Chapter VI

The mechanism of action of lead and cadmium either alone or in combination

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Introduction

Increasing evidence suggests that multifactorial mechanism might be involved in metal induced toxicity and it is suggested that one of the well-known mechanisms is metal induced reactive oxygen species (ROS). These metals have electron-sharing affinities that can result in formation of covalent attachments (Bondy, 1996). These attachments are mainly formed between heavy metals and sulfhydryl groups of proteins (Quig, 1998). Thus depletion of a cell's major sulfhydryl reserves seems to be an important indirect mechanism for oxidative stress that is induced by redox inactive metals such as lead and cadmium. Several mechanisms are proposed for lead induced oxidative stress: 1) direct effect of lead on cell membranes (Waldron, 1966; Lawton and Donaldson, 1991), 2) Lead-hemoglobin interactions (Ribarov et al., 1981; Carrell et al., 1975), 3) δ-aminolevulininic acid (δ-ALA)-induced generation of reactive oxygen species (Bechara, 1996; Monteiro et al., 1991), and 4) Effect of lead on the antioxidant defense system of cells (Christie and Costa, 1998; Adler et al., 1993). Proposed mechanisms for cadmium induced oxidative stress are 1) Adverse effects of cadmium on cellular defense systems and thiol status (Gong and Hart, 1997; Shaikh et al., 1999), Enhancement of lipid peroxidation by cadmium (Yiin et al., 2001; Hussain et al., 1987), 3) Deleterious effects of cadmium on cellular enzymes (Hussain et al., 1987; Shaikh et al., 1999). These ROS result in modulation of intracellular oxidized sate, membrane damage, DNA damage, altered gene expression and apoptosis (Bagchi et al., 1996; Xu et al., 1996; Rossman, 2000; de la Fuente et al., 2002). While neither O^{2-} nor H_2O_2 react directly with DNA, in vivo transition metal ions like Fe^{2+} or Cu^{1+} are believed to catalyze their conversion into the highly reactive $OH^{\cdot}$ radical, which in turn provokes a broad spectrum of DNA lesions (Halliwell

and Gutteridge, 1989). The free radicals thus generated by the metal toxicity are known to cause alterations on membrane structure and functions such as changes in permeability, activity of enzymes, channels, transport proteins, membrane fluidity and receptors (Amoruso et al. 1987; Nivsarkar et al. 1998; Thevenod and Friedmann 1999; Jarrar and Mahamoud 2000). Although there are various reports on the oxidative damage by lead and cadmium on hepatic tissue, no reports are there on their toxic effects on pituitary membrane. Pituitary membrane integrity is very important for normal endocrine functioning as hypothalamic gonadotropin regulating hormone (GnRH) action is initiated by binding to specific cell surface receptors in pituitary, leading to increased synthesis and release of gonadotropins from pituitary which inturn regulate gonadal function..

Most of the animal studies performed to understand biochemical toxicity were carried out with very high concentration of metals and little attention was given on simultaneous exposure of more than one metal. On the other hand, population in real life always has multiple exposures, indicating the need for experimental work with combination of substances. In the present study an attempt has been made to under stand the effects of lead and cadmium either alone or in combination on hepatic as well as pituitary antioxidant defense mechanism.

Experimental design

Experiments were carried out in two ways.

1. Mechanism of action of lead and cadmium on liver and pituitary - *in vivo study*

There were four groups of animals in the study: control (sodium acetate), lead acetate, cadmium acetate and lead acetate and cadmium acetate in combination. The animals were treated intraperitonially with 0.05 mg/kg body wt. dose per day for 15 days. The combined

treated group was also exposed to same dose by taking half concentration of each metal. Animals of all groups were sacrificed in proestrous stage after the metal exposure; liver and pituitary wee excised off. Liver homogenate (10% in 0.175 M KCl) was used for the determination of reduced glutathione (Beutler and Gelbart, 1985) and thiobarbituric acid reactive substances (TBARS) (Braughler et al., 1986). The supernatant obtained after removing mitochondria was used for estimating catalase (Hugo, 1987) and superoxide dismutase (Kakkar et al., 1984) activities. In pituitary homogenate (10% in 0.175 M KCl) various antioxidant parameters such as reduced glutathione content, TBARS levels, catalase and superoxide dismutase activities were determined. Apart from antioxidant parameters, pituitary membrane was used for the determination of fluidity (Shinitzky and Barenholz, 1978), Na^+K^+ATPase activity (Floreani et al., 1981), Schiffs base formation (Tappel et al., 1975) and inorganic peroxide levels (Bernt and Bergmeyer, 1965).

2. Mechanism of action of lead and cadmium on liver and pituitary - *in vitro study*

In vitro experiments were done to understand the inhibitory mechanism of metal exposure with the concentration of metal that is present in the liver and pituitary after *in vivo* exposure. Normal rats were sacrificed, liver and pituitary were removed and homogenates (10% in 0.175 M KCl) were prepared. Liver homogenate was centrifuged at 10000 g to get mitochondrial and postmitochondrial fraction and incubated at 37^0C for 20 min with 0.25 µM lead acetate and/or 1.5 µM cadmium acetate in a mixture containing 0.175 M KCl, 25 mM Tris-HCl at pH 7.4 in a total volume of one ml. Antioxidant capacity of Vitamin E and Mn were also evaluated. Vitamin E (200 µM dissolved in 25 µl dimethyl sulfoxide) and MnSO4 (30 µM), when used, was added 30 s before the addition of metal (Casalino et al., 2002). The reaction was stopped by keeping the tubes in ice. Aliquots of the suspension

were used to determine lipid peroxidation and enzyme activities. In case of pituitary whole homogenate was used (tissue being very small) and incubated with 0.012 μM lead acetate and/or 0.003 μM cadmium acetate in a similar manner as in the case of liver and TBARS and enzyme activities were determined.

Results

1. Mechanism of action of lead and cadmium on liver and pituitary - *in vivo study*

The treatment of animals with lead acetate was without influence on the hepatic GSH content and on the level of lipid peroxidation (Table 1) as compared to control. On cadmium exposure GSH content was decreased and lipid peroxidation elevated. In pituitary, metal exposure either alone or in combination caused decrease in GSH content and increase in lipid peroxidation level (Table 1). Cadmium treated group was showing more effect compared to lead and combined treatment groups.

Data in Table 2 indicates the activities of different antioxidant enzymes. Significant inhibition in the activity of hepatic superoxide dismutase was found in lead exposed animals whereas catalase activity was not changed by lead treatment. Cadmium exposure resulted in marked changes in the antioxidant defense system. The activity of superoxide dismutase was significantly inhibited and catalase activity was increased. In combined metal exposed groups the activity of superoxide dismutase was significantly inhibited and catalase activity was increased. These values were intermediate between individually exposed groups of lead and cadmium. In pituitary, catalase activity was unaffected in lead treated group whereas cadmium and combined metal treatment groups showed significant increase in the catalase activity (Table 2). There was no change in the superoxide dismutase activity in any of the metal treated group as compared to control (Table 2).

Table 1: Hepatic and pituitary GSH and TBARS levels of female rats exposed to lead acetate and cadmium acetate alone and in combination.

Groups	Liver		Pituitary	
	GSH	TBARS	GSH	TBARS
Control	1.34±0.08	24.04±1.4	5.13±0.11	5.53±0.14
Pb	1.2±0.04	28.9±1.93	2.63±0.17*	6.31±0.114*
Cd	0.57±0.03* **	34.74±2.8*	1.66±0.09* **	5.537±0.047*
Pb+Cd	0.89±0.03* ** #	32±0.89*	1.86±0.13* **	6.13±0.11*

*$P \leq 0.001$ vs. control; **$P \leq 0.01$ vs. lead and #$P \leq 0.001$ vs. cadmium exposed group ($n = 5$).

Table 2: Hepatic and pituitary catalase and superoxide dismutase activities of female rats exposed to lead and cadmium alone and in combination.

Groups	Liver		Pituitary	
	Catalase	Superoxide dismutase	Catalase	Superoxide dismutase
Control	204.8±12.9	0.38±0.03	32.75±6.583	0.464±0.031
Pb	223.86±5.47	0.26±0.01*	46.75±6.57	0.419±0.029
Cd	396.2±21.16* **	0.19±0.01* **	84±6.27* **	0.438±0.022
Pb+Cd	282.67±5.95* ** #	0.25±0.02* #	52.1±2.89* #	0.455±0.01

*$P \leq 0.001$ vs. control; **$P \leq 0.001$ vs. lead and #$P \leq 0.01$ vs. cadmium exposed group. ($n = 5$).

The fluidity study in pituitary shows an increase in fluorescence polarization ratio indicative of a decrease in pituitary membrane fluidity, with the probe DPH in metal exposed groups (Table 3). The results show that all the treatment groups had polarization values (i.e., decreased fluidity) significantly higher than that of controls. Among the three groups cadmium treatment was showing more effect compared to other treatments and the combined treatment was showing intermediate values. The formation of Schiffs base was also measured in this study to assess lipid peroxidation. The fluorescence intensity increased approximately 25%, 23% and 19% in cadmium, lead + cadmium and lead groups respectively (Table 3) compared with control. The metal treatment caused increase in inorganic peroxide level in the pituitary membrane. Cadmium exposure results in maximum increase in the inorganic peroxide level compared to lead and lead + cadmium (Table 3). The Na^+K^+ATPase activity was decreased significantly in cadmium and combined metal treated groups as compared to control (Table 3). i.e., among the three groups cadmium treatment was showing more inhibitory effect compared to other treatments.

2. Mechanism of action of lead and cadmium on liver and pituitary - *in vitro study*

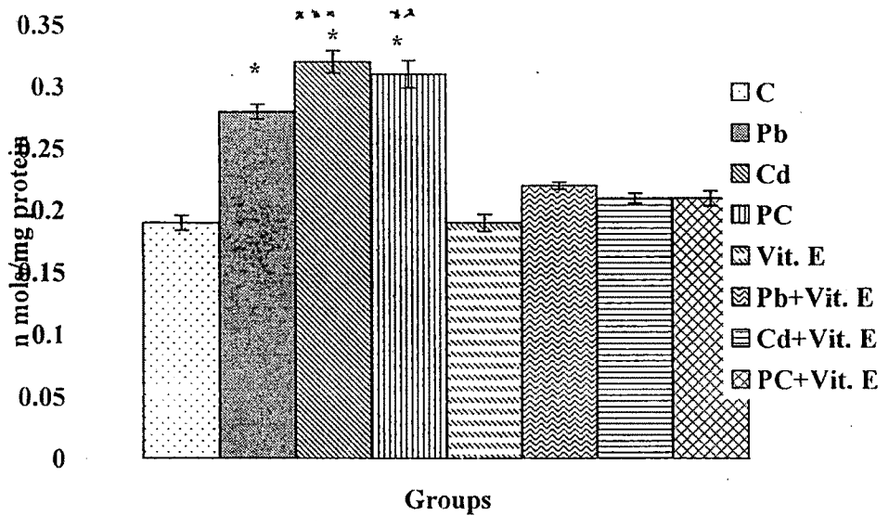
The effect of vitamin E on hepatic post-mitochondrial lipid peroxidation following metal incubation is shown in Fig. 1. The TBARS level found in fractions following incubation with metals in the presence of vitamin E is equal to that of control. The activities of CuZnSOD (Fig. 2) and catalase (Fig. 3) are reduced when hepatic postmitochondrial supernatant were incubated with lead, cadmium and lead+cadmium. But vitamin E could not protect the antioxidant enzymes from metal intoxication. The effect of Mn^{2+} ions on rat liver mitochondria lipid peroxidation following metal incubation is shown in Fig. 4. The

Table 3: Effect of lead and cadmium alone and in combination on pituitary membrane.

Group	Schiffs base (Fluorescence intensity)	Inorganic peroxide level	Na^+K^+ATPase activity	Polarization
Control	29.95±1.98	3.13±0.138	0.147±.009	0.088±0.007
Pb	38.85±1.57*	4.81±0.438*	0.126±0.005	0.120±0.003**
Cd	40.17±1.73*	5.69±0.28**	0.081±0.005** #	0.138±0.008**
Pb+Cd	36.78±0.80*	5.07±0.188**	0.112±0.006* ##	0.126±0.006**

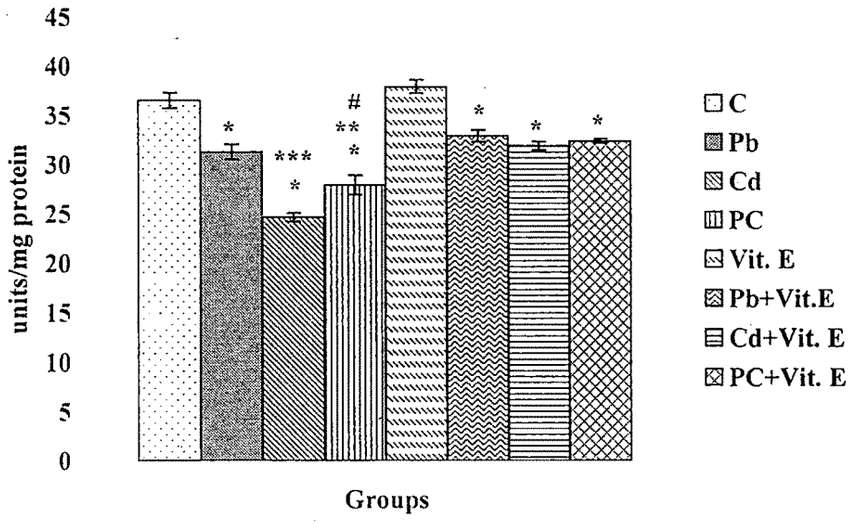
*$P \leq 0.01$; **$P \leq 0.001$ vs. control; #P<0.001 vs. lead; ##P< 0.01 vs. cadmium group (n = 5).

Fig 1: TBARS levels in hepatic post-mitochondria fraction in the presence of lead a: cadmium either alone or in combination: the role of Vitamin E.



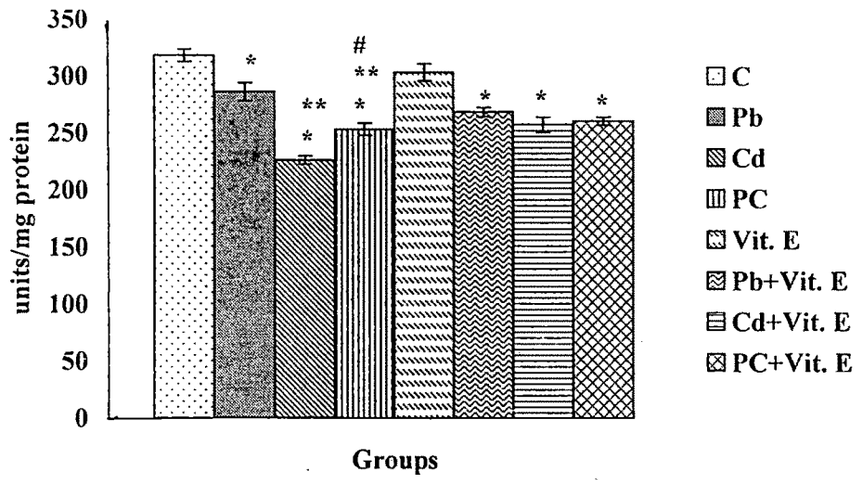
$^{*}P$ <0.001 vs. control; $^{**}P$ <0.01, $^{***}P$ <0.001 vs. lead exposed group (n=6)

Fig 2: Cu Zn SOD activity in hepatic post-mitochondria fraction in the presence of lead and cadmium either alone or in combination: the role of Vitamin E



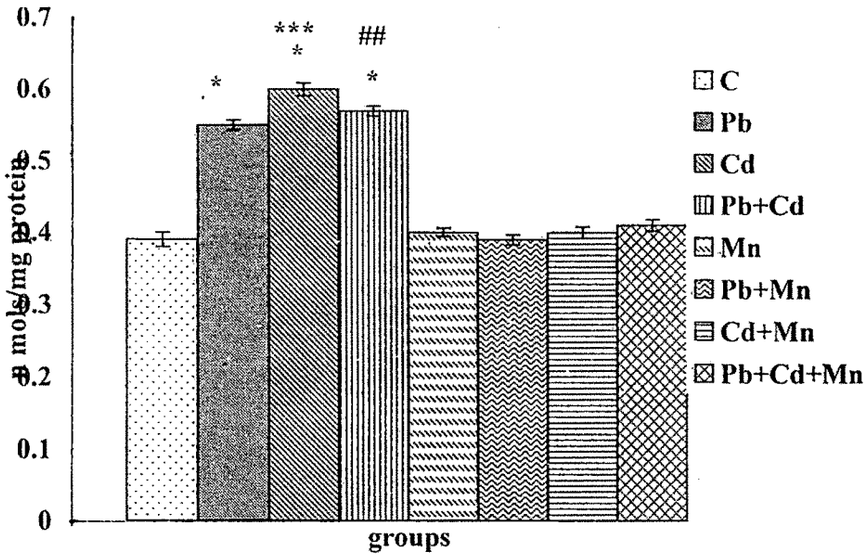
*P<0.001 vs. control: ** P<0.01,*** P<0.001 vs. lead and # P<0.01 vs. cadmium exposed group (n=5)

Fig 3: Catalase activity in hepatic post-mitochondria fraction in the presence of lead and cadmium either alone or in combination: the role of Vitamin E



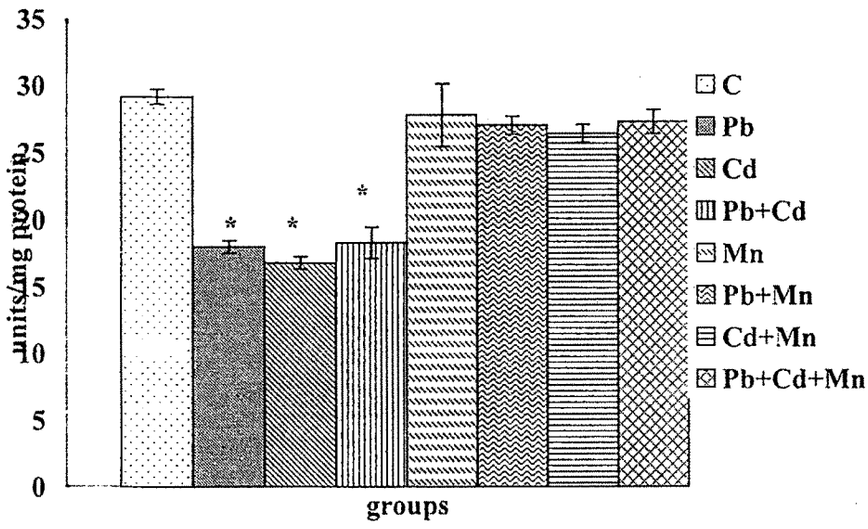
*P<0.001 vs. control; ** P<0.01,*** P<0.001 vs. lead and # P<0.001 vs. cadmium exposed group (n=5)

Fig 4: TBARS levels in hepatic mitochondria fraction in the presence of lead and cadmium either alone or in combination: the role of Mn2+.



*P <0.001 vs. control;*** P<0.001 vs. lead; ## P<0.001 vs cadmium group.(n = 5)

Fig 5: Superoxide dismutase activity in hepatic mitochondria fraction in the presenc of lead and cadmium either alone or in combination: the role of Mn2+



*P <0.001 vs. control group (n = 5)

data indicate that Mn^{2+} ions protect the mitochondria from lipid peroxidation, as the TBARS levels found in mitochondrial fractions following incubation with metal in the presence of Mn^{2+} is equal to that of control. In contrast to vitamin E, Mn^{2+} could completely restore MnSOD activity following metal intoxication (Fig. 5). Pituitary being small, we could perform the *in vitro* studies in whole pituitary homogenate only. Since there was no significant change in any of the antioxidant parameters studied data has not been shown.

Discussion

Both lead and cadmium are sulfhydryl reactive metals. Recent studies indicate that these transition metals act as catalysts in the oxidative reactions of biological macromolecules therefore the toxicities associated with these metals might be due to oxidative tissue damage (El-Maraghy et al., 2001; Moreira et al., 2001). Generation of highly reactive oxygen species such as hydrogen peroxide, superoxide radicals, hydroxyl radicals and lipid peroxides on heavy metal exposure are known to damage various cellular components including membrane lipids, protein, DNA and thereby contribute to cellular dysfunction. The products of lipid peroxidation react with amino acid residues such as cysteine and lysine and disturb protein function (Stanimirovic et al., 1995; Rohn et al., 1996). Both in vivo and in vitro studies have suggested generation of ROS and alteration of antioxidant system in animals as one of the mechanisms for the toxic effects by lead and cadmium (Shafiq-ur-Rehman, 1984; Monterio et al., 1991; Sandhir et al., 1994; Yiin et al., 1998; Shaikh et al., 1999). Our results show that after 15 days of metal exposure hepatic GSH content is decreased in cadmium and combined metal treated groups whereas pituitary GSH content is decreased in all metal exposed groups. GSH constitute the first

line of defense against free radical induced damage. It accounts for about 90% of the intra cellular non-protein thiol content. The decrease in GSH content observed in the present study might be due to the binding of these divalent metals with –SH groups (Bagchi et al., 1996; Shibasaki et al., 1996; Karmakar et al., 1998; Nigam et al., 1999). It has been reported earlier that thiol group inactivation causes oxidative stress, permeability transition, and hepatic dysfunction (Rikans and Yamano, 2000). Metallothionein is another protective agent that play important role in detoxification processes. Cadmium exposure has been shown to increase metallothionein synthesis in various tissues (Onosako and Cherian, 1981). There was a decrease in the level of zinc in hepatic fraction corresponding to metallothionein in animals exposed to lead and cadmium either alone or in combination (Chapter 1), indicating that both lead and cadmium can displace zinc from the metallothionein fractions. In fact there is a direct correlation between GSH depletion and enhanced lipid peroxidation. The increase in TBARS in the present study indicates failure of antioxidant defense mechanism, which prevents the formation of excess free radicals.

The antioxidant enzymes superoxide dismutase and catalase are potential targets for both lead and cadmium as these enzymes depend on various transition metals for proper molecular structure and activity. Both lead and cadmium can readily displace zinc and copper, which are cofactors for superoxide dismutase causing a decrease in the enzyme activity (Hussain et al., 1987; Kofod et al., 1991; Adler et al., 1993; Ariza et al., 1998). Copper ions appear to have a functional role in the reaction by undergoing alternate oxidation whereas zinc ions seem to stabilize the enzyme. We have observed the inhibition in superoxide enzyme activity in hepatic tissue, but no change was seen in the pituitary after the metal exposure. It is interesting to note that basal activity of catalase is very low

in pituitary as compared to liver. Although, we could not compare pituitary catalase activity with any published report, there are evidences, which suggest variable activity of catalase in various tissues (Wohaieb and Godwin, 1987; Matkovics, 1997). After 15 days of metal exposure we have observed a significant increase in catalase activity in cadmium and combined treatment groups. This could be due to the early displacement of the transition metals present in the active site of superoxide dismutase by the heavy metals with no significant inhibition in the catalase activity. Casalino et al. (2002) reported in their *in vitro* experiments on hepatic postmitochondrial supernatant that the order of cadmium's inhibitory effect on antioxidant enzyme activities is MnSOD>CuZnSOD>catalase. To counter the deleterious action of ROS, antioxidant enzymes are also synthesized in response to the higher production of ROS. Thus the increased level of catalase activity observed after metal exposure for 15 days is probably in response of higher production of ROS.

Since the free radicals generated after the metal exposure are known to cause alterations on membrane structure and functions, we were interested to know whether these radicals can cause alterations on pituitary membrane integrity. The results indicate that the plasma membrane of pituitary is very sensitive to exposure to lead and cadmium. It is known that biological membranes, besides their function as selective boundaries play a fundamental role in the regulation of many biochemical processes. Modulation of membrane activity may depend on membrane physical properties, in particular fluidity (Chautan et al., 1990). The results of the present study show that both lead and cadmium individually and in combination cause changes in the biophysical properties of the pituitary membrane. i.e., membrane fluidity is decreased by the metals.

Hannan et al. (1989) suggested that changes in membrane fluidity by heavy metals could result from oxidation of double bonds of the membrane fatty acids. The double bonds in the cis configuration bring about the formation of kinks in the acyl chains and prevent them from packing tightly. But oxidation of the double bond leads to tighter packing. Lipid peroxides and other phospholipid catabolites disrupt membrane bilayer organization (Sevanian et al. 1988), promote nonbilayer organization (Thompson et al. 1987) and affect the packing of phospholipids and thus increase the membrane viscosity (Sevanian et al. 1988). This indicates that an increase in lipid peroxide level due to metal exposure causes appearance of Schiffs products followed by decrease in the plasma membrane fluidity. The increased levels of Schiffs products and inorganic peroxides found after the treatment with metals in our study agrees with the results observed by others (Ribarov et al. 1983; Berndt and Ansari 1990).

Gwozdzinski (1991) showed that mercury and copper increased the rigidity of the RBC membrane and attributed this change to the conformational changes of the membrane proteins brought about by these metallic ions. It is known that most membrane bound enzymes require membrane lipids to be in a "fluid" state for optimum activity. Changes in membrane fluidity as a result of lipid peroxidation can affect membrane proteins since permeability and function of membrane bound protein is known to be intimately associated with the dynamic state of the membrane lipids (Kaplan et al. 1995). The correlation between lipid peroxidation, fluidity and membrane function has been documented in several studies (Dinis et al. 1983; Kaplan et al. 1995). The present study also demonstrates such correlation between lipid peroxidation and membrane fluidity along with significant reduction in the activity of important membrane bound enzyme $Na^{+}K^{+}$ATPase by metal

exposure. The relationship between membrane lipids and enzyme activity was explored for ATPases (Moller et al. 1982; Mishra et al. 1989) and adenylate cyclase (Moller et al. 1982; Schachter 1984). Various other studies have shown the inhibition of Na^+K^+ATPase enzyme by lead and cadmium (Thevenod and Friedmann 1999; Jarrar and Mahamoud 2000) and other metals (Rohn et al. 1996; Stanimirovic et al. 1995) due to oxidative stress and increased lipid peroxidation.

In vitro experiments were carried out with the concentration of lead and cadmium reaching the tissues after *in vivo* exposure for 15 days. In contrast to the *in vivo* results on 15 days metal exposure, *in vitro* experiments did not demonstrate any change in pituitary with respect to both antioxidant and membrane parameters. Such results could be due to the very low concentration of lead and cadmium in the present study and that too for short time of exposure. The results on liver post mitochondrial supernatant, which are again in contrast to the *in vivo* results, indicate that different kind of mechanism exists between *in vivo* and *in vitro* metal-enzyme interaction.

To have further understanding of metal interaction, CuZnSOD, MnSOD and TBARS were estimated in post mitochondrial and mitochondrial fraction of liver and effect of vitamin E and Mn^{2+} ions were also studied. The results clearly demonstrate increase in TBARS in both mitochondrial and postmitochondrial fraction of metal treated groups, which is partially or completely resored to control value by vitamin E and Mn^{2+}. CuZnSOD activity was decreased in both single and combined metal treated groups as compared to control. The alterations in the enzyme activity could be due to the replacement of Zn/Cu ions by the metals. There are various reports that vitamin E can reverse the inhibitory effect of metals on antioxidant enzymes (Farris, 1991; Chaurasia and

Kar. 1997; Hsu et al., 1998; El-Missiry and Shalaby, 2000). The main biological function of vitamin E is to directly influence oxidative stress through modulation of signal transduction pathways (Azzi et al., 1992). However, in the present study the enzyme activities in metal treated groups are not restored to control values in postmitochondrial supernatant when pre-exposed to vitamin E. Our findings agree with the previous reports indicating that decrease in lipid peroxidation by α-lipoic acid (Bludovska et al., 1999) or vitamin E (Casalino et al., 2002) does not restore cadmium inhibited antioxidant enzyme activities. Mitochondrial respiration, the major source of ROS is promoted by lipid peroxidation and therefore increases oxidative stress by cadmium exposure (Karmakar et al., 1998). Mitochondrial SOD, MnSOD protects mitochondria against oxidative stress. MnSOD activity like CuZnSOD activity was significantly decreased in both single and combined metal treated groups. This could be due to a nonspecific interaction between the metals and MnSOD since the enzyme activity was completely restored when mitochondria was incubated with lead, cadmium or lead and cadmium along with Mn^{2+}. A recent study has shown that the transport system for Mn is used for cadmium uptake in mammalian cells (Himeno et al., 2002) that indicates a competition between the two metals if they are present together.

These results demonstrate that both lead and cadmium have pronounced effect on antioxidant defense system. When equal concentration of metals was used in combination, there seems to be competition between the two metals leading to intermediate effects. Both metals are causing depletion in GSH (thiol containing enzymes) along with various antioxidant enzymes leading to the induction of free radicals which inturn increase lipid

peroxidation both at subcellular and membrane level and finally resulting in disruption of membrane integrity.

Thus oxidative tissue damage seems to be basic cause of various dysfunctions observed in earlier chapters in relation to hypothalamus, pituitary and liver.

Summary

Adult female rats were treated intraperitonially with either lead acetate or cadmium acetate alone or in combination at a dose of 0.05 mg/kg daily for 15 days. After the metal exposure hepatic GSH content was decreased in cadmium and combined metal exposed groups whereas pituitary GSH content was decreased in all metal exposed groups. Cadmium and combined metal exposed animals showed significant increase in TBARS levels in both liver and pituitary gland. Superoxide enzyme activity was inhibited in hepatic tissue, but no change was seen in the pituitary after the metal exposure. After 15 days of metal exposure a significant increase in catalase activity was observed in cadmium and combined treatment groups. The alterations in the pituitary antioxidant system were followed by significant changes in the pituitary membrane also. The membrane fluidity was decreased after the heavy metal treatment with cadmium showing more effect compared to other treatments. Na^+K^+ATPase activity was decreased significantly by cadmium and combined treatments along whereas Schiffs base and inorganic peroxide levels were increased in all metal exposed groups. From in vitro experiments it appeared that TBARS removal by vitamin E did not restore the antioxidant enzyme activities to that of control. Mn^{2+} ions protect the mitochondria from lipid peroxidation, as Mn^{2+} could completely restore MnSOD activity following metal intoxication. These data suggest that both lead and cadmium either alone or in combination disrupts the hepatic as well as pituitary antioxidant

defense mechanisms where the effects produced by the combined treatment of metals are not additive. The metal induced free radicals lowered the membrane fluidity, which may affect membrane function and cause alterations in receptor binding and secretory mechanism(s) of pituitary hormones.