Chapter 5 Biochemical, cellular and molecular mechanism of lead and cadmium co-exposure on male reproductive system

5.1 Biochemical mechanism

5.1.1 Introduction

Free radicals can be defined as chemical species possessing an unpaired electron, which is formed by homolytic cleavage of a covalent bond of a molecule, either by the loss of a single electron from a normal molecule or by the addition of a single electron to a normal molecule (Ray and Husain 2002). The main free radicals formed in the body are reactive oxygen species (ROS) and reactive nitrogen species (RNS), and these radicals in excess result in the oxidative stress, which has been implicated in the pathogenesis of several diseases.

However about 5% or more of the inhaled O_2 is converted to reactive oxygen species (Harman 1993). Most of the molecular oxygen consumed by aerobic cells during metabolism is reduced to water by using cytochrome oxidase in mitochondria. However, when the oxygen is partially reduced it becomes 'activated' and reacts readily with a variety of biomolecules such as proteins, carbohydrates, lipids and DNA. In the sequential univalent process by which O_2 undergoes reduction, several reactive intermediates are formed such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and extremely reactive hydroxyl radical (·OH). These three are collectively known as ROS. The process can be represented as

$$O_2 \xrightarrow{e^-} O_2^- \xrightarrow{e^-} H_2O_2 \xrightarrow{e^-} \cdot OH \xrightarrow{e^-} H_2O$$

The endogenous sources of ROS are mitochondrial electron transport chain, respiratory burst by phagocytes, beta-oxidation, auto-oxidation, etc. Reactive oxygen species can be produced by exogenous processes also.

Environmental agents including nongenotoxic carcinogens can directly generate or indirectly induce reactive oxygen species in cells (Rice-Evans and Burdon 1993). The induction of oxidative stress and damage has been observed following exposure to UV, gamma rays, cigarette smoke and xenobiotics. Chlorinated compounds, radiation, metal ions (Pb and Cd), barbiturates,

phorbol esters, and some peroxisome proliferating compounds are among the classes of compounds that have been shown to induce oxidative stress and cause *in vitro* and *in vivo* damage (Klaunig and Kamendulis 2004). The endogenous and exogenous sources of ROS are summarized in Table 1.

Table 1. Endogenous and exogenous sources of ROS

Cellular oxidants	Source	Oxidative species
Endogenous	Mitochondria	O_2^-, H_2O_2, ·OH
	Cytochrome P450	O_2^-, H_2O_2
	Macrophage/Inflammatory cells	O_2^-, ·NO, H_2O_2, OCl^-
	Peroxisomes	H_2O_2
	Redox cycling compounds	O_2^-
Exogenous	Metals (Fenton reaction)	·OH
	Radiation	·OH

Cells are protected by antioxidant defense mechanisms that remove these free radicals to prevent oxidative damage. The antioxidant system comprises of different types of functional components such as enzymatic and nonenzymatic antioxidants. The enzymatic antioxidants comprise of superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione *S* transferase. The non-enzymatic antioxidants include reduced glutathione, vitamin C, vitamin E (α tocopherol), uric acid, carotenoids, flavanoids ubiquinol etc.

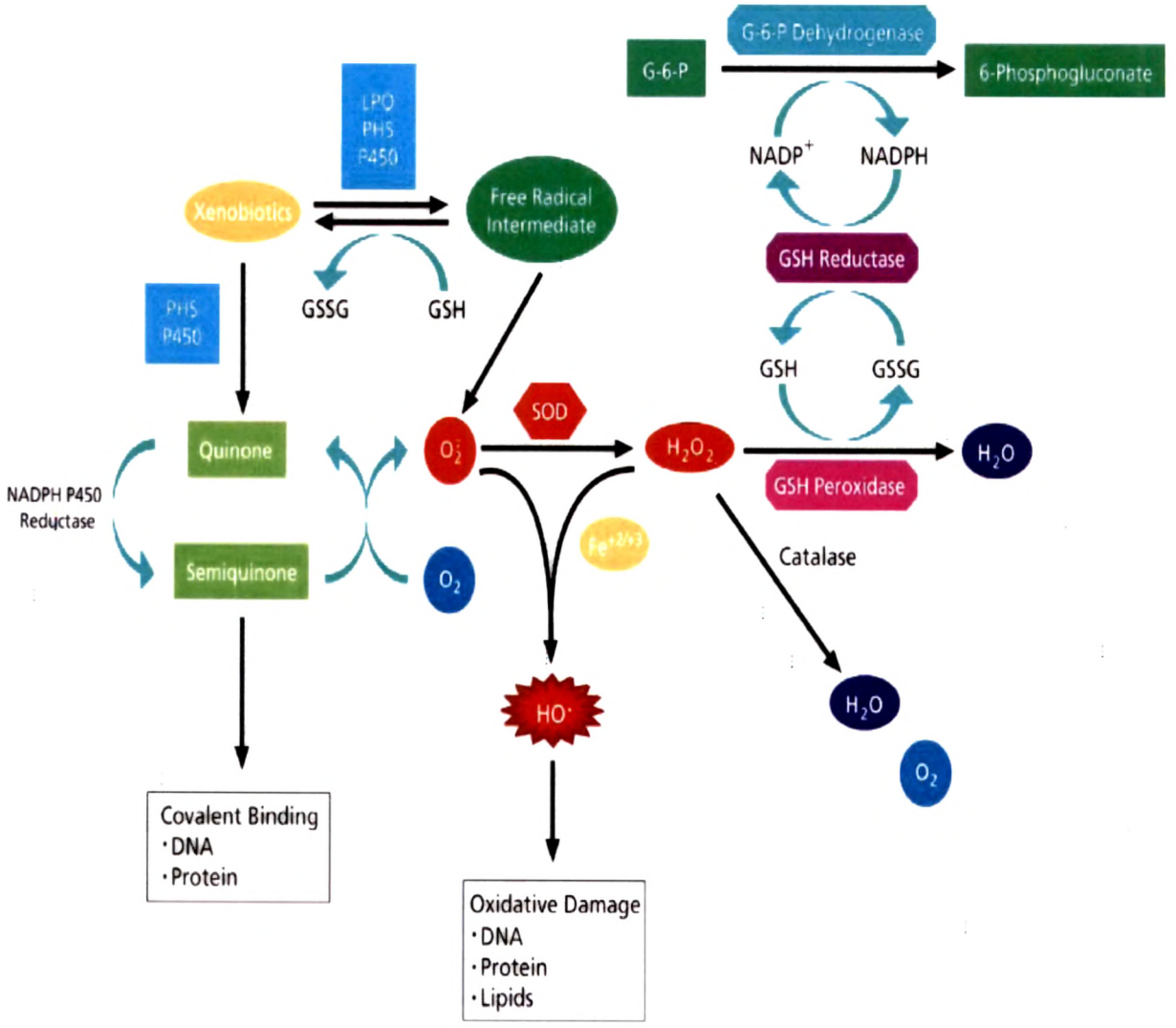
Superoxide dismutase (SOD, EC 1.15.1.1) scavenge the superoxide ions, by rapid dismutation and forms hydrogen peroxide, which are again catalyzed by catalase (CAT, EC 1.11.1.6) to form water and oxygen. Apart from catalase; glutathione peroxidase also (GPx, EC 1.11.1.9) catalyzes the reduction of various organic hydroperoxides, as well as hydrogen peroxide, with glutathione as hydrogen donor. Glutathione peroxidase can directly reduce phospholipids and cholesterol hydroperoxides in cellular membranes and thus plays major role in protecting cells against the damaging effect of lipid peroxidation. Glutathione reductase (GR, EC 1.8.1.7) catalyses the reduction of oxidized glutathione

(GSSG) to reduced GSH, with the concomitant conversion of NADPH to $NADP^+$ (Beutler and Yeh, 1963). Glutathione S transferases (GSTs, EC 2.5.1.18) play an important role in the detoxification and elimination of xenobiotics. This process involves conjugation of glutathione with electrophilic metabolites and extrusion of the conjugate out of the cell for further metabolism.

The intracellular redox potential is determined by the concentrations of oxidants and reductants. A critical modulator of the redox potential is NADPH, the principal intracellular reductant in all cell types. Glucose 6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49), the rate-limiting enzyme of the pentose phosphate pathway (PPP) determines the amount of NADPH by controlling the metabolism of glucose via PPP (Kletzien et al 1994). The amount of NADPH maintains an adequate level of reduced glutathione (GSH).

Glutathione (γ-glutamylcysteinylglycine, GSH) is a sulfhydryl (-SH) non enzymatic antioxidant, antitoxin, and enzyme cofactor. Glutathione is found mainly in the cell cytosol and other aqueous phases of the living system. It is the most abundant (millimolar concentration) non protein thiol compound in mammalian cell. Glutathione exists in two forms: The antioxidant "reduced glutathione" tripeptide is conventionally called glutathione (GSH) and the oxidized form is a sulfur-sulfur linked compound, known as glutathione disulfide (GSSG). The GSSG/GSH ratio is a sensitive indicator of oxidative stress (Rahman et al 2005). Glutathione status is homeostatically controlled both inside the cell and outside, being continually self-adjusting with respect to the balance between GSH synthesis (by GSH synthetase enzymes), its recycling from GSSG (by GSH reductase), and its utilization (by peroxidases, transferases, transhydrogenases, and transpeptidases). Glutathione is an essential cofactor for antioxidant enzymes, namely the GSH peroxidases (both Se-dependent and non-Se-dependent forms). The reaction catalysed by all antioxidant enzymes are illustrated in figure 1

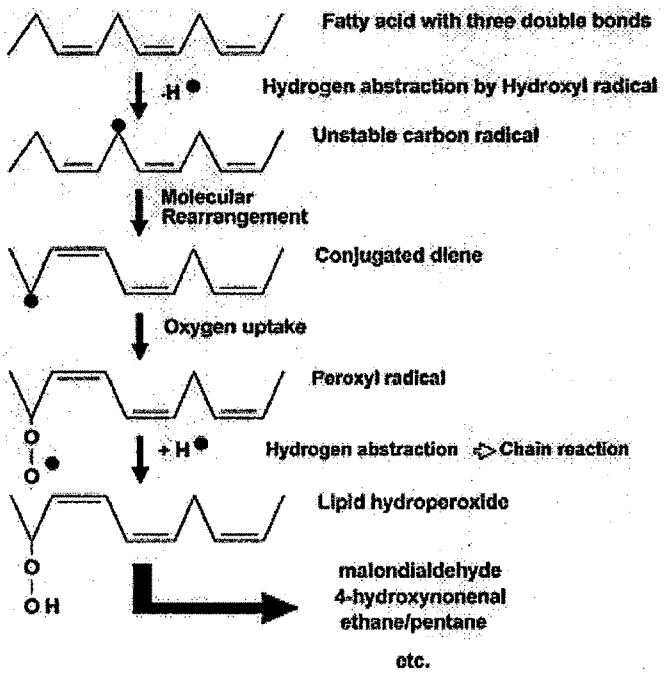
Figure 1. Antioxidant enzymatic reactions.



Oxygen radicals catalyze the oxidative modification of lipids (Gardner 1989). This peroxidation reaction is given in the Figure 2. The presence of double bond adjacent to a methylene group makes the methylene C-H bonds of polyunsaturated fatty acids (PUFA) weaker and therefore the hydrogen becomes more prone to abstraction. Lipid peroxidation is initiated by ·OH, alkoxy radicals (RO·) and peroxy radicals (ROO·) (Turrens and Boveris 1980). This can lead to a self-perpetuating process since peroxy radicals are both reaction initiators as well as the products of lipid peroxidation. Lipid peroxy radicals react with other lipids, proteins and nucleic acids; propagating thereby the transfer of electrons

and bringing about the oxidation of substrates. All membranes, which are structurally made up of large amounts of PUFA, are highly susceptible to oxidative attack, which results in the changes in membrane fluidity, permeability, and cellular metabolic functions. The mechanism of lipid peroxidation is given in the Figure 2.

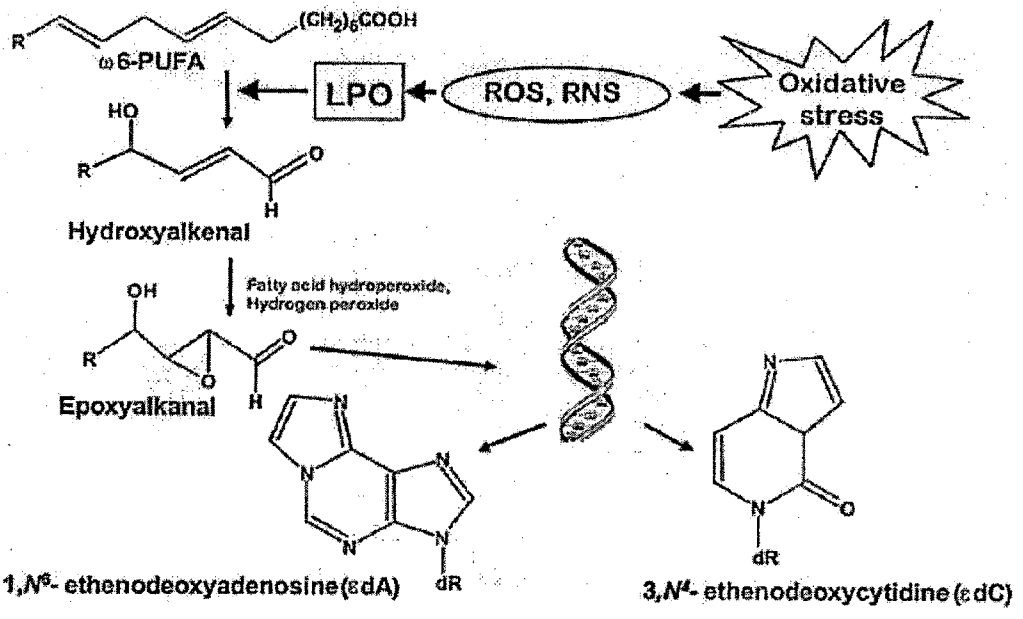
Figure 2. Lipid peroxidation mechanism



Oxidative stress occurs when the production of ROS exceeds the natural antioxidant defence mechanisms, causing damage to macromolecules such as DNA, proteins and lipids. The oxidation of lipids by ROS, notably LPO of polyunsaturated fatty acids (PUFA), results in reactive products such as croton aldehyde, malondialdehyde and 4-hydroxyalkenals. These intermediates can react with DNA bases in vitro and in vivo to form exocyclic DNA adducts. characterized as propano and etheno DNA-base adducts. The evidence (Chung et al., 1996; Bartsch, 1999) indicates that etheno adducts are generated by reaction of DNA bases with lipid peroxidation products, as depicted in Figure 3, involving the following steps: LPO produces fatty acid hydroperoxides and then the reactive trans-4-hydroxy-2-nonenal, a major product in vivo, which can be

oxidized by hydrogen peroxide or fatty acid hydroperoxides to form its epoxide intermediate. This bifunctional agent attacks the nitrogen atom in DNA bases to form the etheno ring in cytidine, adenosine and guanosine. In support of this mechanism, etheno adducts were formed after peroxidation of arachidonic acid and microsomal membranes in the presence of iron (II) ions or cumene hydroperoxide (El Ghissassi et al., 1995). Three etheno adducts 1, N6-ethenodeoxyadenosine (odA) 3, N4-ethenodeoxycytidine (odC) (shown in Figure 3) and N2,3-ethenodeoxyguanosine (odG) have been detected in vivo. Thus various exogenous and endogenous sources can contribute to the mutagen burden imposed ROS.

Figure 3 Generation of etheno-adducts.



Increasing evidence suggests that multifactorial mechanism might be involved in metal induced toxicity. It is also suggested that one of the well-known mechanisms is metal induced reactive oxygen species (ROS). It was supposed that metal compounds would be of particular interest because of coordination capacity of the metal center and their ability to catalyze redox processes involving (di)oxygen and active oxygen species as well as biogenic substrates. 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; Monterio et al., 1991), and 4) Effect on the enzymatic and nonenzymatic antioxidant defense system of cells (Nampoothiri et al., 2007; Pillai et al., 2005; 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 (Nampoothiri et al., 2007; Gong and Hart, 1997; Shaikh et al., 1999), 2) Enhancement of lipid peroxidation by cadmium (Pillai et al., 2005; Yiin et al., 2001; Hussain et al., 1987), 3) Deleterious effects of cadmium on cellular enzymes (Hussain et al., 1987; Shaikh et al., 1999), 4) Modulation of intracellular oxidized sate, membrane damage, DNA damage, altered gene expression and apoptosis (Bagchi et al., 1996; Rossman, 2000; de la Fuente et al., 2002). The free radicals thus generated by the metal toxicity are known to cause alterations on membrane structure and functions, activity of enzymes, channels, transport proteins, membrane fluidity (Pillai et al., 2002) and receptors (Nampoothiri et al., 2004; Jarrar and Mahamoud 2000).

Major reports on the oxidative damage by lead and cadmium 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. Our present study is an attempt to understand the mechanism of lead and cadmium exposure on testicular, hepatic, pituitary as well as prostatic antioxidant defense mechanism. Exposure of rats to lead and cadmium in isolation and combination has demonstrated decrease

neurotransmitter content, steroidogenic synthesizing and catabolic enzymes, testosterone level and testicular function (chapter 3 & 4), since metals can affect oxidant - antioxidant balance.

5.1.2 Experimental design

Study was performed in four groups of animals having 6 animals in each group: control (sodium acetate), lead acetate, cadmium acetate and lead acetate and cadmium acetate in combination. The animals were treated intraperitonially with 0.025 mg/kg body wt. dose per day intraperitonealy for 15 days. The combined treated group was also exposed to same dose by taking half concentration of each metal. At the end of the experimental period the animals were sacrificed by decapitation testis (decapulated), liver, pituitary and prostate was quickly removed and placed in beakers containing chilled (0-4 °C) isolation medium: 0.25 M sucrose, 10 mM Tris-HCl buffer (pH 7.4), 1 mM EDTA and 250 µg BSA/ml. The isolation of mitochondria and post-mitochondrial fractions was done as described previously (Swegert et al., 1999; Kaushal et al., 1999) and fractions used for all biochemical assays.

Glutathione S-transferase activity was measured as described earlier (Pabst et al., 1974). Determination of lipid peroxidation (LPO) was according to method of Ohkawa et al., 1979. Superoxide dismutase (SOD) activity was determined according to the methods described by Marklund and Marklund 1974. Catalase activity was assayed by monitoring the decrease of H_2O_2 at 240 nm (Hugo 1987). Glutathione peroxidase (GPox) activity was determined by the procedure described by Hafeman et al., 1974. Glutathione reductase activity was assayed by method of Smith et al. (1988). Glucose-6-phosphate dehydrogenase (G6PDH) activity was determined by Cohen and Rosemeyer 1975. Content of glutathione (GSH) was measured by the method of Beutler and Gelbart (1985). Protein was estimated by the method of Lowry et al. 1951. Details of the methods have been discussed in chapter 2.

Statistical analyses of log transformed data was done by one-way analysis of variance and all groups were compared by means of Dunnett's test, with

significance set at $p<0.05$. All values represent the mean ± S.E.M. Data were analyzed using PRISM version 3, Graph Pad software.

5.1.3 Results

The oxidative stress related parameters were assessed from rat tissues like testis, liver, pituitary and prostate. Mitochondrial and post mitochondrial fractions were used for testis, liver and prostate samples, while 10% homogenate was directly used for pituitary due to the less sample size.

The cellular damage induced by ROS was estimated by monitoring lipid peroxidation, a well known indicator of cellular damage from oxidative stress (Slater, 1984). In our experiments, significant elevated levels of TBARS were observed highest in cadmium and lead treated group followed by co-exposure treated groups in testis (Figure 4 and 5) and liver (Figure 6 and 7). The testicular TBARS level was higher in the mitochondria than in the post-mitochondrial fraction. Pb, Cd and co-exposed groups exhibited 57%, 152% and 51% increase in mitochondrial TBARS level respectively. Post-mitochondrial fraction demonstrated 33%, 70% and 29% higher peroxidation in Pb, Cd and combined metal treated groups respectively compared to control. Liver mitochondrial and post mitochondrial fractions were equally effective for elevation levels of TBARS. In pituitary, significant increase in TBARS levels were observed only in Cd treated group compared to corresponding control group (Figure 8).

To evaluate the activities of antioxidant defense system by lead and/or cadmium exposure, we assayed SOD, catalase, GPx, GR, GST and G6PDH. The SOD, catalase, GR, GST and G6PDH enzyme activities decreased in mitochondrial and post mitochondrial fraction of testis (Table 2) and liver (Table 3) when compared with the corresponding group of control animal. In cadmium and lead metal exposed groups the activities of antioxidant enzymes were significantly decreased, where as the combined metal exposure groups seems to be least effective. Cadmium treated group exhibited highest decrease in all antioxidant enzymes activity. The testicular and hepatic GPx activity increases in all treated groups and co-exposed group showed highest increase followed by

lead and cadmium exposed groups. Pituitary SOD and GPx activity was significantly increased in all metal treated groups, and maximum increase was observed in combined metal exposed group followed by Pb exposed animal. Both mitochondrial and post mitochondrial SOD activity did not show any significant difference in prostate. Redox status was also measured in prostate (Table 4) and pituitary (Table 5) tissue samples. Metal exposure exhibited significant decrease in catalase activity both in pituitary and prostate samples, whereas GPx activity decreases in both fractions of prostate. On metal exposure both mitochondrial and post mitochondrial GSH content was decreased in testis (Figure 9 and 10), liver (Figure 11 and 12), prostate (Figure 13 and 14) and pituitary (Figure 15). Cadmium treated group showed maximum decrease in testis, liver and pituitary, while co-exposure group exhibited maximum decrease in prostate tissue.

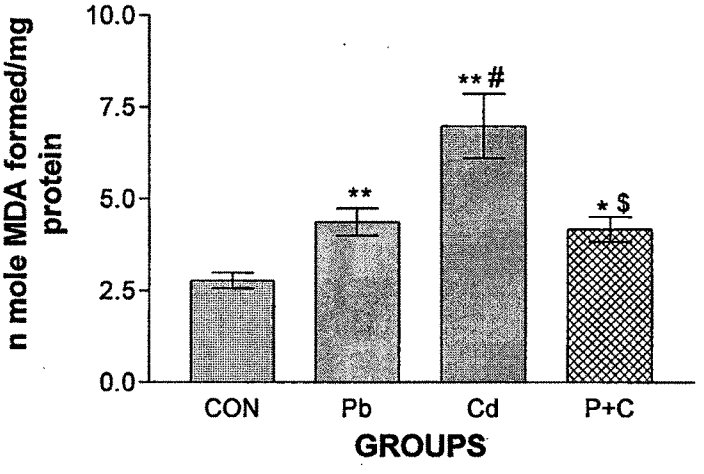
5.1.4 Discussion

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).

Hence, present study was carried out to understand the biochemical mechanism of lead and cadmium induced toxicity. Activity of enzymes involved in reactive oxygen species metabolism was examined. Both mitochondrial and post mitochondrial fraction was used to determine the compartment specific enzyme activity in testis, liver and prostate tissue.

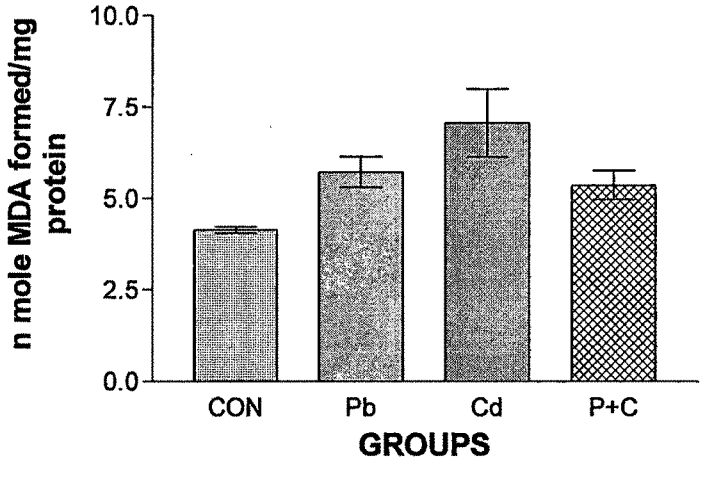
We observed that lead and cadmium even with low level exposure for 15 days exhibited increased lipid peroxidation in testis, liver and pituitary. Since lead and cadmium interacts with cell membranes, lipid peroxidation may be direct consequence of membrane damage (Casalino et al., 1997). The products of lipid peroxidation react with amino acid residues such as cysteine and lysine and

Figure 4. Effect of lead and cadmium in isolation and co-exposure on mitochondrial lipid peroxidation levels in testis.



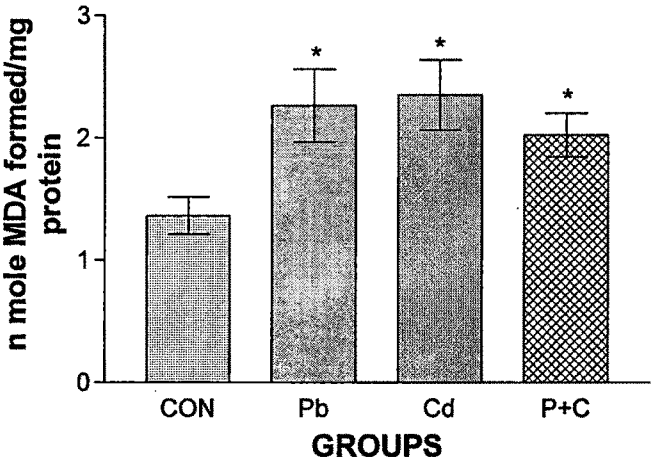
Values are expressed as mean ± SEM (n=6 in each group). *P<0.05, ** P<0.01 vs. control; # P<0.05 vs. lead and $P<0.05 vs. cadmium group.

Figure 5. Effect of lead and cadmium in isolation and co-exposure on post-mitochondrial lipid peroxidation levels in testis.



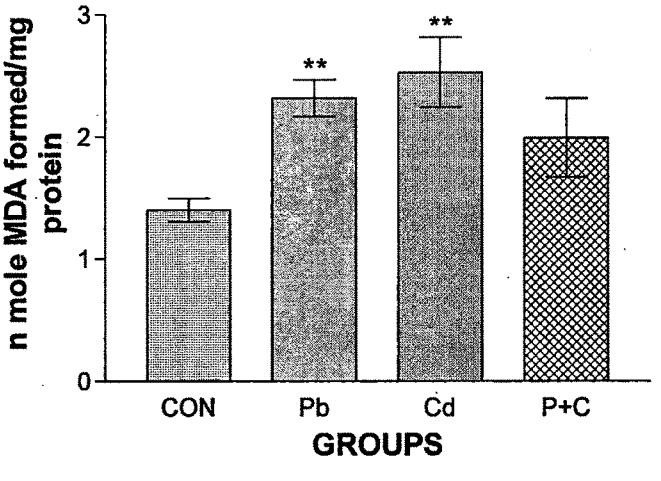
Values are expressed as mean ± SEM (n=6 in each group).
*P<0.05 vs. control group.

Figure 6. Effect of lead and cadmium in isolation and co-exposure on mitochondrial lipid peroxidation levels in liver.



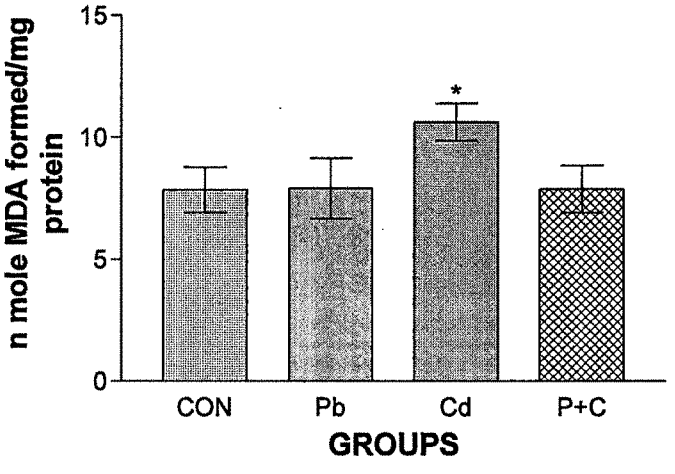
Values are expressed as mean ± SEM (n=6 in each group).
*P<0.05 vs. control group.

Figure 7. Effect of lead and cadmium in isolation and co-exposure on post-mitochondrial lipid peroxidation levels in liver.



Values are expressed as mean ± SEM (n=6 in each group).
** P<0.01 vs. group.

Figure 8. Effect of lead and cadmium in isolation and co-exposure on lipid peroxidation levels in pituitary.



Values are expressed as mean ± SEM (n=6 in each group).
*P<0.05 vs. control group.

Table 2. Effect of lead and cadmium in isolation and co-exposure for 15 days (0.025 mg/kg body weight) on antioxidant enzymes activities in testis of adult male rats.

Parameters	Groups			
	Control	Pb	Cd	Pb + Cd
SOD[a]				
Mitochondrial	2.64 ± 0.05	2.31 ± 0.12*	2.29 ± 0.01***	2.31 ± 0.03**
Post-mitochondrial	2.90 ± 0.23	1.35 ± 0.19**	0.60 ± 0.14$^{***\#}$	1.48 ± 0.21$^{**\psi}$
Catalase[b]	81.16 ± 1.95	36.02 ± 4.71***	28.88 ± 3.19***	61.69 ± 3$^{**\#\#\psi\psi}$
GPx[c]				
Mitochondrial	709.8 ± 143.3	1855 ± 223.6**	1812 ± 105.6**	2402 ±105$^{***\#\psi}$
Post-mitochondrial	245.4 ± 53.16	695.5 ± 46.07**	667.7 ± 31.14$^{***\#\#}$	887.9 ± 105$^{\#\ \psi\psi}$
GST[d]				
Mitochondrial	145.1 ± 6.1	111.6 ± 6.42**	60.04 ± 11.65$^{***\#\#}$	141.8 ± 6.05$^{\#\psi\psi}$
post-mitochondrial	164.9 ± 12.24	138.5 ± 14.56	86.28 ± 6.080$^{**\#}$	137 ± 15.77$^{\psi}$
G6PDH[e]	6.67 ± 0.67	3.95 ± 0.06**	3.19 ± 0.19$^{**\#\#}$	4.13 ± 0.23$^{*\psi}$

The data are presented as mean ± SEM. of 4-6 independent observations. *P<0.05, **P<0.01, ***P <0.001 vs. control; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. lead and ψP<0.05, $\psi\psi$p<0.001 vs. cadmium group.

[a] Amount of enzyme required for 50 % inhibition of pyrogallol autoxidation at 37°C.

[b] µ mole H_2O_2 decomposed/min/mg protein at 37°C.

[c] Δ log [GSH]/min/mg protein at 37°C.

[d] µ mole CDNB conjugated/min/mg protein at 37°C.

[e] µ mole NADPH formed/min/mg protein at 37°C.

Table 3. Effect of lead and cadmium in isolation and co-exposure on antioxidant enzymes activities in liver of adult male rats.

Parameters	Groups			
	Control	Pb	Cd	Pb + Cd
SOD[a]				
Mitochondrial	1.771 ± 0.284	0.845± 0.175*	0.5281 ± 0.086**	0.8310 ± 0.09*
Post-mitochondrial	3.346 ± 0.327	1.635 ± 0.167**	1.213 ± 0.09**	1.454 ± 0.26**
Catalase[b]	226.8 ± 24.10	148.6 ± 11.19*	111.2 ± 8.025**	210.3 ± 16.6$^{\Psi\Psi}$
GPx[c]				
Mitochondrial	4114 ± 341.6	4848 ± 233.5	4370 ± 259.4	6377 ± 389$^{**\#\Psi\Psi}$
Post-mitochondrial	1415 ± 74.5	1643 ± 121.2	1523 ± 111.4	1761 ± 69.91*
GR[d]				
Mitochondrial	71.38 ± 5.21	63.40 ± 1.71	34.46 ± 5.35$^{**\#\#}$	63.87 ± 6.66$^{\Psi\Psi}$
Post-mitochondrial	26.22 ± 1.24	22.00 ± 1.72	9.708 ± 3.3$^{**\#\#}$	20.69 ± 2.01$^{*\Psi}$
GST[e]				
Mitochondrial	177.8 ± 14.58	199.2 ± 14.72	170.9 ± 19.01	183.0 ± 33.69
post-mitochondrial	303.4 ± 13.24	259.9 ± 5.518	155.7 ± 2.881$^{**\#\#}$	240.5 ± 26.23$^{*\Psi\Psi}$
G6PDH[f]	6.039 ± 0.27	4.449 ± 0.5*	3.809 ± 0.34**	4.573 ± 0.27

The data are presented as mean ± SEM. of 4-6 independent observations.
*P<0.05, ** P<0.01 vs. control; $^{\#}$ P<0.05, $^{\#\#}$ P<0.01 vs. lead and $^{\Psi}$ P<0.05, $^{\Psi\Psi}$ p<0.01 vs. cadmium group.

[a] Amount of enzyme required for 50 % inhibition of pyrogallol auto oxidation at 37°C.

[b] μ mole H_2O_2 decomposed/min/mg protein at 37°C.

[c] log [GSH]/min/mg protein at 37°C.

[d] Amount of enzyme that catalyzes the reduction of 1 μmole of NADPH per minute per mg protein.

[e] μ mole CDNB conjugated/min/mg protein at 37°C.

[f] μ mole NADPH formed/min/mg protein at 37°C.

Table 4. Effect of lead and cadmium in isolation and co-exposure for 15 days (0.025 mg/kg body weight) on antioxidant enzymes activities in prostate of adult male rats.

Parameters	Groups			
	Control	Pb	Cd	Pb + Cd
SOD[a]				
Mitochondrial	2.08 ± 0.28	1.53 ± 0.49	1.46 ± 0.34	1.67 ± 0.36
Post-mitochondrial	1.18 ± 0.19	0.98 ± 0.19	1.39 ± 0.18	1.12 ± 0.13
Catalase[b]	3.30 ± 0.07	2.36 ± 0.24*	2.66 ± 0.12**	1.96 ± 0.39*
GPx[c]				
Mitochondrial	13320 ± 395.5	12290 ± 1111	11360 ± 859.8*	11070 ± 436.5**
Post-mitochondrial	11720 ± 987.9	10620 ± 909.1	9929 ± 534.8	8657 ± 907.8

The data are presented as mean ± SEM. of 4-6 independent observations.
*P<0.05, **P<0.01, ***P <0.001 vs. control;
#P<0.05, ##P<0.01 vs. lead and ΨP<0.05, ΨΨp<0.001 vs. cadmium group.

[a] Amount of enzyme required for 50 % inhibition of pyrogallol autoxidation at 37°C.

[b] μ mole H_2O_2 decomposed/min/mg protein at 37°C.

[c] Δ log [GSH]/min/mg protein at 37°C.

Table 5. Effect of lead and cadmium in isolation and co-exposure on antioxidant enzymes activities in pituitary of adult male rats.

Parameters	Groups			
	Control	Pb	Cd	Pb + Cd
SOD[a]	2.22 ± 0.75	7.60 ± 1.15*	5.37 ± 0.42*	9.38 ± 0.28**
Catalase[b]	2.99 ± 0.27	1.92 ± 0.23*	1.75 ± 0.25*	2.18 ± 0.25*
GPx[c]	1828 ± 204.3	2899 ± 266.1*	2810 ± 71.94*	3161 ± 220.9*

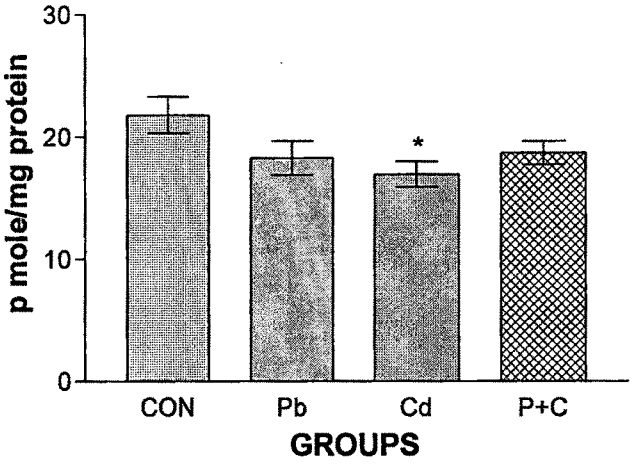
The data are presented as mean ± SEM. of 4-6 independent observations.
*P<0.05, **P<0.01 vs. control;
#P<0.05, ##P<0.01 vs. lead and ψP<0.05, ψψp<0.01 vs. cadmium group.

[a] Amount of enzyme required for 50 % inhibition of pyrogallol auto oxidation at 37°C.

[b] μ mole H_2O_2 decomposed/min/mg protein at 37°C.

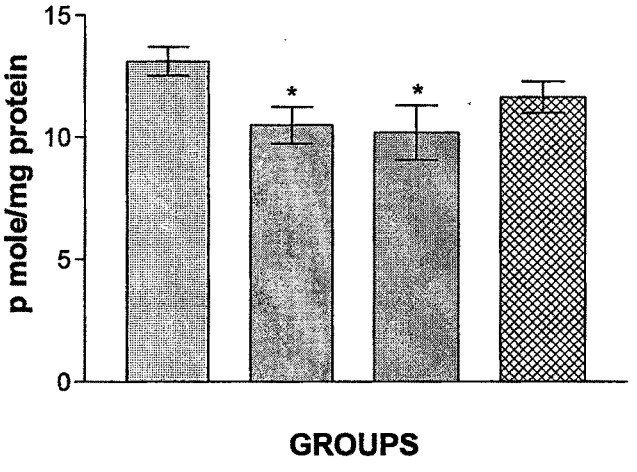
[c] Δ log [GSH]/min/mg protein at 37°C.

Figure 9. Effect of lead and cadmium in isolation and co-exposure on mitochondrial reduced glutathione levels in testis.



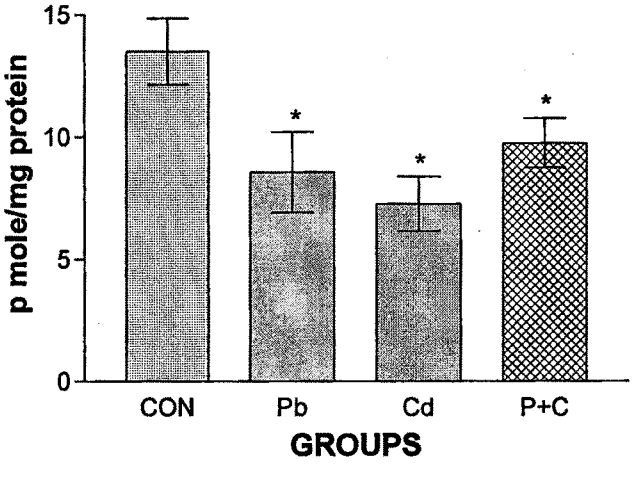
Values are expressed as mean ± SEM (n=6 in each group).
*P<0.05 vs. control group.

Figure 10. Effect of lead and cadmium in isolation and co-exposure on post-mitochondrial reduced glutathione levels in testis.



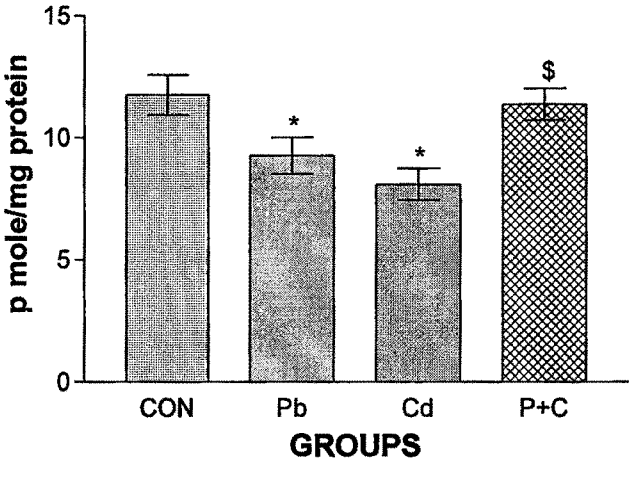
Values are expressed as mean ± SEM (n=6 in each group).
*P<0.05 vs. control group.

Figure 11. Effect of lead and cadmium in isolation and co-exposure on mitochondrial reduced glutathione levels in liver.



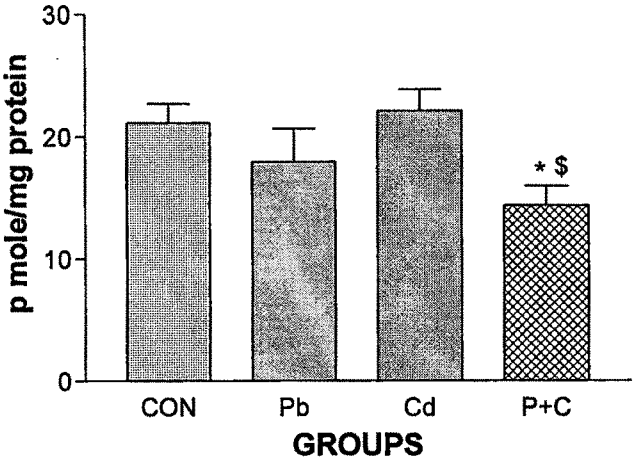
Values are expressed as mean ± SEM (n=6 in each group).
*P<0.05 vs. control group.

Figure 12. Effect of lead and cadmium in isolation and co-exposure on post-mitochondrial reduced glutathione levels in liver.



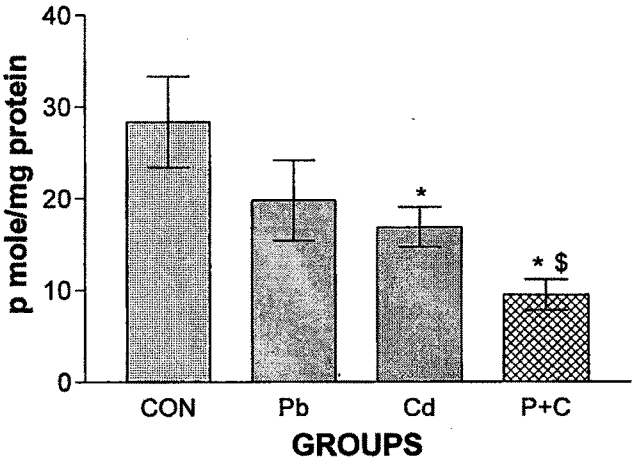
Values are expressed as mean ± SEM (n=6 in each group).
*P<0.05 vs. control and $P<0.05 vs. cadmium group.

Figure 13. Effect of lead and cadmium in isolation and co-exposure on mitochondrial reduced glutathione levels in prostate.



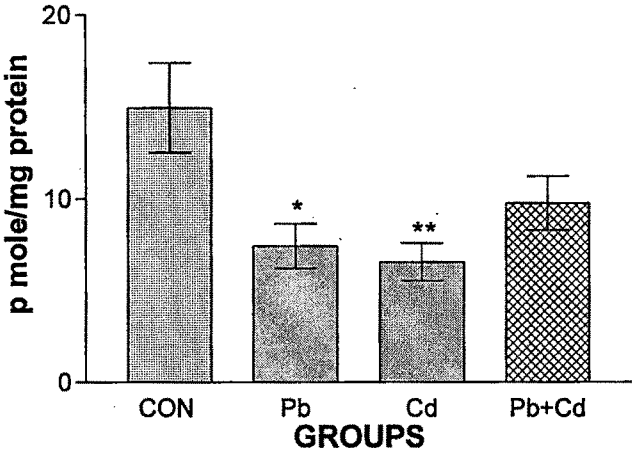
Values are expressed as mean ± SEM (n=6 in each group).
*P<0.05 vs. control and $P<0.05 vs. cadmium group.

Figure 14. Effect of lead and cadmium in isolation and co-exposure on post-mitochondrial reduced glutathione levels in prostate.



Values are expressed as mean ± SEM (n=6 in each group).
*P<0.05 vs. control and $P<0.05 vs. cadmium group.

Figure 15. Effect of lead and cadmium in isolation and co-exposure on reduced glutathione levels in pituitary.



Values are expressed as mean ± SEM (n=6 in each group).
*P<0.05, ** P<0.01 vs. control group.

disturb protein function (Stanimirovic et al., 1995; Rohn et al., 1996). It has been also demonstrated that DNA adducts can be formed from reactive LPO products (Bartsch, 1999). Earlier studies from our laboratory have reported changes in membrane fluidity with both lead and cadmium in female rats (Pillai et al., 2002). Higher TBARS in the mitochondria than in the post-mitochondrial fraction of testis, suggests more ROS generation in mitochondrial fraction. The enhanced lipid peroxidation in the present study indicates failure of antioxidant defense mechanism. This is further supported by our results of antioxidant enzymes activity.

In mitochondrial and/or post mitochondrial fraction of testis and liver, the activities of antioxidant enzymes super oxide dismutase, catalase, GR, GST and G6PDH were decreased whereas the activity of GPx was increased. 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. The decreased SOD activity is due to replacement of the zinc (Zn) and manganese (Mn) of the SOD molecule in post-mitochondrial and mitochondrial fraction respectively by lead (Kagi and Vallee,

1961) and cadmium (Jacobson and Tumer, 1980). Zn is an essential component of the oxidant defense system, functioning at many levels (Sato and Bremner, 1993). Reduction in the activity of catalase may reflect inability of testis cytosol to eliminate H_2O_2 produced. This may also be due to enzyme inactivation caused by ROS production in cytosol (Pigeolet et al., 1990). Hence an imbalance between O_2^- and H_2O_2 might occur in the testis and liver of lead and cadmium treated rats; the decrease in SOD and catalase activities thus might indirectly lead to an increase in oxidative stress, causing ROS induced damage to macromolecules such as DNA, proteins and key enzymes involved in testicular steroidogenesis and spermatogenesis. Alternate route of H_2O_2 elimination would seem to be via GPx. To counter the deleterious action of ROS, antioxidant enzymes are also synthesized in response to the higher production of ROS. Thus from the data, there is increased activity of GPx in metal treated animals suggesting its role in protection from oxidative stress in response of higher production of ROS. Such observations were also demonstrated by Jian-Ming et al., 2003; An-Sik and Mahin, 1987 using leydig and sertoli cells. GSH, the substrate for GPx activity is also depleted. 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. One of the mechanism for the observed decrease in GSH content in the present study could be the binding of these divalent metals with -SH groups (Hsu, 1981; 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). Less availability of GSH in the testis and liver is further compounded by low G6PDH activity. Although NADPH, the substrate for glutathione reduction can be generated by systems other then G6PDH, such as isocitrate dehydrogenase or glutamate dehydrogenase (Satav and Katyare, 2004). It is not clear at this stage whether these alternate systems are also affected by 15 days metal exposure.

In pituitary, only cadmium exposed group exhibited significant increase in TBARS suggesting altered antioxidant defense mechanism. The low level in Pb and co-exposed group was insufficient to cause oxidative stress. This could

be due to the significantly increased activity of SOD and GPx in Pb and co-exposed treated animal as observed in the present study. Decreased GSH content could be due to the sulfhydryl reactive nature of Pb and Cd (Hsu, 1981; Nigam et al., 1999). It is interesting to note that basal activity of catalase is very low in pituitary and prostate as compared to testis and liver (Zini et al., 1996). In our studies, basal activity of catalase is pituitary<prostate<testis<liver. These kinds of tissue specific drastic differences were not observed in any other antioxidant enzyme activity. 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). The low levels of catalase mRNA in reproductive organs was compared with the high levels of expression detectable in rat liver (Zini et al., 1996). Effect obtained on the activity of liver catalase is significantly low as observed earlier in female reproductive system (Pillai et al., 2005). Significant decrease in catalase activity could be due to the replacement of Fe^{+2} present at the active site of enzyme.

Results thus 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 least effects (discussed in Chapter 3). Both metals are causing depletion in GSH (thiol containing enzymes) along with various antioxidant enzymes leading to the induction of free radicals which in turn increase lipid peroxidation both at mitochondrial and postmitochondrial fraction and finally resulting in disruption of various functions.

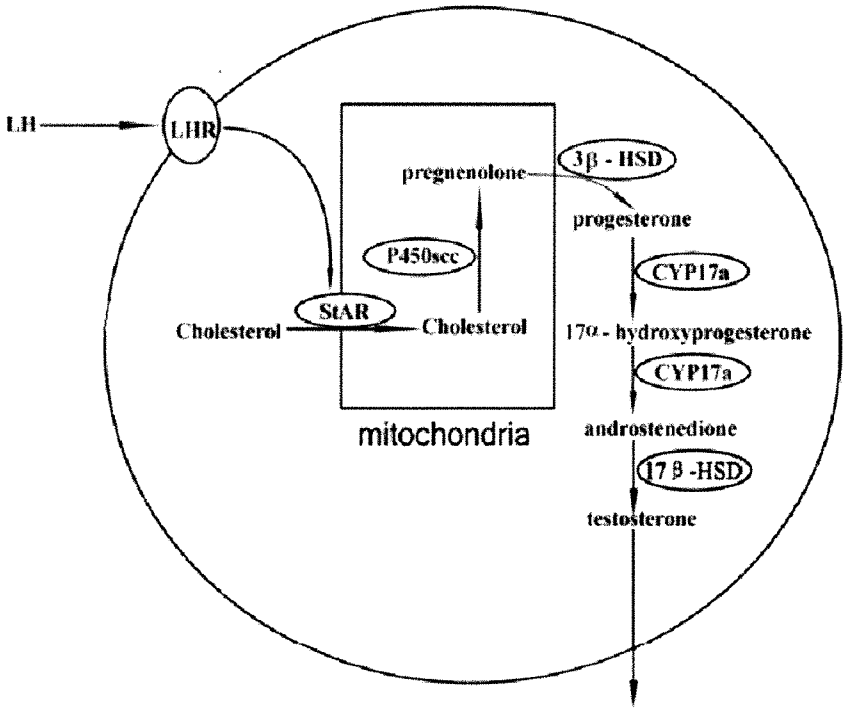
Thus oxidative tissue damage seems to be one of the basic causes of various dysfunctions observed in earlier chapters in relation to hypothalamic-pituitary-testicular-hepatic axis. Tissue specific difference in alteration of antioxidant enzyme status can also be correlated with accumulation of metals in various tissues as noted in earlier chapter 2.

5.2 Molecular mechanism

5.2.1 Introduction

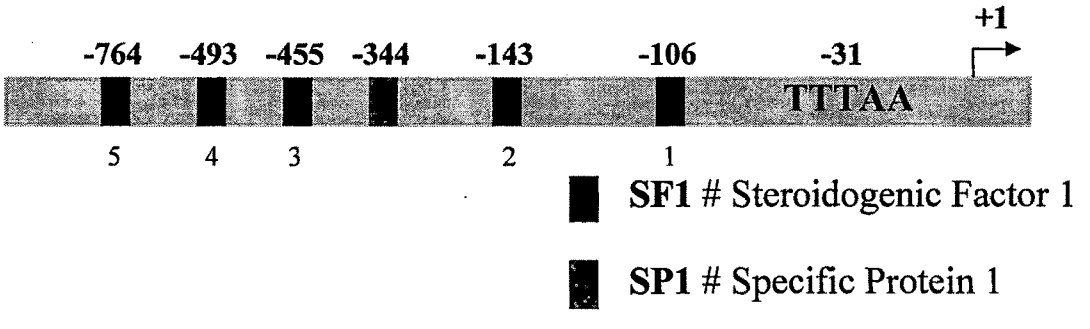
Steroid hormones play a crucial role in the differentiation, development, growth, and physiological function of most vertebrate tissues. The major pathways of steroid hormone synthesis are well established, and the sequence of the responsible steroidogenic enzymes has been elucidated (Miller, 1998; Grumbach and Auchus 1999) (Figure 16). In testis, biosynthesis of testosterone occurs in leydig cells. At leydig cells, transportation of cholesterol from cytosol to mitochondria is governed by StAR protein, followed by the action of two different classes of enzymes like cytochrome P450s and hydroxysteroid dehydrogenases. One of the major differences between the P450 enzymes and the hydroxysteroid dehydrogenases is that each of the P450 enzymes is a product of a single gene, whereas there are several isoforms for the 3βHSDs and several isozymes of the 17βHSDs, each a product of a distinct gene. The number of isoforms or isozymes varies in different species, in tissue distribution, catalytic activity (whether they function predominantly as dehydrogenases or reductases), substrate, cofactor specificity, and subcellular localization.

Figure 16. Steroidogenic pathway



Steroidogenic acute regulatory (StAR) protein is synthesized in response to tropic hormones. Gonadotropins activate expression of their target genes via the cAMP second messenger system. It has been demonstrated that cAMP administration to rat luteal cells stimulates expression of both StAR messenger RNA and protein (Tood et al., 1998). Rat StAR promoter was characterized and sequence analysis demonstrated that the transcription start site was located 82 bp upstream of the translational start codon (ATG) of the rat StAR gene. This site is 27 bp downstream of the TATA-like element. A TATA-like element was identified 31 bp upstream of the StAR transcription start site. Promoter sequence analysis revealed the presence of multiple regulatory elements similar to the consensus sequences for the Steroidogenic Factor -1 (SF-1) binding site (CCTTG), the estrogen receptor half-site (AGGTCA), and the AP-1 site (GTCGTCA). These regulatory elements included five putative SF-1 binding sites at positions -764, -493, -455, -143 and -106, a putative estrogen receptor half-site at position -137, a putative SP1 site at position -344, and two putative AP-1 elements at positions -1561 and -187. Studies also provide the evidence that the rat StAR gene is regulated by SF-1 at the transcriptional level. The three high affinity sites and two low affinity sites are shown in Figure 17. High affinity sites have been designated SF-1 binding sites 1, 3, and 5 (SFB-1, SFB-3, and SFB-5) and the low affinity designated SF-1 binding sites 2, 4 (SFB-2 and SFB-4). Both high and low affinity SF-1 motifs are used by SF-1 to mediate rat StAR gene transcription, both at a basal level and in response to stimulation with cAMP.

Figure 17. Rat StAR promoter (Todd et al., 1998; Busygina et al., 2003)



The promoter regions of all steroidogenic P450 genes contain regulatory elements that have similar AGGTCA motifs. These motifs interact with a common DNA-binding protein, alternatively designated adrenal-4 binding protein (Ad4BP) or steroidogenic factor 1 (SF-1) (Honda et al., 1990; Morohashi et al., 1992; Clemens et al., 1994). SF-1 has a 2 zinc finger domain and a putative ligand binding/dimerization domain, is an orphan member of the steroid/thyroid hormone receptor superfamily (Evans, 1988). All steroidogenic tissues examined (adrenal, ovary, testis, placenta, adipocyte and brain) express SF1 mRNA (Lala et al., 1992; Honda et al., 1993). In situ hybridization (Ikeda et al., 1993) and immunohistochemical staining (Morohashi et al., 1994; Hatano et al., 1994) of the adrenal glands, testes and ovaries of adult rat or mice localized SF1 expression to the specific steroid hormone-producing cells in the tissues, i.e. adrenocortical cells in the adrenal gland, leydig cells in the testis and granulosa and theca cells in the ovary.

Many laboratories showed that SF-1 acts at multiple levels of the hypothalamic-pituitary-steroidogenic organ axis to regulate the expression of many genes that are important for regulated steroidogenesis (Bakke et al., 2001). SP1 binds to GC box promoters elements and selectively activates mRNA synthesis from genes that contain functional recognition sites. Chain length of SP1 is 786 amino acids. Three zinc fingers are presents at 627 to 656, 657 to 686 and 687 to 714 amino acid residues. Sp1 mediates cAMP-dependent transcription of the P450scc gene (Liu and Simpson, 1997). Sp1 and SF-1 interact and cooperate in the regulation of human steroidogenic acute regulatory protein gene expression.

Once cholesterol is transported across mitochondria, the key enzyme cholesterol side-chain cleavage cytochrome P-450 (P-450scc or CYP11a) catalyzes the first rate-limiting step in steroid hormone biosynthesis (Privalle et al., 1983). The entire rat P-450scc gene has been cloned by (Ria et al., 1990). The rat P-450scc gene, includes nine exons, the intron-exon boundaries and the unusual sequences delineating the exon-intron boundary of exon VI, is highly conserved with that of the human gene (Morohashi et., 1987). Sequence analysis of the 940

bp of 5'-flanking DNA of the rat gene revealed a putative TATA box (TATAA) beginning 71 bp upstream from the translational ATG start codon. Also, five putative CRE-like elements in the 5' upstream region and a putative SP-1 binding element (GC box) is present in the nucleotide sequence of the rat P-450scc gene. The transcriptional start site of the rat P-450scc gene was mapped, and the predominant extended product had a size of 103 nucleotides, which mapped the transcription start site to 32 bp upstream of the initiator methionine codon and 24 bp downstream from the TATA box.

Once cholesterol is converted by CYP11a, the two key enzymes that play a role in testosterone steroidogenesis are 3β-HSD and 17β-HSD.

Multiple 3β-HSD isoenzymes have been cloned from several other species, further illustrating that the 3β-HSD gene family is conserved in vertebrate species. Table 6 summarizes the kinetic parameters and major expression sites of 3β-HSDs from rat.

Table 6. Expression sites of 3β-HSDs from rat.

Species	Type	K_m PREG/DHEA (μM)	Cofactor	Major sites of expression
Rat	I	<1	NAD^+	Adrenals and gonads
	II	>10	NAD^+	Adrenals and gonads
	III	3-KSR	NADPH	Male liver
	IV	<1	NAD^+	Placenta and skin

The tissue-specific expression of multiple members of the 3β-HSD family was first demonstrated in the rat (Zhao et al., 1990). The structures of four members of the rat 3β-HSD family have been characterized (Zhao et al., 1990; Zhao et al., 1991; Simard et al., 1993). With the exception of type III, all isoenzymes catalyze the transformation of 5-pregnen-3β-ol and 5-androsten-3β-ol steroids into the corresponding Δ^4-3-ketosteroids as well as the interconversion of 3β-hydroxy- and 3-keto-5α-androstane steroids. The rat type I and II 3β-HSD share 93.8% identity. The type III protein shares 80% identity with the type I and II proteins but, in contrast to other types, is a specific 3-KSR

(ketosteroid reductase). The rat type IV protein shares 90.9, 87.9, and 78.8% identity with that of types I, II, and III proteins, respectively, and is the prominent mRNA species detectable in the placenta and the skin (Simard et al., 1993). The activities of rat types I and IV are similar (Simard et al., 1993), whereas there is much lower enzyme activity for the type II compared with the type I, which could be due to a change in four amino acid residues located in a putative membrane-spanning domain, between residues 75 and 91 (Zhao et al., 1990).

17β-hydroxysteroid dehydrogenase (17β-HSD) is one of the essential enzymes involved in the regulation of intracellular levels of biologically active androgens and estrogens in gonadal and extragonadal tissues (Labrie et al., 1994). Seven distinct 17βHSD isoenzymes have been cloned and characterized from various tissues and are designated types I, II, III, IV (Penning, 1997; Andersson, 1995; Luu-The, 1995); V (5); VI (Biswas and Russell, 1997); and VII (Nokelainen et al., 1998). Types I-IV, VI, and VII are members of the short chain alcohol dehydrogenase super family (Penning, 1997; Biswas and Russell, 1997; Nokelainen et al., 1998). However, type V belongs to the aldo-keto reductase family (Deyashiki et al., 1995). The 17β-HSD isoenzymes differ in their tissue/cellular localization, substrate specificities, cofactor requirements, and preference for oxidation or reduction reactions. Three of the isoforms, types I, III, and VII, catalyze the reductive reaction, whereas the other four preferentially catalyze the oxidation reaction.

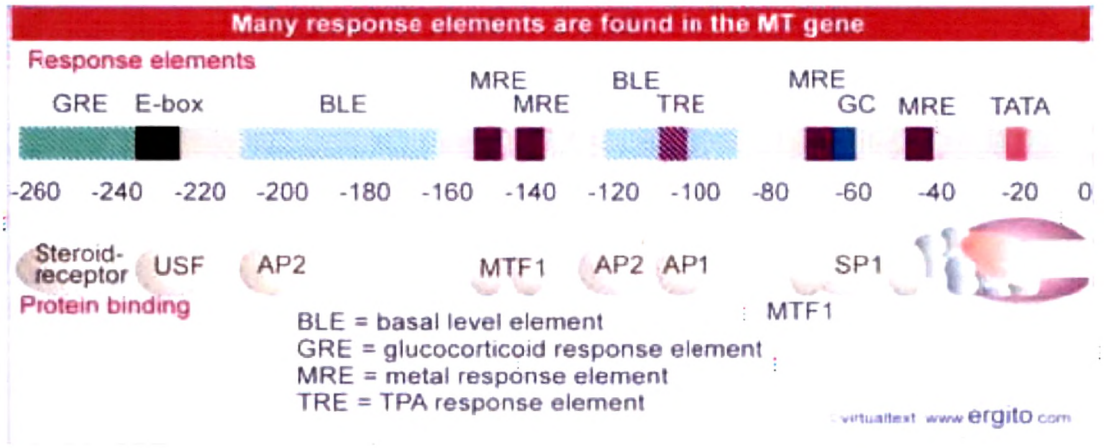
A full-length cDNA (1111 bp) of 17β-HS III was cloned from a rat Leydig cell cDNA library by Chon et al., 1999. The type III enzyme appears to be testis specific (Geissler et al., 1994). It contains 21 bases of 5'-untranslated sequence, an open reading frame of 918 nucleotides encoding an hydrophobic protein of 306 amino acids with an apparent molecular mass of 33.7 kDa, and 172 bases of 39-untranslated region.

Apart from these genes or enzymes needed to maintain steroid homeostasis, various other genes are importantly elucidated for maintenance of

cellular function when exposed to stress conditions like heavy metals. One of major protein is metallothionein.

The metallothionein (MT) gene provides an example of how a single gene may be regulated by many different circuits. The metallothionein protein protects the cell against excess concentrations of heavy metals, by binding the metal and removing it from the cell. The gene is expressed at a basal level, but is induced to greater levels of expression by heavy metal ions (such as cadmium) or by glucocorticoids. The control region combines several different kinds of regulatory elements. The organization of the promoter for a MT gene is summarized in Figure 18. A major feature of this map is the high density of elements that can activate transcription. The TATA and GC boxes are located at their usual positions fairly close to the startpoint. Also needed for the basal level of expression are the two basal level elements (BLE), which fit the formal description of enhancers. Although located near the start point, they can be moved elsewhere without loss of effect. They contain sequences related to those found in other enhancers, and are bound by proteins that bind the SV40 enhancer.

Figure 18. Promoter organization of the MT gene.

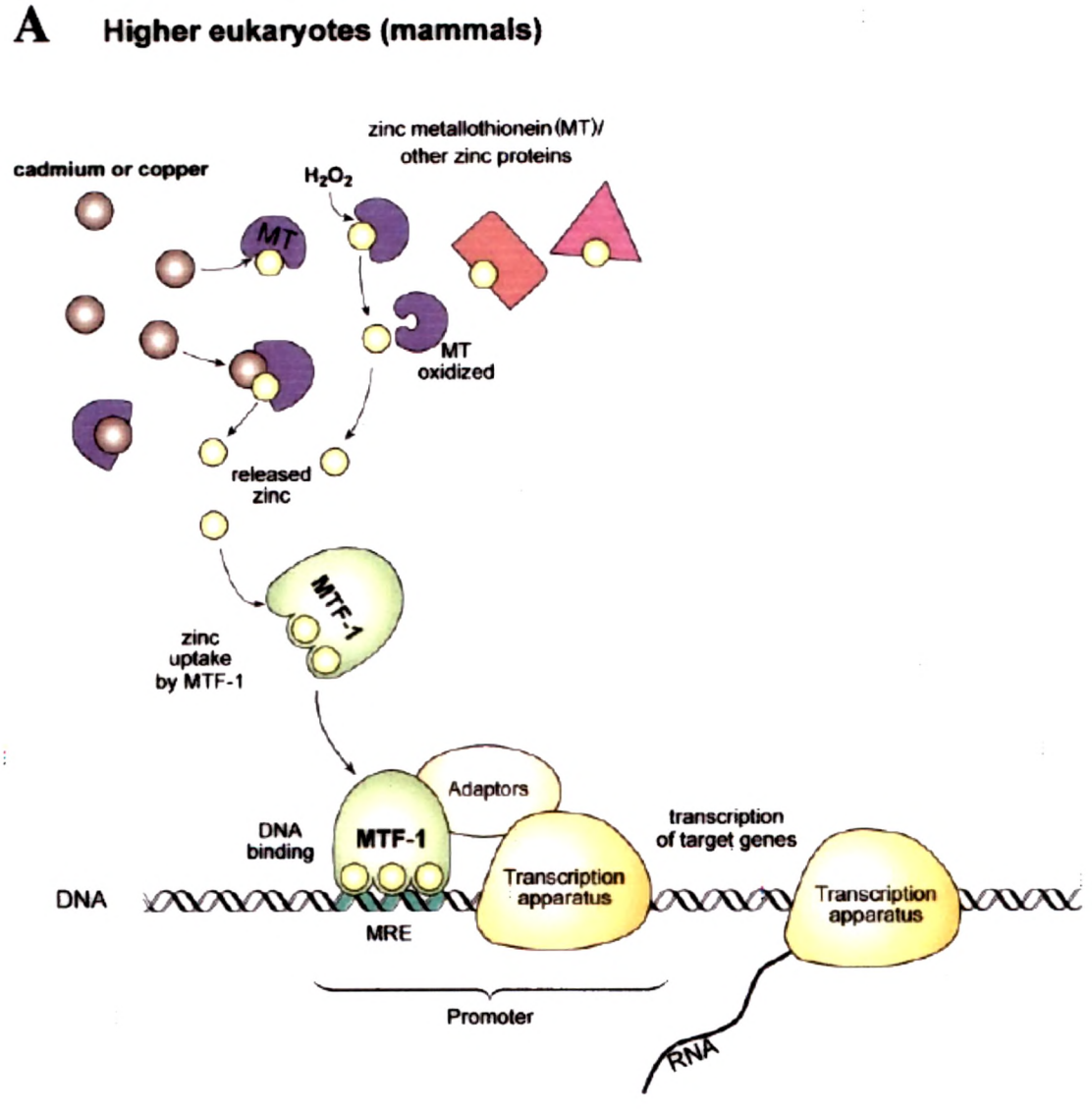


The TRE has a binding site for factor AP1; this interaction is part of the mechanism for constitutive expression, for which AP1 is an activator. However, AP1 binding also has a second function. The TRE confers a response to phorbol

esters such as TPA (an agent that promotes tumors), and this response is mediated by the interaction of AP1 with the TRE.

The inductive response to metals is conferred by the multiple MRE sequences, which function as promoter elements. The presence of one MRE confers the ability to respond to heavy metal; a greater level of induction is achieved by the inclusion of multiple elements. The factor MTF1 binds to the MRE in response to the presence of metal ions. The response to steroid hormones is governed by a GRE, which behaves as an enhancer.

Figure 19. Mechanism of MTF-1 activation by heavy metal.



Heavy metal-responsive transcription in mammals is recently demonstrated by Zhang et al., 2003. Figure 19 showed model for MTF-1 activation by cadmium, copper, and hydrogen peroxide (H2O2) via zinc-loaded metallothionein (Zn7-MT). Cadmium and copper bind to metallothionein (MT) with a much higher affinity than zinc, but due to the great abundance of the latter, the majority of metallothionein under physiological conditions is present as Zn7-MT. Upon cadmium or copper loading, zinc is released from metallothionein and presumably from other cellular proteins and allows zinc saturation of MTF-1, which requires a higher zinc concentration for DNA binding than other typical zinc finger proteins such as Sp1. H2O2 also induces zinc release from metallothionein via oxidation of sulfhydryl groups. In contrast to cadmium and other agents, which are postulated to activate MTF-1 indirectly, elevated zinc concentrations activate MTF-1 directly, i.e., without the need for zinc release from other proteins. (Palmiter, 1994).

5.2.2 Experimental design

The groups of animals and the treatment of the metal were similar as described (5.1.2 Experimental design). After 15 days of exposure animals were sacrificed and testis were removed. Semiquantitative RT-PCR was carried out for gene expression analysis. Total RNA was extracted from the testes by the basic protocol of Chomczynski and Sacchi, 1987 (Figure 20). RNA samples of 7.5 μg were reverse transcribed for 50 min at 42°C in a 40-μl reaction mixture with 400 units of Superscript II reverse transcriptase and 1 μg of oligo(dT) 12-18 primer according to the standerd protocol of the supplier. Each PCR reaction mixture (50μl) contained 0.2 mM of each dNTP mixture, 0.4 μM of each primer, and 1 μl of the cDNA. Taq polymerase was added to the reaction tube as 2.5 units. PCR was performed by denaturing at 94°C for 30 sec, annealing for 30 sec, and extension at 72°C for 45 sec. Table 7 shows the primer sequences, PCR-product sizes, optimized cycles, annealing temperature and Gene Bank accession numbers for all genes examined in this study. The genes coding Steroid acute regulatory protein (StAR; Figure 21), cytochrome P450 side chain cleavage

(P450scc or CYP11; Figure 22), 3β-hydroxysteroid dehydrogenase type I (3βHSD; Figure 23), 17β-hydroxysteroid dehydrogenase type III (17βHSD; Figure 24) were examined. Metallothionein I (MT I; Figure 25), Metallothionein II (MT II; Figure 26) and β-actin expression analysis were also carried out. The PCR products (5 µl) were then separated on 1.5% agarose gel. The bands on the UV-transilluminated gel were converted into digital images with a gel analyzer and the amounts of RT-PCR products were quantified with Images software (Image J138). PCR product of β-actin was used as an internal standard.

Table 7. Primer sequences, PCR-product sizes, optimized cycles, annealing temperature and GenBank accession numbers for all genes studied.

Sr. No.	Gene name		Primer sequence (5' to 3')	Product length (bp)	Accession No.	Annealing Temp.
1.	CYP11a	F	AGA TCC CTT CCC CTG GTG ACA ATG	510	NM_017286	57°C
		R	CCA GGC GCT CCC CAA ATA CAA CA			
2.	StAR	F	AGG CAG GGG GAT CTT TCT AA	330	NM_031558	61°C
		R	TGC CTG ACT AGG GTT TCG TT			
3.	3β-HSD I	F	ATG CCC AGT ACC TGA GGA GA	427	M38178	61°C
		R	TTG AGG GCC GCA AGT ATC A			
4.	17β-HSD III	F	CCTCCGTAGTCAAGATGACA	333	NM_054007	57°C
		R	CAAGGCAGCCACAGGTTTCA			
5.	MT I	F	ACT GCC TTC TTG TCG CTT A	310	NM_138826	55°C
		R	TGG AGG TGT ACG GCA AGA CT			
6.	MT II	F	CCA ACT GCC GCC TCC ATT CG	300	XM_001070713	56°C
		R	GAA AAA AGT GTG GAG AAC CG			
7.	β-actin	F	CCT GCT TGC TGA TCC ACA	505	V012017	57°C
		R	CTG ACC GAG CGT GGC TAC			

5.2.3 Results

In the present study the effects of Pb and Cd exposure on mRNA expression of genes involved in cholesterol transport and steroidogenesis in testis of male rats were determined. Expression of StAR gene responsible for cholesterol transport to the inner mitochondrial membrane, were significantly reduced to 52%, 44% and 40% of the controls on treatment with Pb, Cd and co-exposure (Pb and Cd) 0.025mg /kg/15days, respectively ($p < 0.05$). Compared to the control, expression of P450scc, an enzyme that catalyzes cholesterol side

chain cleavage to form pregnenolone, was markedly reduced to 62% (Pb exposure), 76% (Cd exposure) and 61% (co-exposure of Pb and Cd) ($p < 0.01$). A significant reduction in mRNA levels was observed for 3β-HSD and 17β-HSD and maximum reduction (40% & 38% respectively) was observed in case of cadmium treated group ($p < 0.05$). Similarly, metallothionein, which plays an important role in metal ion detoxification, especially Cd. Metallothionein I and metallothionein II expression, was also notably increased by 16% and 11% respectively of the controls when Cd is given ($p < 0.05$). Pb and co-exposed groups does not exhibit any significant changes compared to control.

Figure 20. Total RNA extraction from testis (Chomczynski P and Sacchi N, 1987).

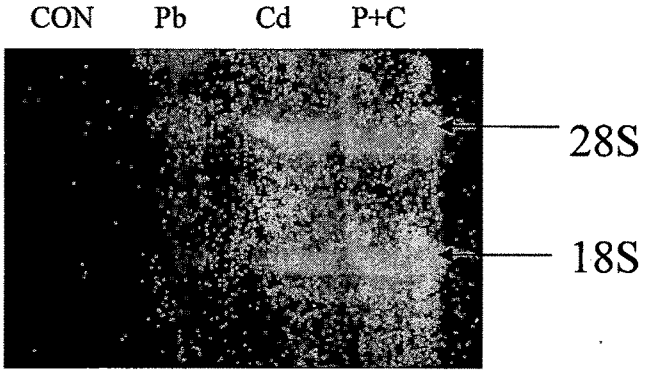
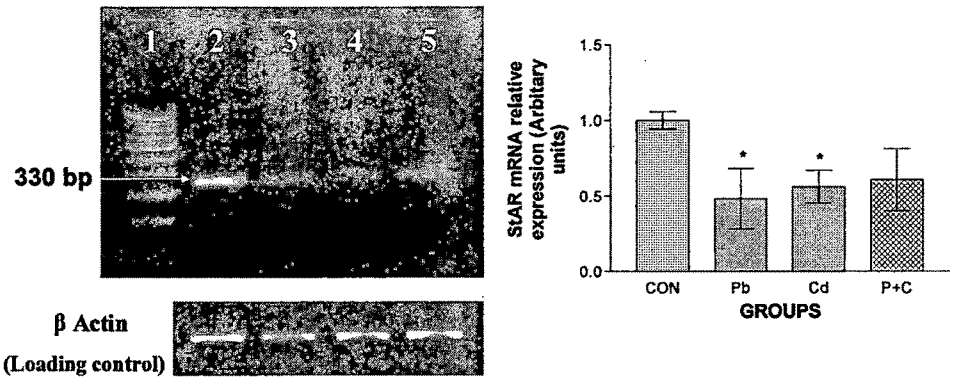


Figure 21. Effect of lead and cadmium in isolation and co-exposure on StAR protein expression analysis in testis.

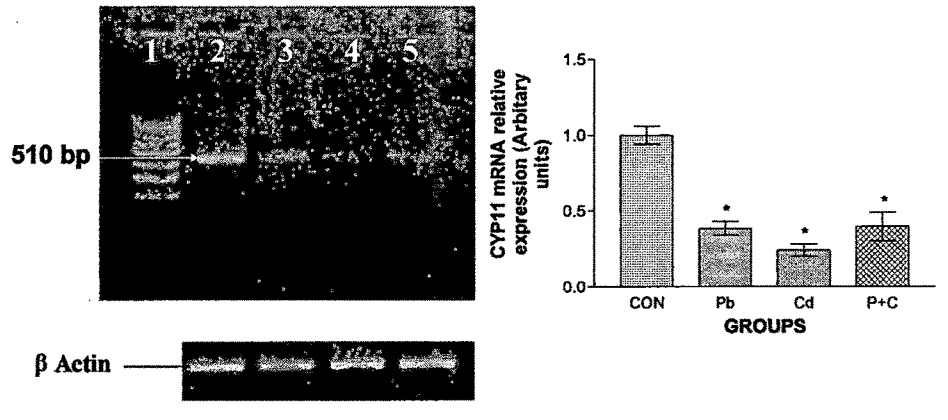
1	2	3	4	5
100 bp ladder	CON	Pb	Cd	P+C



Values are expressed as mean ± SEM (n=3 in each group). *P<0.05 vs. control group.

Figure 22. Effect of lead and cadmium in isolation and co-exposure on CYP11 expression analysis in testis.

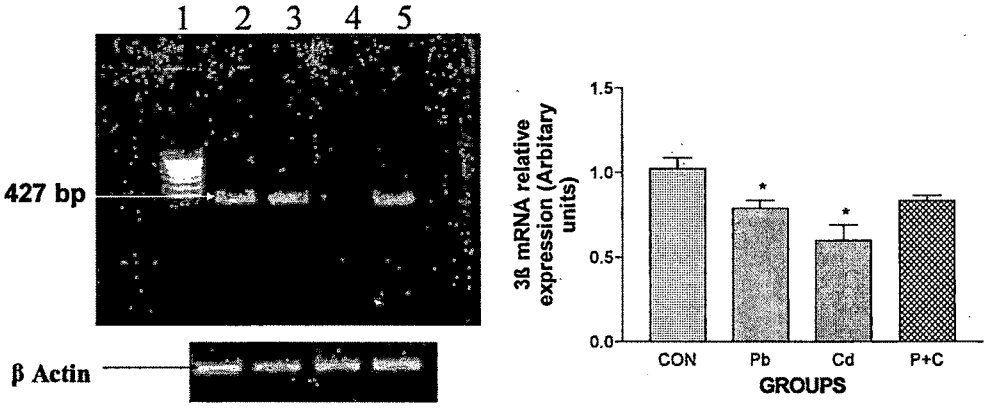
1	2	3	4	5
100 bp ladder	CON	Pb	Cd	P+C



Values are expressed as mean ± SEM (n=3 in each group). *P<0.01 vs. control group.

Figure 23. Effect of lead and cadmium in isolation and co-exposure on 3β-HSD expression analysis in testis.

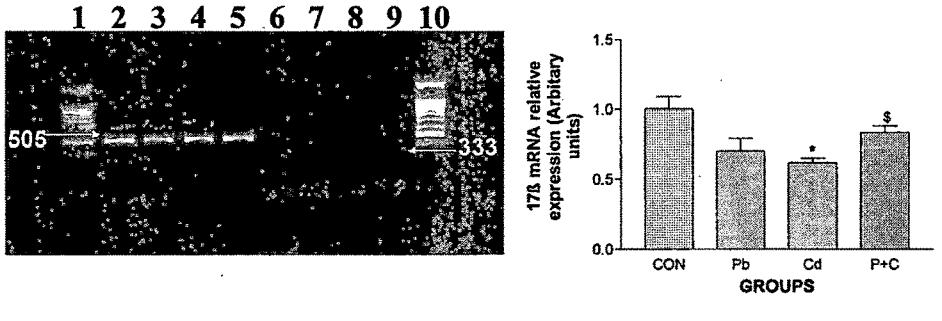
1	2	3	4	5
100 bp ladder	CON	Pb	Cd	P+C



Values are expressed as mean ± SEM (n=3 in each group). *P<0.05 vs. control group.

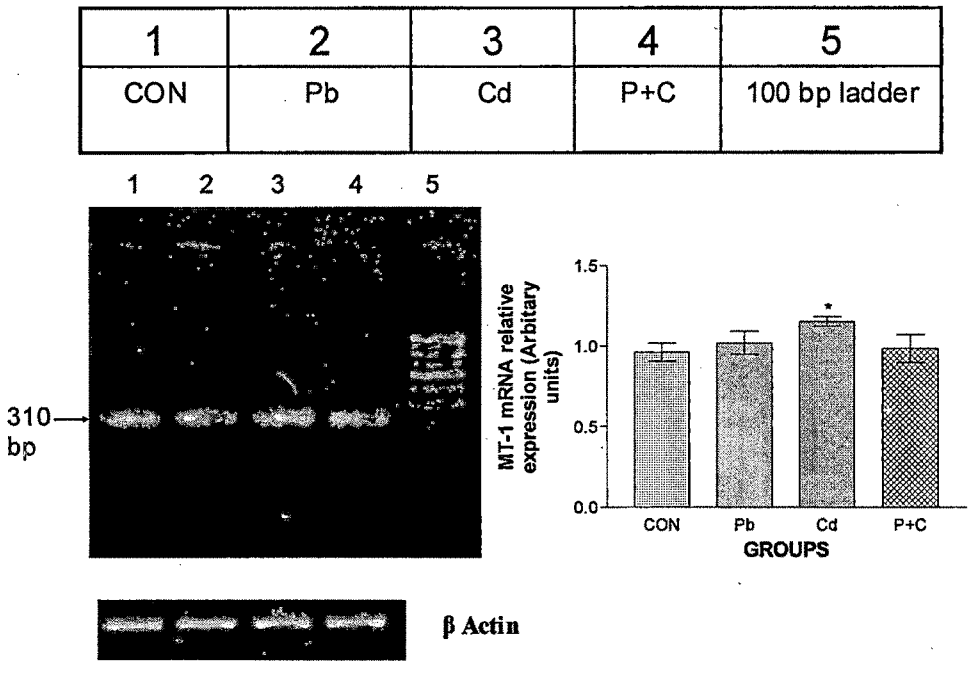
Figure 24. Effect of lead and cadmium in isolation and co-exposure on 17β-HSD expression analysis in testis.

1	2	3	4	5	6	7	8	9	10
	β-Actin				17β-HSDH				
100 bp plus ladder	CON	Pb	Cd	P+C	CON	Pb	Cd	P+C	100 bp plus ladder



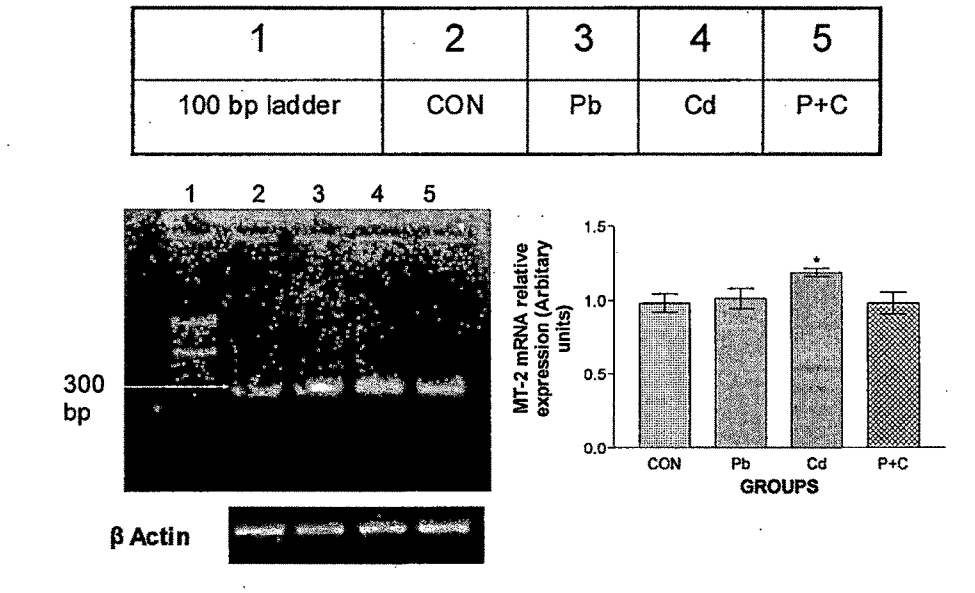
Values are expressed as mean ± SEM (n=3 in each group). *P<0.05 vs. control and $P<0.05 vs. cadmium group.

Figure 25. Effect of lead and cadmium in isolation and co-exposure on metallothionein I (MT-I) expression analysis in testis.



Values are expressed as mean ± SEM (n=3 in each group). *P<0.05 vs. control

Figure 26. Effect of lead and cadmium in isolation and co-exposure on metallothionein II (MT-II) expression analysis in testis.



Values are expressed as mean ± SEM (n=3 in each group). *P<0.05 vs. control

5.2.4 Discussion

In this study, an accompanying decline was observed for mRNA levels of genes responsible for cholesterol transport and steroidogenesis including StAR, P450scc, 3β-HSD and 17β-HSD in rats exposed to Pb and Cd in isolation and in combination. Induction of metallothionein I and II was observed in cadmium exposed rats. The ability of Pb and Cd co-exposure to disrupt steroidogenesis and the mechanisms by which the compound interferes with the function of steroidogenic enzymes is a relatively unexplored area of toxicology. This report is the first to thoroughly investigate the possible molecular mechanisms of serum testosterone reduction and to report alterations in steroidogenic enzyme activity in the testes following co-exposure to Pb and Cd (as reported in chapter 3).

Xenobiotic metals such as cadmium and lead can induce a variety of adverse physiological responses in rodents and humans, including carcinogenesis and reproductive dysfunction (Goyer, 1996). These effects are thought to be mediated through metal ion-protein interactions of a variety of cellular targets; in some cases, the metal ions are complexed with certain proteins in detoxification mechanisms (Goyer, 1983, 1984). Because cysteine amino acids in proteins are highly reactive to electrophilic metal ions, proteins containing such residues are proposed to be primary targets for metal ions, especially those in the heavy-element category (Thomas and Wofford, 1983). An important class of cysteinerich proteins are the regulatory factors that contain Cys2His2 zinc-binding domains first identified in transcription factor IIIA (TFIIIA) and referred to as "zinc fingers" (Hanas et al., 1983; Miller et al., 1985). Cysteine-rich zinc finger proteins are proposed to be cellular targets for many xenobiotics, including metal ions like Pb and Cd (Sunderman and Barber, 1988). Mechanistic effects of cadmium ions on zinc finger structure were examined in the steroid hormone receptor and transcription factor IIIA (Simons et al., 1990; Predki and Sarkar, 1992; Hanas and Gunn, 1996). Both of these proteins are prototypes of transcription factor superfamilies. Members of the hormone receptor family contain two Cys2Cys2 zinc-binding domains and members of

the TFIIIA superfamily contain various numbers of Cys2His2 zinc-binding domains. Divalent metal ions such as cadmium and arsenic display increased avidity for two closely spaced thiols in a vicinal orientation (Joshi and Hughes, 1981). Micromolar concentrations of cadmium ions were found to inhibit the DNA binding mechanism of the prototypical Cys2His2 zinc finger protein, TFIIIA (Hanas et al., 1996; Hanas and Gunn, 1996). Metals such as lead interfere with the DNA binding properties of transcription factors, Sp-1 and Egr-1, both *in vivo* and *in vitro* (Zawia et al., 1998). Lead could also directly interfere with the DNA binding of a recombinant human Sp 1 protein. The action of lead on Sp1, Egr-1 and TFIIIA suggests that it can also target other cellular proteins that contain the zinc finger motif and reveals this protein domain as a potential mediator for lead induced alterations in protein function. It was also found that the zinc finger motif accommodates metals such as cadmium. Cd has been shown to change the binding characteristics of the SP1 transcription factor (Thiesen and Bach, 1991). Cd can replace Zn in the ER finger and still bind functionally to DNA. However, the cooperativity values for Cd are different from those of the normal dimer (Sarkar, 1995). A change in this cooperativity can result in "recognition of a sequence that the protein would not normally bind". This has been suggested a mechanism leading to differences in transcription, as well as DNA redox damage (Sarkar, 1995).

Reports suggests that transcription factors such as steroidogenic factor 1 (SF1), specific protein 1 (SP1) or activated protein 1 (AP1) are required for proper expression of steroidogenic mRNA (Teruo et al., 2000).

Expression pattern of StAR and other steroidogenic genes i.e. CYP11 (P450scc), 3βHSD and 17βHSD exhibited reduction in metal treated groups. Organization of StAR and CYP gene clearly demonstrated binding sites for SF1 and SP1, the important transcriptional factor for their transcription. Thus interaction of metals with above transcriptional factors seems to be the major mechanism for decreased mRNA level in metal treated groups. As mentioned earlied gonadotropins cause cAMP mediated regulation of StAR protein for cholesterol mobilization. CYP11 also has CRE element. Thus disruption of

hypothalamic pituitary axis (as reported in chapter 3 & 4) further will lead to decreased gonadotropin mediated effect (cAMP) and will lead to reduced transcription of steroidogenic genes. The increase in the mRNA levels of MT I and MT II could be due to the increase in free concentration of zinc, cadmium, which activates the MTF-1 leading to increase transcription as suggested by Zhang et al., 2003.

Thus lead and cadmium in isolation and combination affects steroidogenesis both at molecular level and activity level (Chapter 3) and thus completely disrupt HPG axis and reproductive functions.

5.3 Cellular mechanism

5.3.1 Introduction

Testis is composed of two major cells, namely sertoli cells and leydig cells. These cells control the testicular functions, by the production of various steroid hormones and spermatogenesis. The principal function of testicular leydig cells (LCs) is the production of androgens. The expression of steroidogenic enzymes involved in testosterone synthesis is a functional characteristic of mature LCs in the testis. Morphological structure and biochemical function of LCs are dependent upon luteinizing hormone (LH) secreted from the pituitary (Teerds et al., 1989). The receptor for LH (LHR) and FSH (FSHR) are located on the plasma membrane and belongs to a G-protein-coupled receptor family. Thus LCs are very useful in the investigation of regulatory mechanisms of synthesis and secretion of testosterone.

Lead is known to affect the binding of Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH) (Wiebe *et al.*, 1988) and also decrease the gonadal steroid levels (Paksy *et al.*, 2001). Similarly, cadmium is also known to decrease the serum gonadotropins (Paksy *et al.*, 1989) and gonadal steroids (Piasek and Laskey, 1999).

In earlier chapter, it is demonstrated that testicular steroidogenesis is inhibited by lead and cadmium, both in isolation and in combination at a dose of 0.025 mg/kg body wt daily for 15 days. In the present study, an attempt has been made to study the effects of lead and cadmium either in isolation or in

combination at cellular level (Leydig cells). Parameter evaluated includes gonadotropin binding and steroidogenic activity of leydig cells (*in vitro*). To the best of our knowledge, there is no study on simultaneous effect of lead and cadmium on hormone binding on leydig cells.

5.3.2 Experimental design

Testis was removed from normal rats and leydig cells were isolated (Bermúdez et al., 1988). Cells were purified using percoll density gradient centrifugation (Bermúdez et al., 1988). The purity of isolated Leydig cells was ascertained by staining for 3β-HSD activity as previously described (Aldred and Cooke, 1983). Leydig cells were plated in 24-well culture dishes, $2x10^5$ cells per well, and placed in a 5% CO2 incubator and 95% O_2 at 34 °C for 2 h. After incubation, most of the Leydig cells were attached to the substrate of the culture dishes. Media were changed and the fresh media containing Pb, Cd and Pb+Cd were added at a final concentration of 3 µM, 0.5 µM and 2 µM + 0.015 µM respectively. The doses designed were referring to the metal concentration equivalent to concentration reaching the testis in *"in vivo"* experiment (Chapter-2). After 24 h, media were collected for analysis of testosterone level by commercially available RIA kit (Immunotech, France). Leydig cells were harvested to assay cell viability by trypan blue exclusion. Each metal exposed group and the control group contained six parallel wells and all experiments were repeated three times.

For measurement of steroidogenic enzymes activity isolated and purified leydig cells were devided in to four groups each containing 1×10^5 cells/ml. Pb, Cd and Pb+Cd were added at a final concentration of 3 µM, 0.5 µM and 2 µM + 0.015 µM respectively for 1 hour. These cells were then assayed for 3β-HSD and 17β-HSD enzyme activity by spectrophotometeric method (Shivanandappa and Venkatesh., 1997).

Similarly, for the measurement of LH and FSH binding Radio Receptor Assay was performed (Thanki and Steinberger, 1976) with 1×10^5 purified leydig

cells incubated with pb, Cd and Pb + Cd. Percentage binding of LH and FSH to receptor was calculated by considering control as 100% binding.

5.3.3 Results

In the present study, we determined the effects of Pb and Cd exposure on both Δ^5-3β-HSD (Figure 27) and 17-β-HSD (Figure 28) enzyme activities in leydig cells as the markers of steroidogenesis. Cd exposed group exhibited maximum reduction in 3β-HSD ($P<0.05$) and 17-β-HSD ($P<0.05$) enzyme activities, while co-exposure showed antagonist effect, hence least toxic as compared to control group.

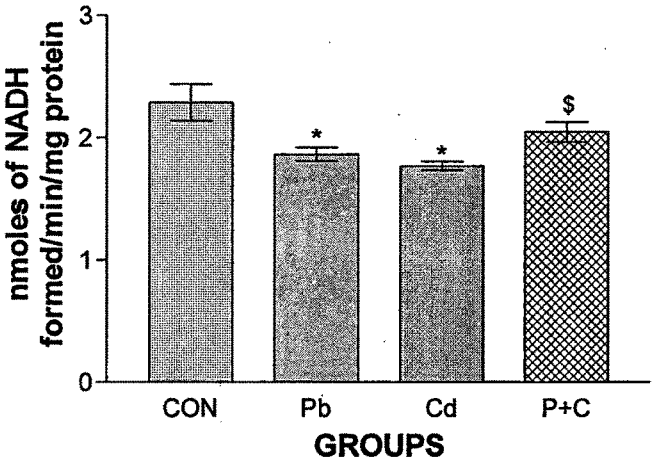
All the metal exposed groups showed significant reduction in the binding of LH to its receptors. As shown in Figure 29, Cd exposed cells showed a maximum reduction (33%, $P<0.01$) while Pb and combined metal (Cd + Pb) exposed groups demonstrated 21% ($P<0.01$) and 16% ($P<0.01$) reduction respectively in binding of ^{125}I-rLH as compared to control. Pb, Cd and combined exposed cells exhibited 10%, 24% ($P<0.01$) and 4% decrease respectively in ^{125}I-rFSH binding as compared to control groups (Figure 30).

The animals in the lead and cadmium exposed groups showed significant ($p < 0.05$) decrease in the testosterone secretion in media (Figure 31) as compared to the control group. Amongst all the treated groups, cadmium shows maximum decrease while co-exposure group did not exhibit significant reduction in testosterone levels compared to control. However, the cadmium exposed group exhibited significant decrease in testosterone secretion compared to co-exposed group.

5.3.4 Discussion

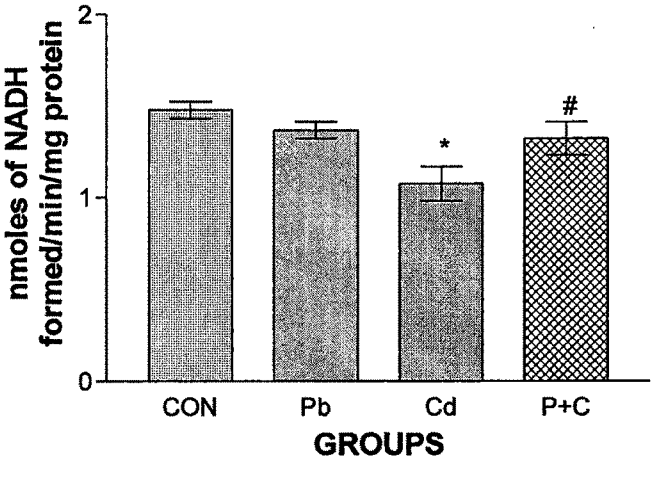
Pb and Cd are known reproductive toxicants. Pb and Cd are known to affect Hypothalamus pituitary testicular axis (HPT axis), but mechanisms governing a modulation at cellular level is not clearly elucidated. As we have observed, several changes at molecular level upon 15 days exposure, it was of great interest to study direct cellular effects of Pb and Cd on testicular leydig cells at concentration that directly reaches the testis.

Figure 27. ***In vitro*** **effect of lead and cadmium in isolation and co-exposure on 3β-HSD activity in leydig cells.**



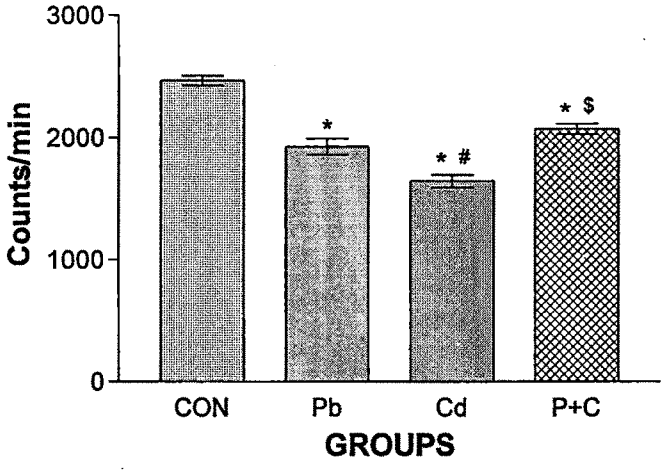
Values are expressed as mean ± SEM (n=5 in each group). *P<0.05 vs. control; and $P<0.05 vs. cadmium group.

Figure 28. ***In vitro*** **effect of lead and cadmium in isolation and co-exposure on 17β-HSD activity in leydig cells.**



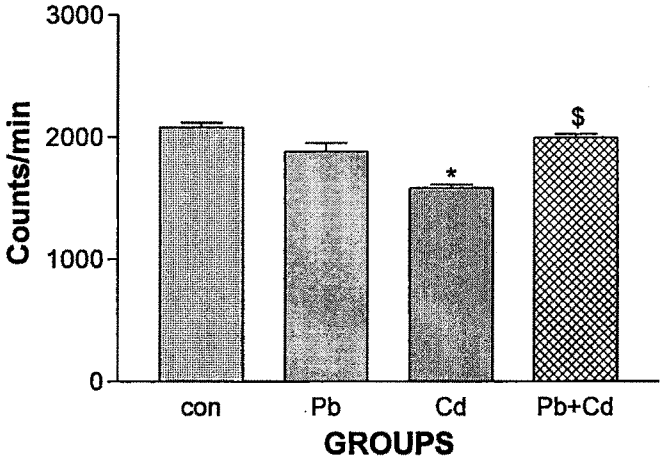
Values are expressed as mean ± SEM (n=5 in each group). *P<0.05 vs. control and #P<0.05 vs. cadmium group.

Figure 29. ***In vitro*** **effect of lead and cadmium in isolation and co-exposure on LH binding to leydig cells.**



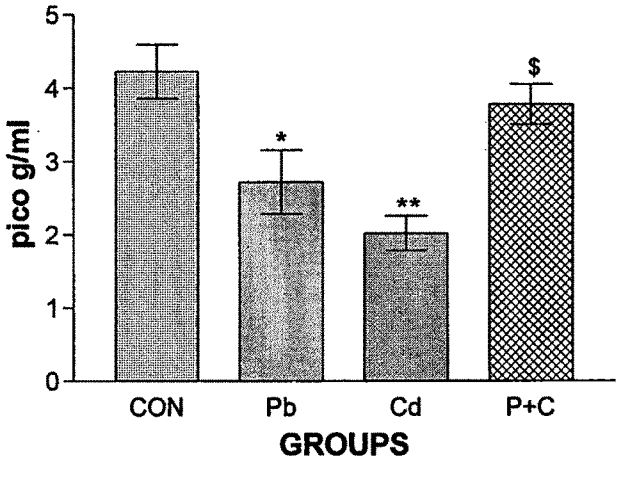
Values are expressed as mean ± SEM (n=5 in each group). *P<0.01 vs. control; # P<0.05 vs. lead and $P<0.01 vs. cadmium group.

Figure 30. ***In vitro*** **effect of lead and cadmium in isolation and co-exposure on FSH binding to leydig cells.**



Values are expressed as mean ± SEM (n=5 in each group). *P<0.01 vs. control and $P<0.01 vs. cadmium group.

Figure 31. ***In vitro*** **effect of lead and cadmium in isolation and co-exposure on testosterone secretion by leydig cells.**



Values are expressed as mean ± SEM (n=5 in each group). *P<0.05, ** P<0.01 vs. control and $P<0.05 vs. cadmium group.

We found significant decrease in both 3β-HSD and 17β-HSD enzyme activities in leydig cells exposed to Pb and Cd in isolation and in combination. Immediate decrease with in an hour in the activity obtained on exposure to Pb and Cd or both is much less as compared to in vivo 15 days exposure (chapter 3). Hence, this can be explained by the mechanism which cause direct effect at protein level. One mechanism is the binding of metal/s to the amino acids present at the active site of the enzyme which contain the Tyr-X-X-X-Lys at its active site (Persson et al., 1991). Secondly both Pb and Cd can bind to -SH groups of cysteine residue present at the NAD binding domain (Persson et al., 1991) and alter the structure of the substrate binding and thus enzyme function get significantly affected. Because cysteine amino acids in proteins are highly reactive to electrophilic metal ions, proteins containing such residues are proposed to be primary targets for metal ions, especially those in the heavy-element category (Thomas and Wofford, 1983) as discussed earlier (chapter 3).

Further effect of metal/s on gonadotropin binding (the major regulators in steroidogenesis) was evaluated. LH and FSH binding to its receptors, was found to be decreased in all metal exposed groups with Cd exhibiting maximum reduction. Possible reasons for decreased binding could be (1) co-operation of metal ions and receptor, (2) binding to amino acids like cysteine residues of the receptors or (3) interferences of metal with the stability of hormone-receptor complex. These are supported by some of the earlier reports where it was shown that Cd can bind to SH group of the receptor and cause internalization leading to down regulation (Klug et al., 1988). Cd can also disrupt the cytoskeletal assembly and thus affect receptor stability (Amsterdam and Rotmensch, 1987). Apart from decreased gonadotropin binding and steroidogenic enzyme activity, testosterone level was also decreased. Decrease in the hormone-receptor binding (LH/FSH binding) would thus result in the decreased formation of cAMP. Also, substitution of calcium ions with cadmium can activate calmodulin dependent phosphodiesterase causing a decrease in cAMP (Nimura et al., 1987). Which in turn there is decrease the transcription

levels of the steroidogenic genes. This leads to reduced secretion of testosterone from isolated leydig cells after 24 hours of metal exposure, as shown in the present study. This was further supported by earlier results where reduction in transcription of steroidogenic genes had been demonstrated in metal exposed animals (Gupta S et al., 2004).

Cd seems to be more toxic than Pb. Although protective mechanism exists; this suggests that Cd in lesser concentration is a harmful eco-toxicant compared to Pb. In combined exposed group, accumulations of metals were least toxic indicating the competitive nature between divalent for binding sites in enzymes or at receptor level.

Thus, direct cellular effect clearly indicated that metals can interact at protein level leading to disruption in steroid synthesis. This effect along with disruption at molecular level will lead to reproductive dysfunction.

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