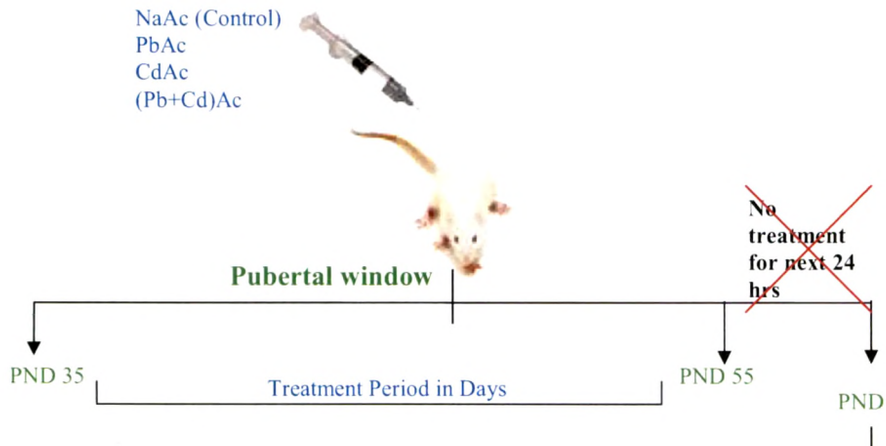
Chapter 4 **Biochemical and molecular effects of pubertal co-exposure to lead and cadmium on hypothalamic-pituitary-ovarian axis and hepatic steroid metabolism**

4.1 General Introduction & Schematic Experimental Design

General Introduction

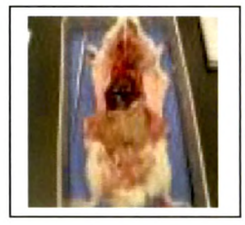
Puberty is a period of dramatic neuroendocrine development that culminates in reproductive maturation. It is marked by the reactivation of the HPG axis manifested by an increase in frequency and amplitude of gonadotropin releasing hormone (GnRH) pulses in the hypothalamus, leading to a rise in pulsatile secretion of the gonadotropins from the anterior pituitary whose release stimulates the gonads. It is also a period of increased sensitivity to environmental agents (Dearth *et al.,* 2002; Louis *et al.,* 2008). A growing body of evidence suggests that the developing organism presents a 5-fold greater absorption of heavy-metals alongwith other xenobiotics and lacks a functional blood brain barrier (Lockitch, 1993; Webb, 1975). Moreover, in the developing brain, effects may be permanent and result in functional deficits. Low-level exposures to endocrine-disrupting chemicals are ubiquitous in today's environment. But, most of the work till date has been carried out with higher concentrations of lead and cadmium in isolation. Higher concentrations of cadmium were reported in children with mental retardation (Jiang *et al.,* 1990; Marlowe *et al.,* 1983), and learning difficulties or dyslexia (Capel *et al.,* 1981; Pihl and Parkes 1977). Pb promotes apoptosis in newborn rat cerebellar neurons and impairs CNS functions by altering synaptogenesis in the neonatal rat brain (Alessandra *et al.,* 1996). However, little is known about the effects of Pb and Cd co-exposure during pubertal window in relation with dysregualtion of reproductive axis function on reaching adulthood. Hence, the present study was conducted to evaluate the sensitivity of pubertal co-exposure to lead and cadmium in relation to HPG axis function and hepatic steroid metabolism. ***In this chapter, effort has been made to analyze the effects of pubertal co-exposure to lead and cadmium on*** a.) Hypothalamic-pituitary axis function (discussed in Section 4.2), b.) Ovarian steroidogenesis (discussed in Section 4.3) and c.) Hepatic steroid biotransformation enzymes (discussed in Section 4.4).

Schematic representation of experimental design

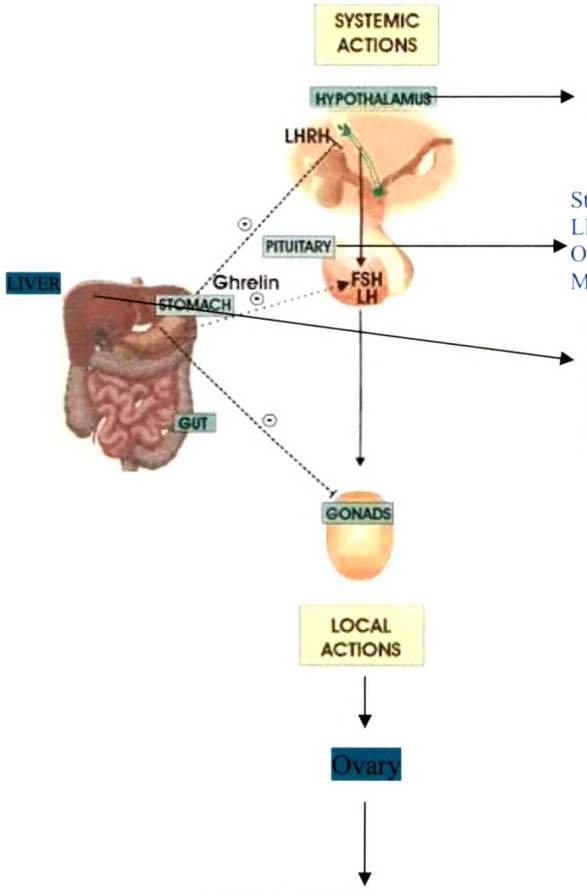


Animal strain: Charles Foster
Dosage: 0.05 mg/kg/day
Mode of administration: Intra peritoneal (i.p.)

PND: Post Natal Day; Ac: Acetate



Parameters evaluated after pubertal exposure study



Steroid metabolism (3α-HSD)
Neurotransmitters (NE, DA); GnRH mRNA expression)
Oxidative stress (GSH, LPO, GPx, SOD, CAT)
Metal accumulation (Pb, Cd)

Steroid metabolism (3α-HSD)
LH β & FSH β mRNA expression
Oxidative stress (GSH, LPO, GPx, SOD, CAT)
Metal accumulation (Pb, Cd)

Steroid metabolism (Phase-I & Phase-II enzymes)
Biochemical parameters (Glycogen, Cholesterol, DNA, RNA)
Oxidative stress (GSH, LPO, GPx, SOD, CAT, GR)
Histopathology
Metal accumulation (Pb, Cd)

17β-HSD, 3β-HSD activities
(StAR, CYP11, CYP19, 3β-HSD) mRNA expression
StAR protein expression
Oxidative stress (GSH, LPO, GPx, SOD, CAT, GR)
Metal accumulation (Pb, Cd)
Biochemical parameters (Cholesterol, Vitamin C)
Serum estradiol & progesterone, granulosa cell count, Histology

4.2 Hypothalamic-pituitary axis function

4.2.1 Introduction

Perinatal exposure to low levels of lead as well as cadmium has been involved in behavioral and neurochemical alterations detected in both suckling and adult rats (Moreira *et al.*, 2001; Lafuente *et al.*, 2000). Apart from the regulatory role of hypothalamus in activation of pituitary gonadotrope regulation of gonadal functions by pituitary hormones, both hypothalamus and pituitary has a very crucial role in metabolism of steroids. 3α-oxidoreductase (3α-OR) catalyzes the conversion of pregnenolone to progesterone and further dehydroepiendrosterone to androstenedione. 3α-HSOR is widely distributed in large number of peripheral tissue (Marte *et al.*, 1992) skin, adipose tissue and liver etc (Bleau *et al.*, 1974; Weisntein *et al.*, 1968; Kaufmann *et al.*, 1995). Both hypothalamic and pituitary (3α-OR) catalyzes the oxido-reduction at carbon 3 of steroid hormones. It converts 5-alpha-pregnane-3, 20-dione (DHP) into 5 alpha-pregnane-3 alpha-ol-20-one (3αHP). 3αHP is a neuroactive steroid and has been shown to inhibit GnRH activity on gonadotropes and selectively suppress FSH release from pituitary cells, without an effect on LH (Gao *et al.*, 2002).

It was shown that metals such as lead and cadmium can affect the activity of hypothalamus-pituitary-ovarian axis by acting at the hypothalamus (Das *et al.*, 1993; Andersson *et al.*, 1997; Antonio *et al.*, 1999), the pituitary (Lorenson *et al.*, 1983; Ronis *et al.*, 1998; Lafuente *et al.*, 1999). Lead exposure has been shown to cause changes in catecholaminergic functions (Winder and Kitchen, 1984; Nation *et al.*, 1989). Even low-level Cd exposure results in increased catecholamine neurotransmission (Cooper and Manalis, 1983; Nation *et al.*, 1989).

Eventhough the neurotoxic effects mediated by lead and cadmium are well known there are no reports available on the effect of pubertal exposure to lead and cadmium in isolation and in combination on hypothalamic-pituitary axis function. In view of this, present study was performed to evaluate the

biochemical and molecular basis of neuroendocrine disruptions after low level pubertal co-exposure to lead and cadmium in hypothalamus and pituitary of PND 56 female rats.

4.2.2 Experimental design

Following the experimental regime as shown in the schematic representation in section 4.1 of this chapter; the animals were sacrificed by decapitation on PND 56; the procedure was completed within 5 to 10 sec to avoid stressors. The hypothalamus, pituitary from all the experimental groups was immediately excised, rinsed thoroughly in saline and weighed before processing for biochemical estimations. 3α-hydroxy steroid dehydrogenase (3α-HSD) activity was assayed from hypothalamus and pituitary (Shivanandappa and Venkatesh, 1997). Dopamine and norepiniephrine were estimated in the hypothalamus samples by the flourimetric method of Shellenberger and Gordon, 1971. Hypothalamus and pituitary were analyzed for lead and cadmium levels by GBC 902 Atomic Absorption Spectrophotometer. Total RNA was extracted from hypothalamus and pituitary by the standard protocol of (Chomczynski and Sacchi, 1987) as described earlier. RT-PCR was carried out for gene expression analysis of GnRH and (LHβ, FSHβ) from hypothalamus and pituitary respectively alongwith β-actin (internal control) expression analysis was also carried out. The PCR products (5 μl) were then separated on 1.5% agarose gel. The bands on the UV-transilluminated gel were converted into digital images with a gel analyzer and the amounts of RT-PCR products were quantified with Alpha imager software. Hypothalamus and pituitary homogenates were used for the determination of various oxidative parameters (GSH, TBARS, SOD, CAT, and GPx) and details of the same are described in Chapter 2.

4.2.3 Results

Table 1 shows the effect of metals in isolation and in combination on body weight, absolute and relative organ weights. There was no significant change either in body weight or in relative organ weights as far as

hypothalamus is concerned. However, relative pituitary weight was significantly affected in combined exposure group.

The analysis of metal concentrations in hypothalamus and pituitary showed both lead and cadmium getting significantly accumulated in the isolated and combined metal exposure groups (Table 2)

Table 1: Effect of pubertal co-exposure to lead and cadmium on body weight, absolute and relative weights of hypothalamus, pituitary.

Parameters	Groups			
	Control	Pb	Cd	Pb + Cd
Body weight (g)	131.3±4.16	123.5±6.95	130.4±5.24	138.9±4.13
AHW (mg)	9.4±0.30	8.92±0.40	8.22±0.38*	9.17±0.25
RHW (mg/g)	0.072±0.003	0.073±0.004	0.064±0.004	0.066±0.002
APW (mg)	4.63±0.20	4.05±0.15*	3.86±0.23*	3.93±0.22*
RPW (mg/g)	0.035±0.001	0.033±0.002	0.030±0.002	0.028±0.001*

BWG = Body weight gain; AHW=Absolute hypothalamus weight; RHW=Relative hypothalamus weight; APW=Absolute pituitary weight; RPW= Relative pituitary weight
The data are presented as mean ± SEM. of 8 independent observations.
*p <0.05 vs. control

Table 2: Lead and Cadmium levels in hypothalamus and pituitary of PND 56 female rats

Groups	Metal Content			
	Hypothalamus		Pituitary	
	Pb (µg/g)	Cd(µg/g)	Pb(µg/g)	Cd (µg/g)
Control	1.217± 0.15	0.313±0.02	1.68±0.10	0.230±0.015
Lead	3.30±0.25***	0.280±0.01	2.63±0.08**	0.206±0.006
Cadmium	1.09±0.06###	0.820±0.035***###	1.30±0.15##	0.423±0.014***###
Combined	2.03±0.26#	0.643±0.03***###@@	1.46±0.20##	0.350±0.010*** ###@

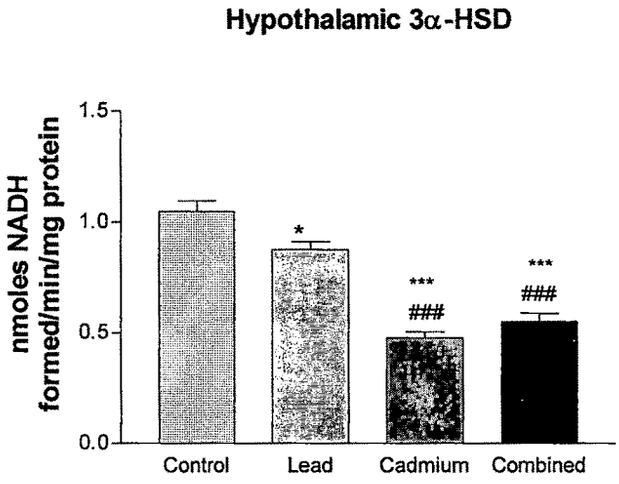
The data are presented as mean ± SEM. of 4 independent observations.
** p<0.01, *** p<0.001 vs. control; #p<0.05, ##p<0.01, ###p<0.001 vs. lead and @p<0.05, @@p<0.01 vs. cadmium group.

Figure 1 and Figure 2 shows the 3α-hydroxy steroid dehydrogenase activity in hypothalamus and pituitary respectively. In case of both hypothalamus and pituitary, activity of hypothalamic 3α-HSD was most affected in cadmium treated group. Combined exposure group showed intermediate effect whereas lead exposed group showed the least significant effect as compared to the control.

Figure 3 and Figure 4 shows the concentration of hypothalamic dopamine and norepinephrine respectively. The dopamine content was decreased significantly in the metal exposed groups. Similarly, the norepinephrine content was also decreased significantly in all the metal exposed groups. Cadmium exposed group exhibited the most significant decrease in both the neurotransmitter content. Combined exposure group showed an intermediate effect whereas the lead exposed group showed the least significant effect.

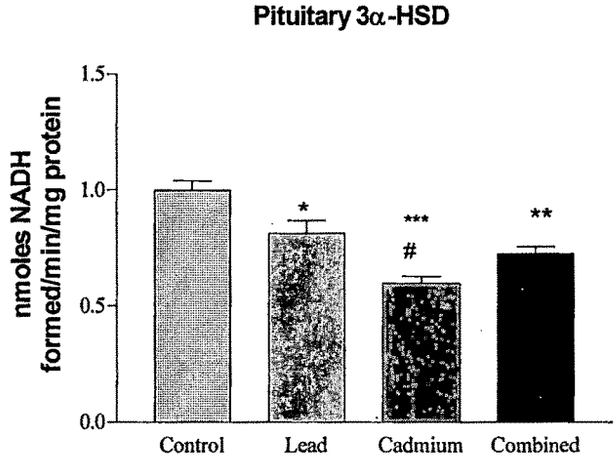
Figure 5A shows the effect of Pb and Cd on PND 56 hypothalamic GnRH mRNA expression after pubertal exposure. Expression of GnRH mRNA was significantly affected in cadmium and combined metal treated groups as compared to the control group. Figure 5B shows the effect of Pb and Cd on PND 56 pituitary LHβ and FSHβ mRNA expression after pubertal exposure. A significant reduction in the mRNA levels of LHβ was observed only in cadmium metal treated group. Whereas, mRNA expression of FSHβ was found to be significantly affected only in combined metal treated group compared to the control. In most cases, Pb exposed group did not demonstrate any significant change in mRNA expression levels of selected genes.

Figure 1: Effect of pubertal co-exposure to lead and cadmium on hypothalamic 3α-hydroxy steroid dehydrogenase activity in PND56 female rats



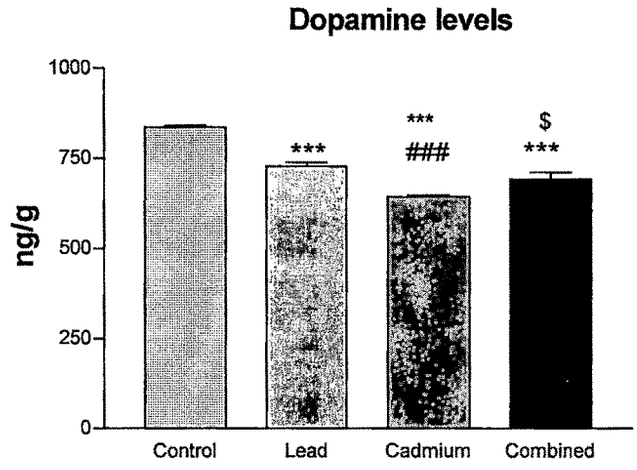
*p<0.05, *** p<0.001 versus control; ###p<0.001 versus lead. The data are presented as mean ± SEM. of 4 independent observations.

Figure 2: Effect of pubertal co-exposure to lead and cadmium on pituitary 3α-hydroxy steroid dehydrogenase activity in PND 56 female rats



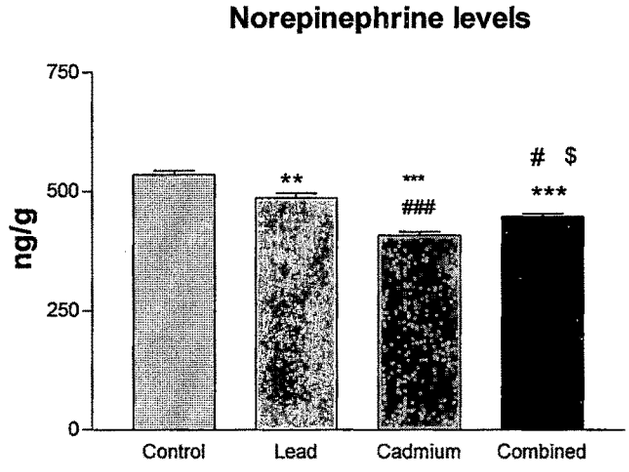
*p<0.05, **p<0.01, *** p<0.001 versus control; #p<0.05 versus lead. The data are presented as mean ± SEM. of 4 independent observations.

Figure 3: Effect of pubertal co-exposure to lead and cadmium on hypothalamic dopamine levels in PND56 female rats



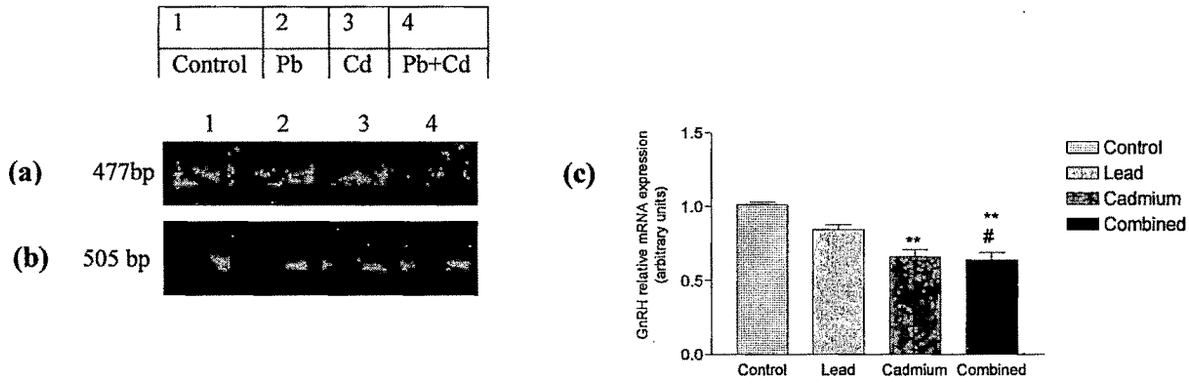
*** p<0.001 versus control; ###p<0.001 versus lead;
$ p<0.05 versus cadmium. The data are presented as mean ± SEM. of 6 independent observations.

Figure 4: Effect of pubertal co-exposure to lead and cadmium on hypothalamic norepinephrine levels in PND56 female rats



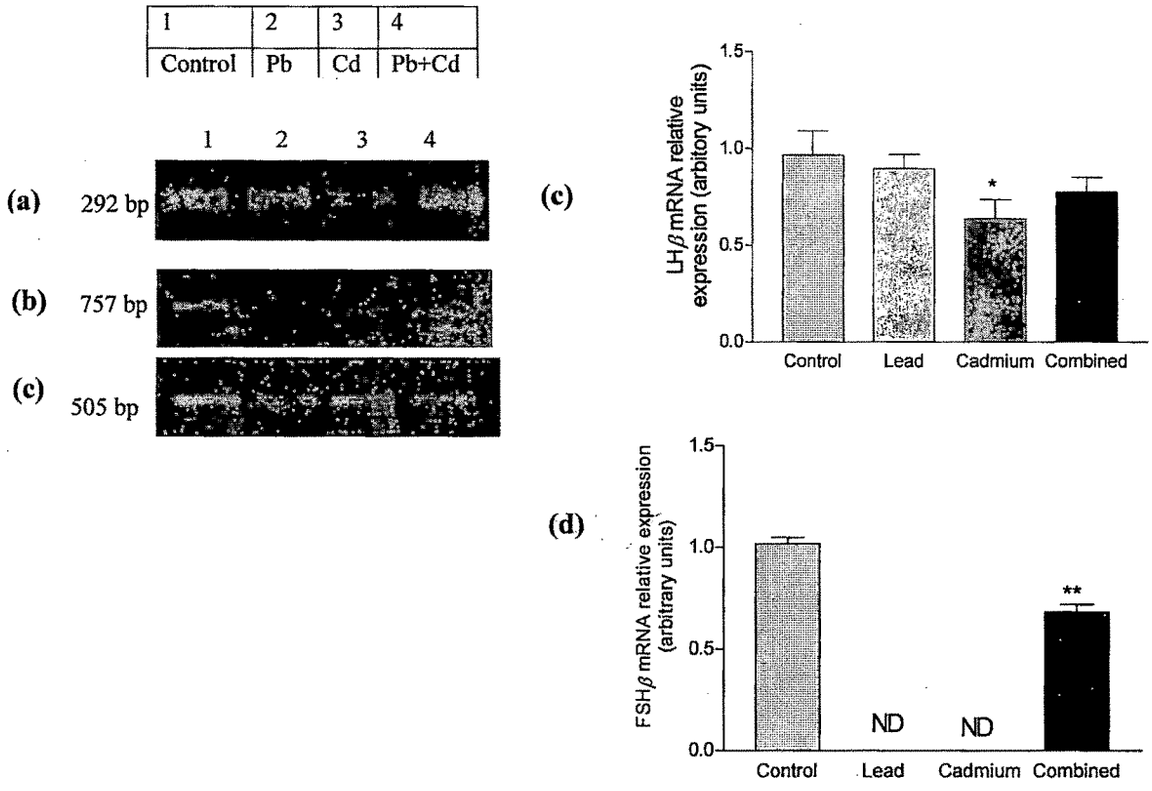
**p<0.01, *** p<0.001 versus control; #p<0.05, ###p<0.001 versus lead;
$ p<0.05 versus cadmium. The data are presented as mean ± SEM. of 6 independent observations.

Figure 5A: Effect of pubertal co-exposure to lead and cadmium on mRNA expression level of hypothalamic GnRH gene. (a) GnRH (b) β-actin (internal control). (c) The relative mRNA expression levels (arbitrary units).



(N=3) The values are mean± SEM. ** $p<0.01$ compared to the control group; and # $p<0.05$ compared to the lead group

Figure 5B: Effect of pubertal co-exposure to lead and cadmium on mRNA expression levels of pituitary LH and FSH genes. (a) LHβ (b) FSHβ (c) β-actin (internal control). (d) The relative mRNA expression levels (arbitrary units). (ND= Not detected)



(N=3) The values are mean ± SEM. ** $p<0.01$, *$p<0.05$ compared to the control group

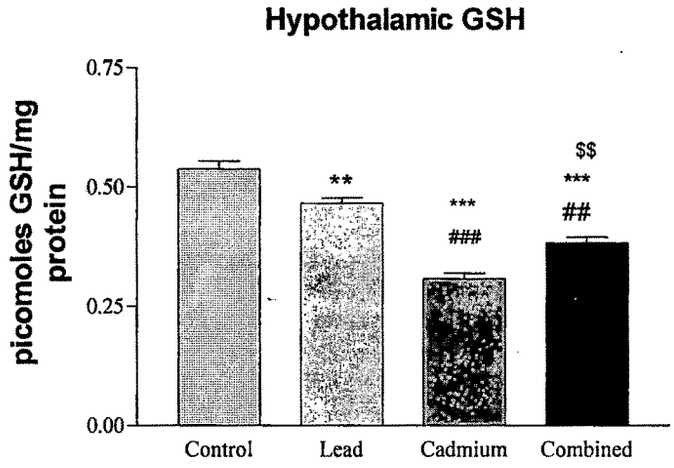
Figure 6 represents reduced glutathione (GSH) levels in hypothalamus of PND 56 rats after pubertal exposure to Pb and Cd alone and in combination. Cadmium exposure group exhibited maximum depletion in reduced glutathione levels and combined metal treated group showed an intermediate effect as compared to the control group. Figure 7 shows the effect of pubertal co-exposure to lead and cadmium on TBARS (Thiobarbituric acid reactive substances) levels i.e an indicative of lipid peroxidation, in hypothalamus of PND 56 rats after pubertal exposure to metals.

Table 3 indicates the activities of different antioxidant enzymes in hypothalamus of PND 56 rats after metal exposure. Cadmium exposure resulted in maximum changes in the antioxidant defense system as compared to the control. CAT, SOD and GPx activities of PND 56 hypothalamus were significantly affected in all the metal treated groups.

Figures 8 & 9 indicate the reduced glutathione (GSH) and TBARS levels in PND 56 pituitary of all the experimental groups after metal exposure. Cadmium exposure group exhibited the maximum alterations in both GSH and TBARS levels of pituitary. Significant elevation in lipid peroxidation was found in all the metal treated groups. While, there was significant depletion in the GSH content in the pituitary of metal treated groups.

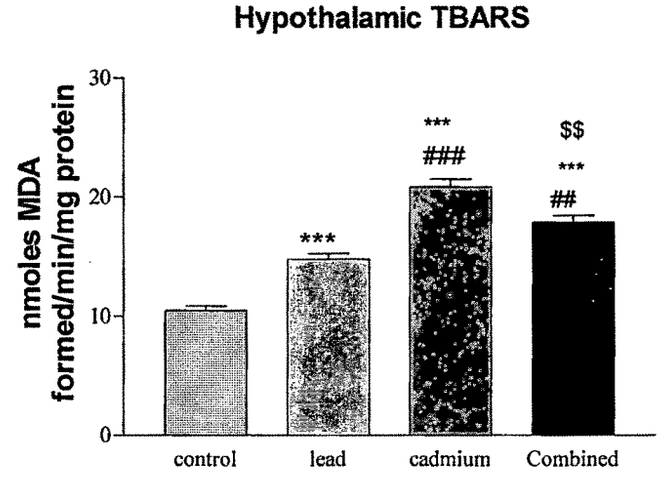
Table 4 indicates the activities of different antioxidant enzymes in PND 56 pituitary after pubertal exposure to metals. Suppressed antioxidant enzyme activities (CAT, SOD, and GPx) were observed in all the metal treated groups. Cadmium exposure showed the maximal effect and the combined metal exposure group exhibited an intermediate effect. Lead exposure group showed the least but significant effect as compared to the control.

Figure 6: Effect of pubertal co-exposure to lead and cadmium on hypothalamic reduced glutathione levels in PND 56 female rats



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

Figure 7: Effect of pubertal co-exposure to lead and cadmium on hypothalamic TBARS levels in PND56 female rats



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

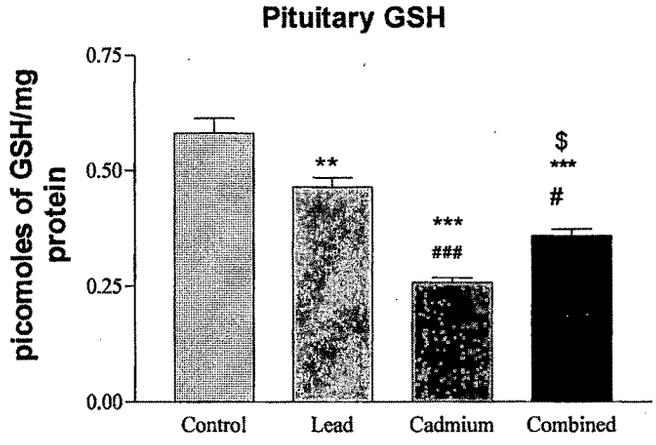
Table 3: Effect of pubertal co-exposure to lead and cadmium on hypothalamic enzymatic antioxidants

Parameters	Groups			
	Control	Pb	Cd	Pb + Cd
Catalase [a]	10.43±0.30	7.54±0.18***	4.79±0.28*** ###	6.01±0.24*** ## $
SOD[b]	42.58±0.57	36.90±0.86***	30.73±0.66*** ###	33.80±0.52*** # $
GPx[c]	47.13±0.85	41.49±0.85*	36.57±1.57*** #	40.03±1.24**

[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.

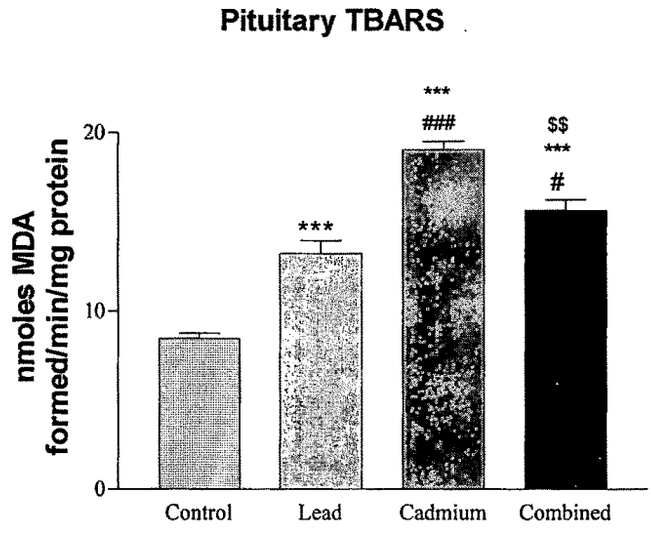
*p<0.05, **p<0.01, *** p<0.001 versus control; #p<0.05, ##p<0.01, ###p<0.001 versus lead; $ p<0.05 versus cadmium. The data are presented as mean ± SEM. of 6 independent observations.

Figure 8: Effect of pubertal co-exposure to lead and cadmium on pituitary reduced glutathione levels in PND56 female rats.



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

Figure 9: Effect of pubertal co-exposure to lead and cadmium on pituitary TBARS levels in PND56 female rats.



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

Table 4: Effect of pubertal co-exposure to lead and cadmium on pituitary enzymatic antioxidants in PND56 female rats.

Parameters	Groups			
	Control	Pb	Cd	Pb + Cd
Catalase [a]	9.71±0.53	8.30±0.23*	5.69±0.28*** ###	6.53±0.20*** ##
SOD[b]	31.98±0.93	28.17±0.66**	20.70±0.52*** ###	24.75±0.59*** # $$
GPx[c]	39.61±1.29	33.43±0.61*	27.98±1.25 ***#	33.17±1.53 ** $

[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.

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

4.2.4 Discussion

Puberty results from withdrawal of the "gonadostat" mechanisms and from increased gonadotropin sensitivity to GnRH. Modifications of neuropeptides, neurotransmitters, and neurosteroids may underlie the onset of pubertal processes. The data presented here show that low doses of lead and cadmium co-exposure during critical developmental window such as pubertal period alter the rat hypothalamic-pituitary axis at multiple molecular and biochemical targets. The accumulation of both metals increased in hypothalamus and pituitary on PND 56 after the pubertal exposure in the present study.

Earlier studies have shown a significant positive correlation between lead dose and expression of mRNA GnRH levels in the hypothalamus (Ronis *et al.*, 1996, Klein *et al.*, 1994). It also demonstrated that lead exposure initially induces an increase in intracellular levels of GnRH mRNA in a dose-related manner, but with attenuation in message production at higher concentrations of blood lead. Previous published studies in other systems also support the hypothesis that adaptation to lead toxicity occurs with prolonged exposure. However, our results indicate significant decrease in the GnRH mRNA expression in hypothalamus after pubertal exposure to metals. Studies carried out in monkeys suggest that chronically exposed animals may develop compensatory mechanisms (Franks *et al.*, 1989). Previous studies evaluating higher exposure levels for shorter periods of time showed that although lead exposure alters the production of GnRH in the hypothalamus at the molecular level, circulating GnRH and LH levels are not altered significantly (Klein *et al.*, 1992, Ronis *et al.*, 1998, Foster, 1993, Nathan *et al.*, 1992). Our results alongwith other published reports suggest that the signals within and between the hypothalamus and pituitary gland appear to be disrupted by lead and cadmium co-exposure. Because the hypothalamic-pituitary axis is a dynamic system and the actions of lead and cadmium at these central nervous system sites are not completely suppressive (Sokol *et al.*, 1998), adequate amounts of GnRH are released to maintain LH synthesis and secretion resulting in

steady-state serum levels. As exposure time increases, adaptation at the molecular level intensifies. The findings of perturbed GnRH mRNA expression at low levels of lead and cadmium exposure in the present study are of clinical importance. Cysteine-rich zinc finger proteins are proposed to be cellular targets for many xenobiotics, including metal ions like Pb and Cd (Sunderman and Barber, 1988). Mechanistic effects of cadmium ions on zinc finger structure were examined in the steroid hormone receptor and transcription factor IIIA (Simons *et al.*, 1990; Predki and Sarkar, 1992; Hanas and Gunn, 1996). Divalent metal ions such as cadmium and arsenic display increased avidity for two closely spaced thiols in a vicinal orientation (Joshi and Hughes, 1981). Metals such as lead interfere with the DNA binding properties of transcription factors, Sp-1 and Egr-1, both *in vivo* and *in vitro* (Zawia *et al.*, 1998). Lead could also directly interfere with the DNA binding of a recombinant human Sp1 protein. It was also found that the zinc finger motif accommodates metals such as cadmium. Cd has been shown to change the binding characteristics of the SP1 transcription factor (Thiesen and Bach, 1991). Cd can replace Zn in the ER finger and still bind functionally to DNA. However, the cooperativity values for Cd are different from those of the normal dimer (Sarkar, 1995). A change in this cooperativity can result in "recognition of a sequence that the protein would not normally bind". This has been suggested as a mechanism leading to differences in transcription, as well as DNA redox damage (Sarkar, 1995). Reports suggests that transcription factors such as steroidogenic factor 1 (SF1), specific protein 1 (SP1) or activated protein 1 (AP1) are required for proper expression of steroidogenic mRNA (Teruo *et al.*, 2000). The pituitary mRNA expression levels of LHβ and FSHβ were decreased significantly in cadmium and combined treatment groups respectively. In contrast, lead exposure failed to cause any change in mRNA expression levels of LHβ and FSHβ levels. Organization of GnRH and gonadotropin genes clearly demonstrates binding sites for SF1 and SP1, the important transcriptional factor for their transcription. Thus interaction of metals with above transcriptional factors seems to be the major mechanism

for decreased mRNA levels in metal treated groups. Altogether, these data suggest that the metal accumulation disrupts the regulatory mechanisms of the hypothalamic-pituitary axis but the effects produced by the combined treatment of metals are never additive.

Norepinephrine 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. In the present study we have observed decrease in NE in both isolated and combined metal treated groups. Dopamine (DA) content was significantly lowered in all the metal treated groups. Similar findings are reported elsewhere where subchronic exposure of low level of lead resulted in significant reduction in DA and its metabolites (Jadhav and Kala, 1994). Lead has also 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 *in vitro* studies have shown that lead enhances calcium activated release in brain transmitters (Minnema *et al.*, 1988). Cadmium is shown to inhibit calcium entry and the attendent release of peripheral catecholamines (Hirning *et al.*, 1988). Therefore the changes observed in the co-treatment 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, 1984).

Work was also extended to evaluate the enzymes involved in neurosteroid production that is mainly responsible for key functions within hypothalamus and pituitary. The results of the present study indicate that pubertal exposure to lead and cadmium inhibit the activity of the enzyme 3α-oxidoreductase in hypothalamus as well as pituitary. The possible mechanism of inhibitory effect of metals on 3α-HSD has already been discussed in earlier chapter-3.

Generation of highly reactive oxygen species such as hydrogen peroxide, superoxide radicals, hydroxyl radicals and lipid peroxides on heavy

metal exposure are known to damage various cellular components including membrane lipids, protein, DNA and thereby contribute to cellular dysfunction. Results of the present study show that after pubertal co-exposure to lead and cadmium, hypothalamic and pituitary GSH content is decreased in all metal exposed groups. GSH constitute the first line of defense against free radical induced damage. It accounts for about 90% of the intra cellular non-protein thiol content. The decrease in GSH content observed in the present study might be due to the binding of these divalent metals with –SH groups (Bagchi *et al.*, 1996; Shibasaki *et al.*, 1996; Karmakar *et al.*, 1998; Nigam *et al.*, 1999). In fact there is a direct correlation between GSH depletion and enhanced lipid peroxidation. The increase in hypothalamic and pituitary TBARS levels indicates failure of antioxidant defense mechanism, which prevents the formation of excess free radicals. The antioxidant enzymes superoxide dismutase and catalase are potential targets for both lead and cadmium as these enzymes depend on various transition metals for proper molecular structure and activity. Both lead and cadmium can readily displace zinc and copper, which are cofactors for superoxide dismutase causing a decrease in the enzyme activity (Hussain *et al.*, 1987; Kofod *et al.*, 1991; Adler *et al.*, 1993; Ariza *et al.*, 1998). We have observed the inhibition in superoxide enzyme activity in hypothalamus, but no change was seen in the pituitary after the metal exposure. It is interesting to note that basal activity of catalase is very low in pituitary as compared to hypothalamus. Although, we could not compare pituitary catalase activity with any published report, there are evidences, which suggest variable activity of catalase in various tissues (Wohaieb and Godwin, 1987; Matkovics, 1997). We have also observed a significant decrease in catalase activity in cadmium and combined treatment groups. These findings clearly indicate oxidative stress as one of the major biochemical mechanism involved in neurotoxic effects of pubertal administration of lead and cadmium in isolation and in combination.

4.3 Ovarian steroidogenesis

4.3.1 Introduction

Environmental pollutants disrupting endocrine function and affecting reproductive functions have been documented in both animals and humans (Colborn *et al,* 1993; Crisp *et al,* 1998). Heavy metals such as lead and cadmium are important environmental contaminants and their harmful effects on human being are of great concern today. Lead and cadmium are two metal pollutants, which has shown to be accumulated in reproductive tissues (Bires *et al.,* 1995; Kumar and Pant, 1984) and their effect is manifested in form of reproductive malfunctioning. Accumulation of heavy metals in the ovarian tissue causes atresia of follicles (Junaid *et al.,* 1997; Bires *et al.,* 1995), hemorrhage and necrosis in estrus cycling rats on cadmium exposure (Parizek, 1983). However, few reports are contradictory where no change in ovarian structure could be demonstrated (Parizek, 1983; Paksy *et al.,* 1989). Administration of single dose of cadmium chloride (2.2 μmol/ 100g of .b. wt) caused a disturbance and prolongation in estrus cycle (Godowicz and Pawlus, 1985). Several reports have shown that lead and cadmium interfere with steroidogenesis (Wiebe *et al.,* 1988a; Paksy *et al.,* 1989; Piasek and Laskey, 1994) and thus affect the hormone levels. Estrogen and progesterone levels have been shown to decrease with lead and cadmium exposure (Wiebe *et al.,* 1988; Piasek and Laskey, 1994; Paksy *et al.,* 1997). 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).

Present study is an effort to elucidate the effects of low-level exposure of lead and cadmium both alone and in combination during pubertal developmental window on ovarian steroidogenesis. In view of this, an attempt has been made to evaluate the effect of lead and cadmium on the key enzymes of ovarian steroidogenesis (17β-HSD & 3β-HSD), serum sex-steroid levels (Estradiol & Progesterone), ovarian antioxidant system along with

ovarian morphology and distribution of metals. Molecular targets of endocrine disruption within ovarian compartment were analyzed by evaluating the mRNA expression levels of key ovarian steroidogenesis pathway genes.

4.3.2 Experimental Design

Following the experimental regimen as discussed in 4.1.2, after 24 hours of the last dosage of metal treatment on PND 55, the animals in the proestrous stage of estrous cycle were sacrificed. Animals were sacrificed immediately after blood collection from the orbital sinus and the ovaries were removed and processed for assessment of steroidogenic enzymes- 3β-HSD and 17β-HSD (Shivanandappa & Venkatesh, 1997). Both ovaries and blood were also analyzed for lead and cadmium levels by GBC 902 Atomic Absorption Spectrophotometer. Western-blot analysis of ovarian mitochondrial protein was performed as described previously (Materials and Methods Section). Histology of ovary and uterus, stages was done using standard histological techniques. Total RNA was isolated from PND 56 ovary using Tri Reagent (Sigma) and intact RNA with an A260/280 ratio 1·6 and above was used for RT-PCR analysis. PCR was carried out with specific primers within the linear range of amplification to assess the mRNA expression levels for steroidogenic acute regulatory protein (StAR), Aromatase (CYP19), Cytochrome P450scc (CYP11), 3β-HSD and β-actin The intensity of the products following electrophoretic analysis were analyzed using an Alpha image software Gel Documentation system and represented as mRNA relative expression (arbitrary units). Serum estradiol and progesterone levels were measured following standard ELISA protocol as discussed previously in the Materials and Methods section. Various biochemical parameters like Vitamin C, Acid phosphatase, Cholesterol and oxidative stress parameters were analyzed in PND 56 ovary and details of each method is described already in the Chapter 2.

4.3.3 Results

Table 1 summarizes the effect of pubertal co-exposure to lead and cadmium on body weights, organ weights of PND 56 female rats. There were no significant alterations in either body weight or relative organ weights of metal treated animals as compared to the control group. Metal analysis in PND 56 ovary revealed significant accumulation of lead and cadmium after pubertal exposure to metals (Table 2).

Table 3 represents the granulosa cell count after pubertal co-exposure to lead and cadmium. Granulosa cell number showed significant decrease in all the metal treated groups. Cadmium treated group showed maximum reduction in granulosa cell number whereas the animals that received combined metal treatment exhibited intermediate effect. Lead exposed animals showed the least but significant reduction in granulosa cell count.

Table 4 summarizes the data on ovarian cholesterol and vitamin C contents. Ovarian cholesterol content was significantly decreased in all the metal treated groups. Metals alone and in combination caused a significant decrease in the ovarian vitamin C content also.

Table 1: Effect of pubertal co-exposure to lead and cadmium on body weights and organ weights of PND 56 rats

Parameters	Groups			
	Control	Pb	Cd	Pb + Cd
Body weight (g)	131.3±4.16	123.5±6.95	130.4±5.24	138.9±4.13
Ovary weight (mg)	62.50±1.92	59.75±3.75	65.88±3.98	65.75±3.86
Relative Ovary Weight (mg/g)	0.482±0.027	0.498±0.045	0.513±0.039	0.473±0.022
Uterus weight (mg)	492.6±9.43	457.3±7.03	526.3±11.17 ###	504.4±12.23 #
Relative Uterus weight	3.77±0.107	3.78±0.224	4 .07±0.167	3.65±0.137

The data are presented as mean ± SEM. of 8 independent observations.
##p<0.05; ###p<0.001 vs lead

Table 2: Lead and Cadmium levels in PND 56 ovary after pubertal co-exposure to metals

Groups	Metal Content	
	Pb (µg/g)	Cd(µg/g)
Control	0.058±0.10	0.028±0.001
Lead	0.118±0.004 **	0.026±0.004
Cadmium	0.044±0.003###	0.256±0.005 *** ###
Combined	0.077±0.010#	0.245±0.002 *** ###

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

Table 3: Effect of pubertal co-exposure to lead and cadmium in isolation and in combination on ovarian total granulosa cell count of PND 56 rats

Parameters	Groups			
	Control	Pb	Cd	Pb + Cd
Total granulosa cell count *(No. of cells x10⁴ per mg ovary)*	3.82±0.12	3.14±0.11*	2.20 ±0.13 ***###	2.08±0.15 *** ###

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

Table 4: Effect of pubertal co-exposure to lead and cadmium on ovarian biochemical parameters (Cholesterol & Vitamin C) of PND 56 rats

Parameters	Groups			
	Control	Pb	Cd	Pb + Cd
Cholesterol [a]	11.51±0:88	8.79±0.38 *	5.95±0.20 *** ##	7.70±0.27 ***
Vitamin C [b]	8.51±0.26	7.10±0.20 **	5.02±0.20 *** ###	6.40±0.22 *** $$

a µg/mg microsomal protein
b nmoles/g tissue

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, ###p<0.001 vs lead;
$$ p<0.01 vs cadmium

Figure 1 represents the effect of pubertal exposure to lead and cadmium on ovarian Δ^5-3β-HSD & 17β-HSD steroidogenic enzyme activities. Both the key steroidogenic enzyme activities showed significant increase as compared to the control animals in cadmium and combined exposure groups. However, the lead-treated group exhibited significant decrease in the key steroidogenic enzyme activities.

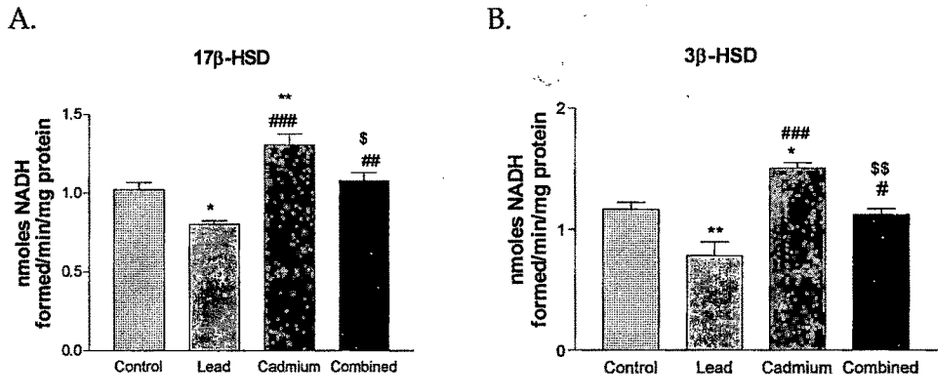
Figure 2 represents the effect of pubertal exposure to lead and cadmium either alone or in combination on mRNA expression levels of key ovarian steroidogenic proteins (3β-HSD, StAR, Cytochrome P450arom (CYP19), and Cytochrome P450scc (CYP11a) and β-actin gene (served as internal control). RT-PCR analysis revealed decrease in mRNA expression of all the candidate genes selected in the present study in all the metal treated groups. Cadmium treated animals exhibited maximal decrease in the mRNA expression levels of the selected genes.

Figure 3 represents the effect of pubertal co-exposure to lead and cadmium on basal level ovarian StAR protein expression. Densitometric quantification of StAR protein following western blotting analysis showed significant increase in StAR protein expression in cadmium and combined metal treated group. However, no such change was observed in the case of lead treated animals.

Figure 4 represents the effect of pubertal exposure to lead and cadmium either alone or in combination on serum estradiol and progesterone levels. Both estradiol and progesterone were significantly decreased in all metal treated groups. Cadmium treated groups showed the maximum decrease whereas the combined exposure group exhibited the intermediate effect.

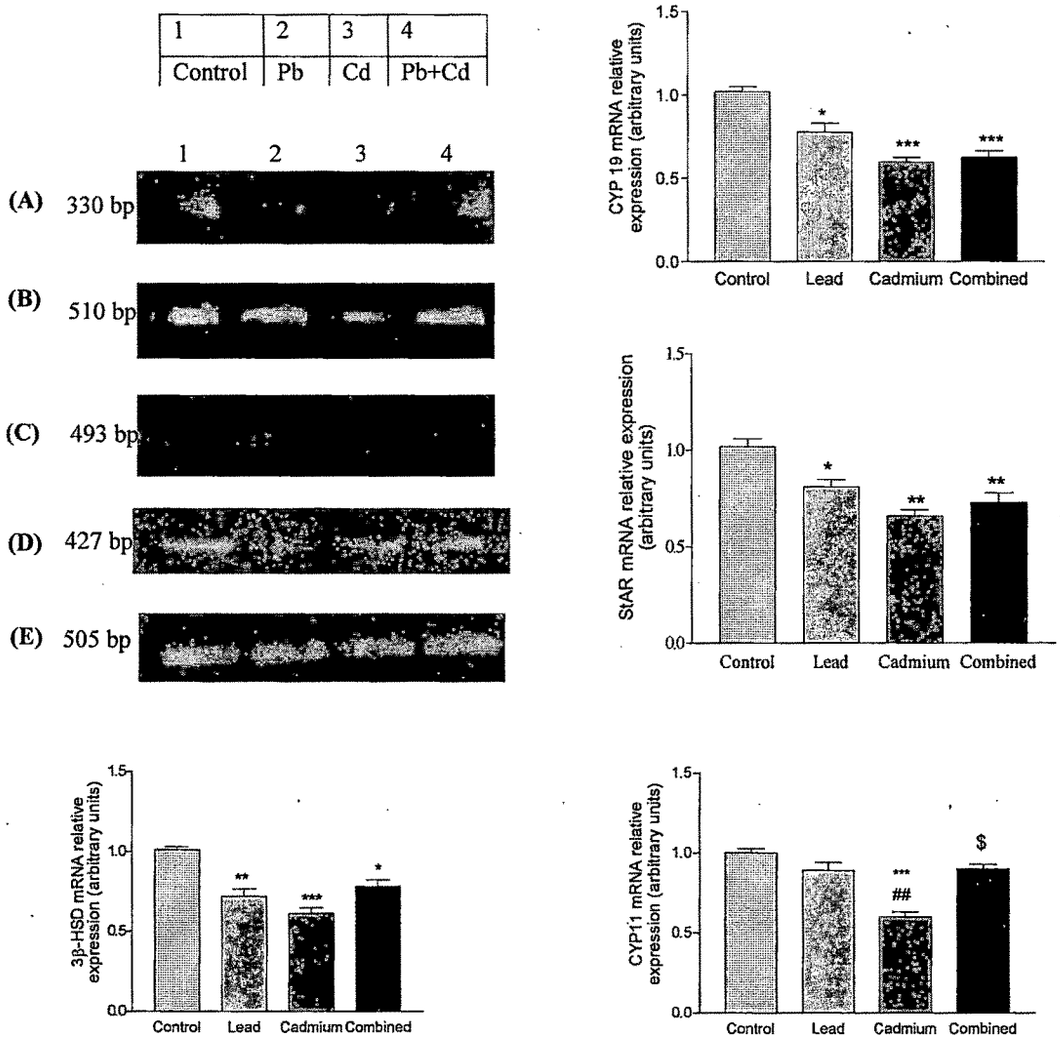
Under histological evaluation, significant structural differences were seen between the control PND 56 ovary and those exposed to lead and cadmium (Figure 5). Metal treatment between PND 35 to PND 55 caused a reduction in the size of ovary as compared to the ovary of control animals. The number of atretic follicles was significantly higher after cadmium administration.

Figure 1: Effect of pubertal co-exposure to lead and cadmium on (A) ovarian 17β-hydroxy steroid dehydrogenase activity and (B) 3β-hydroxy steroid dehydrogenase activity.



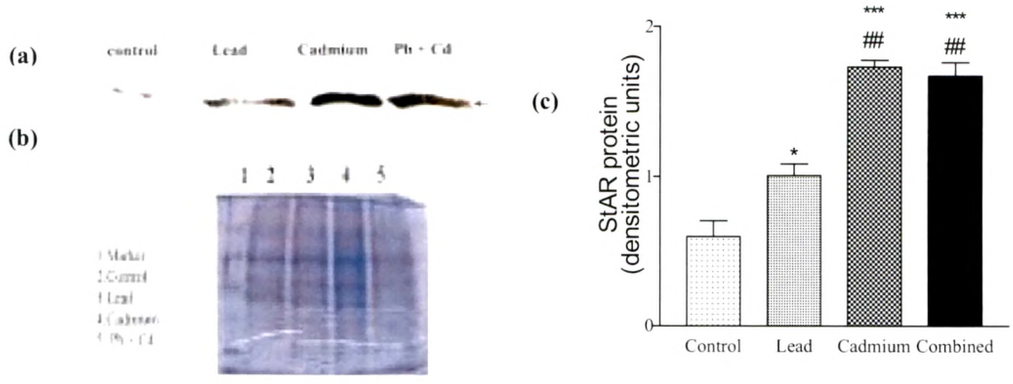
(n=6) The values are mean±SEM. *** p<0.001, ** p<0.01, *c p<0.05 compared to the control group; ### p<0.001, ## p<0.01, # p<0.05 compared to the lead group; $$ p<0.01, $ p<0.05 compared to the cadmium group

Figure 2: Effect of pubertal co-exposure to lead and cadmium on the mRNA expression levels of ovarian (A) StAR, (B) CYP11a (C) CYP19 (D) 3β-HSD and β- Actin (internal control).



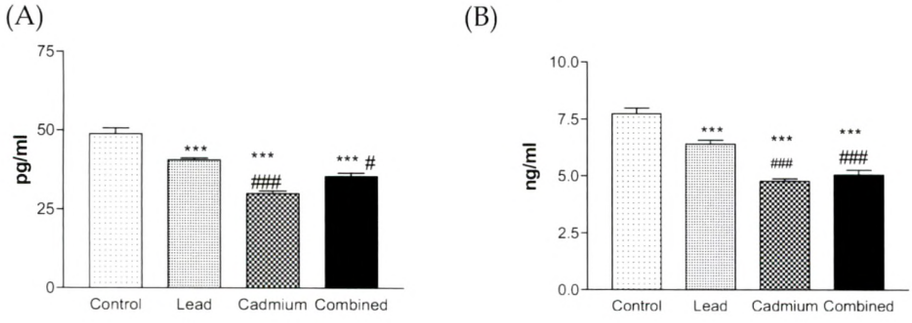
Values are expressed as mean ± S.D. (n = 3). N=6. The values are mean ± SEM.
*** p<0.001, ** p<0.01, * p<0.05 compared to the control group, ##p<0.01 versus lead; $p<0.05 versus cadmium

Figure 3: Effect of pubertal 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) Representative SDS-PAGE of ovarian mitochondrial proteins from animals exposed to experimental groups (c) Composite graph showing the mean (±SEM) densitometric quantitation of the bands from three blots corresponding to the StAR protein.



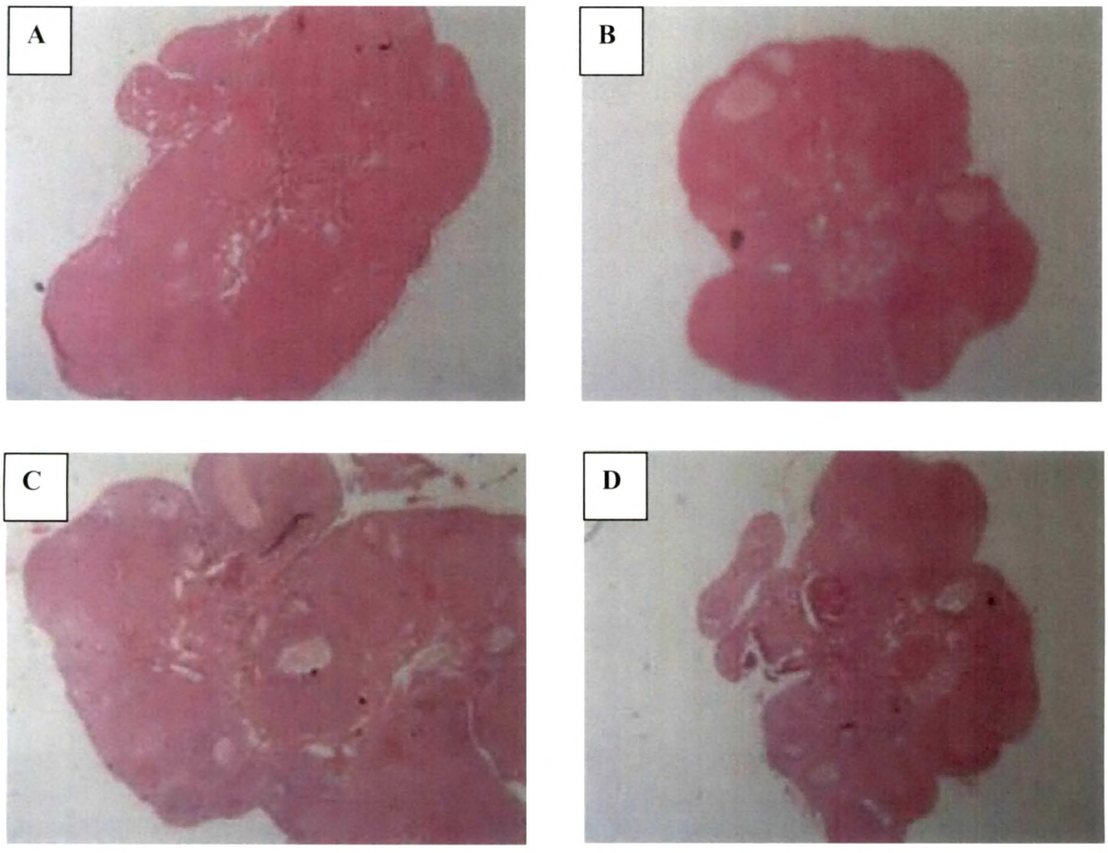
Values are expressed as mean ± S.D. (n = 3). N=6. The values are mean ± SEM.
*p<0.05, *** p<0.001 vs. control group, ##p<0.01 versus lead

Figure 4: Effect of pubertal co-exposure to lead and cadmium on serum estradiol levels (A) and serum progesterone levels (B)



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

Figure 5: Histology of PND 56 Ovary (10X magnification)



A=Control; B=Lead treated; C=Cadmium treated;
D= (Lead+Cadmium) treated

Table 5: Effect of Pubertal co-exposure to lead and cadmium on reduced glutathione (GSH) and TBARS levels in PND 56 ovary

Parameters	Groups			
	Control	Pb	Cd	Pb + Cd
GSH [a]	4.88±0.39	9.56 ±0.24***	6.46±0.40***###	8.16±0.21*** # $$$
TBARS [b]	25.08±0.35	31.20±1.53*	42.06±1.57*** ###	34.72±1.58*** $$

[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<0.001 vs. control; #p<0.05, ###p<0.001 vs. lead;
$$$ p<0.001, $$ p<0.01 vs. cadmium

Table 6: Effect of pubertal co-exposure to lead and cadmium on enzymatic antioxidants of PND 56 ovary

Parameters	Groups			
	Control	Pb	Cd	Pb + Cd
CAT [a]	39.90±0.96	33.26±0.86***	27.32±0.46*** ##	29.40±1.28***
Cu-Zn SOD[b]	12.39±0.67	10.92±0.48	7.64±0.43 *** ###	8.52±0.62**
Mn SOD[b]	6.48±0.33	5.14±0.31*	3.78±0.26*** #	4.66±0.21**
GPx[c]	7444±115.7	6998±91.72	5742±131.7 *** ###	6444±128.9***# $$
GR[d]	15.71±0.26	12.55±0.64***	8.54±0.300***###	11.06±0.42*** $$

[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<0.05, ## p<0.01, ### p<0.001 vs. lead;
$$ p<0.01 vs. cadmium

4.3.4 Discussion

In this chapter, we present the biochemical and molecular targets of endocrine disruptions in relation to ovarian steroidogenesis after pubertal co-exposure to lead and cadmium. We did not observed any change in the reproductive cyclicity in either of the exposure groups. The body weight gain and absolute/relative ovarian weights also remained unaffected in all the experimental groups in the present study. However, absolute/relative uterine weights were significantly increased in cadmium treated group as compared to the control, whereas the lead and combined treated group did not show any significant alterations. The present findings are in line with earlier reports on the estrogenic mode of action of cadmium (Smida *et al.,* 2004; Safe, 2003; Johnson *et al.,* 2003). Distribution pattern shows that toxicants-lead and cadmium get accumulated in ovaries significantly when compared to the control group. Lead and cadmium levels found in the combined exposure group indicate that there is some kind of competition among these metals. Several workers have shown the accumulation of metal salts in ovary (Bires *et al.,* 1995; Saksena and Salmonsen, 1983).

Post natal day 56 in female rat ontogeny marks the beginning of series of periodic events such as changes in ovarian functions, release of ova following changes in the secretion of estrogen and progesterone that further regulates periodic changes in uterine cycle which altogether is very essential for successful fertilization and implantation. It is also well known that granulosa cell continue to increase in number after the onset of puberty. Hence, all these periodic reproductive events are highly vulnerable to lead and cadmium following pubertal exposure (Kumar, 2008). In normal ovarian cycle, as the mass of granulose cells rises, estrogen levels also rises and a shift to positive feedback of estrogen causes a rise in LH that in turn results in cyclic ovulation. We observed significant decrease in granulose cell number in cadmium and combined metal treated groups. The present findings are well correlated with suppressed serum sex-steroid levels and indicate the

possibility of disruption of cyclic ovulation. In this context, a very systematic study was designed to draw attention to the possible biochemical and molecular targets of endocrine disruption in relation to the periodic changes in ovarian function attained on reaching PND 56. 3β-HSD is needed for the production of progesterone while 17β-HSD and P450 aromatase (P450arom) enzyme is essential for estradiol production. We observed an increase in the activity of the key ovarian steroidogenic enzymes unlike the outcome of gestational and lactational exposure regime. Although, we could not compare the increase in the activity of these steroidogenic enzymes with any published report following *in vivo* exposure to metals during early developmental stages, there are evidences, which showed the stimulatory and inhibitory effects of cadmium on progesterone synthesis using the steroidogenically stable JC-410 porcine granulosa cells line, genetically modified with gene constructs containing the promoter region of the cytochrome P450 side chain cleavage gene linked to a luciferase reporter gene (Smida et al., 2004). They showed that stimulatory effect of Cd^{2+} appears to be mediated via a *cis*-acting element located 100 bp upstream of the P450scc gene transcription start site. Thus, our present in vivo findings are in agreement with these reports on stimulatory potential of low dose of cadmium. However, Pb-treated groups demonstrated inhibition in the activities of these key steroidogenic enzymes as compared to the control animals. There involves various factors that determine the induction or suppression of steroidogenic enzyme activity and in this regard, it is also reported that cellular redox state plays a crucial role in regulating hydroxysteroid dehydrogenase activity, and intracellular hormone potency (Agarwal and Auchu, 2005). This differential effect could be related to difference in expression of the enzymes, responsible for synthesis of sex steroids. Both serum sex-steroids ie estradiol and progesterone, demonstrated a significant decrease in all the metal treated groups, which further confirms the net effect of endocrine disruption within steroidogenic pathway by lead and cadmium, despite of stimulatory effect observed in the cadmium and combined exposure group. The central "event" in puberty is the reduction in

sensitivity of the hypothalamus-pituitary part of the axis to negative feedback of estrogens. Hence, the suppressed serum estradiol levels after pubertal exposure to metals also suggest the possible alterations of negative feedback system during early reproductive age, ie PND 56, which may have further serious implications as far as reproductive axis function is concerned. However, decreased levels of gonadal hormones could be due to an indirect effect of these metals on hypothalamic-pituitary axis function as discussed in section 4.2. The inhibition of the steroid dehydrogenases as observed in the lead treated group could be attributed to the direct interaction of Pb with the enzymes or competition with divalent ions like zinc and calcium (Flora *et al.*1982; Waakles & Poirier 1985; Paksy *et al.* 1996). Both the key enzymes belong to the class of short chain alcohol dehydrogenases, which contain the Tyr-X-X-X-Lys at its active site (Persson *et al.*, 1991). Moreover, the metal ions lead and cadmium can interact with these amino acid residues or get bound to the -SH groups of cysteine residue present at the NAD binding domain (Persson *et al.*, 1991) and alter the structure so that the substrate binding and function gets significantly affected.

It has become clear that StAR, the 30-kDa mitochondrial protein, is the rate-limiting step in gonadotropin-stimulated steroid production (Luo *et al.*, 1998; Stocco and Clark, 1997). Several recent studies have shown a direct correlation between the synthesis of steroids and the synthesis of the 30-kDa StAR protein in steroidogenic cells and thus, represent an essential component in steroidogenesis (Huang *et al.*, 1997; Lin *et al.*, 1995; Luo *et al.*, 1998; Orly and Stocco, 1999; Stocco and Clark, 1997). In the present study, we demonstrated that administration of Cd both alone and in combination with Pb during pubertal development induced ovarian StAR protein expression at post natal day 56. This observation is well correlated with depleted levels of cholesterol content in ovary of metal treated groups. From the present results, it can be speculated that StAR protein synthesis is up-regulated for meeting the need of cholesterol which is a precursor molecule for the steroidogenesis

pathway. During the late juvenile phase of pubertal development, the release of LH from the pituitary plays an important role in ovarian maturation by increasing gonadotropin receptor numbers and facilitating production of E2 (Ojeda *et al.*, 1986a). This trophic stimulation of receptors on the ovary causes, through several signaling pathways, activation of the StAR gene, thus producing an increase in StAR protein expression and, subsequently, ovarian steroids (Chung *et al.*, 1998; Clark *et al.*, 1995; Pescador *et al.*, 1997; Sandhoff and McLean, 1996). These observations clearly show that both Pb and Cd exposure during pubertal developmental window may interfere in the signaling pathways responsible for StAR mediated critical functions within ovarian compartment.

Understanding the mechanisms underlying the disruption of regulatory mechanisms of key ovarian steroidogenic pathway genes after early developmental exposure effects of Pb and Cd is very essential. Henceforth, efforts were also made to check the expression profile of key steroidogenic pathway genes that includes (StAR, 3β-HSD, CYP11a, CYP19). The present study demonstrated altered gene expression profiles for these key steroidogenic pathway molecules and thereby suggesting inhibitory potential of lead and cadmium both alone and in combination in relation to transcriptional machinery of steroidogenesis pathway after pubertal exposure. A significant decrease in mRNA coding for P450scc, an enzyme catalyzing cholesterol side chain cleavage, was observed in the present study. This has raised cholesterol metabolism as a putative target of lead and cadmium interference within ovarian steroidogenic pathway. However, in the present study the depression of CYP11a mRNA levels apparently is not severe enough to interfere with pregnenolone formation since we also observed an induction in 3β-HSD activity in cadmium and combined treatment group. In lead treatment group, 3β-HSD activity was significantly decreased and is well correlated with depressed CYP11a gene expression profile. Nevertheless, the progesterone levels were significantly affected in all

the experimental groups. The cytochrome P450 aromatase enzyme is the rate-limiting and FSH-dependent factor in estradiol synthesis. The results of the present study indicate suppressed levels of P450aromatase (CYP19) mRNA which correlate well with low estradiol levels in all the metal exposed groups. Thus, aromatase gene expression also seems to be a sensitive target of both lead and cadmium. Suppression of ovarian mRNA levels of StAR and other steroidogenic enzymes (CYP11a, 3β-HSD) also correlate well with the decreased serum sex-steroid levels in all the metal treated groups. The present study also explains the outcome of endocrine disruption when these metals are present together. Data from the combined exposure group clearly shows the inhibitory potential is showing a trend of intermediate effect as seen in most of the other parameters throughout this study.

Histological studies of PND 56 ovary in the present study indicate negative effects of cadmium in particular on the ovarian follicle development. With regard to the number of follicles, the lowest number of primary follicles was found after i.p. administration of cadmium. The number of atretic follicles was significantly higher in cadmium administered group as compared to the control animals. The diameter of the follicles was significanlty smaller in the primary follicles of cadmium treated group in comparison with control group. Percentage of growing follicles was significantly higher and that of stroma significantly lower in the control group in comparison with all experimental groups. Similar alterations were reported earlier (Massányi et al. 2000) with cadmium as metal toxicant.

It is known that lead and cadmium, both have electron sharing affinities, which can result in the formation of covalent attachments (Bondy, 1996) with sulfhydryl groups of proteins (Ouig, 1998) as discussed in previous chapters. Results from the present study clearly demonstrate oxidative stress as one of the possible biochemical mechanism behind disruption of key ovarian steroidogenic functions at both transcriptional and activity level. Oxidative insults mediated by lead and cadmium alone and in

combination within ovarian compartment are quite evident from the suppressed activities of non-enzymatic and enzymatic antioxidant system (GPx, CAT, SOD, and GR). Mechanism of such alterations has been discussed in earlier chapter (Chapter 3, section 3.3). When the antioxidant control mechanisms are exhausted, the cellular redox potential shifts toward an oxidative stress.

From the above results, metal exposure during pubertal window results in serious structural and functional deficits as far as ovarian steroidogenesis is concerned on post natal day 56.

4.4 Hepatic xenobiotic/steroid metabolism

4.4.1 Introduction

Liver is the main organ where metabolism of hormones as well as toxic elements occurs. One route through which normal hormonal homeostasis could be disrupted is through xenobiotic induction of sex steroid metabolizing cytochrome P450 enzymes. Inducing or blocking these liver metabolizing enzymes conceivably alters the natural balance of circulating sex steroids. Several mechanisms are used for hormone biotransformation in the liver. Major pathway of biotransformation includes direct conjugation, in which the steroid is conjugated to glucuronic acid or sulphate, produces a more water-soluble product that can then be excreted in urine (Bethizy and Hayes, 1994). Oxido-reduction of estradiol to estriol is another route of hepatic biotransformation pathway that influences circulating concentrations of estradiol. The complexity of estrogen synthesis and metabolism provides many potential sites for the regulation of steroid action. Both phase-I & -II xenobiotic/steroid metabolizing enzymes might play an important role in detoxifying or eliminating the endocrine disruptors that got accumulated during early developmental exposure. Therefore, the adverse effects on their activities after pubertal exposure could be candidate markers of liver toxicity. Although drug metabolizing enzymes of hepatic origin were proved to be suppressed by heavy metals, (Arizono *et al.*, 1993; Ueng *et al.*, 1991) it remains to be determined whether the hepatic enzymes are susceptible to the suppressive effects of heavy metals after pubertal exposure regime.

Both lead and cadmium have been reported to disrupt reproductive physiology and steroid metabolism at different levels, along the hypothalamic-pituitary-gonadal-hepatic axis (Sokol, 1997). All the above stated data have illustrated effects of single metal exposure. Also the metal exposure has been used in high concentration but in environment, population receives simultaneous multiple exposures, at low dose indicating the need for experimental work with combinations of toxicants. Even though the general toxicity of lead and cadmium in isolation are very well known, only a few

reports are available on the effect of pubertal co-exposure to these metals on hepatic steroid metabolic enzymes (Murashow, 1966; Neshkow, 1971; Odenbro *et al.*, 1982). In view of this, present study was performed to analyze the effects of low level pubertal co-exposure to lead and cadmium on phase-I and phase-II hepatic xenobiotic/steroid metabolizing enzyme activities of PND56 female rats.

4.4.2 Experimental design

Following the experimental regime as discussed earlier, the animals were sacrificed on PND 56 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 1000 g for 15 min at 4^{0}C. The sediment was discarded and supernatant was centrifuged for 8000 g for 30 min at 4^{0}C. For further fractionation the above supernatant was centrifuged at 15000 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 as described in the chapter 2. Phase-I and Phase-II xenobiotic/steroid metabolizing enzymes from appropriate hepatic fractions were estimated along with various oxidative stress parameters (GSH, LPO, GPx, CAT, MnSOD, Cu-Zn SOD, GR) and biochemical parameters which is described in detail in the Chapter 2.

4.2 Results

Pubertal exposure to metals either alone or in combination did not demonstrate any significant effect on body weights, absolute and relative liver weights of PND 56 female rats (Table 1). The hepatic concentration of lead, cadmium in PND56 female rats are shown in Table 2. Both lead and cadmium accumulated significantly in the liver after pubertal exposure as compared to the control.

Figures 1 & 2 represents the hepatic phase-I xenobiotic metabolizing enzyme activities. Activity of both NADPH-& NADH-cytochrome c reductase were decreased in all the metal treated groups. Cadmium treated group exhibited the maximum change while lead treated group showed the least but significant change, as compared to the control. Combined metal group showed an intermediate effect as compared to the control.

Figures 3, 4, 5 & 6 represent the hepatic phase-II xenobiotic/steroid metabolizing enzyme activities. Activities of hepatic phase-II enzymes (γGT, UDPGT, GST, 17β-HSOR) were significantly inhibited in all the metal treated groups. Cadmium exposed group demonstrated maximal change as compared to the control group in all the cases.

Figure 7 shows the histology of PND 56 liver after pubertal exposure to lead and cadmium alone and in combination. Histological changes such as degenerative damage, focal inflammatory changes and hepatic necrosis were observed in metal treated groups and were significant as compared to the control group.

Table 1: Effect of pubertal co-exposure to lead and cadmium on the body weight, absolute liver weight and relative weight of PND 56 female rats

Parameters	Groups			
	Control	Pb	Cd	Pb + Cd
Body weight	131.3±4.16	123.5±6.95	130.4±5.24	138.9±4.13
Absolute liver weight (g)	5.00±0.094	4.96±0.108	4.83±0.141	4.75±0.158
Relative liver Weight (g)	0.038±0.001	0.041±0.002	0.037±0.001	0.034±0.002

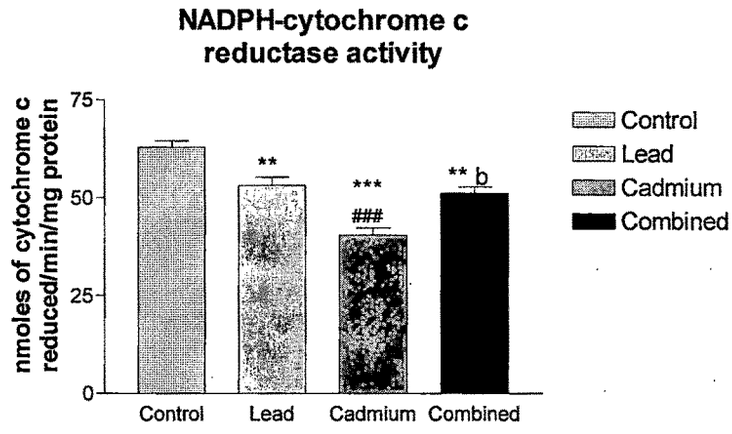
The data are presented as mean ± SEM. of 6 independent observations.

Table 2: Metal accumulation in liver of PND56 rats after pubertal co-exposure to lead and cadmium

Groups	Metal Content	
	Pb(μg/g)	Cd(μg/g)
Control	0.255±0.005	0.045±0.002
Lead	0.787±0.021***	0.039±0.003
Cadmium	0.228±0.011 ###	0.092±0.003*** ###
Combined	0.635±0.008*** ### $$$	0.056±0.005 $$$

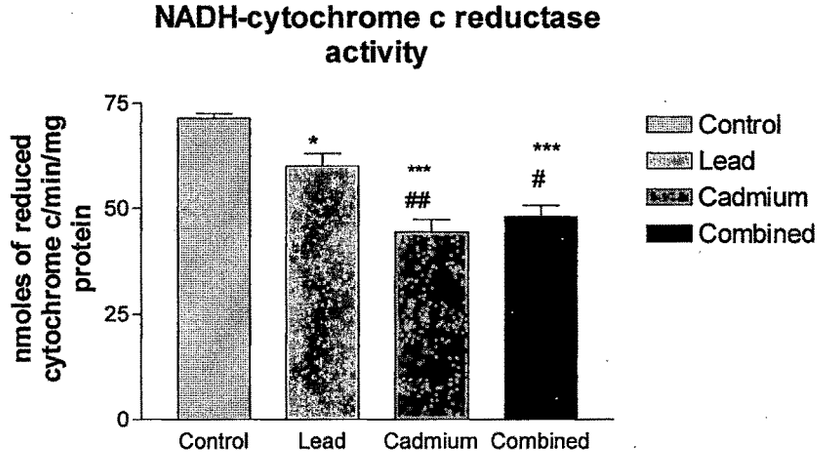
The data are presented as mean ± SEM. of 4 independent observations.
*** p<0.001 vs. control; ###p<0.001 versus lead; $$$p<0.001 versus cadmium

Figure 1: Effect of Pubertal exposure to lead and cadmium alone and in combination on NADPH- cytochrome c reductase activity in PND 56 female rats



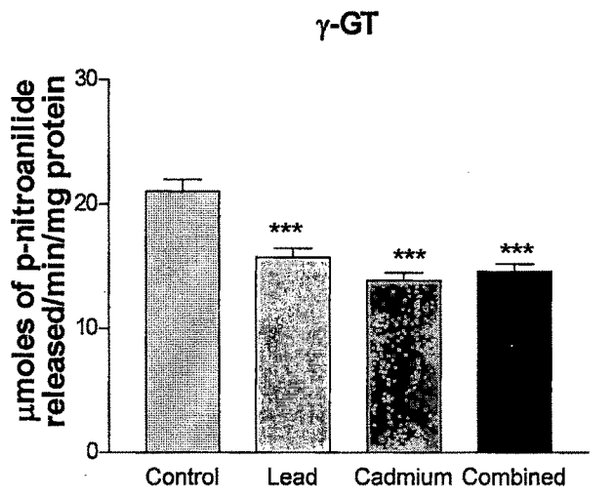
The data are presented as mean ± SEM. of 6 independent observations.
** p<0.01, *** p<0.001 vs. control; ### p<0.001 vs. lead; b p<0.01 vs. cadmium

Figure 2: Effect of pubertal exposure to lead and cadmium alone and in combination on NADH- cytochrome c reductase activity in PND 56 female rats.



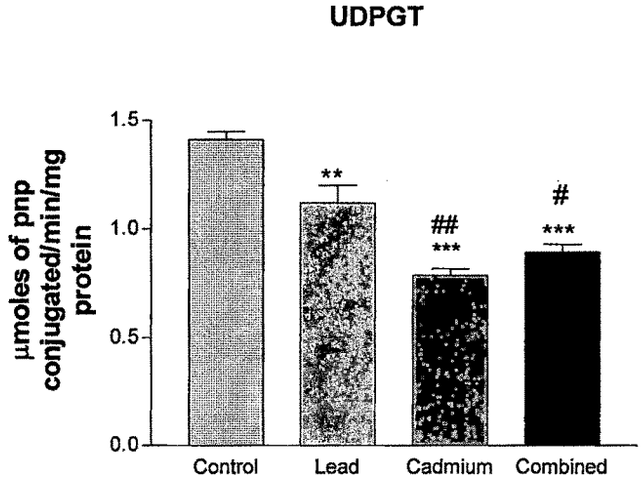
The data are presented as mean ± SEM. of 6 independent observations.
*p<0.05, *** p<0.001 vs. control; # p<0.05, ## p<0.01 vs. lead

Figure 3: Effect of pubertal exposure to lead and cadmium alone and in combination on Gamma-glutamyl transferase (γ-GT) activity in PND 56 female rats



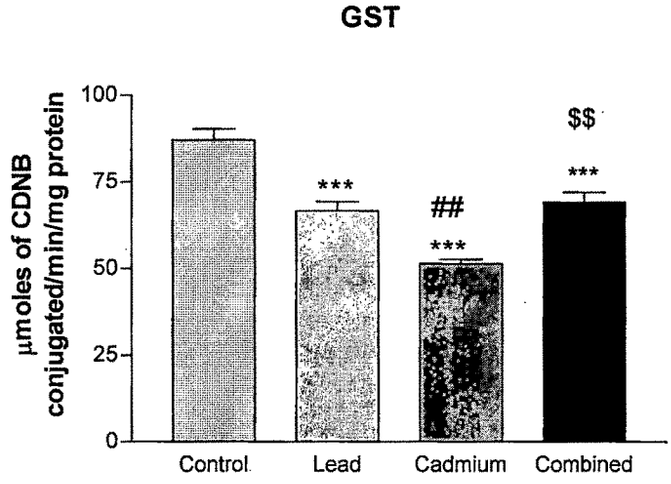
The data are presented as mean ± SEM. of 6 independent observations.
*** p<0.001 vs. control

Figure 4: Effect of Pubertal exposure to lead and cadmium alone and in combination on UDP-glucoronyl transferase (UDPGT) activity in PND 56 female rats



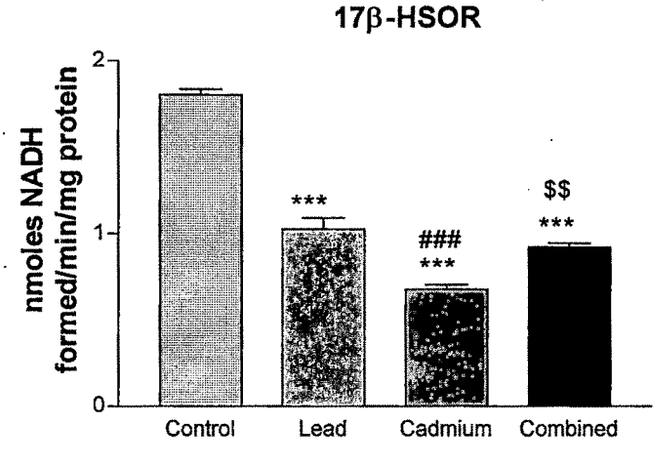
The data are presented as mean ± SEM. of 6 independent observations.
** p<0.01, *** p<0.001 vs. control;
# p<0.05, ## p<0.01 vs. lead.

Figure 5: Effect of pubertal exposure to lead and cadmium alone and in combination on Glutathione-S-transferase (GST) activity in PND 56 female rats



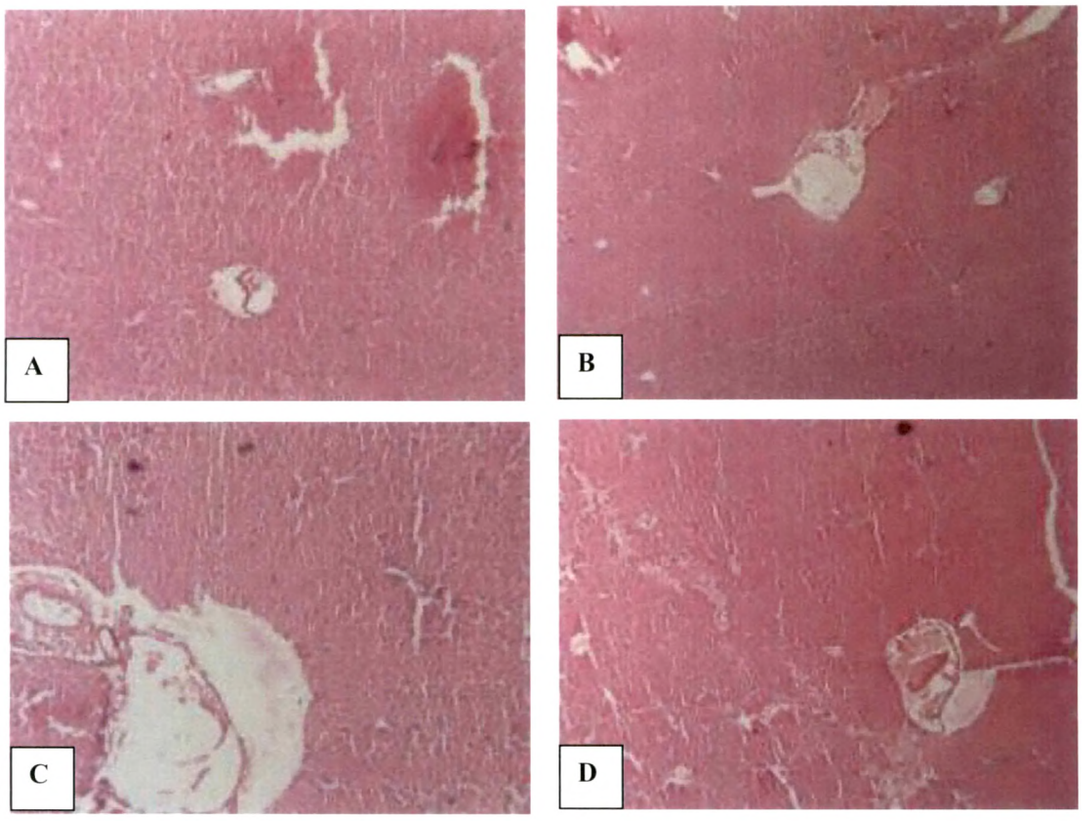
The data are presented as mean ± SEM. of 6 independent observations.
*** p<0.001 vs. control; ##p<0.01 vs. lead; $$p<0.01 vs. cadmium

Figure 6: Effect of Pubertal exposure to lead and cadmium alone and in combination on 17β-hydroxy steroid oxidoreductase (17β-HSOR) activity in PND 56 female rats



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

Figure 7: Histopathology of PND 56 Liver of female rats



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

Table 3 summarizes the data on hepatic Cholesterol, glycogen, DNA and RNA. Hepatic DNA and RNA content were decreased in cadmium and combined metal exposed groups; with no change in lead exposed group. Metals alone and in combination caused a decrease in the hepatic cholesterol level in all the metal treated groups.

Figures 8 & 9 represents the hepatic reduced glutathione (GSH) and lipid peroxidation levels (TBARS) respectively. Results shows that reduced glutathione levels were significantly depleted in all the metal treated groups. The results also clearly demonstrate significant increase in TBARS in all the metal treated groups. Table 4 shows the effect of metals on enzymatic antioxidants (CAT, Cu-Zn SOD, Mn SOD, GPx, and GR) of liver after pubertal exposure. Results demonstrate significant inhibitory effect of metals on antioxidant enzymes. Among the three metal treated groups, cadmium treatment was showing more inhibitory effect compared to other metal treatment groups.

Table 3: Effect of pubertal exposure to lead and cadmium on the biochemical parameters (Cholesterol, Glycogen, DNA, and RNA) of liver in PND56 female rats.

Parameters	Groups			
	Control	Pb	Cd	Pb + Cd
Total Cholesterol [a]	32.28±0.74	26.94±1.10*	24.76±1.26**	25.20±1.29**
Glycogen [b]	117.9±1.45	110.6±1.77	90.08±2.51*** ###	97.64±3.10*** ##
DNA [c]	4.67±0.20	3.98±0.17	3.74±0.19*	3.8±0.21*
RNA[d]	8.64±0.24	7.5±0.36	6.53±0.34 **	7.14±0.36 *

[a] µg/mg microsomal protein

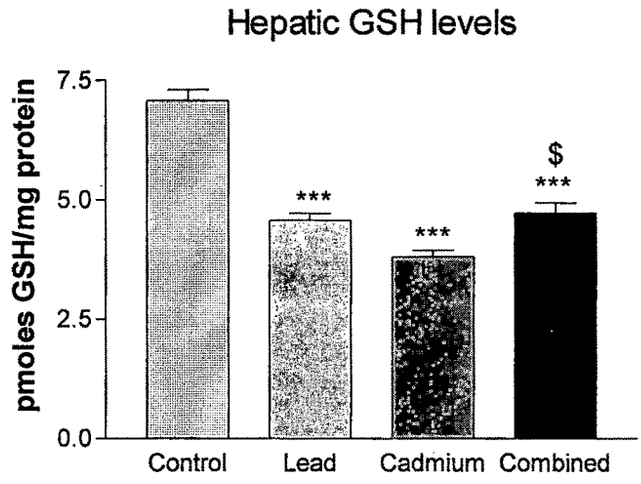
[b] mg/g tissue

[c] µg/g tissue

[d] µg/g tissue

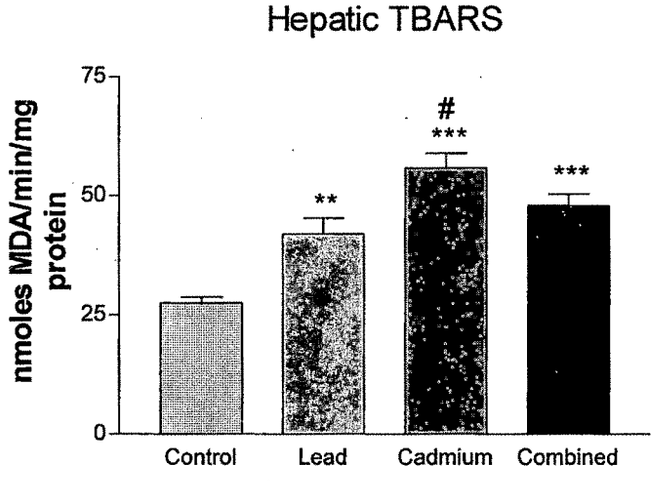
The data are presented as mean ± SEM. of 6 independent observations.
*p<0.05, ** p<0.01, *** p<0.001 vs. Control; ##p<0.01, ###p<0.001 vs lead

Figure 8: Effect of pubertal exposure to lead and cadmium alone and in combination on hepatic reduced glutathione levels (GSH) in PND 56 female rats



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

Figure 9: Effect of pubertal exposure to lead and cadmium alone and in combination on hepatic TBARS levels in PND 56 female rats



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

Table 4: Effect of pubertal exposure to lead and cadmium on enzymatic antioxidants of liver in PND56 female rats

Parameters	Groups			
	Control	Pb	Cd	Pb + Cd
Catalase [a]	185.4±5.09	162.6±2.53 **	152.5±3.26 ***	157.0±3.45 ***
Cu-Zn SOD[b]	24.54±0.56	20.06±0.55 **	16.26±0.62 *** ##	19.48±0.99 *** $
Mn SOD[b]	12.68±0.61	11.21±0.57	8.42±0.37 *** ##	7.8±0.23*** ###
GPx[c]	4942±72.55	4632±63.83*	4293±41.22 *** ##	4610±53.29 ** $$
GR[d]	149.2±2.94	146.0±2.82	136.0±2.89 *	147.6±2.92

[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, ### p<0.001 vs. lead;
$ p<0.05, $$ p<0.01 vs. cadmium

4.4.4 DISCUSSION

The results of the present study indicates that pubertal exposure to lead and cadmium either alone or in combination is inhibitory against phase I and phase II hepatic xenobiotic/steroid metabolizing enzymes. Although inhibitory potential of cadmium, lead and few other heavy metals against phase-I and phase-II enzymes have been already reported (Takayuki *et al.*,2001), very few biochemical studies have been done on the chronic exposure effects and no study has been yet reported showing the consequences of co-exposure effects of lead and cadmium after pubertal exposure. One route through which normal hormonal homeostasis could be disrupted is through xenobiotic induction of sex steroid metabolizing cytochrome P-450 enzymes. Inducing or blocking these liver metabolizing enzymes conceivably alters the natural balance of circulating sex steroids.

The basal levels and adult estrogen responsiveness of specific hepatic cytochromes P450 and steroid 5a-reductase in the rat can be irreversibly programmed or "imprinted" by prior exposure to estrogen or estrogen mimic. Imprinting of these enzymes can occur not only following exposure to estrogen/androgen during the neonatal period (Einarsson et al., 1973), but also during puberty (Pak et al., 1984; Cadario et al., 1992). The present study demonstrated that pubertal exposure to lead and cadmium alone and in combination decreased the activities of phase-I enzymes metabolizing xenobiotics and endogenous steroids in PND 56 rats, but among the two metals cadmium showed more inhibitory effect than lead. The present findings of inhibitory effects of metals on phase-1 enzyme activities after exposure to metals during pubertal developmental window suggest that hepatic biotransformation enzymes are compromised on reaching adulthood. The possible mode of mechanism involved in inhibition of phase-I enzymes have been discussed in earlier chapter 3, section 3.5

Phase-II xenobiotic/steroid metabolizing enzymes were also found to be significantly suppressed in all the metal groups suggesting the susceptibility of steroid biotransformation enzymes to metals even during pubertal development and thereby implicating serious consequences on circulating sex-steroid levels. Our findings are in line with other reports that have shown the deleterious effect of lead and several other environmental toxicants on both phase-I and phase-II hepatic xenobiotic/steroid metabolizing enzymes both in young and adult rats (Mudipalli, 2007; Aykin-Burns et al., 2003). The mechanism of suppression of these enzymes by metal exposure has been discussed in Chapter 3.

Histological observations of liver in PND 56 rats of metal treated groups showed marked alterations. Histological changes in liver such as massive fatty degeneration in hepatocytes and large vacuoles in cytoplasm were very clear evidence in all metal treated groups. Moreover, 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. This clearly suggests that early pubertal exposure to heavy metals can alter the structural integrity of liver. These observations are in agreement with previous reports of hepatotoxic effects of lead and cadmium as observed in adult rats (Brzóska *et al.*, 2003; Pillai *et al.*, 2002).

We investigated the status of hepatic antioxidant defense system of PND 56 rats in order to explore whether oxidative stress is one of the possible biochemical mechanism responsible for compromised phase-I and phase-II hepatic xenobiotic/steroid biotransformation enzyme activities as observed in the present study after pubertal administration of metals. Metal treated groups, demonstrated severe suppressive effects on antioxidant enzymes (CAT, Cu-Zn SOD, Mn SOD, GPx, GR) as compared to the control group. Similar observations have been demonstrated for other metal toxicants showing the ability to induce oxidative stress within hepatic tissues (Monteiro *et al.*, 1991; El-Maraghy *et al.*, 2001). Mechanism of such alteration has been discussed in earlier chapter (chapter 3; section 3.5). As the antioxidant control mechanisms are exhausted or overrun, the cellular redox potential shifts toward an oxidative stress, in turn, increasing the potential for damage to cellular components and resulting in liver dysfunction and thereby possibly affecting steroid biotransformation process as is the case shown in the present study.

The changes in the various biochemical parameters observed in the liver of PND 56 rats co-exposed to lead and cadmium throughout pubertal 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. In most of the studies reporting on combined exposure to metals, researchers have used the same concentrations of the metals both in

individual and combined treatment groups (Nation et al., 1990; Zikic et al., 1998). The results from such 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, whereas in the present study, the total concentration of the metals in the combined exposure group is the same as that in the individual-metal treatment group.

In conclusion, the suppressed hepatic xenobiotic/steroid biotransformation enzyme activities following pubertal exposure signifies the severity of early pubertal administration of metals on the hepatic steroid metabolism and eventually affecting the circulating sex-steroid levels indirectly as shown in the previous sections of this chapter.

4.3 Summary

Weaned pre-pubertal rats were treated intraperitonially with lead acetate and cadmium acetate separately and in combination at a dosage of (0.05 mg/ kg body wt/day) from PND 35 till PND 55 (throughout pubertal developmental window).

The hypothalamic and pituitary steroid metabolizing enzyme (3α-hydroxy steroid dehydrogenase) activity was significantly inhibited in all the metal exposed groups. Hypothalamic dopamine and norepinephrine content were also significantly decreased in all the metal treated groups as compared to the control. The accumulation of both metals increased in hypothalamus and pituitary after the treatment. 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. Hypothalamic GSH content was decreased in all the metal treated groups with cadmium exposed group showing maximum decrease as compared to the control. Pituitary GSH content was also 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 injury. Cadmium and combined metal exposed animals showed significant increase in TBARS levels in both hypothalamus and pituitary whereas lead exposed group showed significant increase in TBARS levels as compared to control but was minimal in comparison to other metal treated groups. SOD 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 SOD 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 CAT activity only in cadmium treated groups as compared to control in both hypothalamus and pituitary.

The key enzymes of ovarian 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 alteration in steroidogenic enzyme activities was followed with decrease in gonadal steroid levels. In all treatments, combined treated group showed intermediate results suggesting competition between the two metals. As observed in chapter 3, 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 function of pubertally metal exposed 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 levels were altered but were found within the normal range. Biochemical effects are correlated with metals accumulated in blood, reproductive tissues like ovary. Histopathological observation of ovary for cytotoxic changes did demonstrate marked alteration in histology of ovary. Number of atretic follicles was higher mainly in cadmium exposed group as compared to the control whereas the growing follicles were minimum as compared to the control. RT-PCR studies

revealed that mRNA levels of genes encoding protein responsible for cholesterol transport and steroidogenesis (StAR, CYP11a, 3β-HSD, CYP19), were decreased significantly compared to control. GSH content was decreased in all metal exposed groups. Cadmium and combined metal exposed animals showed significant increase in TBARS levels whereas lead treated groups showed the least effect. Antioxidant enzyme activities (CAT, SOD, GPx, and GR) were also significantly suppressed in all the metal exposed groups as compared to the control.

Hepatic phase-I and phase-II xenobiotic/steroid metabolizing enzymes were inhibited by the metal exposure. 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. 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 were also significantly inhibited as compared to the control. γ-GT 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. 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.