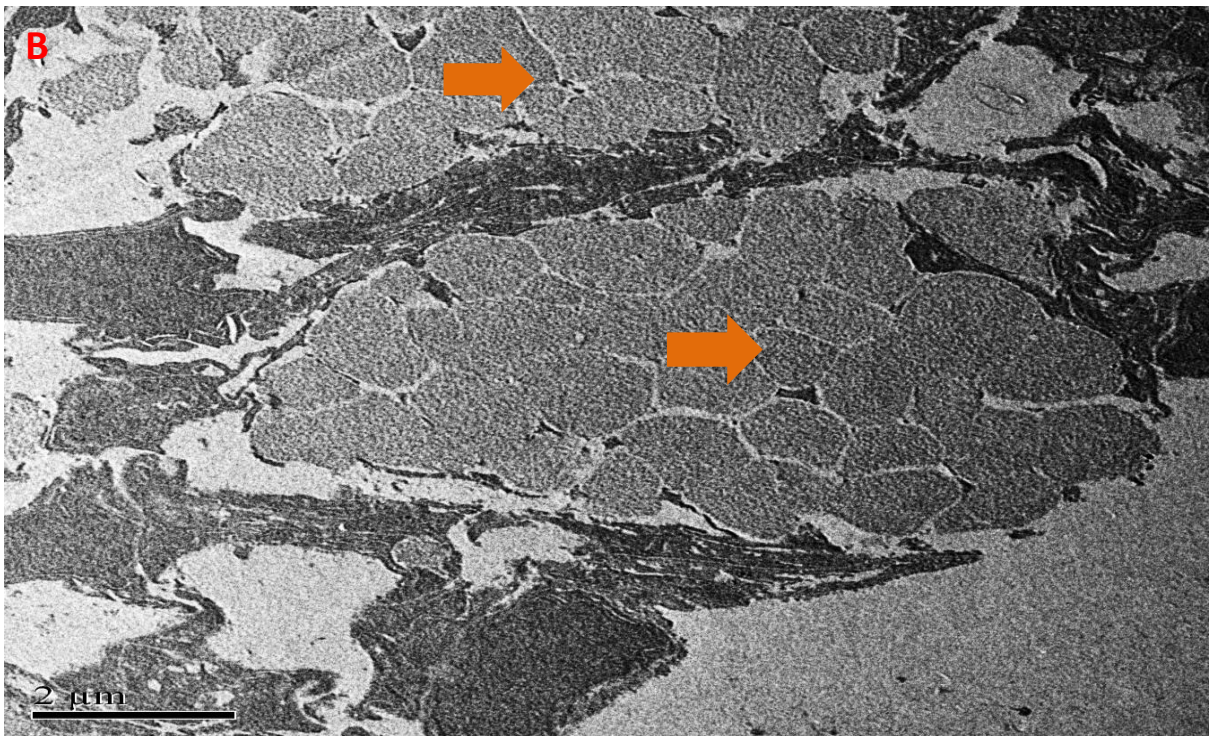


Fig.5.4. Ultramicroscopic photographs of cadmium treated fish showing inflammatory damage in epithelium (white arrow), disarrangement and fragmentation of mucosal foldings (blue arrow) and secretion of mucus after 4 days (E) and 7 days (F) of exposure.

5.1.1.4. Ultra structural analysis of tissue by transmission electron microscope





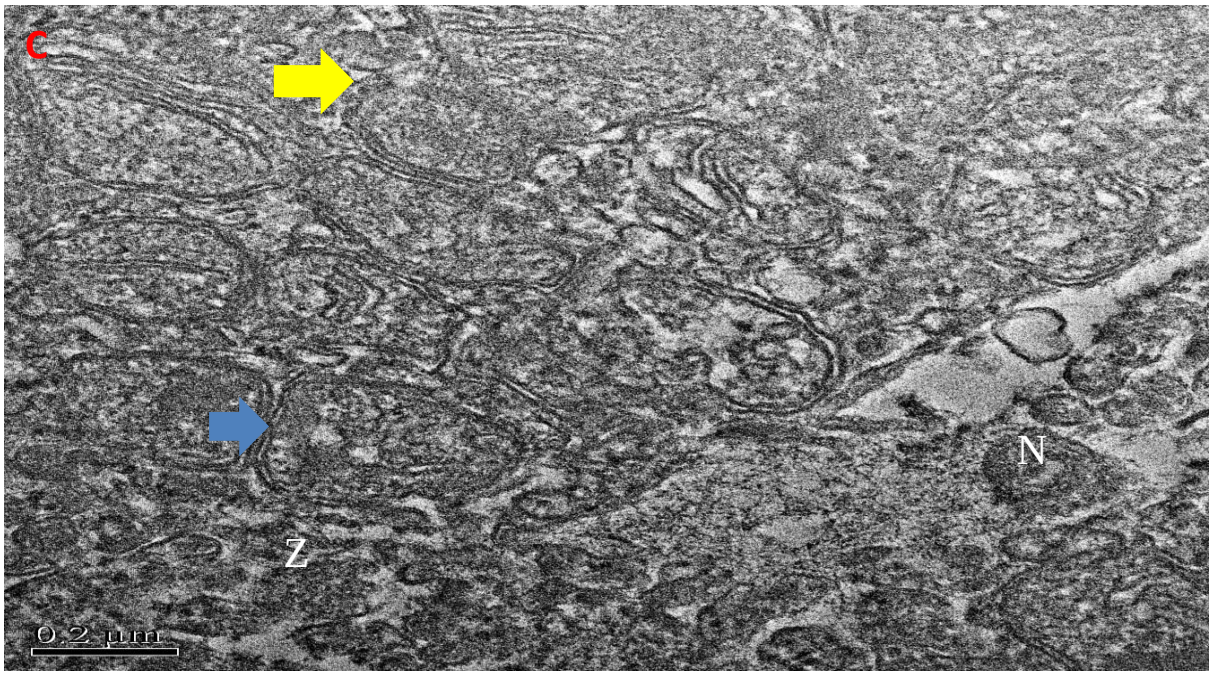


Fig. 5.5: Plate (A) Transmission electron micrograph (TEM) of the intestinal tissue of control fish showing regularly arranged microvilli (red arrow), intact rough endoplasmic reticulam (RER) of epithelial cells (yellow arrow).

Plate (B) Transmission electron micrograph (TEM) of the intestinal tissue of control fish showing goblet cells (orange arrow),

Plate (C) Transmission electron micrograph (TEM) of the control fish showing intact rough endoplasmic reticulam (RER) of epithelial cells (yellow arrow), zona occludens (Z), gap junction (blue arrow) and elongated nucleus with well-developed nucleolus (N).

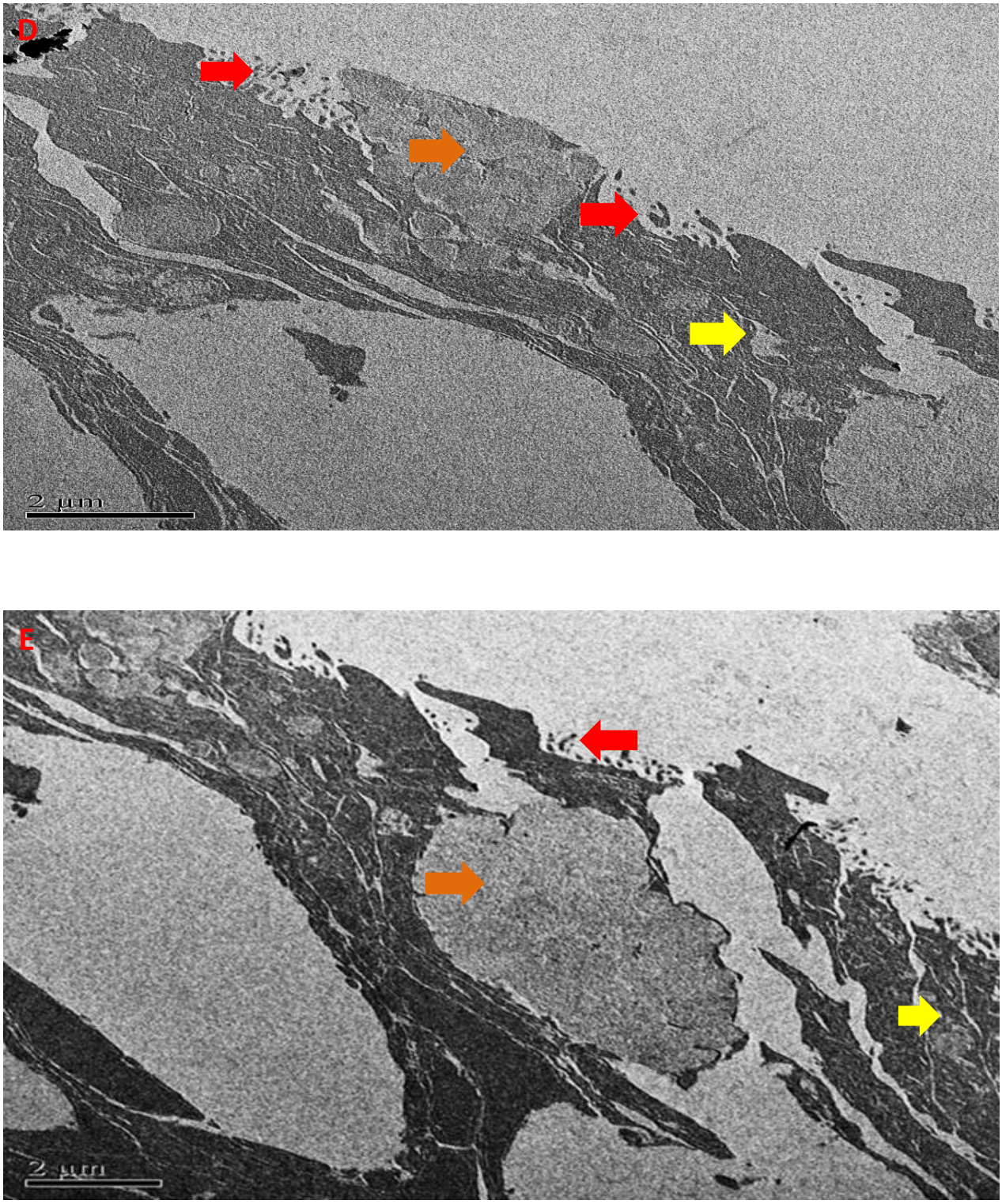


Fig. 5.6: Transmission electron micrograph (TEM) of *C. punctatus* Bloch. after 4 days (D) and 7 days (E) days of mercury exposure showing denudation of goblet cells (orange arrow), disrupted microvilli (red arrow) disrupted RER (yellow arrow), lack of gap junction and zona occludens (Z) as compared to the control fish.

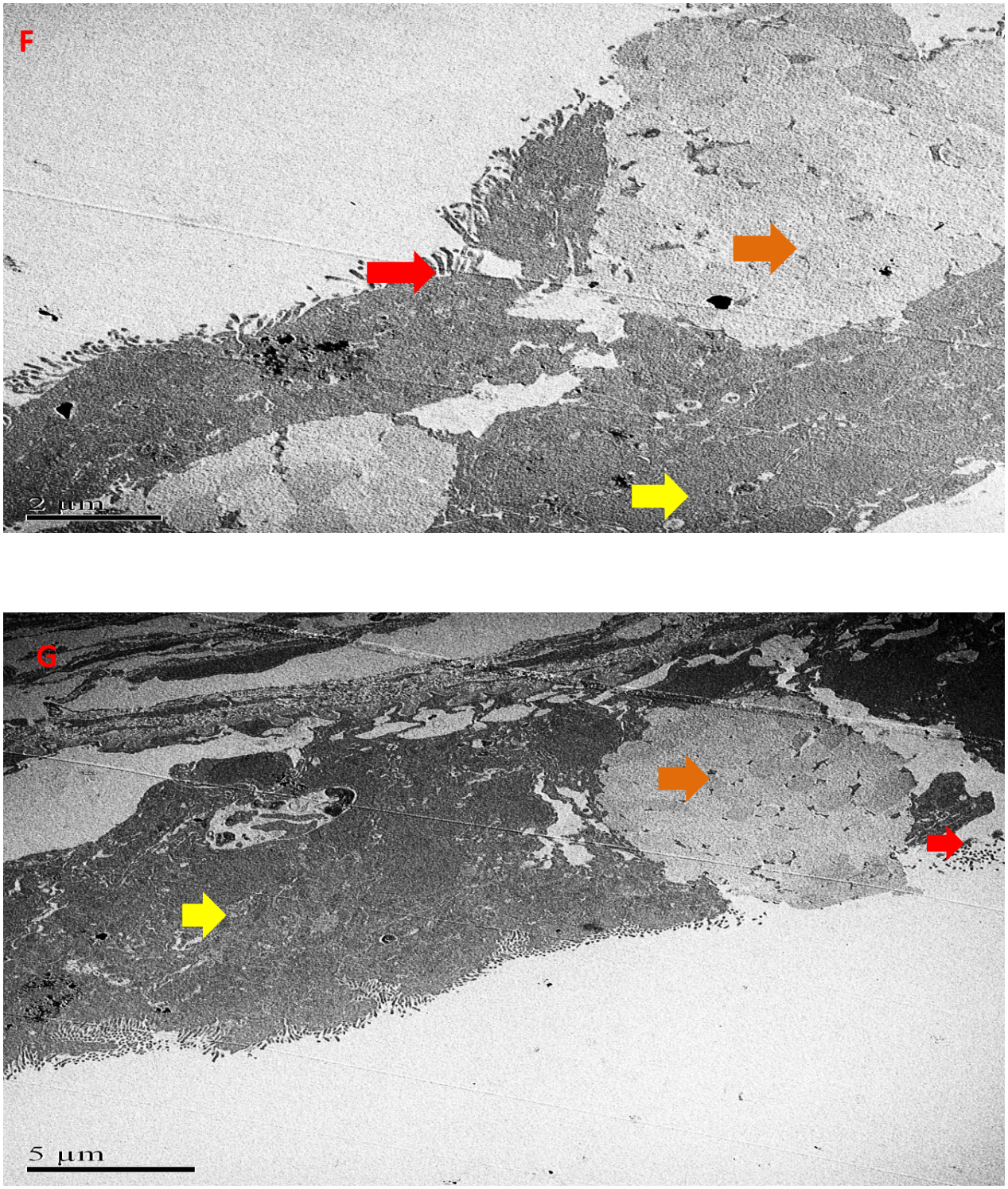


Fig. 5.7: Transmission electron micrograph (TEM) of *C. punctatus* Bloch. after 4 days (F) and 7 days (G) days of cadmium exposure showing denudation of goblet cells (orange arrow), disrupted microvilli (red arrow) disrupted RER (yellow arrow), lack of gap junction and zona occludens (Z) as compared to the control fish.

5.1.2. Study of effect of mercuric chloride and cadmium chloride singly on oxidative stress and antioxidant defenses in macrophages from fish *Channa punctatus*

5.1.2.1. Effect of mercury and cadmium on respiratory burst activity of fish macrophages

There was a significant increase in respiratory burst activity from 0.271±0.09 (4 days), 0.313 ±0.02 (7 days) in control group to 0.735±0.13 (4 days), 0.969±0.03 (7 days) in mercury treated and 0.414±0.09 (4 days), 0.601±0.02 (7 days) in cadmium treated group.

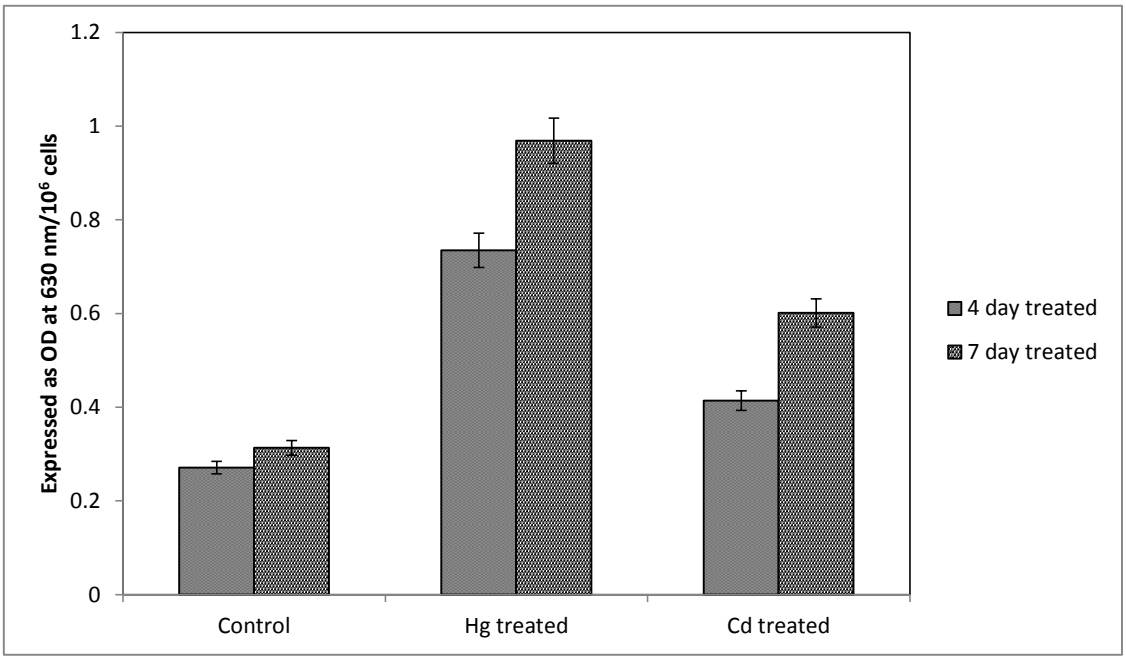


Fig. 5.8: Respiratory burst activity in macrophages of fish treated with mercury and cadmium Hg, Cd (4 days) P< 0.05; Hg, Cd (7 days) P< 0.025.

5.1.2.2. Effect of mercury and cadmium on lipid peroxidation of fish macrophages

There was a significant increase in lipid peroxidation from 0.243± 0.02 nmoles/hr (4 day), 0.215±0.04 nmoles/hr (7 day) in control group to 0.575±0.18 nmoles/hr (4 day), 0.716±1.191 nmoles/hr (7 day) in mercury treated and 0.471±1.22 nmoles/hr (4 day), 0.526±1.11 nmoles/hr (7 day) in cadmium treated group.

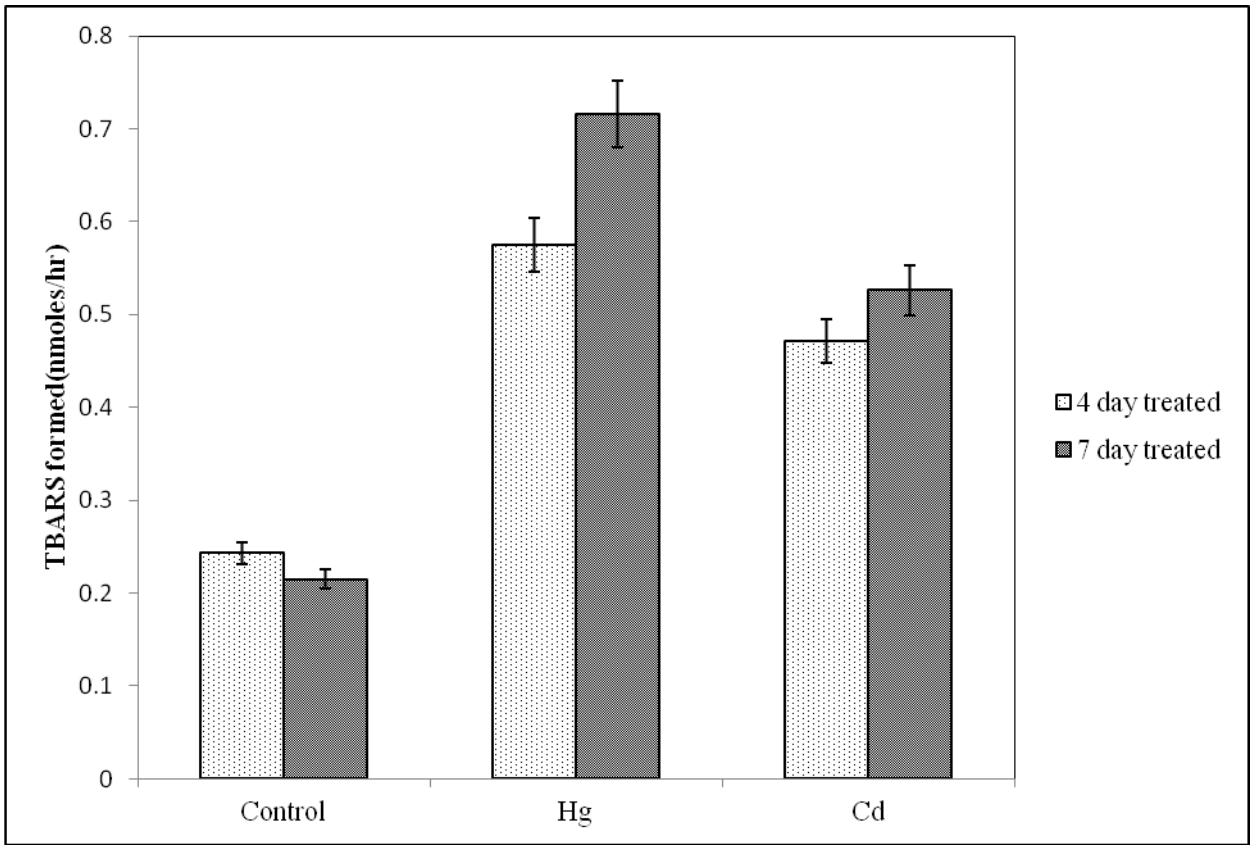


Fig. 5.9: Estimation of lipid peroxidation (LPO) in macrophages of fish treated with mercury and cadmium for 4 days and 7 days respectively. Hg, Cd (4 day) $P< 0.025$; Hg, Cd (7 day) $P< 0.05$.

5.1.2.3. Effect of mercury and cadmium on catalase (CAT) activity of fish macrophages

There was a significance decrease in catalase activity from 5.43 ±1.22 U/mg protein (4 day), 5.69±1.82U/mg protein (7 day) in control group to 5.106±1.144 U/mg protein (4 day), 4.39±1.26 U/mg protein (7 day) in mercury and 5.063±1.54 U/mg protein (4 day), 4.05±1.69 U/mg protein (7 day) in cadmium treated group.

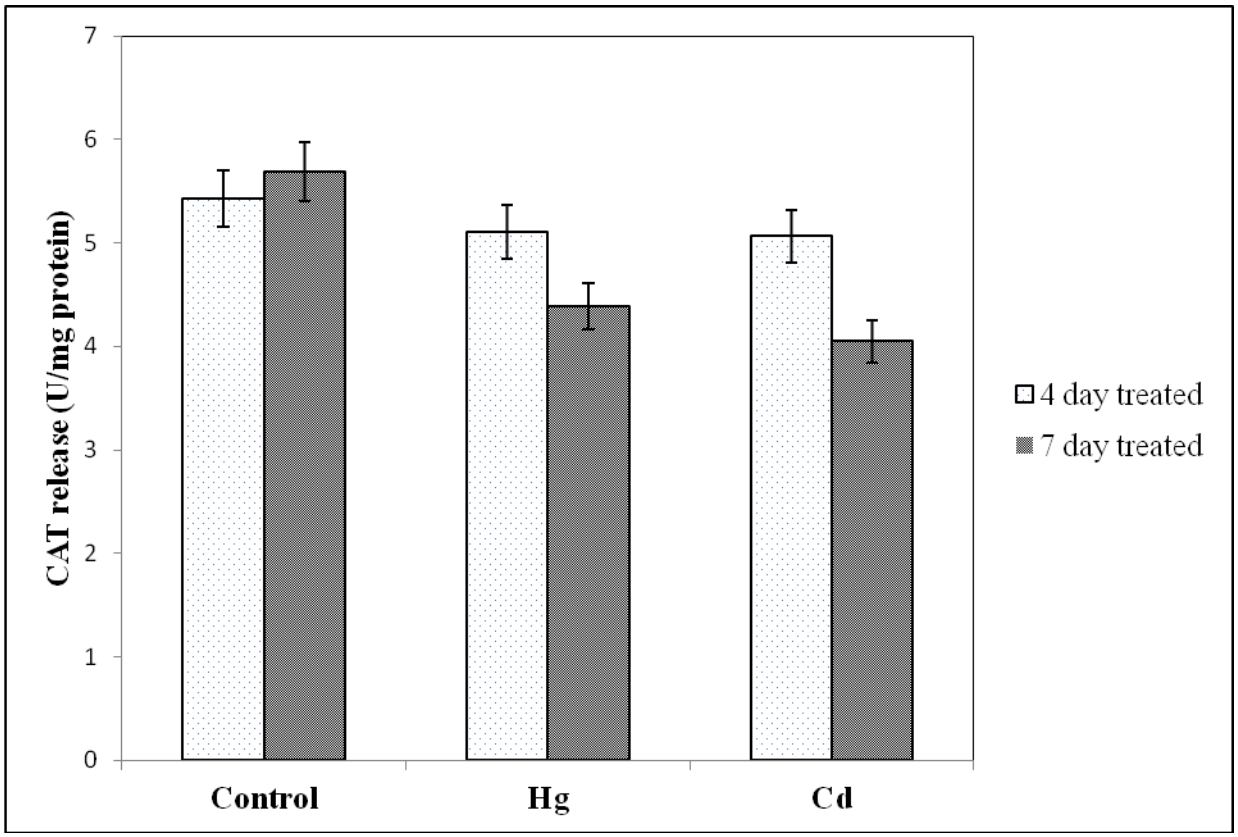


Fig. 5.10: Catalase (CAT) activity in control and heavy metal (mercury and cadmium) exposed fish macrophages after the exposure of 4 days and 7 days respectively. Hg, Cd (4 day) $P< 0.05$; Hg, Cd (7 day) $P< 0.02$.

5.1.2.4. Effect of mercury and cadmium on superoxide dismutase (SOD) activity of fish macrophages

There was a significance increase in superoxide dismutase activity from 0.0096±.001 U/mg protein (4 day), 0.00953±0.018 U/mg protein (7 day) in control group to 0.0157±0.008 U/mg protein (4 day), 0.033±0.001 U/mg protein (7 day) in mercury treated and 0.010±0.001 U/mg protein (4 day), 0.026±0.004 U/mg protein (7 day) in cadmium treated group.

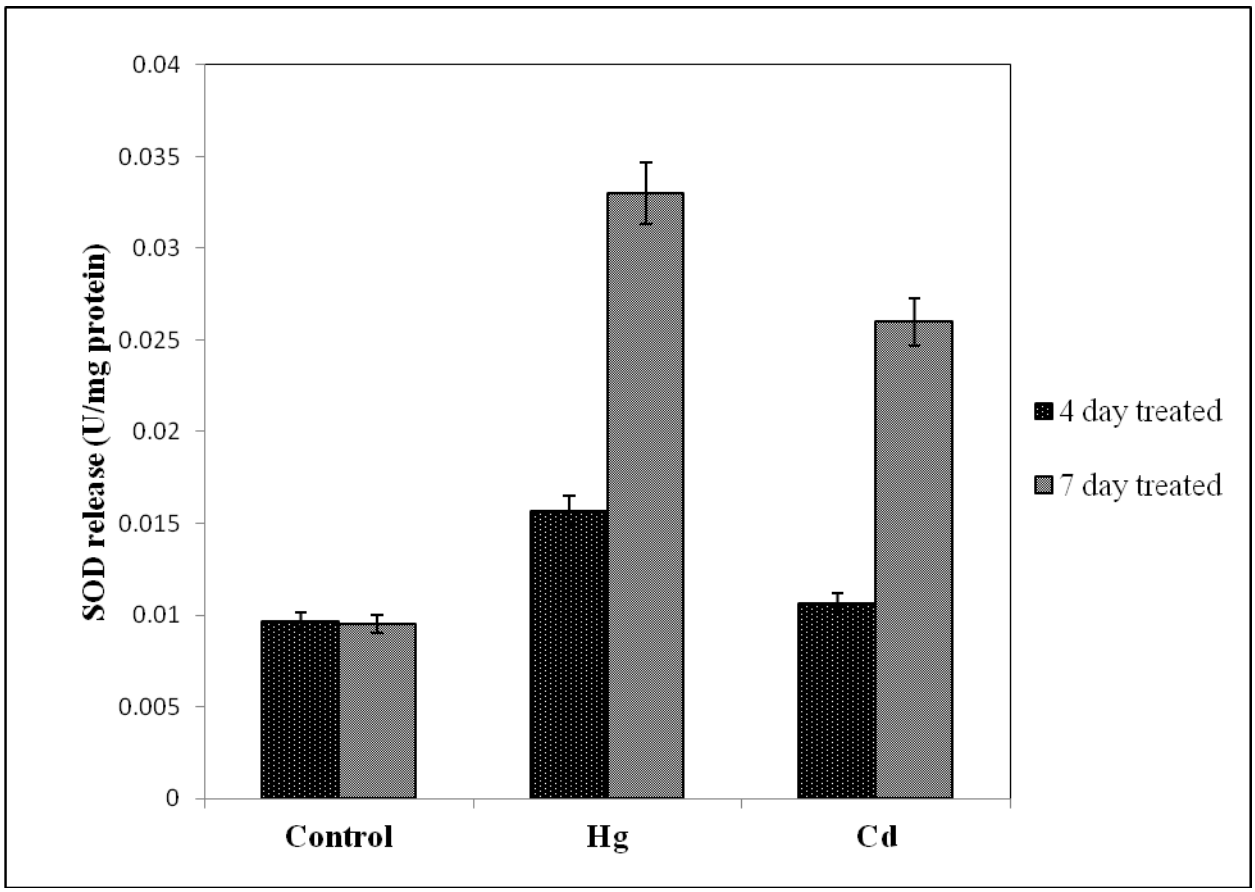


Fig. 5.11: Superoxide dismutase (SOD) activity in control and heavy metal exposed fish macrophages. Hg (4 day) P< 0.02; Cd (4 day) P< 0.05; Hg, Cd (7 day) P< 0.01.

5.1.2.5. Effect of mercury and cadmium on glutathione S- transferase (GST) activity of fish macrophages

There was a significance decrease in glutathione S- transferase activity from 31.61±1.76 nmoles/min/mg protein (4 day), 30.68±0.50 nmoles/min/mg protein (7 day) in control group to 25.94±2.46 nmoles/min/mg protein (4 day), 22.18±0.24 nmoles/min/mg protein (7 day) in mercury treated and 29.32±0.51 nmoles/min/mg protein (4 day), 26.29±0.99 nmoles/min/mg protein (7 day) in cadmium treated group.

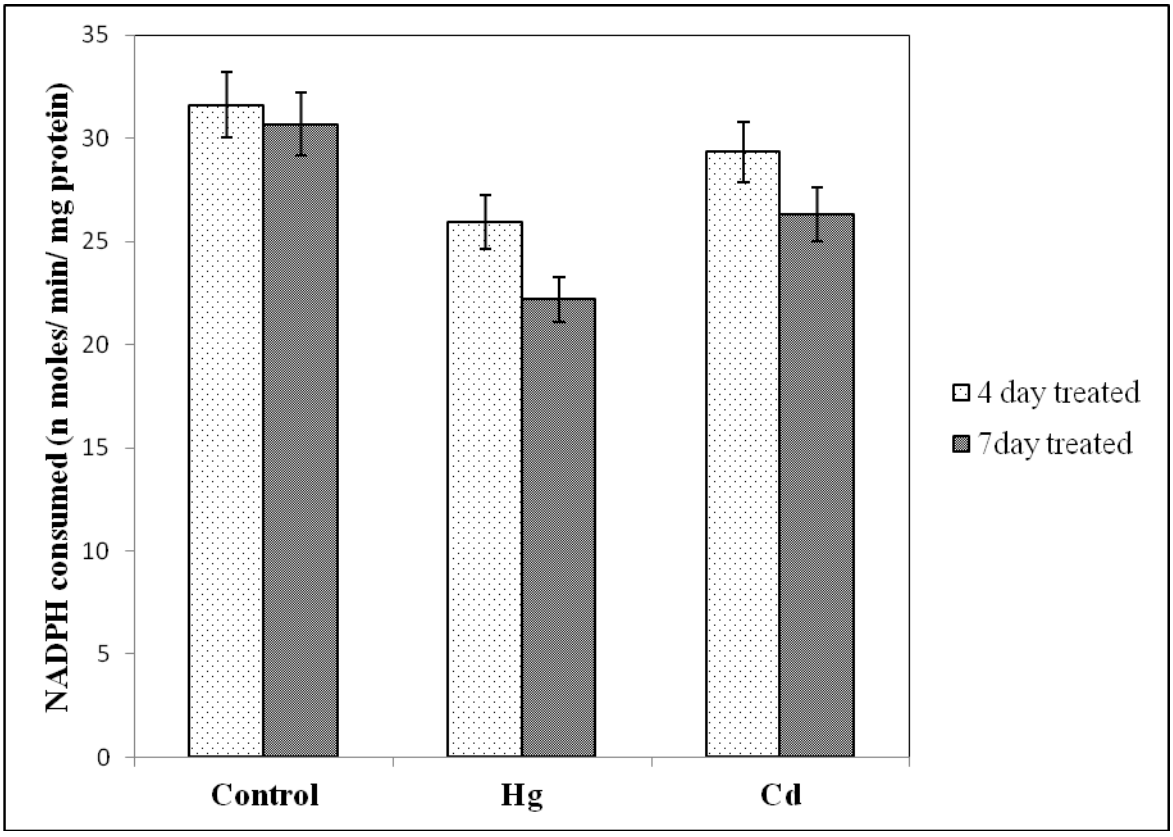


Fig. 5.12: Glutathione S-transferase (GST) activity in macrophages of fish treated with mercury and cadmium. Hg, Cd (4 day) P< 0.01; Hg, Cd (7 day) P< 0.05.

5.1.2.6. Effect of mercury and cadmium on glutathione peroxidase (GPx) activity of fish macrophages

There was a significance decrease in glutathione peroxidase activity from 7.60±0.77 nmoles/min/mg protein (4 day), 7.72±1.05 nmoles/min/mg protein (7 day) in control group to 5.48±0.38 nmoles/min/mg protein (4 day), 4.31±0.25 nmoles/min/mg protein (7 day) in mercury treated and 6.36±0.39 nmoles/min/mg protein (4 day), 5.06±0.05 nmoles/min/mg protein (7 day) in cadmium treated group.

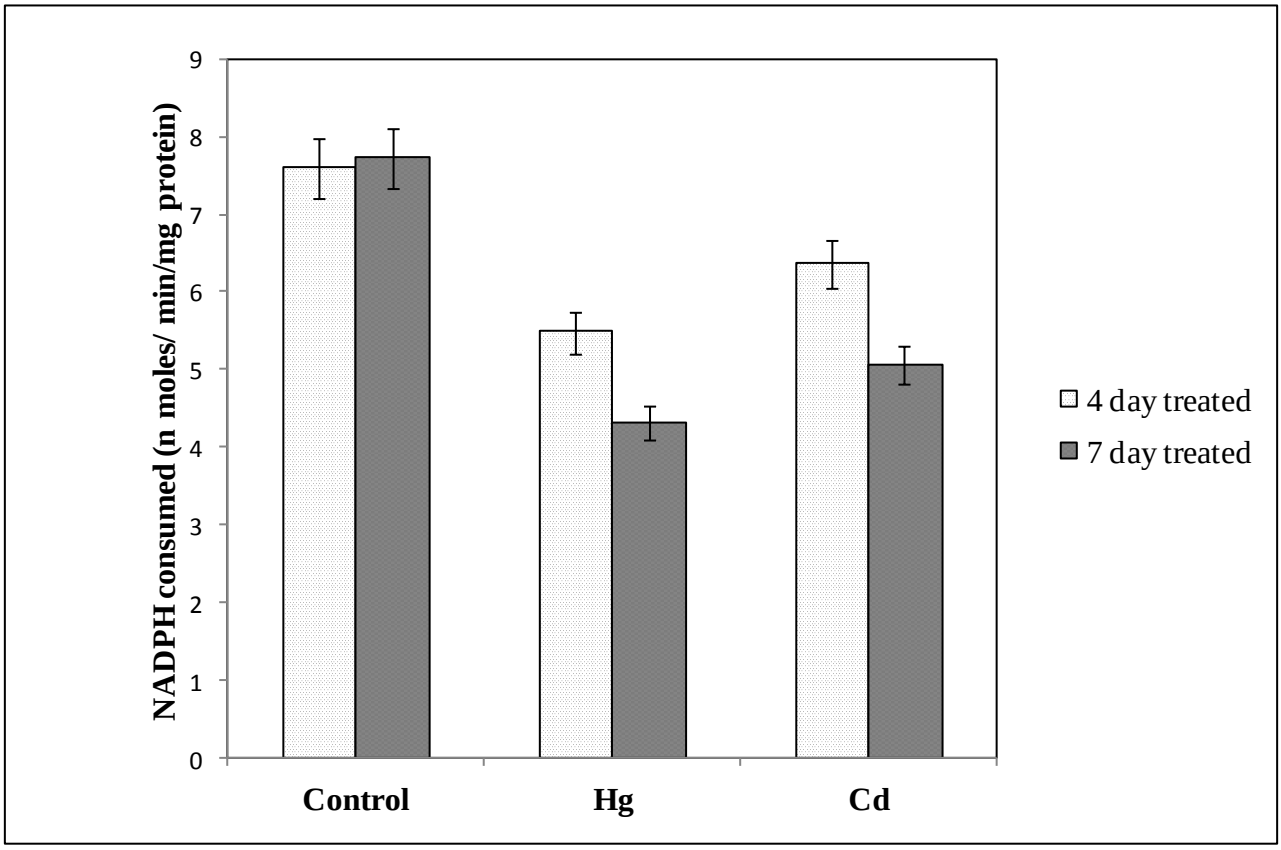


Fig. 5.13: Glutathione peroxidase (GPx) activity in macrophages of fish treated with mercury and cadmium. Hg, Cd (4 day) $P< 0.01$; Hg, Cd (7 day) $P< 0.025$.

5.1.2.7. Effect of mercury and cadmium on glutathione reductase (GR) activity of fish macrophages

There was a significance decrease in glutathione reductase activity from 6.32±0.53 nmoles/min/mg protein (4 day), 6.13±0.51 nmoles/min/mg protein (7 day) in control group to 4.82±0.21 nmoles/min/mg protein (4 day), 3.83±0.28 nmoles/min/mg protein (7 day) in mercury treated and 5.23±0.38 nmoles/min/mg protein (4 day), 4.12±0.12 nmoles/min/mg protein (7 day) in cadmium treated group.

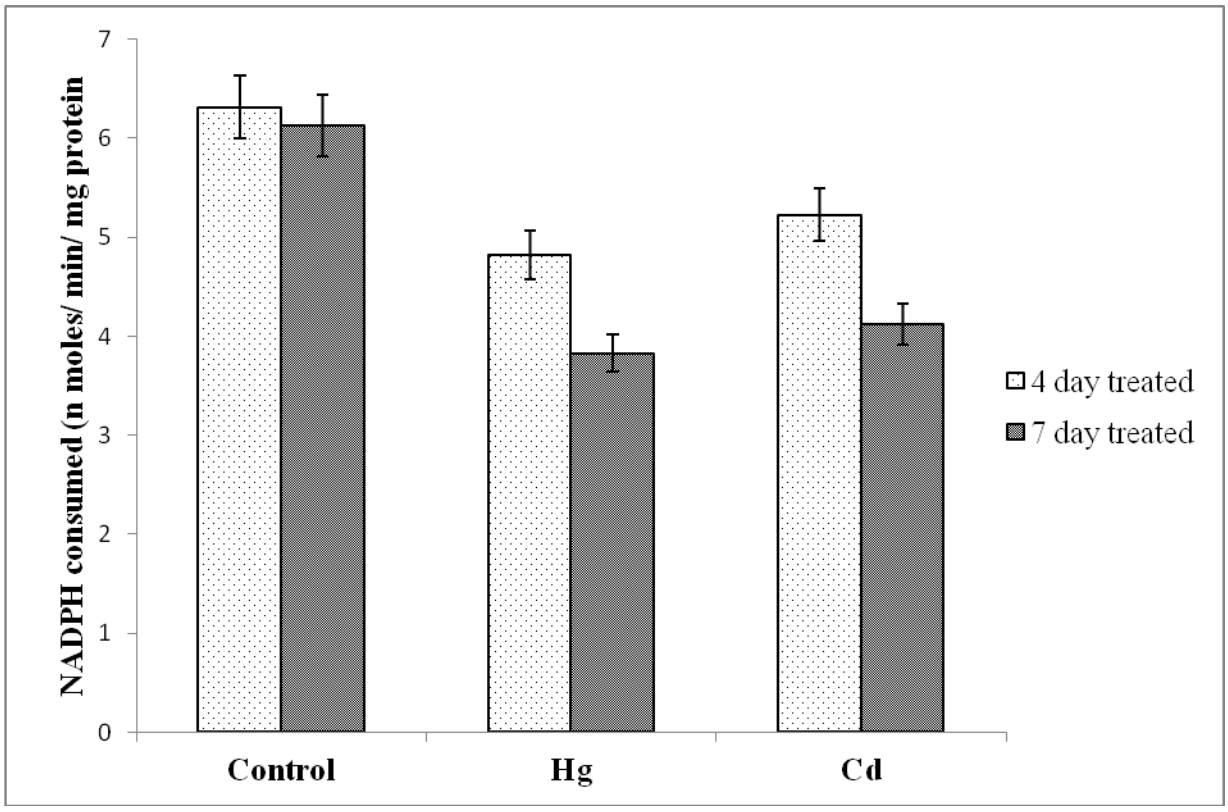


Fig. 5.14: Glutathione reductase (GR) activity in macrophages of fish treated with mercury and cadmium. Hg, Cd (4 day) P< 0.025; Hg, Cd (7 day) P< 0.05.

5.1.2.8. Effect of mercury and cadmium on reduced glutathione (GSH) activity of fish macrophages

There was a significance decrease in reduced glutathione activity from 0.338±0.05 nmoles/min/mg protein (4 day), 0.329±0.01 nmoles/min/mg protein (7 day) in control group to 0.249±0.05 nmoles/min/mg protein (4 day), 0.111±0.008 nmoles/min/mg protein (7 day) in mercury treated and 0.31±0.01 nmoles/min/mg protein (4 day), 0.104±0.007 nmoles/min/mg protein (7 day) in cadmium treated group.

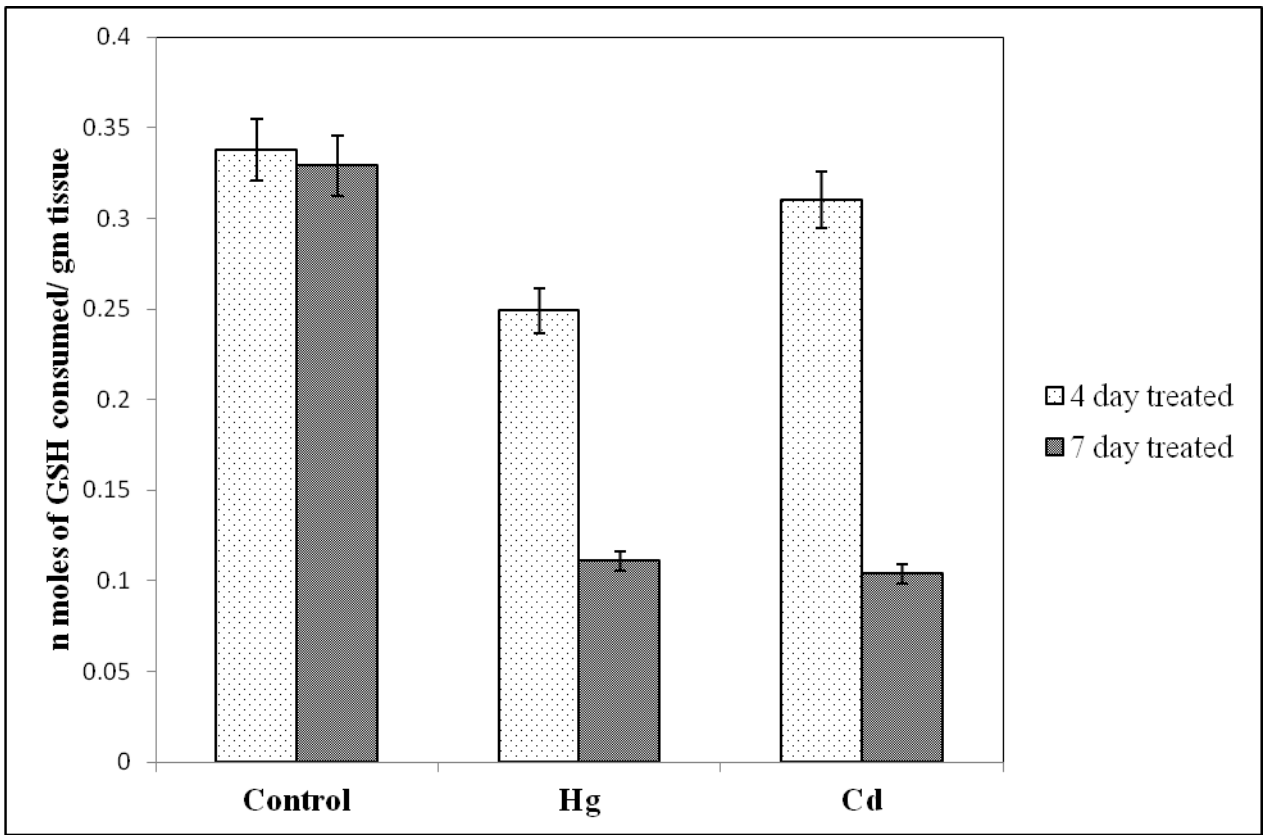


Fig. 5.15: Reduced glutathione (GSH) consumed in macrophages of fish treated with mercury and cadmium. Hg, Cd (4 day) P< 0.25; Hg, Cd (7 day) P< 0.001.

5.1.3. Study of effect of mercuric chloride and cadmium chloride singly on innate immune responses in macrophages from fish *Channa punctatus*

5.1.3.1. Effect of mercury and cadmium on phagocytic activity of fish macrophages

There was a significant decrease in phagocytic activity from 21739±780.63 % (4 day), 21618±548.26 % (7 day) in control group to 14520±716.89 % (4 day), 10781±1057.87 % (7 day) in mercury treated and 17074.41±781.12 % (4 day), 14290.67±674.20 % (7 day) in cadmium treated group.

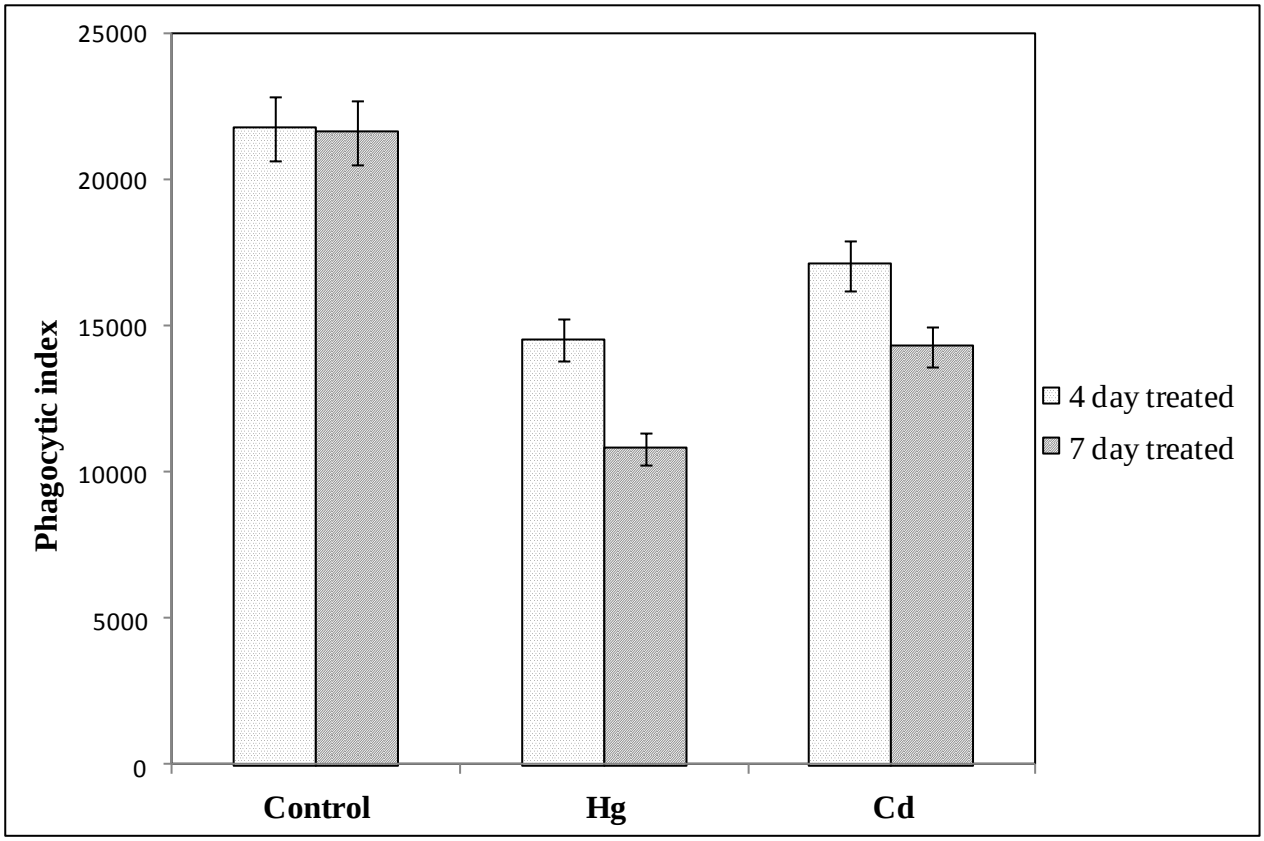


Fig. 5.16: Phagocytic activity in macrophages of fish treated with mercury and cadmium. Hg (4 day) P<0.02; Hg (7 day) P< 0.01; Cd (4 day) P< 0.05; Cd (7 day) P< 0.05.

5.1.3.2. Effect of mercury and cadmium on intracellular killing activity of fish macrophages

There was a significant increase in bacterial viability (%) at different time interval in both mercury and cadmium treated group as compared to the control group.

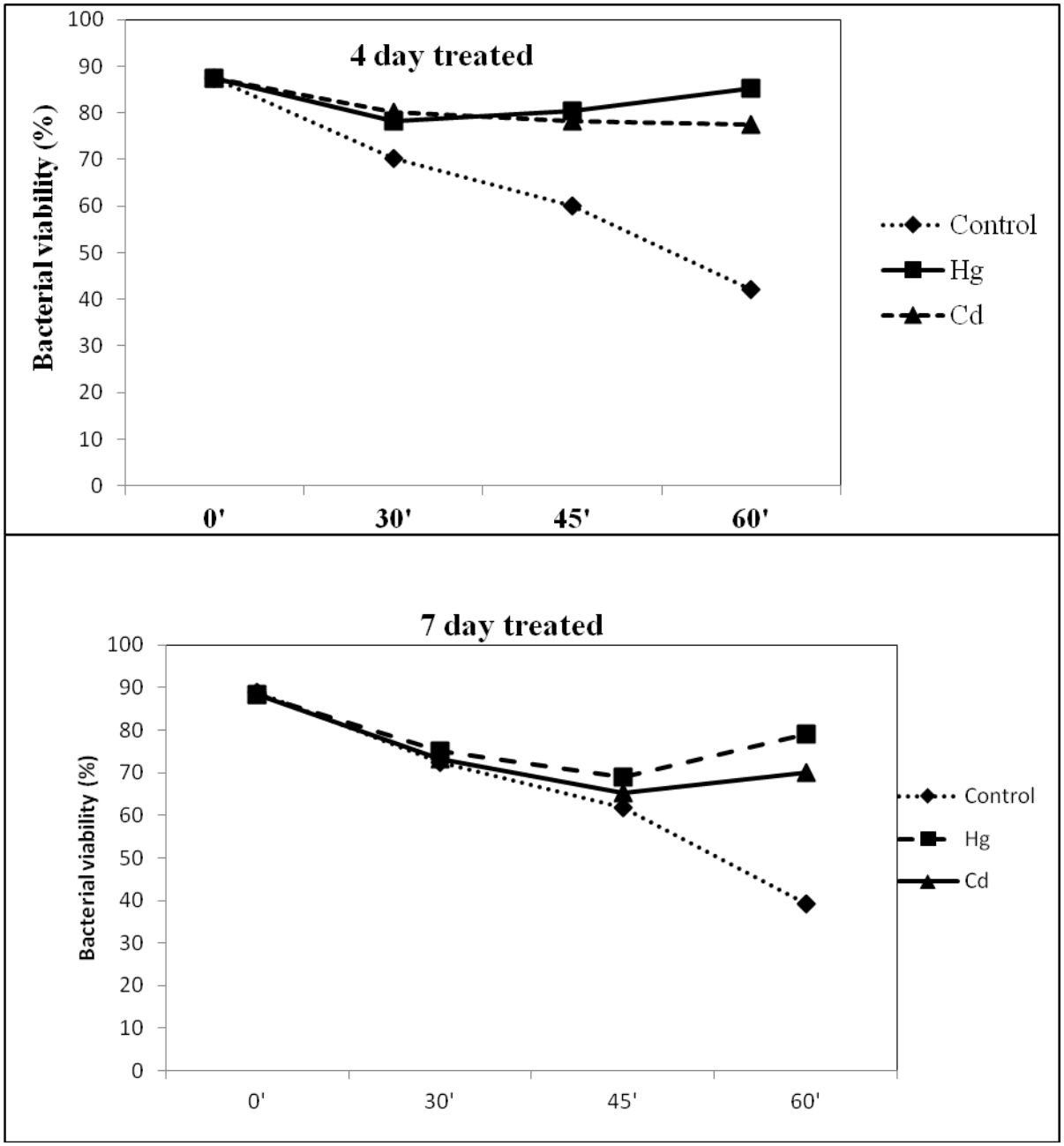


Fig 5.17: Intracellular killing activity in macrophages of fish treated with mercury and cadmium. 4 day- Hg ($P<0.05$); Cd ($P<0.005$), 7 day- Hg ($P<0.01$); Cd ($P<0.025$).

5.1.3.3. Effect of mercury and cadmium on the cell adhesion property of fish macrophages

There was a significant decrease in the cell adhesion property of cells at different time interval in both mercury and cadmium treated group as compared to the control.

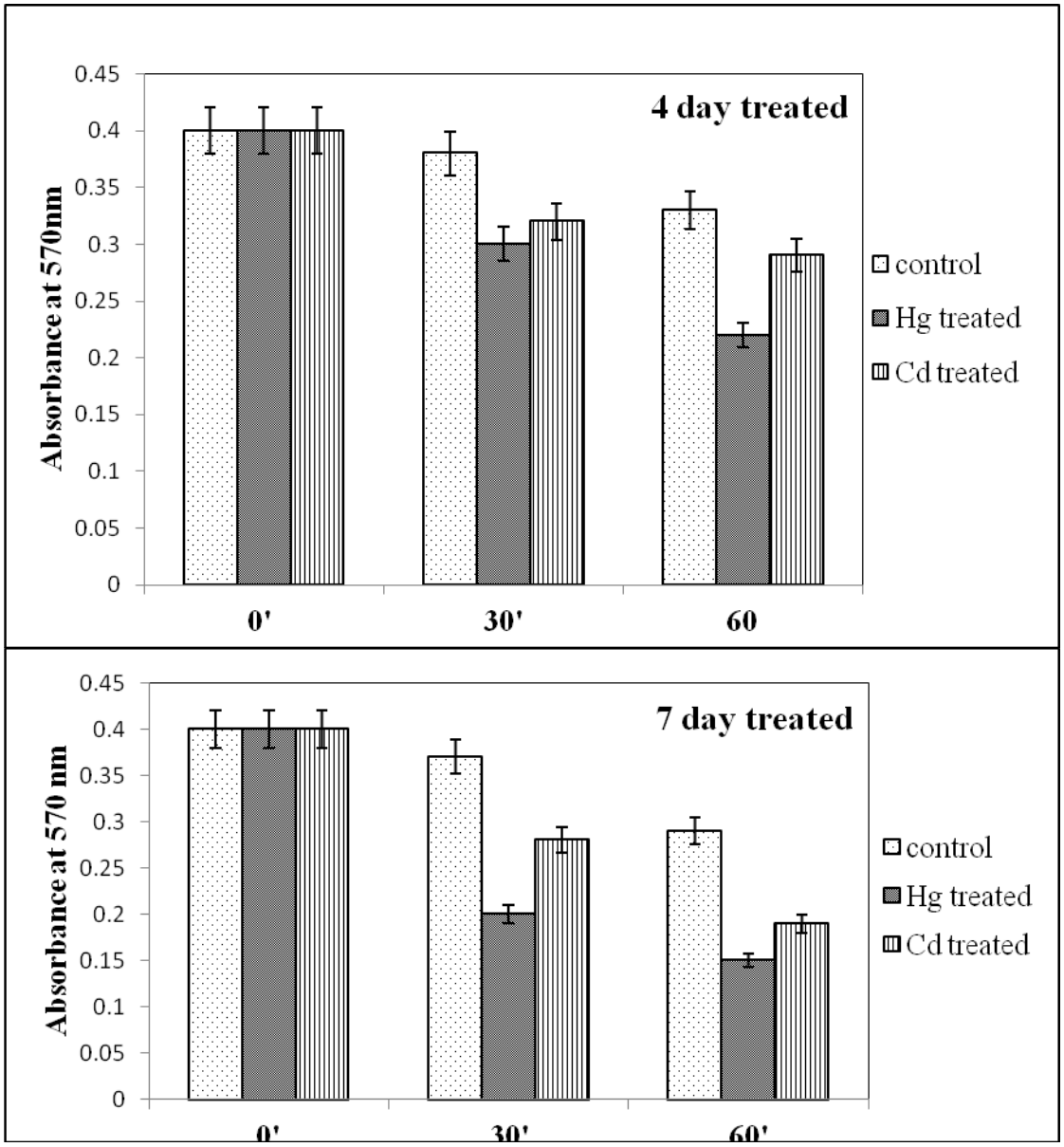


Fig. 5.18: *In vitro* cell adhesion in macrophages of fish treated with mercury and cadmium. 4 day- Hg ($P<0.025$); Cd ($P<0.004$), 7 day- Hg ($P<0.01$); Cd ($P<0.025$).

5.1.3.4. Effect of mercury and cadmium on nitric oxide (NO) release of fish macrophages

There was a significant decrease in nitric oxide release from 52.48±2.42 µM (4 day), 52.94±1.56 µM (7 day) in control group to 23.45±4.75 µM (4 day), 17.56±3.65 µM (7 day) in mercury treated and 29.12±1.11 µM (4 day), 24.03±1.09 µM (7 day) in cadmium treated group.

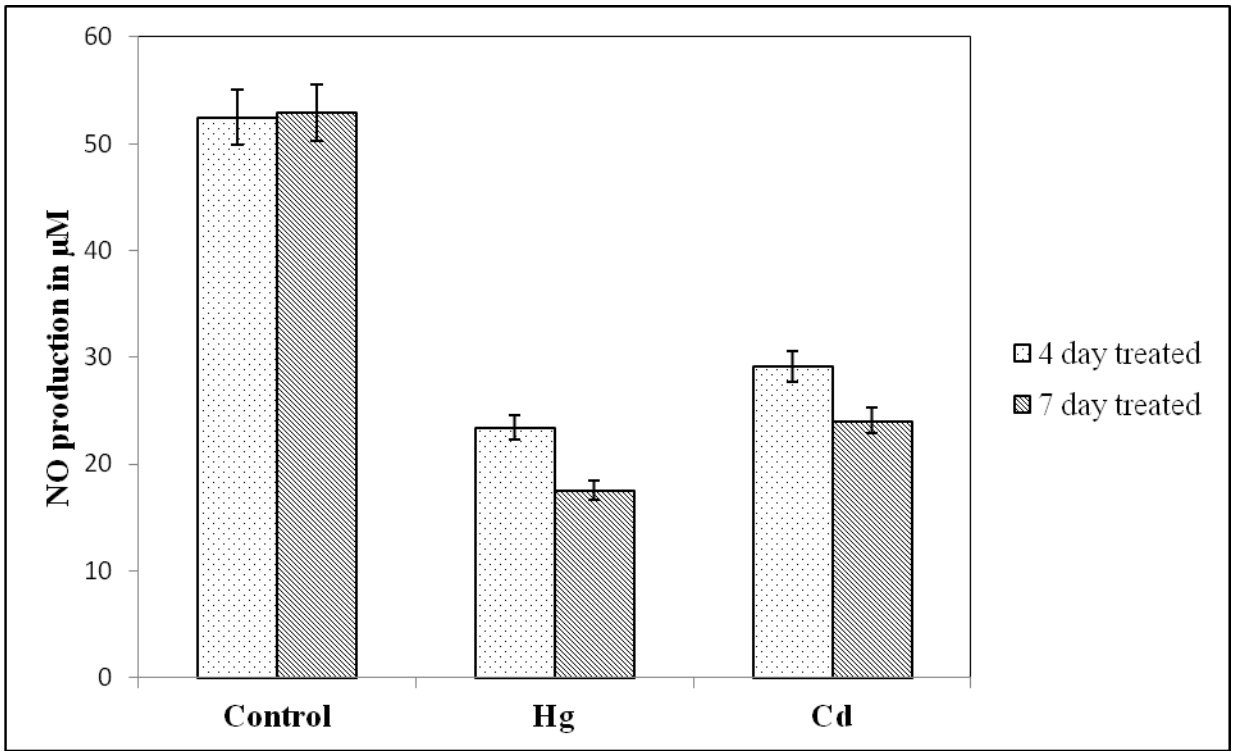


Fig. 5.19: Nitric oxide release in macrophages of fish treated with mercury and cadmium. 4day- Hg ($P< 0.01$) Cd ($P<0.02$); 7 day- Hg, Cd ($P< 0.05$).

5.1.3.5. Effect of mercury and cadmium on myeloperoxidase (MPO) release of fish macrophages

There was a significant decrease in myeloperoxidase release from 42.85±0.36% (4 day), 43.26±0.89 % (7 day) in control group to 29.66±0.68 % (4 day), 24.77±1.62 % (7 day) in mercury treated and 35.76±2.89 % (4 day), 31.37±1.26 % (7 day) in cadmium treated group.

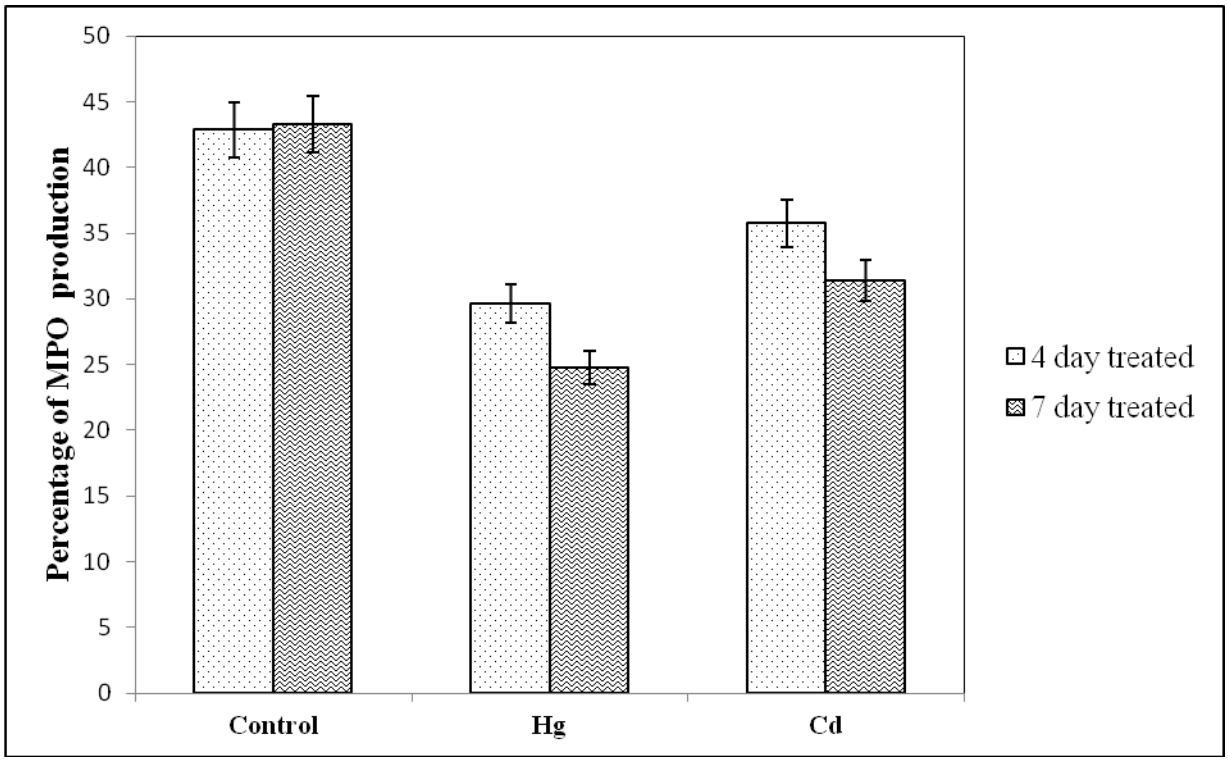


Fig. 5.20: Myeloperoxidase release in macrophages of fish treated with mercury and cadmium. Hg, Cd (4 day) P<0.02; Hg (7 day) P< 0.05; Cd (7 day) P< 0.04.

5.1.3.6. Effect of mercury and cadmium on TNF-α release of fish macrophages

TNF-α release both from cell lysate and plasma was significantly increased in mercury treated and cadmium treated group as compared to the control group.

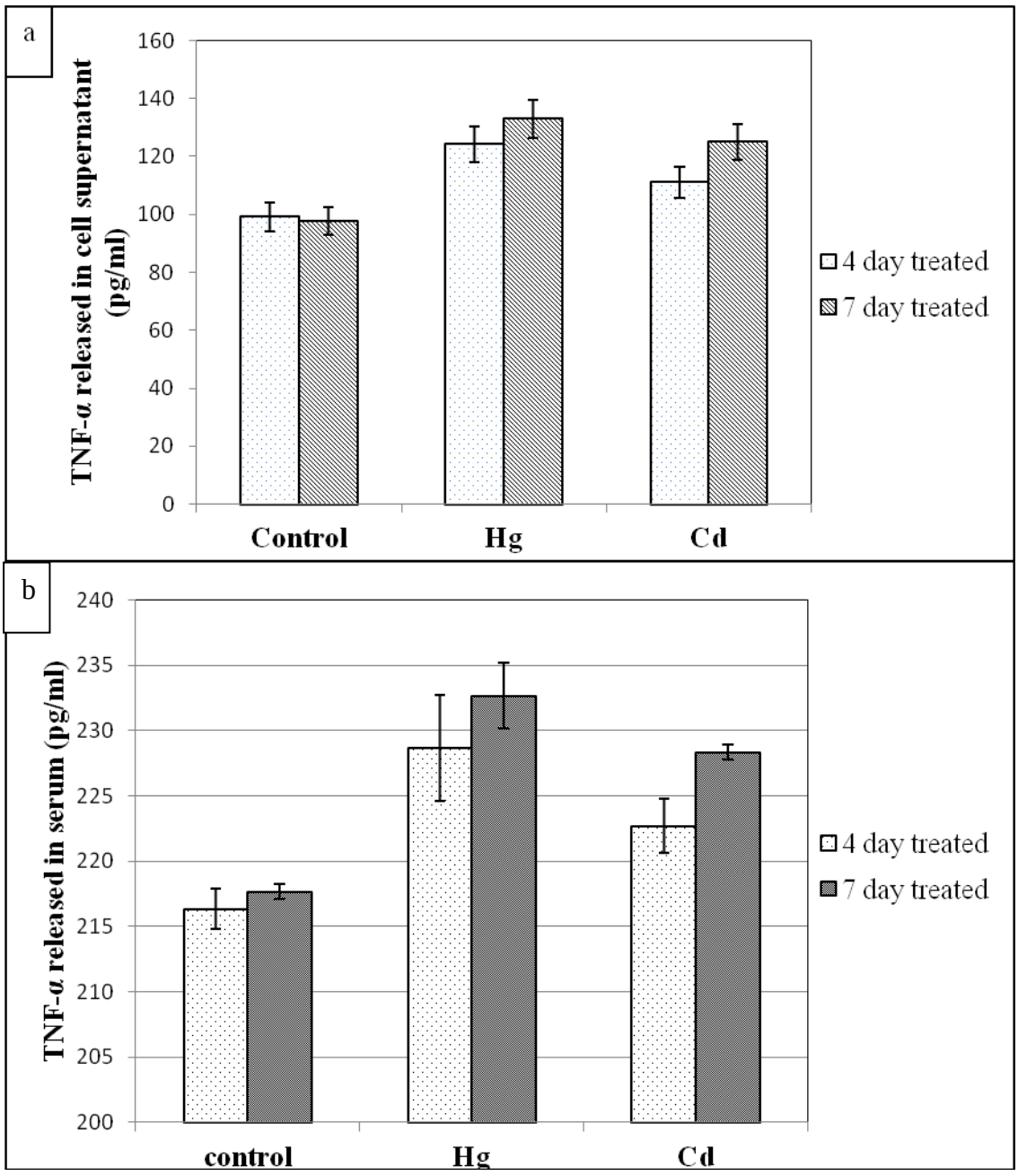


Fig. 5.21 (a): TNF-α released in cell supernatant of mercury and cadmium treated group (b) TNF-α released in serum of mercury and cadmium treated group. Hg (4 day) P<0.02; Hg, Cd (7 day) P< 0.05; Cd (4 day) P< 0.01.

5.1.3.7. Effect of mercury and cadmium on IL- 6 release of fish macrophages

There was a significant increase in IL-6 release from 0.042±0.005 pg/ml (4 day), 0.047±0.002 pg/ml (7 day) in control group to 0.055±0.002 pg/ml (4 day), 0.064±0.004 pg/ml (7 day) in mercury treated and 0.050±0.044 pg/ml (4 day), 0.058±0.034 pg/ml (7 day) in cadmium treated group.

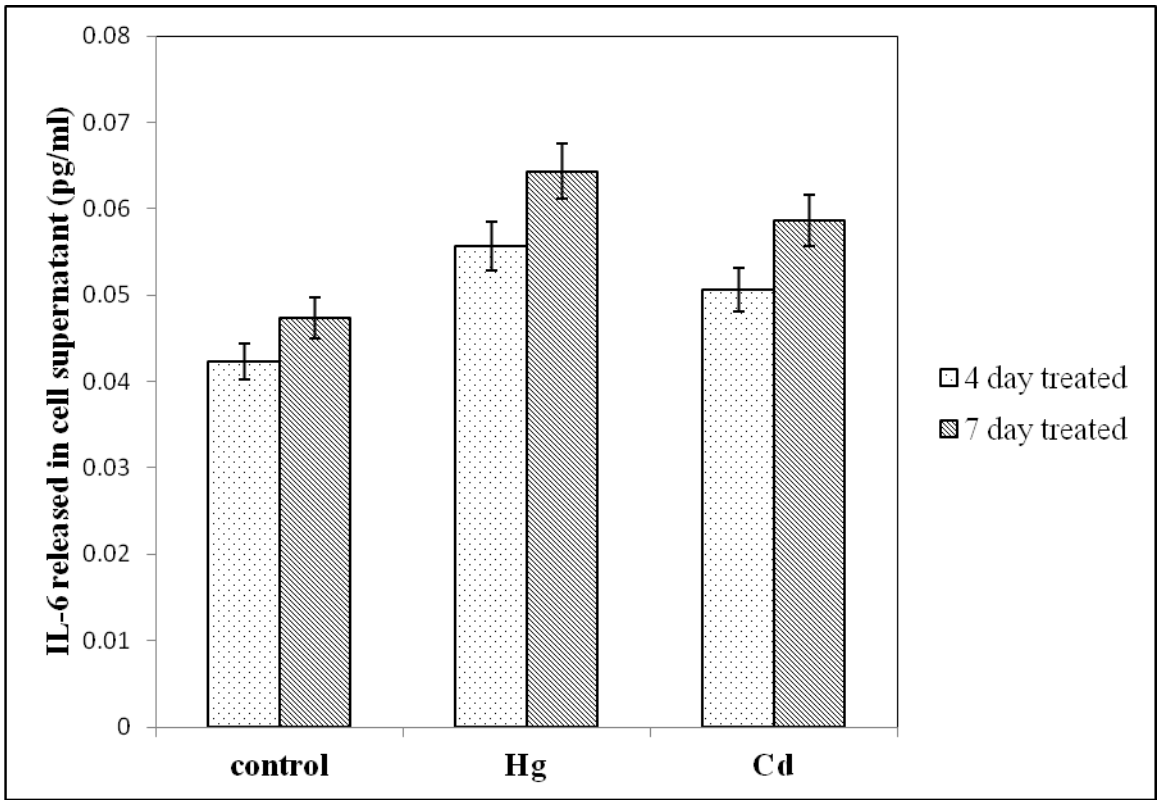


Fig 5.22: IL-6 released from plasma of mercury and cadmium treated group. Hg (4 day) P<0.02; Hg, Cd (7 day) P< 0.05; Cd (4 day) P< 0.01.

5.2. Study of mercuric chloride and cadmium chloride exposure simultaneously on intestinal of fish *Channa punctatus.*

5.2.1. Effects from combined mercuric chloride and cadmium chloride exposure on ultrastructural changes, bioaccumulation and morphological alteration in macrophages from fish *Channa punctatus*

5.2.1.1. Heavy metal analysis in different organs of untreated and treated multi-metal group (ppm/g tissue)

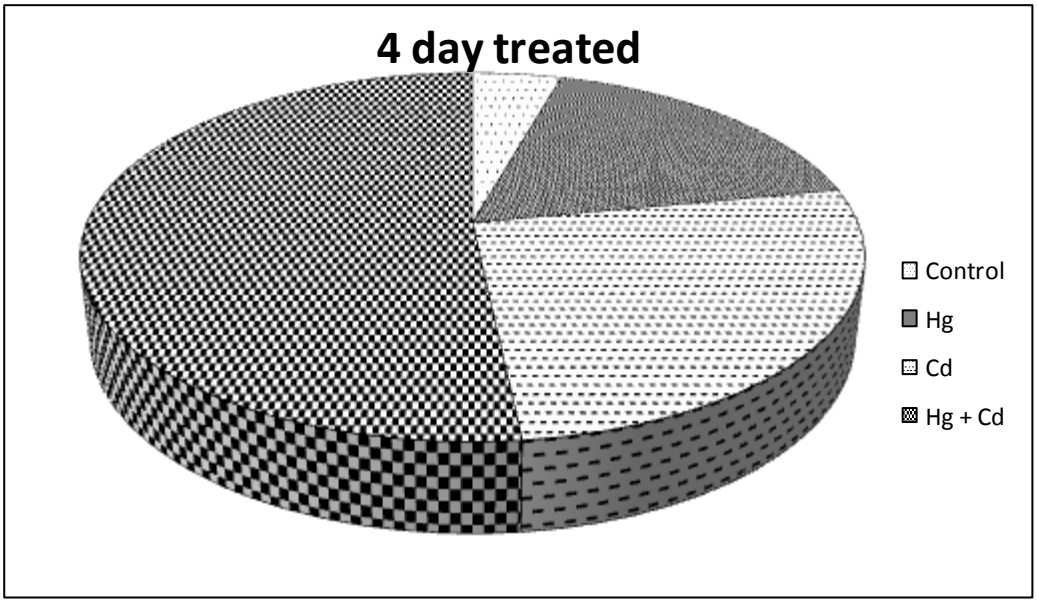
Table 5.2: Heavy metal analysis in different organs of untreated group and group with simultaneous treatment of mercury and cadmium after 7 days of exposure (ppm/g tissue).

UNTREATED GROUP	**INTESTINE**	**LIVER**	**GILLS**	**MUSCLES**
Mercury accumulation	**0.072±0.001**	**0.039±0.02**	**0.044±0.023**	**0.025±0.017**
Cadmium accumulation	**0.041±0.01**	**0.046±0.016**	**0.0388±0.014**	**0.027±0.07**

TREATED GROUP	**INTESTINE**	**LIVER**	**GILLS**	**MUSCLES**
Mercury accumulation	**1.10±0.003**	**1.15±0.004**	**1.061±0.14**	**0.59±0.01**
Cadmium accumulation	**0.99±0.001**	**0.972±0.017**	**1.0131±0.003**	**0.83±0.002**

Note: The values were statistically significant at $P< 0.005$.

5.2.1.2. Morphological alteration of macrophages in multi- metal treated group



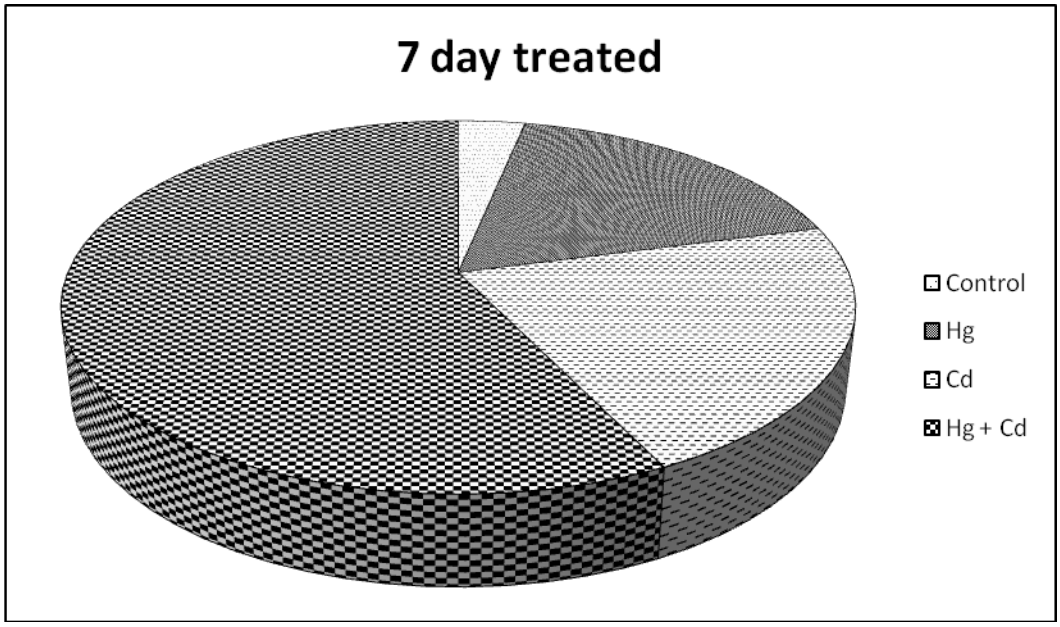


Fig. 5.23: Combined effect of mercury and cadmium on the morphological alteration of intestinal macrophages for 4 days (a) and 7 days (b) respectively. Hg, Cd (4 day) $P< 0.05$; Hg, Cd (7 day) $P< 0.05$.

5.2.1.3. Ultra structural analysis of tissue by scanning electron microscope

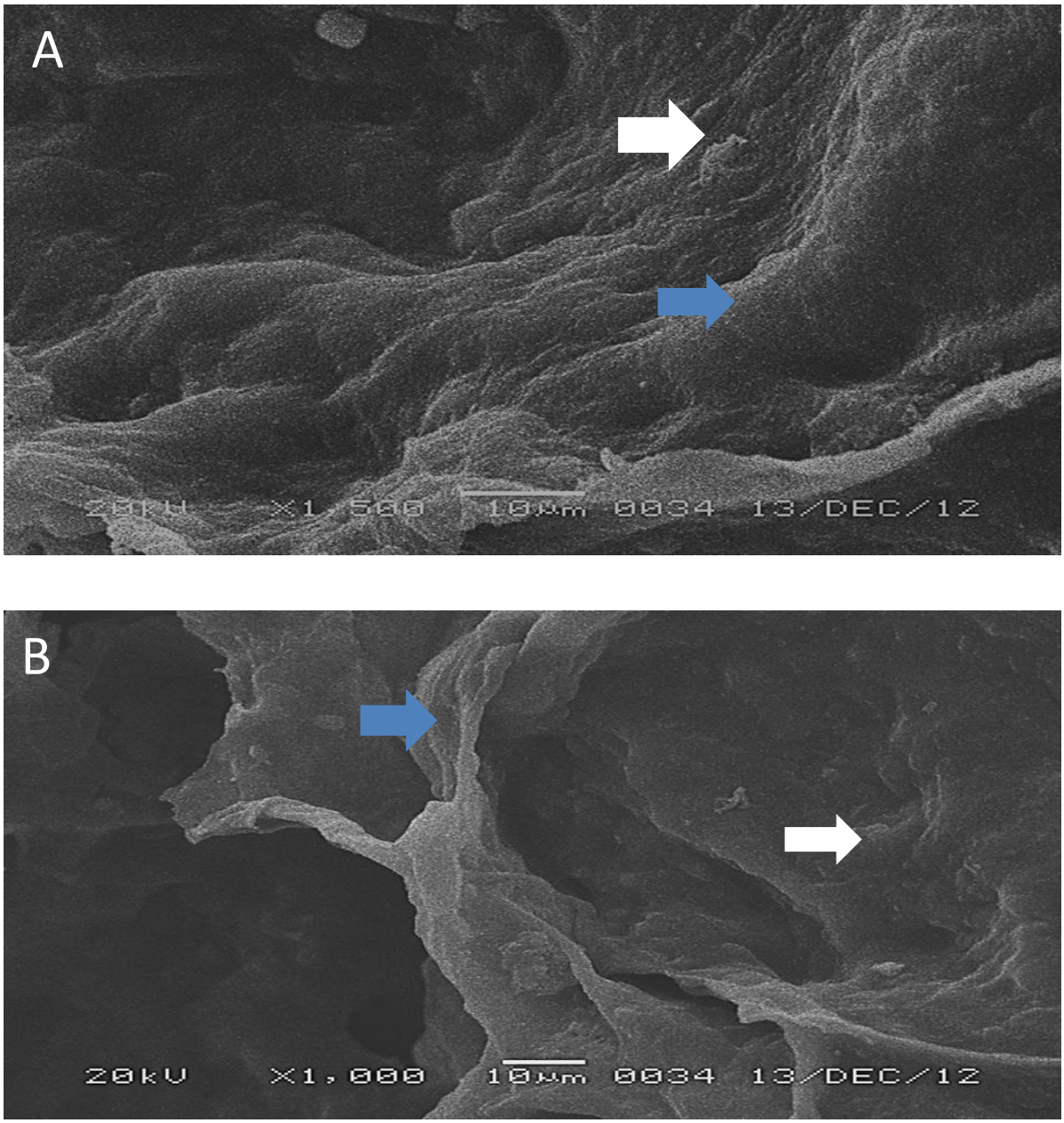


Fig.5.24: Ultramicroscopic photographs of untreated fish showing prominent mucosal foldings (blue arrow) and normal epithelium (white arrow) after 4 days (A) and 7 days (B) repectively.

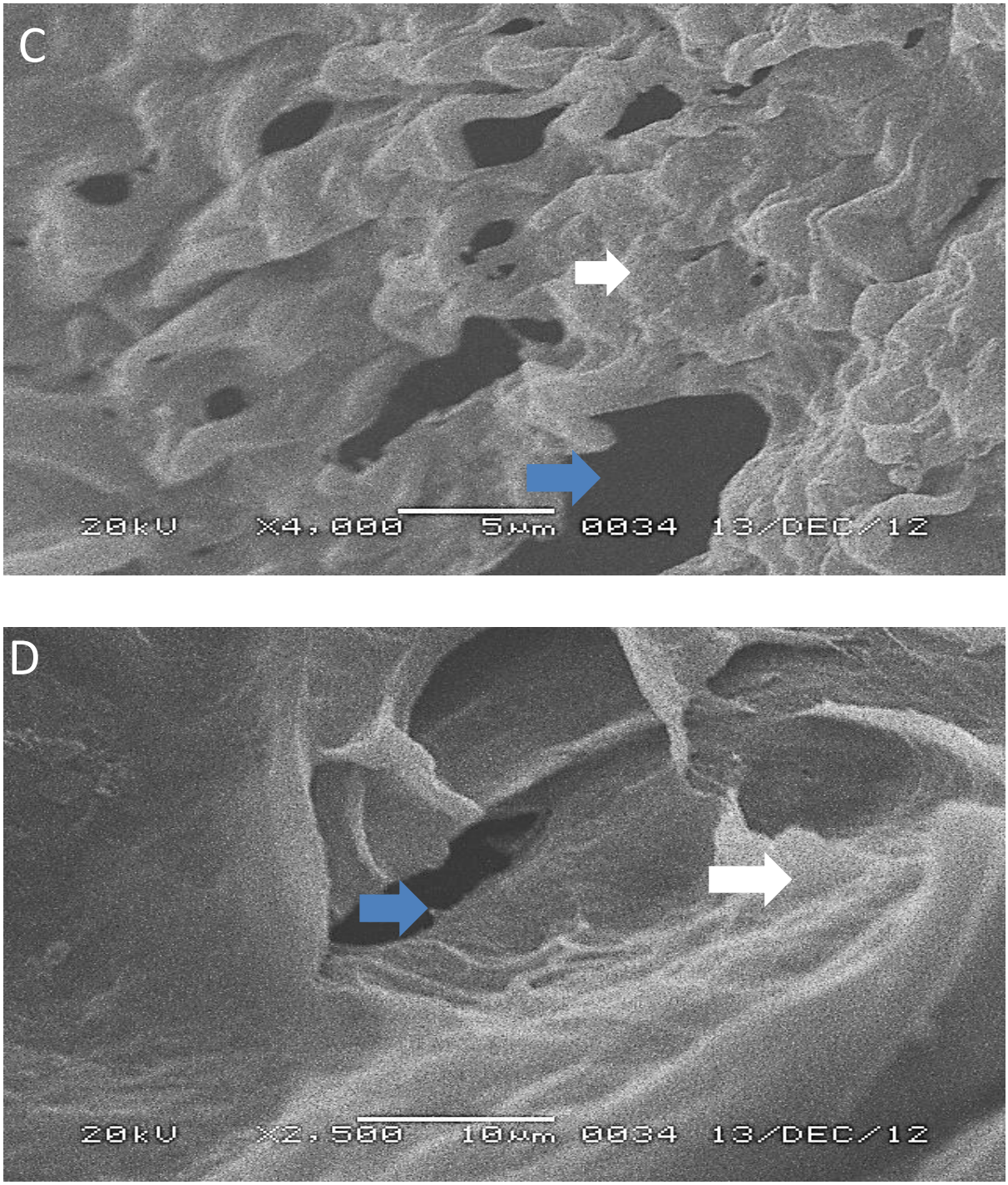


Fig. 5.25: Ultramicroscopic photographs of mercury treated fish showing debris of mucosal folds (blue arrow), damaged and degenerated epithelium (white arrow) after 4 days (C) and 7 days (D) of exposure.

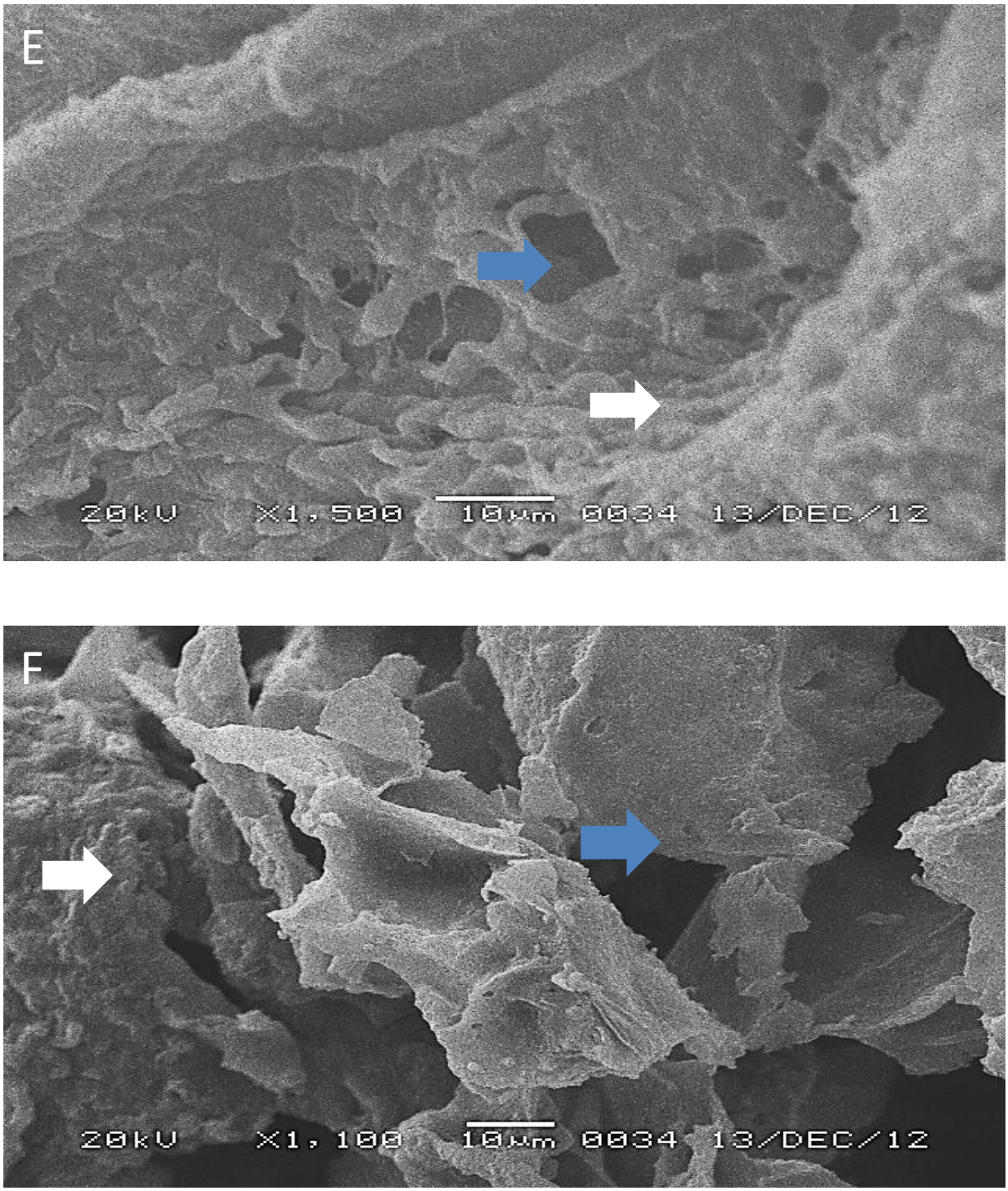


Fig. 5.26: Ultramicroscopic photographs of cadmium treated fish showing inflammatory damage in epithelium (white arrow), disarrangement and fragmentation of mucosal foldings (blue arrow) and secretion of mucus after 4 days (E) and 7 days (F) of exposure.

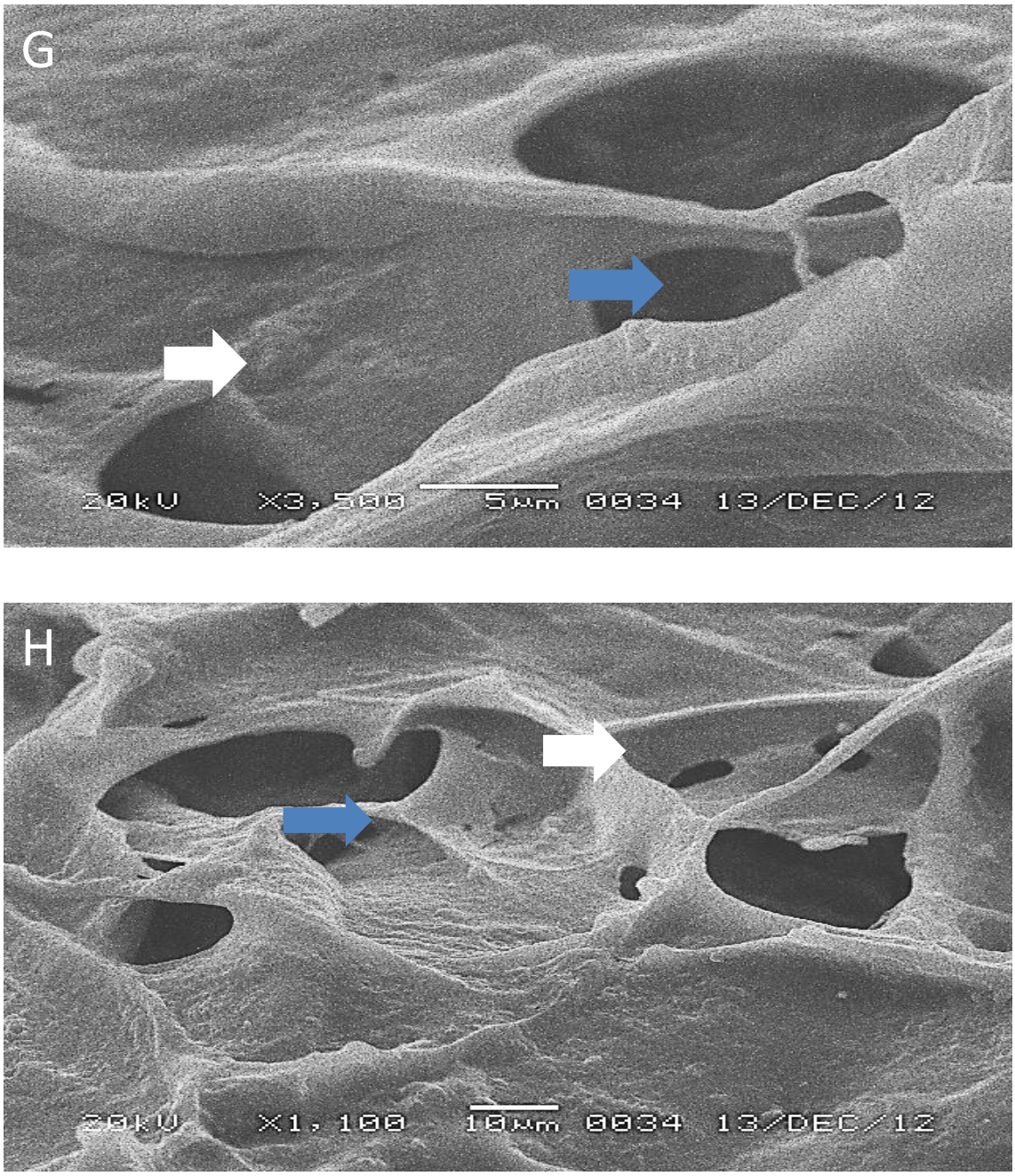


Fig. 5.27: Ultramicroscopic photographs of multi metal (Hg+Cd) treated fish showing inflammatory damage in epithelium (white arrow), disarrangement and fragmentation of mucosal foldings (blue arrow) and secretion of mucus after 4 days (G) and 7 days (H) of exposure.

5.2.1.4. Ultra structural analysis of tissue by transmission electron microscope

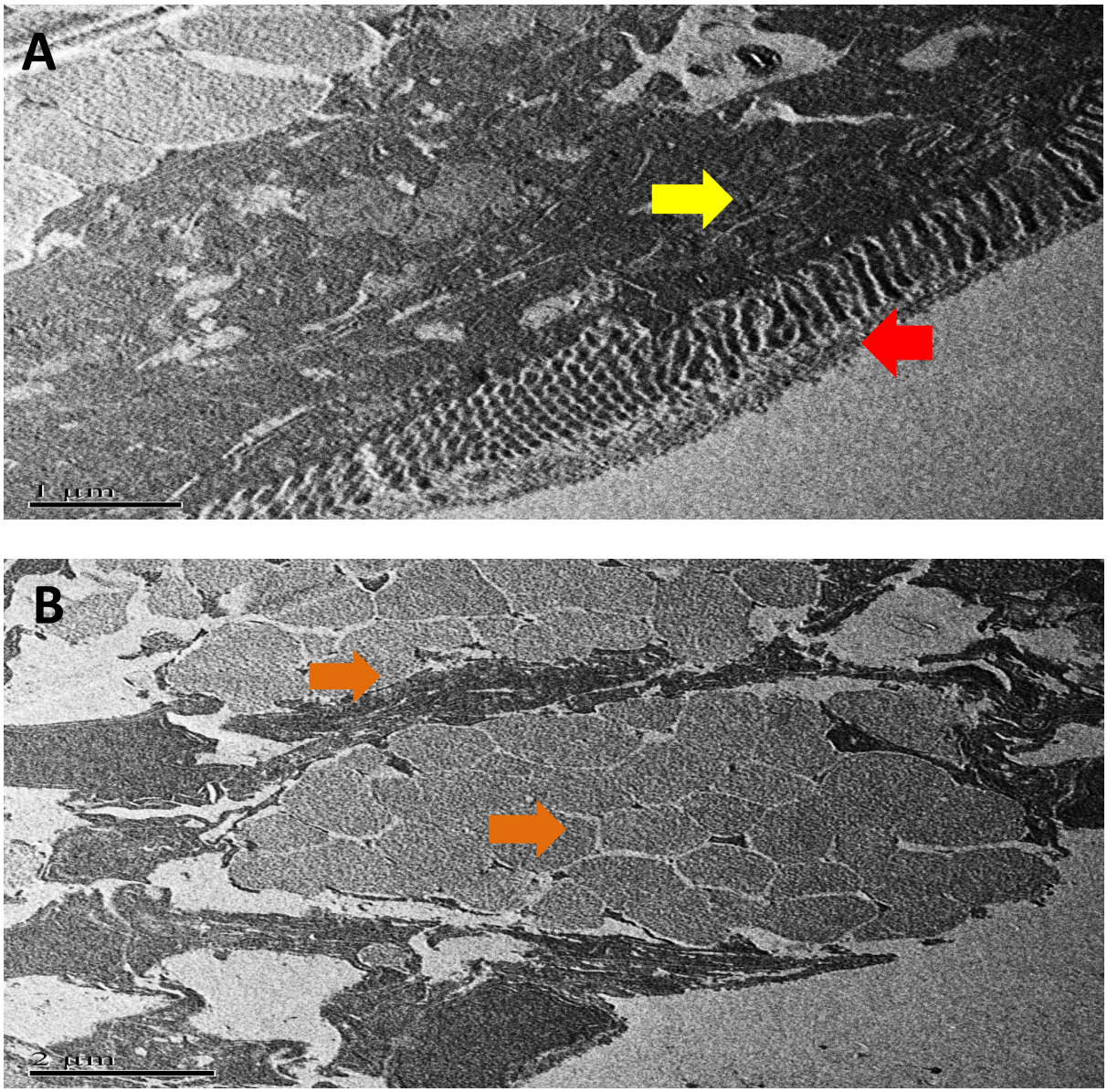


Fig. 5.28: Plate (A) Transmission electron micrograph (TEM) of the intestinal tissue of control fish showing regularly arranged microvilli (red arrow), intact rough endoplasmic reticulam (RER) of epithelial cells (yellow arrow).

Plate (B) Transmission electron micrograph (TEM) of the intestinal tissue of control fish showing goblet cells (orange arrow).

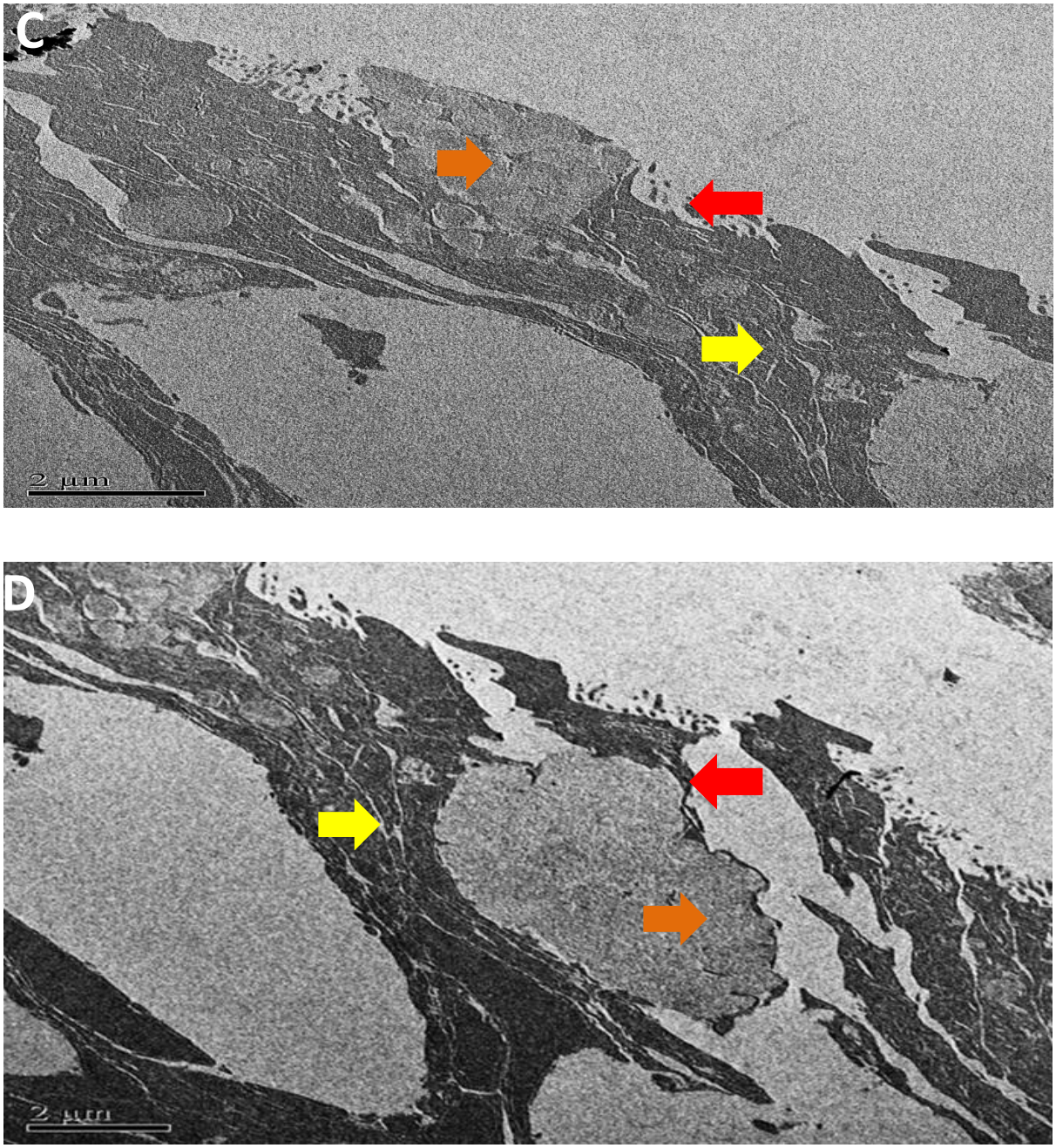


Fig.5.29: Transmission electron micrograph (TEM) of *C. punctatus* Bloch. after 4 days (C) and 7 days (D) days of mercury exposure showing denudation of goblet cells (orange arrow), disrupted microvilli (red arrow) disrupted RER (yellow arrow), lack of gap junction and zona occludens (Z) and disrupted RER (yellow arrow), as compared to the control fish**.**

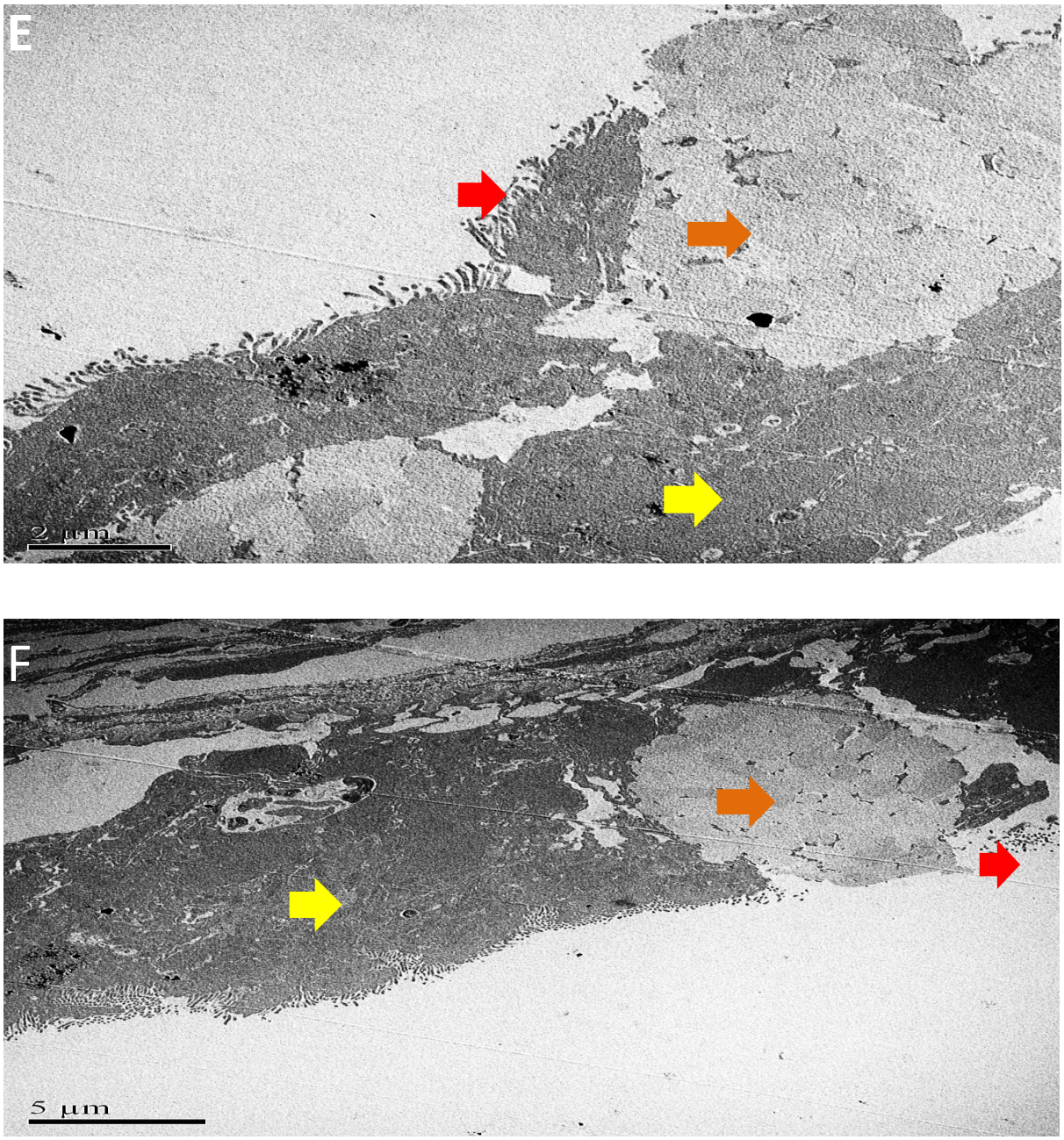


Fig.5.30: Transmission electron micrograph (TEM) of *C. punctatus* Bloch. after 4 days (E) and 7 days (F) days of cadmium exposure showing denudation of goblet cells (orange arrow), disrupted microvilli (red arrow) disrupted RER (yellow arrow), lack of gap junction and zona occludens (Z) as compared to the control fish.

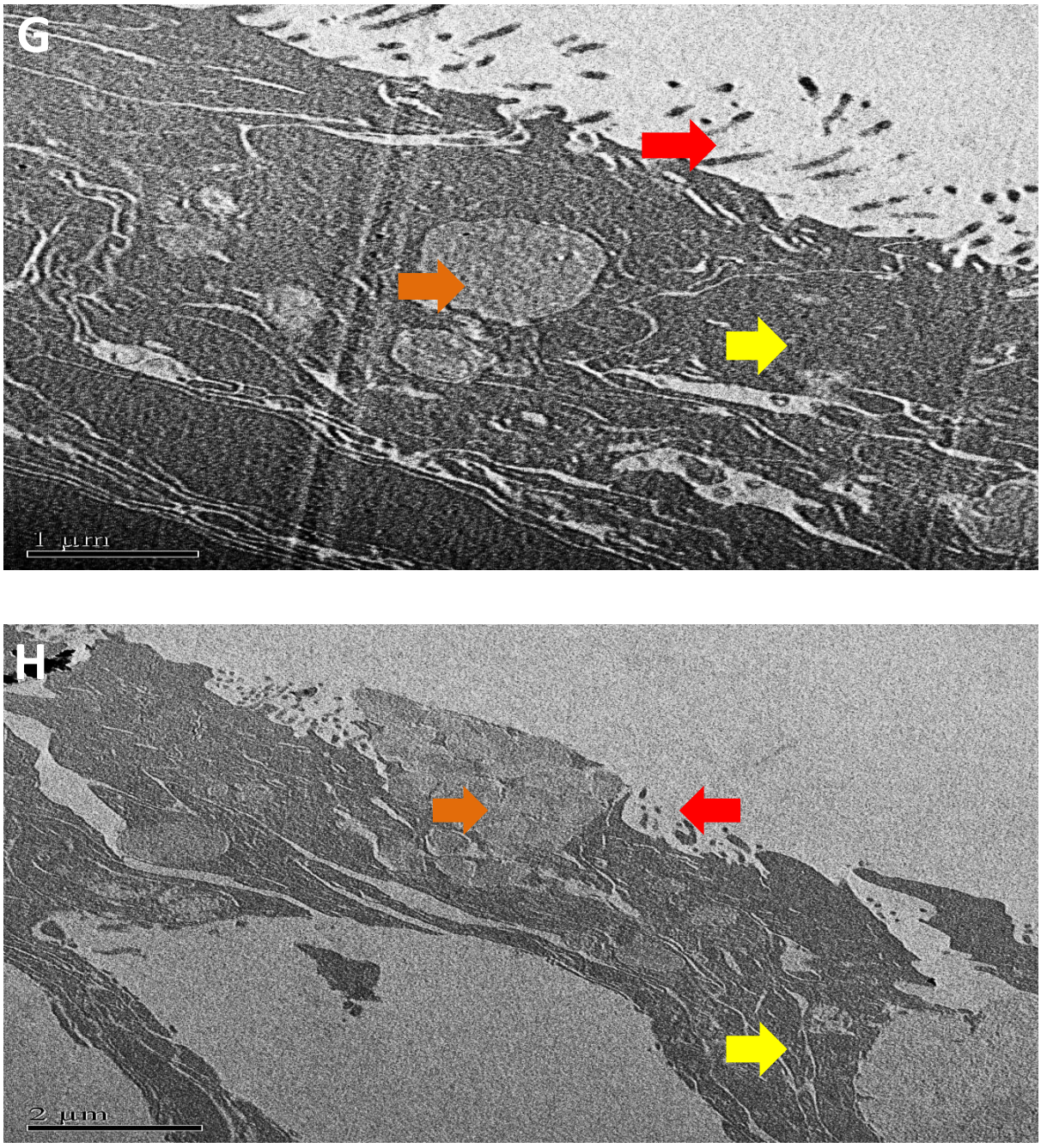


Fig.5.31: Transmission electron micrograph (TEM) of *C. punctatus* Bloch. after 4 days (G) and 7 days (H) days of multi metal exposure (Hg+Cd) exposure showing denudation of goblet cells (orange arrow), disrupted microvilli (red arrow) disrupted RER (yellow arrow), lack of gap junction and zona occludens (Z) as compared to the control fish.

5.2.2. Study of effect of mercuric chloride and cadmium chloride exposure simultaneously on oxidative stress and antioxidant defenses in macrophages from fish *Channa punctatus*

5.2.2.1. Effect of multi-metal exposure on respiratory burst activity of fish macrophages

There was a significant increase in respiratory burst activity from 0.271±0.09 (4 day), 0.313±0.02 (7 day) in control group to 1.105±0.09 (4 day), 1.50±0.07 (7 day) in multi-metal treated group.

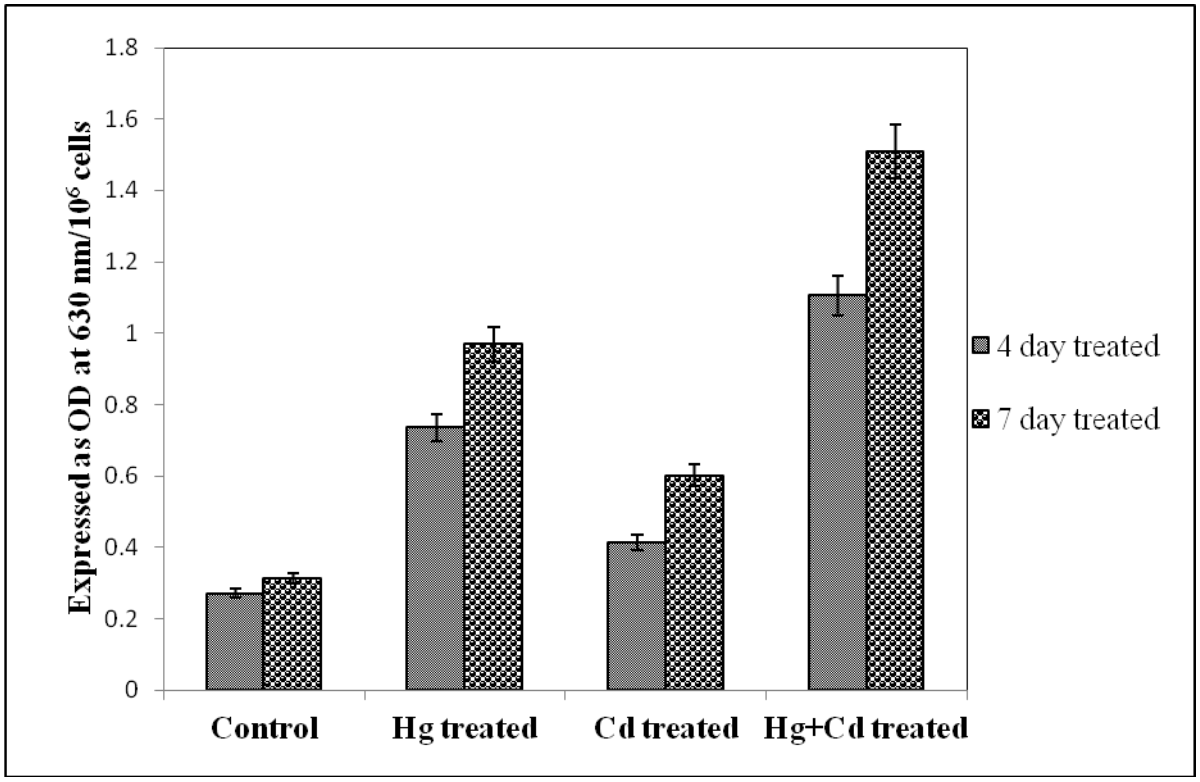


Fig. 5.32: Respiratory burst activity in intestinal macrophages of fish treated simultaneously with mercury and cadmium. Hg, Cd (4 day) $P< 0.05$; Hg, Cd (7 day) $P< 0.025$.

5.2.2.2. Effect of multi- metal exposure on lipid peroxidation of fish macrophages

There was a significant increase in lipid peroxidation from 0.243±0.033 nmoles/hr (4day), 0.215±0.003 nmoles/hr (7day) in control group to 0.875±0.059 nmoles/hr (4 day) and 1.214±0.176 nmoles/hr (7 day) in multi-metal treated group.

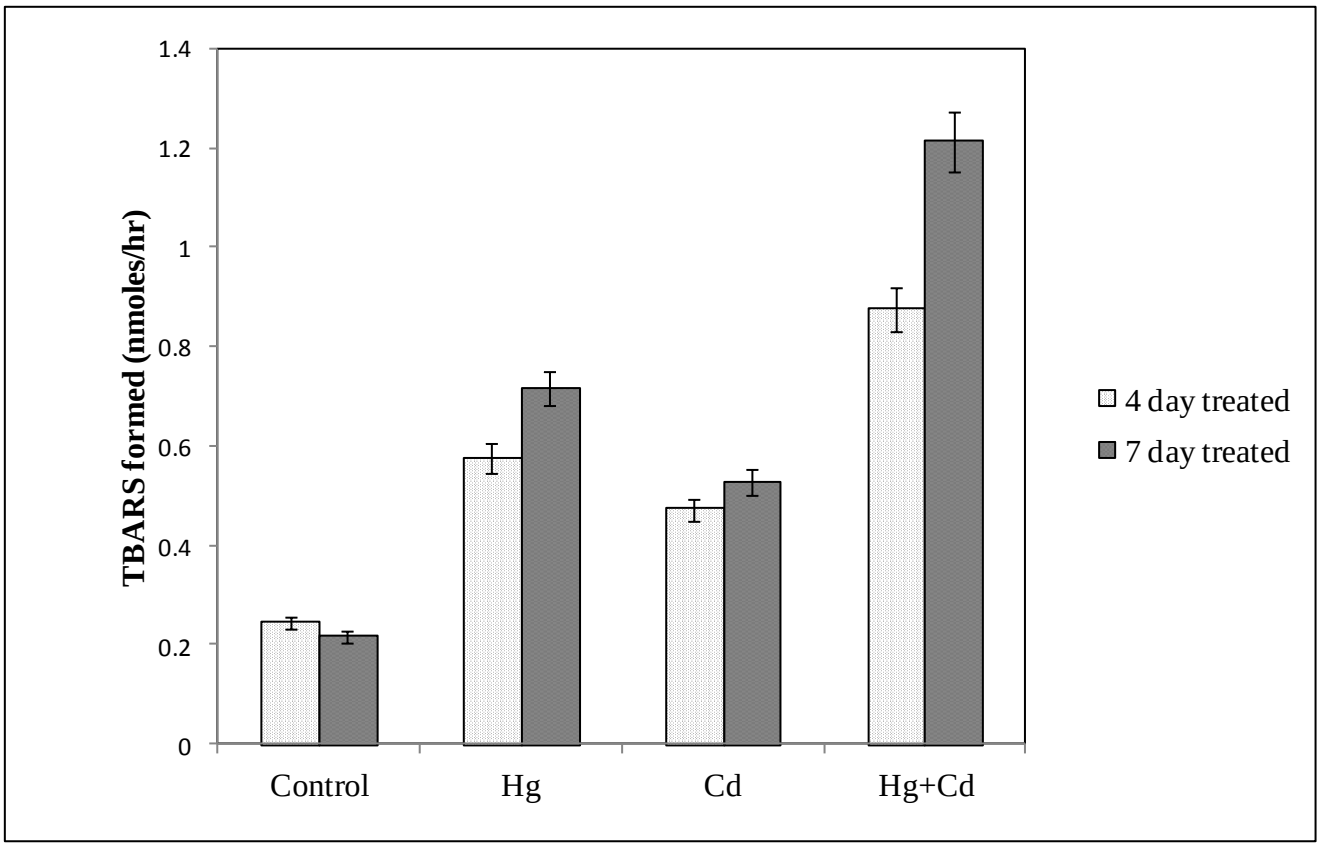


Fig. 5.33: Estimation of lipid peroxidation (LPO) in multi- metal exposed fish macrophages. Hg, Cd (4 day) $P< 0.05$; Hg, Cd (7 day) $P< 0.01$.

5.2.2.3. Effect of multi-metal exposure on catalase (CAT) activity of fish macrophages

There was a significance decrease in catalase activity from 5.43±0.508U/mg protein (4 day), 5.69±0.805 U/mg protein (7 day) in control group to 2.89±0.77 U/mg protein (4 day), 1.92±0.266 U/mg protein (7 day) in multi-metal treated group.

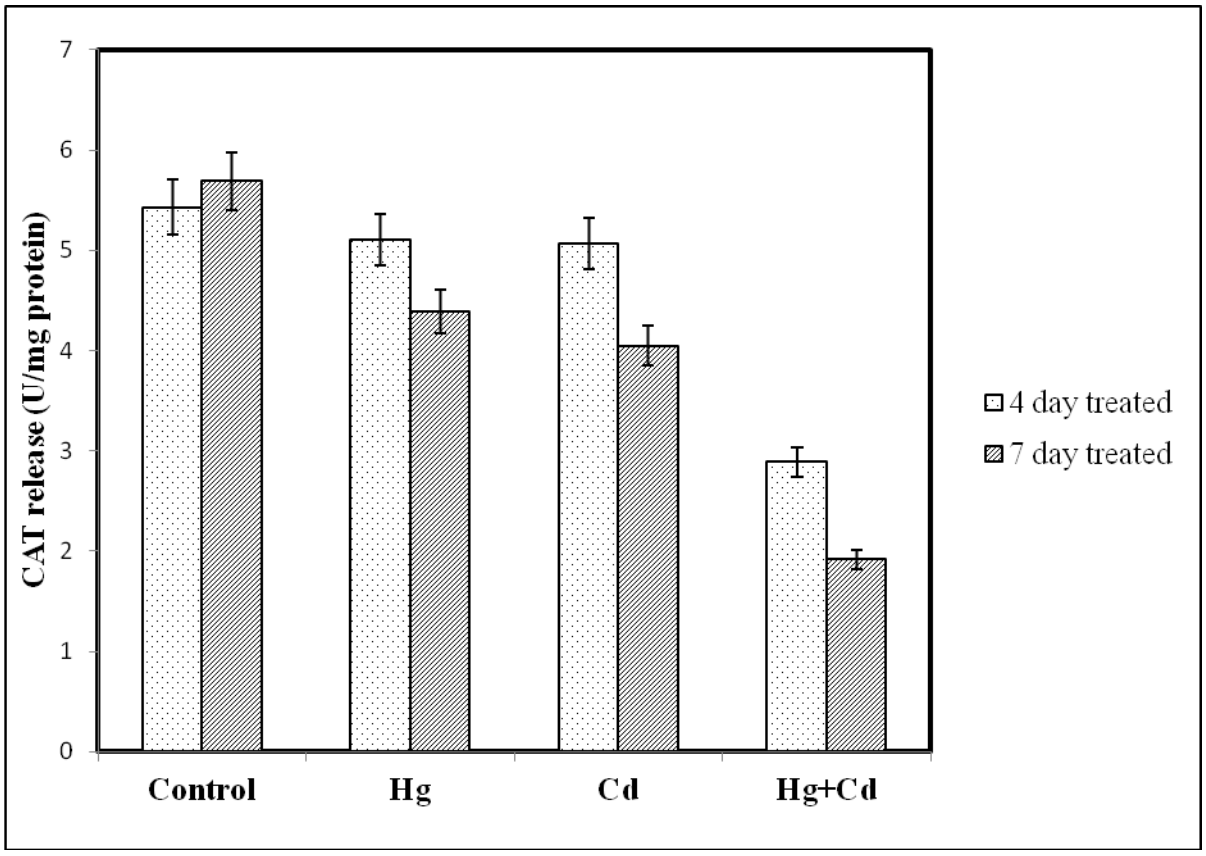


Fig. 5.34: Catalase (CAT) released from control and multi-metal (Hg+Cd) exposed fish macrophages. Hg, Cd (4 day) P< 0.05; Hg, Cd(7day) P< 0.02.

5.2.2.4. Effect of multi- metal exposure on superoxide dismutase (SOD) activity of fish macrophages

There was a significance increase in superoxide dismutase activity from 0.0096±0.0004 U/mg protein (4 day), 0.0095±0.0004 U/mg protein (7 day) in control group to 0.195±0.017 U/mg protein (4 day), 0.276±0.027 U/mg protein (7 day) in multi-metal treated group.

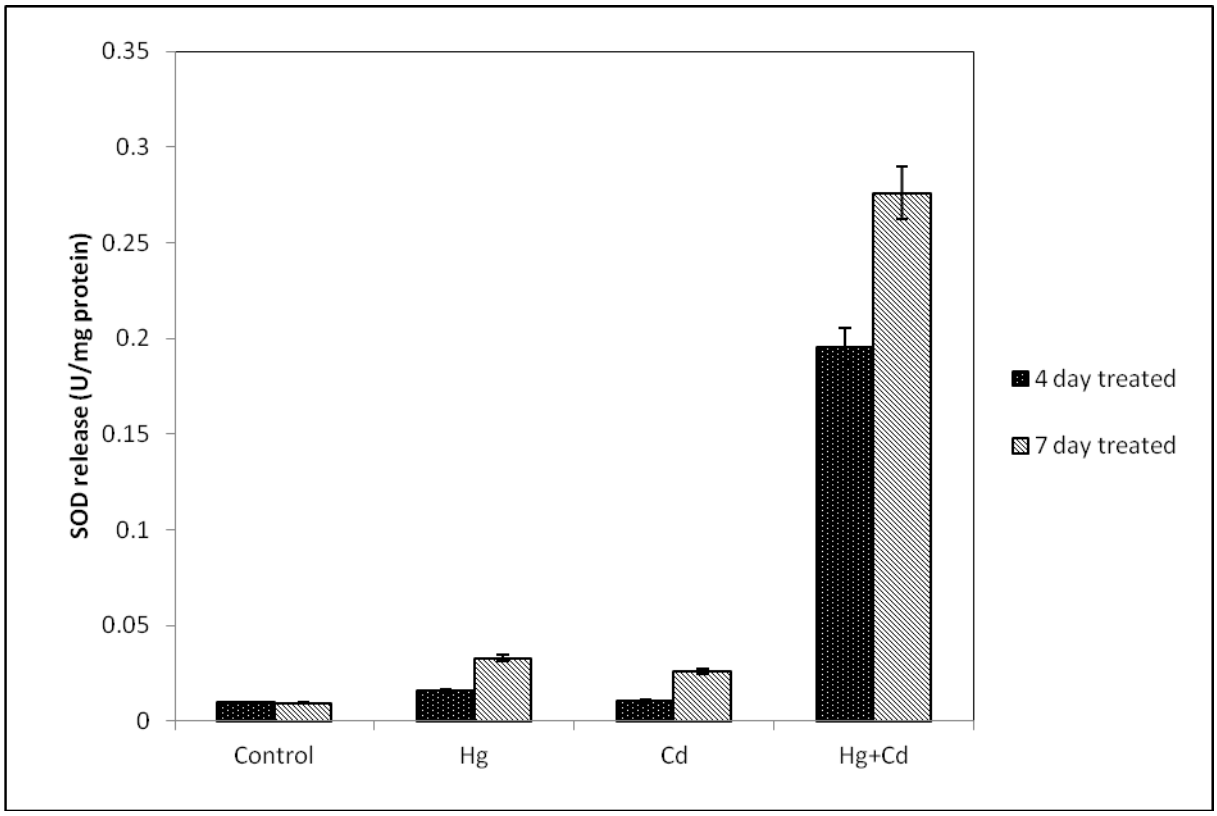


Fig. 5.35: Superoxide dismutase (SOD) released from control and multi-metal exposed fish macrophages. Hg (4 day) P< 0.02; Cd (4 day) P< 0.05; Hg, Cd (7 day) P< 0.01.

5.2.2.5. Effect of multi- metal exposure on glutathione S- transferase (GST) activity of fish macrophages

There was a significance decrease in glutathione S-transferase activity from 31.61±1.76 nmoles/min/mg protein (4 day), 30.68±0.50 nmoles/min/mg protein (7 day) in control group to 20.13±0.11 nmoles/min/mg protein (4 day), 17.07±3.27 nmoles/min/mg protein (7 day) in multi-metal treated group.

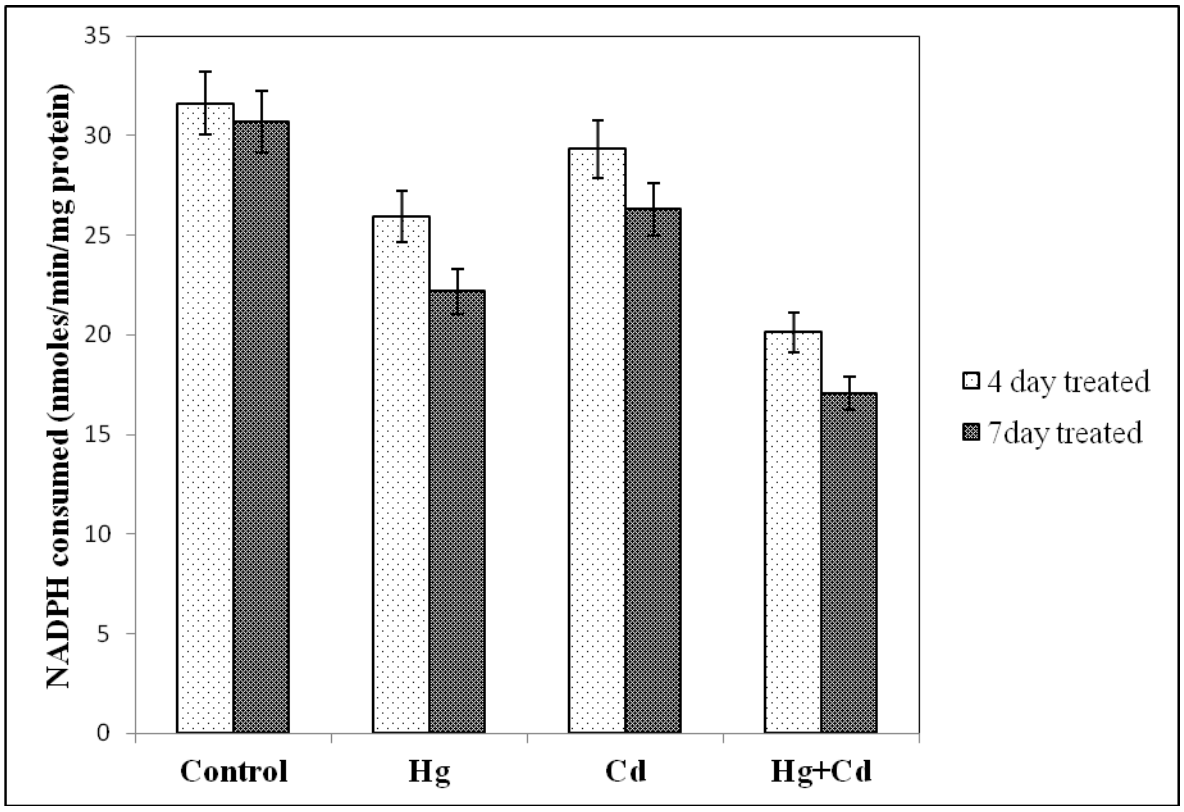


Fig. 5.36: Glutathione S-transferase (GST) activity in macrophages of fish treated simultaneously with mercury and cadmium. Hg, Cd (4 day) P< 0.01; Hg, Cd (7 day) P< 0.05.

5.2.2.6. Effect of multi- metal exposure on glutathione peroxidase (GPx) activity of fish macrophages

There was a significance decrease in glutathione peroxidase activity from 7.60±0.77 nmoles/min/mg protein (4 day), 7.72±1.05 nmoles/min/mg protein (7 day) in control group to 3.34±0.28 nmoles/min/mg protein (4 day), 2.60±0.29 nmoles/min/mg protein (7 day) in multi-metal treated group.

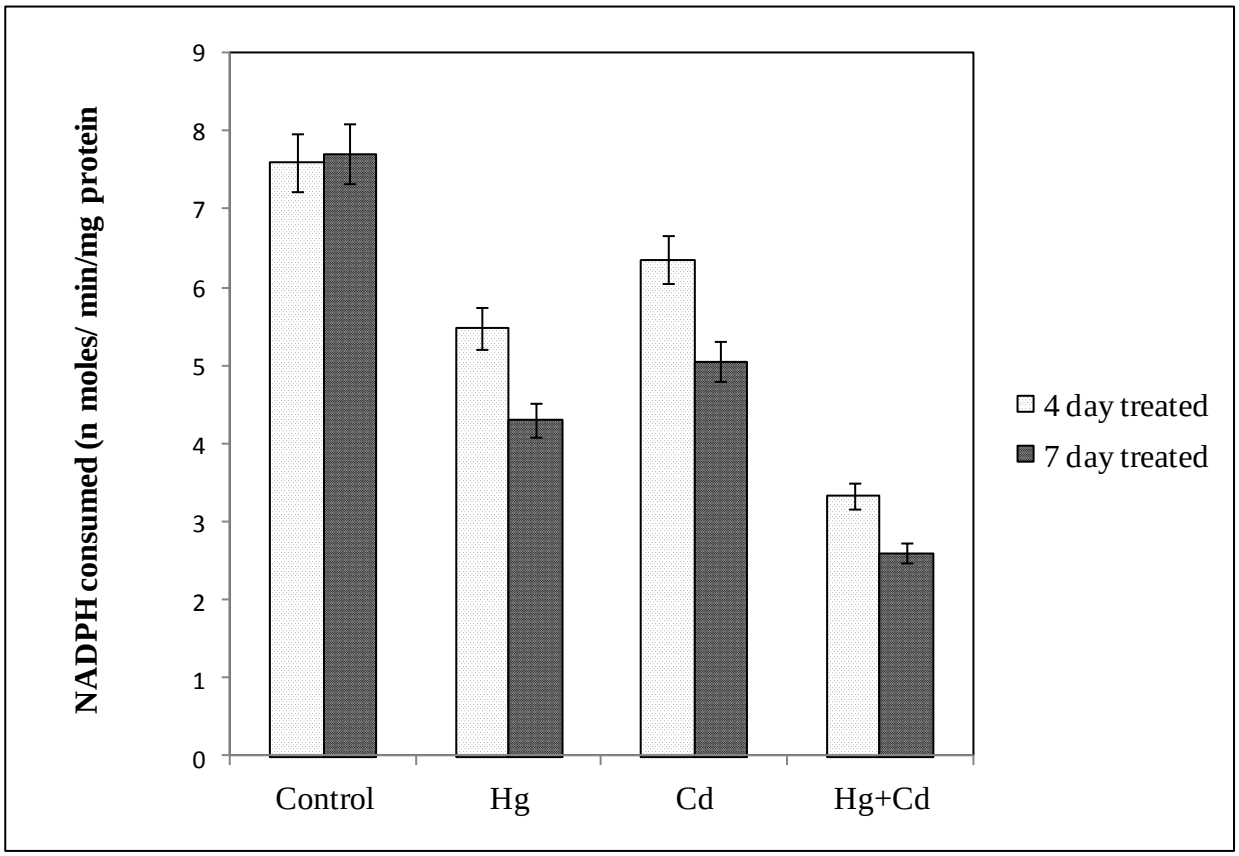


Fig.5.37: Glutathione peroxidase (GPx) activity in macrophages of fish treated simultaneously with mercury and cadmium. Hg, Cd (4 day) P< 0.01; Hg, Cd (7 day) P< 0.025.

5.2.2.7. Effect of multi- metal exposure on glutathione reductase (GR) activity of fish macrophages

There was a significance decrease in glutathione reductase activity from 6.32±0.538 nmoles/min/mg protein (4 day), 6.13±0.511 nmoles/min/mg protein (7 day) in control group to 2.40±0.11 nmoles/min/mg protein (4 day), 1.33±0.489 nmoles/min/mg protein (7 day) in multi-metal treated group.

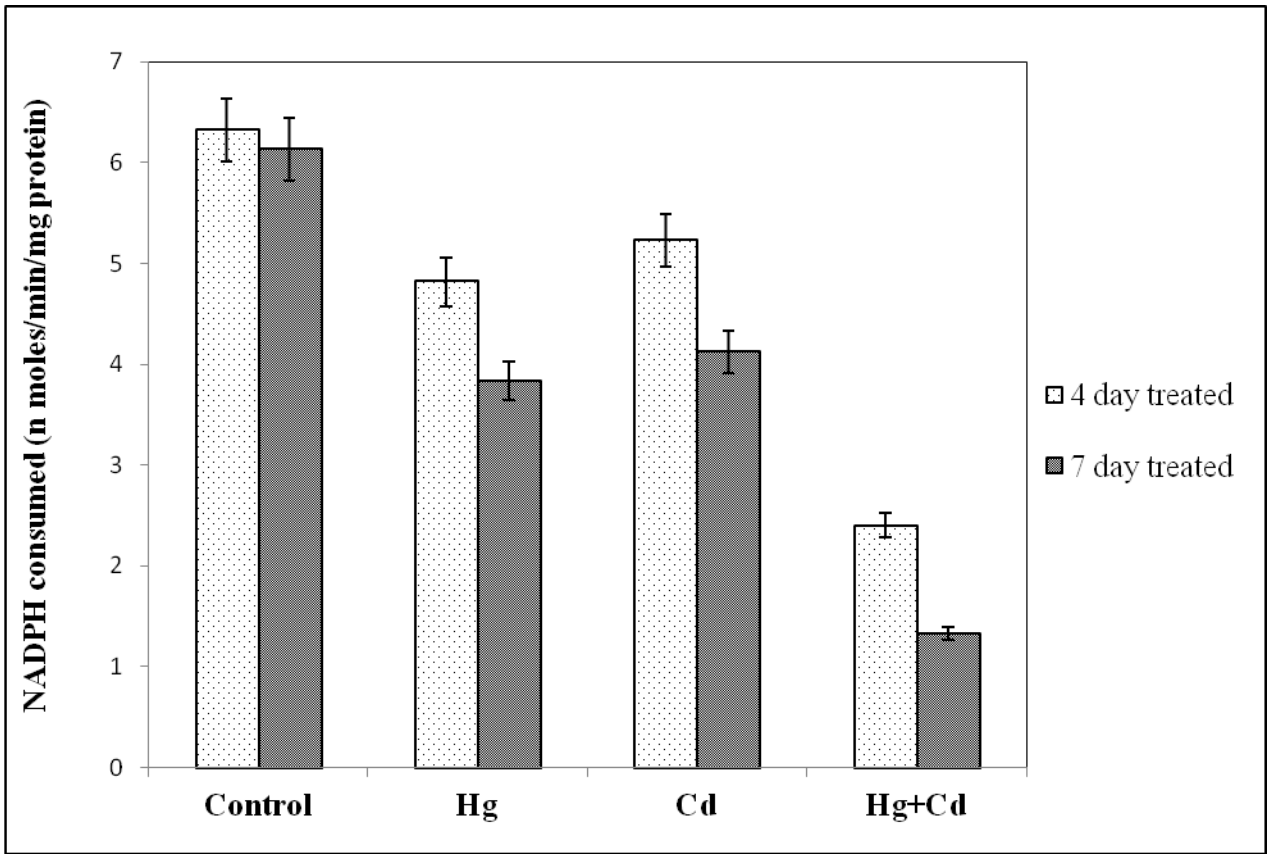


Fig. 5.38: Glutathione reductase (GR) activity in macrophages of fish treated simultaneously with mercury and cadmium. Hg, Cd (4 day) P< 0.025; Hg, Cd (7 day) P< 0.05.

5.2.2.8. Effect of multi-metal exposure on reduced glutathione (GSH) activity of fish macrophages

There was a significance decrease in reduced glutathione activity from 0.338±0.053 nmoles/g tissue (4 day), 0.329±0.014 nmoles/g tissue (7 day) in control group to 0.155±0.028 nmoles/g tissue (4 day) and 0.096±0.003 nmoles/g tissue (7 day) in multi-metal treated group.

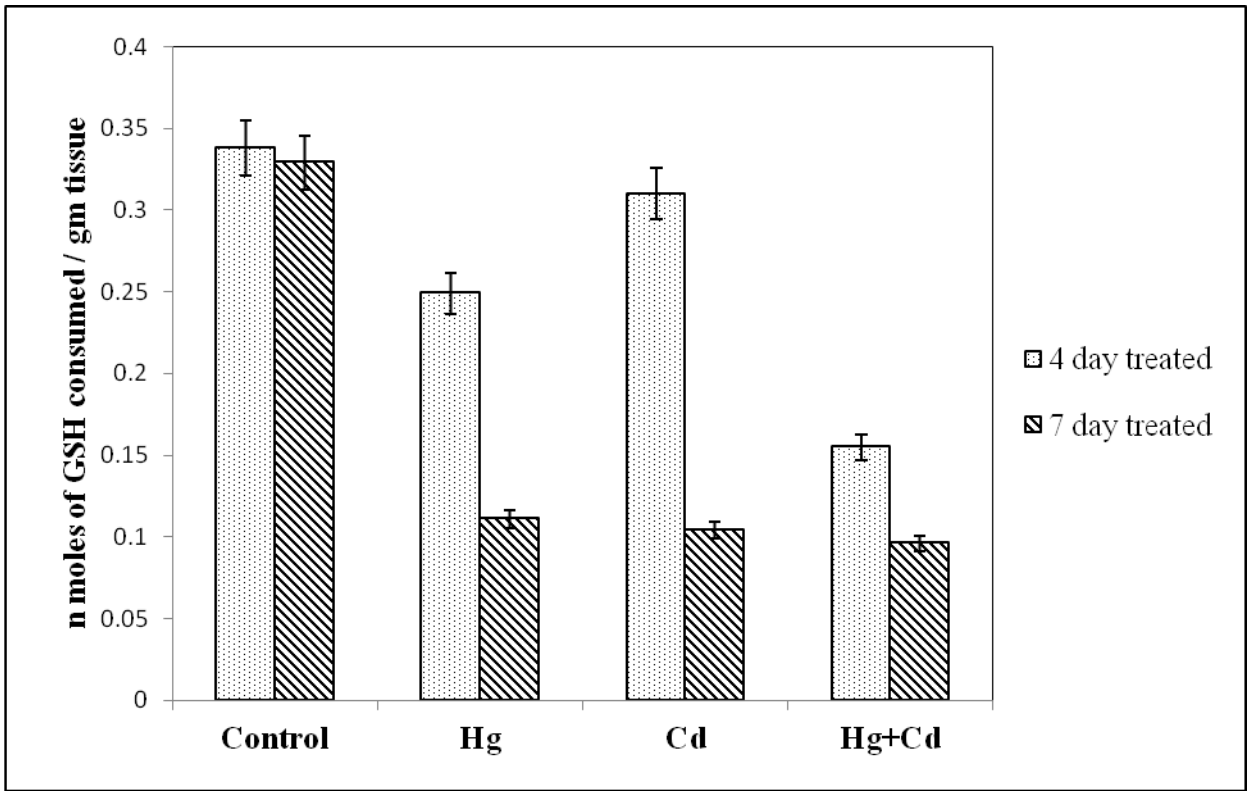


Fig. 5.39: Reduced glutathione (GSH) consumed in macrophages of fish treated simultaneously with mercury and cadmium. Hg, Cd (4 day) $P< 0.05$; Hg, Cd (7 day) $P< 0.01$.

5.2.3. Study of effect of mercuric chloride and cadmium chloride simultaneously on innate immune responses in macrophages from fish *Channa punctatus*

5.2.3.1. Effect of multi-metal exposure on phagocytic activity of fish macrophages

There was a significant decrease in phagocytic activity from 21739±780.63 % (4 day), 21628±548.26 % (7 day) in control group to 11212.8±481.30 % (4 day), 8378.81±1213.94 % (7 day) in multi-metal treated group.

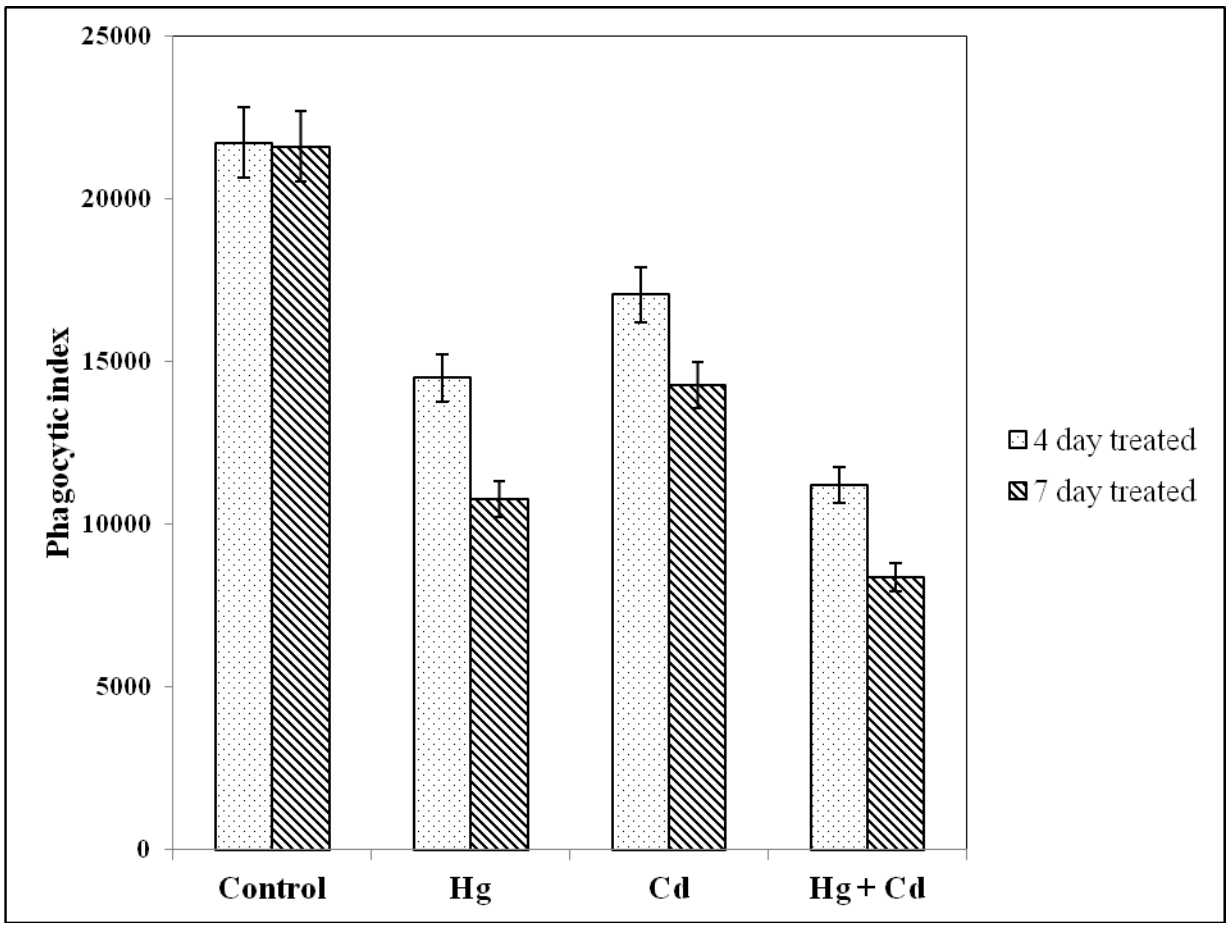


Fig 5.40: Phagocytic activity in macrophages of fish treated simultaneously with mercury and cadmium. Hg (4 day) P<0.02; Hg, Cd (7 day) P< 0.05; Cd (4 day) P< 0.01.

5.2.3.2. Effect of multi-metal exposure on intracellular killing activity of fish macrophages

There was a significant increase in bacterial viability in multi-metal treated group at 60 min as compared to the control group.

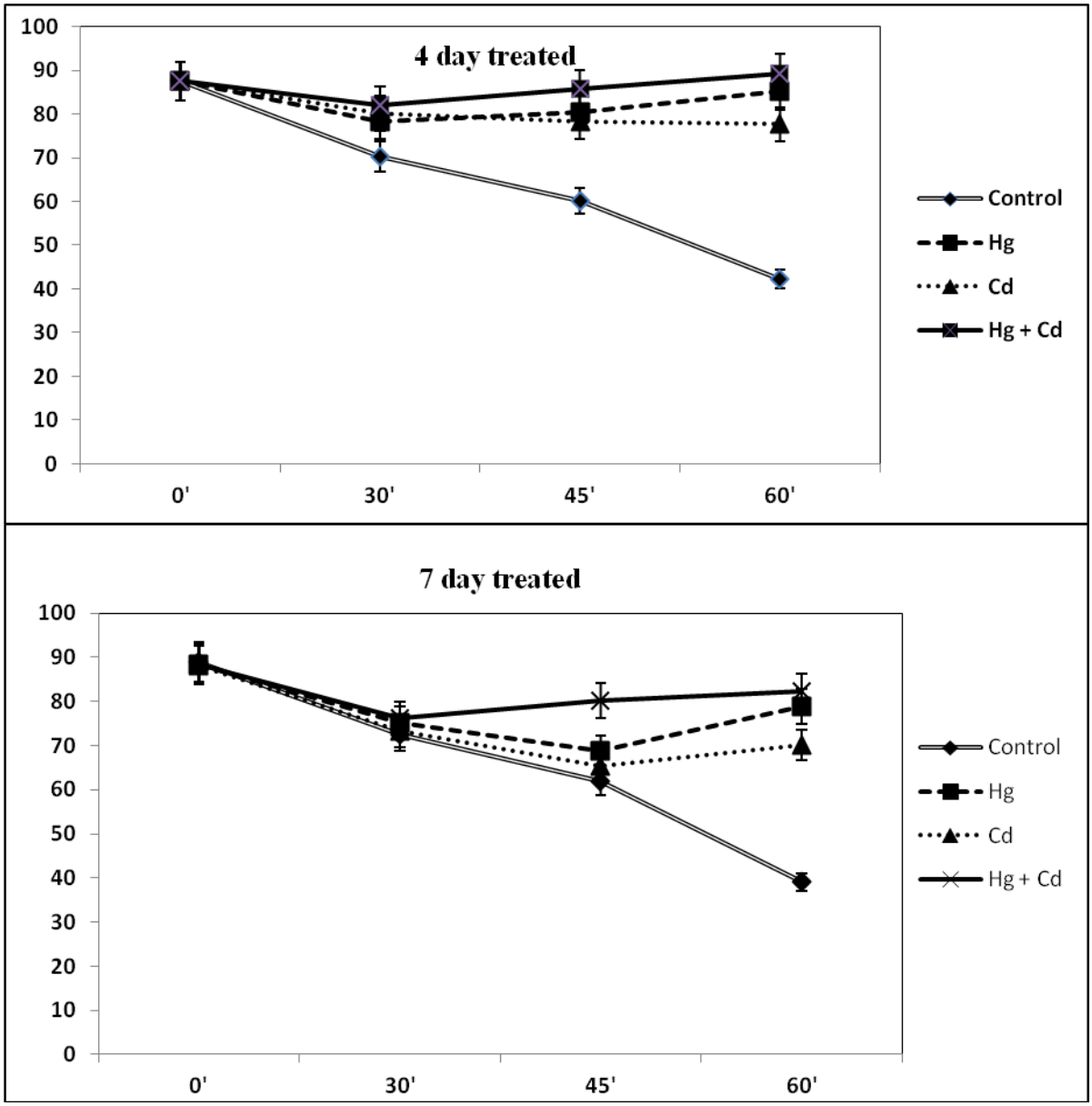


Fig. 5.41: Intracellular killing activity in macrophages of fish treated simultaneously with mercury and cadmium. 4 day- Hg ($P<0.05$); Cd ($P<0.0057$ day- Hg ($P<0.01$); Cd($P<0.025$).

5.2.3.3. Effect of multi- metal exposure on cell adhesion of fish macrophages

There was a significant decrease in cell adhesion in multi-metal treated group at 60 min as compared to the control group.

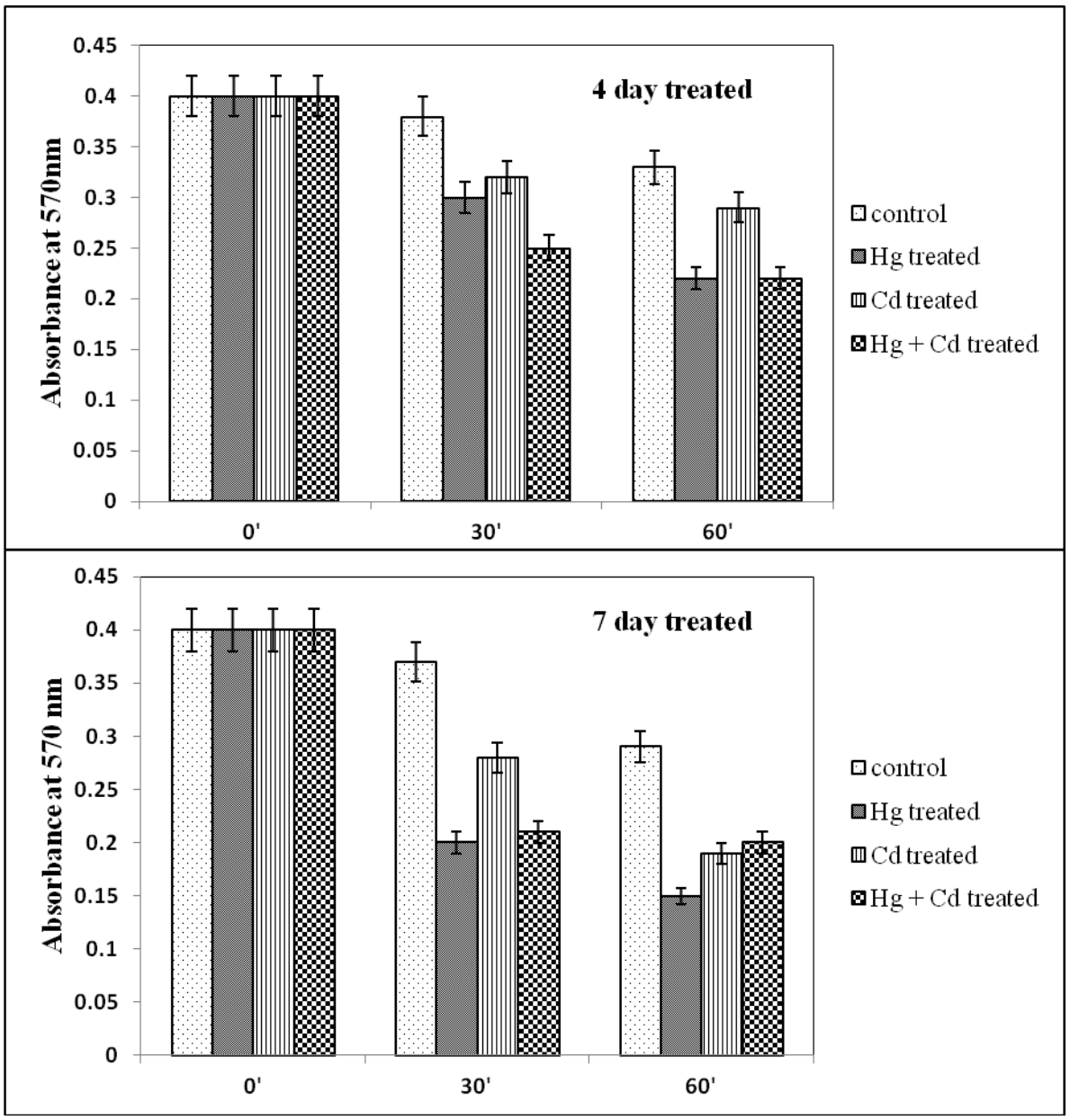


Fig. 5.42: Cell adhesion in macrophages of fish treated simultaneously with mercury and cadmium. 4 day- Hg ($P<0.025$); Cd ($P<0.004$) 7 day- Hg ($P<0.01$); Cd ($P<0.025$).

5.2.3.4. Effect of multi-metal exposure on nitric oxide (NO) release of fish macrophages

There was a significant decrease in nitric oxide release from 52.48±2.42 μM (4 day), 52.94±1.96 μM (7 day) in control group to 16.60 ±2.61 μM (4 day) and 10.65-±1.35 μM (7 day) in multi-metal treated group.

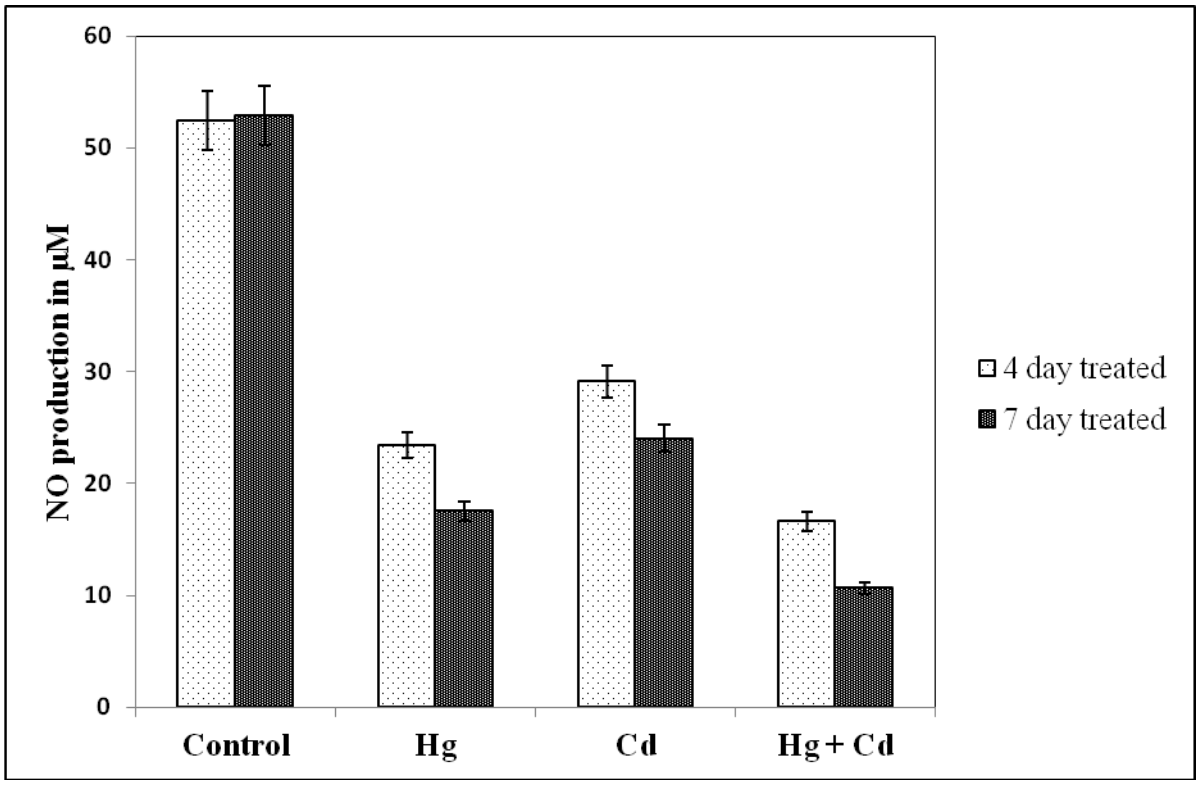


Fig. 5.43: Nitric oxide release in macrophages of fish treated simultaneously with mercury and cadmium. Hg (4 day) P<0.02; Cd (7 day) P< 0.01; Hg, Cd (7 day) P< 0.05.

5.2.3.5. Effect of multi-metal exposure on myeloperoxidase (MPO) release of fish macrophages

There was a significant decrease in myeloperoxidase release from 42.85±0.36 (4 day), 43.26±0.36 (7 day) in control group to 17.83±1.60 (4 day), 12.02±1.76 (7 day) in multi-metal treated group.

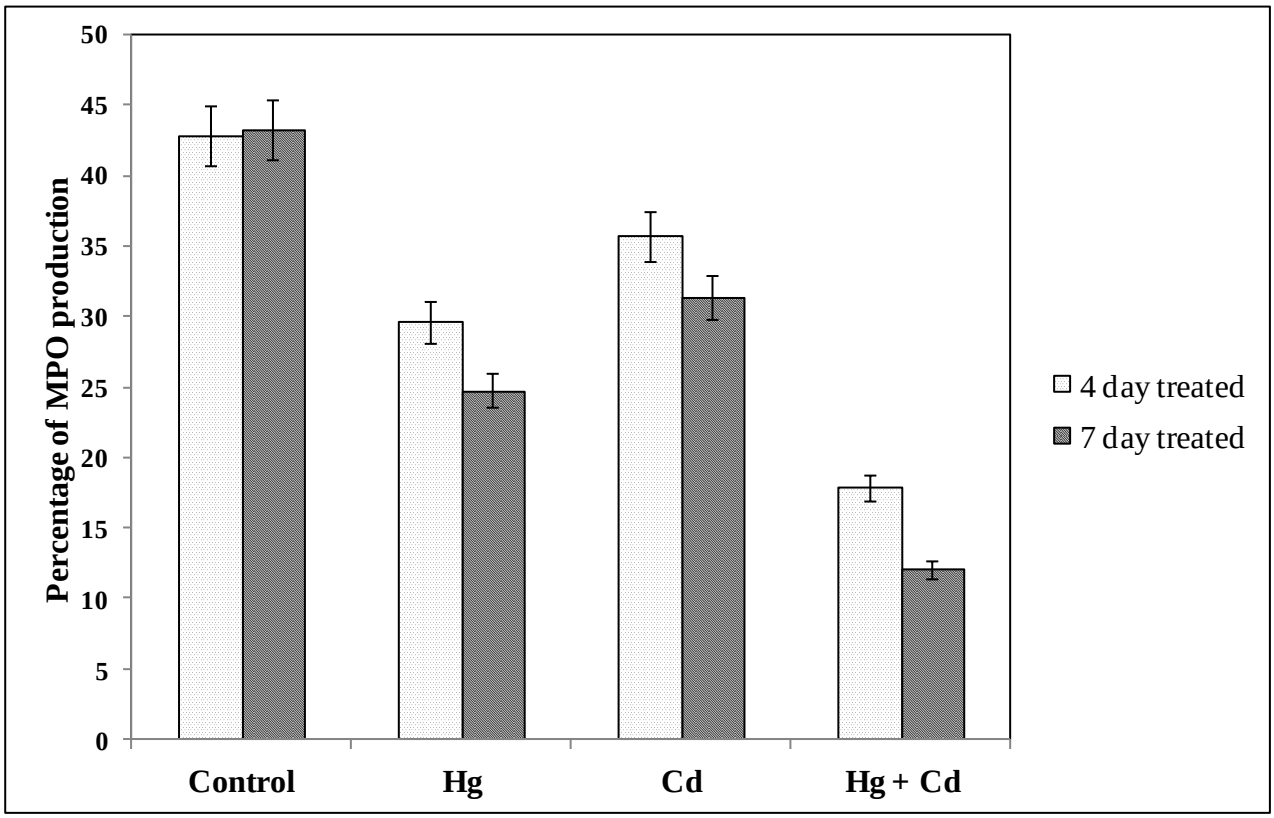


Fig. 5.44: Myeloperoxidase release in macrophages of fish treated simultaneously with mercury and cadmium. Hg, Cd (4 day) P<0.02; Hg (7 day) P< 0.05; Cd (7 day) P< 0.04.

5.2.3.6. Effect of multi- metal exposure on TNF-αrelease of fish macrophages

There was a significant increase in TNF-α release in multi-metal treated group both from cell supernatant and serum as compared to the control group.

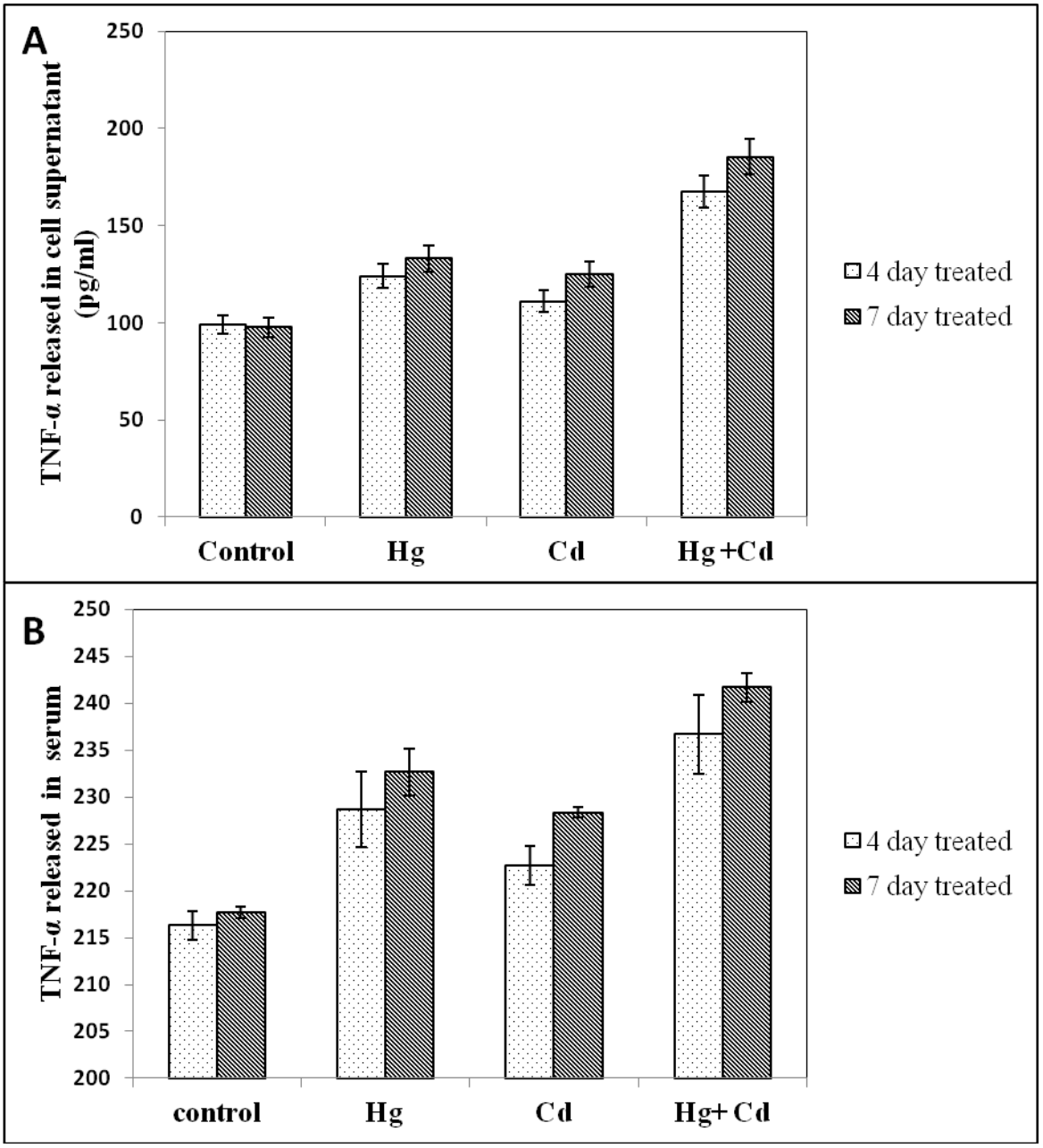


Fig 5.45(A): TNF-α released from cell supernatant of multi- metal treated group. (B) TNF-α released from serum of multi- metal treated group. Hg (4 day) P<0.02; Hg, Cd (7 day) P< 0.05; Cd (4 day) P< 0.01.

5.2.3.7. Effect of multi- metal exposure on IL- 6 release of fish macrophages

There was a significant increase in IL-6 release from 0.0423±0.005 (4 day), 0.0473±0.002 (7 day) in control group to 0.069±0.002 (4 day), 0.078±0.003 (7 day) in multi-metal treated group.

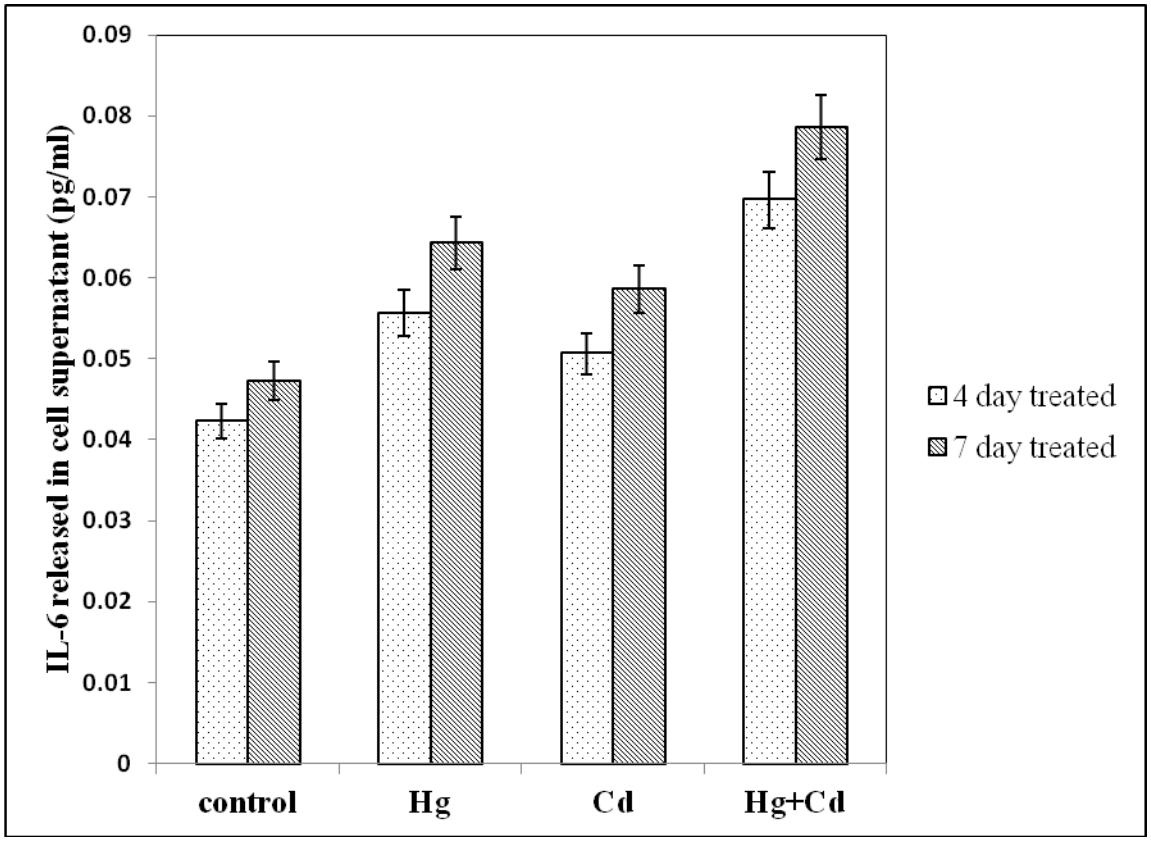


Fig 5.46: IL-6 released from plasma of multi- metal treated group. Hg (4 day) P<0.02; Hg, Cd (7 day) P< 0.05; Cd (4 day) P< 0.01.

5.3. Statistical analysis (one way ANOVA and isobologram)

5.3.1. One way ANOVA

Table 5.3: Dose levels of Hg (mg/L) and NO (μM) release after the exposure of Hg for 7 days

Dose Level	1.2	0.6	0.3	0.15	0.075	**No Dose**
Obs	**D1**	**D2**	**D3**	**D4**	**D5**	**Control**
1	54	48	18	38	48	53
2	55	49	18	35	49	53
3	57	50	17	39	48	52
4	55	53	17	36	46	53
Mean NO	55.25	50	17.5975	37	47.75	52.775

Table 5.4: Dose levels of Cd (mg/L) and NO (μM) release after the exposure of Cd for 7 days

Dose Level	7.84	3.92	1.96	0.98	0.49	**No Dose**
Obs	**D1**	**D2**	**D3**	**D4**	**D5**	**Control**
1	68	50	24	43	51	53
2	61	49	23	44	50	53
3	62	44	25	45	50	52
4	65	41	24	44	49	53
Mean NO	64	46	24	44	50	52.775

Table 5.5: ANOVA single factor for NO release of Hg without control as separate group

Null hypothesis: No effects due to treatments

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	4	221	55.25	1.583333
Column 2	4	200	50	4.666667
Column 3	4	70.39	17.5975	0.135892
Column 4	4	148	37	3.333333
Column 5	4	191	47.75	1.583333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3567.81	4	891.9526	394.58	5.36E-15	3.055568
Within Groups	33.90768	15	2.260512			
Total	3601.718	19				

Table 5.6: ANOVA single factor for NO release of Hg with control as separate group

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	4	221	55.25	1.583333
Column 2	4	200	50	4.666667
Column 3	4	70.39	17.5975	0.135892
Column 4	4	148	37	3.333333
Column 5	4	191	47.75	1.583333
Column 6	4	211.1	52.775	0.275567

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3990.098	5	798.0196	413.5486	6.47E-18	2.772853
Within Groups	34.73438	18	1.929688			
Total	4024.832	23				

Table 5.7: ANOVA single factor for NO release of Cd without control as separate group

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	4	256	64	10
Column 2	4	184	46	18
Column 3	4	96	24	0.666667
Column 4	4	176	44	0.666667
Column 5	4	200	50	0.666667

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3308.8	4	827.2	137.8667	1.23E-11	3.055568
Within Groups	90	15	6			
Total	3398.8	19				

Table 5.8: ANOVA single factor for NO release of Cd with control as separate group

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	4	256	64	10
Column 2	4	184	46	18
Column 3	4	96	24	0.666667
Column 4	4	176	44	0.666667
Column 5	4	200	50	0.666667
Column 6	4	211.1	52.775	0.275567

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3480.402	5	696.0804	137.9489	1.06E-13	2.772853
Within Groups	90.8267	18	5.045928			
Total	3571.229	23				

Table 5.9: Dose levels of Hg (mg/L) and TNF-α (pg/ml) release after the exposure of Hg for 7 days

Dose Level	1.2	0.6	0.3	0.15	0.075	**No Dose**
Obs	**D1**	**D2**	**D3**	**D4**	**D5**	**Control**
1	55	142	138	123	90.13	97
2	59	144	131	130	98.99	98
3	60.5	119	130	115	110	99
4	61.2	123	135	128	103	97
Mean TNF-α	58.925	132	133.5	124	100.53	97.75

Table 5.10: Dose levels of Cd (mg/L) and TNF-α (pg/ml) release after the exposure of Cd for 7 days

Dose Level	7.84	3.92	1.96	0.98	0.49	**No Dose**
Obs	**D1**	**D2**	**D3**	**D4**	**D5**	**Control**
1	83	138	125	110	92.13	97
2	72	133.2	122	109	89.22	98
3	61.5	130	128	123	85	99
4	85	131	126	117	98	97
Mean TNF-α	75.375	133.05	125.25	114.75	91.0875	97.75

Table 5.11: ANOVA single factor for TNF- α release of Hg without control as separate group

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	4	235.7	58.925	7.689167
Column 2	4	528	132	164.6667
Column 3	4	534	133.5	13.66667
Column 4	4	496	124	44.66667
Column 5	4	402.12	100.53	68.77113

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	15721.47	4	3930.368	65.62419	2.54E-09	3.055568
Within Groups	898.3809	15	59.89206			
Total	16619.85	19				

Table 5.12: ANOVA single factor for TNF- α release of Hg with control as separate group

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	4	235.7	58.925	7.689167
Column 2	4	528	132	164.6667
Column 3	4	534	133.5	13.66667
Column 4	4	496	124	44.66667
Column 5	4	402.12	100.53	68.77113
Column 6	4	391	97.75	0.916667

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	16204.76	5	3240.952	64.73769	7.17E-11	2.772853
Within Groups	901.1309	18	50.06283			
Total	17105.89	23				

Table 5.13: ANOVA single factor for TNF- α release of Cd without control as separate group

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	4	301.5	75.375	118.2292
Column 2	4	532.2	133.05	12.67667
Column 3	4	501	125.25	6.25
Column 4	4	459	114.75	42.91667
Column 5	4	364.35	91.0875	29.80489

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	9284.013	4	2321.003	55.29427	8.43E-09	3.055568
Within Groups	629.6322	15	41.97548			
Total	9913.645	19				

Table 5.14: ANOVA single factor for TNF- α release of Cd with control as separate group

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	4	301.5	75.375	118.2292
Column 2	4	532.2	133.05	12.67667
Column 3	4	501	125.25	6.25
Column 4	4	459	114.75	42.91667
Column 5	4	364.35	91.0875	29.80489
Column 6	4	391	97.75	0.916667

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	9627.591	5	1925.518	54.80756	2.91E-10	2.772853
Within Groups	632.3822	18	35.13234			
Total	10259.97	23				

Inference: The null hypothesis of no effects due to treatment is rejected at 0.001 percent level. Thus, treatments are significant.

5.3.2. **Isobologram: A mathematical model to study interactivity**

Based on the nature of the experimental data, a linear functional form ($Z = \alpha + \beta X$) was chosen to represent the pure and interactive effects of Hg and Cd on TNF-α release.

For pure effects, $Z_1 = \alpha_1 + \beta_1.X_1$ and $Z_2 = \alpha_2 + \beta_2.X_2$ were estimated for Hg and Cd respectively and for the interactive effects, $Z_3 = \alpha_3 + \beta_3.(X_1.X_2)$ was estimated. All three are linear regressions run on the basis of experimental data.

Final total effect,

$Z = Z_1 + Z_2 + Z_3$, which is the sum of pure and interactive effects.

Setting Z = 400 pg/ml, we get the isobologram for TNF-α. This is shown in the following figure. The nature of the curve is suggestive of synergy.

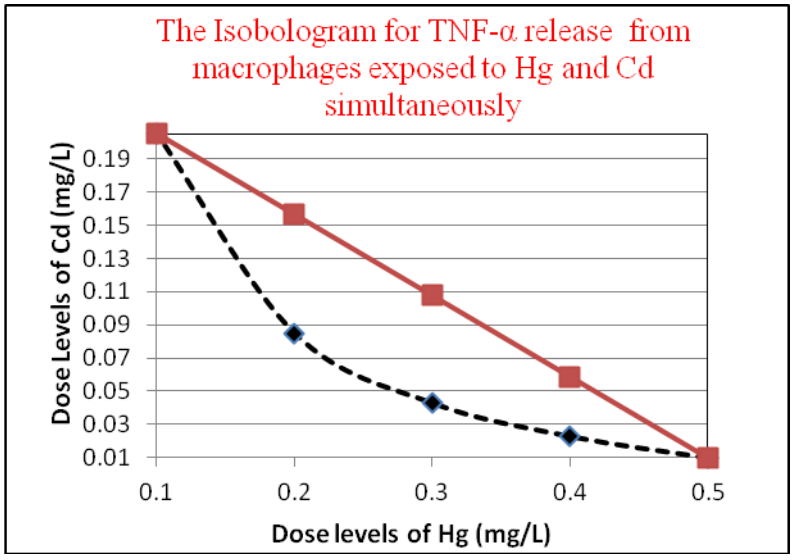


Fig. 5.47: Isobologram for TNF-α release

5.4. Relative gene expression quantification of tumor necrosis factor (TNF-α) and interleukin-6 (IL-6) gene using 18S gene as endogenous control in the intestine of *Channa punctatus* after the simultaneous exposure of mercury and cadmium for 7 days.

5.4.1. Quantitative and Qualitative analysis of total RNA

Table 5.15: Nanodrop reading of total RNA

Sl. No.	**Sample**	**Conc.(ng / µl)**	**$OD_{A260-280}$**
1	S1 (Control)	**415.6**	**2.03**
2	S2 (Hg treated)	**562.3**	**1.98**
3	S3 (Cd treated)	**623.4**	**2.01**
4	S4 (Hg + Cd treated)	**510.5**	**2.02**

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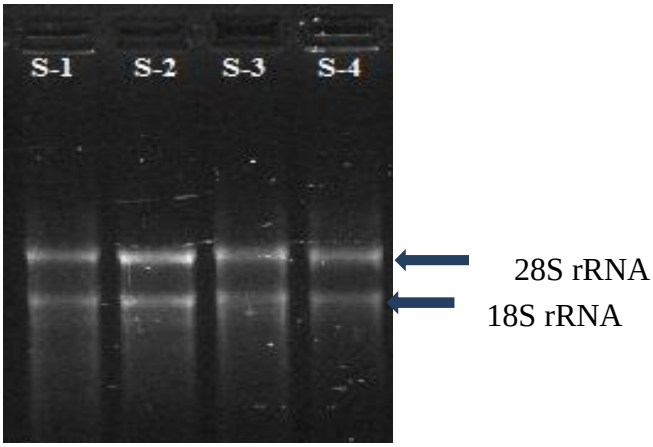

Fig. 5.48: 1 % denaturing agarose gel electrophoresis of total RNA of all 4 samples

Table 5.16: Sequences of primers used in gene expression analysis and accession numbers. (Teles *et al*., 2011).

Gene	Forward Primer	Reverse Primer	Accession number
TNF-α	CGCTGACACAGTGCAGTGGA	TCCCCGATGGAGTCCGAATA	AJ401677
IL-6	TTTCAGAAGCCCGTGGAAGAGA	TCTTTGACCAGCCCTATCAGCA	DQ866150
18S	CGAGCAATAACAGGTCTGTG	GGGCAGGGACTTAATCAA	AF243428.2

5.4.2. Effect of mercury and cadmium on TNF-α, IL-6 and 18S (as reference) expression after 7 days of exposure

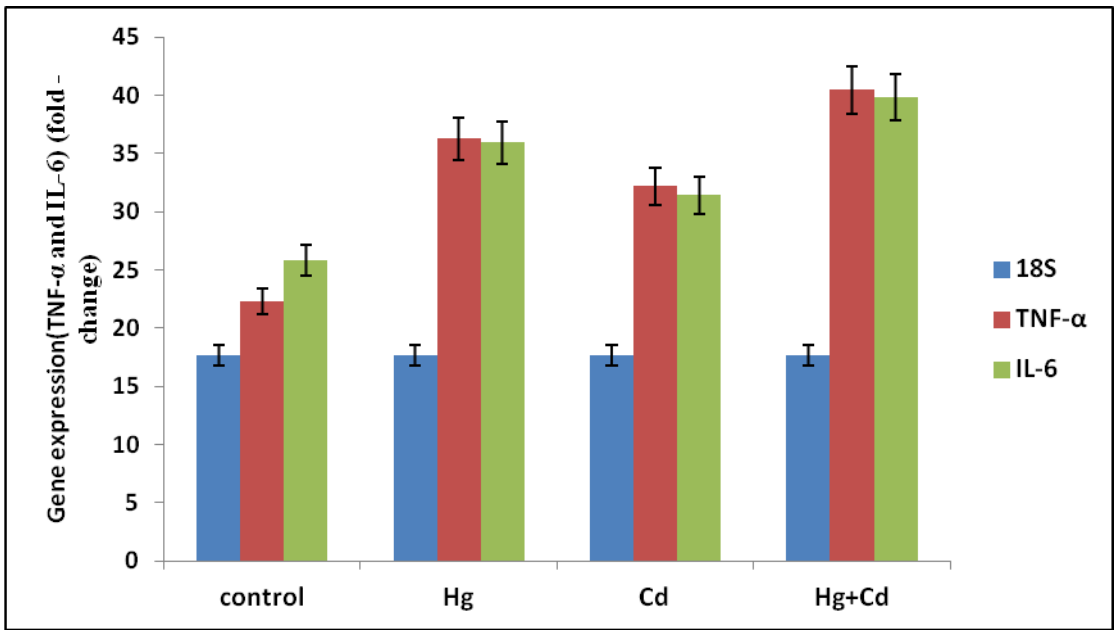

Fig. 5.49: TNF-α, IL-6 and 18S (as reference) expression after the 7 days exposure of mercury and cadmium.

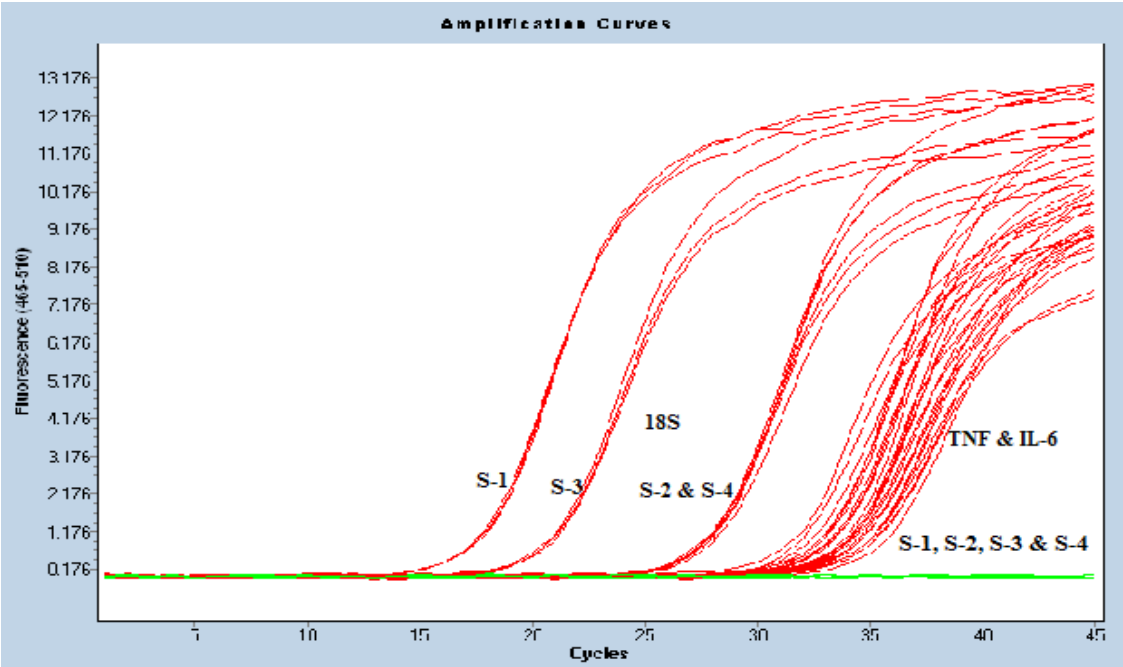

Fig. 5.50: Amplification curves of TNF-α, IL-6 mRNA (targets) and 18S mRNA (reference). Figure shows fluorescence of TNF-α, IL-6 (targets) and 18S (reference) mRNA amplification in S1- untreated group, which served as control, S2- Hg treated, S3- Cd treated and S4- Hg+ Cd treated in intestinal tissues of *C. punctata* after exposure to 0.3 mg/L ($^{96}LD_{50}/6$) Hg and 1.96 mg/L ($^{96}LD_{50}/6$) mg/L Cd for 7 days.

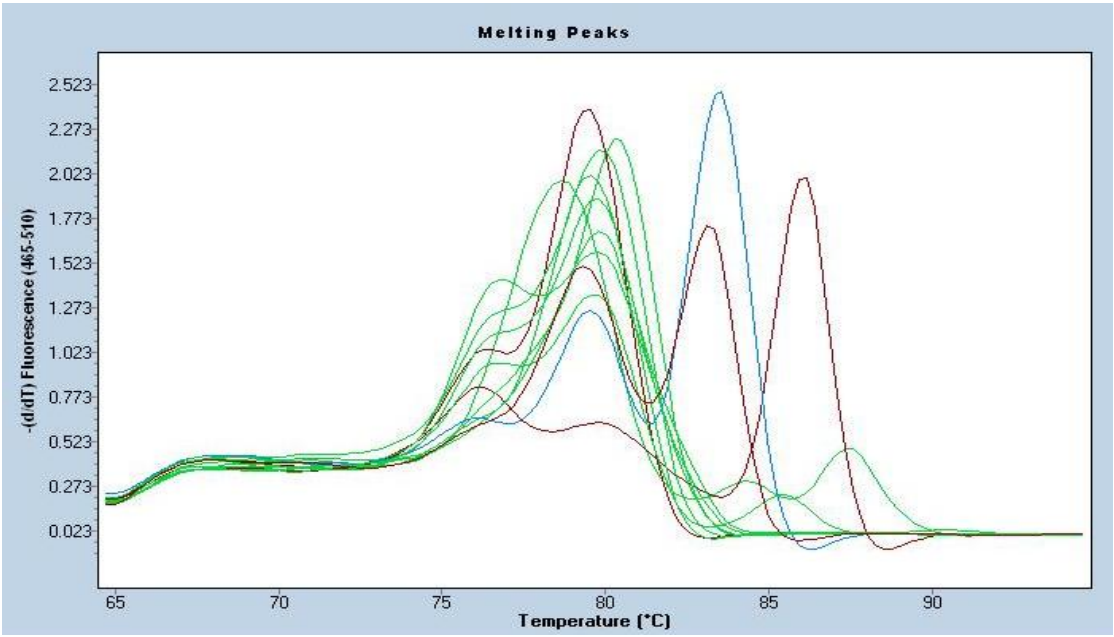

Fig. 5.51: Melt Curve of TNF-α (Target)

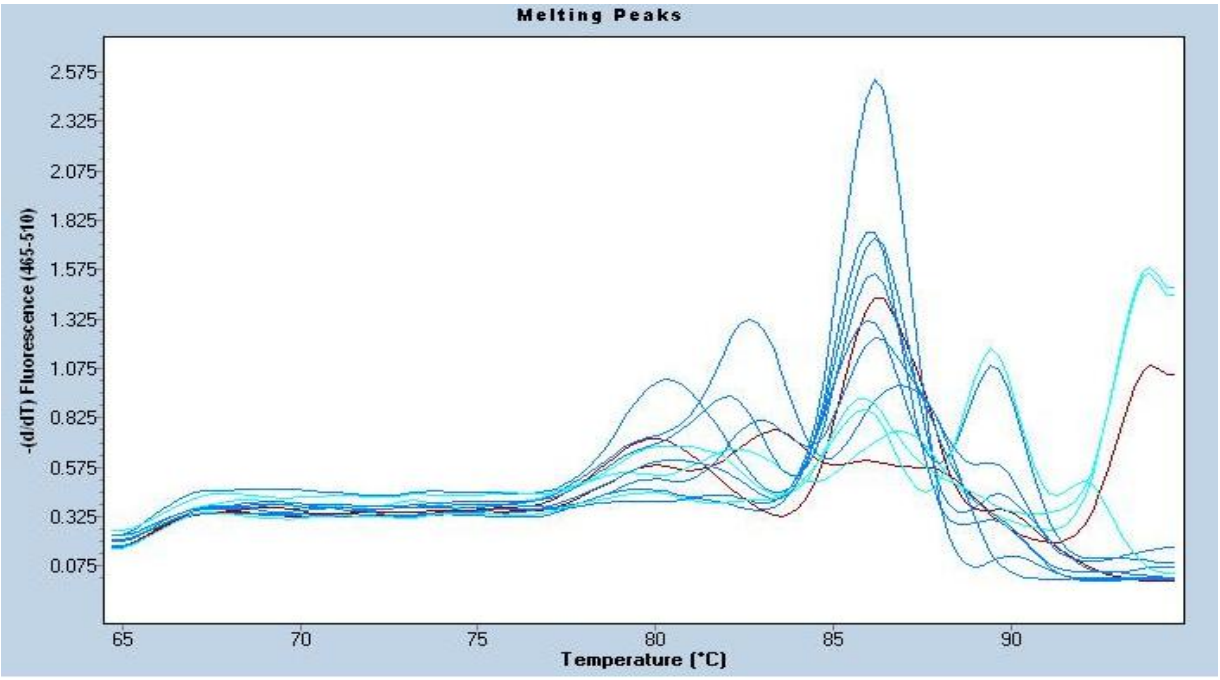

Fig. 5.52: Melt Curve of IL-6 (Target)

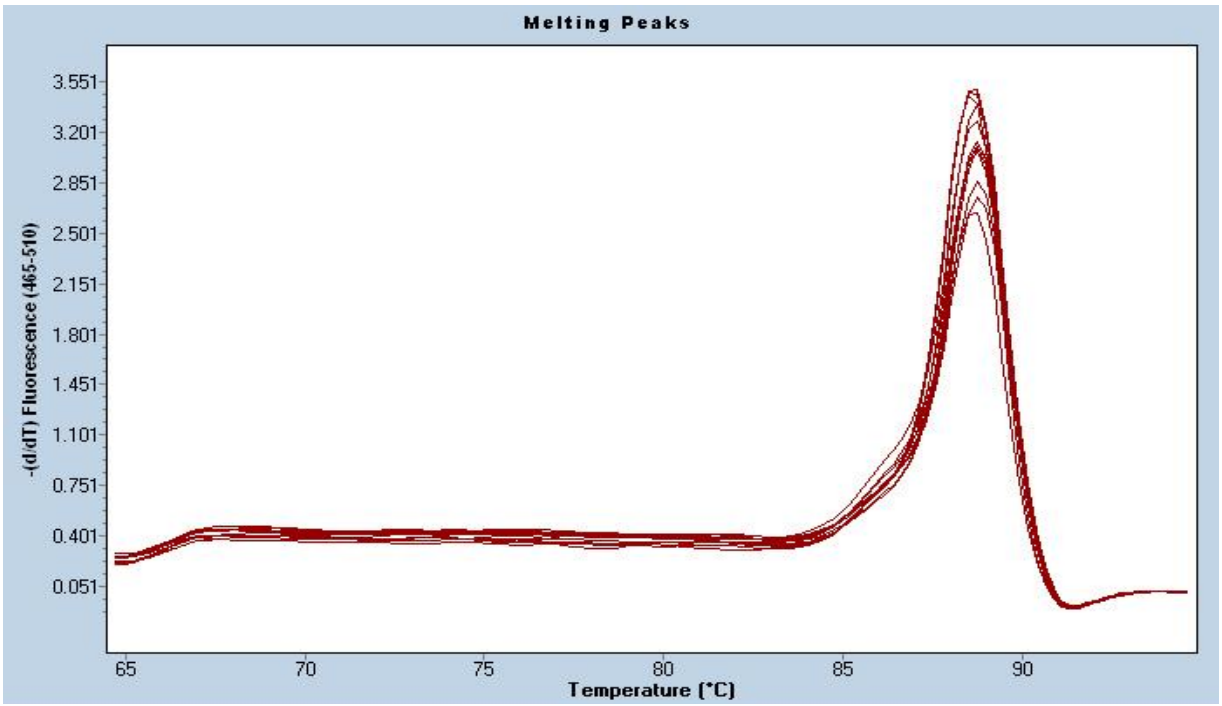

Fig. 5.53: Melt Curve of 18S (Reference)