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An *in vitro* study of metal ion-induced lipid peroxidation in giant fresh water prawn *Macrobrachium rosenbergii* (de MAN)

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Though metal ions are essential components of many cellular functions, their overexposure to organisms lead to oxidative stress through the formation of reactive oxygen species (ROS). Lipid peroxidation (LPX) is the oxidative deterioration of membrane lipids and considered as an index of oxidative stress. In the present study *in vitro* effect of various metals ($FeCl_3$, $FeSO_4$, $CuSO_4$, $CdCl_2$, and $ZnSO_4$) on the lipid peroxidation of gills and hepatopancreas of Giant Freshwater prawn, *Macrobrachium rosenbergii*, was compared with respect to dose and duration. The results clearly indicate that among all the metals investigated, $FeCl_3$ and $CdCl_2$ are more potent in inducing LPX, and $FeCl_3$ is more toxic than $FeSO_4$ in inducing LPX in the hepatopancreas. $ZnSO_4$ exhibits a moderate toxicity while $CuSO_4$ is least toxic and also inhibits LPX at higher concentration. Thus results of the present investigation suggest that all the metal ions investigated in the present study are capable of inducing oxidative stress in gills and hepatopancreas of *M. rosenbergii*.

Keywords: oxidative stress, lipid peroxidation (LPX), metal ions, freshwater prawn (*Macrobrachium rosenbergii*).

Introduction

Reactive oxygen species (ROS) such as superoxide radical ($\dot{O}_2$) hydroxyl radical ($\dot{O}H$), hydrogen peroxide (H_2O_2) etc. are continuously generated in the organisms during normal cellular functions (Halliwell & Gutteridge 1985). Under normal physiological state ROS are effectively disactivated by the antioxidant enzymes and small antioxidant molecules present in the cells (Winston & Di Giulio 1991, Gille & Sigler 1995). If ROS are not neutralised efficiently, they attack almost all macromolecules of cells including proteins and DNA, but membrane lipids are more susceptible to their attack triggering lipid peroxidation (Elstner 1991). Polyunsaturated fatty acids of cell membranes are of special importance because they maintain membrane fluidity and secure biological functions. Lipid peroxidation (LPX) leads to deterioration of membrane structure and integrity, and therefore, LPX is considered as an index of tissue oxidative stress (Kappus & Sies 1981).

It is now established that toxicity caused to aquatic organisms, by heavy metals, particularly the transitional group is partly due to excess generation of ROS (Thomas & Wofford 1993, Doyotte *et al*. 1997). Further it is suggested that the health of aquatic organisms is also linked to the overproduction of ROS in their tissues (Di Giulio *et al*. 1989). The aquatic environment serves as a major sink for various metal ions which are the byproduct of different industrial activities and their concentration is increasing constantly (Waldichuk 1974). Since heavy metals are stable and nonbiodegradable they accumulate in the tissue of aquatic organisms and cause physiological disorder.

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Although several studies have demonstrated the effect of xenobiotics including heavy metals on the antioxidant system and LPX in several marine species (Livingstone *et al.* 1990, Lemaire & Livingstone 1993, Doyotte *et al.* 1997) our knowledge of freshwater invertebrates, particularly crustacea, is relatively scanty. The present investigation, therefore, is designed to compare the kinetics of LPX in gills and hepatopancreas of a commercially important freshwater prawn *Macrobrachium rosenbergii* (crustacea, decapoda), in response to some metal salts such as $FeSO_4$, $FeCl_3$, $CdCl_2$, $ZnSO_4$ and $CuSO_4$, under *in vitro* condition.

Two important organs, gills and hepatopancreas are chosen for the present study because gills are the respiratory organs and are directly exposed to the aquatic environment while hepatopancreas serves as the main digestive gland and lipid storage organ in crustacea (Muriana *et al.* 1993).

Materials and methods

Animals

Adult males of *M. rosenbergii* (total wt. 37.28 ± 2.68 g., total length 146.60 ± 3.78 mm, carapace length 76.60 ± 1.94 mm) were collected from the culture ponds of the freshwater prawn complex located at Central Institute of Freshwater Aquaculture, Kausalyaganga, Bhubaneswar. The prawns were acclimatised to laboratory condition for a period of two weeks in 100 litre capacity of fiber glass reinforced plastic tanks, (FRP tanks) under constant aeration prior to experimentation. Various hydrobiological parameters of acclimatised water were as follows: dissolved oxygen 5.00 ± 0.5 ppm; NH_3-N 0.2 ± 0.05 ppm; pH 7.8 ± 0.2 and temperature 28.5 ± 0.4°C. During acclimatisation water was changed daily and prawns were fed *ad libitum* with a standard pelletised diet (30–35% protein content). In the present study prawns only in the intermoult stage were used.

Chemicals

All chemicals were of analar grade. Thiobarbituric acid (TBA) and bovine serum albumin (BSA) were obtained from Sigma Chemical Co., USA.

Tissue preparation and experimental protocol

The prawns were sacrificed by decapitation, gills and hepatopancreas were rapidly removed with proper care. They were washed thoroughly with ice cold normal saline solutions, wiped dry using blotting paper and weighed with the help of an electrical balance and processed immediately for biochemical analyses. Tissues were minced and 10% homogenates of the tissues were prepared in 1.15% ice-cold KC1 solution with the help of a motor driven glass Teflon homogeniser in ice. Crude homogenates of tissue (150 μl containing approximately 1 mg. protein) samples were incubated with various concentrations of freshly prepared salt solutions (50 μl) for 30 min at 37°C. After the end of incubation time, LPX of the samples were estimated as described below. To study the time kinetics, tissue homogenate samples were incubated with salt solutions for various time intervals at 37°C and LPX were estimated.

Lipid peroxidation assay

Lipid peroxidation of the tissue samples was assayed according to the method of Ohkawa *et al.* (1979) by monitoring the formation of thiobarbituric acid reactive substances (TBA-RS). In brief, the reaction mixture containing approximately 1 mg of protein, 0.3 ml of 8.1% sodium dodecyl sulphate (SDS), 1.5 ml of acetic acid (pH 3.5), 1.5 ml of 0.8% aqueous solution of TBA and 0.6 ml of double distilled water was heated at 95°C for 60 min, then cooled to room temperature and centrifuged at 4000 rpm for 10 min. The absorbance of the supernatant was read at 532 nm in a UV-VIS spectrophotometer. The amount of TBA-RS formed was calculated by using an extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ (Wills 1969), and expressed as nmol malondialdehyde (MDA) formed per mg protein. Protein content of the samples were measured according to the method of Lowry *et al.* (1951) using BSA as standard.

Statistical analysis

Results are presented as means ± standard error of means (SEM). Difference among the means were analysed by one way analysis of variance (ANOVA) followed by Duncans new multiple range test. Differences were considered statistically significant when $P < 0.05$.

Results

In Figure 1, endogenous LPX level in crude homogenates of gills and hepatopancreas of *M. rosenbergii* was compared at 0 and 30 min after incubation at 37°C. Results revealed that basal LPX value in hepatopancreas was about 8 times more than that of gill and incubation for 30 min at 37°C did not produce any significant effect on basal LPX values of the tissues.

$FeSO_4$ induced LPX in gills and hepatopancreas of *M. rosenbergii* with and without ascorbic acid (ASA) was depicted in Figure 2. In case of gills, incubation of homogenate with 1000 μM $FeSO_4$ resulted in 44% elevation in LPX value which remained same when concentration of $FeSO_4$ raised to 2000 μM. Incubation of gill homogenate with $FeSO_4$ and ascorbic acid did not induce LPX further (Figure 2A). On

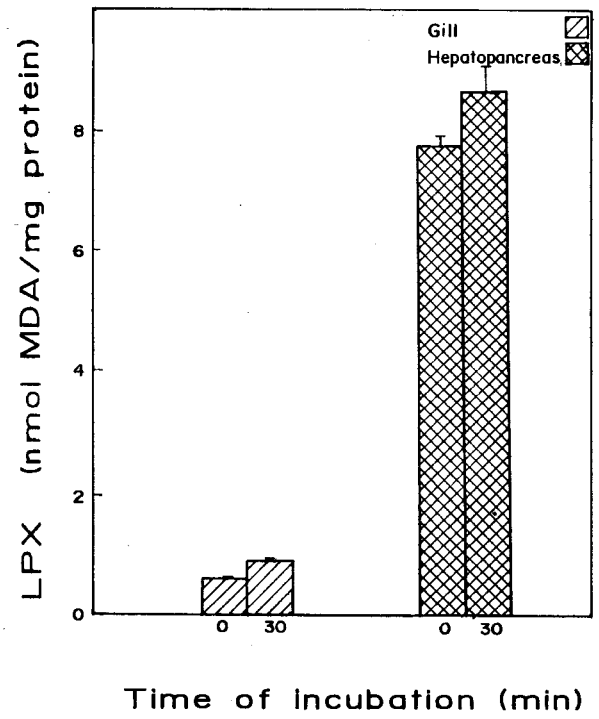

Figure 1. Comparison of endogenous levels of lipid peroxidation (LPX) in crude homogenates of gill and hepatopancreas of prawn *M. rosenbergii* at 0 and 30 min of post-incubation at 37°C. Data are mean ± standard error of mean (SEM) of 5 observations.

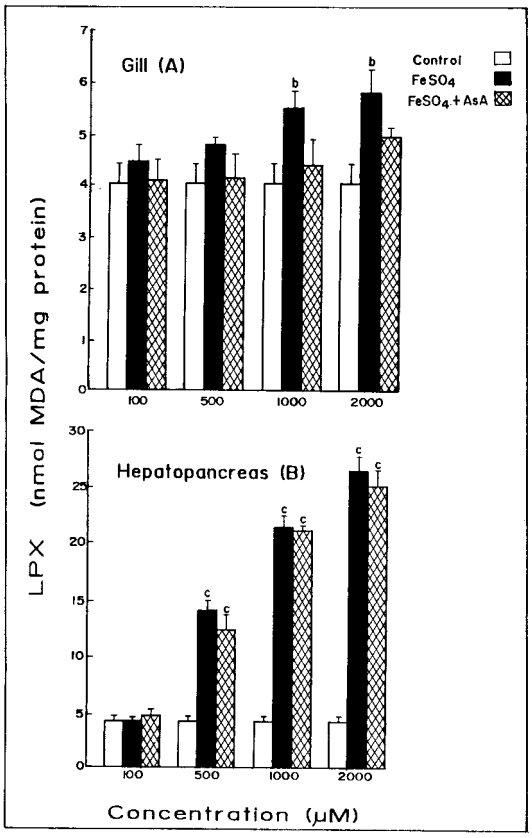

Figure 2. Effect of different concentrations of $FeSO_4$ with and without ascorbic acid (ASA, 500 μM) on lipid peroxidation (LPX) in gill(A) and hepatopancreas(B) of *M. rosenbergii* after 30 min. of incubation at 37°C. Data are mean ± SEM (n = 5), superscripts indicate significant differences ($^b p<0.01$, $^c p<0.001$) from the respective controls as determined by ANOVA.

the other hand, incubation of crude homogenates of hepatopancreas with 500, 1000 and 2000 μM, $FeSO_4$ resulted in 237%, 412% and 529% elevation, respectively. The magnitude of elevation was more or less same when homogenate was incubated along with $FeSO_4$ and ascorbic acid (Figure 2B).

Effect of incubation of various concentrations of $FeCl_3$, $CuSO_4$, $CdCl_2$ and $ZnSO_4$ on LPX values of gills was presented in Figure 3. Except $CuSO_4$, LPX value of the tissue homogenate increased in a dose-dependent manner from 100 to 2000 μM. A 323%, 537% and 259% increase in LPX value was recorded in response to 2000 μM of $FeCl_3$, $CdCl_2$ and $ZnSO_4$, respectively (Figure 3A, 3C, 3D). In the case of $CuSO_4$, maximum induction in LPX value was recorded for 500 μM which decreased in a dose-dependent manner when concentration of salt was increased (Figure 3B).

In Figure 4 the effect of incubation of various concentrations of $FeCl_3$, $CuSO_4$, $CdCl_2$ and $ZnSO_4$ on LPX values of hepatopancreas was presented. 408% and 569% increase in LPX value of hepatopancreas homogenate were recorded in response to 1000 and 2000 μM of $FeCl_3$, respectively (Figure 4A). In the case of $CuSO_4$, no significant increase in LPX value was noticed till 500 μM concentration but subsequent increase in the concentration of the salt resulted in reduction in LPX value (Figure 4B). A 27% increase in LPX value was noticed in response to 500 μM $CdCl_2$ which remained more or less the same when the concentration was increased to 2000 μM (Figure 4C). Similar results were observed in the case of $ZnSO_4$ (Figure 4D).

The time kinetics of LPX induction in homogenates of gills and hepatopancreas are presented in Figure 5 and Figure 6, respectively. Although maximum induction was observed after 45 min incubation of gill homogenates with $FeCl_3$ (2000 μM; Figure 5A) and $CuSO_4$ (500 μM; Figure 5B), respectively, in the case of $CdCl_2$ (2000 μM; Figure 5C) and $ZnSO_4$ (2000 μM; Figure 5D) maximum induc-

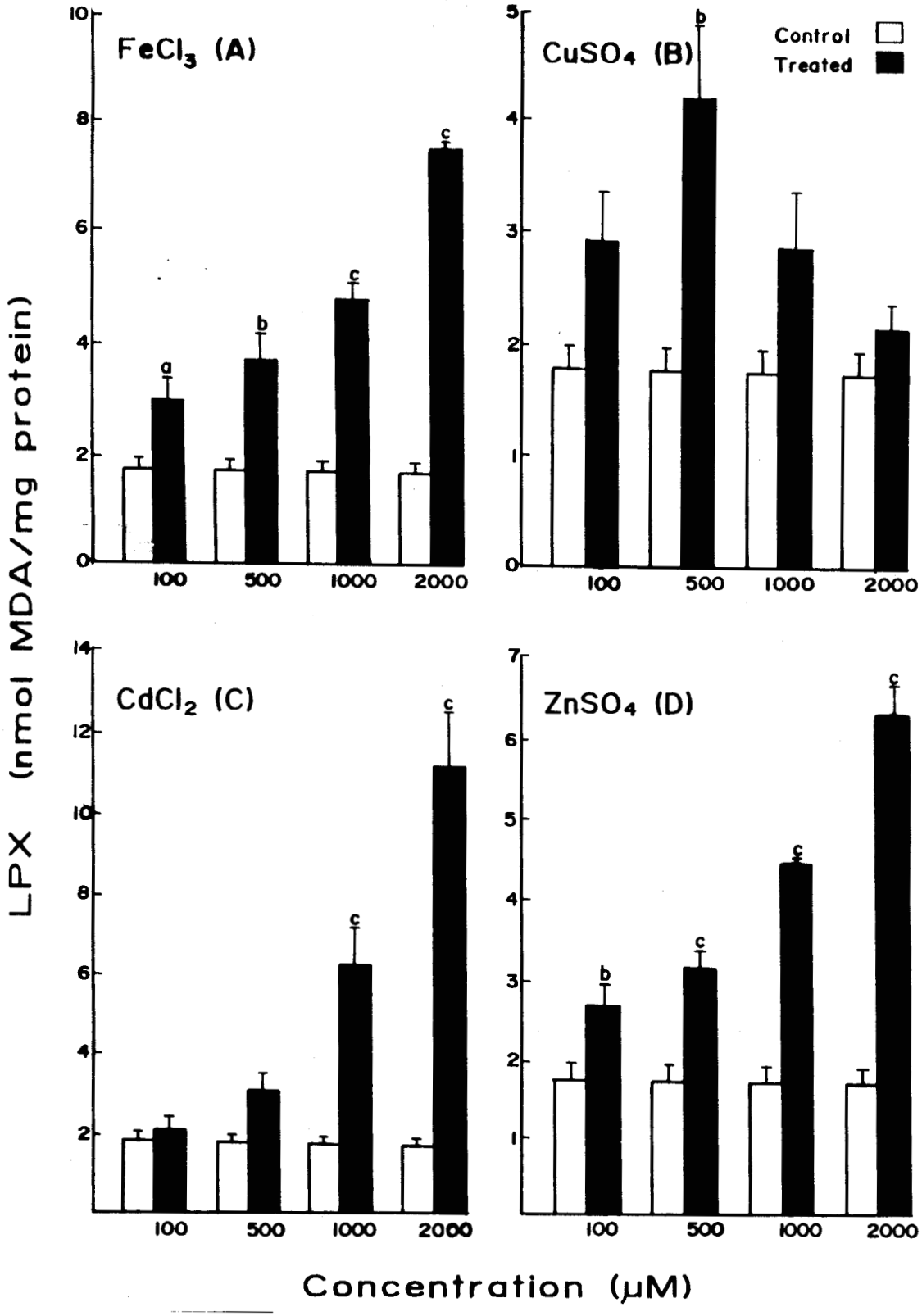

Figure 3. *In vitro* effect of different metals $FeCl_3$(A), $CuSO_4$(B), $CdCl_2$(C) and $ZnSO_4$(D) on lipid peroxidation (LPX) in crude homogenate of gill of *M. rosenbergii*. Data are means of ± SEM (n = 5) superscripts a, b & c indicate significant differences from respective controls ($^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$).

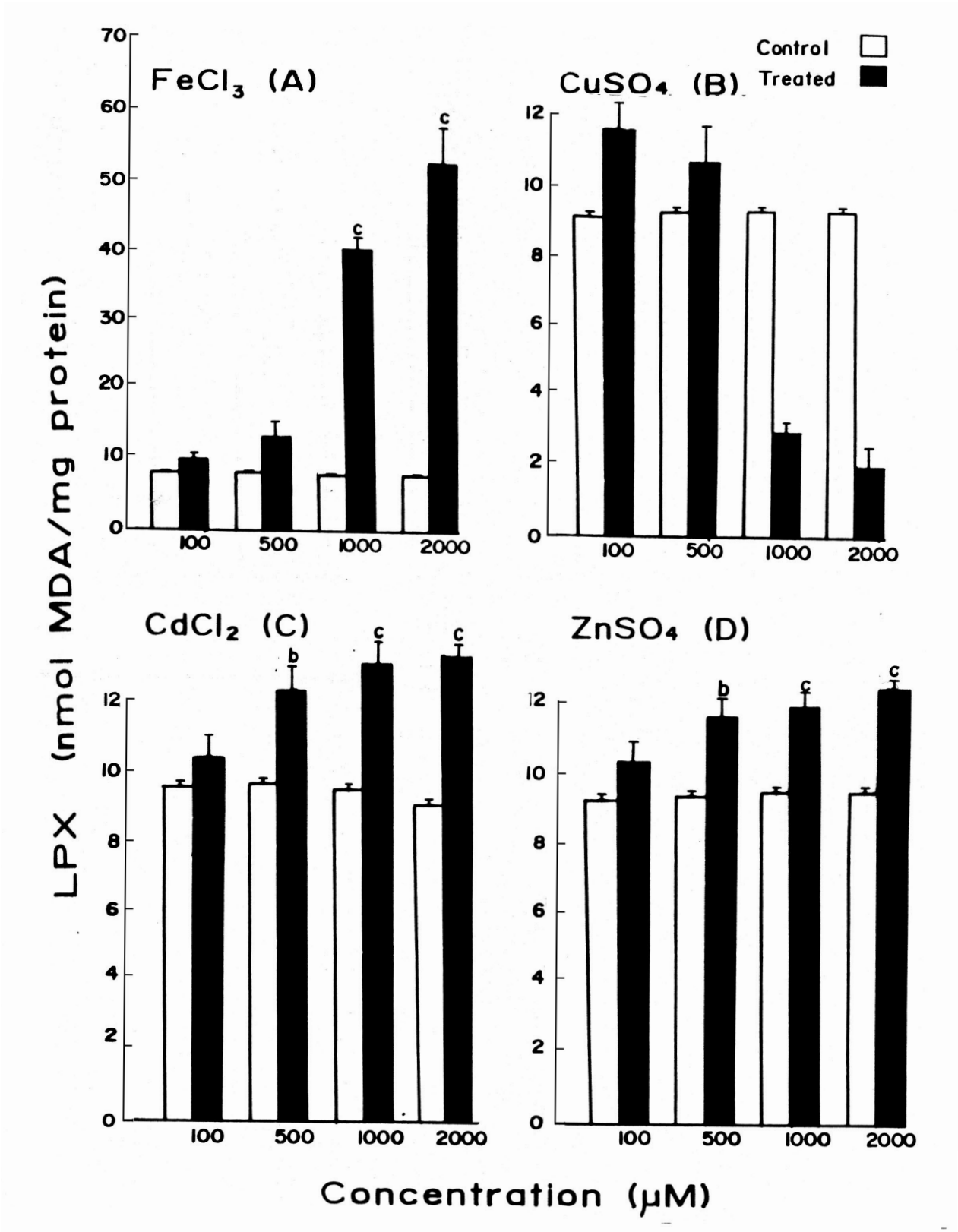

Figure 4. Effect of various metals $FeCl_3$(A), $CuSO_4$(B), $CdCl_2$(C), $ZnSO_4$(D) on lipid peroxidation (LPX) in crude homogenate of hepatopancreas of *M. rosenbergii*. Data are mean ± SEM (n = 5) superscripts a, b & c indicate significant differences from respective controls ($^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$).

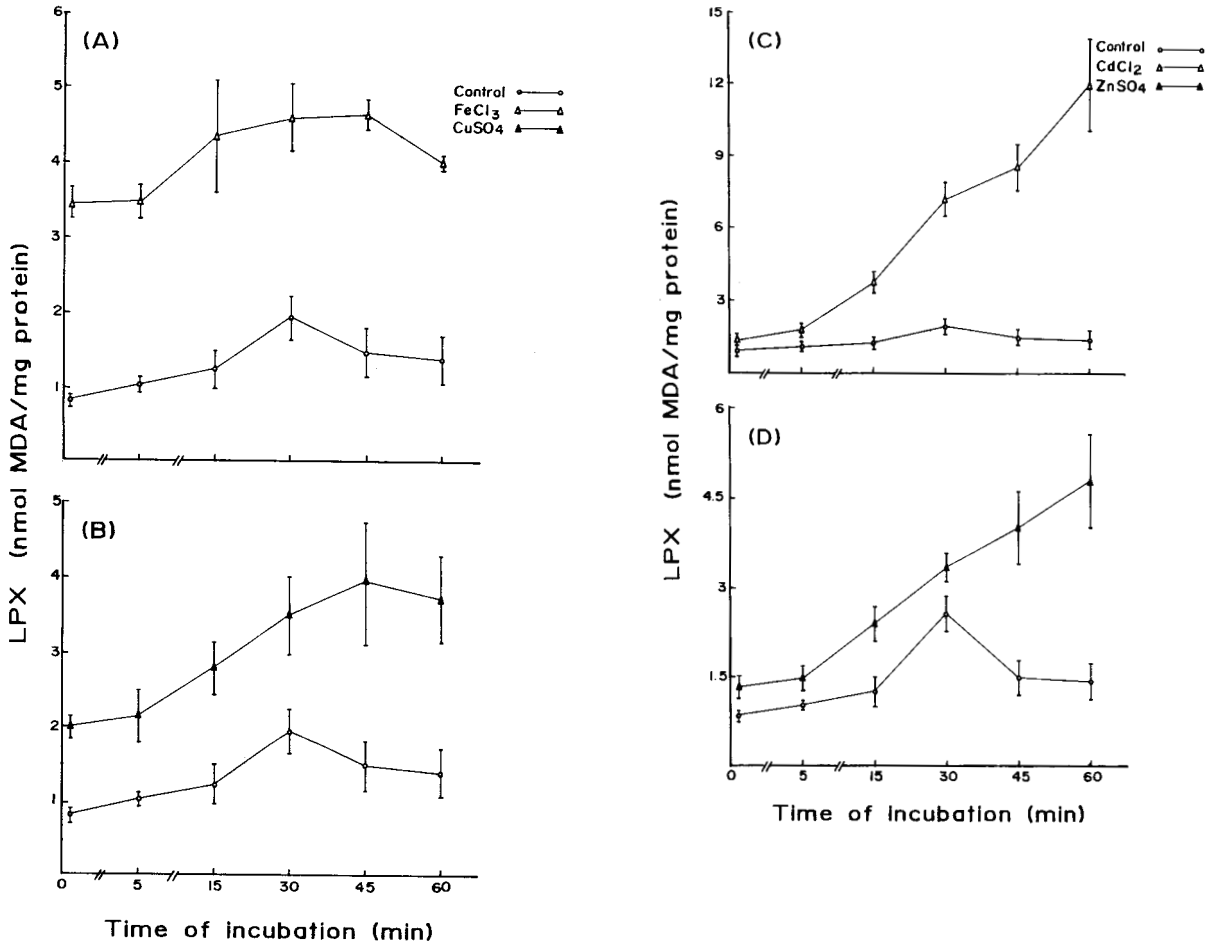

Figure 5. Changes in levels of lipid peroxidation in crude homogenate of gill of *M. rosenbergii* with different metal ions $FeCl_3$(A), $CuSO_4$(B), $CdCl_2$(C), $ZnSO_4$(D) with respect to time. Data are mean ± SEM (n = 4).

tion was recorded after 60 min of incubation. A similar time kinetics was recorded for hepatopancreas (Figure 6, A–D). However, incubation of homogenate with 500 μM $CuSO_4$ for 15 min resulted in 12% decrease in LPX value which remained more or less the same till 60 min of incubation (Figure 6B).

Discussion

The results of the present study clearly indicate that endogenous LPX level is higher in hepatopancreas in comparison to gills. Since LPX depends upon the biochemical composition of membranes, the observed differences in LPX values in hepatopancreas and gills may be due to qualitative and quantitative differences in their respective lipid profiles. It is reported that hepatopancreas of marine shrimp (*Paenius japonicus*) is rich in lipids, particularly in n-3 polyunsaturated fatty acids (PUFA) such as eicosapentaenoate (20:5) and docosahexaenoate (22:6) (Muriana *et al.* 1993). Liu *et al.* (1997) also opined that an abundance of PUFA increases the sensitivity of the tissue to peroxidative damage.

In the present study *in vitro* effects of four metal ions on lipid peroxidation of gill and hepatopancreas were examined. It is observed that both $FeSO_4$ and $FeCl_3$ are capable of inducing LPX in gills and hepatopancreatic tissues of prawn, however, $FeCl_3$ is more potent than $FeSO_4$. Earlier reports in this context indicated that ferrous iron is a more potent catalyst of LPX than ferric iron in mammalian tissues (Chavapil *et al.* 1974, Lee *et al.* 1981).

In comparison to gill, hepatopancreas is found to be more susceptible to iron in the case of *M. rosenbergii*. It is reported that in the biological system iron induces oxidative stress by facilitating the

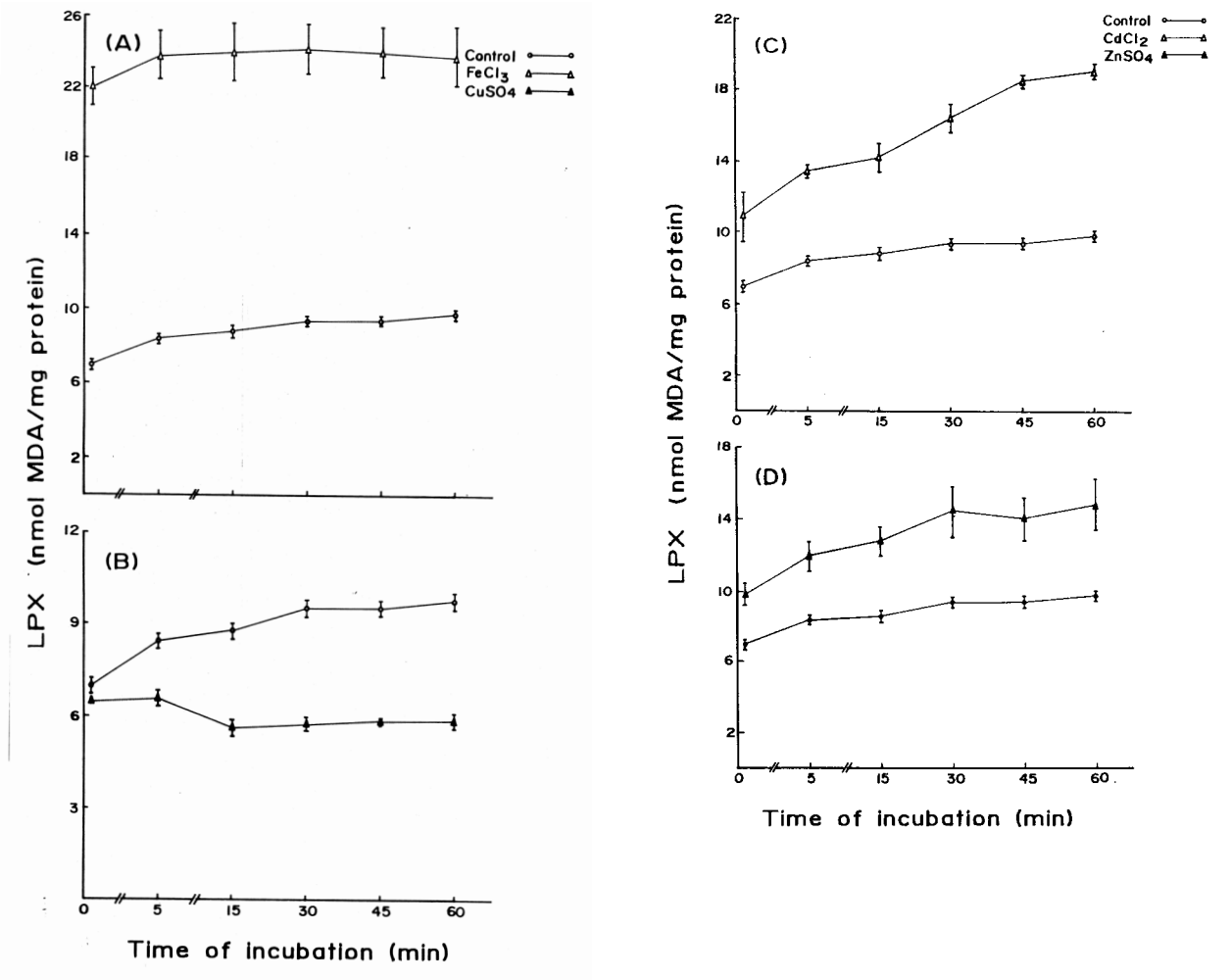

Figure 6. Changes in levels of lipid peroxidation in crude homogenate of hepatopancreas of *M. rosenbergii* with different metal ions $FeCl_3$(A), $CuSO_4$(B), $CdCl_2$(C), $ZnSO_4$(D), with respect to time. Data are mean ± SEM (n = 4).

decomposition of lipid peroxides and formation of hydroxyl radicals from hydrogen peroxide (Halliwell & Gutteridge, 1985). Further, Minotti and Aust (1992) suggested that iron-induced LPX also depends on certain factors like redox cycling of iron, the amount of lipids and degree of polyunsaturated fatty acids. It is possible that the presence of a higher amount of lipids and PUFA in the hepatopancreas may be one of the causes which make the tissue more susceptible to iron-induced LPX. On the other hand, gill tissue is more susceptible to $CdCl_2$ than hepatopancreas. Cadmium is reported to induce LPX in various organisms like fishes (Wofford and Thomas 1988) and mammals (Sarkar *et al.* 1995). Cadmium-induced cellular hyperplasia and tissue necrosis in the gills of banana shrimp (*Penaeus merguiensis*) has also been reported by Darmono *et al.* (1990). They also reported that between gills and hepatopancreas, gills are comparatively more susceptible to cadmium toxicity. The results suggest that $CuSO_4$ at low concentration acts as prooxidant while at high concentration it acts as antioxidant. Induction of LPX in liver, gill and white muscle of carp by $CuSO_4$ has also been reported previously (Radi & Markovics 1988). Viarengo *et al.* (1998) also reported elevation of LPX in the gills and digestive glands of *Mytilus edulis* by copper salts. Doyotee *et al.* (1997) observed that very low concentration of copper in the presence of thiram in the ambient water induced LPX in the gill and digestive gland of mussel *Unio tumidus*. Copper being a transition metal can enhance LPX through the formation of ROS via redox cycling between Cu^{2+} and Cu^{+1} oxidation state. However, the antioxidant behaviour of copper at high doses is an important finding. Though copper induces free radical generation, it is an essential trace element and the chief constituent of crustacean body fluid (Vijayram & Geraldine, 1996).

Although $ZnSO_4$ enhances LPX in gill and hepatopancreas, the magnitude of induction was more in the case of gills than hepatopancreas. Zinc is reported to elevate tissue LPX in case of carp (Radi & Markovics 1988) but failed to induce LPX in tissues of *Mytilus* (Viarengo *et al*. 1988). Zinc, unlike copper, is not a redox active metal, therefore, its induction of LPX in tissues of prawn is difficult to explain. However, the possibilities of its inhibiting some radical reaction by displacing other transition metals from the binding sites of proteins cannot be ruled out (Halliwel & Gutteridge 1985). The results of the present investigation clearly indicate that among all the metals studied $CdCl_2$ and $FeCl_3$ are more potent in inducing LPX in the homogenate of gills and hepatopancreas than other metals. Fe^{3+} is more toxic than Fe^{2+} in inducing LPX in hepatopancreas. $ZnSO_4$ exhibits a moderate toxicity and $CuSO_4$ is the least toxic and also inhibits LPX at high concentration. Since LPX is an index of oxidative stress and enhancement of LPX in tissues of *M. rosenbergii* by iron, copper, cadmium and zinc in the present study suggest that above metals ions can cause toxicity to prawn by inducing oxidative stress, however, the degree of toxicity of each metal is tissue specific.

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