Chapter 5 Biochemical and molecular mechanism of cellular toxicity by lead and cadmium in luteinized granulosa cells

- *"In vivo"* exposure studies
- *"In vitro"* exposure studies

5.1 Introduction

Understanding the mechanisms by which dominant follicles develop in the ovary is a major goal of reproductive research because of its vital role in female fertility. FSH and LH regulate the growth and differentiation of granulosa cells (Chun *et al.* 1994), with FSH action predominating before the midcycle gonadotropin surge (Hillier, 2001). Moreover, growth factors form a controlling network to ensure the proper spatial and temporal pattern of FSH-dependent gene expression in the granulosa cells (GCs) during folliculogenesis (Erickson, 2001). FSH drives the proliferation, growth and differentiation of granulosa cells. It alone promotes activation of a number of "differentiation" target genes in granulosa cells, such as aromatase, LH receptor (Mary and Evelyn, 2006).

The importance of the growth factors is underscored by the fact that any impairment of FSH function cause infertility. In rodents, one of the growth factor known to be involved in regulating FSH action is IGF-I produced by the granulosa cells, has an essential role in FSH signaling (Minegishi et al., 2000; Bicsak *et al.*, 1990; Baker *et al.*, 1996). IGFs are some of the most prominent apoptosis inhibitory cytokines and their regulated expression in various tissues play a pivotal role in regulating cellular homeostasis (Yin *et al.*, 1998).

In the rat ovary, estrogens act as survival factors, whereas androgens promote apoptosis (Billig *et al.* 1993). Moreover, the dependence on transcription and translation for ovarian apoptosis has also been demonstrated (Svanberg and Billig, 1999). Granulosa cells are one of the major ovarian cell types that undergo apoptosis, with granulosa cell apoptosis accounting in part for the atresia of ovarian follicles (Pru, 2002). Numerous studies have shown that peptide hormones, steroids, and growth factors preserve the viability of granulosa cells (Amsterdam, 1997; Hsu, 1998; Peluso, 2003). Conversely, a variety of stimuli, including oxidative stress and

deprivation of growth factors, trigger granulosa cell apoptosis (Quirk *et al.*, 2006; Amsterdam, 1997; Hsu, 1998; Hsueh *et al.*, 1994). It has been shown that few classes of endocrine disruptors other than heavy-metals caused the decrease of the number of ovulated ova in superovulation induced in mice by treatment with pregnant mare's serum gonadotropin(PMSG) and human chorionic gonadotropin (Sekiguchi *et al.*, 2003; Ushinohama *et al.*,2001).

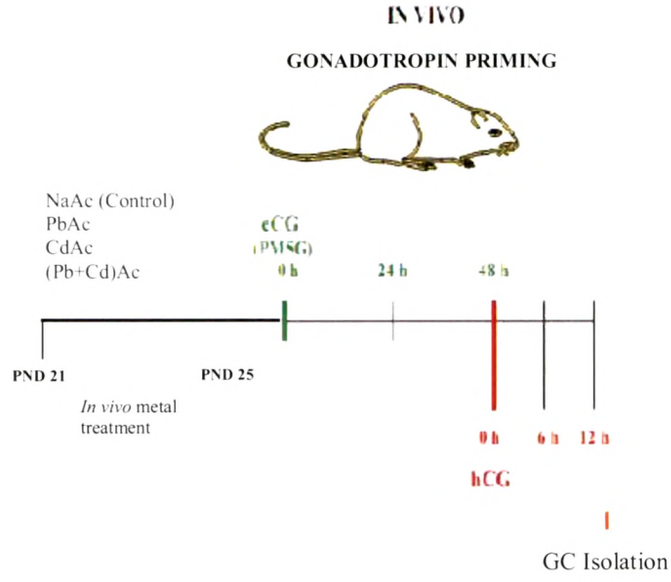
Among the members of an expanding list of environmental pollutants, lead and cadmium, are considered to be the most toxic chemicals found in various sources. In this regard, earlier studies done in our laboratory (Priya *et al.*,2004; Nampoothiri and Gupta, 2006; Nampoothiri *et al.*,2007), indicate that the adverse effects of lead and cadmium include a depression of sex-steroid levels, altered gonadotropin binding, disruption of key steroidogenic enzyme activities, oxidative stress generation in granulosa cells. In earlier chapters, we demonstrated that long-term lead and cadmium co-exposure in vivo during critical developmental periods (pubertal and gestational/lactational) decreased ovarian steroidogenesis and induced follicular arrest. However, there is a need of procedures for examining reproductive dysfunctions utilizing induced ovulation as an index of reproductive toxicity. In light of the above reports, we investigated a simple method using induced ovulation in immature rats to detect ovarian injury due to lead and cadmium using granulosa cells as a model system.

Thus in the present study the aim was firstly, to examine the influence of "*in vivo*" exposure to lead and cadmium on ovulation in immature rats primed with exogenous gonadotropins. Secondly, to elucidate the molecular and biochemical mechanism of cellular toxicity by lead and cadmium in luteinized granulosa cells with special emphasis on its susceptibility towards undergoing apoptosis.

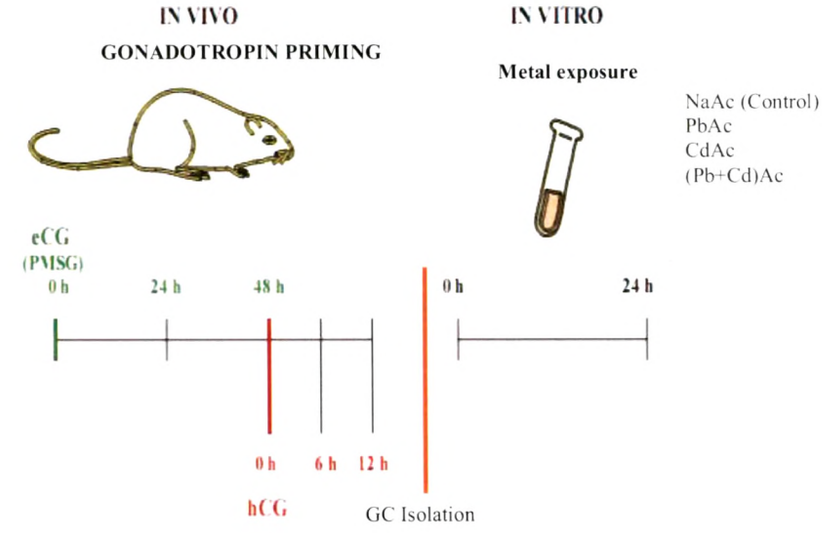
5.2 Experimental design

Experiments were performed following PMSG-hCG priming of immature Charles Foster female rats on ovarian granulosa cells in two ways:"*in vivo* treatment" and "*in vitro* treatment".

Schematic representation of *in vitro* exposure study



Schematic representation of *in vivo* exposure study



weighed immediately and used for various biochemical and molecular analysis. Granulosa cells were then isolated from the ovary following Campbell et al., 1970. Viability of the cells was checked by trypan blue staining. Purity of granulosa cells was evaluated by assessing 3β hydroxy steroid dehydrogenase (3β-HSD) activity (pure preparation of cells shows undetectable 3β-HSD activity). Total granulosa count was calculated using Naeubaur chamber. Each group comprised of $5x10^5$ cells incubated in DMEM-F12 medium. The *"in vitro"* experiment was divided into following treatment groups. *(1) Control:* cells were treated with sodium acetate *(2)* cells were treated with lead acetate *(3)* cells were treated with cadmium acetate *(4)* cells were treated with (Lead acetate+Cadmium acetate). Studies were carried out for two different concentrations ie 50μM and 200 μM and two different incubation periods ie 6 h and 12 h.

After the treatment, cells were harvested using 0.1% trypsin-EDTA and processed for following assays. % Viability of cells was determined by Trypan blue staining method. Cell injury was evaluated by LDH assay after 6h and 12h exposure period for both the concentrations (50μM and 200 μM). Estradiol and progesterone levels were also measured in conditioned media by ELISA after both 6 h and 12 h incubation period for both the concentrations (50μM and 200 μM). Total RNA was isolated using TriZol and processed for RT-PCR analysis for selected genes (FSH-R, IGF-1, PR, Aromatase, 17β-HSD, StAR) in treatment groups exposed for 12h to various metals at a concentration of 200 μM. Apoptosis of metal treated granulosa cells was quantitated by caspase-3 activity in the treatment groups exposed to various metals at concentration of 200 μM for a period of 12 h exposure. Comet Assay was performed for determining DNA damage at 12 h, following exposure to various metals at 200 μM concentration. For analyzing the antioxidant parameters, treated cells were sonicated at 5cycles/min and these sonicated cells were used to assess oxidative parameters (GSH, LPO, CAT and SOD). To define the role of oxidative stress from lutenized granulosa cells in metal-induced toxicity, intracellular reactive oxygen species (iROS) were measured by 2′, 7′-

dichlorofluorescein (DCF) staining, and the intensity of fluorescence in the granulosa cells after metal exposure was compared with cells from control group.

5.3 Results

There were no significant differences in body weight in metal-treated animals compared to control animals following both *"in vivo"* and *"in vitro"* treatment regimens.

Table 1 shows the influence of *"in vivo"* exposure to lead and cadmium on ovulation induced by exogenous gonadotropins in immature Charles foster female rats. Ovulation was induced in 5/6 rats (83.3%) in the control group, while in the experimental groups ovulation was induced in 4/6 (66.7%), 3/6 (50%) and 3/6 (50%) rats in lead–treated, cadmium-treated and combined group respectively. Among the ovulation induced rats, the control animals exhibited 3/5(60%) superovulation while the lead-treated, cadmium-treated and combined exposure group exhibited 1/4(25.0%), 0/3(0%), 0/3(0%) respectively.

Table 2 shows the *"in vivo"* effects of lead and cadmium on body and reproductive organ weights of immature female rats primed with gonadotropins. Neither absolute nor relative organ-weights were affected significantly in any of the metal treated groups.

Table 1: Effect of *"in vivo"* exposure to lead and cadmium in isolation and combination on ovulation induced by gonadotropins in immature rats.

Parameters	Groups			
	Control	Pb	Cd	Pb + Cd
Total no. of rats	6	6	6	6
Ovulation				
Induced rats	5/6 (83.3%)	4/6 (66.7%)	3/6 (50.0%)	3/6 (50.0%)
Ovulated ova	19.00±3.34	11.503.27	6.33±0.88	8.66±0.33
Superovulation				
Induced rats	3/5(60%)	1/4(25.0%)	0/3(0%)	0/3(0%)
Ovulated ova	24.33±2.33	21	-	-

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

Table 2: Effect of *"in vivo"* exposure to lead and cadmium in isolation and combination on body and reproductive organ weights, and ovulation in gonadotropin primed immature rats.

Parameters	Groups			
	Control	Pb	Cd	Pb + Cd
Total no. of ovulated rats	5	4	3	3
Initial body weights (g)	34.33±1.40	33.67±0.95	31.67±0.98	33.17±1.42
Final body weights (g)	61.00±1.23	59.50±1.40	60.50±1.56	58.50±1.25
Growth rate(%)	178.5±4.25	177.2±4.85	191.6±5.39	177.4±4.99
Ovary weights (mg)	79.17±1.42	75.83±1.04	74.17±1.64	76.0±1.71
Relative Ovary weight (mg/g b.wt)	1.301±0.039	1.280±0.047	1.229±0.037	1.301±0.028
Uterus weights (mg)	155.2±3.69	149.5±3.28	144.5±3.07	146.7±2.52
Relative uterus weight (mg/g b.wt)	2.549±0.083	2.525±0.109	2.393±0.057	2.512±0.064
Uterus(mg)/Ovary(mg)	1.965±0.072	1.971±0.033	1.951±0.042	1.934±0.045

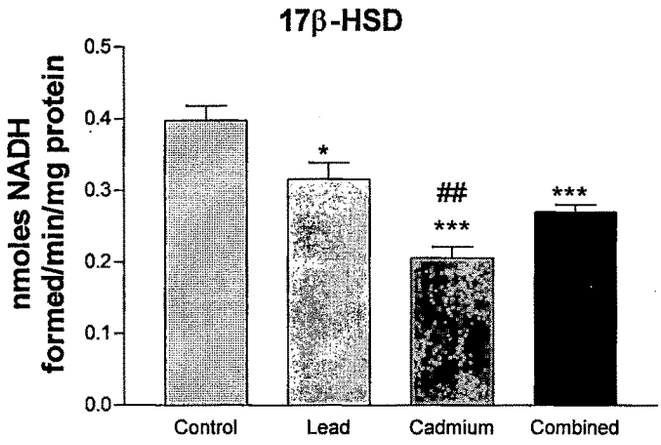
The data are presented as mean ± SEM.

Figure 1 represents the effect of in vivo exposure to lead and cadmium on 17β-HSD activity of granulosa cells. Cells of cadmium treated animals showed a maximum decrease in the activity compared to cells of control animals. Cells of animals co-exposed to lead and cadmium showed an intermediate change while cells of lead treated animals showed minimum change.

Figure 2 represents the in vivo effect of pre-pubertal co-exposure to lead and cadmium on serum sex steroid levels (estradiol and progesterone) in gonadotropins primed immature female rats. Serum estradiol levels were significantly decreased only in cadmium and combined exposed groups whereas progesterone levels were affected only in cadmium treated groups.

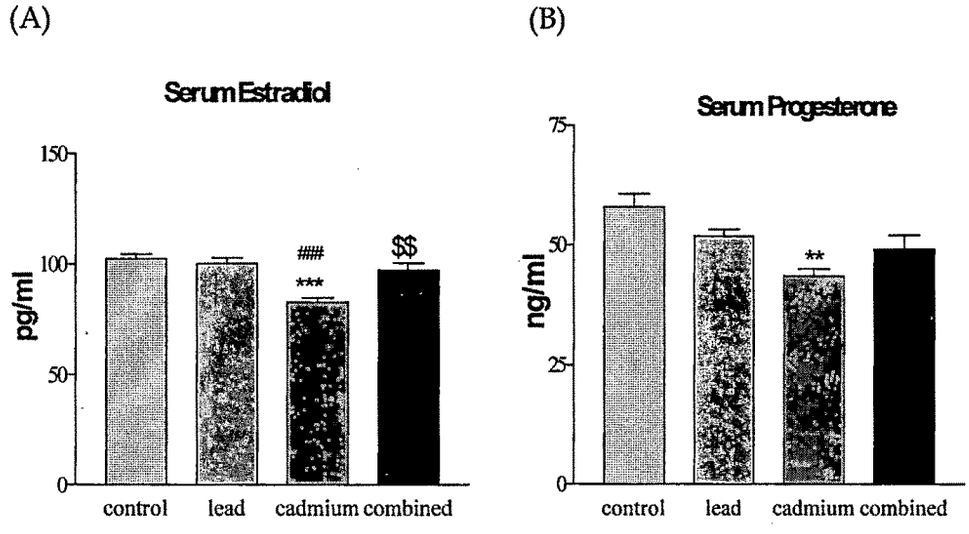
Figure 3, 4, 5, and 6 represents effect of "in vivo" exposure to lead and cadmium on reduced glutathione, TBARS, and antioxidant enzymes (CAT & SOD) of luteinized granulosa cells isolated from gonadotropin primed immature female rats. The antioxidant enzyme activities (CAT, SOD, GPx) in luteinized granulosa cells were found to be significantly suppressed in all the metal treated groups. Significant depletion in GSH levels with enhanced levels of lipid peroxidation (increased TBARS levels) was also observed in luteinized granulosa cells isolated from ovaries of animals that were prior administered both metals and gonadotropins.

Figure 1: Effect of *"in vivo"* exposure to lead and cadmium in isolation and combination on 17β-hydroxysteroid dehydrogenase activity of granulosa cells in gonadotropin primed immature rats



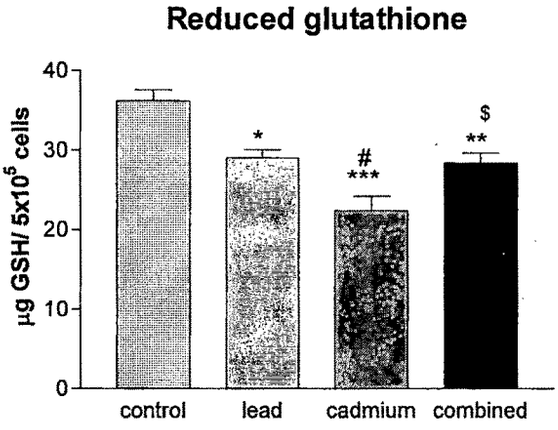
The data are presented as mean ± SEM of 6 independent observations.* p<0.05, **p<0.001 vs. control; ##p<0.01 vs. lead

Figure 2: Effect of *"in vivo"* exposure (PND 21 to PND 25) to lead and cadmium in isolation and combination on (A) serum estradiol and (B) progesterone levels in gonadotropin primed immature female rats.



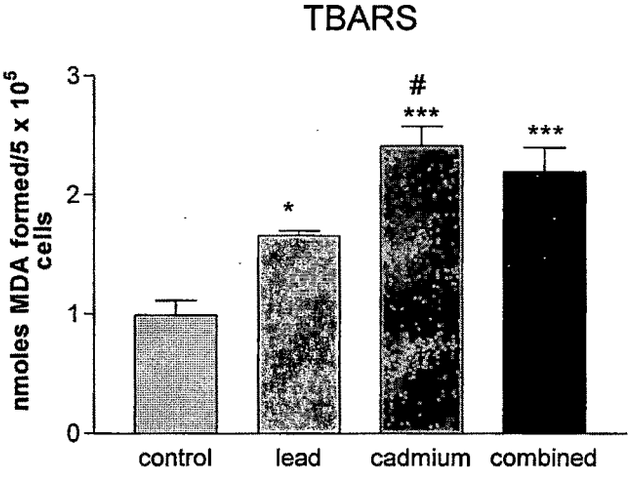
The data are presented as mean ± SEM of 6 independent observations.
***p<0.001 trol, **p<0.01 vs. control
###p<0.001 vs. lead and $$ p<0.01 vs. cadmium group.

Figure 3: Effect of *"in vivo"* exposure (PND21 to PND25) to lead and cadmium in isolation and combination on reduced glutathione (GSH) levels of luteinized granulosa cells from gonadotropin primed immature rats.



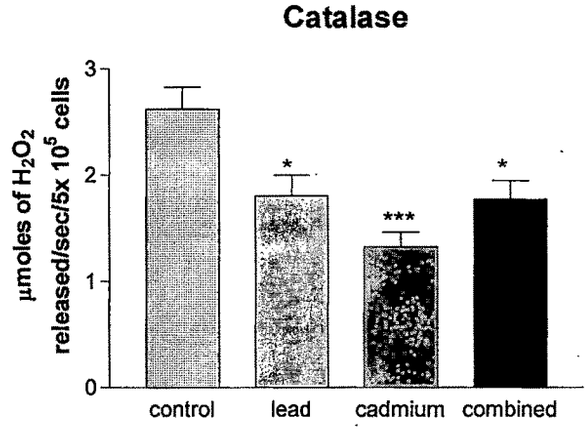
The data are presented as mean ± SEM of 6 independent observations. *p<0.05, **p<0.01, ***p<0.001 vs. control; #p<0.05 vs. lead and $ p<0.05 vs. cadmium group.

Figure 4: Effect of *"in vivo"* exposure (PND 21 to PND 25) to lead and cadmium in isolation and combination on TBARS levels of luteinized granulosa cells from gonadotropin primed immature rats.



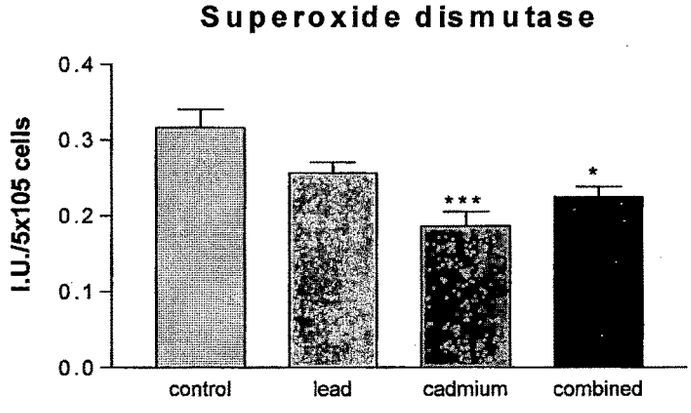
The data are presented as mean ± SEM of 6 independent observations. *p<0.05, **p <0.001 vs. control; #p<0.05 vs. lead

Figure 5: Effect of *"in vivo"* exposure (PND 21 TO PND 25) to lead and cadmium in isolation and combination on Catalase activity of luteinized granulosa cells obtained from gonadotropin primed immature rats.



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

Figure 6: Effect of *"in vivo"* exposure (PND 21 to PND 25) to lead and cadmium in isolation and combination on SOD activity of luteinized granulosa cells obtained from gonadotropin primed immature rats.



I.U = Amount of enzyme required for 50 % inhibition of pyrogallol autoxidation at 37°C.

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

Figures 7 & 8 represents the % viability of luteinized granulosa cells after *"in vitro"* exposure to metals alone and in combination for 6 h and 12 h respectively. Percentage viability of granulosa cells was maximally affected following 12 h exposure to metals at 200μM concentration. Though, % viability was also significantly affected in cadmium and combined metal treated groups at metal concentration of 50 μM, but the effect observed was less pronounced as compared to the metal concentration of 200 μM. *"in vitro"* exposure for 6 h did not cause any change in the viability of luteinized granulosa cells exposed to metals alone and in combination.

Effect of *"in vitro"* exposure to lead and cadmium alone and in combination on the estradiol levels in the conditioned media of luteinized granulosa cells exposed for 6 h and 12 h, shown in Figures 9 and 10 respectively. The maximum inhibition in estradiol levels were seen in luteinized granulosa cells exposed for 12 h to metals at a concentration of 200 μM.

Figures 11 & 12 shows the progesterone levels in the conditioned media of luteinized granulosa cells after *"in vitro"* exposure to lead and cadmium alone and in combination for 6 h and 12 h respectively. Cells from cadmium treated group showed maximal inhibition in progesterone production after both 6 h and 12 h exposure regimen. Cells of lead treated group showed minimal inhibition as compared to the cells of control group. Inhibition in progesterone production was also observed at higher concentration (200 μM) even for 6 h exposure regimen.

Figure 13 demonstrates the effect of lead and cadmium on activity of 17 β-HSD in luteinized granulosa cells. Cells of cadmium treated group showed maximum inhibition while cells of combined treated group showed intermediate results. Cells of lead treated group did not demonstrate any significant inhibition in enzyme activity.

Figure 14 demonstrates the effect of *"in vitro"* exposure (12 h) to lead and cadmium on activity of Caspase-3 in luteinized granulosa cells. Cells of cadmium treated group showed maximum inhibition ($p< 0.001$) while cells of

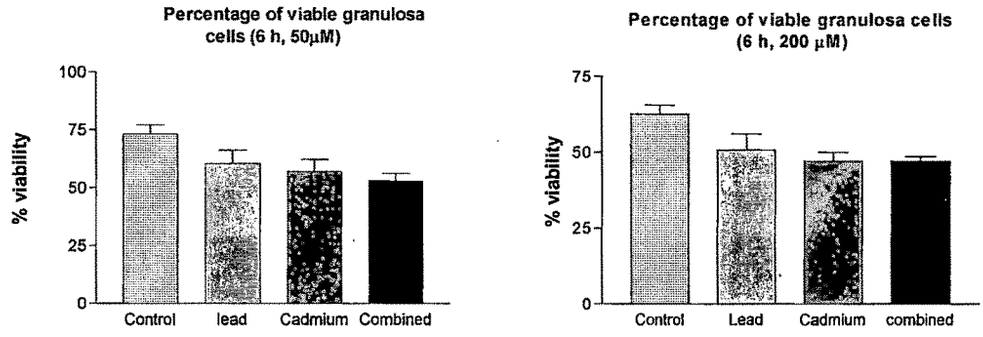
combined treated group showed intermediate results ($p<0.001$). Cells of lead treated group showed minimal inhibition ($p< 0.01$) as compared to cells of control group.

Figure 15 represents the % LDH release in the conditioned media of luteinized granulosa cells after *"in vitro"* exposure (12 h) to lead and cadmium in isolation and in combination. Cells of cadmium treated group showed maximum inhibition ($p<0.001$) while combined treated group showed intermediate effect ($p<0.001$) as compared to the cells of the control group. Cells of the lead treated group did not demonstrate any significant inhibition in the % LDH release as compared to control group. Whereas luteinized granulosa cells exposed to metals at a concentration of 50μM did not demonstrate any significant change in % LDH release as compared to the control group.

Figure 16 represents the DCF fluorescence staining of luteinized granulosa cells after "in vitro" exposure to lead and cadmium in isolation and in combination for 12 h at a final concentration of 200 μM. Photomicrographs clearly shows maximal fluorescence staining intensity in cadmium treated group followed by combined metal treated group. Cells of lead treated group showed the least fluorescence intensity.

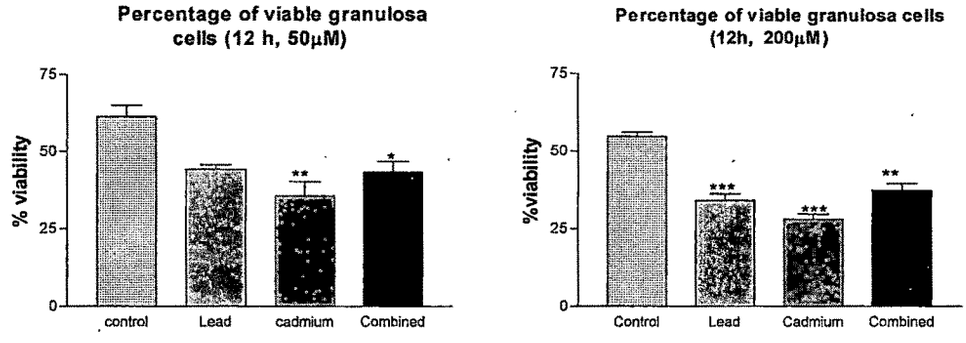
Figure 17 represents the COMET staining of DNA of luteinized granulosa cells after *"in vitro"* exposure to lead and cadmium in isolation and in combination for 12 h at a concentration of 200 μM. Cadmium treated group exhibited the maximal DNA damage as evident from the COMET tail formation. Cells of lead and combined metal treated group did not demonstrate any severe DNA damage.

Figure 7: Effect of *"in vitro"* exposure to lead and cadmium on % viability of luteinized granulosa cells after 6 h exposure at (50μM & 200μM) concentration of metals alone and in combination.



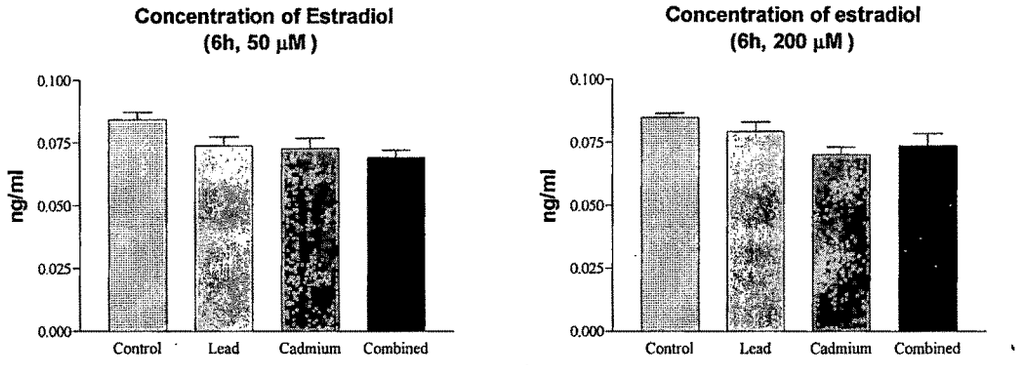
The data are presented as mean ± SEM of 3 independent observations.

Figure 8: Effect of *"in vitro"* exposure to lead and cadmium on % viability of luteinized granulosa cells after 12 h exposure at (50μM & 200μM) concentration of metals alone and in combination.



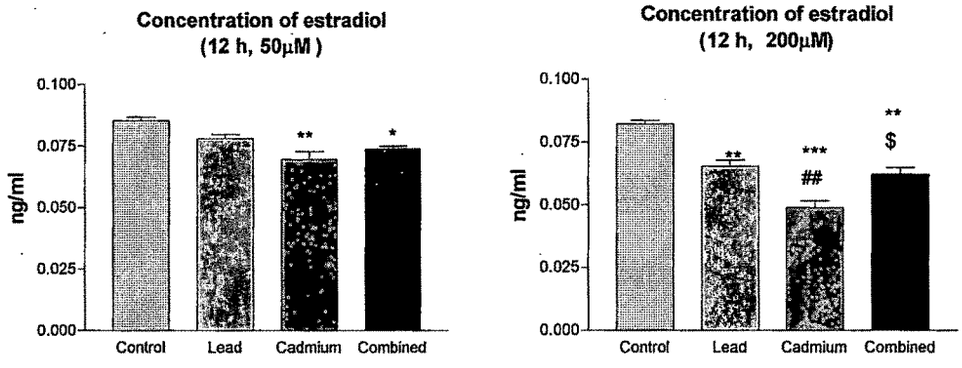
The data are presented as mean ± SEM of 3 independent observations.
*p<0.05, **p<0.01, ***p<0.001 vs. control.

Figure 9: Effect of *"in vitro"* exposure to lead and cadmium (6 h exposure at concentrations of 50μM & 200μM) on estradiol levels in conditioned media of luteinized granulosa cells isolated from gonadotropin primed immature rats.



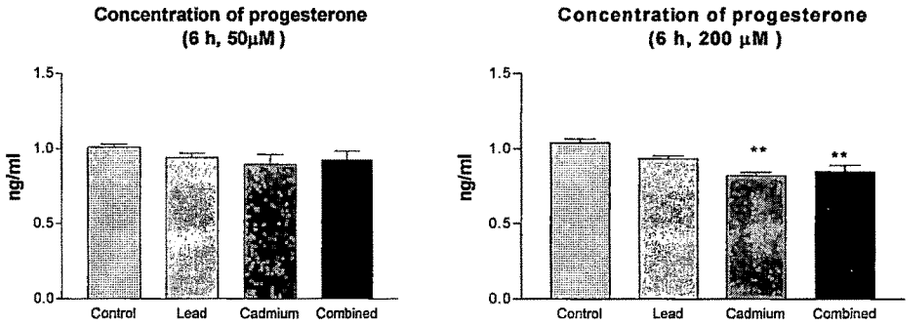
The data are presented as mean ± SEM of 3 independent observations.

Figure 10: Effect of *"in vitro"* exposure to lead and cadmium (12 h exposure at concentrations of 50μM & 200μM) in isolation and combination on estradiol levels in conditioned media of luteinized granulosa cells isolated from gonadotropin primed immature rats.



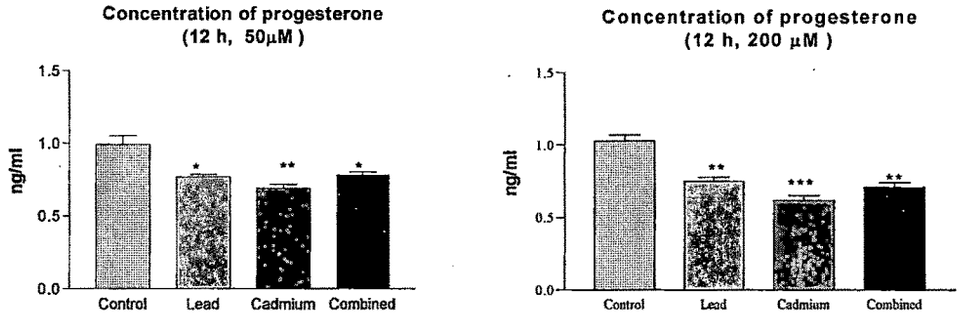
The data are presented as mean ± SEM of 3 independent observations.
*p<0.05, **p<0.01, ***p <0.001 vs. control; ##p<0.01 vs. lead and
$ p<0.05, vs. cadmium group.

Figure 11: Effect of *"in vitro"* exposure to lead and cadmium (6 h exposure at concentrations of 50μM & 200μM) on progesterone levels in conditioned media of luteinized granulosa cells isolated from gonadotropin primed immature rats.



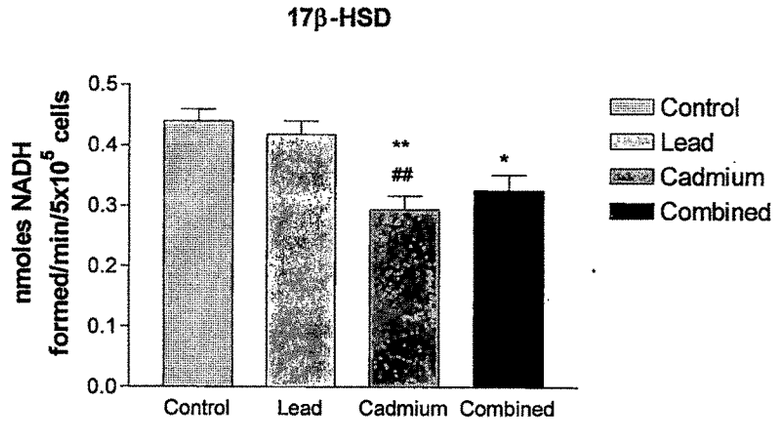
The data are presented as mean ± SEM of 3 independent observations.
**p<0.01, vs. control

Figure 12: Effect of *"in vitro"* exposure to lead and cadmium (12 h exposure at concentrations of 50μM & 200μM) on progesterone levels in conditioned media of luteinized granulosa cells isolated from gonadotropin primed immature rats.



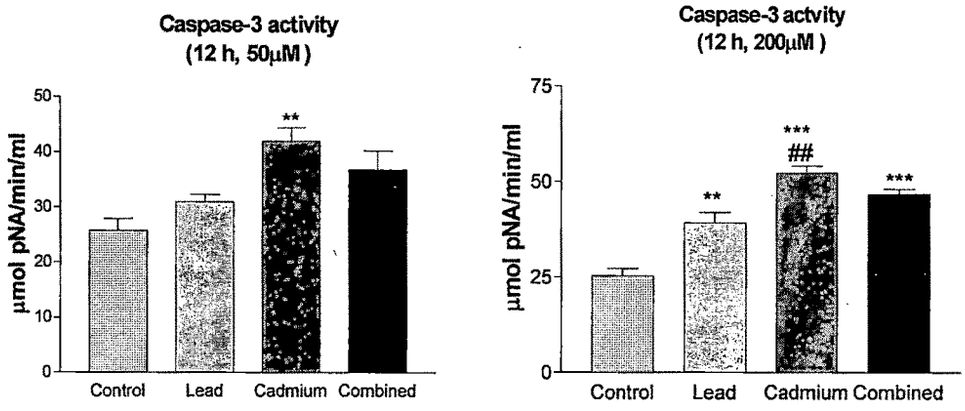
The data are presented as mean ± SEM of 3 independent observations.
*p<0.05, **p<0.01, ***p <0.001 vs. control

Figure 13: Effect of *"in vitro"* exposure to lead and cadmium (12 h exposure at concentration of 200μM) on 17β-hydroxysteroid dehydrogenase activity in luteinized granulosa cells isolated from gonadotropin primed immature rats.



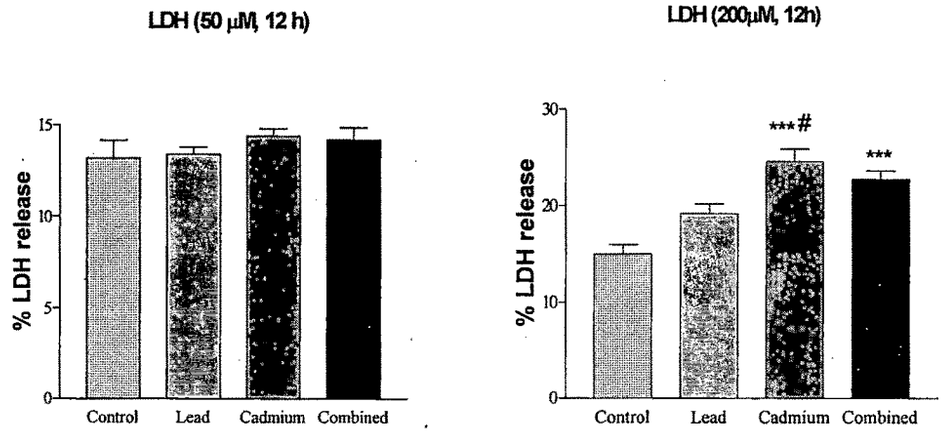
The data are presented as mean ± SEM of 3 independent observations.
*p<0.05, **p<0.01, vs. control, ##p<0.01, vs. lead

Figure 14: Effects of *"in vitro"* exposure to lead and cadmium (12 h exposure at concentrations of 50μM and 200μM) in isolation and in combination on caspase-3 activity in the luteinized granulosa cells from gonadotropin primed immature rats.



The data are presented as mean ± SEM of 3 independent observations.
**p<0.01, ***p<0.001 vs. control; ##p<0.01 vs. lead

Figure 15: Effects of *"in vitro"* exposure to lead and cadmium for 12 h at concentrations of (50μM and 200μM) in isolation and in combination on % LDH release measured in conditioned media of luteinized granulosa cells.



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

Table 3 represents the effects of *"in vitro"* exposure to lead and cadmium either alone or in combination for 12 h on reduced glutathione, TBARS and antioxidant enzymes in luteinized granulosa cells. Cells that were exposed to cadmium showed a maximum decrease in reduced glutathione content and elevation in lipid peroxidation compared to cells of control animals. Luteinized granulosa cells which were exposed to combination of metals showed an intermediate change in both the parameters while cells of lead treated animals showed minimum change. Significant inhibition in the activity of SOD and CAT activity was also seen in cells exposed to cadmium treated group and combined metal treated.

Table 3: Effect of *"in vitro"* exposure to lead and cadmium in isolation and combination on antioxidant system in luteinized granulosa cells from gonadotropin primed immature rats. (200μM, 12 h).

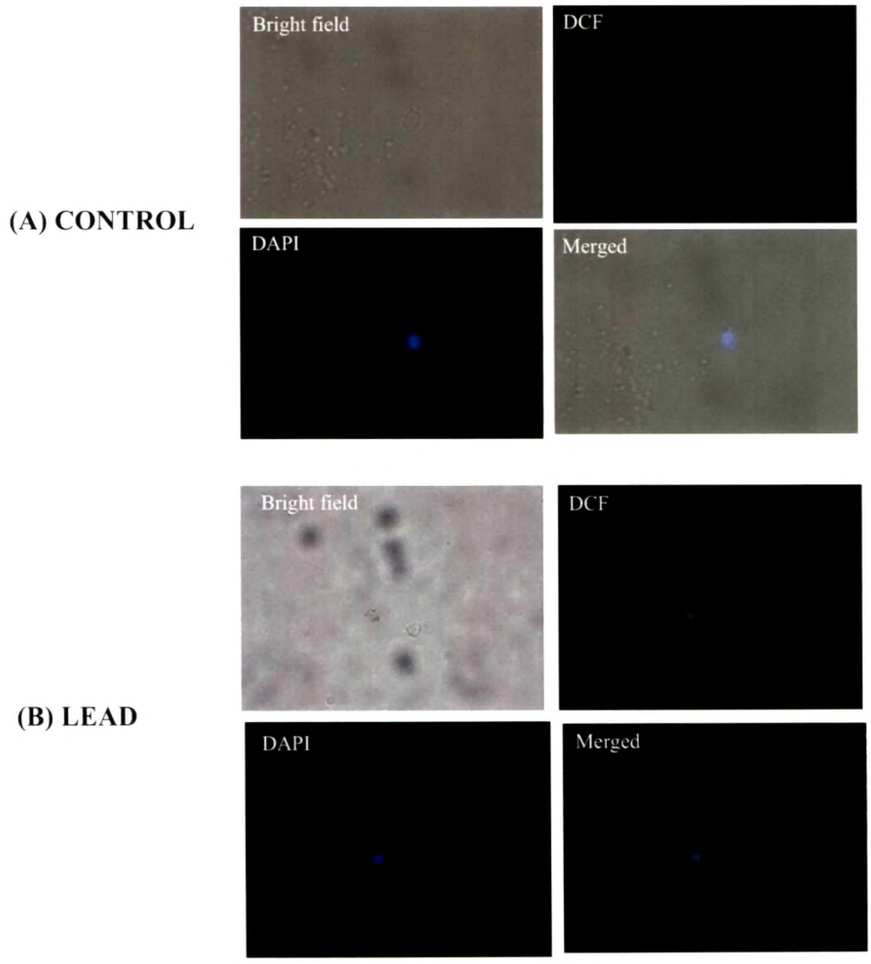
Parameters	Groups			
	Control	Pb	Cd	Pb + Cd
GSH[a]	33.00±0.99	28.74±0.98 *	19.44±1.09*** ###	27.72±0.77** $$$
TBARS[b]	0.88±0.02	1.10±0.04 *	1.45±0.02*** ###	1.14±0.05** $$$
CAT [c]	4.48±0.25	4.04±0.15	3.04±0.21*** #	4.00±0.14 $
SOD [d]	0.47±0.03	0.37±0.02	0.32±0.03*	0.34±0.02 *

a pmoles/mg protein
b nmoles MDA/min/mg protein
[c]μ mole H_2O_2 decomposed/min/mg protein at 37°C.

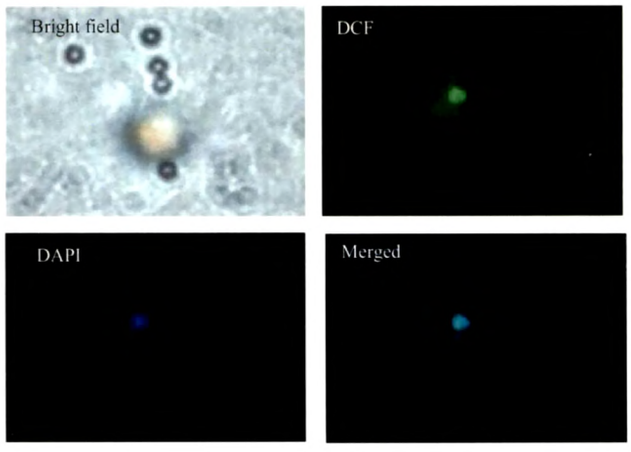
[d]Amount of enzyme required for 50 % inhibition of pyrogallol autoxidation at 37°C.

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

Figure 16: ROS levels in luteinized granulosa cells, as measured by DCF fluorescence intensity, increase upon incubation with lead and cadmium for 12 h at the concentration of 200μM. A, B, C, D shows representative images. (A) Control luteinized granulosa cells with very faint DCF fluorescence, (B) granulosa cells from lead treated group, (C) granulosa cells from cadmium-treated, with intense fluorescence and (D) granulosa cells from combined metal-treated cells respectively.



(C) CADMIUM



(D) COMBINED

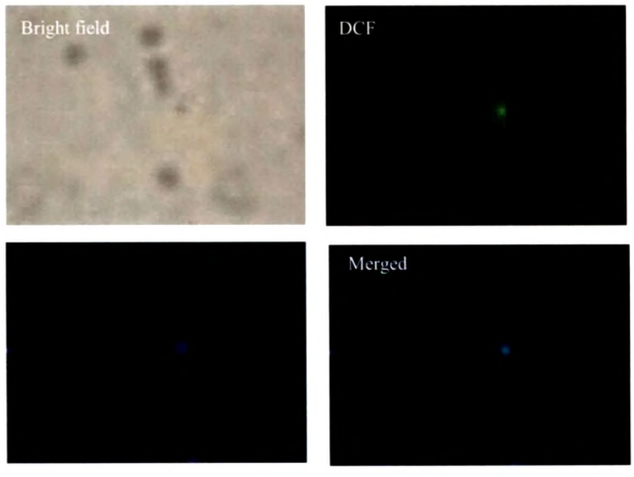


Figure 17: Comet staining of DNA from luteinized granulosa cells after being exposed to lead and cadmium for 12 h at concentration of 200μM in isolation and in combination.

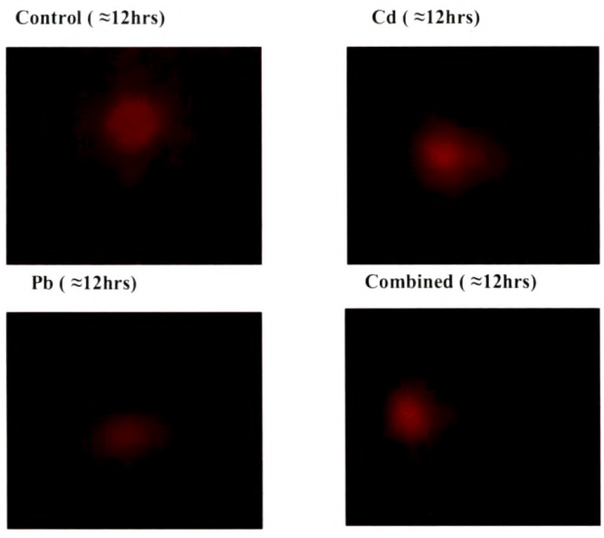
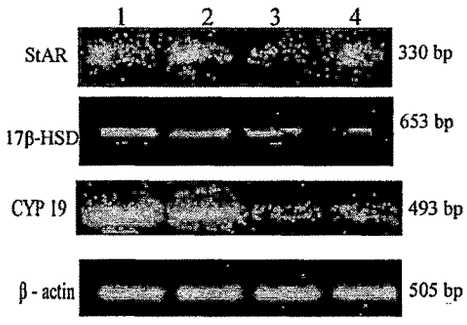
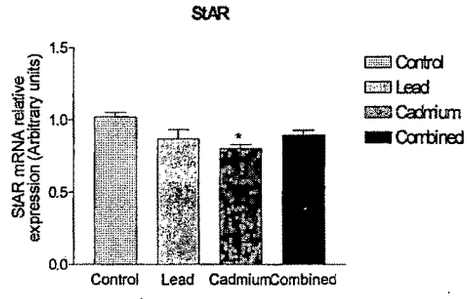


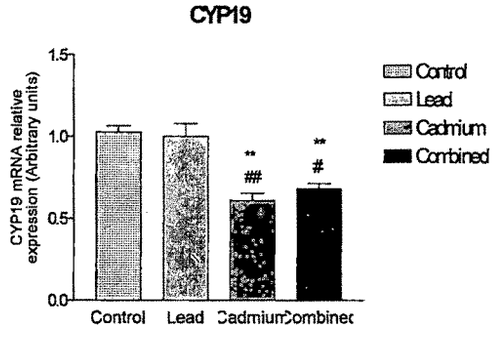
Figure 18 represents the mRNA expression levels of key steroidogenic genes (StAR, CYP19, 17β-HSD), proliferative and growth factors (FSHR,PR,IGF-1), apoptotic/anti-apoptotic genes (Bcl-2, Bax) in luteinized granulosa cells by RT-PCR analysis following "in vitro" exposure to lead and cadmium for 12 h at concentration of 200 μM. In the present study the effects of "in vitro" exposure to Pb and Cd on mRNA expression of genes involved in cholesterol transport, steroidogenesis, growth-factors, apoptotic proteins in luteinized granulosa cells obtained from gonadotropin primed immature rats were determined. Expression of StAR gene responsible for cholesterol transport to the inner mitochondrial membrane, were significantly reduced only in cadmium treated group as compared to that of controls. Significant reductions in mRNA levels of CYP19 and 17β-HSD were observed in both cadmium and combined metal treated groups whereas lead treated group did not demonstrate any statistically significant change as compared to the control group. FSHR and IGF-1 mRNA levels were affected significantly only in cadmium treated group. PR mRNA expression did not demonstrate any significant alterations as compared to the control group. Bcl-2 and Bax expression was also notably altered in metal treated groups. Pb and co-exposed groups does not exhibit any significant changes compared to control.

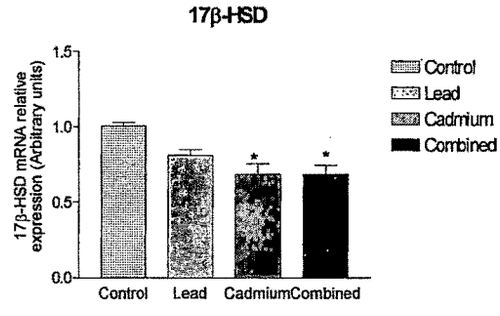
Figure 18A: Effect of *"in vitro"* exposure to lead and cadmium on mRNA expression of StAR, 17β-HSD, Cyp19 and β-actin (internal control) in luteinized granulosa cells after 12 h exposure to 200 μM concentration of each metal.

1	2	3	4
Control	Pb	Cd	Pb+Cd



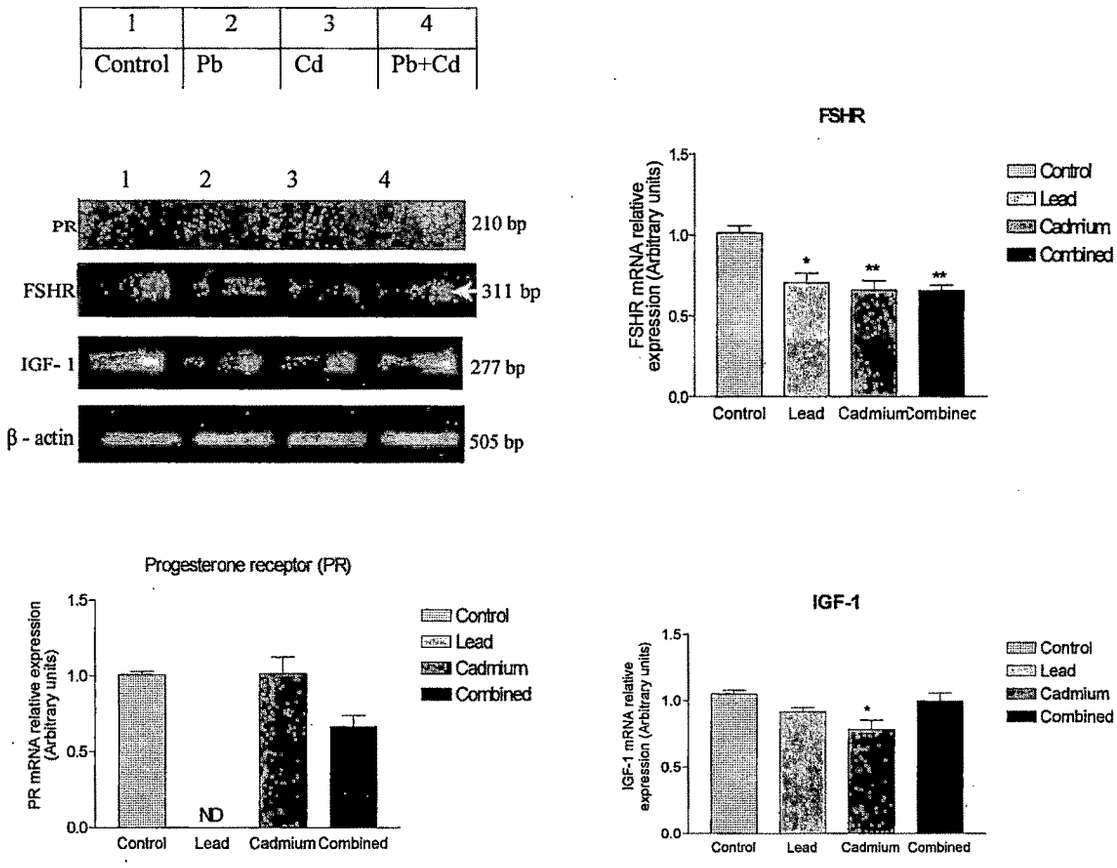






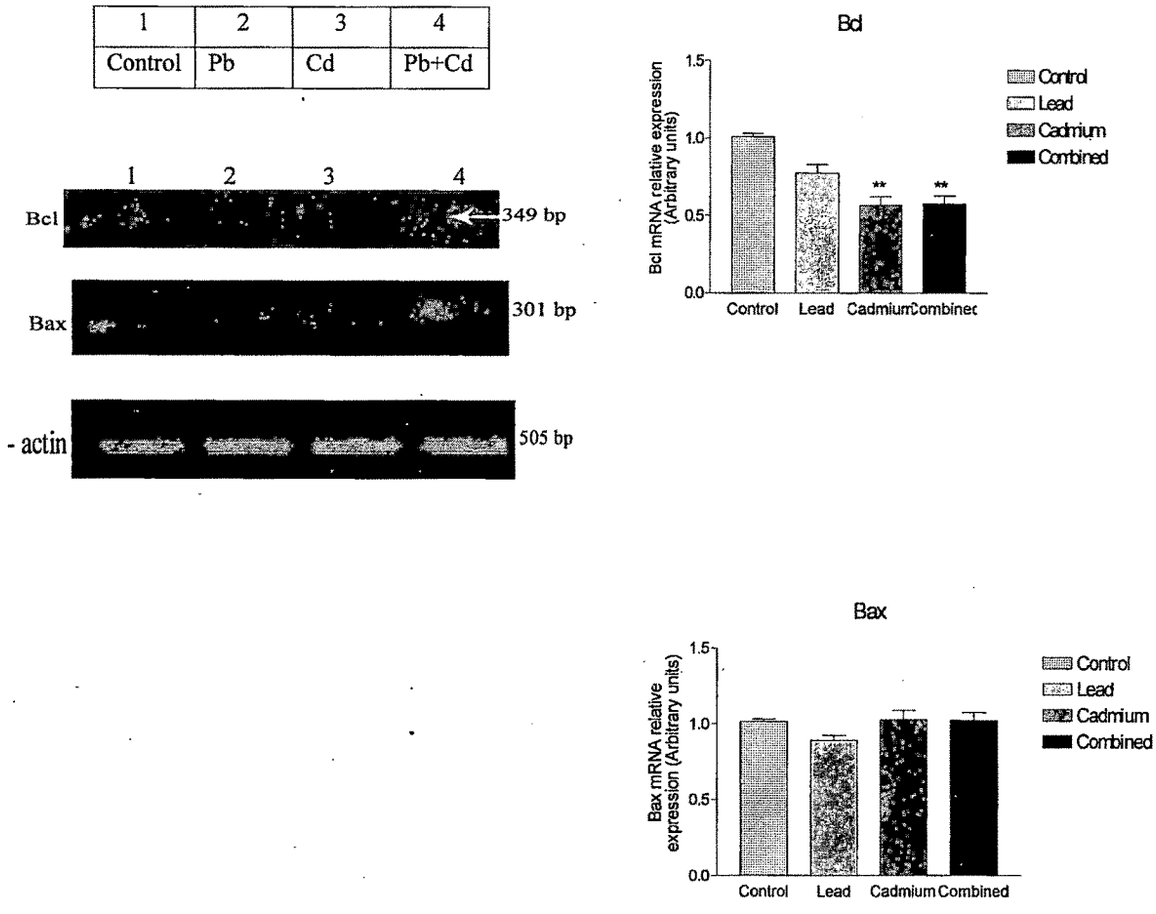
The data are presented as mean ± SEM of 3 independent observations.
*p<0.05, **p<0.01 vs. control; # p<0.05; ## p<0.01vs. lead

Figure 18B: Effect of *"in vitro"* exposure to lead and cadmium on mRNA expression of progesterone receptor, FSH receptor, IGF-1, and β-actin (internal control) in luteinized granulosa cells after 12 h exposure to metals alone and in combination.(ND=not detected)



The data are presented as mean ± SEM of 3 independent observations.
*p<0.05, **p<0.01 vs. control

Figure 18C. Effect of *"in vitro"* exposure to lead and cadmium on mRNA expression of Bcl and Bax genes in luteinized granulosa cells after 12 h exposure to metals alone and in combination



The data are presented as mean ± SEM of 3 independent observations.
**p<0.01 vs. control

5.4 Discussion

In the present study a series of experiments were performed using the ovulation model in immature Charles Foster rats to examine reproductive toxicity of lead and cadmium alone and in combination. In *"in vivo"* experiments following the experimental strategy as discussed earlier, no differences were found in indices of body and reproductive weights between the metal treated and control groups of animals that were primed with gonadotropins. PMSG is known to induce follicular development that leads to estradiol (E2) secretion, and as a result, forced ovulation occurs in immature females. Results from *"in vivo"exposure* studies indicate that inhibition of ovulation as observed in the metal exposed animals that were earlier primed with PMSG-hCG administration, is probably due to the interference of lead and cadmium in follicular development. We also observed a significant decrease in serum estradiol levels in all the metal treated groups as compared to the control animals. Considering these findings, the suppressed ovulation observed in our present study in metal exposed groups may have been due to the disruption of ovarian steroidogenesis and/or follicular growth induced by lead and cadmium. 17β-HSD is the marker enzyme for steroidogenesis in granulosa cells (Ghersevich *et al.,* 1994). Hence, we evaluated the 17β-HSD activity in the granulosa cells after *in vivo* metal exposure to gonadotropins primed immature rats. Our *in vivo* results showed that metal treatment significantly suppressed the steroidogenic enzyme activity in granulosa cells. It is also shown that binding of metals directly to the amino acids present on the active site of the enzyme also may be one of the mechanisms behind decreased steroidogenic enzyme activity as discussed in earlier chapters.

Metals alone and in combination exhibited inhibitory effect on progesterone secretion along with estradiol secretion following *"in vivo"* exposure. Thus, we could show the negative effects of lead and cadmium in general and cadmium in particular to decrease the number of ovulated ova in the present experimental model. The concomitant decrease of estradiol, progesterone secretion suggested interference with the first steps of

steroidogenesis (StAR, P450scc activity). Because progesterone synthesis was affected, it is likely that the in vivo metal exposure during pre-pubertal period has the potential to interfere with cholesterol transport to the inner mitochondrial membrane in pre-ovulatory granulosa cells.

Earlier studies carried out by Smida *et al.*, 2004 showed that low (0.6-3μM) and high (5 μM) concentrations of Cd^{2+} have a dual action in the granulosa cells wherein low concentrations activate, and high concentrations inhibit expression of the P450scc gene. In cultured human placental trophoblast cells, cadmium at concentrations between (5-20) μM inhibited expression of the steroidogenic genes, including LDL-R (Henson and Anderson 2000; Jolibois *et al.*, 1999). In the present study, pre-ovulatory granulosa cells obtained from gonadotropin primed immature female rats were incubated for 6hr and 12hr with concentrations of 50 μM and 200 μM of metals alone and in combination to evaluate the susceptibility of luteinized granulosa cells to undergo apoptosis after metal exposure. The dose most commonly used in several in *vitro* experiments reported till date ranges from 1μM-20 μM for both lead and cadmium in isolation (Smida *et al.*, 2004). Although it is quite difficult to compare the doses used in the *in vitro* experiments to that of lead and cadmium levels measured in the blood of humans exposed to these metals, health effects associated with both lead and cadmium exposure have been correlated to blood levels from 3 to 5 μM for chronic exposure and up to 20 μM for acute exposure.

Studies have also shown that the balance between estradiol and progesterone can be a good predictor of whether a follicle will proceed to maturity or to undergo atresia (Jolly *et al.*, 1997; Rosales-Torres *et al.*, 2000; Huet *et al.*, 1997). We, therefore, measured the concentration of estradiol and progesterone in the conditioned media and found that the level of estradiol and progesterone were significantly lowered mainly in the cells exposed for 12h & 200 μM concentration. However, the levels of estradiol and progesterone were also affected in cultured luteinized granulosa cells exposed

to 50 μM for 12h but the percentage decrease was lesser as compared to the cells exposed for 12h and 200 μM concentrations.

To test whether these changes in steroid secretion are caused by effects on cell viability and apoptosis, we evaluated LDH and caspase-3 activity. Both LDH and caspase-3 activity showed significant alterations in luteinized granulosa cells exposed for 12h to 200 μM concentration of metals in isolation and in combination whereas the activities of both LDH and caspase-3 were not affected in luteinized granulosa cells exposed for 6hr exposure period. In addition to this, we also evaluated the % viability of luteinized granulosa cells using trypan blue exclusion staining method after 6 h and 12 h exposure time for 50 μM and 200 μM concentrations of metals in isolation and in combination. We observed that % viability was significantly affected in luteinized granulosa cells exposed for 12 h with 200 μM concentrations of each metal in isolation and in combination. From these findings, it clearly suggest that an imbalance between estradiol and progesterone level as observed in the present study is involved in the initiation of granulosa cell death possibly by apoptosis (as evident from the caspase-3 activity). The present data also explains the phenomenon of follicular atresia that we had shown particularly in cadmium exposed group in our pubertal exposure studies which is discussed in the earlier chapter.

The concomitant decrease of estradiol, progesterone secretion suggested action on the first steps of steroidogenesis that involves StAR, 17β-HSD, Cytochrome P450aromatase (CYP19) after *in vitro* exposure to lead and cadmium and is in line with earlier findings (Zhang *et al.*, 2008; Paksy *et al.*, 1992). So we analyzed m-RNA expression of StAR, 17β-HSD and CYP19 in cultured luteinized granulosa cells after being exposed to lead and cadmium for 12hr at 200 μM concentrations of metals. The present study demonstrates that mRNA expression of CYP19, StAR, and 17β-HSD were significantly decreased in the metal exposed groups. This may be caused by specific alteration of the transcription process. Apart from this, other possible mechanism is the direct inhibitory effect of these metals. It maybe mediated

by blocking the ferroxy radical involved in all three steps of P450 aromatase action via iron interaction (Watanabe, 2001; Ahmed, 1997). It may also be mediated by the formation of a complex with the cysteine residues, because lead and cadmium has a strong affinity for sulfhydryl groups (Goering, 1993).

Numerous factors inhibit granulosa cell apoptosis and thereby maintain the viability of ovarian follicles (Hsueh et al., 1994; Martimbeau and Tilly, 1997). These anti-apoptotic factors represent a diverse group of biological regulators, including peptide hormones, growth factors, cytokines, and steroids (Hsu and Hsueh, 1997). We therefore investigated the key regulatory genes (FSHR, PR, IGF-1 and Bax) that serve as signals for growth, proliferation and death respectively. The expression of IGF-1 mRNA in pre-ovulatory granulosa cells decreased in cadmium and combined metal exposed group. On the other hand, we did not observed any significant change in the expression pattern of PR gene in metal treated groups. However, the present study demonstrated significant inhibition in the mRNA expression of FSHR in all the metal exposed cells wherein maximal inhibition was found in cadmium treated group. Up-regulation of Bax gene was demonstrated in cadmium treated cells indicating the increased sensitivity of granulosa cells to undergo apoptosis after metal exposure for 12 h, whereas lead and combined exposure groups did not demonstrate any significant increase in mRNA expression of bax gene. Studies were also carried out to evaluate the extent of DNA damage by staining of DNA using the COMET assay following *"in vitro"* exposure to metals. Visual scoring of DNA damage was analyzed based on the photomicrographs of the comet obtained following *"in vitro"* exposure to metals and it revealed maximal damage in cadmium treated group followed by combined exposure and lead exposed group exhibited the minimal damage.

Toxic metals act as a catalyst in the generation of reactive oxygen species and the increase in free radicals can attack the lipid molecules causing lipid peroxidation and alterations in antioxidant status of the cells (Yiin and Lin, 1995; Shafiq-ur-Rehman, 1984; Stohs et al., 2001; El-Maraghy et al., 2001).

Luteinized granulosa cells exposed to cadmium alone and combined metal exposure showed higher lipid peroxidation as compared to cells of the control group. Present study exhibited suppression of all key antioxidant enzymes following *"in vivo"* exposure as well as *"in vitro"* exposure pinpointing the strong endocrine disrupting potential of lead and cadmium in gonadotropin primed immature rats. Our biochemical data showing the induction of oxidative stress in luteinized granulosa cells exposed to metals alone and in combination for 12h period is also supported by DCF fluorescence staining that is again a sensitive measure for the intracellular ROS production as demonstrated in cultured preovulatory rat follicular cells (Tsai-Turton *et al.*, 2007).We demonstrate here a significant increase in the fluorescence intensity measured in the metal treated luteinized granulosa cells as compared to the cells from control group.

In conclusion, our results indicate that (i) the suppressed ovulation observed in our present study is due to the disruption of ovarian steroidogenesis and possibly also involving disruption of follicular growth induced by lead and cadmium (ii) increased apoptosis sensitivity, as evident from the induction of caspase activity and altered mRNA expression of Bax – Bcl-2 genes in metal treated groups, is related to the imbalance between estradiol and progesterone levels in the conditioned media and increased oxidative stress. Taken together, the findings of the present study indicates that luteinized granulosa cells sensitivity to undergo cell death after metal exposure is closely linked to alterations in the expression levels of survival factors such as FSH-R, IGF-1 , key steroidogenic pathway candidates such as StAR, Aromatase.

In a wider perspective, decoding of multiple pathways of endocrine disruptions by lead and cadmium, both alone and in combination, in luteinized granulosa cells as demonstrated in the present study would be of great importance in the screening of possible targets associated with a wide range of reproductive disorders caused by a disturbance in the apoptosis/proliferation equilibrium. In conclusion, the present work has

evaluated the underlying biochemical and molecular mechanism of cellular toxicity by metals such as lead and cadmium and extends its plausible role to female infertility.

5.5 Summary

Studies were carried out in luteinized granulosa cells as the model system to study the underlying biochemical and molecular mechanism of cellular toxicity by lead and cadmium. Following *in vivo* exposure to lead and cadmium in isolation and in combination from PND 21 to PND 25 (pre-pubertal exposure), the immature female rats were primed with exogenous gonadotropins for induction of ovulation. The suppressed ovulation observed in metal treated groups in the present study was accompanied by altered steroidogenic enzyme activity, sex steroid production, and increased oxidative stress in the luteinized granulosa cells. Granulosa cells isolated from gonadotropin primed immature rats were exposed to lead and cadmium in isolation and in combination at a final concentration of 50μM and 200μM of metals for 6 h and 12 h. Results from *in vitro* exposure of luteinized granulosa cells to Pb and Cd exposure clearly demonstrated altered gonadotropin receptor (FSHR) mRNA expression, suppressed key steroidogenic enzyme activity (17β-hydroxy steroid dehydrogenase) and decreased estradiol and progesterone production in all the experimental groups. Increased oxidative stress in luteinized granulosa cells is very well evident from the suppressed antioxidant system and DCF staining in the present study. The present study thereby provided insight into modulation of some important events regulating apoptosis in ovarian luteinizing granulosa cells after metal exposure.