Chapter –V

EFFECT OF LEAD AND CADMIUM AT THE CELLULAR LEVEL USING GRANULOSA CELL AS THE MODEL

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Introduction

Ovary is composed of two major cells, namely theca and granulosa cells. These cells control the ovarian functions, by the production of various steroid hormones. Studies on the steroidogenic capacities of isolated granulosa cells and theca cells led to the proposal of two-cell gonadotropin theory, which stated that theca cell produces C_{19} steroids which then diffuse into granulosa cell to produce estradiol (Hseuch et al., 1984). This difference in steroid production is due to differential expression of key steroidogenic enzymes. 3β Hydroxy steroid dehydrogenase (3β HSD) is mainly expressed in theca cells while 17β Hydroxy steroid dehydrogenase (17β HSD) in the granulosa cells. Granulosa cell produces estradiol under the influence of LH and FSH. It is known that in the adult female, granulosa cells have both FSH and LH receptors, which are maximally expressed during early and late proestrous stage of ovarian cycle respectively (Hseuch *et al.*, 1984). Interference in their binding could reflect a change in female physiology. Lead is known to affect the binding of Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH) in ovarian homogenate (Wiebe *et al.*, 1988) and also decrease the gonadal steroid levels (Paksy *et al.*, 2001). Similarly, cadmium is also known to decrease the serum gonadotropins (Paksy *et al.*, 1989) and gonadal steroids (Piasek and Laskey, 1999). In earlier chapter, it is demonstrated that ovarian steroidogenesis is inhibited by lead and cadmium, both in isolation and in combination at a dose of 0.05 mg/kg. body wt daily for 15 days.

In the present study, an attempt has been made to study the effects of lead and cadmium either in isolation or in combination at cellular level (Granulosa cells). Parameters evaluated includes gonadotropin binding and steroidogenic activity of granulosa cells (both *in vivo* and *in vitro*). To the best of our knowledge, there is no study on simultaneous effect of lead and cadmium on hormone binding on granulosa cells.

Experimental design

Experiments were performed in two ways: "*in vivo* treatment" and "*in vitro* treatment".

***"In vivo"* treatment**

Animals were divided into four groups containing 12 animals each: Group I animals served as control and Group II, III and IV animals received lead acetate, cadmium acetate and lead, cadmium in combination respectively at a dose of 0.05 mg/kg body wt daily for 15 days. Six animals from each group were sacrificed at early proestrous stage for FSH binding and at late proestrous stage for LH binding. Ovaries were then removed and granulosa cells were isolated by the modified method of Campbell (1979). Viability of cells was checked by Trypan blue staining. Purity of granulosa cells was evaluated by assessing 3β hydroxy steroid dehydrogenase (3 βHSDH) activity (pure preparation of cells should have undetectable 3βHSD activity). 17 β Hydroxy steroid dehydrogenase (17 βHSDH) was estimated in granulosa cells by the method of Shivanandappa and Venkatesh (1997). Rat LH and Rat FSH was iodinated by the Chloramine-T method (Greenwood et al., 1963). Radio-receptor assay was performed according to the method of Guerrero et al (1993).

"*In vitro*" treatment

Control adult female rats were sacrificed at early proestrous stage for FSH binding and at late proestrous stage for LH binding respectively. Ovaries were removed, granulosa cells were isolated and purity was checked as mentioned above. These cells were exposed to lead acetate and cadmium acetate alone or in combination for 1 h. Concentration was selected as per the metal kinetic concentration with dose of 0.05 mg/kg.b.wt/day for 15 days. Viability of the cells was checked after 1 hour of exposure. To study the protective effect of zinc, cells were pretreated with zinc (concentration same as lead and cadmium for 30 min), before the metal exposure. Following the incubations, radio-receptor assay for all groups were performed according to the method of Guerrero et al (1993).

Another set of " *in vitro*" experiments were performed to study the direct effect of lead and cadmium alone and in combination, with and without zinc and sulfhydryl protectants on 17 βHSDH activity of granulosa cells. Four group of 4 x 10^5 cells/ml were taken and treated with sodium acetate, lead acetate, cadmium acetate and lead and cadmium in combination for 1 hour. In addition, other set of cells were pre-incubated with zinc or sulfhydryl group protectant (DTT or GSH) for 30 min before metal exposure and assayed for 17 β hydroxy steroid Dehydrogenase (17 βHSDH) by the method of Shivanandappa and Venkatesh (1997).

Results

There were no significant differences in body weight in treated animals compared to control animals.

Figures 1 and 2 shows the gel filtration profile of iodinated r-LH and r-FSH. The fraction 6 (10μl) showed highest counts for both hormones, which is the iodinated fraction of the gonadotropins. 100 μg of unlabelled r-LH and 1000 ng of

unlabelled r-FSH showed better displacement and these concentrations were used as unlabelled hormones in non-specific binding tubes. Concentration curve shows that 30,000 (0.043 nM) counts of iodinated r-LH and 20,000 (0.027 nM) counts of iodinated r-FSH gave maximum binding and hence, in all further experiments, this concentration of labelled hormones were used (Figures 3 and 4).

Table 1 shows the effect of lead and cadmium either alone or in combination on granulosa cell number. Cells of lead treated group showed maximum decrease in granulosa cell number compared to control.

Effects of lead and cadmium on binding of I^{125}-rFSH and I^{125}-rLH to granulosa cells are shown in Figures 5. Cells of cadmium treated animals showed a maximum decrease in binding of both gonadotropins compared to combined treatment; while lead treated cells showed minimum inhibition in binding compared to cells of control group.

Figure 6 demonstrates the effect of lead and cadmium on activity of 17 βHSDH in granulosa cells of ovary. Cells of cadmium treated group showed maximum inhibition (55.5 %, $P< 0.001$) while cells of combined treated group showed intermediate results with 49% inhibition ($P<0.001$). Cells of lead treated group showed minimal inhibition (43.2%, $P < 0.001$) as compared to cells of control group.

" *In vitro*" treatment for one hour did not cause a change in viability in cells exposed to metals. Figures 7 and 8 shows the binding of I^{125}-rLH and I^{125}-rFSH to granulosa cells " *in vitro*". Cadmium treated cells showed a maximum reduction in

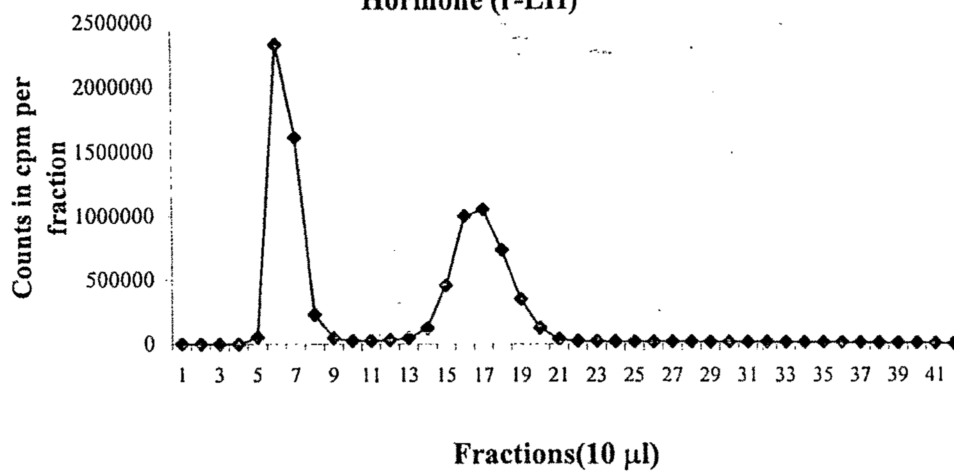


Figure 1: Gel filtration profile of iodinated Luteinising Hormone (r-LH)
Counts in cpm per fraction
2500000
2000000
1500000
1000000
500000
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41
Fractions(10 μl)

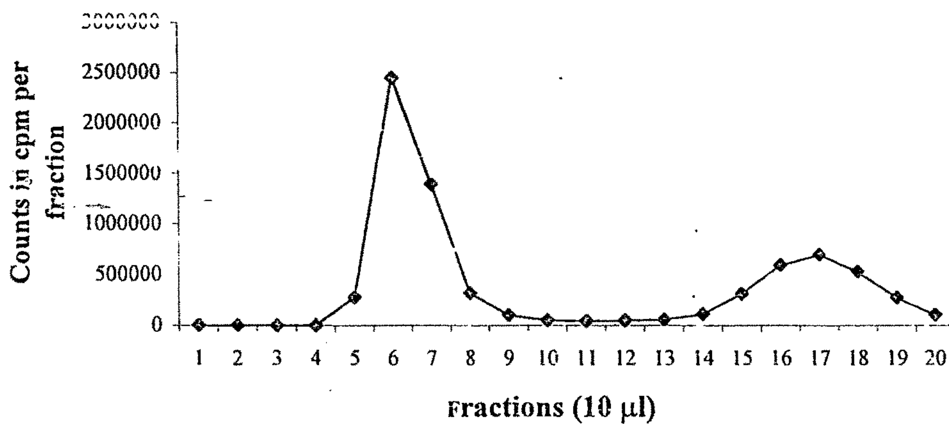


Figure 2: Gel filtration profile of iodinated Follicle Stimulating hormone(r-FSH)
Counts in cpm per fraction
2500000
2000000
1500000
1000000
500000
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Fractions (10 μl)

Fig 3 : Concentration curve for rLH on rat granulosa cells

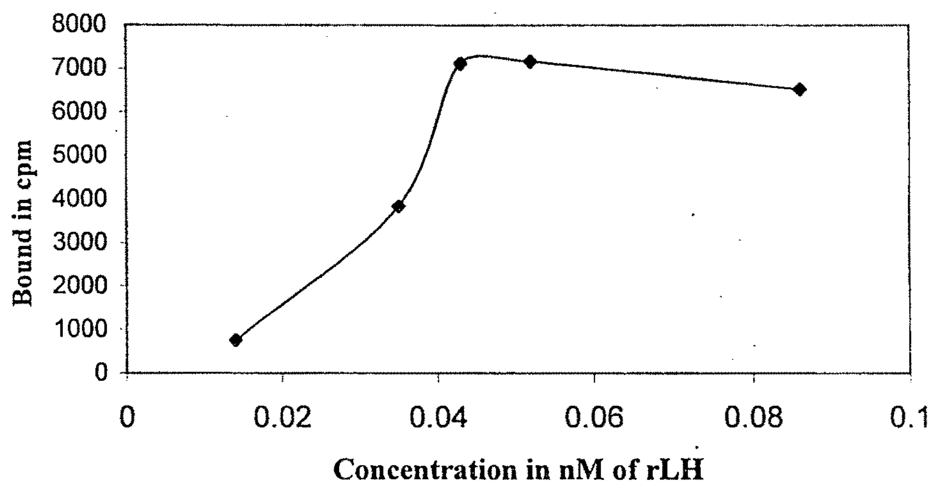


Fig 4 : Concentration curve for rFSH on rat granulosa cells

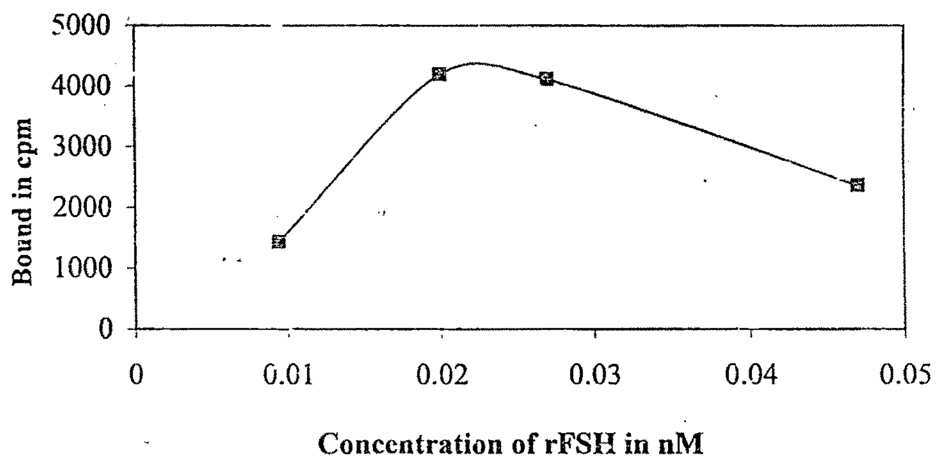
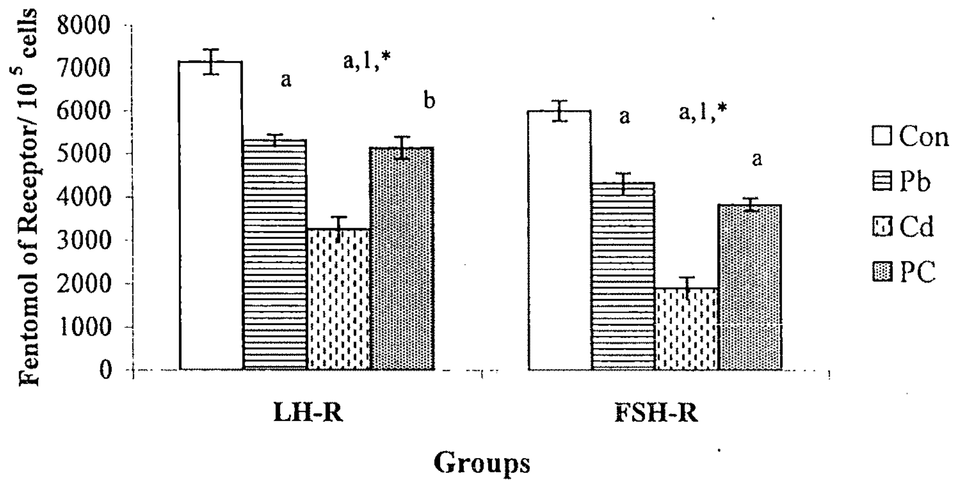


Table 1 : Effect of lead and cadmium either alone or in combination on granulosa cell count.

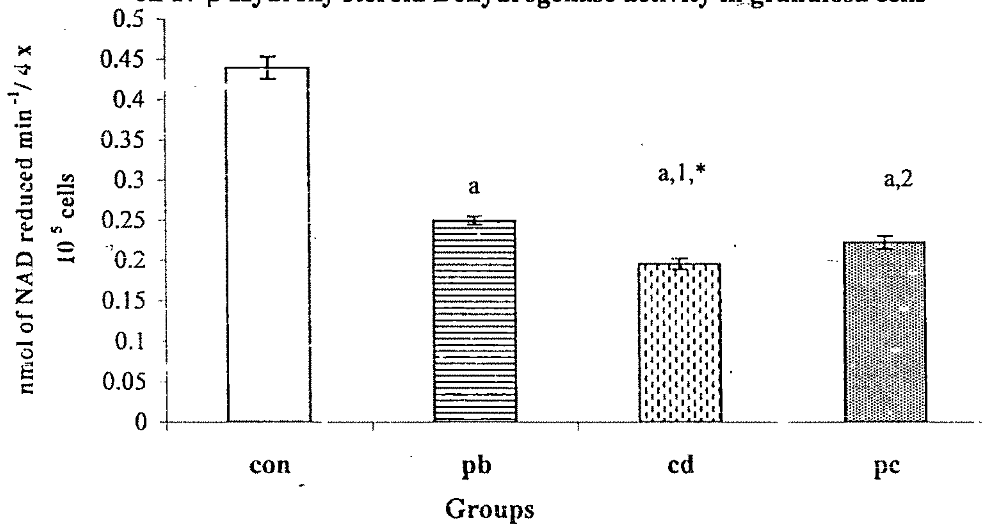
GROUPS	Number of Cells x 10^6 per mg ovary
Control (NaAc)	0.028 ± 0.045
Lead	0.017 ± 0.035 [b]
Cadmium	0.228 ±0.041 [c]
Lead +Cadmium	0.241 ± 0.045

N=5-6. The values are Mean ± SEM.

b $p<0.01$, c $p<0.05$ compared to control



n=6. The values are mean ±SEM.
a p<0.001, b p<0.01 compared to control
1 p<0.001 compared to lead group
* P<0.001 compared to combined treated group



n=6. The values are Mean + SEM.
a P<0.001 compared to control group
1 P<0.001, 2 P<0.05 compared to lead group.
* P<0.05 compared to combined treated group

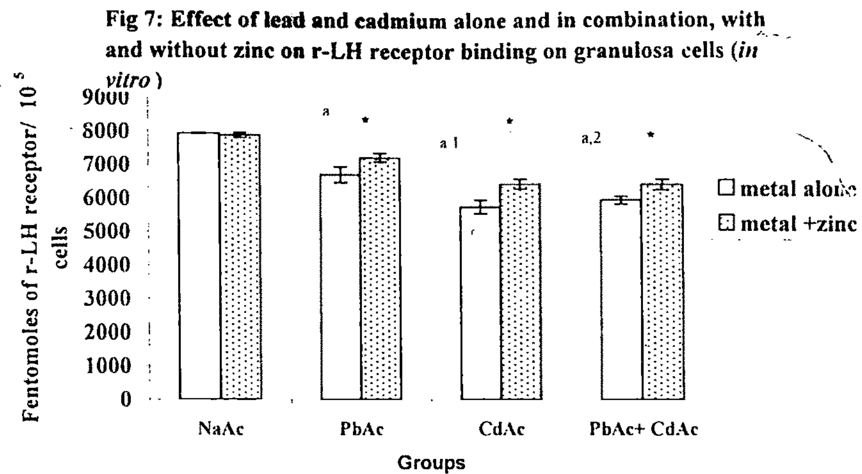


Fig 7: Effect of lead and cadmium alone and in combination, with and without zinc on r-LH receptor binding on granulosa cells (*in vitro*)

N=4. The values are mean +SEM.
a p<0.001 compared to control
1 p<0.001, 2 P<0.01 compared to lead group
* P<0.05 compared to protectant with corresponding metal treatment

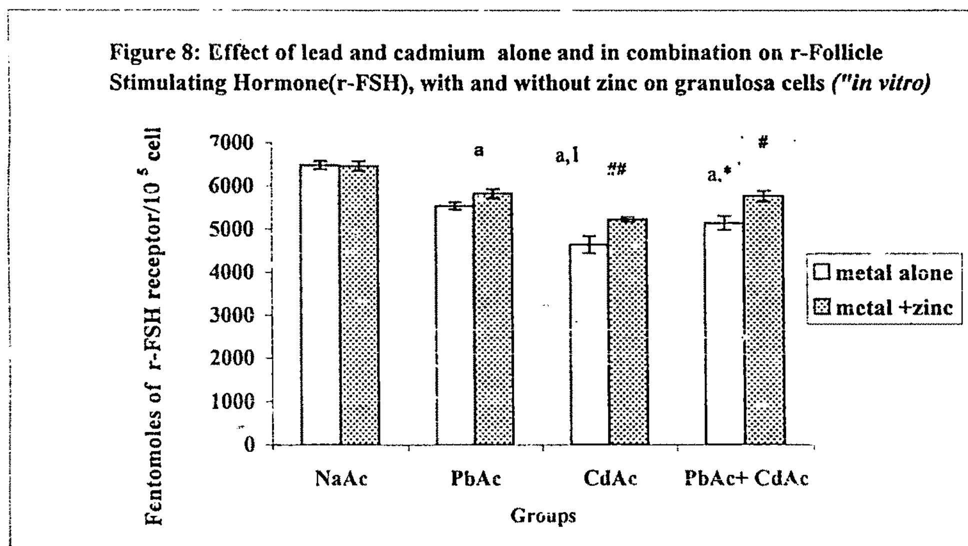
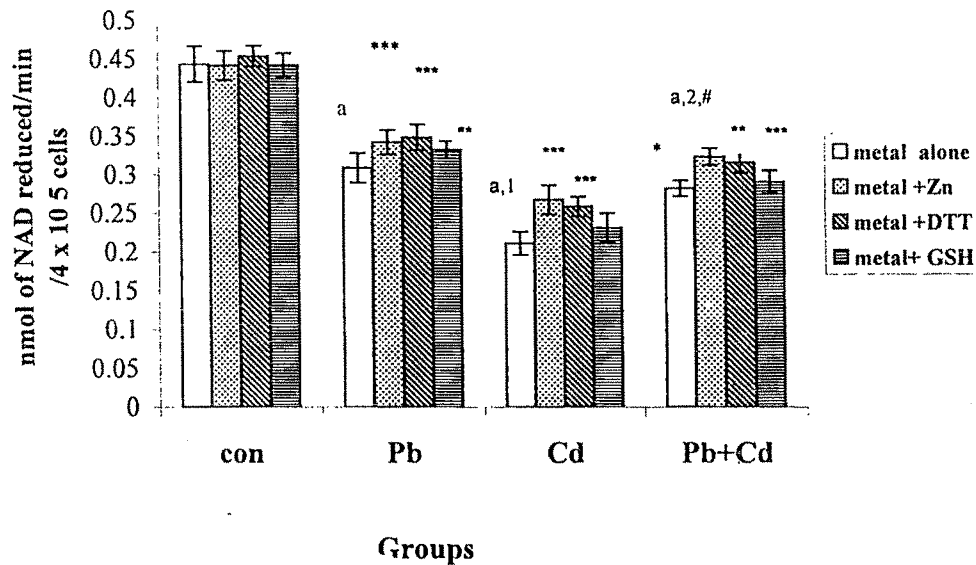


Figure 8: Effect of lead and cadmium alone and in combination on r-Follicle Stimulating Hormone(r-FSH), with and without zinc on granulosa cells *("in vitro)*

N=4. The values are mean ±SEM.
a p<0.001 compared to control
1 p<0.001 compared to lead group
* P<0.01 compared to cadmium group
$P < 0.01$, # $P < 0.05$ compared to protectant with corresponding metal treatment

Fig 9: Effect of both lead and cadmium exposure in isolation and in combination, both with and without Zinc and -SH protectants preexposure, on 17 βHydroxy Steroid dehydrogenase activity on



N=4. The values are mean ± SEM

a -p<0.001 compared to control

1 p<0.001, 2 <0.01 compaɪed to lead group

# p<0.01 compared tc cadmium group

*** p<0.001, **p <0.01, * p<0.05 compared to corresponding metal

binding of both gonadotropins compared with combined treatment; while lead treated cells showed minimum inhibition. Pre-exposure to Zn along with metal toxicants ameliorates the binding of LH and FSH to its receptors in all metal treated groups. Maximum amelioration is observed in cadmium treated group when compared with other groups.

Effect of lead and cadmium alone and in combination on 17 βHSDH activities "*in vitro*" is shown in figure 9. The maximum inhibition of 17 βHSDH was seen in cadmium treated cells (52.5%, $P < 0.001$) while combined treatment showed intermediate effect (37%, $P < 0.001$) and lead exposed cells showed minimum inhibition in activity (31%, $P < 0.001$) as compared to control. Pretreatment with Zn, -SH group protectants like reduced glutathione (GSH) and dithiothretol (DTT) are able to ameliorate the activity of enzyme compared with only metal treated cells. Zn is able to increase the enzyme activity in all metal treated groups. DTT and GSH are two sulfhydryl reagents which are able to ameliorate enzyme activity in all treated groups. DTT pretreatment causes a greater increase in activity of enzyme as compared to GSH pretreatment.

Discussion

To the best of our knowledge, this is the first study to demonstrate the effects of both lead and cadmium in combination on gonadotropin binding and their effect on steroidogenesis. Ovary is the vital organ for steroid hormone synthesis that controls the physiology of female origin. The gonadotropins- LH& FSH bind to their receptors present on the granulosa cell surface and mediates the activation of secondary messenger system to produce gonadal steroids. Effect of the reproductive toxicants like lead and cadmium on female physiology is quite difficult to intercept due to

differences in sensitivity of metals (Bull et al., 1979) which could affect the hormone receptor kinetics, enzyme activities and hormone secretion.

The sensitivity also depends on concentration of lead or cadmium present in the tissue and the effects due to the time of exposure to the toxicants (Petruz et al., 1979). Distribution pattern of heavy metals in ovary in the present study shows that intermediate concentration of the toxicants in combined treated animals compared to the control as discussed in chapter 3. Paksy et al. (1990, 2001) reported that lead and cadmium gets concentrated in the granulosa cells of the ovary. Our results also demonstrate that cadmium treatment caused maximum decrease in binding of peptide hormones (both LH and FSH) as compared to the combined treatment; while lead treatment showed minimum inhibition as compared to control. The lesser accumulation of lead and cadmium in the ovaries of combined treated animals can be the reason for intermediate values in binding as discussed in chapter 3. Wiebe et al. (1988a) also reported a decrease in binding of LH and FSH in ovarian homogenate. The decreased binding obtained could be due to the interaction of lead and cadmium with amino acids like cysteine residues of the receptor and might interfere with stability of hormone- receptor complex. It is known that these heavy metals alters membrane integrity (Amorusa et al., 1987; Flora and Sheth, 2000). This alteration in membrane structure could affect receptor structure and thereby contribute to decreased binding of gonadotropins.

LH/ FSH receptors are present on the granulosa cells and are associated with microvilli (actin rich filaments) area at the cell circumference. The receptor molecule has been suggested to be associated with contractile cytoskeletal elements playing a role in receptor mobility (Amsterdam and Rotmensch, 1987). Lead and cadmium is

known to cause disassembly of cytoplasmic microtubule complex (Their et al., 2003; Perino and Chou, 1989) and causing a change in receptor mobility.

Decreased binding of gonadotropins to its receptors also contributes to a decrease in activity of 17 βHSDH. 17 βHSDH is a marker enzyme for granulosa cells (Gherevisch et al., 1994a) and belongs to the class of short chain dehydrogenases (Person et al., 1993). Our study demonstrates that metal salts causes a significant decrease in the activity of the enzyme. This decrease in enzyme activity could be attributed to decreased gonadotropin binding as well as a direct interaction of metal with the amino acids present on the active site of the enzyme or to –SH groups of cysteine residue present at the NAD binding domain (Persson et al., 1991), which further leads to decreased production of steroid hormones (reported in chapter 3). Paksy and his workers (1992) reported that cadmium considerably enters the granulosa cells and causes a dose dependent decrease in estradiol production, which could probably be through the interference of cadmium with the activity of aromatase system. Inhibition of hormone synthesis may partly involve the ability of cadmium and lead to compete with calcium (Wiemann et al., 1999; Sun and Suszkiw, 1995). Veldhius et al. (1984) reported that calcium deficient incubation impedes the capacity of 8-bromo cAMP to stimulate pregnenolone synthesis from endogenous substrate, thereby indicating a plausible role of calcium in steroidogenesis. Calcium-calmodulin systems are known to play a fundamental role in follicular steroidogenesis (Tsang and Carnegie, 1983). Moreover, it has been demonstrated that cadmium and lead can bind with calcium binding site's of calmodulin and disorderly regulate calcium - calmodulin dependent functions (Kern et al., 2000; Akjiyama et al., 1990).

We carried out "*in vitro*" study to understand whether the inhibitory effect seen in the present study was a direct or secondary effect (through changes

gonadotropin binding) of the metals. Gonadotropin binding was decreased in all "*in vitro*" metal exposed groups. The decrease obtained on "*in vitro*" exposure was less than "*in vivo*" exposure. Possible reasons for immediate decrease obtained in "in vitro" experiment could be due to co-precipitation of metal ions and receptor. Pre-treatment of the cells with zinc showed better binding than cells exposed to lead and cadmium either alone or in combination supporting the fact that there exists a competition between the metals (Flora et al., 1982; Waakles & Priorer, 1985) either by reducing or antagonizing the binding sites for lead (Gunn et al., 1968) and cadmium uptake (Shaikh, 1995).

Our results demonstrated that " *in vitro*" metal exposure causes a significant inhibition in the activity of 17βHSD. Immediate decrease in activity obtained on exposure to lead, cadmium or both can be explained by two mechanisms. The first mechanism is the binding of metal/s directly to the amino acids present on the active site of the enzyme. Other mechanism is the decrease in hormone-receptor binding as obtained in our LH/FSH "*in vitro*" binding studies that can result in decrease in cAMP levels leading to decreased steroidogenesis. It is known that cadmium can substitute calcium ions and activate calmodulin dependent phosphodiesterase causing a decrease in cAMP (Nimura et al.,1987).

It is well documented that DTT, GSH contain –SH groups and have ability to maintain protein thiol groups in the reduced state (Jewel et al., 1982). In the present study, GSH and DTT protected the cells against the inhibitory effects of heavy metals. Exogenous GSH is a non-permeable thiol compound and DTT is a permeable -SH group protectant (Graf & Sies, 1984). The different degrees of protection shown by these two sulfhydryl reagents might be due to their differences in their affinities for lead and cadmium. DTT binds with metals with higher affinity than GSH. Our results

also show that DTT is a better protectant than GSH. Zn pretreatment was also able to show the protective effect on 17 βHSDH activity. Zn has been reported to antagonize cellular cadmium transport and can compete for binding to sulfhydryl ligands (Graf & Sies, 1984). It has been reported that Zn can either increase (10-200 μM) or decrease (>40 μM) FSH induced progesterone biosynthesis (Paksy et al., 1996). Heng and coworkers (1993) further reported that an intradermal Zn injection causes an elevation of cAMP levels in the cytosol of epidermal cells of nude mice. The concentration of zinc used in present study increases steroidogenesis possibly by increasing cAMP levels and thereby ameliorating 17 βHSDH activity in granulosa cells.

In all parameters studied, inhibition obtained in " *in vitro*" study was less than that in " *in vivo*" experiments, suggesting both primary and secondary effects of metals. Intermediate results obtained on combined exposure could be related to accumulation of metals as compared to individual metal treated groups. Competition among the metals to bind to a single amino acid site of enzymes and the receptor could also contribute to intermediate results in all parameters studied.

This study thus demonstrated the adverse effect on peptide hormone binding by the simultaneous exposure of toxicants, which is correlated with decrease in steroid dehydrogenase activity and leading to decreased production of the steroid hormone (estradiol). Since hormones are the key regulators of female physiology, their suppression by metal ions is expected to result in altered reproductive function.

Summary

Lead (Pb) and Cadmium (Cd), two known reproductive toxicants known to accumulate in the granulosa cells of ovary. In this chapter, the influence of lead and cadmium on gonadotropin binding on granulosa cells has been studied. Female Charles foster rats were treated with sodium acetate (Control), Lead acetate, Cadmium acetate either alone or in combination at a dose 0.05 mg/kg b. wt for 15 days daily. Animals were killed at proestrous stage and granulosa cells were isolated from ovaries. Binding of I^{125}- Luteinizing Hormone (I^{125}- LH) and I^{125}- Follicle Stimulating Hormone (I^{125}- FSH) and 17 β Hydroxy Steroid Dehydrogenase (17 β HSDH) was measured (both "*in vivo*" and " *in vitro*"). Decreased gonadotropins (LH, FSH), binding along with a decreased activity of 17 βHSDH activity was observed, in both " *In vivo and* "*In vitro*" exposure. In all parameters, decrease obtained was less in "*in vitro*" exposed cells compared to "*in vivo*" cells. Pretreatment with SH groups protectants (Glutathione [GSH], Dithiothretol [DTT]) and zinc caused an amelioration in enzyme activity whereas zinc pretreatment showed an increase in gonadotropin binding in metal exposed cells. These results suggest that both lead and cadmium can cause a reduction in gonadotropin binding, which significantly alters steroid production "in *vitro*" and "*in vivo*". It is clear that lead and cadmium accumulation, causes both primary and secondary effects and thus affecting granulosa cell function leading to reproductive dysfunction.