Chapter 4

To study the effect of cadmium on cell death and expression of steroidogenic and major candidate genes in insulin resistant rat ovarian cells.

4.1 Introduction

Development and function of female reproductive tract depends on coordinated biological processes. Follicular development and ovulation are dependent on proliferation and differentiation of both granulosa and theca cells that undergo steroidogenesis upon stimulation with gonadotropins and intra-ovarian cytokines. Granulosa cells of the ovary are very important components that are involved in the production of sex steroid hormones and a milieu of growth factors involved in interaction with oocyte development. Cellular growth in ovary is controlled by apoptosis of granulosa cells, a phenomenon responsible for follicular atresia. Interference with any of these processes can predispose a women to reproductive dysfunctions ultimately leading to infertility (Diamanti-Kandarakis et al. 2009). It has been estimated that approximately 15% of the population in industrially developed countries are infertile (Homan et al. 2007). Among the various proposed etiological factors for infertility, genetic, immunologic, endocrinology and environmental disorders account for 50% whereas in remaining 50%, the cause remains unknown (idiopathic infertility) (Ford and Schust 2009; Vettriselvi 2012). It is now thought that the idiopathic infertility might be due to life style-environment interaction.

Changes in lifestyle with high carbohydrate diet intake or sedentary habits lead to insulin resistant (IR) condition. It has been reported that approximately 50-75% of women with polycystic ovarian syndrome (PCOS) have some degree of IR. Furthermore, it has been found that women with IR are more likely to have PCOS (Hackbart et al. 2013). Besides the classical target organs for insulin action such as liver, adipose tissue and muscle, the presence of insulin receptors in both stromal and follicular compartments and findings for insulin's ability to stimulate steroidogenesis in ovarian cells *in vitro*, has eventually established ovary as an important target organ for insulin action (Mukherjee and Maitra 2010). Hyperinsulinemia in IR alters the genes, enzymes and proteins that are crucial for the steroidogenic machinery ultimately affecting follicle development and ovulation (Diamanti-Kandarakis et al. 2008). In certain

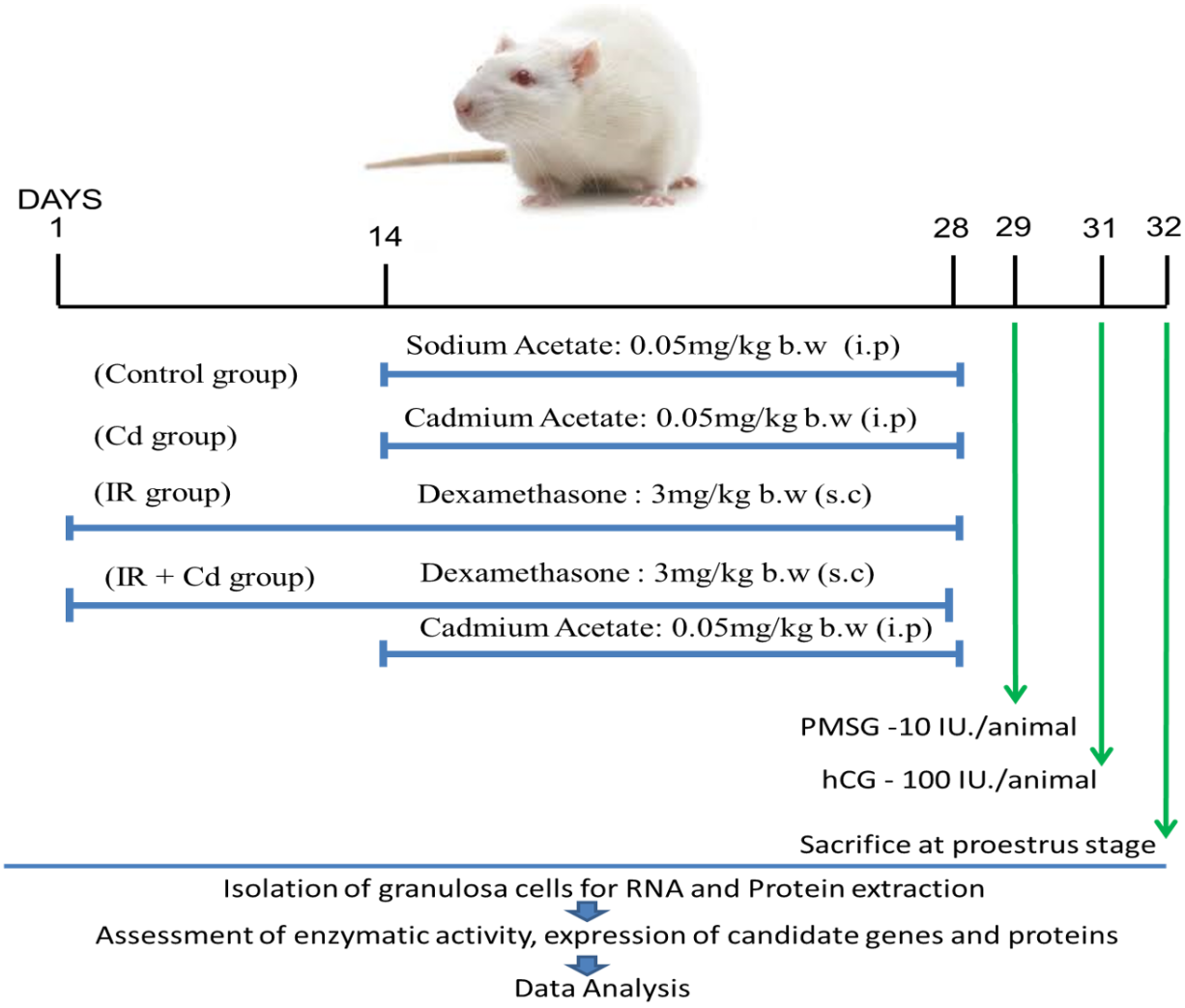
animal models of maternal diabetes, abnormal maternal physiology affects oocytes by showing delayed maturation, smaller size and increased granulosa cell apoptosis and results in adverse embryonic and fetal outcomes, including delayed embryonic development, growth restriction, anatomical defects, and smaller foetuses (Wyman et al. 2008; Jungheim et al. 2010).

Amongst many environmental pollutants, cadmium (Cd) is an endocrine disruptor which is relatively dispersed in the environment mainly because of pollution from a variety of sources, including mining, smelting, fossil fuel combustion, batteries, paints, plastics, tobacco smoke (Kaiping Yang 2006). Because of high rate of soil-to-plant transfer, Cd is a contaminant found in most human foodstuffs, which renders diet a primary source of exposure among non-smoker and non - occupationally exposed population (Soisungwan Satarug 2010). Due to its unknown biological function, low rate of excretion from the body, and long biological half-life, it accumulates over time in blood, kidney, liver, and in the reproductive organs such as placenta, testis, and ovaries (Zhang and Jia 2007). Using the benchmark dose (BMD) -derived urinary Cd threshold, the tolerable weekly intake for Cd was 2.5µg/Kg body weight, which corresponds to 25 µg/day for a person who weighs 70kg (Alexander 2009). The stimulatory or inhibitory effects of Cd on key ovarian steroidogenic enzymes at a sub-clinical dose of 0.05mg/kg body wt. has been very well established in various tissues of rats including granulosa cells of the ovary in our lab (Gupta et al. 1994; Pillai et al. 2002; Priya et al. 2004; Nampoothiri and Gupta 2006). Further, our lab has also demonstrated decrease in expression of StAR in granulosa cells of F1 generation rats that were exposed to Cd during their developmental age (Pillai et al. 2010). Decrease in gene expression of CYP19A1 due to Cd exposure has been established in carp ovarian follicles (Das and Mukherjee 2013). Apart from reproductive disorder, both human and animal studies also suggest an association between Cd exposure and hyperglycemia (Edwards and Prozialeck 2009).

To date studies have mainly focused on investigating the effect of Cd and IR individually on granulosa cells ultimately affecting fertility. It has been observed that Cd and IR are prevalent in today's lifestyle. In light of this, we aim to investigate the effect of Cd and IR in granulosa cells, which could be mimicking present environment. Several animal models for IR such as HFD induced model, transgenic model, models with genetic background and glucocorticoid induced model are available. Amongst all the models, dexamethasone, a potent glucocorticoid

induced model was preferred for the present study as its development was not expensive, short treatment period along with no sophisticated maintenance (Srinivasan and Ramarao 2007). Moreover dexamethasone has been reported to result in increased serum insulin and glucose levels, decrease in insulin-mediated glucose uptake, insulin resistance and anovular conditions in different species (Gao et al. 2007; Qu et al. 2009; Hackbart et al. 2013). Thus in the present study the combined effect of IR and Cd was evaluated on granulosa cell death along with other physiological, biochemical and molecular parameters such as mRNA and protein expression of StAR for cholesterol transport and CYP11A1, 3β-HSD, CYP19A1 and 17β-HSD responsible for steroid synthesis. The study further observed the effect of Cd and IR on mRNA expression of GnRH, CYP19A1 and FSH-β, LH- β located respectively in hypothalamus and pituitary, the organs involved in the feedback regulation of steroidogenesis.

4.2 Experimental Design



4.3 Results

4.3.1 Induction of IR is solely because of dexamethasone

Long term administration of dexamethasone leads to insulin resistance. To delineate the plausible causative agent of insulin resistance, parameters at systemic like FIRI index and serum insulin levels along with insulin receptor protein levels were checked in the experimental groups under study. The groups treated with dexamethasone alone and in combination with Cd, showed significant glucose intolerance as shown in OGTT curve compared to control, whereas Cd alone did not show any change as compared with control. Fasting serum insulin levels and FIRI values were observed to be significantly high ($P < 0.001$) in groups treated with dexamethasone alone and in combination with Cd with respect to control, whereas Cd alone did not show any change. Further, to confirm the status of IR at granulosa cell level, western blot for insulin receptor was performed. We observed significant down regulation of insulin receptor in granulosa cells of dexamethasone alone and co-exposed group as compared to control (Fig. 4.1).

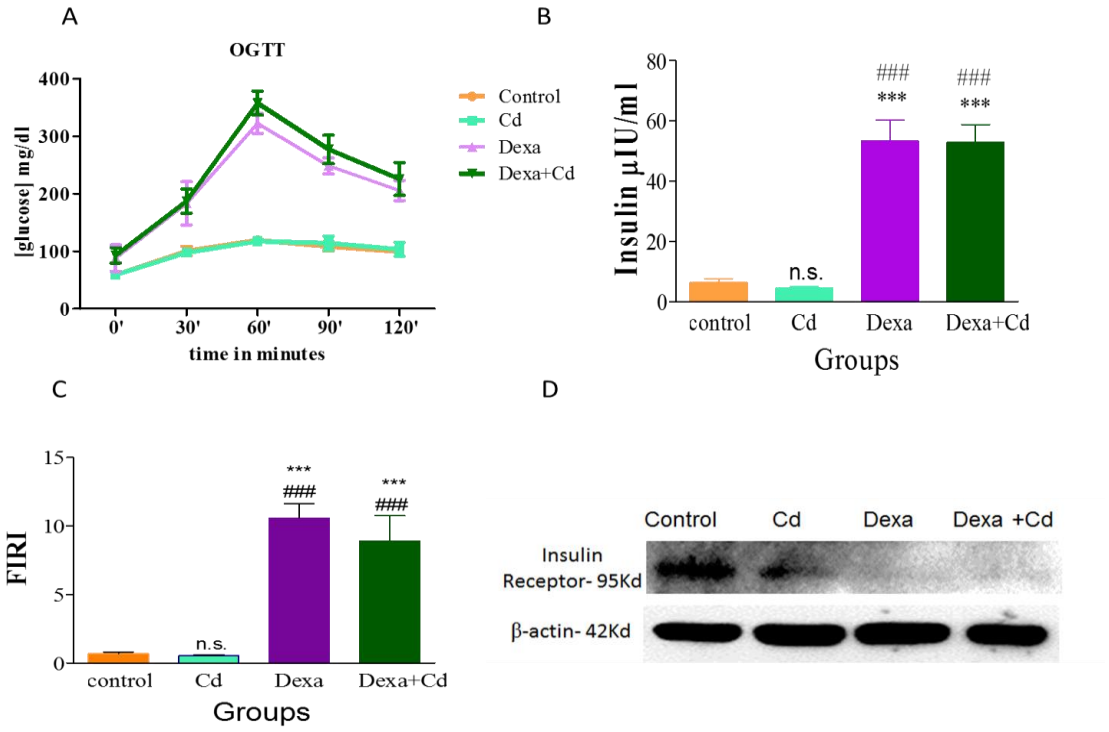


Figure 4. 1: Effect of Cd, Dexamethasone and Dexamethasone + Cd treatment on (A) OGTT profile, (B) serum insulin levels, (C) FIRI and (D) protein expression of insulin receptor in granulosa cell lysate using beta actin as internal control in rats. Serum glucose and insulin levels were measured using GOD-POD and ELISA kits respectively. *** $P < 0.001$ as compared to control, ### $P < 0.001$ as compared to Cd. ns= non-significant. The values are represented as mean ± SEM of three independent observations (n = 6). Dexa=Dexamethasone.

4.3.2 Effect of Cd and IR on Estrous Cyclicity

Daily inspection of vaginal cytology in the groups treated with dexamethasone and its co-exposure with Cd revealed absence of normal estrous cyclicity, with a prolonged period of persistent mixed cells (Metestrus), which last for few days, followed by a period of persistent leucocytes (Diestrus) as compared with control group. However, alone Cd group did not show any change in estrous cyclicity (Fig. 4.2).

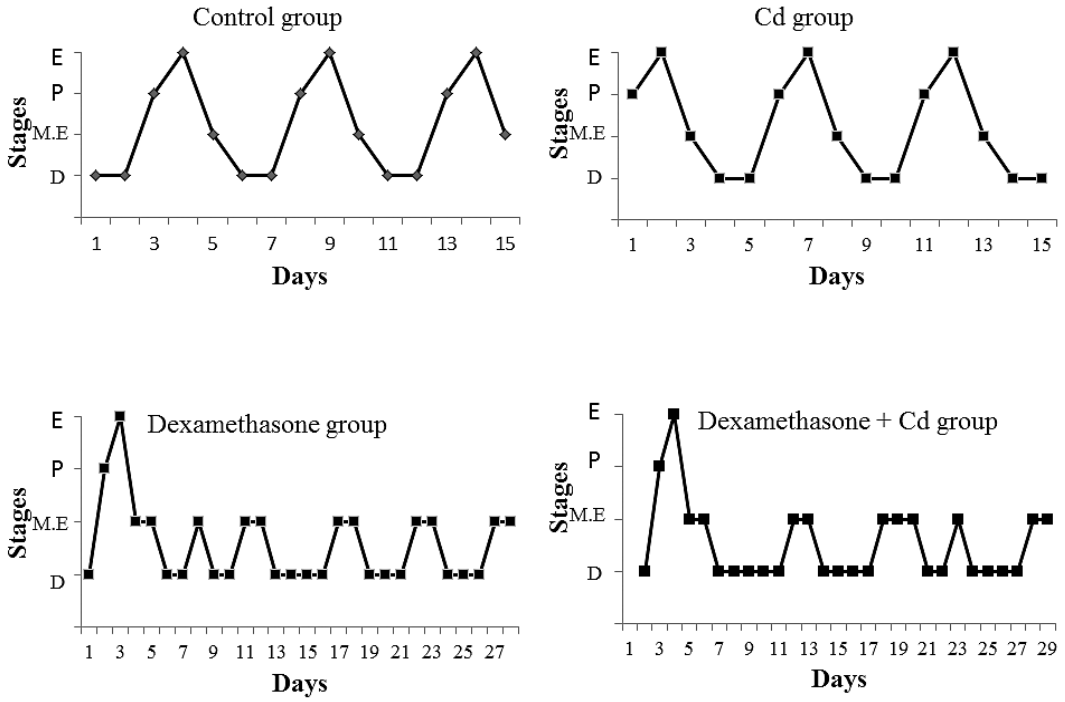


Figure 4. 2: Effect of Dexamethasone and Cd either alone or in combination on estrous cyclicity of rats (n = 6). E=Estrous stage-cornified cells, P=proestrous stage-nucleated epithelial cells, M.E=metestrus-nucleated, cornified and leucocytes, D=diestrous-leucocytes.

4.3.3 Histological Analysis revealed alterations in ovary

Histological examination of ovaries showed absence of mature follicles in all the three groups as compared to control group. Increased amount of fibrosis in the stroma was also observed in all the three groups as compared to control. Thickening of capillary wall was found to be evident in IR and IR+Cd treated group as compared to control group (Fig. 4.3).

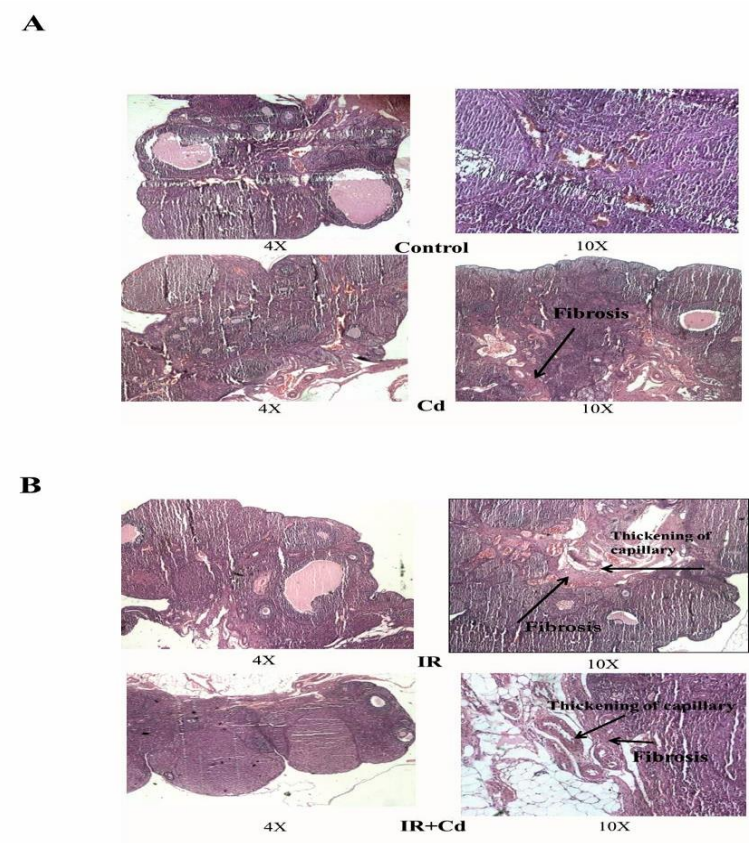


Figure 4. 3: Histological assessment of ovarian structure in Control, Cd, IR and IR+Cd treated rats. n = 4

4.3.4 Granulosa cell viability

Further investigation for granulosa cell viability by trypan blue exclusion dye, demonstrated deleterious effect of Cd and IR either alone or in combination. Percentage cell viability was significantly affected in all the groups as compared to control groups. The Cd treated group in combination with IR exhibited maximum decrease ($P < 0.001$) whereas alone Cd and IR groups exhibited significant decrease ($P < 0.01$) in granulosa cell viability as compared to control group. Within the groups, the combined group showed significant decrease ($P < 0.05$) as compared to Cd alone and IR group and no change was observed between IR and Cd alone group (Fig.4.4).

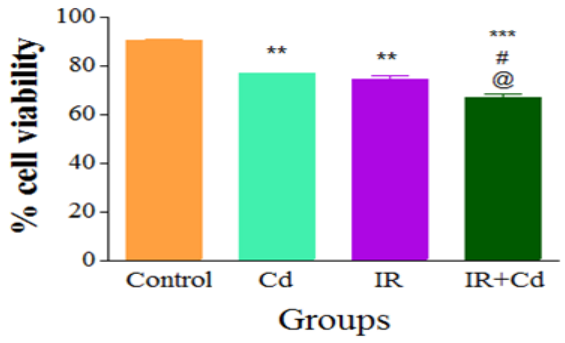


Figure 4. 4: Effect of IR and Cd on % cell viability of luteinized granulosa cells of rats. The values are represented as mean ± SEM of three independent experiments n = 6 ** P < 0.01, ** P < 0.01 and *** P < 0.001vs control[#] P < 0.05 and [@] P < 0.05 vs IR+Cd

4.3.5 Effect of Cd and IR on cell death parameters in granulosa cell

IR and Cd being apoptotic inducers, their combinatorial effect was assessed by mRNA expression of Bax and Bcl2, genes involved in cell death and protein expression of cleaved PARP-F2 and active caspase-3 in granulosa cells exposed to IR and Cd alone and in combination. Binding of AnnexinV/PI in granulosa cells was analyzed by Immunoblotting and confocal microscopy. A significant increase (P<0.05) in mRNA expression of Bax was observed in IR+Cd group as compared to control. Alone Cd and IR groups also showed increase in mRNA expression of Bax, however the increase was not significant. Further, a significant increase (P <0.01 and P < 0.05) was observed in mRNA expression of Bcl2 in IR+Cd and alone IR groups respectively as compared to control and cadmium. However, alone Cd showed a non significant decrease in Bcl2 mRNA expression as compared to control (Fig 4.5) . There was a significant increase ($P < 0.001$) in protein expression of activated caspase-3 in all the groups as compared to control. Within the groups, significant increase ($P < 0.001$) was observed in IR and IR+Cd group as compared to Cd alone and in IR+Cd group as compared to alone IR. A significant increase (P < 0.05, P < 0.01 and P < 0.01) in protein expression of cleaved PARP-F2 in alone Cd, IR and IR+Cd group respectively was observed as compared to control. Whereas within the groups no change in protein expression of cleaved PARP-F2 was observed (Fig 4.6). In AnnexinV/PI staining, all the groups were observed to be positive for the staining of Annexin V as well as PI as compared to control (Fig 4.7).

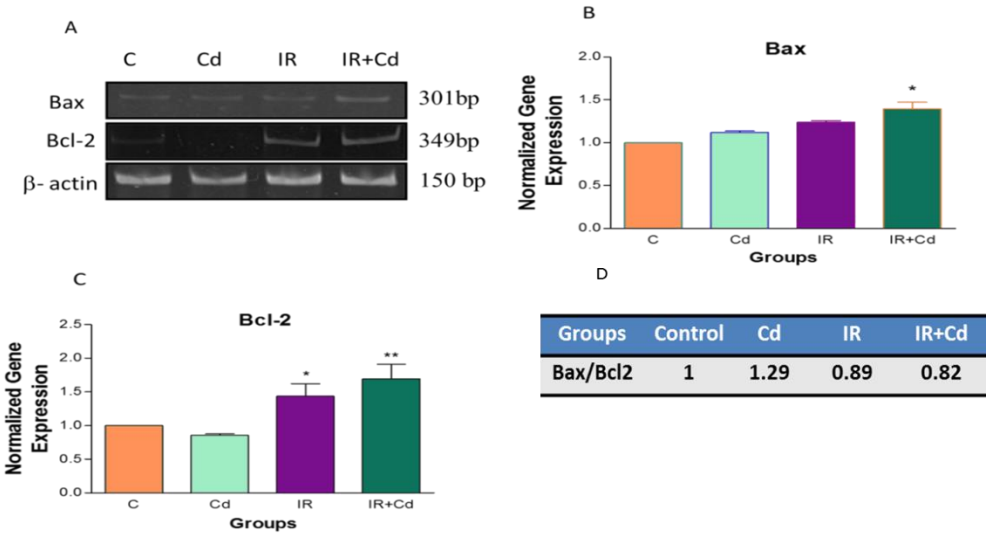


Figure 4. 5: Effect of IR and Cd either alone or in combination on cell death parameters. (A) mRNA expression of Bax and Bcl2 was done by semi-quantitative RT-PCR. (B). Densitometric analysis of Bax (C) Densitometric analysis of Bcl2. * P < 0.05 vs. C, ** P< 0.01 vs. C (n = 6). (D) Bax/Bcl2 ratio. The normalized gene expression values are represented as mean ± SEM of three independent experiments. C= control, ns= not significant.

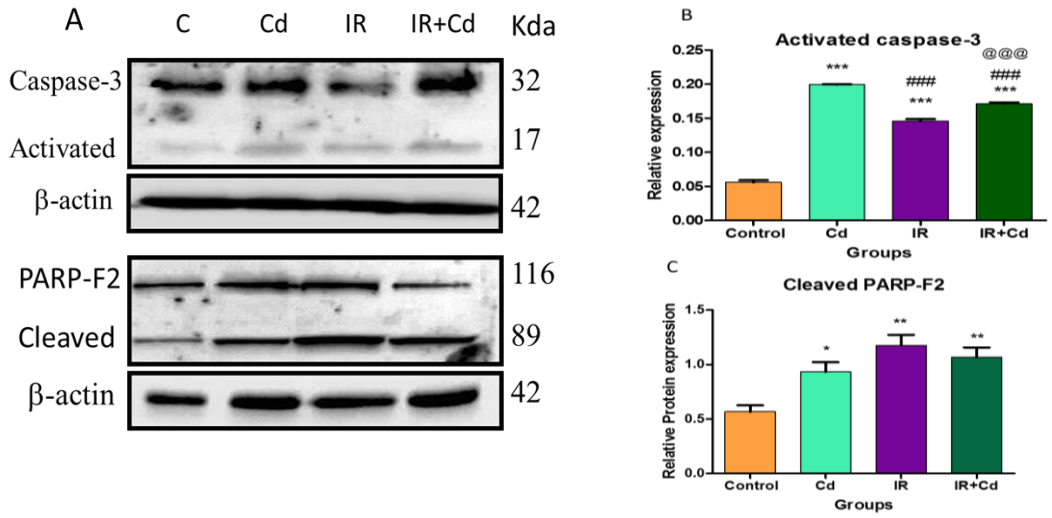


Figure 4. 6: Effect of IR and Cd either alone or in combination on protein expression of PARP and caspase-3. (A) western blot images of PARP and caspase-3. (B). Densitometric analysis of Activated caspase-3. (C) Densitometric analysis of Cleaved PARP-F2. (* $P < 0.01$,** $P < 0.01$ and **$P < 0.01$) vs C, ### $P < 0.001$ vs. Cd, @@@ $P < 0.001$ vs. IR (n = 6). The normalized gene expression values are represented as mean ± SEM of three independent experiments. C= Control, ns= not significant.

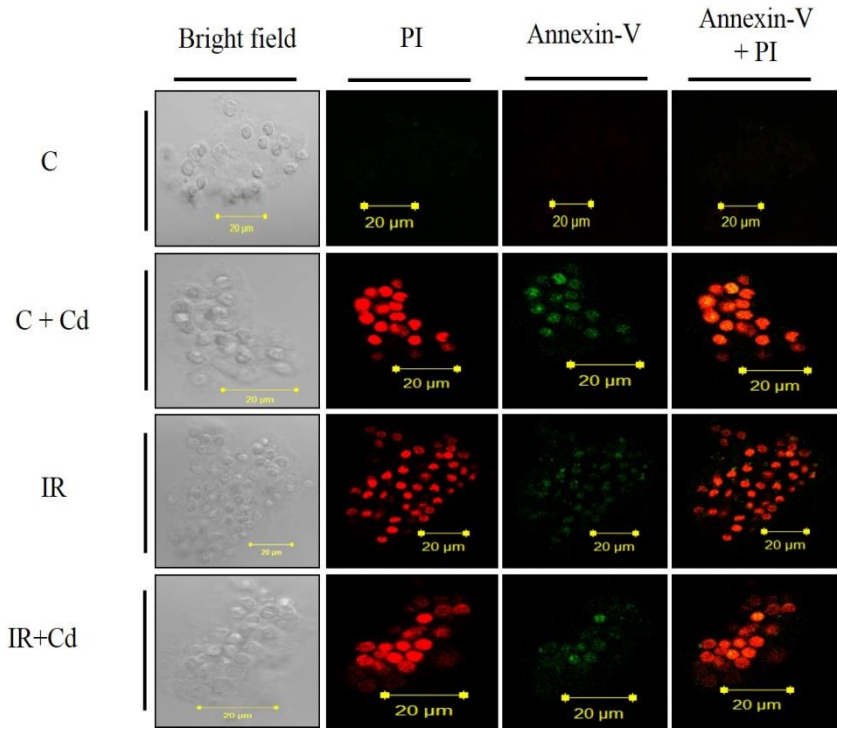


Figure 4. 7: Effect of Cd, IR alone and in combination on Annexin V/PI staining in granulosa cells. Red color indicates presence of PI, green color indicates Annexin V staining.

4.3.6 Effect of Cd and IR on serum estradiol and progesterone concentrations

Steroid hormones play an important role in reproductive system. Serum of the experimental animals were subjected for estimation of estradiol and progesterone. Estradiol concentration was

observed to be significantly decreased in Cd ($P < 0.01$), IR ($P < 0.001$) and IR+Cd ($P < 0.001$) group as compared to control group. When compared between the groups, both IR and IR+Cd demonstrated significant decrease compared to Cd ($P < 0.01$) treated group. Similarly, progesterone concentration was decreased in Cd ($P < 0.01$), IR ($P < 0.001$) and IR+Cd ($P < 0.001$) group as compared to control group. When compared between the groups, both IR and IR+Cd demonstrated significant decrease compared to Cd ($P < 0.05$ and $P < 0.01$ respectively) treated group (Fig.4.8).

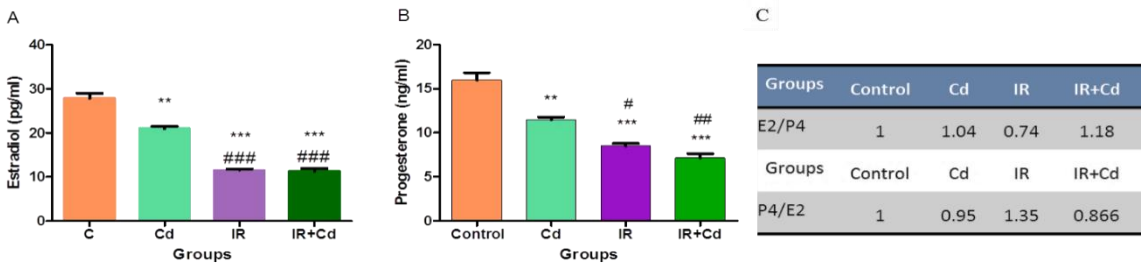


Figure 4. 8: Effect of IR and Cd either alone or in combination on serum (A) estradiol and (B). progesterone levels. (C) E2/P4 ratio. The values are represented as mean ± SEM of three independent experiments (n = 6). ** P < 0.01 vs C, *** P < 0.001 vs. C, # P < 0.05 vs. Cd, ## P < 0.01 vs. Cd, ### P < 0.001 vs. Cd.

4.3.7 Expression of genes and proteins involved in steroidogenesis in rat granulosa cells

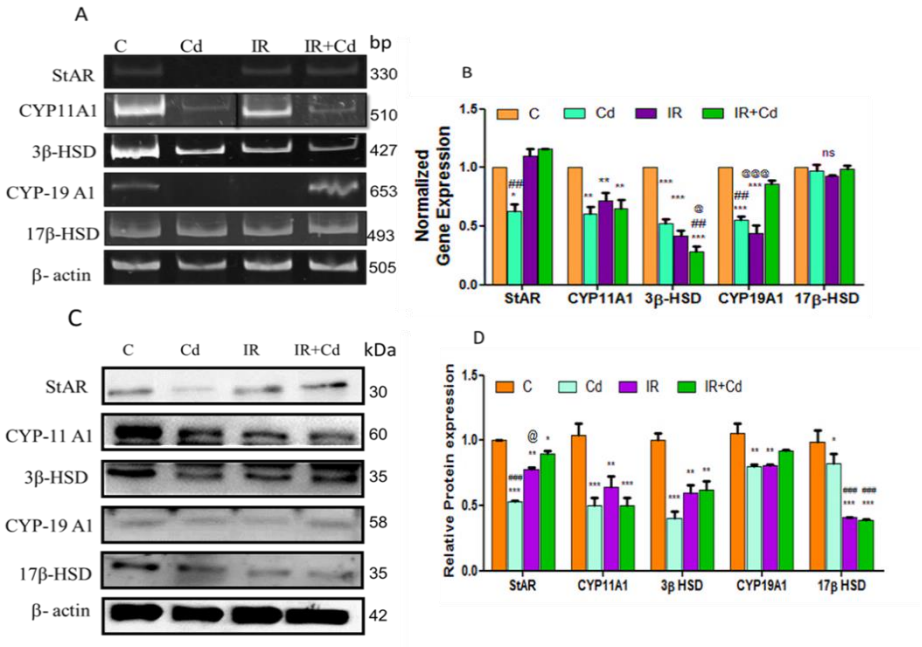


Figure 4. 9: Effect of IR and Cd either alone or in combination on (A) mRNA expression of genes involved in steroidogenesis (B) densitometric analysis for mRNA (C) protein expression of genes invovled in steroidogenesis (D) densitometric analysis for protein. The normalized expression values are represented as mean ± SEM of three independent experiments (n = 6). * P < 0.05, ** P < 0.01, *** P < 0.001 vs. C, # P < 0.05, ## P < 0.01, ### P < 0.001 vs. Cd, @ P < 0.05, @@ P < 0.01, @@@ P < 0.001 vs. IR.

Effect of IR and cadmium and their co-exposures may confer alterations at the molecular level in steroidogenesis, hence mRNA and proteins expression of StAR, CYP11A1, 3β-HSD, CYP19A1 and 17β-HSD of steroidogenic pathways were studied by qPCR and western blot employing β-actin as the internal control. As expected mRNA and protein expression of StAR showed significant decrease ($P < 0.05$) and ($P < 0.001$) respectively in Cd treated group as compared to control. There was slight but significant decrease ($P < 0.05$) in protein expression of StAR in IR+Cd group whereas animals exposed to IR alone demonstrated an intermediate pattern of inhibition ($P < 0.01$) as compared with control. mRNA expression of CYP11A1 revealed a significant decrease ($P < 0.01$) in all the groups as compared to control group whereas within the group there was no significant change. A significant decrease ($P < 0.001$, $P < 0.01$ and $P< 0.001$) was observed in CYP11A1 protein expression in Cd alone, IR and IR+Cd group respectively as compared to control. mRNA expression of 3β-HSD showed a significant decrease ($P < 0.001$) in all the three groups as compared to control. Within the groups IR+Cd showed maximum decrease ($P < 0.01$, $P < 0.05$) as compared to alone Cd and IR group whereas no change was observed in the protein expression of 3β-HSD between alone Cd and IR. Significant decrease ($P < 0.001$ and $P < 0.01$) was observed in mRNA expression of CYP19A1 in Cd alone when compared to control and IR+Cd group respectively. Similarly IR group showed significant decrease ($P < 0.001$) in mRNA expression of CYP19A1 as compared to control and IR+Cd animals. IR+Cd group showed non-significant decrease in CYP19A1 mRNA as compared to control group. CYP19 A1 protein revealed significant decrease ($P < 0.01$) in Cd alone and IR groups respectively as compared to control. CYP19A1 protein showed non-significant decrease in IR+Cd group as compared to control. Further, mRNA expression of 17β-HSD did not reveal any significant difference in any of the group. But, protein expression of 17β-HSD revealed significant decrease ($P < 0.05$) in Cd group, and maximum decrease ($P < 0.001$) in IR and IR+Cd group as compared to control, whereas, IR and IR+Cd revealed significant decrease ($P < 0.001$) as compared to Cd group with no change between themselves (Fig. 4.9).

4.3.8 Effect of Cd and IR on steroidogenic enzyme activity

Alterations in the expression of steroidogenic genes inquisted us further to observe for the effect of Cd and IR on the activity of the key enzymes. Cells of IR+Cd, IR and Cd group showed significant decrease ($P < 0.001$) in 17β-HSD and 3β-HSD activity as compared to control. Within the groups, IR+Cd showed significant decrease ($P < 0.001$ and $P < 0.01$) when compared

to Cd alone and IR group whereas alone IR and Cd groups within themselves did not show any significant change in the activities (Fig. 4.10).

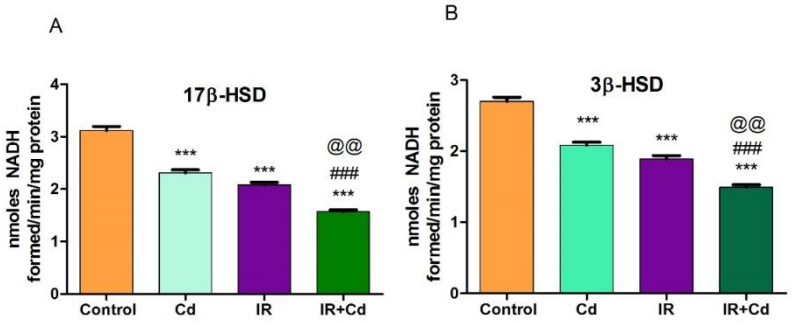


Figure 4. 10: Effect of IR and Cd either alone or in combination on A. 17β-HSD activity and B. 3β-HSD activity in rat luteinized granulosa cells. *** P < 0.001 vs. C, ### P < 0.001 vs. Cd, @@ P <0.01 vs. IR. The values are represented as mean ± SEM of three independent experiments (n = 6).

4.3.9 Expression of genes for gonadotropin receptors in rat granulosa cells

Binding of gonadotropin to their receptors initiates down stream signaling process towards steroidogenesis. In the present study effect of Cd and IR alone and in combination on mRNA expression of FSH-R and LH-R was analysed by real time analysis which revealed significant decrease (P < 0.001) in all the groups as compared to control. Further within the groups, mRNA expression of FSH-R showed significant decrease (P < 0.01) in IR and IR+Cd as compared to Cd whereas IR and IR+Cd did not show any difference between themselves. Significant decrease (P < 0.05) for mRNA expression of LH-R was observed in Cd and IR groups as compared to IR+Cd group (Fig. 4.11)

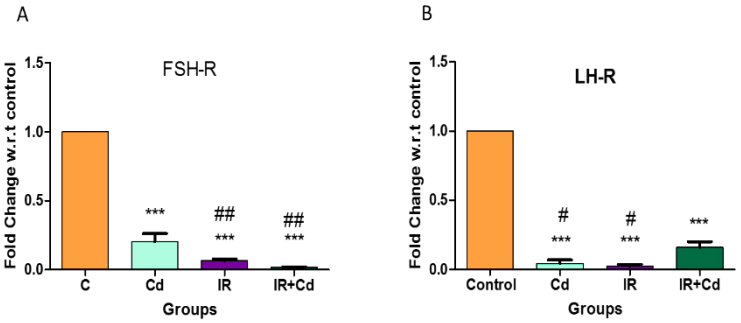


Figure 4. 11: Effect of IR and Cd either alone or in combination on gonadotropin receptors. mRNA of FSH-R and LH-R was analysed by real time PCR. (A) FSH-R. (B) LH-R. *** P < 0.001 vs. C, ## P < 0.01 vs. Cd (n = 6). The normalized gene expression values are represented as mean ± SEM of three independent experiments. ns= not significant.

4.3.10 Expression of genes involved in regulation of steroidogenesis.

Hypothalamus and pituitary are pivotal for regulation of steroidogenesis. To assess the effects of Cd, IR, and their co-exposure on the genes regulating ovarian steroidogenesis through hypothalamus and pituitary, mRNA expression of GnRH and CYP19A1 in hypothalamus and FSH-β and LH- β in pituitary and FSH-R and LH-R in granulosa cells were analyzed by real time PCR employing β-actin as the internal control. mRNA expression of GnRH and CYP19A1 showed significant decrease (P < 0.001 and P < 0.05) in alone Cd, IR and IR+Cd groups

respectively as compared to control. A significant decrease ($P < 0.01$, $P < 0.01$ and $P < 0.001$) in mRNA expression of FSH-β was observed in alone Cd, IR and IR+Cd groups respectively as compared to control. Similarly mRNA expression of LH- β revealed a significant decrease ($P < 0.01$, $P < 0.001$ and $P < 0.01$) in alone Cd, IR and IR+Cd groups respectively as compared to control. The ratio of LH/FSH in Cd, IR and IR+Cd group was 1.14, 2.4 and 2 respectively as compared to 1 in control group (Fig 4.12).

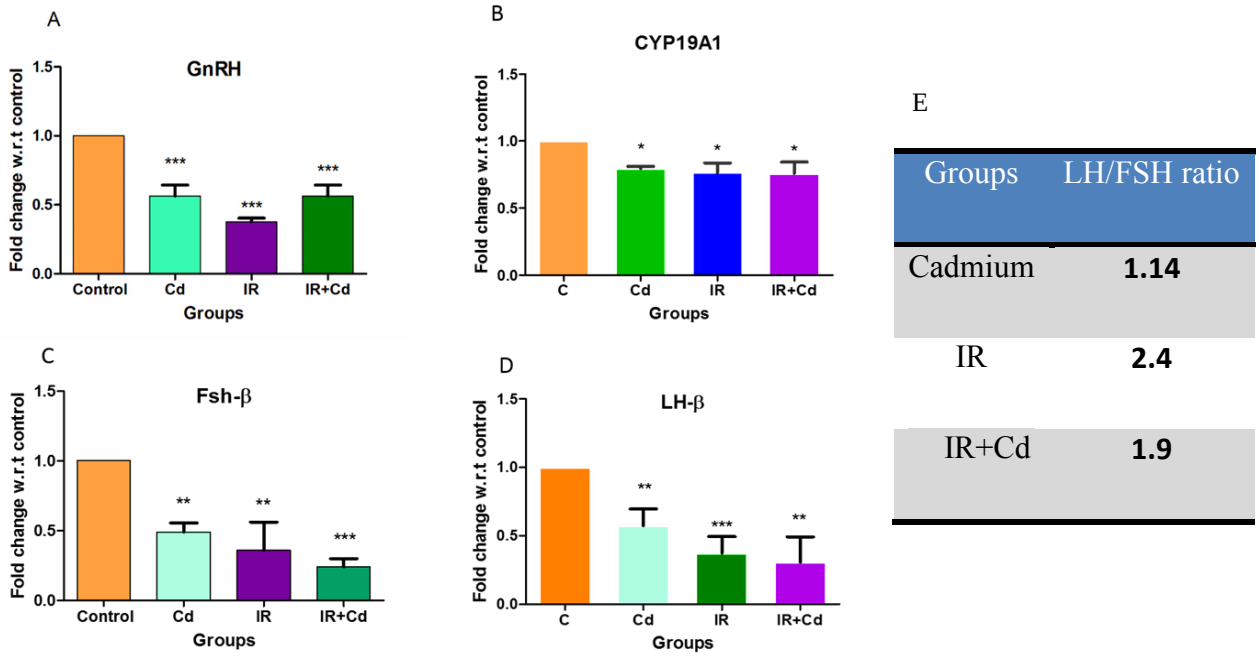


Figure 4. 12: Effect of IR and Cd either alone or in combination on genes involved in regulation of steroidogenesis. mRNA expression of GnRH, CYP19A1, FSH-β and LH- β was done by real time PCR. A. CYP19A1. B. GnRH. C. FSH-β D. LH-β. E. Ratio of LH/FSH. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. C. The normalized gene expression values are represented as mean ± SEM of three independent experiments (n = 6).

4.4 Discussion

Evidences state that because of the change in life style, there are increasing number of women with some degree of IR (Hackbart et al. 2013). Moreover, due to environmental issues,women are also exposed to toxicants such as Cd in their reproductive age (Caserta et al. 2008; Soisungwan Satarug 2010). As evident in present scenario, females are eventually exposed to both Cd & IR condition which may lead to altered reproductive performance and fertility-related problems. In this regard, the present study analyzed the extent of the effect of Cd in the ovary of dexamethasone induced IR rat model. To prove this, at a very first step, we developed an IR female rat model with FIRI index of 2.8 by using dexamethasone which has been previously

reported to exacerbate IR in monovular animal model, such as cow, in isolated and cultured 3T3 adipocytes and in ovarian theca cells from porcine follicles (Qu et al. 2009; Hackbart et al. 2013). Further, studies on Cd exposure (2.0 mg/kg daily for 14 days) have demonstrated it to be a diabetogenic agent in animal model (Edwards and Prozialeck 2009). The results of the present investigation demonstrated glucose intolerance and IR in the groups treated with dexamethasone alone and in combination with Cd but not in alone Cd treated group. These findings thus indicate that dexamethasone is responsible for the development of IR in both dexa alone treated and co-exposed group. Further, western blot analysis in granulosa cell, revealed down regulation of insulin receptor in groups treated with dexamethasone and dexamethasone with Cd as compared to control and Cd alone group. In earlier reports, insulin resistance has been demonstrated in cultured porcine granulosa and theca cells in response to dexamethasone treatment by monitoring protein expression of downstream targets such as IRS-1, GLUT-4 and PPAR-γ (Qu et al. 2009; Yan et al. 2009). We instead demonstrated down regulation of insulin receptor itself in dexamethasone treated granulosa cells confirming the establishment of frank IR condition in rodent model at the cellular level.

Fertile rats show normal estrous cyclicity of 4 days and a normal ovarian morphology with growing follicles and a well defined stroma (Marcondes 2002). Our present result of Cd treated group is similar to earlier reports from our lab which demonstrated reduced serum estradiol and progesterone concentrations and normal estrous cyclicity with sub-clinical Cd exposure (Pillai et al. 2010). However in dexa and dexa + Cd group, prolonged metaestrus and diestrus stages were observed which corelated with less number of mature follicles indicating direct or indirect actions of IR on the follicles along with decreased serum estradiol and progesterone concentration which are responsible for anovulation, reduced reproductive capacity ultimately leading to reproductive failure. Recently glucocorticoid induced insulin resistance has been shown to suppress circulating estradiol and progesterone concentration in serum, affect oocyte development, ovulation, fertilization and implantation thus leading to infertility (Akamine et al. 2010; Hackbart et al. 2013). Our data for reduced cell viability along with altered histological findings and decrease in serum estradiol and progesterone levels in all the groups as compared to control imply that Cd exposure along with IR alters folliculogenesis and granulose cell viability more deleteriously in co exposed group. Hence we further investigated the effect of Cd, IR and IR+Cd on granulosa cell death parameters.

Folliculogenesis and ovulation being the ultimate functions of the ovary are intimately linked to multidirectional communication between oocyte, granulosa cells, and theca cells (Uzumcu and Zachow 2007). Granulosa cells are the first somatic cell type known to interact with germ cells (Pepling and Spradling 2001) and are involved in apoptosis and steroidogenesis (Hsueh et al. 1984). Previous reports have demonstrated decrease in granulosa cell number and change in its morphology with *in-vitro* cadmium exposure (Paksy et al. 1997; Smida et al. 2004; Nampoothiri and Gupta 2006). Action of insulin on granulosa cells has also been implicated in PCOS, where in granulosa cell numbers were found to be decreased relative to follicle size (Poretsky et al. 1999). Apoptotic cell death is physiologic process undertaken by granulosa cells for the process of atresia where Bcl 2 and Bax proteins are the key mediators of instrinsic apoptotic pathway. Results of the present study indicated significant increase in mRNA expression of Bax and Bcl-2 in IR+Cd group. Cd is known to induce cell death by apoptotic mechanism in many cell types involving increase in expression of pro-apoptotic Bax and decrease in expression of anti-apoptotic Bcl2. In the present study increase in mRNA expression of Bcl-2 in IR+Cd and alone IR group can be attributed to the effect of dexamethasone, a glucocorticoid. Glucocorticoid surprisingly induces apoptosis in a hematopoietic cell system to block inflammation whereas in epithelial cells it protects against apoptosis (Sasson and Amsterdam 2002; Wätjen et al. 2002). This function of glucocorticoid explains the rapid healing of the follicular wall after its rupture and luteinization. Moreover presence of the glucocorticoid receptors in granulosa cells imply direct action in the ovary. Further, in the cell death mechanism Bax/Bcl2 ratio is important for determining the sensitivity to apoptotic stimuli. But, in the present study the ratio of Bax/Bcl2 is less than 1.0 in IR and IR+Cd groups. Hence we further examined whether caspase-3 is activated and PARP-F2 is cleaved. Activation of caspase-3 and nuclear poly (ADP-ribose) polymerase (PARP) enzymes are important terminal events which promote apoptosis in cells (Banu et al. 2011). In the process of follicular atresia cleavage of PARP-1 into two fragments by execution of caspases-3 is strongly implicated (Wei and Shi 2013). Moreover, follicles isolated from caspase-3 null ovaries do not show GCs apoptosis in response to serum starvation, suggesting caspase-3 as an effector caspase in this process, and that its activation leads to the final stages of cellular death (Chowdhury et al. 2012). These findings from the literature explain the basal expression of activated caspase-3 in luteinized granulosa cells in control group as observed in the present study. In alliance with our study, Cd induced apoptosis through activation of caspase-3 and cleaved PARP has been observed in many different species, primary

cultures and cell lines. In insulin signaling, PI3K is an essential regulator of apoptotic pathways. Hence low levels of insulin receptors reduce the activity of PI3K leading to activation of caspase-3 (Du et al. 2004; Banu et al. 2011). Results of the present study demonstrate significant increase in activation of caspase-3 in IR+Cd and alone Cd and IR groups when compared to control thus indicating apoptosis as the type of cell death. In the process of apoptosis the upregulation of caspase-3 is accompanied by elevated phosphatidylserine (PS) externalization and an increase in membrane permeabilization (McComb et al. 2010; Dunai et al. 2012). The study observed granulosa cells in Cd alone, IR and IR+Cd to be positive for both annexin V and PI dual staining confirming apoptosis as the type of cell death. Thus a higher incidence of granulosa cells undergoing apoptosis in alone Cd, IR and IR+Cd group would lead to empty follicles, fewer oocyte retrival and poor quality of oocyte.

We observed a significant decrease in serum estradiol and progesterone levels in all the groups as compared to the control. Considering these findings, the suppressed ovulation in the present study might be due to disruption of ovarian steroidogenesis. The rate-limiting step in ovarian steroid hormone synthesis requires StAR protein in order to transport cholesterol to the intra mitochondrial membrane (Clark and Stocco 1995). Cd alone demonstrated decreased expression of StAR at both mRNA and protein level when compared to control group supporting the fact that StAR might be a potent target for Cd to bind and thus alter steroidogenesis (Paksy K 1992; Paksy K 1997; Zhang and Jia 2007; Pillai et al. 2010). IR alone failed to demonstrate any effect on expression of StAR mRNA but showed a significant decrease in its protein expression (Jakimiuk et al. 2001). Similarly little but significant decrease in protein expression of StAR was observed in IR+Cd group in present study, with reduced gonadal steroidogenesis leading to accumulation of cholesterol in lipid droplets supported by earlier reports (Petrescu et al. 2001).

In follicular steroidogenesis, the synthesis of progesterone is dependent on CYP11A1 and 3β-HSD and estrogen is dependent on CYP19A1 and 17β HSD, these expressions are highly dependent on the type of granulosa cells (Miro et al. 1995). In our study, we demonstrated significant decrease in protein expression of CYP11A1, 3β HSD, 17β HSD and a non-significant decrease in protein expression of CYP19A1 in the IR+Cd group as compared to control group. Expression of CYP11A1 reveals contradictory results in the literature. Jaimiuk et al 2001b reported over expression of CYP11A1 in granulosa cells from PCOS whereas down regulation of

CYP11A1 expression was observed in rat adrenal gland treated with dexamethasone by Hu et al 2013. The down regulation of CYP11A1 both at gene and protein level as observed in the present study could be contributed to the effect of dexamethasone. The signaling pathway in granulosa cells of IR ovaries get altered, forcing the cell to luteinize at a premature stage as compared to granulosa cells from control ovaries. This leads to terminal differentiation in these cells because of which they lose their proliferative capacity and thus fail to express CYP19A1 and 17β HSD (Mukherjee and Maitra 2010; Hackbart et al. 2013). Also presence of TGF-β, IGF-1, EGF, TNF-α, and abnormally high levels of 5 alpha-androstane-3, 17-dione in the follicular fluid act as endogenous inhibitors of estrogen production by inhibiting CYP19A1 activity explaining decrease in estradiol synthesis in IR+Cd group. These changes along with attenuation of estradiol and progesterone hormone synthesis disrupt the normal developmental program thereby arresting estrus cyclicity in metaestrus and diestrus stage. Studies in literature have also demonstrated strong inhibition of CYP11A1 and CYP19A1 expression by Cd and other endocrine disruptors in Teleost Fish, human embryonic 293 cells and genetically modified stable porcine granulosa cells thus leading to decreased estradiol and progesterone synthesis thus disturbing reproductive cycle. (Jakimiuk et al. 2001; Smida et al. 2004; Benachour et al. 2007; Cheshenko et al. 2008; Das and Mukherjee 2013). Apart from mRNA and protein expression present study also showed significant suppression of 3β HSD and 17β HSD activity in granulosa cells. Cadmium can directly bind to the amino acids present on the active site leading to decreased activity which has been demonstrated in earlier reports. (Piasek and Laskey 1994; Nampoothiri and Gupta 2006). Moreover, the activity of enzymes may also be affected by external factors, such as diet that leads to insulin resistance like condition which explains decrease in the activity in IR condition as observed in our result (Hu et al. 2010). Gonadotropin receptors FSH-R and LH-R play a pivotal role in the reproductive process. LHR knockout arrests post-natal sexual development and results in compromised ovulation and infertility. Significant decrease was observed in mRNA expression of FSH-R and LH-R in alone Cd treated group. Cd is known to bind to zinc binding domains in G protein coupled receptors (Nemoto et al. 2009). Thus the decrease in steroidogenic proteins along with the gonadotropin receptors would ultimately lead to decrease in steroidogenesis in the combined group.

HPO axis is the central regulator of ovarian steroidogenesis. The sequence of events corresponding to the ovarian steroidogenesis is induced by the action of GnRH in hypothalamus.

In the present study decrease in mRNA expression of GnRH , CYP19A1, and pitutary gonadotrophin LH-β and FSH- β due to the inhibitory effect of cadmium is in accordance with the earlier reports (Varga and Paksy 1991; Lafuente 2013). Insulin is known to stimulate the secretion of GnRH in vivo, thus implicating its role in the regulation of female reproductive function. Significant decrease was observed in the mRNA expression of GnRH and CYP19A1 in IR group. In the IR condition estradiol negative feedback mechanism is inhibited ultimately leading to decrease in pulsatile release of GnRH which in turn leads to decreased secretion of gonadotropins LH-β and FSH- β (Hackbart et al. 2013)., Further significant decrease was also observed in mRNA expression of LH-β and FSH- β in the Cd group correlated well with the literature (Paksy et al. 1997; Pillai et al. 2002).. The present study also revealed decrease in mRNA expression of LH-β and FSH- β along with increase in the ratio of LH: FSH in the IR group. During IR condition in pituitary gonadotrope cells FOXO1, a transcription factor remains localized in the nucleus of the cell itself because of absence of insulin signaling where it functions to block expression of the LH-β gene (Arriola et al. 2012). The decrease in LH pulse frequency because of deceleration in the firing rate of hypothalamic GnRH pulse generator has been observed as a metabolic effect of diabetes in STZ-induced diabetic rats (Dong et al. 1991). Decrease in mRNA expression of LH-β and FSH- β along with increase in LH: FSH ratio in IR+Cd group indicated pre timely LH surge thus disturbing the folliculogenesis and an early transition towards luteal phase.

Summarizing the results as per the insults, Cd alone directly interacts with StAR ultimately leading to decrease in availability of cholesterol. It also decreases CYP11A1, 3β HSD, 17β HSD and CYP19A1 protein expression thus leading to relative decrease in progesterone and estradiol production along with fibrosis. IR alone decreases expression of levels along with fibrosis and thickening of capillaries, the effects seem to be more adverse as compared to Cd alone. In IR+Cd, along with decrease in transcript and protein expression of StAR, CYP11A1, 3β HSD, 17β HSD and CYP19A1 a significant decrease in gonadotropin receptors and steroid hormone suggested overall effect on ovarian steroidogenesis. The decrease in steroidogenesis was observed to be due to effect of IR and Cd on regulators of reproduction in hypothalamus and pituitary axis StAR, CYP11A1, 3β HSD, 17β HSD and CYP19A1 protein leading to decrease in progesterone and estradiol along with increased death of luteinized granulosa cells. The results showed that although individual parameters variedly affected at molecular level in different

groups, overall effect was more deleterious in co-exposed group providing support for their involvement in etiology of reproductive dysfunction as shown in schematic Figure 4.13.

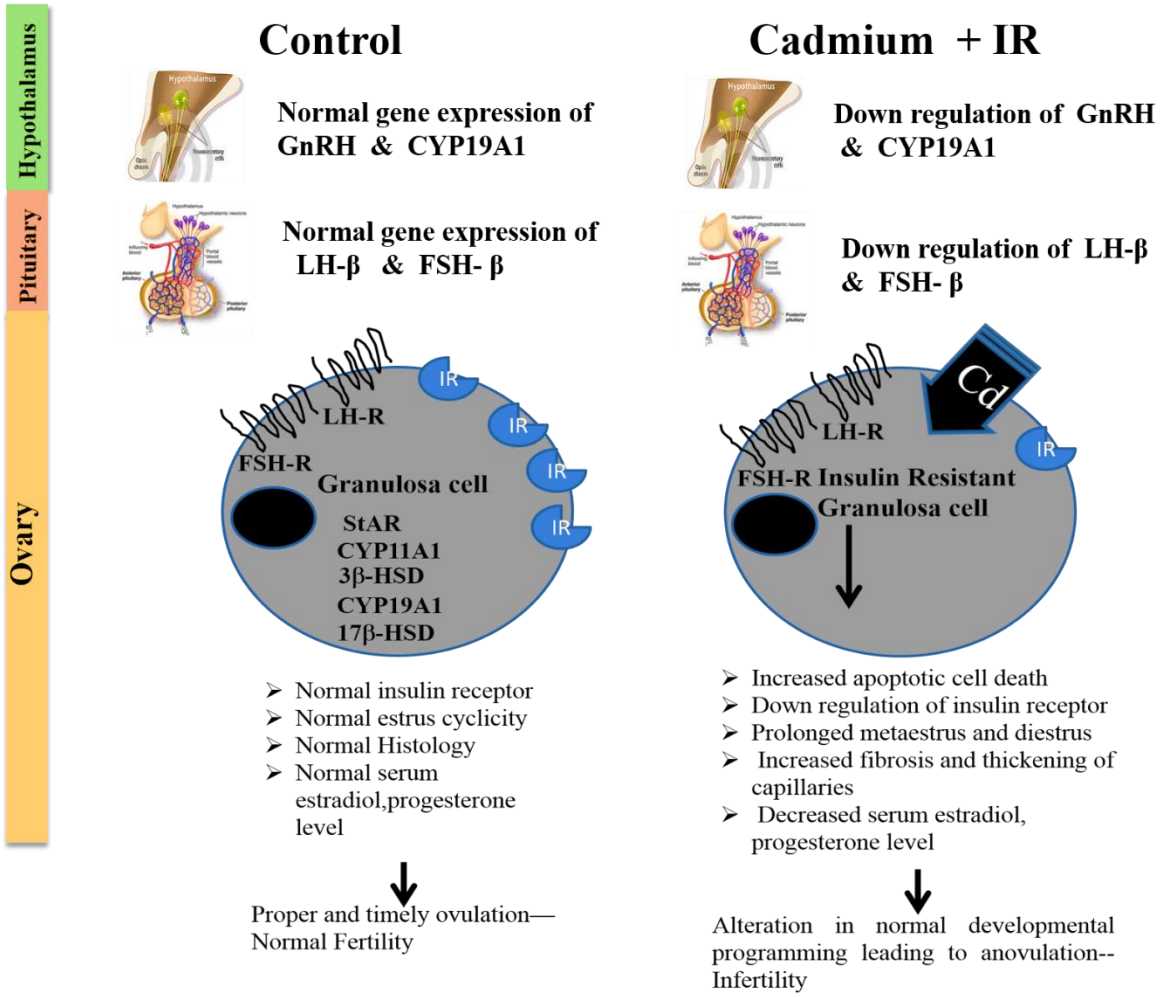


Figure 4. 13: Schematic diagram summarizing potential effect of Cd on dexamethasone induced IR rat model

4.5 References

Akamine EH, Marcal AC, Camporez JP, Hoshida MS, Caperuto LC, Bevilacqua E, Carvalho CR. 2010. Obesity induced by high-fat diet promotes insulin resistance in the ovary. *J Endocrinol* **206**: 65-74.

Alexander J. 2009. Cadmium in food-Scientific Opinion of the Panel on Contaminants in the Food Chain. *EFSA J*: 980.

Arriola DJ, Mayo SL, Skarra DV, Benson CA, Thackray VG. 2012. FOXO1 transcription factor inhibits luteinizing hormone β gene expression in pituitary gonadotrope cells. *Journal of Biological Chemistry* **287**: 33424-33435.

Banu SK, Stanley JA, Lee J, Stephen SD, Arosh JA, Hoyer PB, Burghardt RC. 2011. Hexavalent chromium-induced apoptosis of granulosa cells involves selective sub-cellular translocation of Bcl-2 members, ERK1/2 and p53. *Toxicology and applied pharmacology* **251**: 253-266.

Benachour N, Moslemi S, Sipahutar H, Seralini GE. 2007. Cytotoxic effects and aromatase inhibition by xenobiotic endocrine disrupters alone and in combination. *Toxicol Appl Pharmacol* **222**: 129-140.

Caserta D, Maranghi L, Mantovani A, Marci R, Maranghi F, Moscarini M. 2008. Impact of endocrine disruptor chemicals in gynaecology. *Hum Reprod Update* **14**: 59-72.

Cheshenko K, Pakdel F, Segner H, Kah O, Eggen RI. 2008. Interference of endocrine disrupting chemicals with aromatase CYP19 expression or activity, and consequences for reproduction of teleost fish. *Gen Comp Endocrinol* **155**: 31-62.

Chowdhury I, Garcia-Barrio M, Harp D, Thomas K, Matthews R, Thompson WE. 2012. The emerging roles of prohibitins in folliculogenesis. *Frontiers in bioscience (Elite edition)* **4**: 690.

Clark BJ, Stocco DM. 1995. Expression of the steroidogenic acute regulatory (StAR) protein: a novel LH-induced mitochondrial protein required for the acute regulation of steroidogenesis in mouse Leydig tumor cells. *Endocr Res* **21**: 243-257.

Das S, Mukherjee D. 2013. Effect of cadmium chloride on secretion of 17beta-estradiol by the ovarian follicles of common carp, Cyprinus carpio. *Gen Comp Endocrinol* **181**: 107-114.

Diamanti-Kandarakis E, Argyrakopoulou G, Economou F, Kandaraki E, Koutsilieris M. 2008. Defects in insulin signaling pathways in ovarian steroidogenesis and other tissues in polycystic ovary syndrome (PCOS). *J Steroid Biochem Mol Biol* **109**: 242-246.

Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, Hauser R, Prins GS, Soto AM, Zoeller RT, Gore AC. 2009. Endocrine-disrupting chemicals: an Endocrine Society scientific statement. *Endocr Rev* **30**: 293-342.

Dong Q, Lazarus R, Wong L, Vellios M, Handelsman D. 1991. Pulsatile LH secretion in streptozotocin-induced diabetes in the rat. *Journal of endocrinology* **131**: 49-55.

Du J, Wang X, Miereles C, Bailey JL, Debigare R, Zheng B, Price SR, Mitch WE. 2004. Activation of caspase-3 is an initial step triggering accelerated muscle proteolysis in catabolic conditions. *The Journal of clinical investigation* **113**: 115.

Dunai ZA, Imre G, Barna G, Korcsmaros T, Petak I, Bauer PI, Mihalik R. 2012. Staurosporine induces necroptotic cell death under caspase-compromised conditions in U937 cells. *PLoS One* **7**: e41945.

Edwards JR, Prozialeck WC. 2009. Cadmium, diabetes and chronic kidney disease. *Toxicol Appl Pharmacol* **238**: 289-293.

Ford HB, Schust DJ. 2009. Recurrent pregnancy loss: etiology, diagnosis, and therapy. *Rev Obstet Gynecol* **2**: 76-83.

Gao X, Li B, Jiang H, Liu F, Xu D, Liu Z. 2007. Dioscorea opposita reverses dexamethasone induced insulin resistance. *Fitoterapia* **78**: 12-15.

Gupta S, Bhosale S, Pandya K. 1994. Effect of simultaneous low level exposure of Pb and Cd on delta-ALAD and acetylcholinesterase activity in rats. *Indian J Exp Biol* **32**: 819-821.

Hackbart KS, Cunha PM, Meyer RK, Wiltbank MC. 2013. Effect of glucocorticoid-induced insulin resistance on follicle development and ovulation. *Biol Reprod* **88**: 153.

Homan GF, Davies M, Norman R. 2007. The impact of lifestyle factors on reproductive performance in the general population and those undergoing infertility treatment: a review. *Hum Reprod Update* **13**: 209-223.

Hsueh AJ, Adashi EY, Jones PB, Welsh TH, Jr. 1984. Hormonal regulation of the differentiation of cultured ovarian granulosa cells. *Endocr Rev* **5**: 76-127.

Hu G-X, Zhao B-H, Chu Y-H, Zhou H-Y, Akingbemi BT, Zheng Z-Q, Ge R-S. 2010. Effects of genistein and equol on human and rat testicular 3beta-hydroxysteroid dehydrogenase and 17beta-hydroxysteroid dehydrogenase 3 activities. *Asian J Androl* **12**: 519-526.

Jakimiuk AJ, Weitsman SR, Navab A, Magoffin DA. 2001. Luteinizing hormone receptor, steroidogenesis acute regulatory protein, and steroidogenic enzyme messenger ribonucleic acids are overexpressed in thecal and granulosa cells from polycystic ovaries. *J Clin Endocrinol Metab* **86**: 1318-1323.

Jungheim ES, Schoeller EL, Marquard KL, Louden ED, Schaffer JE, Moley KH. 2010. Diet-induced obesity model: abnormal oocytes and persistent growth abnormalities in the offspring. *Endocrinology* **151**: 4039-4046.

Kaiping Yang LJ, Fran Rubio, Anju Sharma, and Haiyan Guan. 2006. Cadmium reduces 11b-hydroxysteroid dehydrogenase type 2 activity and expression in human placental trophoblast cells. *American journal of physiology endocrinology metabolism* **290**: E135-E142.

Lafuente A. 2013. The hypothalamic–pituitary–gonadal axis is target of cadmium toxicity. An update of recent studies and potential therapeutic approaches. *Food and Chemical Toxicology* **59**: 395-404.

Marcondes FK, Bianchi, F.J. and Tanno, A.P. 2002. Determination of the estrous cycle phases of rats: some helpful considerations. *Braz J Biol* **62**: 609-614.

McComb S, Mulligan R, Sad S. 2010. Caspase-3 is transiently activated without cell death during early antigen driven expansion of CD8 (+) T cells in vivo. *PLoS One* **5**: e15328.

Miro F, Smyth CD, Whitelaw PF, Milne M, Hillier SG. 1995. Regulation of 3 beta-hydroxysteroid dehydrogenase delta 5/delta 4-isomerase and cholesterol side-chain cleavage cytochrome P450 by activin in rat granulosa cells. *Endocrinology* **136**: 3247-3252.

Mukherjee S, Maitra A. 2010. Molecular & genetic factors contributing to insulin resistance in polycystic ovary syndrome. *Indian J Med Res* **131**: 743-760.

Nampoothiri LP, Gupta S. 2006. Simultaneous effect of lead and cadmium on granulosa cells: a cellular model for ovarian toxicity. *Reprod Toxicol* **21**: 179-185.

Nemoto K, Miyajima S, Hara S, Saigusa R, Yamada M, Shikama H, Yotsuya S, Sekimoto M, Degawa M. 2009. Decreased Gene Expression of Testicular Cell-Specific Proteins in Cadmium-Induced Acute Testicular Toxicity. *Journal of Health Science* **55**: 952-956.

Paksy K, Rajczy K, Forgacs Z, Lazar P, Bernard A, Gati I, Kaali GS. 1997. Effect of cadmium on morphology and steroidogenesis of cultured human ovarian granulosa cells. *J Appl Toxicol* **17**: 321-327.

Paksy K VB, Lázár P. 1992. Cadmium interferes with steroid biosynthesis in rat granulosa and luteal cells in vitro. *Biometals 1992 Winter;5(4):245-50* **Winter;5**: 245-250.

Paksy K VB, Lázár P. 1997. Zinc protection against cadmium-induced infertility in female rats. Effect of zinc and cadmium on the progesterone production of cultured granulosa cells. *Biometals 1997 Jan;10(1):27-35* **Jan;10**: 27-35.

Pepling ME, Spradling AC. 2001. Mouse ovarian germ cell cysts undergo programmed breakdown to form primordial follicles. *Dev Biol* **234**: 339-351.

Petrescu AD, Gallegos AM, Okamura Y, Strauss JF, 3rd, Schroeder F. 2001. Steroidogenic acute regulatory protein binds cholesterol and modulates mitochondrial membrane sterol domain dynamics. *J Biol Chem* **276**: 36970-36982.

Piasek M, Laskey JW. 1994. Acute cadmium exposure and ovarian steroidogenesis in cycling and pregnant rats. *Reproductive Toxicology* **8**: 495-507.

Pillai A, Laxmi Priya PN, Gupta S. 2002. Effects of combined exposure to lead and cadmium on pituitary membrane of female rats. *Arch Toxicol* **76**: 671-675.

Pillai P, Pandya C, Gupta S, Gupta S. 2010. Biochemical and molecular effects of gestational and lactational coexposure to lead and cadmium on ovarian steroidogenesis are associated with oxidative stress in F1 generation rats. *J Biochem Mol Toxicol* **24**: 384-394.

Poretsky L, Cataldo NA, Rosenwaks Z, Giudice LC. 1999. The insulin-related ovarian regulatory system in health and disease. *Endocr Rev* **20**: 535-582.

Priya PN, Pillai A, Gupta S. 2004. Effect of simultaneous exposure to lead and cadmium on gonadotropin binding and steroidogenesis on granulosa cells: an in vitro study. *Indian J Exp Biol* **42**: 143-148.

Qu J, Wang Y, Wu X, Gao L, Hou L, Erkkola R. 2009. Insulin resistance directly contributes to androgenic potential within ovarian theca cells. *Fertil Steril* **91**: 1990-1997.

Sasson R, Amsterdam A. 2002. Stimulation of apoptosis in human granulosa cells from in vitro fertilization patients and its prevention by dexamethasone: involvement of cell contact and bcl-2 expression. *The Journal of Clinical Endocrinology & Metabolism* **87**: 3441-3451.

Smida AD, Valderrama XP, Agostini MC, Furlan MA, Chedrese J. 2004. Cadmium stimulates transcription of the cytochrome p450 side chain cleavage gene in genetically modified stable porcine granulosa cells. *Biology of reproduction* **70**: 25-31.

Soisungwan Satarug SHG, Mary Ann Sens, and Donald A. Sens. 2010. Cadmium, Environmental Exposure, and Health Outcomes. *Environmental Health Perspectives* **118**: 182-190.

Srinivasan K, Ramarao P. 2007. Animal model in type 2 diabetes research: An overview. *Indian Journal of Medical Research* **125**: 451.

Uzumcu M, Zachow R. 2007. Developmental exposure to environmental endocrine disruptors: consequences within the ovary and on female reproductive function. *Reprod Toxicol* **23**: 337-352.

Varga B, Paksy K. 1991. Toxic effects of cadmium on LHRH-induced LH release and ovulation in rats. *Reproductive Toxicology* **5**: 199-203.

Vettriselvi V. 2012. A study on genetic polymorphisms associated with unexplained recurrent pregnancy loss in south indian population.

Wätjen W, Cox M, Biagioli M, Beyersmann D. 2002. Cadmium-induced apoptosis in C6 glioma cells: mediation by caspase 9-activation. *Biometals* **15**: 15-25.

Wei Q, Shi F. 2013. Cleavage of poly (ADP-ribose) polymerase-1 is involved in the process of porcine ovarian follicular atresia. *Animal reproduction science* **138**: 282-291.

Wyman A, Pinto AB, Sheridan R, Moley KH. 2008. One-cell zygote transfer from diabetic to nondiabetic mouse results in congenital malformations and growth retardation in offspring. *Endocrinology* **149**: 466-469.

Yan M, Wang J, Wu X, Hou L, Kuang H, Wang Y. 2009. Induction of insulin resistance by phosphatidylinositol-3-kinase inhibitor in porcine granulosa cells. *Fertil Steril* **92**: 2119-2121.

Zhang W, Jia H. 2007. Effect and mechanism of cadmium on the progesterone synthesis of ovaries. *Toxicology* **239**: 204-212.