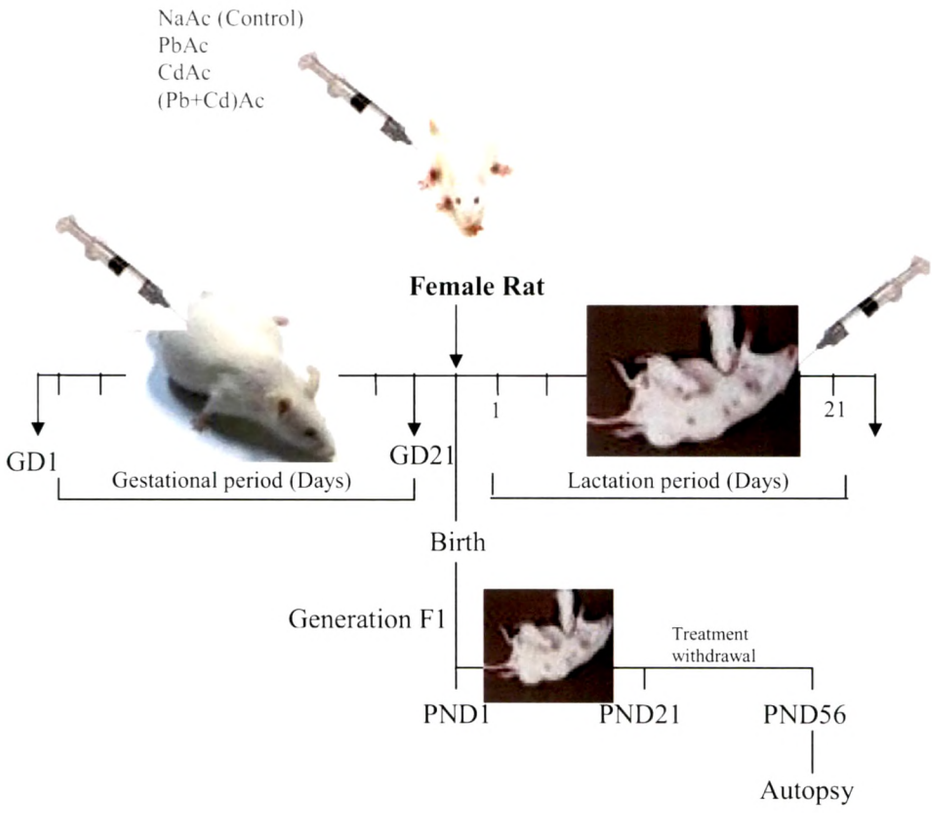
Chapter 3 Biochemical and molecular effects of gestational and lactational co-exposure to lead and cadmium on hypothalamic-pituitary-gonadal axis and hepatic steroid metabolism in F1 generation PND 56 rats.

3.1 General Introduction

Although the reproductive and developmental effects of environmental contaminants have become an issue of great interest recently, there are very few reports demonstrating the endocrine disrupting effects on reaching adulthood after early developmental exposure. Lead and cadmium are known to produce various adverse effects on reproduction. Pregnancy affects mobilization of the tissue level of lead and cadmium and enhances the toxicity of these metals. Lead poisoning during pregnancy, its teratogenicity and fetal toxicity have been extensively studied (Zhang *et al.*, 1999; Pillai and Gupta, 2005a; Dearth *et al.*, 2002). During pregnancy, lead and cadmium have been shown to interfere with placental and essential enzyme function or the availability of essential trace elements or other nutrients (Laxmipriya and Gupta, 2008; Baranski, 1986). Though placenta acts as an important, but not complete barrier to protect the fetus from cadmium exposure (Webster, 1988; Korpela *et al.*, 1989). Moreover, lactational transfer of cadmium to F1 offspring has also been reported in several population studies (Nishijo *et al.*, 2002). However, to the best of our knowledge no studies till date have focused on sex-specific effects of early developmental co-exposure to lead and cadmium on reaching adulthood. Thus, the purpose of the present investigation was to identify the key biochemical and molecular targets of endocrine disruptions mediated by lead and cadmium with special emphasis on neurotransmitter levels, hormone levels, gonadal steroidogenesis, xenobiotic/steroid metabolizing enzymes, non-enzymatic and enzymatic antioxidant defense system.

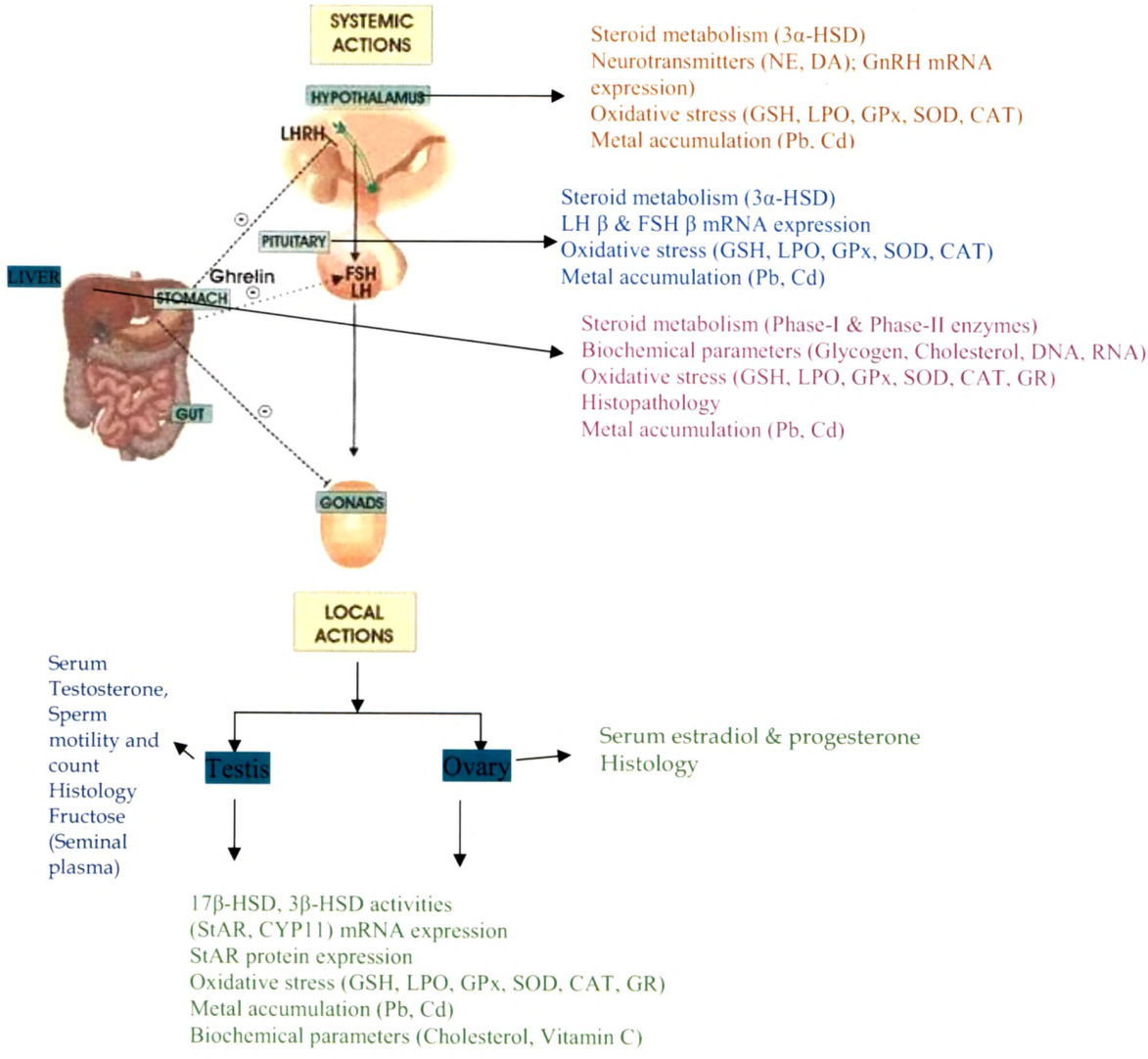
Efforts have been put to analyze the effects of gestational and lactational co-exposure to lead and cadmium on a.) Hypothalamic-pituitary axis function **(discussed in Section 3.2)**, b.) Ovarian steroidogenesis **(discussed in Section 3.3)**, c.) Testicular steroidogenesis **(discussed in Section 3.4)**, d.) Hepatic xenobiotic/steroid biotransformation enzymes **(discussed in Section 3.5)**, e.) Summary of the chapter **(Section 3.6)**

SCHEMATIC REPRESENTATION OF EXPERIMENTAL DESIGN



Animal strain: Charles Foster
Metal Dosage: 0.05 mg/kg
Mode of administration: Sub-coetaneous injection (s.c.)
GD: Gestational Day, PND: Post Natal Day, Ac: Acetate

Parameters evaluated after gestational lactational exposure study



3.2 Hypothalamic-Pituitary Axis Function

3.2.1 Introduction

Steroid hormones play a critical role in the processes of brain sexual differentiation and sexual development. Therefore, these processes may be particularly vulnerable to endocrine disruption by compounds that block or mimic endogenous hormones. Studies have clearly reported the susceptibility of hypothalamus towards heavy metals (Anderson *et al.*, 1997; Antonio *et al.*, 1999; Das *et al.*, 1993), the susceptibility of pituitary towards toxic metals (Lafuente *et al.*, 1999; Lorenson *et al.*, 1983; Ronis *et al.*, 1998). It is also well documented that lead and cadmium can modify plasma levels of luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Lafuente *et al.*, 1997, 1999; Lorenson *et al.*, 1983; Paksy *et al.*, 1989, Pillai *et al.*,2003). Neural systems are shown to be organized by steroid hormones perinatally which can later be activated by the same hormones, yielding sex differences in physiology as well as brain sexual dimorphism (Hotchkiss *et al.*, 2002) Also, catecholamines and indoleamines as well as other neurotransmitters of the central nervous system modulate pituitary hormone secretion (Drouva and Gallo, 1976; Lopez *et al.*, 1989). Exposure to endocrine disrupting chemicals during critical periods in fetal development can alter the organization or differentiation of the neuroendocrine system and may change the regulatory mechanism of hypothalamus-pituitary (H-P) axis on reaching adulthood. Pb poisoning and fetal toxicity is well established in earlier studies (Dearth *et al.*, 2002; Zhang *et al.*, 1999). As compared to Cd, Pb is easily permeable through the placenta, and thereby fetal blood contains very high concentrations of lead (Sabatelli *et al.*, 1995; Sorkun *et al.*, 2007). Maternal exposure to Cd is associated with low birth weight (Kuhnert *et al.*, 1988). Studies have shown that Pb and Cd exposure can cause changes in catecholaminergic functions (Cooper and Manalis, 1983; Arito *et al.*, 1981; Nation *et al.*, 1989; Shih and Hanin, 1978; Winder and Kitchen, 1984, Liuji *et al.*, 2002) and even poses vulnerability of developing brain (Moreira *et al.*, 2001, Christopher *et al.*, 2007, Uzbekov *et al.*, 1999). In humans and other primates, the hypothalamic-pituitary

unit that governs gonadal function becomes operational during fetal and neonatal development, and final differentiation and maturation occurs as puberty approaches (Plant, 2004).

Most of the above cited studies were carried out with single metal. Available data on combined exposure of lead and cadmium shows that these metals show additive effect on acetylcholine release at the frog neuromuscular junction (Cooper and Manalis, 1984) whereas Nation *et al.*, 1989 observed lesser effects of combined exposure than that in isolation. Even our earlier laboratory reports using adult female rats have shown lead and cadmium accumulation disrupts the regulatory mechanisms of the hypothalamic-pituitary axis where the effects produced by the combined treatment of metals are not additive (Pillai *et al.*, 2003). Very few studies have investigated oxidative stress and their possible relationship with neurochemical and endocrine changes, if any, in F1 generation offspring whose dams were exposed to lead and cadmium alone and in combination during pregnancy and lactation. The transfer of such contaminants to the developing embryo and fetus during pregnancy and to the newborn during lactation is not simply a function of recent maternal exposure.

Thus, in the present study an attempt has been made to evaluate the effects of early developmental exposure to lead and cadmium either alone or in combination on the neurotransmitter content (Dopamine, Norepinephrine), steroid metabolizing enzyme activity (3α-hydroxy steroid dehydrogenase), gonadotropin releasing hormone (GnRH) and gonadotropins (FSH β, LH β) gene expression profile, and antioxidant system in the hypothalamus-pituitary axis of post natal day 56 rats.

3.2.2 Experimental design

Following the experimental regime as shown in section 3.1, the male and female rat offspring were sacrificed on PND 56 by cervical dislocation. The hypothalamus and pituitary were quickly excised from the dissected animals, rinsed in ice-cold saline to clear them of blood, weighed and homogenized (10%

w/v). Hypothalamus and pituitary homogenates were then used for various biochemical assays. Dopamine and norepinephrine were estimated in the hypothalamus samples by the flourimetric method of Shellenberger and Gordon, 1971. (3α-HSD) activity was estimated in hypothalamus and pituitary samples following the method of Shivanandappa and Venkatesh, 1997. Lipid peroxidation (LPO) was estimated by thiobarbituric acid (TBA) reaction with malondialdehyde (MDA), Ohkawa *et al.*,1979. Reduced glutathione (GSH) content was determined according to the method of Beutler and Gelbart, 1985. Superoxide dismutase (SOD) activity was assessed according to the method of Marklund and Marklund, 1984. Catalase (CAT) activity was assayed following the method of Hugo, 1987. Both hypothalamus and pituitary were also analyzed for lead and cadmium levels by GBC 902 Atomic Absorption Spectrophotometer (AAS). Details of each method have been already described in chapter 2.

Total RNA was extracted from the hypothalamus and pituitary by the basic protocol of Chomczynski and Sacchi, 1987. The genes encoding for GnRH, LHβ and FSHβ were amplified by PCR with use of a one-step RT-PCR kit (Fermentas). The primers, PCR cycling conditions are shown in Table 1 of Chapter 2. 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 by Alpha imager software.

3.2.3 Results

There were no significant differences in body weight of male and female F1 offspring at Post natal day 56 (PND 56) after the dams were exposed through gestational and lactational window to lead and cadmium (Table 1).

Table 1: Body weight, absolute hypothalamus, pituitary weights of PND 56 F1 offspring after gestational and lactational exposure to lead and cadmium alone and in combination

Parameters	Groups			
	Control	Pb	Cd	Pb + Cd
Body weight (g)				
Male	174.3±6.63	157.6±4.20	163.6±5.61	165.5±5.25
Female	143.8±3.48	132.9±2.70	136.9±2.80	134.2±3.78
Hypothalamus weight (mg)				
Male	9.36±0.39	9.20±0.40	9.15±0.34	8.40±0.23
Female	8.38±0.28	8.20±0.26	8.18±0.26	8.21±0.39
Pituitary weight (mg)				
Male	4.45±0.19	4.55±0.24	4.43±0.22	4.51±0.19
Female	4.65±0.21	4.33±0.18	4.28±0.18	4.43±0.25

The data are presented as mean ± SEM. of 8 independent observations.

Table 2 and Table 3 shows the metal accumulation in hypothalamus and pituitary respectively after gestational and lactational exposure to lead and cadmium in isolation and in combination. Both lead and cadmium were significantly accumulated in hypothalamus and pituitary.

Table 2: Lead and Cadmium levels in hypothalamus of PND 56 F1 offspring after gestational and lactational exposure to lead and cadmium alone and in combination

Groups	Metal Content			
	Male		Female	
	Pb (µg/g)	Cd(µg/g)	Pb(µg/g)	Cd (µg/g)
Control	1.250±0.064	0.187±0.014	1.213±0.042	0.277±0.010
Lead	2.850±0.170***	0.122±0.013	3.663±0.068***	0.200±0.018
Cadmium	1.070±0.056###	0.385±0.017*** ###	1.020±0.044###	0.820±0.073*** ###
Combined	1.875± 0.170* ### @@	0.312±0.024 ** ###	1.83±0.159** ### @@@	0.580±0.037 ** ### @

The data are presented as mean ± SEM. of 4 independent observations.
*p<0.05, ** p<0.01, *** p<0.001 vs. control; ###p<0.001 vs. lead and
@p<0.05, @@p<0.01, @@@p<0.001 vs. cadmium group.

Table 3: Lead and Cadmium levels in pituitary of PND 56 F1 offspring after gestational and lactational exposure to lead and cadmium alone and in combination

Groups	Metal Content			
	Male		Female	
	Pb (µg/g)	Cd(µg/g)	Pb(µg/g)	Cd (µg/g)
Control	1.14±0.140	0.22±0.030	1.52±0.099	0.22±0.005
Lead	2.58±0.295**	0.21±0.014	2.48±0.235**	0.21±0.007
Cadmium	1.33±0.137##	0.44±0.022*** ###	1.163±0.134###	0.48±0.026*** ###
Combined	1.95±0.193	0.39±0.017 *** ###	1.99±0.045@	0.34±0.012** ### @@@

The data are presented as mean ± SEM. of 4 independent observations.
** p<0.01, *** p<0.001 vs. control; ##p<0.01, ###p<0.001 vs. lead
and @p<0.05, @@@p<0.001 vs. cadmium group.

Table 4 shows the steroid metabolizing enzyme, 3α-HSD activity in hypothalamus and pituitary of male and female F1 offspring at PND 56. Activity of hypothalamic 3α-HSD was most affected in cadmium and combined treated groups in male rats whereas in female rats, enzyme activity was decreased in all the metal treated groups. The combined treated group exhibited intermediate effect in case of male rats whereas female rat's demonstrated similar effect as that of cadmium treated group. In case of pituitary 3α-HSD, the enzyme activity was significantly inhibited in cadmium and combined treated groups in both male and female rats whereas lead treated group did not demonstrate any significant inhibition in the enzyme activity.

The concentrations of dopamine in the hypothalamus of F1 generation male and female rats at PND 56 are represented in Figure 1. Dopamine concentrations of cadmium treated group showed a maximum decrease as compared to the control animals, while lead treated group also showed minimum decrease but significant change as compared to control and the combined exposure group showed an intermediate change. The norepinephrine levels in the hypothalamus of F1 generation male and female rats at PND 56 are represented in Figure 2. Norepinephrine levels were maximally decreased in the cadmium group and followed by combined group and least effect seen in the lead treated group. However, the decrease in the norepinephrine levels was found to be significant in all the treated groups.

Figures 3A and 3B shows the effect of gestational and lactational exposure to Pb and Cd on PND 56 hypothalamic GnRH mRNA and pituitary LH & FSH mRNA expression levels respectively. Expression of GnRH mRNA was significantly affected only in combined metal treated groups as compared to the control group. A significant reduction in the mRNA levels of LH was observed in lead and cadmium treated groups. Whereas, mRNA expression of FSH was found to be affected only in combined metal treated group compared to the control.

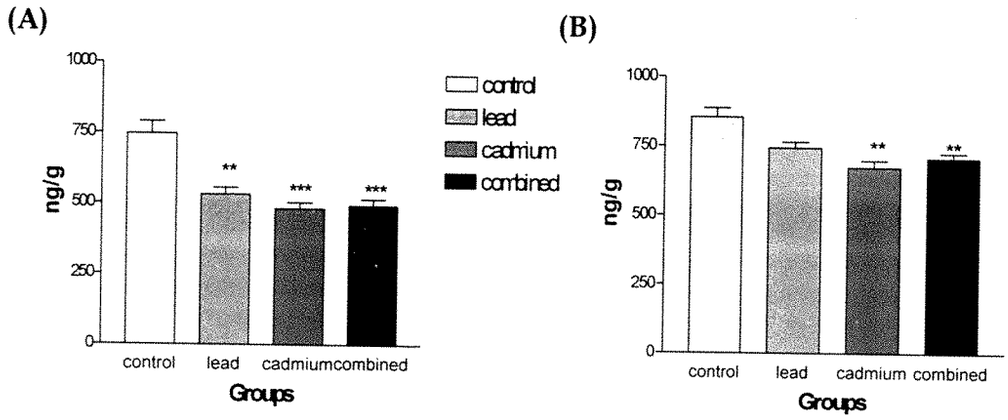
Table 4: Effect of gestational and lactational exposure to lead and cadmium on steroid metabolizing enzyme activity in hypothalamus and pituitary of PND 56 rats

Parameters	Groups			
	Control	Pb	Cd	Pb + Cd
Hypothalamic 3α-HSD[a]				
Male	1.58±0.108	1.38±0.048	0.89±0.028*** ##	1.098±0.047**
Female	1.11±0.058	0.80±0.038*	0.51±0.024*** ###	0.51±0.031*** ###
Pituitary 3α-HSD[a]				
Male	1.18±0.078	1.060±0.041	0.85±0.034**	0.86±0.035**
Female	0.95±0.026	0.89±0.040	0.70±0.025** #	0.76±0.045*

[a] nanomoles NADH formed/min/mg protein at 37°C.

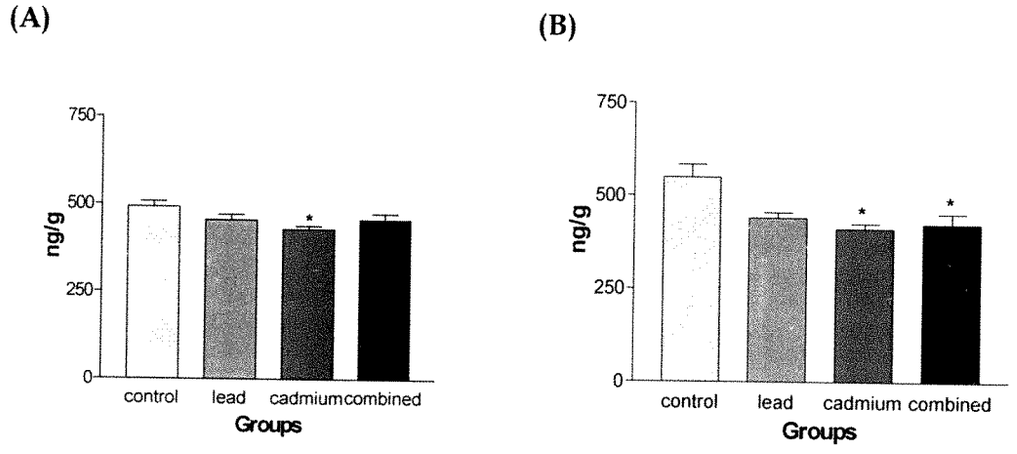
The data are presented as mean ± SEM. of 6 independent observations.
*p<0.05, ** p<0.01, *** p<0.001 vs. control; # p<0.05, ## p<0.01, ### p<0.001 vs. lead

Figure 1: Effect of Gestational and Lactational exposure to lead and cadmium in isolation and combination on Dopamine levels in PND56 male rats (A) and PND56 female rats (B)



The data are presented as mean ± SEM. of 4 independent observations.
** p<0.01, ***p< 0.001 versus control

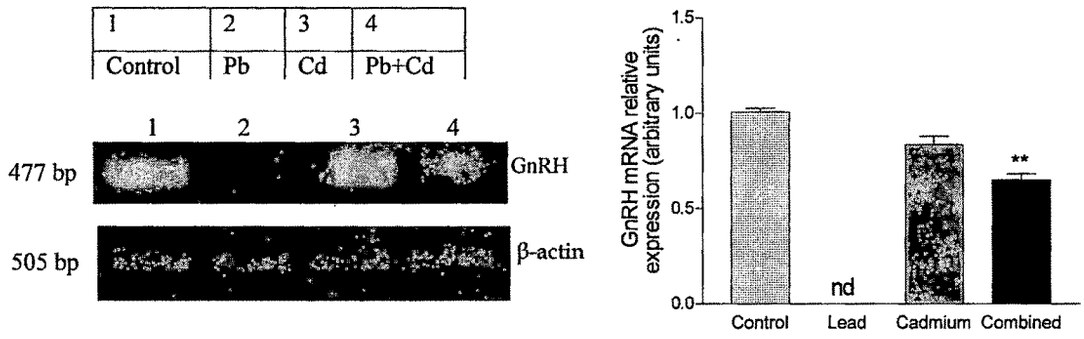
Figure 2: Effect of Gestational and Lactational exposure to lead and cadmium in isolation and combination on norepinephrine levels in PND56 male rats (A) and PND56 female rats (B)



The data are presented as mean ± SEM. of 4 independent observations.

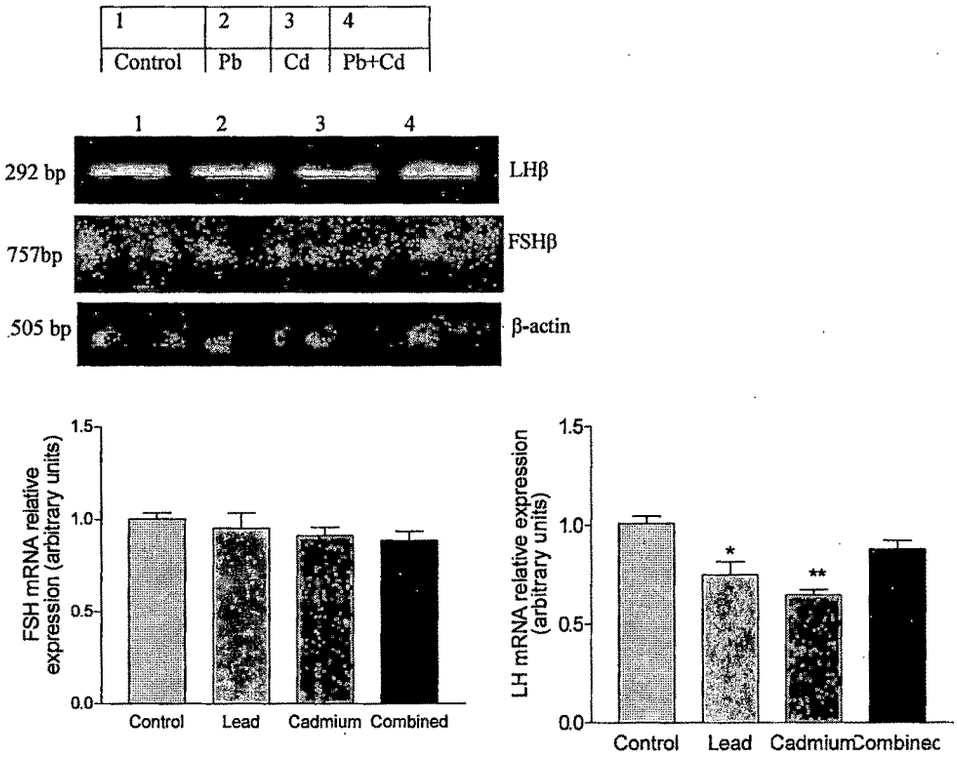
* p<0.05 versus control

Figure 3A: Effect of gestational and lactational co-exposure to lead and cadmium on the mRNA expression of (A) GnRH gene and (B) β-actin (internal control). (nd=not detected)



Values are expressed as mean ± S.D. (n = 3).
**p<0.01 versus control

Figure 3B: Effect of gestational and lactational co-exposure to lead and cadmium on mRNA expression of LHβ &FSHβ genes and β-actin (internal control).



Values are expressed as mean ± S.D. (n = 3)
* p<0.05 compared to the control group

The sex-specific effects of gestational and lactational exposure to lead, cadmium and combined metals on antioxidant system of hypothalamus and pituitary of F1 offspring at Post natal day 56 (PND 56) are shown in Table 5 and 6 respectively. In male and female rats, the reduced glutathione levels were maximally decreased in the cadmium treated group in comparison to lead and combined treated groups with respect to both hypothalamus and pituitary. Significant elevation in lipid-peroxidation levels were also seen in cadmium treated groups followed by combined exposure group in both male and female rats. Changes in hypothalamic (CAT and SOD) activities were not statistically significant when compared to the control group. However, both CAT and SOD of pituitary were significantly affected in cadmium treated group, whereas lead treated group did not demonstrate any significant changes when compared to the control group.

Table 5: Effect of gestational and lactational co-exposure to lead and cadmium on the non-enzymatic and enzymatic antioxidants of hypothalamus in PND 56 rats

Parameters	Groups			
	Control	Pb	Cd	Pb + Cd
GSH [a]				
Male	0.79 ± 0.02	0.65 ±0.03*	0.45 ± 0.03*** ###	0.61 ± 0.03** $$
Female	0.64 ±0.03	0.47 ±0.03**	0.36±0.03***	0.43±0.03**
TBARS [b]				
Male	13.6 ±0.59	14.25 ±0.99	18.77±1.08*#	17.12±0.89
Female	9.15±0.50	13.54±0.97	17.52±1.74**	14.25±0.92*
CAT [c]				
Male	23.49±1.31	19.90±1.29	17.97±0.94	18.18±2.14
Female	20.59±1.69	20.27±1.23	15.32±0.95	18.94±1.37
SOD [d]				
Male	45.65±2.25	42.38±2.00	39.94±1.66	39.91±2.56
Female	42.97±3.15	40.96±2.20	40.55±2.07	42.79±2.60

The data are presented as mean ± SEM. of 6 independent observations.
*p<0.05, ** p<0.01, *** p<0.001 vs. control; #p<0.05, ### p<0.001 vs. lead and $$ p<0.01, vs. cadmium group

a = pmoles/mg protein; b = nmoles MDA/min/mg protein; c=μmoleH_2O_2 decomposed/min./mg protein; d=amount of enzyme required for 50 % inhibition of pyragallol auto oxidation.

Table 6: Effect of gestational and lactational exposure to lead and cadmium on the non-enzymatic and enzymatic antioxidants of pituitary in PND56 rats

Parameters	Groups			
	Control	Pb	Cd	Pb + Cd
GSH[a]				
Male	0.42±0.04	0.29±0.01	0.24±0.04 *	0.26±0.01 *
Female	0.51±0.04	0.38±0.03	0.29±0.02**	0.37±0.02
TBARS[b]				
Male	8.13±0.64	11.65±0.92	13.76±1.21**	12.96±0.87*
Female	5.72±0.24	8.98±1.05	12.00±0.73***	10.54±0.90**
CAT[c]				
Male	16.55±1.32	13.53±1.19	12.52±0.85	13.73±1.22
Female	9.00±0.62	8.46±0.40	5.88±0.38** #	7.79±0.36
SOD[d]				
Male	31.50±1.37	29.85±1.56	27.28±2.03	30.13±2.19
Female	33.13±1.89	27.65±2.74	23.40±1.26*	25.44±2.02

The data are presented as mean ± SEM. of 6 independent observations.
*p<0.05, ** p<0.01, *** p<0.001 vs. control; #p<0.05 vs. lead

a = pmoles/mg protein; b = nmoles MDA/min/mg protein; c=μmoleH_2O_2 decomposed/min./mg protein; d=amount of enzyme required for 50 % inhibition of pyragallol auto oxidation.

3.2.4. Discussion

The present study was mainly carried out to understand the relationship of heavy metal exposure during gestational-lactational period and neuro-endocrine axis vulnerability of F1 generation offspring on reaching adulthood and henceforth the following parameters were examined 1.) Hypothalamic neurotransmitter levels (Dopamine, Nor-epinephrine) that are mainly responsible for GnRH release, 2.) Steroid metabolizing enzyme activity in brain regions (hypothalamus and pituitary), 3.) mRNA expression of GnRH, LHβ & FSHβ genes and 4.) non-enzymatic and enzymatic antioxidant status in brain regions (hypothalamus and pituitary) of F1 generation male and female rats at post natal day (PND) 56, whose dams were exposed to lead and cadmium during pregnancy and lactation. To the best of our knowledge, this is the first report

showing the effects of gestational and lactational exposure to both lead and cadmium alone and in combination on endocrine, neurochemical, antioxidant enzyme activities and redox metabolite changes in hypothalamus–pituitary axis at later stages of life and therefore pinpointing the possibility of alterations in fetal programming of endocrine functions.

The administration of lead and cadmium either alone or in combination appeared to have no effect on the absolute and relative hypothalamus and pituitary weights. The absence of effects of these metals on organ weights indicates that lead and cadmium when administered during early developmental window is less harmful in relation to both organ and body weight gain. The changes in norepinephrine content within the hypothalamus observed in this study agree with those described for the whole brain (Arito, *et al.*, 1981, Pillai *et al.*, 2002). Early developmental effects of lead and cadmium alone and in combination on norepinephrine levels, may be explained by the sensitivity of the hypothalamic-pituitary axis to exogenous stimulation with age (Esquifino *et al.*, 1984). Studies have revealed that metal accumulation in the hypothalamus and pituitary was higher after post-pubertal than after pre-pubertal metal exposure (Lafuente *et al.*, 2001) although earlier studies have shown that changes observed in nor-epinephrine content and serotonin metabolism were more marked in younger than in older rats (Nation *et al.*,1989). Moreover, the developing organism presents a 5-fold greater absorption of Pb and lacks a functional blood brain barrier (Lockitch, 1993). Perinatal exposure to low levels of lead has been involved in behavioral and neurochemical alterations detected in both suckling and adult rats (Moreira *et al.*, 2001). Norepinephrine (NE) plays an important role in modulating luteinizing hormone releasing hormone (LHRH) neurons that are involved in the regulation of gonadotropin secretion by the anterior pituitary. It has been reported that lead can act at the hypothalamic level to alter gonadotropin releasing hormone secretion in the rat (Bratton *et al.*, 1994). It is shown that lead blocks the NE induced release of PGE_2, thereby resulting in diminished LHRH secretion. However, we observed decrease in NE only in

cadmium and combined metal treated groups of female rats whereas levels of norepinephrine were affected only in cadmium treated group in male rats. The discrepancies with other studies could be due to differences in the age of animals, mode of treatment, duration and brain areas analyzed. Dopamine content was affected by lead and combined metal treated group whereas cadmium alone was showing maximum decrease in the DA content. Lead has been reported to alter calcium homeostasis by affecting both voltage dependent and receptor operated calcium channels (Audesirk, 1993; Bressler and Goldstein, 1991; Oortgiesen *et al.*, 1993) whereas the invitro studies have shown that lead enhances calcium activated release in brain transmitters (Minnema *et al.*, 1988). On the other hand, cadmium is shown to inhibit calcium entry and the attendent release of peripheral catecholamines (Hirning *et al.*, 1988). Hence, decrease in NE could be also due to the altered Ca^{2+} mobilisation, and also a limiting step in PGE_2 formation which subsequently leads to alterations in gonadotropin releasing hormone secretion. Therefore the changes observed in the co-exposure group might be due to the fact that lead and cadmium compete with calcium at the channel site and compete with each other for entry through terminal membrane channels (Cooper and Manalis, 1983).

It was worthwhile to study whether the transcriptional machinery of both hypothalamus and pituitary with respect to GnRH and LH, FSH genes respectively, are also susceptible to metal exposure. Any alterations in the transcriptional machinery of hypothalamus and pituitary will thereby indicate the role of lead and cadmium in modulating the GnRH synthesis machinery within hypothalamus that in turn will determine the circulating gonadotropins level. Hypothalamic GnRH gene expression analysis showed that combined treated group significantly inhibited the mRNA expression levels of GnRH gene in post natal day 56 female offspring. Similarly, the mRNA expression levels of pituitary FSH and LH genes were also analyzed and the results showed no significant alterations in mRNA expression levels of FSH in any of the metal treated groups. However, lead and cadmium treated animals showed significant

inhibition in the mRNA expression levels of LH. These observations clearly suggest that hypothalamic and pituitary transcriptional machinery is vulnerable to early developmental exposure to lead and cadmium and is in agreement with earlier findings on altered mRNA expression profile of gonadotropins and gonadotropin releasing hormone (Sokol *et al.*, 2002).

Since many environmental contaminants exercise their action by binding to the body's receptor, sexual differences in receptor density or sensitivity and any modulation by the sex-hormones may result in sexual differences in vulnerability. Receptors for estrogens and androgens are found to be localized in hypothalamic-pituitary axis apart from other parts of the body, and thus modulating toxic response in a sex-specific way. In this regard, the data from the present study highlights the differences in the vulnerability of enzyme activities of steroid metabolism and antioxidant system which further helps to measure the neuro-toxicity induced by lead and cadmium in sex-specific manner. The brain synthesizes neuro-steroids de novo (Compagnone and Mellon, 2000; Tsutsui *et al.*, 2000) but the relative roles of locally produced neuroactive steroids and those converted from circulating precursors remain to be defined. Alterations in levels of locally produced neuro-steroids in the hypothalamus, pituitary may serve as crucial paracrine modulators of essential brain functions (Genazzani *et al.*, 1995; 1996; Calogero *et al.*, 1998; Akwa *et al.*, 2001). The results of the present study indicate that activity of 3α-hydroxy steroid dehydrogenase in hypothalamus and pituitary was decreased after the metal treatment with cadmium showing maximum inhibition in females while an additive effect was observed in case of combined exposure group in males. However, the decrease in activity of 3α-hydroxy steroid dehydrogenase was not significant in the pituitary of both male and female rats as compared to hypothalamus. Thus, the results suggest that hypothalamus which is the higher centre of regulatory pathway, is more susceptible to metal exposure than pituitary and implicating serious suppressive effects on circulating gonadotropins levels.

It was also supposed that oxidative stress was one possible mechanism for lead and cadmium neurotoxicity (Carageorgiou *et al.*, 2005, Moreira *et al.*, 2001; El-Maraghy *et al.*, 2001). Pb-exposure might also induce decrease in activities of free radical scavenging enzymes and this is mainly attributed to high affinity of lead to sulfhydryl-groups in these enzymes. Moreira *et al.* (2001) have demonstrated that brain antioxidant defenses (SOD and GSH-Px) decreased significantly in individuals exposed to lead. The involvement of reactive oxygen species (ROS) in lead poisoning has been addressed by Schwartz *et al.* (2000) who found a decrease in GR and an increase in oxidized glutathione (GSSG) concentrations in lead acetate-treated rats and thus leading to imbalance in GSH/GSSG, an indicator of oxidative stress (Wilson *et al.*, 2000). Glutathione, ascorbic acid and others have specific roles in the mitigation of heavy metal toxicity in general and cadmium in particular. Pb and Cd being divalent heavy metals, has a strong affinity for the thiol groups of proteins and enzymes and thereby causing conformational changes that interfere with their function (Casalino *et al.*, 2002; Hassoum and Stohs, 1996).

So we were interested in investigating whether Pb and Cd mediated neurochemical alterations are due to oxidative damage to the hypothalamus and pituitary. Glutathione (GSH) is the most abundant non-protein thiol that buffers ROS in the brain tissue (Dringern *et al.*, 2000). Present study clearly demonstrated pronounced effect of both lead and cadmium on antioxidant system by depleting the reduced glutathione content and elevating the lipid peroxidation levels in hypothalamus and pituitary of both male and female rats. We also observed a significant increase in lipid peroxidation (LPO) production in both hypothalamus and pituitary which is line with earlier reports (Adonaylo and Oteiza, 1999). However, our findings did not demonstrate any significant alterations of CAT and SOD enzyme activities in both hypothalamus and pituitary and thereby pointing towards some kind of adaptive mechanism within hypothalamic-pituitary axis (Sokol *et al.*, 2002; Hitzfield and Taylor, 1989).

It is very clear from the results of the present study that there exist a sexual dimorphic pattern of endocrine disruptions. Our combined exposure

group in most cases demonstrated intermediate effect and clearly pinpointing some kind of competition among the two metals when present together. Such observations suggest the possibility of multiple ways of manifestation of lead and cadmium toxicity when present together. Though, it is difficult to explain the mechanism of such sexual dimorphic pattern of endocrine disruptions as well as discrepancies about intermediate and additive effect, but it is a clear indication of the possibility of differential vulnerability in male and female population towards environmental pollutants which has been reported elsewhere (Patisaul, 2007).

In conclusion, neurochemical changes in the hypothalamus and pituitary of F1 generation PND 56 male and female rats have been demonstrated through altered neurotransmitter levels of hypothalamus and altered steroid metabolizing enzyme activities in both hypothalamus and pituitary. Present study has also demonstrated the role of oxidative stress in hypothalamic-pituitary axis to be an important biochemical mechanism behind the observed neurotoxic effects of lead and cadmium in rat brain regions after early developmental exposure. These results, taken together, are suggestive of suppressive effects of gestational and lactational co-exposure to lead and cadmium on hypothalamic-pituitary axis function of male and female offspring on reaching adulthood.

3.3 Ovarian Steroidogenesis

3.3.1 Introduction

There is growing evidence that many women usually work during their reproductive years. This will increase the likelihood that women during pregnancy will be exposed to a variety of reproductive toxicants such as heavy metals and other hazardous chemicals at work (Kumar, 2004). Hence exposures to endocrine disruptors during pregnancy may interact with foetal development, resulting in health effects in the offspring that may lead to altered reproductive performance and fertility related problems in their reproductive age.

Lead poisoning and fetal toxicity is well established in earlier studies (Dearth *et al.*, 2002; Zhang *et al.*, 1999). As compared to cadmium, lead is easily permeable through the placenta, and thereby fetal blood contains very high concentrations of lead (Sabatelli *et al.*, 1995). Studies have clearly shown that cadmium is retained in the placenta during pregnancy, which thus acts as an important, but not complete, barrier to protect the fetal development (Sorkun *et al.*, 2007; Webster, 1998). Maternal exposure to cadmium is also associated with low birth weight (Kuhnert *et al.*, 1988, Frery *et al.*, 1993). The stimulatory and inhibitory effects of cadmium on key ovarian steroidogenic enzymes have been well established (Smida *et al.*, 2004; Henson, 2004). Studies have also reported that lead down regulates some of the key proteins involved in ovarian steroidogenesis (Taupeau *et al.*, 2003; Wiebe *et al.*, 1998).

Very few studies have been undertaken with a view to understand the outcome of endocrine disruption in the reproductive age of first generation offspring after early developmental exposure to lead and cadmium. Earlier, our laboratory has already demonstrated the co-exposure effects of lead and cadmium during gestational and lactational period on hypothalamic-pituitary-ovarian axis and hepatic steroid metabolism, reproductive performance, and implantation, ovarian and placental steroidogenesis in post natal day 21 rats. Till date, most of the studies dealt mainly with single metal exposure and in real life scenario, it is generally seen that mixtures of metals are present in the

environment. Henceforth, it is very essential to understand the outcome of endocrine disruption mediated by co-exposure of these metals. Hence, the present study is carried out to analyze the biochemical and molecular basis of disruption of ovarian steroidogenesis at post natal day 56 which is the reproductive age of the first generation offspring.

3.3.2 Experimental design

Following the experimental regime as discussed in the section 3.1, all experiments were carried out by sacrificing animals of all groups at post natal day 56 by decapitation. The activity of key ovarian steroidogenic enzymes (17β-HSD and 3β-HSD) were estimated following standardized protocol as discussed in Chapter 2. Western-blot analysis of ovarian StAR protein was analyzed in all the experimental groups following standardized protocol described in Chapter 2. Total RNA was isolated from ovary using Tri Reagent (Sigma) and intact RNA with an A260/280 ratio 1 6 and above was used for RT-PCR analysis of ovarian (StAR & Cyp11a) genes and β-actin (internal control). 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 Alpha imager software. Oxidative parameters of ovary such as Lipid peroxidation (LPO), Reduced glutathione (GSH), Glutathione peroxidase (GPx), Glutathione reductase (GR), Superoxide dismutase (SOD) and Catalase (CAT) activities were assayed and the details of each are discussed in the Chapter 2. Biochemical parameters such as vitamin C level, Acid phosphatase, Cholesterol were assayed in ovarian homogenate and the details of all the protocols are discussed already in chapter 2. One ovary per animal was fixed in 15–20 volumes of Bouin's fixative for at least 24 h and cleared in three successive 1-h washes in 70% ethanol. Three 5 µm cross-sections from ovary and uterus were stained with hematoxylin and eosin. Sections were mounted on the slide and evaluated microscopically by pathologists blind to the treatment group.

3.3.3 Results

Lead and cadmium either alone or in combination had no significant effect on reproductive performance of dams as shown in Table 1. Further, no change was observed in the fertility rate, litter size, and litter weights in any of the metal-treated groups. Body weight of PND 56 female offspring did not demonstrate any significant alterations as compared to the control animals. Cadmium treated group showed significant decrease in ovarian and uterine absolute weight as compared to the control group (Table 2).

Table 3 summarizes the results on metal content in PND 56 ovary. There was significant increase in ovarian content of lead and cadmium after gestational and lactational exposure to metals.

The ovarian cholesterol and vitamin C contents were decreased significantly in cadmium and combined treatment groups of PND 56 rats following gestational and lactational exposure regime (Table 4). Lead treated group did not demonstrate any significant alterations in these biomolecules.

Table 1: Effect of gestational & lactational co-exposure to lead and cadmium on reproductive performance of dams

	Parameters	Control	Lead	Cadmium	Lead+Cadmium
1.	Maternal Weight at conception (gm)	224.50 ± 7.62	212.20 ± 10.88	212.0 ± 7.42	218.8 ± 13.03
2.	Litter Size	9.67 ± 0.71	8.3 ± 0.55	8.14 ± 0.59	8.33 ± 0.61
3.	Mortality Rate (%)	3.4	4.0	8.77	4.0
4.	FEMALE PUPS (%)	44 ± 5.714	50 ± 3.633	42 ± 7.526	55 ± 9.038
5.	MALE PUPS (%)	57 ± 5.714	50 ± 3.633	58 ± 7.526	45 ± 9.038

N=8. The values are mean±SEM.

Table 2: Effect of gestational and lactational co-exposure to lead and cadmium on body weight, ovary and uterus weights in F1 generation PND 56 rats.

Parameters	Groups			
	Control	Pb	Cd	Pb + Cd
Body weight(g)	143.8±3.48	132.9±2.70	136.9±2.80	134.2±3.78
Ovary (mg)	76.43 ± 3.50	69.67 ± 3.40	60.67±5.74*	74.88 ± 5.74
Uterus (mg)	300.9 ± 16.36	260.3 ± 25.38	219.8 ± 18.03**	242.3 ± 11.56

The data are presented as mean ± SEM. of 6 independent observations.
*p<0.05, ** p<0.01 vs. control

Table 3: Lead and cadmium levels in the ovary of PND 56 F1 offspring after gestational and lactational co-exposure to lead and cadmium

Groups	Lead (µg/g)	Cadmium (µg/g)
Control	0.070±0.007	0.023±0.005
Lead	0.135±0.018 *	0.027±0.008
Cadmium	0.057±0.008 ##	0.167±0.013 *** ###
Lead + cadmium	0.097±0.008	0.105±0.013 ** ## @@

Values are expressed as mean± SEM (n=4 in each group).
*p<0.05, **p<0.01, ***p<0.001 vs. control;
##p<0.01, ###p<0.001 vs. lead and @@p<0.01vs. cadmium group.

Table 4: Effects of gestational and lactational co-exposure to lead and cadmium on the biochemical parameters of Ovary of PND 56 rats

Parameters	Groups			
	Control	Pb	Cd	Pb + Cd
Total Colesterol[a]	11.70±0.65	10.33±0.24	8.00±0.26*** ##	8.73±0.34 ***
Vitamin C [b]	9.1±0.22	8.73±0.21	7.51±0.15 *** ###	8.17±0.14*

[a] µg/g tissue, [b] mg/g tissue

The data are presented as mean ± SEM. of 6 independent observations.
*p<0.05, *** p<0.001 vs. control; ##p<0.01, ###p<0.001 vs. lead

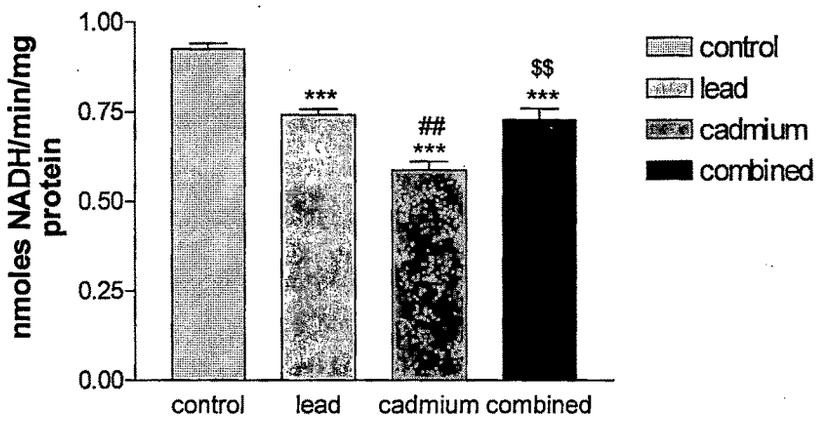
Figure 1 and Figure 2 demonstrates the effect of lead and cadmium either alone or in combination on ovarian Δ^5, 17β-HSD and 3β-HSD specific activities respectively. Activities of both the key steroidogenic enzymes were significantly affected in all the metal treated groups. The cadmium-treated group exhibited maximal change in the key steroidogenic-enzyme activity as compared to the control group. The lead-treated groups showed the least but significant change in the steroidogenic enzyme activity. The animals receiving combined metal treatment demonstrated an intermediate pattern of inhibition as compared to the control.

In order to assess the effect of gestational and lactational co-exposure to lead and cadmium on the ovarian steroidogenic transcriptional machinery, mRNA expression of StAR and CYP11a were assessed by RT-PCR employing β-actin as internal control (Fig.3). Analysis revealed a significant decrease in mRNA expression levels of StAR only in cadmium treated group as compared to control animals. However, CYP11a mRNA expression was significantly inhibited in cadmium and combined metal treated groups.

Figure 4 shows the effect of Pb and Cd alone and in combination on basal level of ovarian StAR protein expression level in metal treated groups as compared to the control. Results indicates a trend towards decrease in the basal level expression of StAR, however, the changes were not statistically significant.

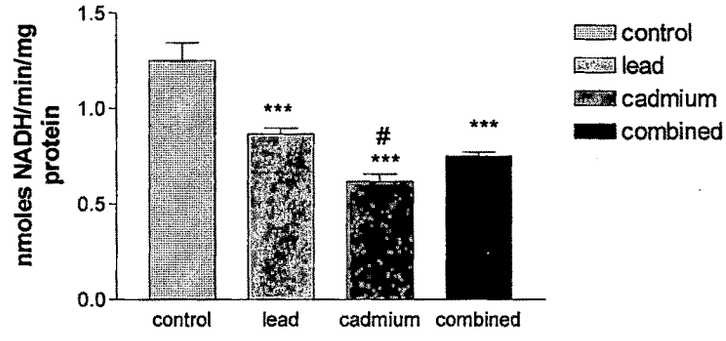
The animals in the cadmium and combined groups showed decrease in the serum estradiol (Fig.5) and progesterone (Fig.6) levels as compared to the control group whereas lead treated group did not demonstrate any significant change in the serum estradiol and progesterone levels as compared to the control.

Figure 1: Effect of gestational and lactational co-exposure to lead and cadmium on ovarian 17β-hydroxysteroid dehydrogenase activity in PND 56 rats.



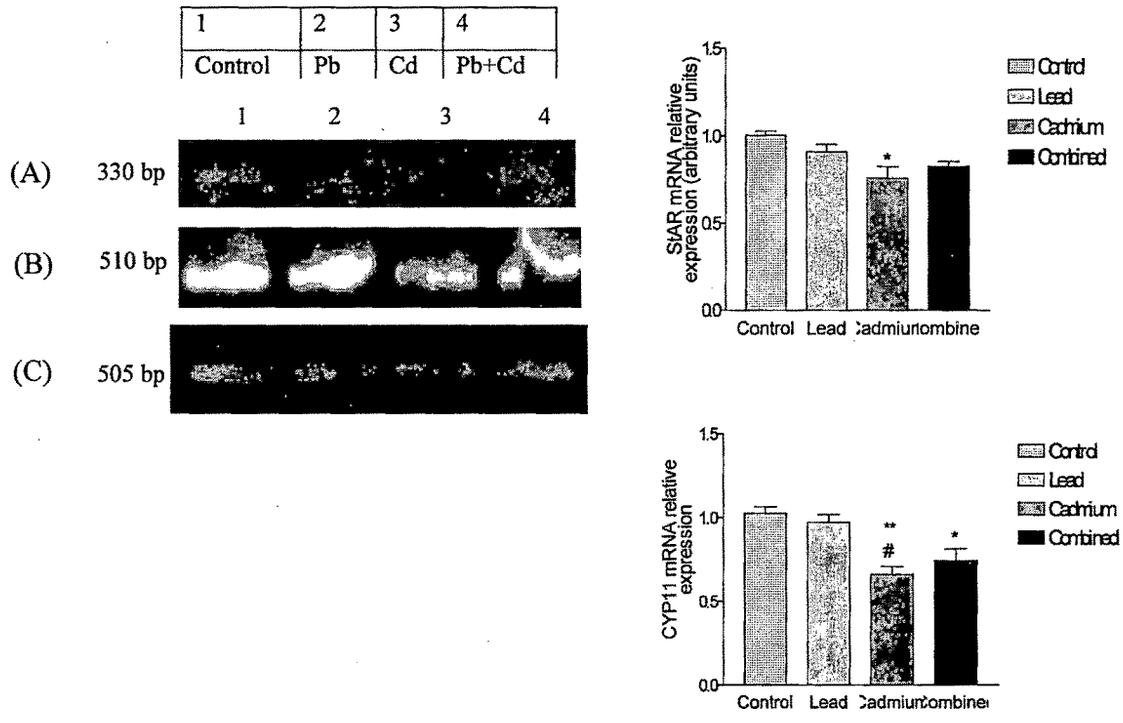
N=6. The values are mean± SEM. *** p<0.001 compared to the control group;
p<0.01 compared to the lead group;
$$ p<0.01, compared to the cadmium group

Figure 2: Effect of gestational and lactational co-exposure to lead and cadmium on ovarian 3β-hydroxysteroid dehydrogenase activity in PND 56 rats.



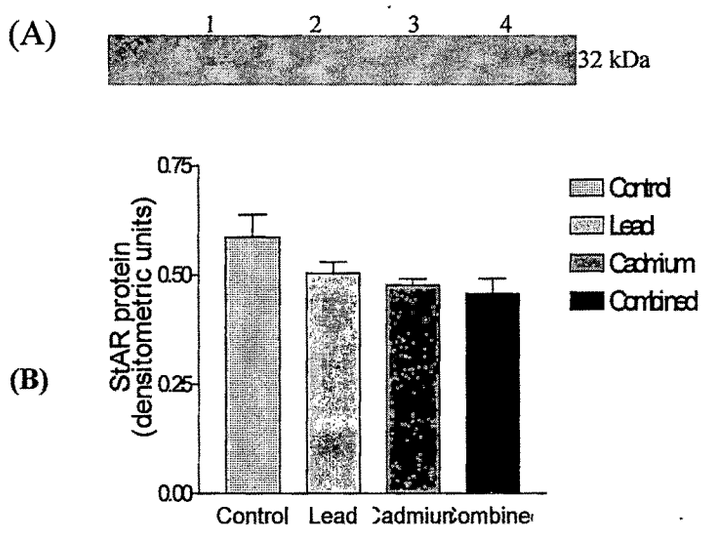
N=6. The values are mean±SEM. *** p<0.001 vs. control group;
#p<0.05 compared to the lead group

Figure 3: Effect of gestational and lactational co-exposure to lead and cadmium on the expression of ovarian (A) StAR gene and (B) Cyp11a gene (C) β-actin.



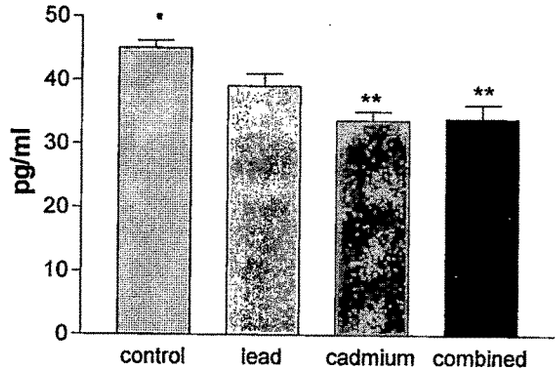
Values are expressed as mean ± S.D. (n = 3).
* p<0.05 **p<0.01 vs. control group;
#p<0.05 vs. lead group

Figure 4: Effect of gestational and lactational co-exposure to lead and cadmium on basal level Ovarian StAR protein expression by western-blot analysis. (A) Representative western immunoblot of ovarian StAR protein. (B) Composite graph showing the mean (±SEM) densitometric quantitation of the bands from three blots corresponding to the StAR protein.



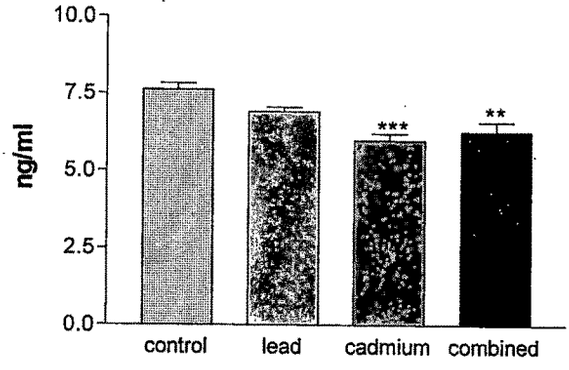
N=3. The values are mean ± SEM.

Figure 5: Effect of gestational and lactational co-exposure to lead and cadmium on serum estradiol levels in PND 56 rats



N=6. The values are mean ± SEM.
** $p<0.01$ compared to the control group

Figure 6: Effect of gestational and lactational co-exposure to lead and cadmium on serum progesterone levels in PND 56 rats

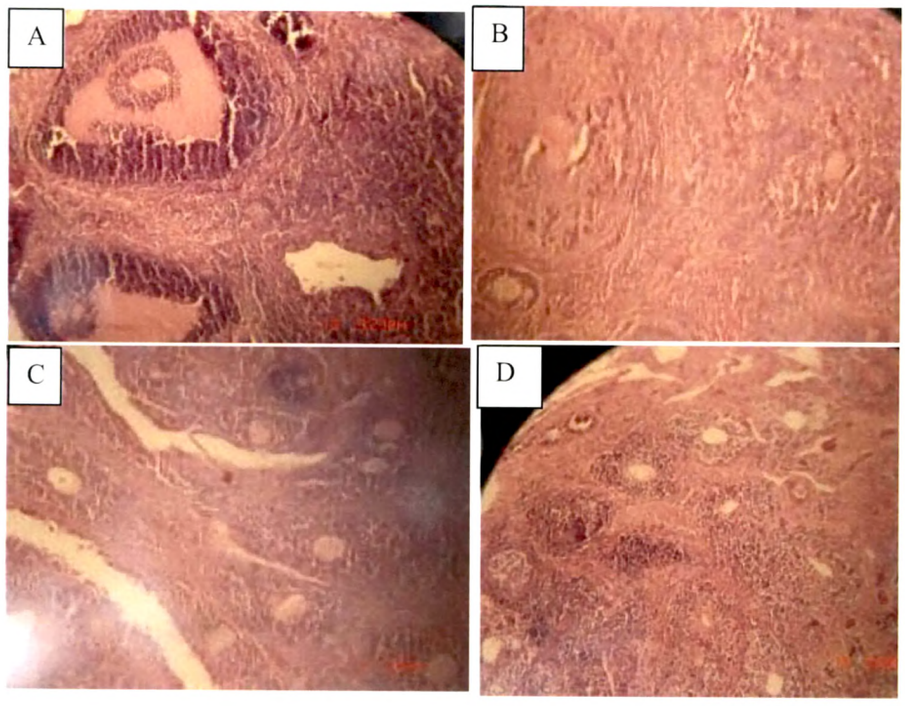


N=6. The values are mean ± SEM. *** $p<0.001$, ** $p<0.01$ vs. control group

Histological observation of ovary after metal exposure showed significant alterations (Figure 7). Basic histological studies showed that in the PND56 ovary cadmium treatment brings about minor alterations in follicular dynamics resulting in reduced number of growing follicles. The percentage of growing follicles was also significantly higher and that of stroma significantly lower in control group in comparison with all the experimental groups in general and cadmium treated group in particular.

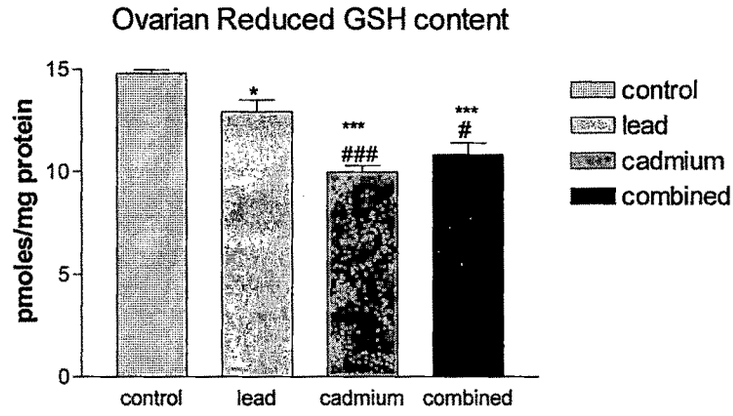
Figure 8 and Figure 9 represents the effects of lead and cadmium either alone or in combination on the reduced glutathione and lipid peroxidation levels within PND 56 ovary respectively. Cadmium treated animals showed a significant depletion in reduced glutathione content and higher levels of lipid peroxidation in the ovary compared to control animals. While the combined treated animals exhibited an intermediate change in both the parameters while lead treated animals showed minimum change. Table 5 represents the effects of lead and cadmium either alone or in combination on the enzymatic antioxidants in the ovary. Significant inhibition in the activity of Cu-Zn SOD, Mn-SOD and CAT were seen in the cadmium treated animals as compared to the control animals. Similar trend was also observed in the GPx and GR activities in cadmium treated animals as compared to the control animals. While the combined exposed animals showed an intermediate effect.

Figure 7: Histology of PND 56 ovary after gestational and lactational exposure to lead and cadmium alone and in combination (Photomicrographs are 10X magnification)



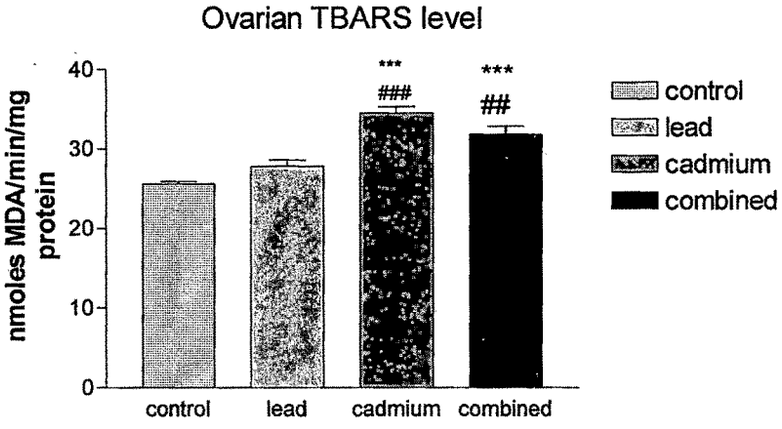
A: Control; B: Lead; C: Cadmium; D: Combined

Figure 8: Effect of gestational and lactational exposure to lead and cadmium on reduced glutathione content in ovary of F1 generation PND 56 rats



N=6. The values are mean±SEM. *** p<0.001, * p<0.05 vs. control group;
# p<0.05, ### p<0.001 vs. lead group

Figure 9: Effect of gestational and lactational exposure to lead and cadmium on TBARS levels in ovary of F1 generation PND 56 rats



N=6. The values are mean±SEM. *** p<0.001 vs. control group;
##p<0.01, ### p<0.001 vs. lead group

Table 5: Effects of gestational and lactational exposure to lead and cadmium on enzymatic antioxidants in ovary of PND 56 rats

Parameters	Groups			
	Control	Pb	Cd	Pb + Cd
Catalase [a]	38.41 ± 0.75	35.90 ±0.59	32.62 ±0.84*** #	32.97± 0.75***
Cu-Zn SOD [b]	13.24±0.89	10.50±0.30*	8.2±0.35***	9.76±0.57**
Mn SOD [b]	6.69 ± 0.16	6.40± 0.32	4.73 ± 0.24*** ###	5.72± 0.16 * $
GPx [c]	7420 ± 25.42	7078± 46.65***	6830 ± 62.32*** #	7044±58.38*** $
GR [d]	14.90 ± 0.23	14.25 ± 0.41	11.75 ± 0.40*** ##	12.97± 0.50*

The data are presented as mean ± SEM. of 6 independent observations.
*p<0.05, ** p<0.01, *** p<0.001 vs. control; # p<0.05, ## p<0.01, ### p<0.001 vs. lead;
$ p<0.01 vs. cadmium

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

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

[c] Δ 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

3.3.4 Discussion

There is growing evidence that many women usually work during their reproductive years. This will increase the likelihood that women during pregnancy will be exposed to a variety of reproductive toxicants such as heavy metals and other hazardous chemicals at work (Gerhard *et al.*, 1998; Xu *et al.*, 1998; Kumar, 2004). Hence exposures to endocrine disruptors during pregnancy may interact with foetal development, resulting in health effects in the offspring that may lead to altered reproductive performance and fertility related problems in their reproductive age. Studies indicating early developmental exposure and effects measures are scanty on endocrine disruptors such as lead and cadmium. In this regard, the present study analyzed the effects of gestational and lactational co-exposure to lead and cadmium on ovarian steroidogenesis in F1 generation post natal day (PND) 56 rats.

Body weight gain was not altered significantly in any of the metal-treated rats in comparison to the controls; thus any deleterious effect of metals on the female reproductive system seen is due to the toxic effect of Pb and Cd itself on this specific system, and not due to the bad health of the animals. The results of the present investigation demonstrate the adverse effect of gestational and lactational exposure to lead and cadmium in isolation and in combination on female gonadal steroidogenic enzyme activity of F1 offspring at post natal day 56. In ovarian steroidogenesis, Δ^5, 3β-HSD and 17β-HSD are the key regulatory enzymes (Hinshelwood *et al.*, 1994); and the suppressed activities of these steroidogenic enzymes by heavy-metals in the present study is in agreement with the previous findings where Pb and Cd treatment was associated with inhibition of ovarian steroidogenesis in adult rats (Piasek and Laskey, 1994; Nampoothiri and Gupta, 2006) and testicular steroidogenesis (Chirayu Pandya, PHD Thesis) in adult rats. One possibility for the alteration in steroidogenic enzymes activity in metal-treated rats may be the result of low serum levels of FSH and LH, since these are the regulators of ovarian 17β-HSD activities. Decreased serum levels of LH and FSH have been reported after administration of higher doses of cadmium to female rats (Paksy *et al.*, 1989; Varga and Paksy, 1991; Pillai *et al.*, 2003).

In this regard, data from the present study showed that both Pb and Cd in isolation and in combination decreased serum levels of estradiol and progesterone. This may be attributed to the inhibition of ovarian steroidogenic enzyme activity, because these enzymes are responsible for the regulation of ovarian estradiol and progesterone synthesis (Hinshelwood *et al.*, 1994).

We also showed significant decrease in granulose cell number in cadmium and combined metal treated groups at post natal day 56. Histological analysis of post natal day 56 ovaries was also carried out so as to assess if any structural changes seen after metal treatment. The decreased number of healthy follicles after metal treatment and the elevation in the number of atretic follicles in metal-treated rats has been attributed to altered sex-steroid levels. Hence, the decreased levels of ovarian steroids in metal-treated rats can be correlated with decreased number of follicles and the granulose cell population in the developing follicles,

and/or impaired ovarian function. 17β-estradiol is not only responsible for follicular maturation, but also regulates secretion of LH and FSH from the pituitary through positive and negative feedback on the hypothalamus and pituitary (Hoyer, 2005). Thus, any imbalance in ovarian steroidogenesis will ultimately lead to reproductive failure. A reproductive toxicant can cause direct ovarian effects, or indirect effects on the hypothalamus and/or pituitary (Hoyer, 2005).

Study was also carried out to analyze the molecular mechanisms involved in suppressed ovarian steroidogenesis. The first step in steroidogenesis is the transport of cholesterol through the inner mitochondrial membrane which is mediated by StAR protein (Clark *et al.*, 1994; Clark and Stocco, 1995). The present study demonstrates that both Pb and Cd in isolation and in combination decreased the StAR protein expression levels in the post natal day 56 female rats. A significant decrease in mRNA expression of StAR and Cyp11a were observed in cadmium treated groups, whereas lead and combined metal treated group did not demonstrated any significant alterations. The reported interference of other endocrine disruptors with P450scc activity in rat granulose cells (Dasmahapatra *et al.*, 2000) and in mouse (Fukuzawa *et al.*, 2004) and rat testis (Kleeman *et al.*, 1990) has raised cholesterol metabolism as a putative target of metal's steroidogenic action in general and cadmium in particular.

Changes in cholesterol and ascorbic acid content in ovary are closely linked to ovarian steroidogenesis (Payne and Hales, 2004). In the present study, a significant decrease in ovarian cholesterol content was observed in all the metal treated groups. The maximal decrease was found in cadmium treated group followed by combined metal treated group. Similarly, we also showed significant decrease in the non-enzymatic antioxidant such as Vitamin C levels in the post natal day 56 ovary in metal treated groups. These results indicate that long-term exposure to moderate levels of lead and cadmium throughout gestational and lactational window has the potential to bring about metabolic derangements.

Suppressed antioxidant system and increased superoxide levels within ovarian compartment are responsible for follicular regression (Cassano *et al.*,

1999; Paszkowski *et al.*, 1995). Both *in vivo* and *in vitro* studies have suggested generation of reactive oxygen species (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; Yiin *et al.*, 1998; Laxmipriya *et al.*, 2007; Pillai and Gupta, 2005). Our results show that ovarian GSH content is decreased in all metal treated groups. 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; Nigam *et al.*, 1998). There exist a direct correlation between GSH depletion and enhanced lipid peroxidation. The increase in ovarian TBARS in the present study indicates the failure of antioxidant defense system after early developmental exposure to metals. The suppressed levels of GPx, GR, Mn-SOD, Cu-Zn SOD and CAT activities in ovarian tissue of animals belonging to cadmium treated groups in particular suggest that these enzymes are potential targets for both lead and cadmium. Most of 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 co-factors 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).

In conclusion, data from the present study suggest that metal-induced oxidative stress is one of the major biochemical mechanisms responsible for altered ovarian steroidogenesis as evident from suppressed steroidogenic enzyme activity and altered steroidogenic transcriptional machinery, on reaching adulthood after early developmental exposure to lead and cadmium.

3.4 Testicular Steroidogenesis

3.4.1 Introduction

Increased environmental estrogenic chemicals or "xenoestrogens" has more or less coincided with the decline in sperm counts (Colborn and Clement 1992; Sharpe, 1994). Concerns about such hormonally active pollutants, such as heavy metals lead and cadmium in general and cadmium in particular, has been voiced past few years (Stoica *et al.*, 2000; Taupeau *et al.*, 2003). Epidemiological evidences suggests that both the quality and the quantity of semen in humans has declined progressively over the past half a century (Carlsen *et al.*, 1992) in concert with a general increase in the incidence of male reproductive pathologies. Understanding the consequences of repeated oxidative stress in the male reproductive milieu is gaining wide attention (Cummins *et al.*, 1994; Sikka, 2001; Saleh and Agarwal, 2002; Agarwal and Said, 2005). Free radical production and lipid peroxidation (LPO) are known to be important mediators in testis physiology (Lamirinde *et al.*, 1997). Recent findings have led to the proposal that oxidative stress can play a vital role in the etiology of male infertility (Aitken, 1995; Aitken *et al.*, 1998; Ong *et al.*, 2002). Further, ascorbic deficiency and smoking, have been shown to cause an increase in oxidation of sperm DNA and poor antioxidant levels in semen, clearly emphasizing a potential relationship between oxidative damage to testis and sperms and male reproductive dysfunctions (Saleh *et al.*, 2002). The maturation of sperm requires a specific luminal environment, which in turn is created and maintained by testosterone (T)-dependent absorptive and secretory activites of the epithelium lining the excurrent ducts (Orgebin-Crist, 1996; Syntin *et al.*, 1999). Post natal day (PND) 56 is considered to be the time around first wave of spermatogenesis and spermiogenesis.

However, data on biochemical dysfunctions in testis and cauda epdidymis following gestational and lactational exposures to lead and cadmium in F1 generation post natal day (PND) 56 is limited. Such studies are vital, as elevated reactive oxygen species (ROS) levels may influence some transcription factors,

enzyme activities, modulate cell proliferation, and various important signal transduction pathways, leading to male reproductive dysfunctions and in turn affects the fertility (Agarwal and Saleh, 2002; Kaur *et al.*, 2006).

The present study was therefore designed to elucidate the biochemical and molecular dysfunctions, such as induction of lipid peroxidation, perturbations in antioxidant defenses (enzymatic/non-enzymatic), alterations in transcriptional, translational machinery concerned with testicular steroidogenesis, and testicular/cauda epididymal sperm numbers and motility that ultimately reflects the male fertility status.

3.4.2 Experimental design

Following the experimental regime as discussed previously in section 3.1, rats were sacrificed by decapitation on PND 56. Blood from orbital sinus was collected just prior to decapitation, in clean, dry eppendorfs and allowed to clot at room temperature for 10–15 min. The clear serum was removed after centrifugation at 1500×*g* for 15min at 4°C and stored at–80°C until the assay of hormone. Serum concentrations of testosterone was measured with commercially available kit (Immunotech, France), following the radioimmunoassay (RIA) with a testosterone I^{125}. Radio activity was determined by gamma scintillation counter. Sample preparation was carried out using the method described by Tohda *et al.*, 2001. The testis, cauda epididymis, prostate gland and seminal vesicle were immediately excised out and processed for various biochemical estimations. Testis was used for the measurement of activities of 17β-and 3β-hydroxy steroid dehydrogenase key steroidogenic pathway enzymes as per method of Shivanandappa and Venkatesh, 1997. Cauda epididymis was separated from testis and were put into 2 ml pre-warmed PBS, pH 7.4. Sperm were allowed to diffuse after the epididymal tubule was pierced with a scalpel blade and sperm was forced out so as to enable maximum mature spermatozoa to be diffused out, not forcing out excess material, i.e., immature cells. The dish was shaken gently and, after 5 min of dispersion, an aliquot of sperm was used for sperm count, viability and motility. An aliquot of sperm was diluted 1:100 with fixative (10% formalin in PBS, pH 7.4) and counted using a haemocytometer. Sperm viability

was performed by the eosin nigrosin staining. One drop of semen was mixed with two drops of 1% eosin Y. After 30 s, three drops of 10% nigrosin were added and mixed well. A smear was made by placing a drop of mixture on a clean glass slide and allowed to air dry. The prepared slide was examined using a phase contrast microscope. Pink-stained dead sperm were differentiated from unstained live sperm, and there numbers were recorded following the method of (Eliasson, 1977). Epididymal sperm motility was evaluated in the PBS, pH 7.4. A 50-µl aliquot was diluted 20 times in PBS 37°C, and transferred to a glass slide. Under a light microscope (10X magnification), a random field was chosen, and sperm classified as motile or immotile. Sperm motility was expressed as the percentage of motile sperm per field. The seminal vesicle was removed, weighed and stored at -20°C to determine the content of fructose following the method of (Motoshima and Settlage, 1978).

Western-blot analysis of testicular mitochondrial protein was performed as described previously in the Materials and Methods section (Chapter 2). The integrated optical density of the bands was quantified using scanning densitometry. Total RNA was also isolated from testis using Tri Reagent (Sigma) and intact RNA with an A260/280 ratio 1 6 and above was used for RT-PCR analysis of testicular (StAR and Cyp11a) genes, β-actin served as internal control. The details of the primers used and PCR cycling conditions, size of the PCR-amplified products are listed in Table 1 of Chapter 2.

Oxidative-stress related parameters such as Lipid peroxidation (LPO), Reduced glutathione (GSH), Glutathione peroxidase (GPx), Glutathione reductase (GR), Superoxide dismutase (SOD), Catalase (CAT) activities were assayed in testis and cauda epididymis following standardized protocols which are described in Chapter 2. Biochemical parameters such as Vitamin C content, acid phosphatase activity in testis and cauda epididymis, Fructose content in seminal plasma, testicular cholesterol content were assayed following standardized protocols and the details of the same are discussed in Chapter 2.

One testis per animal was fixed in 15–20 volumes of Bouin's fixative for at least 24 h and cleared in three successive 1-h washes in 70% ethanol. Wet tissue

was stored in 70% ethanol at room temperature. Each testis was embedded in paraffin, and three 5 μm cross-sections from was stained with hematoxylin and eosin and observed under light-microscopy.

3.4.3 Results

The present study did not showed any significant differences in body weight in experimental animals as compared to control animals in F1 generation PND 56 Rats. Table 1 represents the body weight, absolute organ weights (testis and epididymis) and their relative weights. Alterations of relative epididymal weight in lead and cadmium treated groups were significant as compared to the control.

Table 2 shows the accumulation of lead and cadmium in post natal day 56 testis after gestational and lactational exposure to metals in isolation and in combination. Both lead and cadmium were found to be accumulated in testis significantly as compared to the control group.

Table 3 shows testicular vitamin C levels significantly decreased in all the experimental groups whereas testicular acid-phosphatase activity in all the experimental groups were higher as compared to controls and it well correlates with altered tissue reorganization in testis and similar observations were also made in cauda epididymis. Table 3 also demonstrates the maximal decrease in fructose content in cadmium treated animals and combined exposure groups exhibited an intermediate effect and the lead treated group was the least affected group. Total cholesterol in testis was also significantly decreased in cadmium and combined metal treatment groups.

Table 1: Effect of gestational and lactational co-exposure to lead and cadmium on body weight, testis, and epididymis weights in PND 56 male rats.

Parameters	Groups			
	Control	Pb	Cd	Pb + Cd
Body weight(g)	184.5±8.34	196.5±3.75	162.3±4.09	194.3±9.51
Testis (mg)	1325±85.39	1350±54.01	1320±20.00	1425±85.39
Epididymis (mg)	344.3±21.26	377.3±19.65	222.0±18.81**	324.5±10.97
Relative Testis wt. (mg/g b.wt)	7.36±0.47	6.72±0.08	8.12±0.54	7.16±0.19
Relative Epididymis wt. (mg/g b.wt)	1.86±0.05	1.652±0.04*	1.363±0.09**	1.942±0.04

The data are presented as mean ± SEM. of 6 independent observations.
*p<0.05, ** p<0.01 vs. control

Table 2: Lead and Cadmium levels in testis of PND 56 F1 offspring after gestational and lactational co-exposure to lead and cadmium.

Parameters	Groups			
	Control	Pb	Cd	Pb + Cd
Testis				
Lead[a]	0.282±0.035	1.020±0.090 ***	0.290±0.026###	0.625±0.125 #
Cadmium [a]	0.018±0.003	0.015±0.001	0.136±0.004*** ###	0.090±0.011*** ### @@

a =µg/g tissue

The data are presented as mean ± SEM. of 4 independent observations.
*** p<0.001 vs. control; #p<0.05, ###p<0.001 vs. lead and @@p<0.01 vs. cadmium group

Table 3: Effect of gestational and lactational co-exposure to lead and cadmium on biochemical parameters in testis, cauda epididymis and seminal vesicle of PND 56 rats

Parameters	Groups			
	Control	Pb	Cd	Pb + Cd
Testis				
Vitamin C [a]	4.78±0.97	3.97±0.104**	3.42±0.108*** #	3.82±0.162***
Total Cholesterol [b]	3.53±0.13	3.33±0.09	2.57±0.08*** ##	3.19±0.14 $$
Acid phosphatase [c]	0.34±0.007	0.39±0.015	0.51±0.012*** ###	0.45±0.019 *** #
Cauda epididymis				
Vitamin C [a]	2.57±0.14	1.95±0.15*	0.92±0.07 *** ###	1.6±0.12 *** $$
Acid phosphatase [c]	0.263±0.02	0.206±0.02	0.125±0.01*** #	0.205±0.02 $
Seminal Vesicle				
Fructose [d]	4.87±0.17	3.82±0.07***	2.32±0.08 *** ###	3.30±0.18 *** $$$

The data are presented as mean ± SEM. of 6 independent observations.
*p<0.05, ** p<0.01, *** p<0.001 vs. control, #p<0.05, ##p<0.01, ###p<0.001 vs. lead and $p<0.05, $$p<0.01, $$$p<0.001 vs. cadmium group.

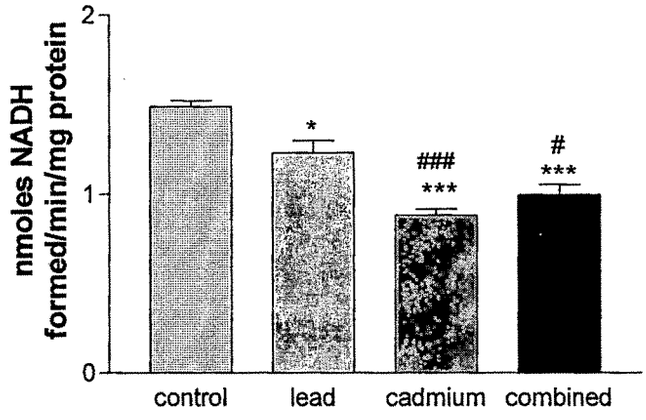
a=µg/gm tissue; b= µg/gm; c=µmoles PNP released/min./mg protein; d=mg/gm tissue

Figure 3 shows the effect of Pb and Cd alone and in combination on basal level testicular StAR protein expression levels as compared to control group. Cadmium treated group showed significant decrease compared to the control group.

In order to assess the effect of gestational and lactational co-exposure to lead and cadmium on the testicular steroidogenic transcriptional machinery, the mRNA expression levels of StAR and Cyp11a were assessed by RT-PCR employing β-actin as internal control. Analysis revealed a significant decrease in mRNA expression of StAR in lead and cadmium treated groups as compared to control animals (Fig. 4). Cyp11a mRNA expression was significantly decreased only in cadmium and combined metal treated groups.

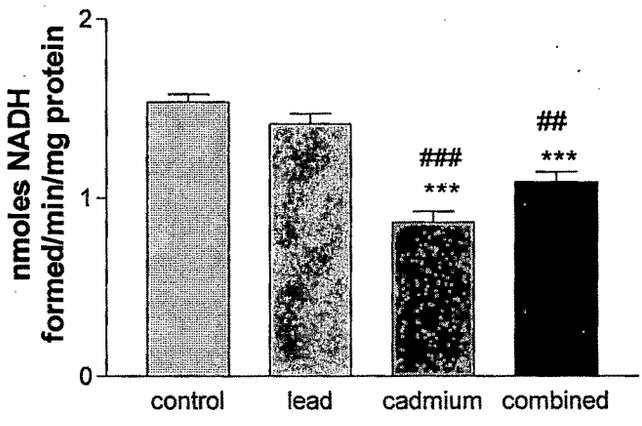
Figure 5 represents serum testosterone levels in all the metal treated groups. Results indicate that serum testosterone was significantly decreased in all the experimental groups as compared to the control animals.

Figure 1: Effect of gestational and lactational exposure to lead and cadmium on testicular 17β- hydroxysteroid dehydrogenase activity of PND 56 rats



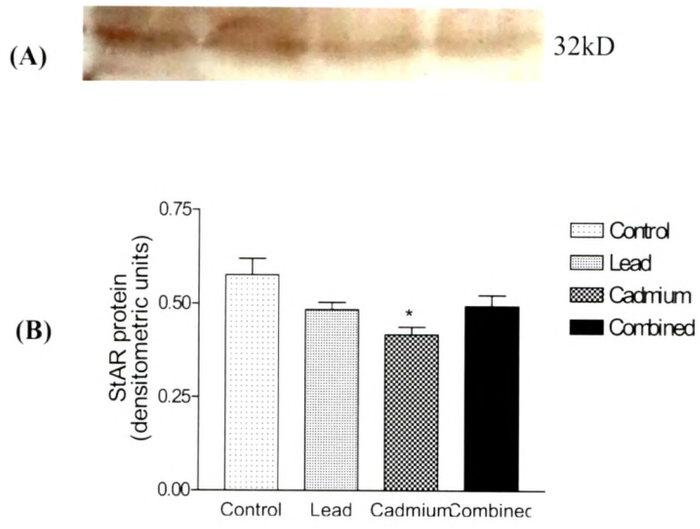
N= 6; the values are mean ± SEM. *p<0.05, *** p<0.001 vs. control; #p<0.05, ###p<0.001 vs. lead

Figure 2: Effect of gestational and lactational exposure to lead and cadmium on testicular 3β-hydroxysteroid dehydrogenase activity of PND 56 rats



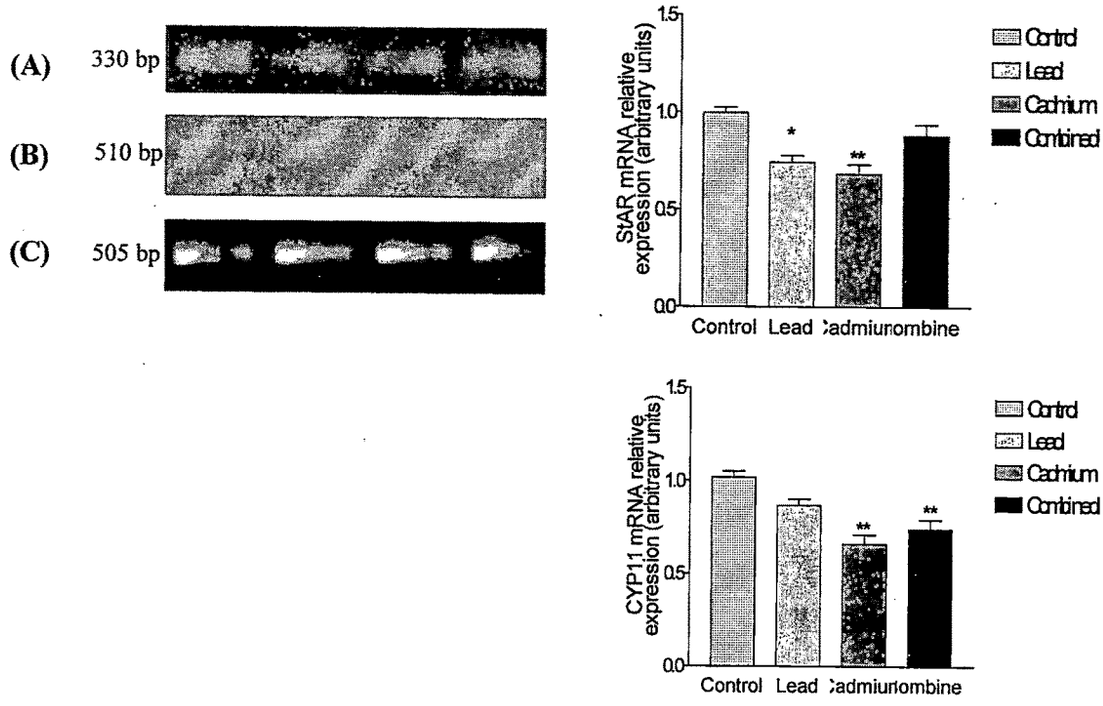
N= 6; the values are mean ± SEM; *** p<0.001 vs. control; ##<p<0.01; ###p<0.001 vs. lead

Figure 3: Effect of gestational and lactational co-exposure to lead and cadmium on basal level testicular StAR protein expression by western-blot analysis. (A) Representative Western immunoblot of StAR protein in testis (B) Composite graph showing the mean (±SEM) densitometric quantitation of the bands from three blots corresponding to the StAR protein



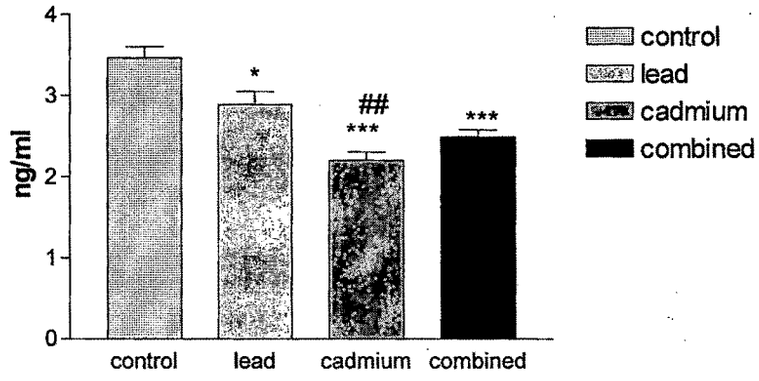
The data are presented as mean ± SEM. of 3 independent observations.
*p<0.05, vs. control

Figure 4: Effect of gestational and lactational co-exposure to lead and cadmium on the expression of testicular (A) StAR gene and (B) CYP11a gene in PND 56 testis.



N=3. The values are mean±SEM.
** p<0.01, * p<0.05 vs. control group

Figure 5: Effect of gestational and lactational exposure to lead and cadmium on serum testosterone levels of PND 56 rats



N= 6; the values are mean ± SEM; *p<0.05, *** p<0.001 vs. control; ##<p<0.01 vs. lead

Table 4 shows the effect of lead and cadmium exposure on testicular and epididymal sperm count as well as epididymal sperm motility. Experimental groups showed significantly lower testicular and epididymal sperm count as compared to controls. Epididymal sperm motility was also significantly lower as compared to control groups. Cadmium group exhibited maximum effect and co-exposure showed lowest reduction in sperm count and motility.

Table 5 shows the effect of gestational and lactational exposure to lead and cadmium on testicular and cauda-epididymal GSH and TBARS levels. Significant depletion in GSH levels were observed in both testis and epididymis of cadmium treated rats as compared to controls. TBARS levels were significantly elevated in both testis and epididymis of experimental groups as compared to controls.

Table 6 demonstrates maximum suppression of specific activities of antioxidant enzymes (SOD, GPx and CAT) in cadmium and combined metal exposed rats. Lead treated rats did not demonstrate any significant alterations in the specific activities of antioxidant enzymes.

Histological observations revealed normal testicular structures, except for the mild disintegration of seminiferous tubular compartment in lead and

cadmium exposed testes as compared to the control. However, testes of combined metal exposure group demonstrated severe disintegration of seminiferous tubular compartment (Figure 6).

Table 4: Effect of gestational and lactational exposure to lead and cadmium on testicular and cauda epididymal sperm numbers, % sperm motility of PND 56 rats

Parameters	Groups			
	Control	Pb	Cd	Pb + Cd
Testicular sperm number (x 10^6)	142.7±3.32	128.7±3.60*	109.2±1.85*** ###	120.7±1.70*** $
Cauda epididymal sperm number (x 10^6)	80.17±4.54	65.50±2.94*	51.33±1.78*** #	59.00±2.82***
% cauda epididymal sperm motility	86.83±1.28	74.67±1.54**	54.33±2.28*** ###	68.17±3.56*** $$

The data are presented as mean ± SEM. of 6 independent observations.
*p<0.05, ** p<0.01, *** p<0.001 vs. control; #p<0.05, ###p<0.001 vs. lead and $p<0.05, $$p<0.01 vs. cadmium group.

Table 5: Effect of gestational and lactational exposure to lead and cadmium on testicular and cauda epididymal non-enzymatic antioxidant and lipid peroxidation

Parameters	Groups			
	Control	Pb	Cd	Pb + Cd
Testis				
GSH[a]	61.33±2.15	51.82±3.60	40.15±2.35*** #	47.46±2.14**
TBARS[b]	70.40±1.50	90.9±2.16*	116.6±2.89*** ##	90.11±8.15* $$
Cauda epididymis				
GSH[a]	29.03±1.06	20.05±0.58***	14.99±0.93*** ##	16.78±0.64***
TBARS[b]	48.19±2.62	67.44±2.22***	79.47±2.04*** #	70.31±3.45***

The data are presented as mean ± SEM. of 6 independent observations.
*p<0.05, ** p<0.01, *** p<0.001 vs. control; #p<0.05, ##p<0.01 vs. lead and $$p<0.01 vs. cadmium group.
a=pmoles/mg protein, b=nmoles MDA/min/mg protein

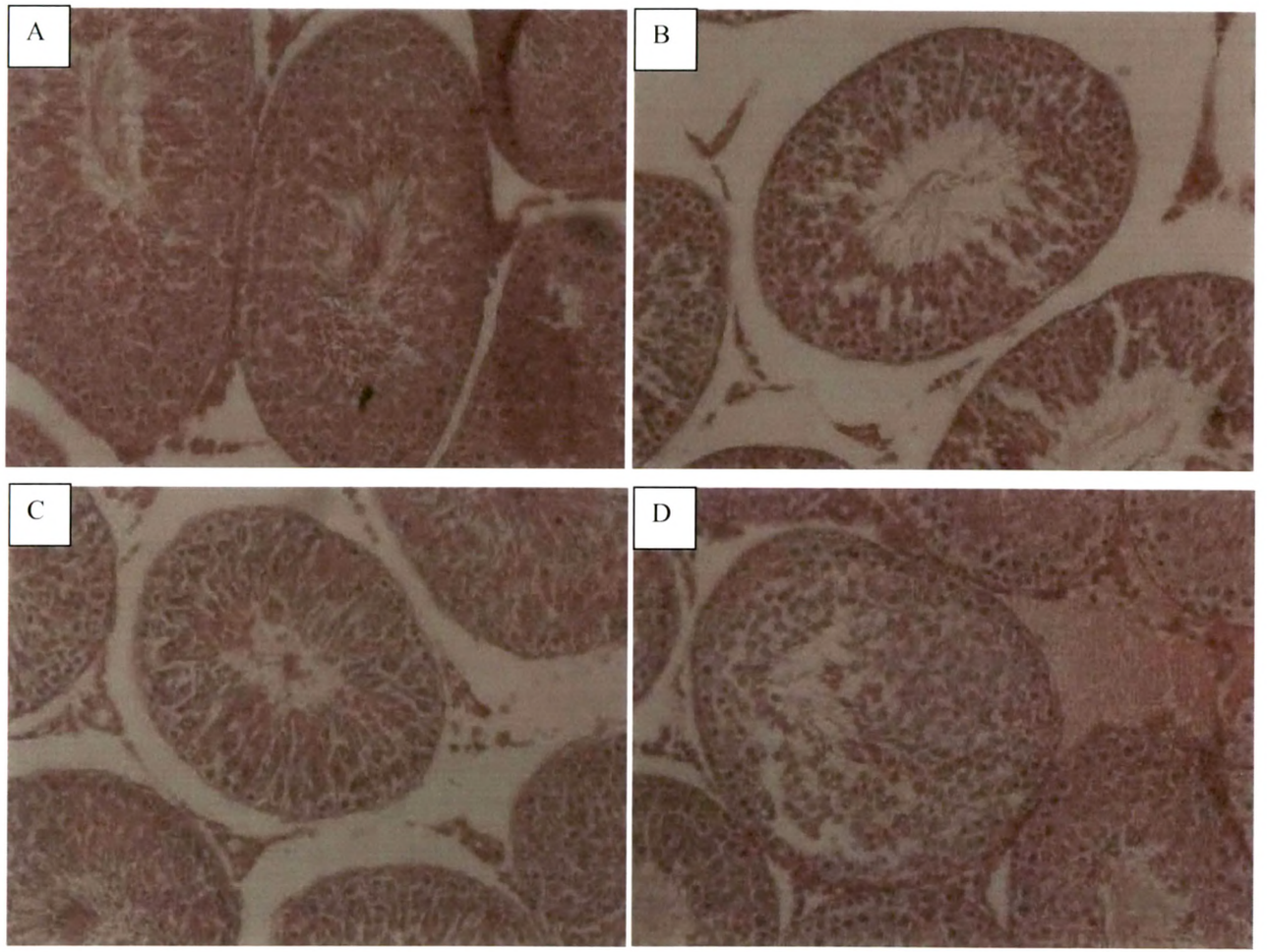
Table 6: Effect of gestational and lactational exposure to lead and cadmium on testicular and cauda-epididymal enzymatic antioxidants

Parameters	Groups			
	Control	Pb	Cd	Pb + Cd
Testis				
CAT[a]	94.32±1.75	80.83±2.49**	48.55±2.33***###	69.04±1.74*** ## $$$
SOD[b]	4.93±0.21	4.58±0.122	3.34±0.15*** ###	3.99±0.14**
GPx[c]	677.5±19.25	651.2±18.27	525.0±12.58*** ###	641.2±13.14 $$$
Cauda epididymis				
CAT[a]	71.47±1.80	62.17±2.0	44.58±1.72*** ###	58.18±3.31** $$
SOD[b]	2.87±0.18	2.70±0.20	1.46±0.18*** ##	2.36±0.21 $
GPx[c]	581.2±11.58	584.0±9.35	452.5±11.53*** ###	565.3±12.51 $$$

The data are presented as mean ± SEM. of 6 independent observations.
** p<0.01, *** p<0.001 vs. control; ##p<0.01, ###p<0.001 vs. Lead and $p<0.05, $$p<0.01, $$$p<0.001 vs. cadmium group.

[a]μ mole H_2O_2 decomposed/min/mg protein at 37°C.
[b]Amount of enzyme required for 50 % inhibition of pyrogallol autoxidation at 37°C.
[c] Δ log [GSH]/min/mg protein at 37°C.

Figure 6: Histology of Testis



A : Control, B: Lead, C: Cadmium, D: Combined

3.4.4 Discussion

Results from the present study indicate that lead and cadmium exposure alone and in combination during gestational and lactational period has an adverse effect in F1 generation male rats as far as relative organ weights are concerned. Exposure to lead and cadmium significantly reduced absolute weights of accessory reproductive organs, but testis did not exhibit any significant differences from controls in terms of absolute weight. However, when organ weights were expressed as relative weights, the decrease in relative weight of the epididymis was significant but not in case of testis. The observations of reduction in weights of testis and epididymis are in agreement with earlier reports (Ng and Liu, 1990). Similar responses have been reported previously in relation to estrogen exposure, where F1 rats sired from P1 rats had significantly lowered epididymal weight and sperm numbers whereas no significant decrease was noted in testis weight (Cook *et al.,* 1998). The epididymal effects observed in the present study may have resulted from estrogenic effects of metals, and cadmium in particular (Oko and Hrudka, 1984).

Steroidogenesis in the testes is under the physiologic control of two dehydrogenases. A constant supply of cholesterol and ascorbic acid is required for the synthesis of steroid hormones. Both dehydrogenases are directly involved in biosynthesis of testosterone from pregnenolone as well as androstenedione. Any alteration in the activity of these two enzymes affects androgen production. Reduced activities of these key steroidogenic enzymes (17β-HSD & 3β-HSD) in F1 generation PND 56 male rats indicate suppressed testicular steroidogenesis. This may be due to a direct effect of lead and cadmium on the testicular steroid biosynthesis, by virtue of metal accumulation in the testis as a consequence of transfer during lactational as well as gestational exposure.

Efforts were put to analyze the molecular mechanisms involved in suppressed testicular steroidogenesis. The first step in steroidogenesis is the transport of cholesterol through the inner mitochondrial membrane which is

mediated by StAR protein (Clark *et al.*, 1994; Clark and Stocco, 1995). The present study demonstrates that Cd treatment decreased the basal level StAR protein expression in testes of post natal day 56 rats. A significant decrease in mRNA expression of StAR and P450scc were also observed in metal treated groups. The mechanism behind susceptibility of transcriptional machinery associated with steroidogenesis has been discussed in earlier section of this chapter (3.3). These findings are in agreement with earlier reports of endocrine disruptor mediated StAR gene and protein inhibition (Sugawara *et al.*, 2001; Sengupta *et al.*, 2004). Such observation has raised cholesterol metabolism as a putative target of metal's steroidogenic action in general and cadmium in particular.

Lower serum testosterone level seen in experimental animals might be due to the direct effect of lead and cadmium on leydig cells (Shalgi *et al.*, 1989) or an indirect effect by altering gonadotropin secretion from pituitary, since, both lead and cadmium have been shown to get accumulated in hypothalamus and pituitary as discussed in section (3.2) of this chapter.

Histology of testes showed marked alterations in seminiferous tubular arrangement in metal treated groups. The disintegration of tubular compartment is well correlated with biochemical alterations induced by lead and cadmium as seen from the increased testicular acid phosphatase activity. Acid phosphatase is very important for tissue reorganization and tissue repair. Intracellularly, acid phosphatase activity is restricted to the lysosomes. Thus, lead and cadmium mediated histological and biochemical alterations suggest derangements in testicular tissue organization and thereby implicating serious consequences on spermatogenesis process.

The most interesting finding of the present study was that lead and cadmium induced alterations in cauda-epididymal sperm contents and motility occurred in the absence of an alteration in sperm morphology. Studies have also shown that both castration and hypophysectomy alter motility and fertility in epidiymal sperm (Dyson & Orgebin-Crist, 1973) suggesting the role of

hypothalamic-pituitary axis in male fertility. Sperm count directly depends upon the hormonal input from the hypothalamic-pituitary-testicular (HPT) axis and its effect on functional testicular epithelium. Significant alterations in testicular and epididymal sperm numbers as found in the present study clearly suggest the spermotoxic effects of lead and cadmium and its interference in HPT axis function. Sperm acquires its characteristic motility by undergoing maturation process which occurs under the influence of epididymal proteins and other substances, in turn producing structural and biochemical changes in the sperms during their passage through the epididymis. Thus, motility is chiefly a parameter of post testicular i.e. epididymal function and is vulnerable towards reproductive toxicants (Kaneto *et al.,* 1999). Lower serum testosterone levels in the experimental groups as observed in the present study is believed to accelerate sperm transport (Sujarit and Pholpramool, 1985) and thus can explain the reduced number of sperm observed in the cauda-epididymis. It is also shown that altered testosterone levels profoundly influence the number of androgen receptors on the epididymis (Goyal *et al.,* 1998). Moreover, fructose is the main energy source for spermatozoa motility and thus decrease in the sperm motility observed in the present study might be due to the metal induced alteration of normal fructolysis. These observations showed similarities with the earlier findings (Viskum *et al.,* 1999; Rosa *et al.,* 2003; Rhemrev *et al.,* 2001).

The essential role of vitamin C in the physiology of the testes appears to be associated with protein metabolism (Levine, 1986). The many enzymatic functions of vitamin C is essential for the normal integrity and function of the testes ie the synthesis, development and maintenance of normal sperm (Dawson, *et al.,* 1987). Additionally, Vitamin C is an excellent radical scavenger due to the reductive properties of the ascorbic acid/dehydroascorbic acid ratio in biological media (Sapper *et al.,* 1982) and it may nullify the possible spermicidal and genotoxic effects of various free radicals that results from oxidative stress condition induced by lead and cadmium. It also plays an important role in testicular homonogenesis (Chowdhury *et al.,* 1984). In the present study,

testicular vitamin C content was found to be depleted in all the metal treated groups and therefore suggesting disturbance in testicular nutritional status necessary for above mentioned functions.

We were interested in investigating whether Pb- and Cd- induced suppression of testicular steroidogenesis and epididymal functions are due to oxidative damage to the testis. Both Pb and Cd compounds are known to induce various forms of oxidative damages, such as increased lipid peroxidation (Manca *et al.*, 1991), a reduction in glutathione peroxidase (Garcia-Fernandez, 2002) and DNA strand breaks (Snyder, 1988). However, Cd is not capable of accepting or donating electrons under physiological conditions (Ochi *et al.*, 1987), and the mechanism by which it induces oxidative stress remains to be clarified. When the parameters of oxidative damage were evaluated, we observed that cadmium treatment alone and in combination led to higher levels of lipid peroxidation in the testis and cauda epdididymis; and so testicular and epididymal TBARS increased, and the activities of the antioxidant enzymes, superoxide dismutase (SOD) and glutathione peroxidase (GPx) were significantly inhibited. On the other hand, Cd is shown to interact with cell membranes; and hence lipid peroxidation may be a direct consequence of membrane damage (Casalino *et al.*, 1997) or of weakened antioxidant defense (Jamall and Sprowls, 1987). The reduction of SOD activity is known to be due to replacement of the zinc (Zn) and/or manganese (Mn) of the SOD by Cd (Jacobson and Turner, 1980). The reduction in activity of glutathione peroxidase might be due to depletion of selenium (Se) by cadmium (Zumkley, 1988). Similarly, CAT activity was also significantly suppressed in testes and cauda-epididymis exposed to cadmium and combined metal treatments. Reduction in the activity of catalase may reflect inability of testicular and cauda-epididymal mitochondria and microsomes to eliminate hydrogen peroxide produced as a consequence of oxidative stress. The reduction may also be due to enzyme inactivation caused by excess ROS production in mitochondria and microsomes (Pigeolet *et al.*, 1990). The changes in oxidant defense systems associated with both lead and cadmium exposure could

increase the steady-state level of oxidants in the testis as well as cauda-epididymis. Moreover, reactive oxygen species (ROS) are produced continuously in cells as a by-product of mitochondrial electron transport and other metabolic processes (Hales, 2002; Liu *et al.*, 2001). In the case of spermatozoa, a shift of the redox status of sperm protein thiol to disulfides occurs as the spermatozoa undergo maturation during their passage through the epididymis (Sies,1991).The epididymal region in which this change occurs differs among species (Maiorino and Ursini, 2002). Studies have also shown the involvement of epididymal GSH in sperm thiol content oxidation during sperm maturation (Seligman *et al.*, 1997). Hence, the reduced glutathione levels and suppressed antioxidant defense system in epididymis as observed in the present study, particularly in cadmium exposed group, might prove deleterious for sperm maturation. This also confirms that reduced sperm motility observed in testes of metal treated groups might be the consequence of impaired sperm maturation within the epididymis.

In conclusion, though the present study do not provide direct evidence of a link between human exposure to environmental endocrine disruptors and falling sperm counts in men, however, the findings do provide some preliminary, indirect evidence that exposure of rats to endocrine disruptors such as lead and cadmium during gestational and lactational life can result in altered epididymal and testicular functions along with decreased sperm count & motility in adulthood. As these effects occurred in rats after only few weeks of exposure, whereas in men the corresponding window of development span several years, and hence there is always a possibility that similar effects in men might be of greater magnitude than the consequences observed in rats.

3.5 Hepatic Xenobiotic/Steroid Metabolism

3.5.1 Introduction

The liver is an essential physiological site for maintaining homeostasis in all vertebrates. In addition to metabolizing toxicants, the liver plays an essential role in hormone homeostasis, as it metabolizes both peptide and steroid hormones (Louis *et al.*, 2001). The hepatic metabolism of many drugs and steroids occurs in a sexually dimorphic manner (Niwa *et al.*, 1995; Wilson *et al.*, 1999) and thus could potentially serve as a biomarker for exposure to both naturally occurring and synthetic hormones. Maintenance of steroid homeostasis involves several variables including rate of hormone synthesis, interactions among hormones, and rates of secretion, transport, biotransformation, and elimination (Wilson and LeBlanc, 2000). The sexual dimorphism seen in expression of sex-steroid metabolizing enyzmes is imprinted during development, a process mediated by sex steroids. Considerable evidence exists demonstrating that many hepatic P450s are regulated by developmental, sex, or hormonal factors in addition to receptors that interact with foreign chemicals (Prough *et al.*, 1996). Drug or xenobiotics metabolizing enzymes play central roles in the biotransformation, metabolism and/or detoxification of xenobiotics or foreign compounds, which are introduced to the human body (Rushmore and Kong, 2002). Our earlier studies reported the effects of gestational and lactational co-exposure to lead and cadmium on hepatic estradiol metabolizing enzymes in pups (PND21) and fetus (Pillai and Gupta, 2005). Apart from this, most of the studies till date have mainly focused on body confronting with high xenobiotic load that result in induction of hepatic phase-I and phase-II enzymes (Park *et al.*, 1996; Manson *et al.*, 1997).

The present study was undertaken to evaluate the effects of gestational and lactational co-exposure to lead and cadmium on hepatic phase-I enzymes (NADPH-and NADH-cytochrome c reductase) and hepatic phase-II enzymes (UDP- glucoronyl transferase, γ-GT, GST) in PND56 male and female rats. We hypothesized that early developmental exposure to lead and cadmium alone and

in combination would alter the sexually dimorphic patterns of hepatic xenobiotic and steroid metabolizing enzyme activities of F1 generation PND56 rats in a sex-dependent manner. We also evaluated the non-enzymatic and enzymatic antioxidants to analyze whether metal induced reactive oxygen species (ROS) is acting as one of the possible biochemical mechanism involved in metal induced hepatotoxicity.

3.5.2 Experimental design

Following the experimental regimen as described in section 3.1, on PND 56, the animals were sacrificed by decapitation and the livers were quickly excised, rinsed in ice-cold saline to clear them of blood, weighed, and finely minced in the same solution and homogenized (10% w/v) in a Potter Elvehjem homogenizer with a Teflon pestle. For biochemical examination, liver was snap-frozen in liquid nitrogen and stored at -80° C until analysis. 10% homogenate was prepared in cold 0.25 M of sucrose buffer (pH 7.4). The homogenate was centrifuged at 1,000 g for 15 min at 4°C. The sediment was discarded and supernatant was centrifuged for 8,000 g for 30 min at 4°C. For further fractionation the above supernatant was centrifuged at 15,000 g for 45 min and the pellet thus obtained was dissolved in minimum amount of 0.25 M of sucrose buffer (crude microsomal preparation). The isolation of mitochondria and cytosolic fractions were carried out according to the procedures described in Chapter 2.

Phase-I and Phase-II enzymes that include NADPH- and NADH-cytochrome c reductase, Glutathione *S*-transferase (GST), Gamma-glutamyl transpeptidase (γ-GT), 17β-hydroxy steroid dehydrogenase activity (17β-HSD), UDP glucoronyl transferase (UDPGT), enzyme activities were estimated in appropriate liver homogenate fractions as described in detail in Chapter 2. Hepatic oxidative stress parameters such as Lipid peroxidation (LPO), Reduced glutathione (GSH) content, Glutathione peroxidase (GPx), Glutathione reductase (GR), Superoxide dismutase (SOD), Catalase (CAT) activities were assayed

following standardized methods as described in detail in Chapter 2. Hepatic biochemical parameters such as Glycogen, Cholesterol, DNA, RNA were estimated following standardized protocols as described in Chaper 2. Toxicity parameters such as serum creatinine levels, serum alkaline phosphatase and serum glutamate pyruvate transaminase (SGPT) were also analyzed to check the toxicity of administered metal dose following the present experimental regime. Details of all the protocols have been described in Chapter 2 (Material and Methods).

3.5.3 Results

Alterations of body-weights, relative liver weights in the metal treated groups, compared to control, were mostly insignificant. (Table 1). The hepatic phase-I xenobiotic metabolizing enzyme (NADH- cytochrome c reductase and NADPH- cytochrome c reductase) activities in both male and female PND 56 rats are represented in Figures (1 &2). Cadmium exposure group showed the maximum suppressive effect while the lead exposure group showed the least suppressive effect and the trend was similar in both male and female PND 56 rats. Phase-I xenobiotic metabolizing enzymes exhibited no sexual dimorphic pattern; however the enzyme activities were significantly suppressed in both the sexes.

Figures 3, 4, 5 & 6 represents the hepatic phase-II xenobiotic and steroid metabolizing enzymes (γ-GT, UDP-glucoronyl transferase, GST & 17β-HSOR) respectively. Results indicate that the enzyme activities were significantly decreased in all metal-exposed groups in both PND 56 F1 generation female and male rats in a sex-specific manner. In most of the parameters, combined exposure showed an intermediate effect as compared to other metal exposure groups in female offspring whereas there was a trend of additive effect observed in combined metal treated group in male offspring.

Histological observations of the liver after gestational and lactational exposure to lead and cadmium alone and in combination showed alterations. The metal treatment caused marked changes in liver such as massive fatty degeneration in the hepatocytes and large vacuoles in cytoplasm. Significant histological changes such as degenerative damage, hepatic necrosis were observed in the cadmium and combined metal exposed animals (Figure 7).

Table 3 shows the effect of lead and cadmium either alone or in combination on markers of general toxicity in both male and female PND 56 rats. SGPT, a marker of liver damage, showed higher activity only in cadmium treated of male rats, whereas, changes observed in creatinine as well as ALP was within the normal range.

Table 4 demonstrates the effect of lead and cadmium either alone or in combination on non-enzymatic antioxidant and lipid peroxidation in both male and female PND 56 rats. Depletion in reduced glutathione was seen in cadmium treated group of both male and female rats. Lipid peroxidation i.e measured as thiobarbituric acid reactive species (TBARS) levels, were higher in cadmium treated groups in male and female rats.

Table 5 demonstrates the effect of lead and cadmium either alone or in combination on enzymatic antioxidants in both male and female PND 56 rats. Cadmium treated group demonstrated maximum decrease in the specific activities of hepatic Cu-Zn SOD, Mn SOD, CAT, GPx, GR, while the combined metal exposed group exhibited an intermediate effect. Lead exposed group exhibited the least effect on the specific activities of antioxidant enzymes.

Table 6 demonstrates the effect of lead and cadmium either alone or in combination on biochemical parameters of liver in both male and female PND 56 rats. In both male and female rats, DNA and RNA content showed a maximum change in liver of cadmium-treated rats, whereas liver of combined metal treated animals exhibited a minimum changes as compared to the control. While the liver of lead treated group exhibited the least effect in females and it was non-

significant in male rats. Cholesterol and Glycogen content were maximally decreased in liver of cadmium treated male and female rats. While the liver of lead treated groups was least affected and the combined metal treated group manifested an intermediate effect.

Table 1: Body weights, absolute liver weights of PND 56 F1 offspring after gestational and lactational exposure to lead and cadmium alone and in combination

Parameters	Groups			
	Control	Pb	Cd	Pb + Cd
Body weight (g)				
Male	174.3±6.63	157.6±4.20	163.6±5.61	165.5±5.25
Female	143.8±3.48	132.9±2.70	136.9±2.80	134.2±3.78
Absolute Liver weight (mg)				
Male	5.14±0.07	4.94±0.09	4.86±0.09	4.91±0.10
Female	5.08±0.12	4.97±0.09	5.00±0.11	4.95±0.05

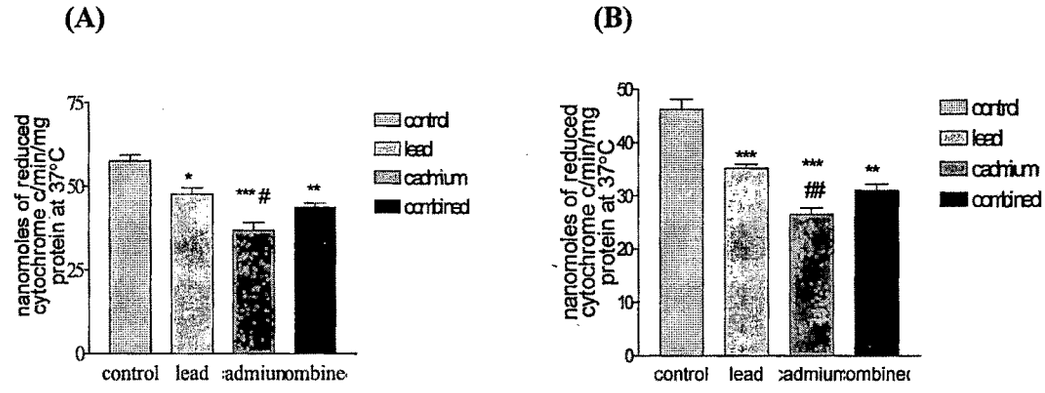
The data are presented as mean ± SEM. of 6 independent observations.

Table 2: Lead and cadmium contents in liver of PND 56 F1 offspring after gestational and lactational exposure to lead and cadmium

Groups	Male		Female	
	Pb	Cd	Pb	Cd
Control	0.117±0.012	0.073±0.002	0.267±0.013	0.037±0.001
Lead	0.465±0.035***	0.063±0.004	0.817±0.067 ***	0.027±0.001
Cadmium	0.104±0.006###	0.772±0.094*** ###	0.152±0.018 ###	0.090±0.004*** ###
Combined	0.132±0.009###	0.607±0.058 *** ###	0.391± 0.034 ### @@	0.068±0.003 *** ### @@

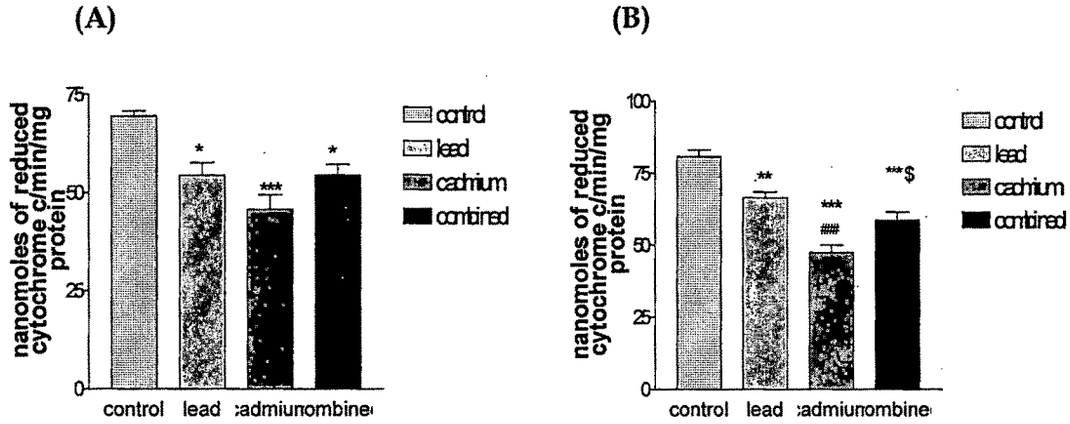
The data are presented as mean ± SEM. of 4 independent observations.
*** $p<0.001$ vs. control; ###$p<0.001$ vs. lead and @@$p<0.01$ vs. cadmium group.

Figure 1: Effect of gestational and lactational exposure to lead and cadmium alone and in combination on NADPH- cytochrome c reductase activity in PND 56 female rats (A) and male rats (B)



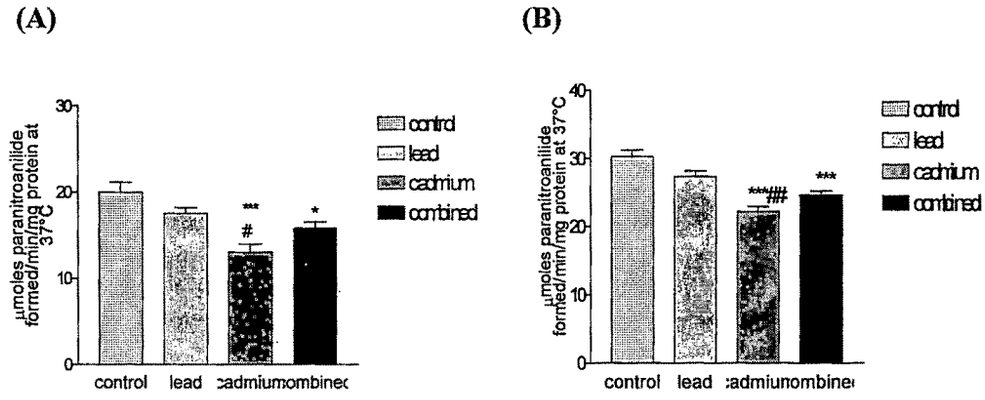
The data are presented as mean ± SEM. of 4 independent observations.
*p<0.05, ** p<0.01, *** p<0.001 vs. control; # p<0.05, ## p<0.01 vs. lead.

Figure 2: Effect of gestational and lactational exposure to lead and cadmium alone and in combination on NADH- cytochrome c reductase activity in PND 56 female rats (A) and male rats (B)



The data are presented as mean ± SEM. of 4 independent observations.
*p<0.05, ** p<0.01, *** p<0.001 vs. control; ### p<0.001 vs. lead and $ p<0.05, vs. cadmium group

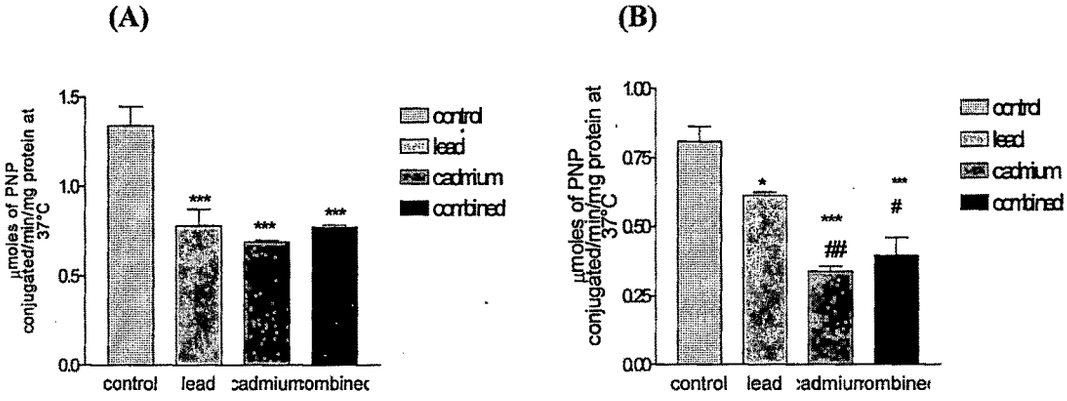
Figure 3: Effect of gestational and lactational exposure to lead and cadmium alone and in combination on γ-glutamyl transferase activity in PND 56 female rats (A) and male rats (B)



The data are presented as mean ± SEM. of 5 independent observations.

* p<0.05, *** p<0.001 vs. control; # p<0.05, ## p<0.01 vs. lead

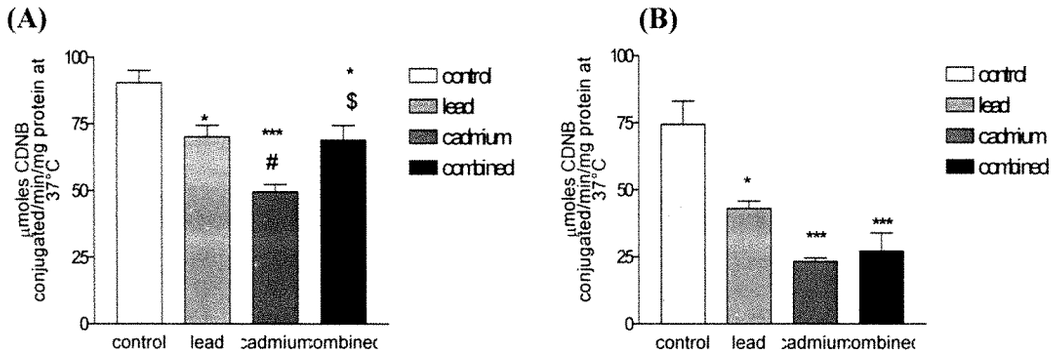
Figure 4: Effect of gestational and lactational exposure to lead and cadmium alone and in combination on UDP-glucoronyl transferase activity in PND 56 female rats (A) and male rats (B)



The data are presented as mean ± SEM. of 5 independent observations.

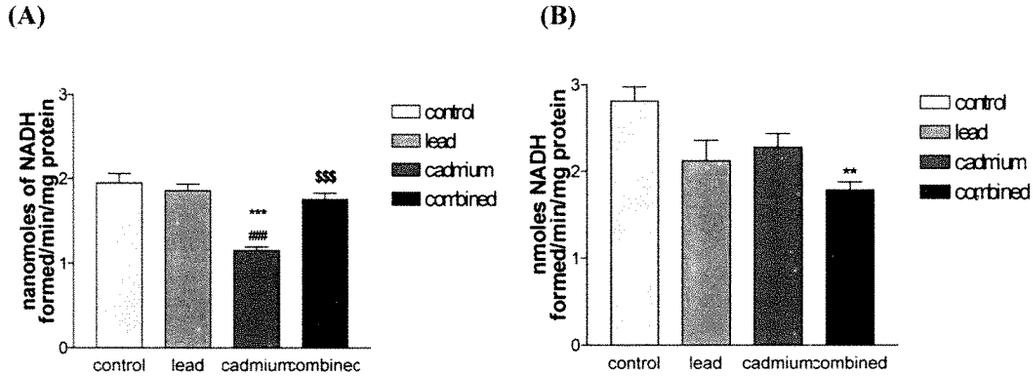
*p<0.05, *** p<0.001 vs. control; #p<0.05 # #p<0.01 vs. lead and $ p<0.05 vs. cadmium group

Figure 5: Effect of gestational and lactational exposure to lead and cadmium alone and in combination on Glutathione-S-transferase activity in PND 56 female rats (A) and male rats (B)



The data are presented as mean ± SEM. of 5 independent observations.
*p<0.05, *** p<0.001 vs. control; # p<0.05 vs. lead and $ p<0.05 vs. cadmium group

Figure 6: Effect of gestational and lactational exposure to lead and cadmium alone and in combination on 17β-hydroxysteroid oxidoreductase activity in PND 56 female rats (A) and male rats (B).



The data are presented as mean ± SEM. of 5 independent observations.
** p<0.01, *** p<0.001 vs. control; ### p<0.001 vs. lead and $$$ p<0.001 vs. cadmium group

Figure 7: Histology of PND 56 liver of female rats

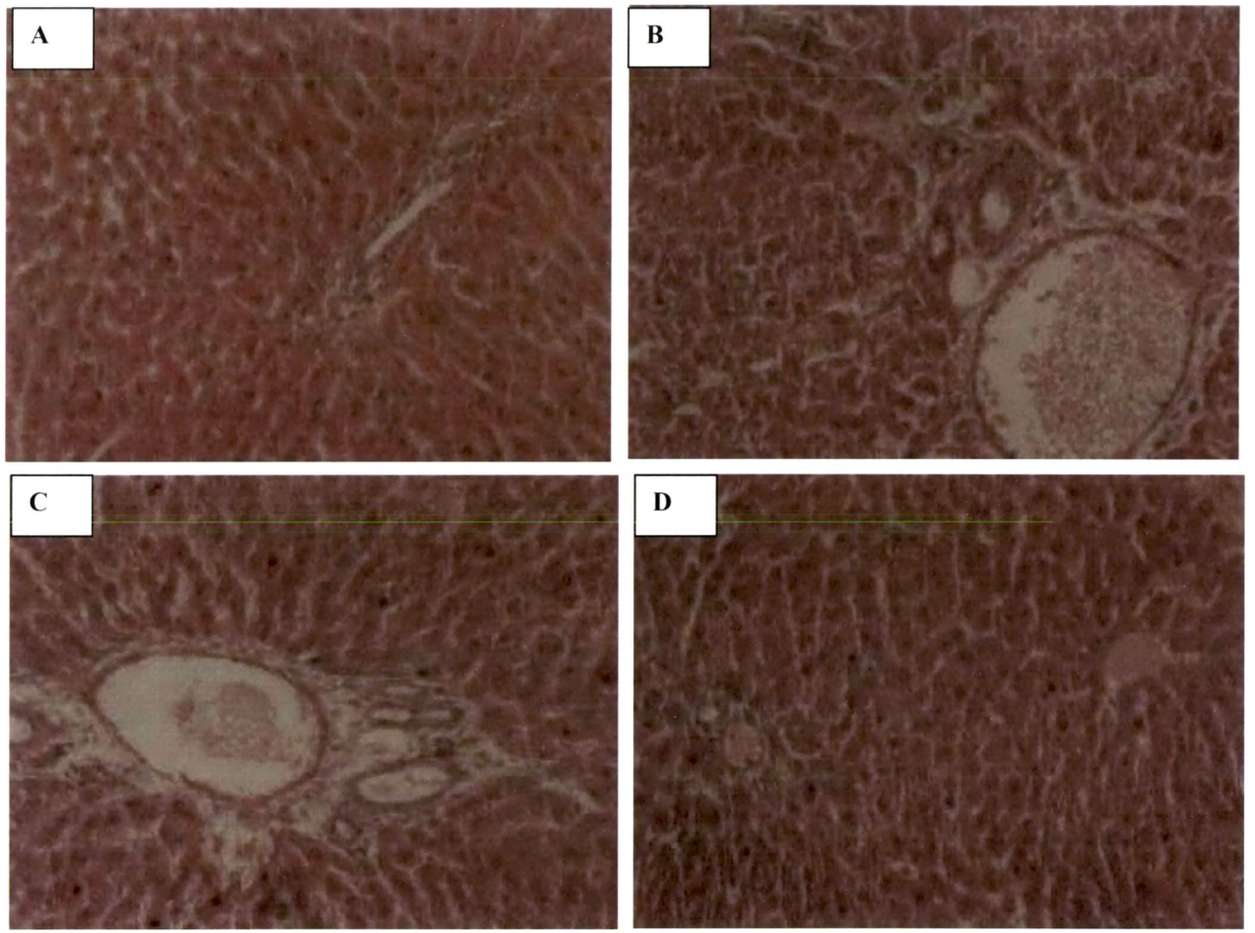


Table 3: Effect of gestational and lactational exposure to lead and cadmium on markers of general toxicity in PND 56 rats

Parameters	Groups			
	Control	Pb	Cd	Pb + Cd
Serum glutamate pyruvate dehydrogenase (IU/L)				
Female	25.16±.749	27.37± 0.952	27.30 ± 1.210	25.79 ± 1.270
Male	32.16 ± 0.617	32.64 ± 0.759	36.00 ± 1.259*	33.88 ± 1.325
Serum creatinine (%)				
Female	0.885 ± 0.025	0.911± 0.030	0.920 ± 0.021	0.949 ± 0.023
Male	1.108 ± 0.018	1.108 ± 0.036	1.269 ± 0.049*	1.211 ± 0.034*
Serum alkaline phosphatase (IU/L)				
Female	76.67 ± 1.205	70.20± 2.598	72.11 ± 2.388	77.24 ± 1.046
Male	85.84 ± 1.297	81.56 ± 2.153	75.74 ± 2.622**	79.11 ± 1.624 *

The data are presented as mean ± SEM. of 6 independent observations.
*p<0.05, ** p<0.01 vs. control

Table 4: Effect of gestational and lactational exposure to lead and cadmium on the non-enzymatic antioxidant and TBARS level of liver in PND 56 rats

Parameters	**Groups**			
	Control	**Pb**	**Cd**	**Pb + Cd**
Reduced Glutathione[a]				
Female	7.1± 0.60	5.74 ± 0.30	4.52 ± 0.56*	5.72 ± 0.39
Male	2.9±0.14	2.6± 0.10	2.04 ± 0.20**	2.42 ± 0.12
TBARS[b]				
Female	29.00 ± 1.61	39.20 ± 2.57	44.00 ± 3.74*	37.60 ± 2.92
Male	22.20± 1.96	26.80± 1.06	32.00 ± 2.07**	28.00 ± 1.59

a pmoles/mg protein, b nmoles MDA/min/mg protein

The data are presented as mean ± SEM. of 6 independent observations.
*p<0.05, ** p<0.01 vs. control

Table 5: Effect of gestational and lactational exposure to lead and cadmium on enzymatic antioxidants of liver in PND 56 rats

Parameters	Groups			
	Control	Pb	Cd	Pb + Cd
CAT [a]				
Female	199.4 ± 7.56	185.6 ± 7.6	167.0 ± 4.98 *	182.8 ± 5.35
Male	580.8 ± 13.29	439.2± 31.88**	353.6 ± 27.85***	386.4 ± 12.45***
Cu-Zn SOD[b]				
Female	24.20 ± 2.28	21.20 ± 3.21	17.20 ± 1.65***##	21.6 ± 2.04**
Male	43.40 ± 2.35	38.00± 2.84	29.60 ± 2.11**	33.80 ± 2.87
Mn SOD[b]				
Female	13.20 ± 1.06	12.00 ± 1.14	9.6 ± 0.97*	11.8 ± 1.28
Male	34.40 ± 0.67	32.00± 1.41	26.00 ± 2.00*	33.00 ± 2.9
GPx[c]				
Female	5145 ± 101.6	4898 ± 99.14**	4670 ± 95.08***##	4777 ± 191.9***
Male	503.4± 10.4	470.2± 9.49	435.2 ± 12.97*	466.2 ± 22.22
GR[d]				
Female	150 ± 2.89	147.0 ± 5.29	134.0 ± 5.63**	143.0 ± 3.76
Male	162.8 ± 3.83	148.0± 6.06	142.2 ± 3.58	147.2 ± 5.52

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

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

[c] Δ 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

The data are presented as mean ± SEM. of 6 independent observations.
*p<0.05, ** p<0.01, *** p<0.001 vs. control; ## p<0.01 vs. lead

Table 6: Effect of gestational and lactational exposure to lead and cadmium on the biochemical parameters (Cholesterol, Glycogen, DNA and RNA) of liver in PND 56 rats

Parameters	Groups			
	Control	Pb	Cd	Pb + Cd
Total Cholesterol [a]				
Female	34.56 ± 0.591	30.99± 0.682**	23.18 ± 1.05***	29.10 ± 0.671***
Male	47.06 ± 1.123	41.85 ± 1.384*	34.58 ± 0.679***	39.95 ± 1.061**
Glycogen [b]				
Female	119.8 ± 2.594	112.0± 1.346*	86.17 ± 2.032***	102.4 ± 1.363***
Male	139.7± 1.237	136.2 ± 1.670	104.5 ± 2.122***	120.6 ± 2.121***
DNA [c]				
Female	2.596 ± 0.027	2.460± 0.022**	2.220 ± 0.032***	2.334 ± 0.022***
Male	4.612 ± 0.137	4.430 ± 0.177	3.382 ± 0.065***	4.300 ± 0.100
RNA[d]				
Female	9.392 ± 0.199	8.556± 0.206*	7.520 ± 0.175***	8.220 ± 0.372*
Male	11.93 ± 0.347	11.67 ± 0.266	9.144 ± 0.205***	10.32 ± 0.309**

[a] µg/mg microsomal protein

[b] mg/g tissue

[c] µg/g tissue

[d] µg/g tissue

The data are presented as mean ± SEM. of 6 independent observations.
*p<0.05, ** p<0.01, *** p<0.001 vs. control

3.5.4 Discussion

Pregnant and lactating female animals absorb and retain substantially more dietary cadmium than do their non-pregnant counterparts (Bhattacharyya *et al.*, 1986; Bhattacharyya *et al.*, 2000; Floris *et al.*, 2000). The present study demonstrated that in utero and lactational exposure to lead and cadmium alone and in combination decreased the activities of phase I enzymes metabolizing xenobiotics and endogenous steroids in both male and female PND 56 rats, but among the two metals cadmium showed more inhibitory effect than lead and similar trend was observed for both male and female rats. This could be due to the fact that cadmium is more hepatotoxic than lead (Cook *et al.*, 1984) and the amount of cadmium acetate absorbed in hepatic tissue is more than lead acetate (Pillai and Gupta, 2005). A sex-related difference in the inhibitory effect of cadmium on hepatic phase-I enzymes observed may be due to lower metallothionein induction in liver of male rats. The combined treatment of lead and cadmium did not further potentiated the suppression of hepatic phase-I enzyme activities as observed in lead alone and cadmium alone experimental groups but instead showed antagonistic effect suggesting competition between lead and cadmium for its inhibitory potential against phase-I enzymes in both male and female rats. However, the fold decrease in the phase-I enzyme activities were greater in female rats as compared to male rats, pinpointing sex-specific differences (Gustafsson, 1983) in susceptibility against inhibitory potential of lead and cadmium. The basal activities of these enzymes were compared amongst male and female rats in terms of specific activity and it clearly demonstrated sex-related differences suggesting hormonal regulation of these hepatic enzymes. The results of the present study are in agreement with previous reports on suppressive effects of heavy metals on hepatic phase-I enzyme activities. NADPH-cytochrome c reductase is a limiting enzyme of drug metabolizing system in liver microsome. Concerning the effects of heavy metals on the hepatic drug metabolizing enzyme activities, Pb has been reported to suppress the activities of hepatic drug and steroid metabolizing enzyme activities (Roomi *et*

al., 1986; Falke and Zwennis, 1990). (Chow and Cornish, 1978) have also demonstrated that lead, *in vitro*, inhibited the hepatic microsomal NADPH-cytochrome *c* and *P*-450 reductase activity in a dose-dependent manner. They also observed significant inhibition of NADPH-cytochrome *c* and *P*-450 reductase activity after the injection of lead acetate *"in vivo"*. Similarly, there are several reports suggesting suppressive effects of cadmium on hepatic drug and steroid metabolizing enzyme activities (Yoshida *et al.*, 1976; Aitio *et al.*, 1978; Means *et al.*, 1979). Howard *et al.*, 1984 reported that cadmium exposure decreased hepatic NADPH-cytochrome *c* and NADPH-cytochrome *P*-450 reductase activities but had no effect on NADH-cytochrome *c* reductase activity. The effects of cadmium on microsomal metabolism persist fully even after removal of approximately 95% of the metal. Phase-I and Phase-II metabolizing enzymes are postulated to share the same receptor mediated and non-receptor mediated regulation pathways (Rushmore and Kong, 2002). Thus, enzyme activity profiles may be compared for phase-I and phase-II metabolizing enzymes.

The changes observed in 17β-HSD activity in both male and female rats when compared to controls can be due to the binding of the divalent metal ions to the lysine and tyrosine residues found in the active site of the enzyme. Glucuronyl transferase activity is responsible for conjugating xenobiotics and steroids and sex differences appear to be hormonally related and imprinted during development. The inhibition in the activity of UDP glucoronyl transferase observed both in male and female liver suggest a similar mechanism, where metals might have interacted with methionine in the active site of enzyme. The changes observed in UDPGT activity in combined group is not following the pattern of inhibition seen in other enzymes in the present study. Moreover, Pb seems to have more inhibitory effect as compared to cadmium in both male and female rats. Xenobiotic nuclear receptor-mediated regulation of UDPGT (Zhou *et al.*, 2005) suggests a possible target of lead and cadmium mediated disruption in this phase II enzyme activity. (Scheri *et al.*, 1981) determined the influence of lead acetate on kinetic parameters of gamma-Glutamyl Transpeptidase (γ-GT) in brain

homogenates from 15 and 30 day old rat pups and demonstrated inhibition of γ-GT activity by lead acetate. (Karmakar *et al.*, 1999) have earlier reported the influence of cadmium intoxication on hepatic lipid peroxidation, glutathione level, and glutathione S-transferase and γ-glutamyl transpeptidase activities. We also observed a significant decrease in both GST and γ-GT activities in experimental rats which suggest inability of liver to cope up against toxic insult of Cd and Pb thus decreasing its detoxifying capacity. (Hamden *et al.*, 2008) on the other hand showed that γ-GT activity was increased significantly in Cd-treated rats compared to the control rats. In male rats, the suppression of hepatic GST activity in co-exposure group is further potentiated as compared to lead alone and cadmium alone group suggesting additive effect while in female rats, antagonistic effect was observed. The discrepancy in the results obtained imply that lead and cadmium exert their in vivo effects by different mechanism when present together. Histological changes in PND 56 liver such as massive fatty degeneration in hepatocytes and large vacuoles in cytoplasm were very clear evidence. Pycnotic nuclei appearance was also a prominent feature in all the treated groups. Infiltration of lymphocytes in liver was seen in all metal treated groups.

The data on non-enzymatic antioxidants and lipid peroxidation in the present study indicates free radical toxicity in the liver of F1 generation PND 56 female and male rats after gestational and lactational exposure to metals. GSH is a major cellular reductant and plays a very important role against the deleterious actions of hydroxyl radicals (Pal *et al.*, 1993). The decreased concentrations of GSH observed in the liver of both male and female rats exposed to metals, cadmium in particular, suggest underlying mechanism of hepatotoxicity of these metals in general and cadmium in particular. Liver exposed to either cadmium alone or in combination showed higher lipid peroxidation as compared to control. Several reports have accounted for such an elevation in lipid peroxidation upon cadmium exposure (Stohs *et al.*, 2001, Wang *et al.*, 2002). Antioxidant enzymes play a very crucial role in maintaining the homeostasis of

free oxygen radicals Present study also showed that SOD activity was inhibited in the metal treated groups suggesting displacement of zinc by lead and cadmium from its active site. Similar reports have been reported earlier by other workers (Hussain *et al.,* 1987; Ariza *et al.,* 1998). Apart from zinc displacement mediated inhibition of enzyme activity, heavy metal inactivation of an enzyme may be attributed to dysfunctional conformational changes which lead to loss of catalytic activity. Both CAT and GR exhibited significant reduction in activity after following the present experimental regime. At the physiological pH of 7.00 and which is also the pH at which CAT activity was assayed, the imidazole N of His-74 (pKa = 6.5) is substantially unprotonated thus making it more likely for Cd2+ to interact with this essentially basic N, and reducing enzyme activity (Ikediobi *et al.,* 2004). GR and GPx are FAD-dependent and Se-dependent oxidoreductases, respectively, which are supposed to protect the cellular biomolecules and structures from oxidative damage induced by lead and cadmium intoxication. That these enzymes have significantly reduced activities in the presence of lead and cadmium alone and in combination indicates the severity of metal-induced oxidative stress and the price the liver has to pay to protect itself from performing other vital functions such as biotransformation activity. Given that the intracellular redox status is generally maintained by the coordinated activities of GR and GPx, it is not surprising that exposure to large fluxes of H_2O_2 and hydroxyl radical (arising from oxidative stress) might result in an imbalance in GSH/GSSG ratio which can affect intracellular GPx and GR activities (Ikediobi *et al.,* 2004).

The changes in the various biochemical parameters observed in the liver of PND 56 rats co-exposed to lead and cadmium during early developmental period might result from an independent effect of lead and/or cadmium and also from their interaction. The present study clearly demonstrated a decrease in DNA, RNA, and cholesterol content in all treated groups suggesting. The interactive effect may involve changes in metal accumulation and concentration of various essential elements such as Zn, Cu and Fe in the serum and liver. Sex-specific

response was clearly seen in terms of intermediate effects of combined exposed groups in female hepatic enzymes while male hepatic enzymes were suppressed in an additive manner in combined exposed group. Till date, the results from combined metal exposure studies showed either additive effect in the combined exposure group as the concentration of the metals are increased, or antagonistic effects depending on the nature of the metals used (Nation *et al.,* 1990; Zikic *et al.,* 1998)

It has been demonstrated in rodents that the hypothalamo-pituitary-liver axis is influenced by hormonal signaling during development (Legraverend *et al.,* 1992) and the vulnerability of hepatic steroid metabolism after lead and cadmium exposure in the adult male and female in the present study is in accordance with the observations done in adult female rats which has been well documented in our earlier laboratory studies (Pillai *et al.,* 2002; Pillai and Gupta, 2005).There is also a possibility of genetic differences playing a role in differences in hepatic biotransformation observed in this study which can be further explored. It is interesting to note that few of the parameters studied in the present study (17β-HSOR, GST) exhibited differential pattern of inhibition in liver of male and female rats. Although it is difficult to explain these results, it certainly points out that the hepatic xenobiotic and steroid metabolizing enzymatic system is highly complex, and influenced by myriad regulatory mechanism in sex-specific manner.

In conclusion, our results indicate that long-term exposure of pregnant rats to moderate levels of lead and cadmium, when given throughout pregnancy and lactational period, suppresses steroid metabolizing enzyme activities in both male and female rats. Thus, it clearly suggests serious implications of early developmental exposure to heavy metals on drug and steroid metabolizing capacity of an organism on reaching adulthood.

3.6 SUMMARY

Pregnant rats were treated subcutaneously (0.05 mg/ kg body wt/day) with lead acetate and cadmium acetate separately and in combination throughout the gestational and lactational period. The metal treatment was thereafter withdrawn till PND 55 and all experiments were performed on PND 56 male and female F1 offspring. Efforts were also made to monitor the reproductive performance of dams at birth. Frequency of pregnancy was equally distributed over all metal exposure groups and no effect was observed on reproductive performance. The litter size, pup weights, sex ratio, % mortality, maternal weights did not differ significantly.

Hypothalamic dopamine levels decreased in individually and combined metal treated groups in both PND 56 male and female rat offspring. However, norepinephrine levels decreased in individually and combined metal treated groups only in PND 56 male rat offspring whereas in PND 56 female rats, norepinephrine levels decreased only in cadmium treated group. The accumulation of both metals increased in hypothalamus and pituitary after the treatment. Activity of 3α-hydroxy steroid dehydrogenase in hypothalamus and pituitary was decreased after the metal treatment with cadmium showing maximum inhibition in both male and female rat offspring at post natal day 56. Lead exposed rats did not demonstrate any significant decrease in 3α-hydroxy steroid dehydrogenase activity in both male and female rat offspring. Hypothalamic GnRH mRNA levels were decreased in cadmium and combined treated groups in female rat offspring. Pituitary LH and FSH mRNA levels decreased only in cadmium treated groups in female rat offspring. In both male and female rat offspring, hypothalamic GSH content was decreased in all the metal treated groups with cadmium exposed group showing maximum decrease as compared to the control. Similarly, in both male and female rat offspring, pituitary GSH content was decreased in all metal exposed groups with cadmium showing the maximum decrease as compared to the control. Depletion in the reduced glutathione in the metal exposed groups is a hallmark of oxidative damage. Cadmium and combined metal exposed animals showed significant

increase in TBARS levels in both hypothalamus and pituitary of male and female rat offspring at PND 56 whereas lead exposed group showed significant increase in TBARS levels as compared to control but was minimal in comparison to other metal treated groups. The increase in TBARS levels were more pronounced in female rat offspring than male rat offspring. Superoxide dismutase enzyme activity was inhibited only in pituitary cadmium treated group, but no change was seen in any other metal treated groups in the pituitary. Hypothalamic superoxide dismutase activity was not inhibited in any of the metal treated groups in both male and female rats. Both male and female rat offspring showed significant decrease in catalase activity only in cadmium treated groups as compared to control in both hypothalamus and pituitary.

The key enzymes of ovarian and testicular steroidogenesis (3β-HSD and 17-βHSD) were affected the most in cadmium and combined treated animals while lead treated animals showed minimum change compared to control group. This decrease was correlated to decrease in gonadal steroid levels. In all treatments, combined treated group showed intermediate results suggesting competition between the two metals. Simultaneous exposure of metal toxicants at this level neither showed any additive effect nor caused clinical signs of toxicity but still able to manifest biochemical effects and thus affects the ovarian and testicular function of treated animals. Biomolecules like glycogen, protein, RNA, DNA content were affected in all metal treated groups. Cadmium treated animals showed greater effect on cholesterol content compared to other groups. Toxic parameters like ALP, SGPT and creatinine were altered but were within the normal range. Biochemical effects are correlated with metals accumulated in blood, reproductive tissues like ovary and testis. Histopathological observation of ovary and testis for cytotoxic changes did not demonstrate any marked alteration in histology features. However, number of atretic follicles was higher mainly in cadmium exposed group as compared to the control. Results also revealed that mRNA levels of genes encoding protein responsible for cholesterol transport and steroidogenesis (StAR, CYP11), were decreased significantly compared to control in both male and female rat offspring at PND 56.

Hepatic phase-I and phase-II xenobiotic/steroid metabolizing enzymes were inhibited by the metal exposure in a sex-specific manner. NADPH-cytochrome c reductase enzyme activity showed maximal inhibition in cadmium exposed group, whereas combined metal exposed group showed an intermediate effect. Lead exposed group demonstrated the minimal decrease as compared to the control in both male and female rat offspring. Similarly, NADH-cytochrome c reductase activity showed maximal inhibition in cadmium treated group with combined group showing an intermediate effect.17β-HSOR and GST enzyme activities exhibited differential pattern of inhibition in liver of male and female rat offspring at PND 56. γ-glutamyl transferase activity also demonstrated maximal inhibition in cadmium treated group whereas combined metal treated group showed an intermediate effect. Interestingly, lead exposed group did not demonstrate any alterations in the enzyme activity in both the sexes. UDP-glucoronyl transferase activity was also inhibited in all the metal treated groups with cadmium showing the maximum inhibition followed by combined metal treatment group.