# Chapter –VI

## MECHANISM OF LEAD AND CADMIUM EITHER ALONE OR IN COMBINATION.

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### Introduction

Free radicals are generated continuously in aerobic cells (Clance et al., 1979). Cellular respiration involves the reduction of molecular oxygen to water in the electron transport chain. This reduction involves the production of reactive oxygen species ($O^2$ $H_2O_2$ „ $OH^-$), which can damage cellular systems by attacking the lipid components of the membrane causing lipid peroxidation. Cells are protected by defense systems that remove these free radicals to prevent oxidative damage. Superoxide dismutase (SOD) scavenge the superoxide ions, by rapid dismutation and forms hydrogen peroxide, which are again catalyzed by catalase (CAT) to form water and oxygen. It has been suggested that cells may maintain a homeostatic level of free radicals and lipid peroxides to provide a suitable oxidative environment for controlling metabolic pathways throughout the ovarian cycle (Nelson et al., 1994). A six fold rise in superoxide anion ($O^{2-}$) were seen during proestrous stage along with decreased superoxide dismutase (SOD) activity (Duran Reyes et al., 1988). Whereas, the activities of anti-oxidative enzymes like superoxide dismutase (SOD) and catalase (CAT) are known to increase during the process of ovulation, particularly in late proestrous to estrous stage (Sato et al., 1992; Miyazaki et al., 1991). Several reports have indicated that activities of SOD and CAT increases along with a decrease in glutathione levels during luteal phase of ovarian cycle particularly in mid corpus luteal stage (Sevanian et al., 1988; Vega et al., 1994). To best of our knowledge, there are no reports suggesting antioxidant status of granulosa cells. During pregnancy, feto-placental unit being in the environment of the materno-fetal circulation is

very much susceptible to the danger of reactive oxygen species-induced oxidative damage. As the pregnancy elapse the lipoperoxides products increases and at the end of the pregnancy, the antioxidants exceed peroxidative phenomena (Qanungo et al., 1999). Sugino et al. (1993) reported that SOD and CAT plays an important role in regulating luteal function during pregnancy, by reducing the lipid peroxidation levels.

Both lead and cadmium belongs to the class of redox inactive metals and the proposed mechanism for these metals for inducing toxicity, is through their role in the generation of reactive oxygen species and their effect on the antioxidant system (Christie and Costa, 1998; Adler et al., 1993). 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). This results in depletion of glutathione, cell's major sulfahydryl reserve.

The membrane structure is maintained by two components namely cholesterol and phospholipid . ROS generated by the lead and cadmium alters membrane composition Cook et al., 1987; Chen et al., 2002) which then can affect receptors, activity of enzymes (Thevnod and Friedmann, 1999; Satyavathi and Prabhakara Rao, 2000) and membrane fluidity (Mishra et al., 1989; Nivsarkar et al., 1998).

It is clear from earlier chapters that lead and cadmium causes decreased gonadotropin binding, leading to decreased steroid production. Moreover, there are several reports on the mechanism of lead and cadmium in relation to oxidative stress and changes in membrane characteristics in other tissues, but little is known about mechanism behind the toxic effects of metal salts on the granulosa cells of the ovary and placenta. Membrane integrity is important for ovarian function as the gonadotropin receptors are

present on the cell membrane of granulosa cells in non pregnant stage. During pregnancy, placenta is the major organ involved in hormone synthesis and hormone action. Hence, the aim of the present study was to understand the mechanism behind the effects caused by lead and cadmium either alone or in combination on granulosa cells of ovary in non pregnant stage as well as placenta of pregnant rat.

**Experimental Design**

Experiments were carried out in two different stages:

1. ***Mechanism of action of lead and cadmium either alone or in combination in granulosa cells of ovary***

   a)" *In vivo" Study*

   There were four group of animals in the study: control (sodium acetate), lead acetate, cadmium acetate and lead acetate and cadmium acetate in combination. The animals were treated intraperitoneally at a dose of 0.05 mg/kg. body wt./ day for 15 days daily. The combined treated group was treated with same dose by taking half concentration of each metal. After 15 days of metal treatment, the animals in late diestrous stage of estrous cycle received 75 I.U. of hCG to increase the yield of cells by super ovulation and they were sacrificed after 24 hours at proestrous stage of estrous cycle. Ovaries were removed and granulosa cells were isolated (Campbell, 1979). Sonicated granulosa cells were used to assess oxidative parameters- reduced glutathione (Beulter and Gelbart, 1985), Lipid peroxidation (Ohkawa et al., 1979) and antioxidant enzymes – catalase (Hugo , 1987) and superoxide dis mutase (Kakkar et al., 1984). Granulosa cell membrane was prepared from 4 x $10^5$ cells (Riordan and Ling, 1979) and membrane parameters were studied. Total cholesterol (Leffler and McDougald, 1963), Total phospholipids (Folch, 1957), Fluorescence

polarization (Shinitzky and Barenholz, 1978) and $Na^+K^+$ATPase activity (Floreani et al. 1981) were analyzed.

b) *"In vitro" Study*

Adult female virgin rats were treated with 75 I.U. hCG at late diestrous stage and were sacrificed at proestrous stage and granulosa cells were isolated from the ovaries and exposed to metals for one hour, at a concentration of metals that reach the ovary when exposed to 0.05mg/kg body wt. /day for 15 days. These cells were then sonicated at 5 cycles per minute and were then assayed for all above parameters.

2. ***Mechanism of action of lead and cadmium either alone or in combination in placenta during pregnancy.***

Adult virgin female rats (200-220 g) were divided into four groups each consisting of 8 animals. First group received subcutaneous injections of sodium acetate (control), second and third groups were treated with lead acetate and cadmium acetate respectively. The group four animals were treated with combined dose of lead acetate and cadmium acetate at a dose of 0.05 mg/kg. body wt/ day. On the fifth day of treatment, those animals that were in late diestrous - early proestrous stage of estrus cycle were allowed to mate with males. Presence of thick cornified smear, along with sperm on the next day confirmed the mating and was considered as a day 1 of pregnancy. Exposure to metals continued till the end of gestation.

Animals were sacrificed on gestation day 18 or 19 and placenta was removed. Placental homogenate was used for determination of reduced glutathione (Beutlar and Gelbart, 1985), lipid peroxidation (Ohkawa et al., 1979), catalase activity (Hugo, 1987) and superoxide dismutase activity (Kakkar et al., 1984). Placental membrane was prepared (Parkkila et al., 1997) and assessed for fluidity (Shinitzky and

Barenholz (1978), $Na^+$ $K^+$ ATPase activity (Floreani et al., 1981), Schiff' base formation (Tappel et al., 1975) and inorganic peroxide levels (Bent and Bergmeyer, 1965).

**Results**

1. *Mechanism of action of lead and cadmium either alone or in combination in granulosa cells of ovary*

Table 1 represents the effects of lead and cadmium either alone or in combination on reduced glutathione, TBARS and antioxidant enzymes both "*in vivo*" and " *in vitro*". Cells of cadmium treated animals showed a maximum decrease in reduced glutathione content and elevation in lipid peroxidation compared to cells of control animals. Cells of animals which were exposed to both lead and cadmium showed an intermediate change in both the parameters while cells of lead treated animals showed minimum change. Significant inhibition in the activity of superoxide dismutase and marked elevation in catalase activity was seen in cells of cadmium exposed animals. The activity of superoxide dismutase was inhibited to similar extent as in cadmium exposed group while the activity of catalase was intermediate between individually exposed groups of lead and cadmium.

In case of "*in vitro*" study, significant decrease in glutathione content has been demonstrated in cadmium and combined treated group, whereas elevation in lipid peroxidation was seen only in cadmium treated groups (Table 1).

Figure 1 shows the effect of lead, cadmium in isolation and combination on membrane $Na^+$ $K^+$ ATPase activity with "*in vivo*" and "*in vitro*" exposure. In both the studies, granulosa cell membrane of cadmium treated animals showed a maximum inhibition in the activity of $Na^+$ $K^+$ ATPase while animals exposed to lead showed a

**Table 1: Effect of lead and cadmium either alone or in combination on GSH, TBARS levels and on antioxidant enzymes in ovarian granulosa cells ("*in vivo*" and "*in vitro*")**

| Parameters | GSH (μg GSH/3x $10^5$ cells) | TBARS (nmol of MDA formed/4 x $10^5$ cells) | Catalase (μmoles of $H_2O_2$ decomposed /sec/ 4 x $10^5$ cells) | Superoxide dismutase (I.U/ 3 x $10^5$ cells) |
|---|---|---|---|---|
| **In vivo** | | | | |
| **Control** | 33±1.47 | 1.24 ± 0.073 | 3.8 ± 0.23 | 0.32 ±0.02 |
| **Lead** | 24 ±1.056 [a] | 1.73 ±0.22[b] | 6.8 ± 0.158.[a] | 0.26 ±0.01[b] |
| **Cadmium** | 16.32 ±0.702[a,2,*] | 3.07 ± 0.143 [a,2,*] | 10.18 ± 0.53[a,1,**] | 0.17 ± 0.015 [a,1,**] |
| **Lead +Cadmium** | 20.8 ±1.38 [a] | 2.59 ±0.17 [a] | 8.5 ± 0.088[a] | 0.11 ± 0.03 [a,1] |
| **In vitro** | | | | |
| **Control** | 33 .4 ±1.67 | 1 ± 0.032 | 3.3 ± 0.34 | 0.35 ± 0.02 |
| **Lead** | 29.8 ±0.77 | 1.052 ± 0.03 | 3.33 ± 0.25 | 0.36 ± 0.02 |
| **Cadmium** | 25.2 ±1.05 [b] | 1.362 ± 0.02 [b,2,**] | 3.27 ± 0.29 | 0.35 ± 0.04 |
| **Lead +Cadmium** | 27.8 ±1.23 [c] | 1.12 ± 0.08 | 3.21 ± 0.35 | 0.36 ± 0.025 |

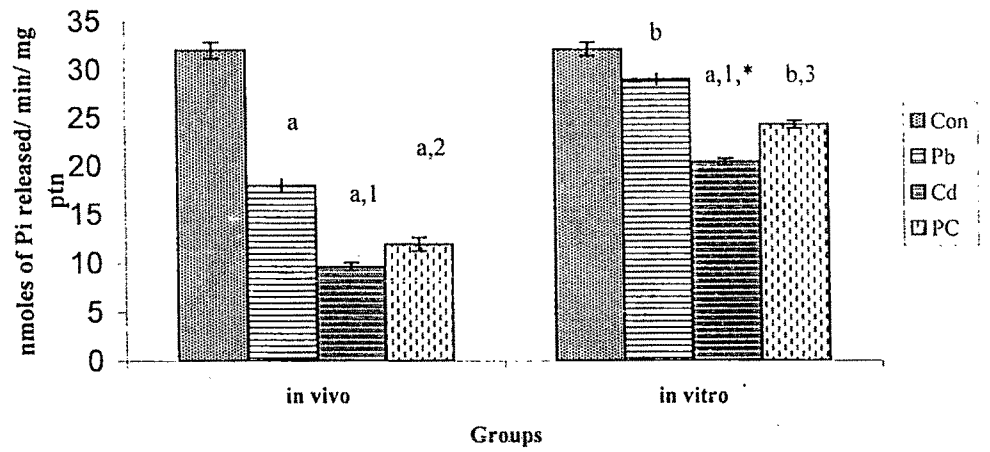
N=4-5. The values are Mean ± SEM.

a- p<0.001, b-p<0.01, c- P <0.05 compared to control

1- p<0.001, 2-P<0.01 compared to lead group

** P<0.01, *P<0.05 compared to lead+ cadmium group

**Figure 1: Effect of lead and cadmium either alone or in combination on Na+K + ATPase activity in granulosa cell membrane**

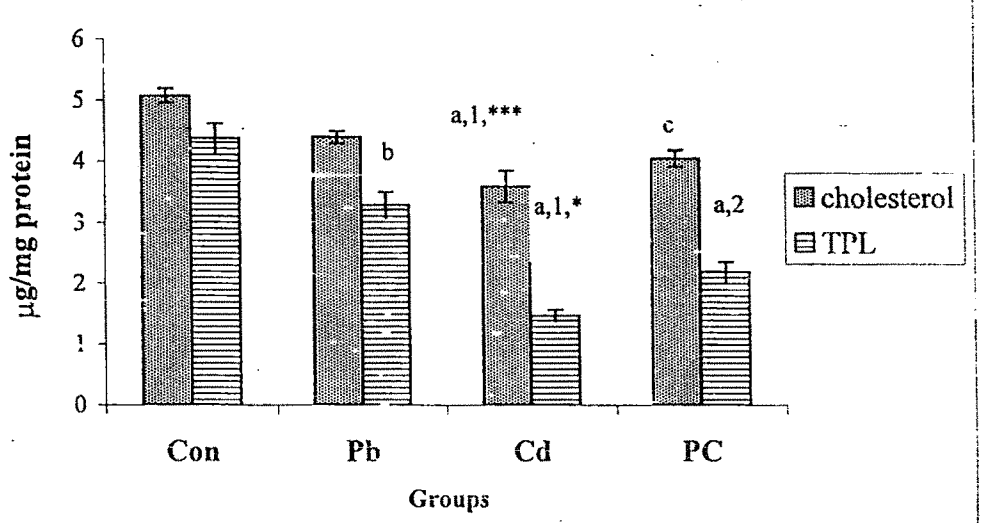



N=5-6.The values are mean ±SEM.

a p<0.001, b p< 0.05 compared to control

1 P<0.001, 2- p<0.01, 3 p<0.05 compared to lead group.

**Figure 2: Effect of lead and cadmium either alone or in combination on membrane parameters of granulosa cell**




N=5-6.The values are mean ±SEM.

a p<0.001, b p<0.01, c p<0.05 compared to control

1 p<0.001, 2 p< 0.01compared to lead group.

*** p<0.001, * p<0.05 compared to lead+cadmium group.

minimal inhibition compared to control. However, inhibition obtained *in vitro* study was less compared to "*in vivo*" study in all metal exposed membrane (Figure 1).

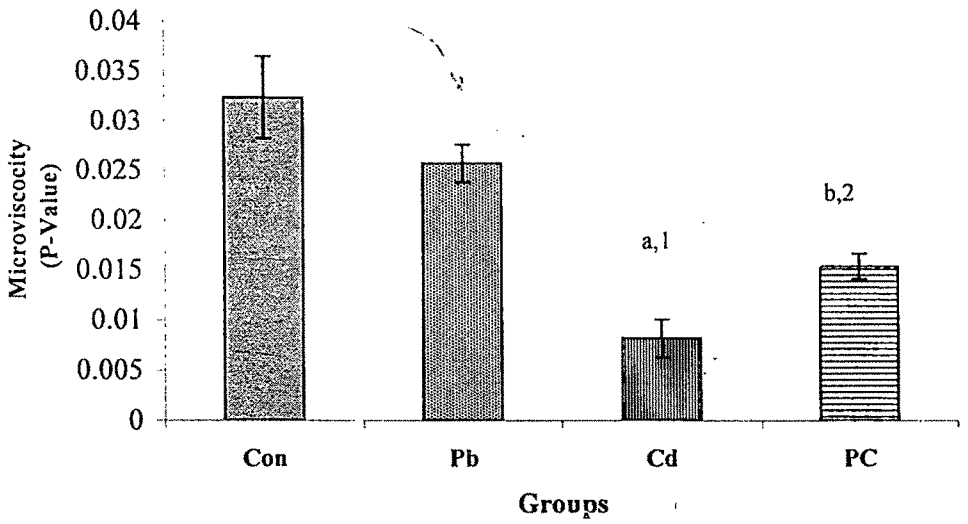
Both cholesterol and phospholipid content was maximally decreased in the granulosa cell membrane of cadmium exposed animals, while both the components showed a minimum decrease in lead treated groups (Figure 2). Membrane fluidity was increased maximally in membrane of cadmium exposed animals compared to control. The membrane of granulosa cells of lead treated animals showed minimal change in both fluidity and cholesterol to total phospholipid ratios. Combined treated animals showed an intermediate change in fluidity (Figure 3). "*In vitro*" exposure of lead and cadmium did not cause any change in membrane parameters-cholesterol, total phospholipid content and fluidity (Table 2).

2. ***Mechanism of action of lead and cadmium either alone or in combination in placenta.***

Reduced glutathione content and lipid peroxidation was significantly altered in all metal treated groups. Placental reduced glutathione content was decreased significantly, along with maximum elevation in lipid peroxidation levels in cadmium exposed animals while animals exposed to lead showed minimal change in both the parameters compared to control. Data in this table shows an inhibition of superoxide dismutase activity, with an increase in catalase activity in all groups treated with metal. Alteration again being maximum in cadmium treated and intermediate in combined animals (Table 3).

Placenta shows an increase in fluorescence polarization ratio, suggesting a decrease in fluidity in all metal treated groups. Placenta of cadmium exposed animals

**Fig 3: Effect of lead and cadmium either alone or in combination on granulosa cell membrane fluidity**




n=4-5. the values are mean ± SEM.
a $p<0.01$, b $p<0.01$ compared to control group
1 $p<0.001$ compared to lead group

**Table 2: Effect of lead and cadmium either alone or in combination on membrane parameters of granulosa cell membrane (*in vitro study*)**

| Groups | Cholesterol (μg/mg protein) | Total phospholipid (μg/mg protein) | P-value (Microvisocity) |
|---|---|---|---|
| **Control** | 5.11 ± 0.0.4 | 4.4 ± 0.147 | 0.032±0.014 |
| **Lead** | 5.1 ± 0.08 | 4.3 ± 0.13 | 0.031 ± 0.025 |
| **Cadmium** | 5.03 ± 0.06 | 4.3 ±0.11 | 0.29 ±0.031 |
| **Lead + Cadmium** | 5 ± 0.07 | 4.4 ±0.11 | 0.32 ± 0.03 |

**Table 3: Effect of lead and cadmium either alone or in combination on GSH, TBARS levels and on antioxidant enzymes in placental homogenate.**

| Groups | GSH (μg GSH/mg protein) | TBARS (nmol of MDA formed/mg protein) | Catalase (μmoles of $H_2O_2$ decomposed /sec/ mg protein) | Superoxide dismutase (I.U/mg protein) |
|---|---|---|---|---|
| **Control** | 14.19 ± 0.67 | 7.2 ± 0.58 | 33.3 ± 5.4 | 0.293 ±0.022 |
| **Lead** | 10.4 ± 0.76 [a] | 10.1 ± 0.37 [c] | 56.8 ±4.4 [a] | 0.24 ±0.013 [c] |
| **Cadmium** | 6.15 ± 0.59 [a,2] | 16.5 ±0.61 [a,1,**] | 65.2 ±3.8 [a,1] | 0.137 ± 0.008 [a,1] |
| **Lead + Cadmium** | 7.2 ± 0.29 [a,2] | 13.6 ± 0.92 [a,1] | 60.78± 4.1 [a,1] | 0.207 ± 0.008 [b,**] |

N=4. The values are mean ± SEM.

a $p<0.001$, b $p<0.01$, c $p<0.05$ compared to control

1 $p<0.001$, 2 $p<0.01$ compared to lead group

** $p<0.01$ compared to lead +cadmium group.

**Table 4: Effect of lead and cadmium either alone or in combination on placental membrane parameters.**

| Groups | Schiff's Base (Fluorescence intensity) | Inorganic peroxide level (μg/mg protein) | $Na^+ K^+$ ATPase activity (μmol/min/mg protein) | Microvisocity (P-Value) |
|---|---|---|---|---|
| Control | 43.2 ± 2.65 | 11.31 ± 0.366 | 0.069 ±0.004 | 0.103 ± 0.01 |
| Lead | 59.7 ± 4 [b] | 12.7 ± 0.62 | 0.051 ± 0.004 [b] | 0.25 ± 0.017 [a] |
| Cadmium | 113.91 ± 7.9 [a,1,**] | 17.51 ± 0.91 [a,2,*] | 0.035 ±0.002 [a,1] | 0.36± 0.016 [a,1] |
| Lead + Cadmium | 93.9 ± 4.4 [a,1] | 14.77 ± 0.75 [c,3] | 0.039 ±0.002 [a,2] | 0.34 ±0.006 [a,1] |

N=4. The values are mean ±SEM.

a $p<0.001$, b $p<0.01$, c $p<0.05$ compared to control group

1 $p<0.001$,2 $p<0.01$, 3 $p<0.05$ compared to lead group.

** $p<0.01$ compared to lead +cadmium group.

**Table 5: Effect of lead and cadmium either alone or in combination on placental membrane characteristics**

| Groups | Cholesterol (μg/mg protein) | Total phospholipid (μg/mg protein) |
|---|---|---|
| Control | 774.7 ±18.42 | 761 ± 22.5 |
| Lead | 561.2 ±22.1 a | 573 ±5.2 [a] |
| Cadmium | 377.1 ±10.9 [a,1,*] | 382.8 ±10.47 [a,1] |
| Lead + Cadmium | 442.1 ±19.4 [a,1] | 421.1 ± 14.1 [a,1] |

N=4. The values are mean ±SEM.

a $p<0.001$ compared to control group

1 $p<0.001$ compared to lead group

* $p<0.05$ compared to lead +cadmium group.

showed maximum increase in Schiff's base, inorganic peroxide levels along with a significant decrease in Na+ K+ ATPase activity compared to control. Lead exposed animals shows minimum change while animals that received both lead and cadmium showed intermediate values in all parameters (Table 4). Similar type of change has been demonstrated on placental membrane cholesterol and total phospholipid content (Table 5).

**Discussion**

Toxic metals acts as a catalyst in generation of reactive oxygen species (Monteiro et al., 1991; El-Maraghy et al., 2001). 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). Granulosa cells and placenta exposed to cadmium either alone or in combination showed higher lipid peroxidation as compared to control. Several reports have accounted for such an elevation in lipid peroxidation on cadmium exposure (Wang et al., 2002; Stohs et al., 2001). Antioxidant enzymes of the cells plays a role in maintaining the homeostasis of free oxygen radicals in the steroidogenic tissues (Quanango et al., 1999). Superoxide dismutase requires Zn as factor for its activity. Present study exhibits inhibition of SOD activity in both granulosa cells and placental tissue, suggesting displacement of zinc by lead and cadmium from its active site. Similar kind of inhibition was obtained by several other workers (Hussain et al., 1987; Kofod et al., 1991; Adler et al., 1993; Ariza et al., 1998). Sugino et al. (2000) reported that decreased SOD expression and the increase in lipid peroxides in placenta could be involved in causing spontaneous abortion. Our present study also exhibits significant

increase in catalase activity in all metal treated groups. This increase in activity could be due to the early displacement of the transition metals from the active site of SOD by the lead and cadmium, thereby causing no inhibition in catalase activity. Increase in catalase activity could be due to higher production of ROS by these heavy metals. Similar kind of increase in catalase activity has been reported in different tissues on metal exposure (Kostic et al., 1993; Mousa et al., 2002). A recent study by Strehlow (2003) reported that estrogen upregulates SOD expression, without any change in CAT activity. It is clear from earlier chapters that estrogen production is decreased on lead and cadmium exposure. Thereby, it could be plausible that decreased estrogen observed upon metal exposure, could also contribute to decreased SOD expression.

An important protein involved in interaction of toxic elements is glutathione, which accounts for more than 90% of the total non protein sulfur (Meister, 1988). Present study shows a decrease in GSH content in both placental and granulosa cells. This could be attributed to the interaction of these divalent elements with sulfhydryl groups of GSH as suggested by several workers (Christie and Costa, 1984; Monterio et al., 1985; Nigam et al., 1999; Stohs et al., 2001). Various studies have indicated that increase in lipid peroxidation also causes an alteration in glutathione status, thus indicating direct correlation between the two components (Struzynska et al., 2002; Stohs et al., 2001).

Free radicals produced by these heavy metals are known to cause change in membrane structure, which then can affect receptors, activity of enzymes and membrane fluidity (Mishra et al., 1989; Nivsarkar et al., 1998). Gonadotropins mediate their action through binding with their receptors present on the membrane of

granulosa cells and placenta. Thereby, any alteration in the change in membrane structure would reflect a change in gonadotropin binding, leading to change in hormone action. Membrane integrity are maintained by two major components - cholesterol and phospholipids. Our studies have shown that both lead and cadmium decreases the total cholesterol and total phospholipid content. Present study indicates an increase in fluidity of granulosa cell membrane of all treated groups, which is partly correlated with decreased cholesterol content. Placental membrane showed a decrease in fluidity, along with significant change in cholesterol. Cadmium treated group shows maximum increase in fluidity whereas combined metal treated group shows intermediate value. Amurosa et al. (1987) has reported an increase in fluidity of erythrocyte membrane on exposure to lead. This could be due to interaction of lead with negatively charged groups of phospholipid as suggested by Fiorini et al. (1982). Heavy metals are known to cause kinks in acyl chains and prevent them from packing tightly (Hannan et al. 1989), Decrease in fluidity of placental membrane could be due to an increase in lipid peroxides that results in increased amounts of inorganic peroxides and Schiff's base, that could disrupt the membrane bilayer (Sevanian et al., 1988), which in turn could increase the membrane microviscocity. Increase in these peroxides, Schiff's base was maximum in placental membrane of cadmium treated compared to lead treated animals. Similar kind of increase in these catabolites on metal exposure was observed by many workers (Ribarov et al., 1983; Berndt and Ansari, 1990).

Most membrane bound enzymes require membrane lipids in a "fluid state" for its activity. Several authors have reported that free radicals can alter membrane proteins

including $Na^+K^+$ ATPase (Dinis et al., 1993; Kaplan et al., 1995) The present study shows cadmium treated animals showed maximum inhibition in the activity compared to other metal treated groups, in both the granulosa and placental membrane. Alteration in the activity of ATPase can be correlated to the lipid peroxidation and a change in fluidity. In addition, several other reports have also indicated that lead inhibits K+ dephosphorylation step of $Na^+K^+$ATPase (Siegal and Fopgt, 1977; Bertoni and Sprenkle, 1988) and cadmium interacts with enzyme-phosphate complex (Ahmaddsahib et al., 1989), thus inhibiting the enzyme activity directly. It was also reported that cadmium was showing greater inhibition of $Na^+K^+$ATPase activity compared to lead (Carfagna et al., 1996) in a "*in vitro*" simultaneous exposure.

" *In vitro*" experiments were performed with the concentration of lead and cadmium reaching the tissues after *in vivo* exposure for 15 days on granulosa cell membrane. Present study demonstrates no change in membrane parameters and antioxidant parameters. Only cadmium treated membrane showed a slight elevation in lipid peroxidation and decrease in reduced glutathione level. This could be due to very low concentration of lead and cadmium and exposure for only 1 hour. There is a significant inhibition of enzyme by the metal exposure. However, percent inhibition obtained in *"in vitro"* study was less than that in the "*in vivo* " study, thus indicating both direct interaction of metal with enzyme as well as an altered membrane structure by the metals, responsible for the change in "*in vivo*" treated groups of animals. Metals when used in combination gave intermediate results, which could be due to

competition between the two metals. This competition could be due to similarity in electronic affinities.

Present study indicates that both lead and cadmium alters membrane components, along with antioxidant enzyme status, causing an increase in free radicals in both granulosa cell and placental membrane. Thereby, it is clear that increase free radicals along with membrane damage in both tissues, causes all the effects caused by metals as discussed in earlier chapters.

**Summary**

Adult female rats were treated with lead acetate, cadmium acetate or both (i.p) for 15 days daily at a dose of 0.05 mg/kg. body wt during non-pregnant stage and at a dose of 0.05 mg/kg. body wt (S.C.) from the day of premating till the end of gestation. After the metal treatment, animals were sacrificed, ovarian granulosa cell and placental membrane were prepared. Metal exposure caused a decrease in reduced glutathione content along with elevated lipid peroxidation in all groups. Granulosa cells and placenta of cadmium and combined treated groups showed a maximum increase lipid peroxidation, with decreased glutathione status and decreased SOD and increased catalase activities. Granulosa cell membrane and placenta membrane also showed an alteration in cholesterol, total phospholipid along with alteration in fluidity on metal exposure. Activity of membrane bound $Na^+$ $K^+$ ATPase was inhibited in all metal exposed groups, in both membranes. Combined treated animals showed intermediate effects in both membrane parameters and antioxidant status. "*In vitro*" exposure showed no significant change in antioxidant enzymes and membrane parameters in all metal exposed cells. However, a significant inhibition of Na+ K+ ATPase activity was seen in membrane exposed to metals. Data from the present study indicates that lead and cadmium in isolation and in combination affects membrane components, through free radical generation. Lead and cadmium in combination do not show additive or synergistic effect indicating the competition between them due to similarity in electronic affinities. Toxic metals disturbs membrane integrity via ROS, thereby leading to alteration in receptor binding, steroidogenesis and hormone production which has been observed in earlier chapters.