3 Assessment of the key regulators of early pregnancy in letrozole-induced PCOS mouse model

3.1 Rationale of the study

Polycystic Ovary Syndrome, a type of female endocrinopathy has been now recognized as one of the major infertility disorders around the world (Wolf et al., 2018). Etiopathology of this disorder is mainly linked to hyperandrogenism, hyperinsulinemia, infrequent ovulation, and ovarian dysfunction (Robin et al., 2012; Broekmans et al., 2008; Qu et al., 2012). This multi-etiological pathology is associated with clinical pregnancy complications, an increased rate of spontaneous abortion/early pregnancy loss, and preterm delivery (Palomba et al., 2015; Azizia & Hardiman, 2011). Most of the studies elucidating the complications are in a relationship with pregnancy outcomes. However, molecular alterations in PCOS pregnancy that originates from the mother, embryo, or both are still under debate.

To achieve a successful pregnancy, the first step is embryo implantation, wherein two-way communication between a competent blastocyst and receptive uterus gives rise to attachment and invasion of the embryo to the uterine epithelium, following the decidualization of the uterine stroma (Lee et al., 2007). Each step of the initial pregnancy involves an interplay of the various signalling pathways in which synchronized production of ovarian estrogen and progesterone mediates structural and functional changes in the uterus (Wang et al., 2013). These steroid hormones are synthesized in the primary steroidogenic organs, ovary under the regulation of gonadotropins and key proteins like steroidogenic acute regulatory protein (STAR), cytochrome P450-17α-hydroxylase/C17, 20-lyase (CYP17A1), and cytochrome P450 aromatase (CYP19A1). Further, gonadal hormones exert their effect through their receptors, progesterone receptor (PGR) and estrogen receptor α & β (ESR1 & 2) respectively, and control cell differentiation, proliferation, and excretion of protein in the uterus (Pawar et al., 2014b). In addition, androgen and its receptor (AR) could modulate uterine growth, antagonize the expression of estrogen-regulated genes, and also helps in the decidualization of the uterine stroma (Kowalski et al., 2004). During the adhesion phase of early pregnancy, integrins (e.g., α4, αv, β1, β3) are considered to be endometrial markers, and their expression is synchronized with the blastocyst attachment to the endometrium (Singh & Aplin, 2009a). As a sequel to blastocyst-uterine attachment, transcription factors such as homeobox 10A

(HOX10A) genes are known to involve in the proliferation and differentiation of stromal cells surrounding the implanting blastocyst into a decidual cell (Du & Taylor, 2016). Further, invasion starts with penetration of the embryo to the uterine wall which involves degradation of the extracellular matrix (ECM) through matrix metalloproteases (MMP-2 & 9). Activities of metalloproteases are firmly regulated by their endogenous inhibitors, TIMPs (tissue inhibitors of MMPs). The elaborated balance between the stimulation of MMPs and their repression by TIMPs is crucial for the synchronization of embryo implantation (Nothnick et al., 2004). Latterly, leukemia inhibitory factor (LIF, pleiotropic cytokine of the IL-6 family) is examined to influence ranges from embryo attachment to the management of stromal cell proliferation (Salleh & Giribabu, 2014). LIF transduces its signal through the formation of a heterodimer with specific LIFR and the common co-receptor for the IL-6 family, glycoprotein 130 (gp130). The binding of the LIF to its receptor leads to the activation of signal transducer and activator of transcription factor 3 (STAT3), which further has an impact on the modulation of embryo-uterine functions during implantation (Suman et al., 2013).

These key modulators of the implanting embryo interacting with the uterus establish an appropriate milieu that is crucial for the development and survival of the fetus during pregnancy. However, ethical restrictions and a lack of mechanistic studies have excluded studies on embryo-endometrium interlinkage in PCOS patients. To address these problems several attempts are being made. Transgenic mouse models turned out to be used in understanding the mechanistic roles of many key determinants in uterine biology and implantation. Even so, the role of crucial genes remains undetermined because their constitutive deletion could lead to systemic deficiencies and embryonic lethality (Cha et al., 2012b). Additionally, the implantation phase of pregnancy is complex, and overlapping expression patterns make it challenging to mimic their stage-specific roles. Thereby, there exists a need for a model system wherein molecular events of early pregnancy could be studied with ease and dissection of pathways. Hence, in a study of the early pregnancy stage, rodent models have been employed as they exhibit similar anatomical and physiological features of pregnancy as humans (Adamson et al., 2002). Thereby, molecular changes in the materno-fetal interface were studied in the rodent model.

Our previous lab study has shown that 0.5 mg/kg/day of letrozole when administrated orally for 21 days, was adequate to induced PCOS in adult female Balb/c mice (Dey et al., 2022; Maharjan et al., 2010). Also, the preliminary study exhibited abnormal fetal growth and development in the letrozole-treated animals compared to the untreated animals. This

suggests that there could be some alteration in the early window of pregnancy that directs pregnancy outcomes. Thus, the current study was attempted to investigate the probable regulatory mechanism for the organization of early pregnancy events in a letrozole-induced PCOS mouse model.

3.2 Material and methods

The detailed methodology used in this study is explained in chapter 2. In brief, the PCOS mouse model was developed using letrozole and the pregnancy complications associated with PCOS pathology have been studied. The procedure for drug treatment regimens is provided in **Figures 3.1** and **3.2.**

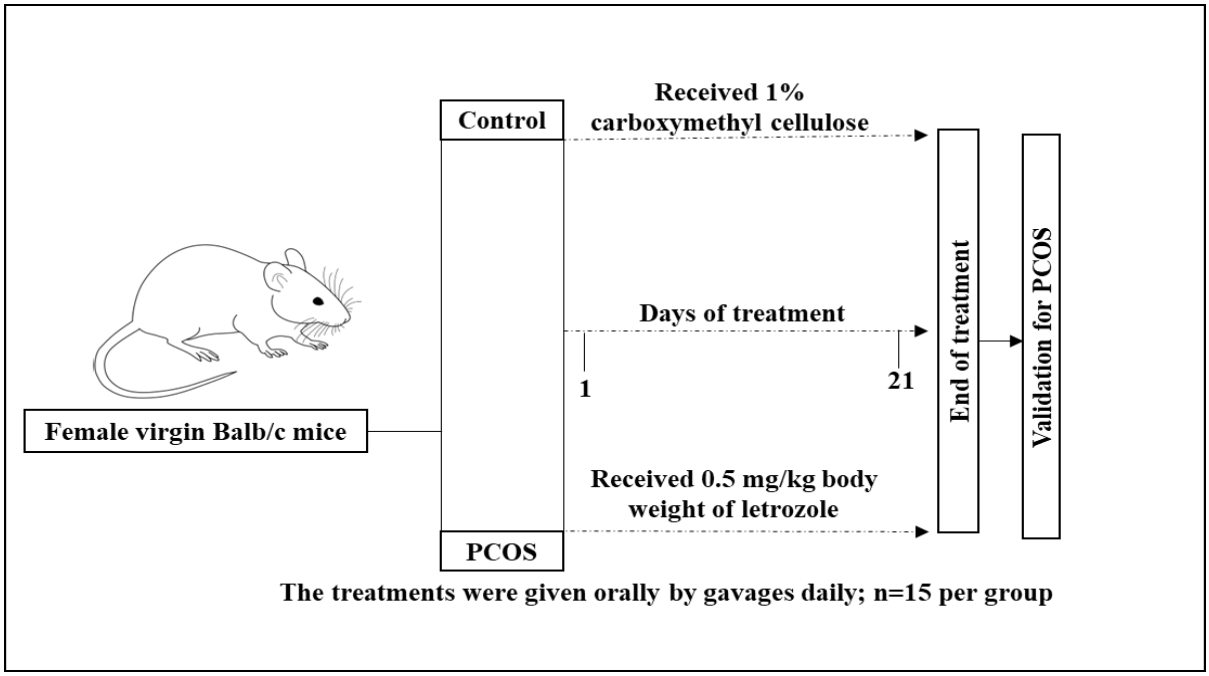


Figure 3.1. Plan of work for the development of the PCOS mouse model.

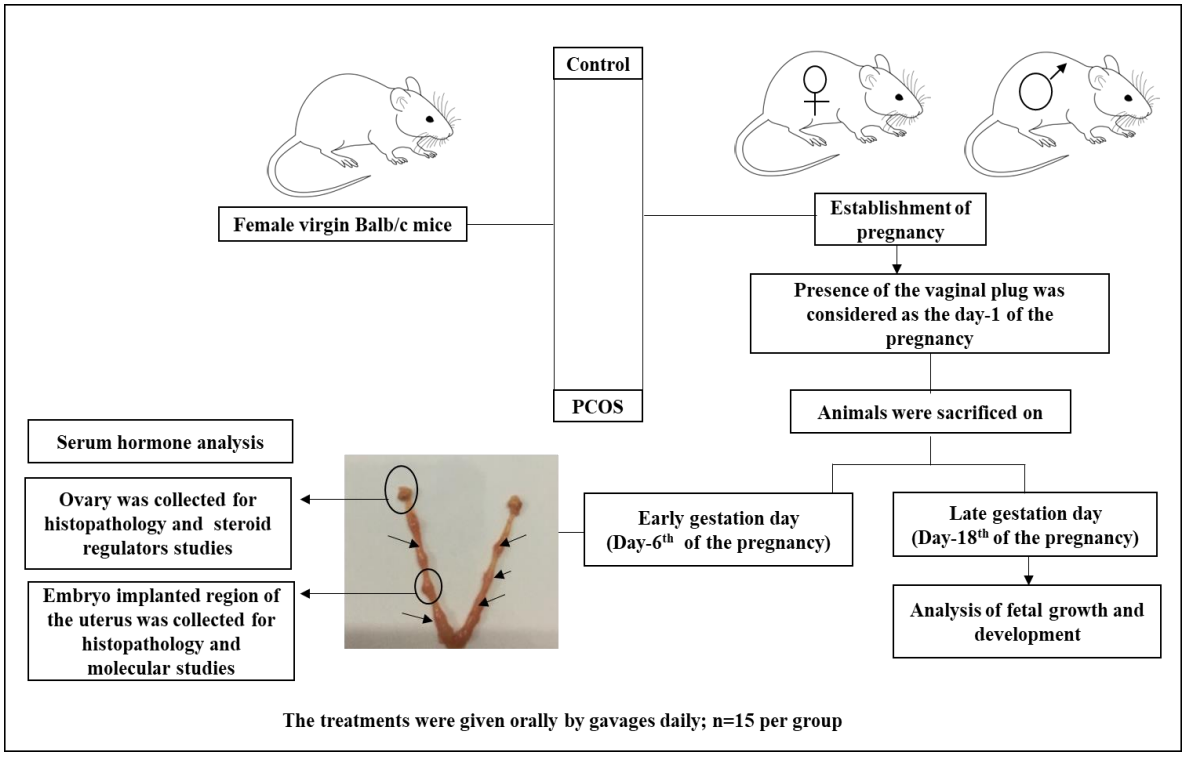


Figure 3.2. Plan of work for investigating the pregnancy loss of letrozole-induced PCOS mouse model.

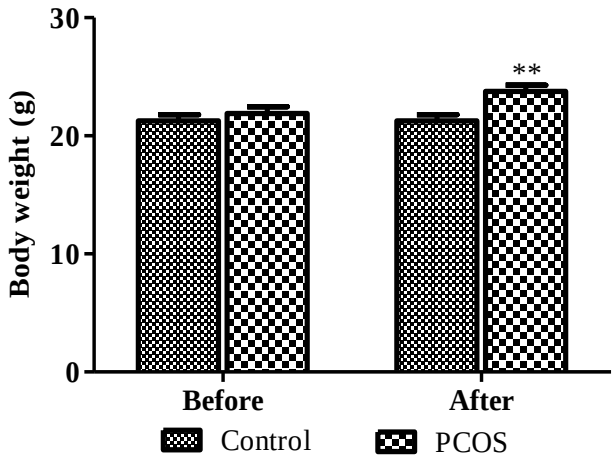
3.3 Results

3.3.1 PCOS induction in mice using letrozole

For the development of the PCOS mouse model, the animals were categorized into two groups (Control: Received 1% of carboxymethyl cellulose and PCOS: 0.5 mg/kg body weight of letrozole orally daily for 21 days). The detailed drug administration and experiment design have been provided in figures 3.1 and 3.2. After 21 days of treatment, the PCOS phenotype was validated according to Rotterdam's criteria (2003). The effect of letrozole treatment on body weight, estrus cyclicity, hormone profile, and ovarian histology was analysed. The figure 3.3a shows a significant increase (**P<0.01) in the body weight of the letrozole-treated animals as compared to day 0 of treatment. However, control animals did not show any difference. Further, to monitor the preliminary ovarian function, estrus cyclicity has been monitored in both groups. The control group of the animals exhibited a normal cycle while treatment with letrozole exhibited a disturbed estrus cycle (Figure 3.3b). One of the criteria for validation of PCOS is an elevation in serum testosterone levels. In this regard, the serum testosterone in the PCOS model group was significantly higher (**P<0.01) than that in the

control group, which can be correlated with hyperandrogenaemia in PCOS. Also, a decrease (***P<0.001) in progesterone content was observed in letrozole-treated mice whereas estradiol levels remained unchanged in both groups (Figure 3.3d). Since it is known that maintenance of the steroid milieu is vital for the ovarian structure and function, the histology profile of the ovary using a Haematoxylin-Eosin stain was carried out. In the untreated group, normal ovarian morphology (Matured follicles & corpora lutea) was observed. While PCOS group demonstrated reduced mature follicles, fewer corpus luteum and multiple large peripheral cysts as compared to control group, thus representing clinical features of PCOS development (Figure 3.3c).

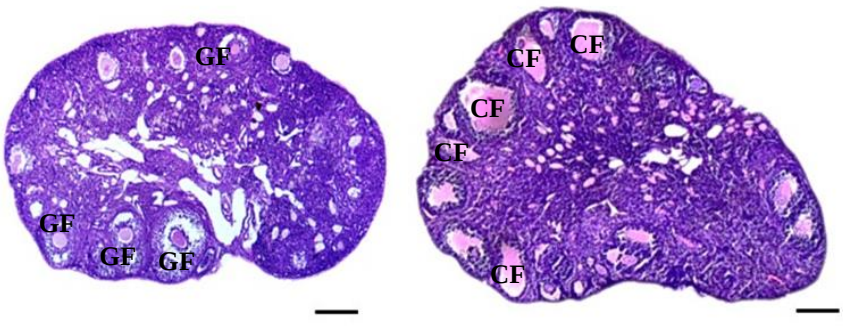
a



b

	Control	PCOS
Normal Cycle	100%	-
Extended Proestrus	-	-
Extended Estrus	-	-
Extended Metaestrus	-	35%
Extended Diestrus	-	65%

c



d

	Control	PCOS
Testosterone (ng/ml)	0.2650 ± 0.05334	0.5763 ± 0.06027**
Progesterone (ng/ml)	6.940 ± 0.6554	1.203 ± 0.2583***
Estradiol (pg/ml)	23.25 ± 1.306	25.00 ± 3.433 ^{ns}

Figure 3.3. Validation of PCOS phenotype in mice after 21 days of letrozole treatment. **a.** Change in body weight **b.** Estrus cycle profile **c.** Hematoxylin Eosin-stained ovarian section. Cystic follicle (CF); Graafian follicle (GF), magnification 4X. **d.** Hormone profile. All the values are represented as mean ± SEM; N = 6 per group; **P < 0.01; ***P < 0.001, ns not significant as compared to control group.

Data in the current study denoted that letrozole (0.5 mg/kg/day orally for 21 days) was able to generate PCOS-like features in the PCOS mouse model which is similar to the human clinical features of PCOS. This pathology has been associated with various pregnancy complications that have translated to poor fetal development and loss. Thus, after the development of the PCOS mouse model, pregnancy was established and fetal growth/number of pups was monitored during the late gestation period.

3.3.2 Fetal growth and development during the late gestation period in PCOS mice

After the induction of PCOS, female mice from both groups were kept for mating, and the presence of a vaginal plug was considered as the day 1st of pregnancy. Further, the animals were sacrificed on the day-18th of pregnancy. The detailed plan of work has been provided in figures 3.1 and 3.2. The body weight of animals throughout the gestation period was monitored and the number, weight, and morphology of fetuses have been examined. The body weight of letrozole-treated animals was found to be gradually decreased during the late gestation period compared to the control (Figure 3.4a). Moreover, the control group of animals showed a healthy growing fetus on the day-18th of pregnancy while the letrozole-treated animals exhibited retarded fetal growth, and development with the decreased (**P<0.01) weight of growing fetuses compared to the control animals (Figure 3.4 b, c, d).

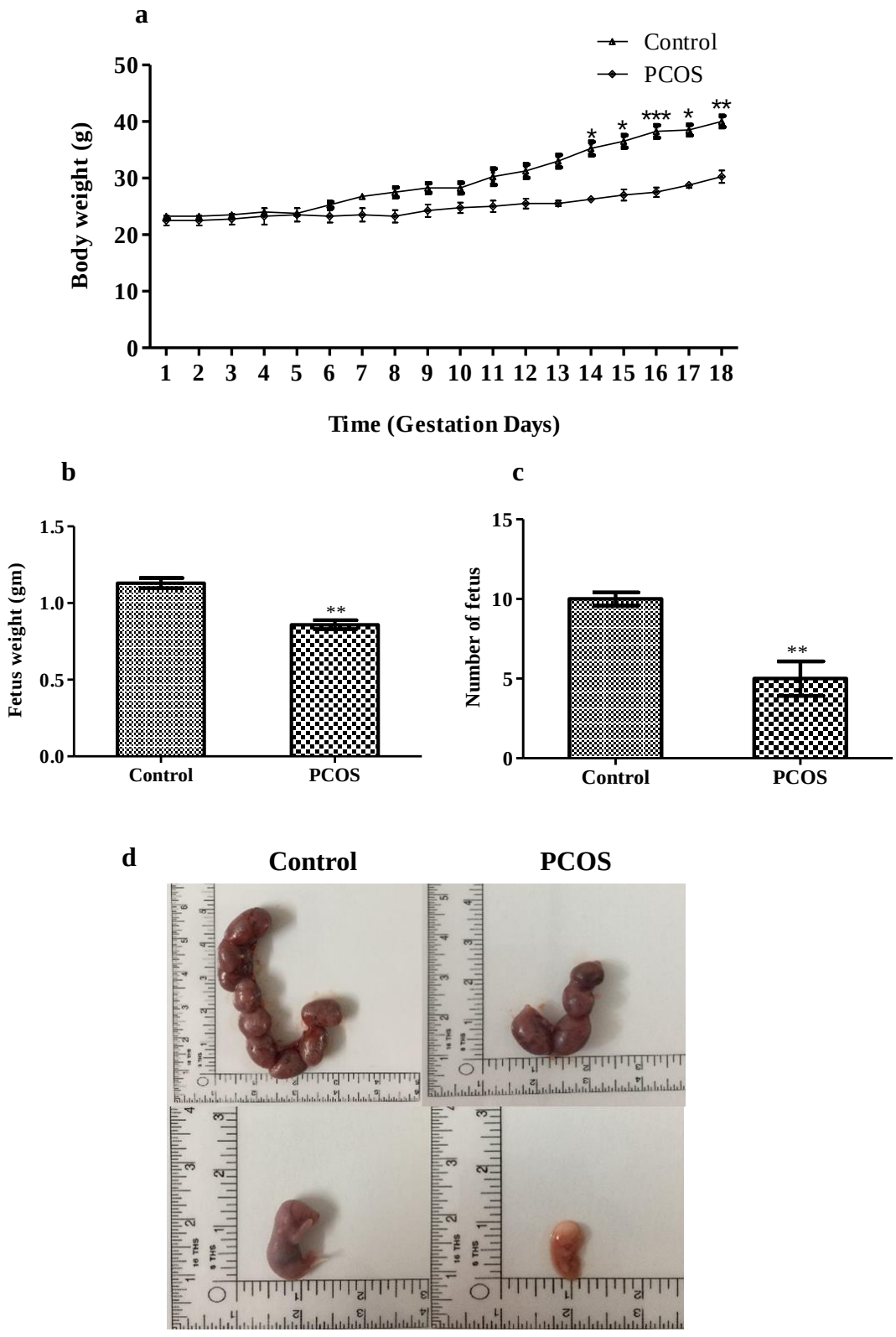


Figure 3.4. Effect of letrozole on the day 18th of pregnancy. **a.** Changes in the body weight during the gestation period **b.** Fetus weight **c.** The number of fetuses/pups delivered to animals **d.** Pictorial representation of uterus with growing fetuses/pups. All the values are

represented as mean ± SEM; N = 6 per group; **P < 0.01; ***P < 0.001, ns not significant as compared to the control group.

The late gestation studies in the letrozole-induced mouse model have clearly indicated abnormal pregnancy outcomes. This suggests that there could be some alteration in the early gestation events that direct proper fetal outcomes by numerous molecular pathways. Therefore, the present study attempts to prove the probable regulatory mechanism in the establishment of early pregnancy events of a letrozole-induced PCOS mouse model. After the induction of PCOS, female mice from both groups were kept for mating, and the presence of a vaginal plug was considered as the day 1^{st} of pregnancy. Further, the animals were sacrificed on the day-6^{th} of pregnancy (The plan of work is given in Figures 3.1 and 3.2). Initially, the body weight, hormone profile, blood glucose levels, and ovarian histology were studied. Further, to study the early pregnancy outcomes, reproductive performance, number, and histology of embryo implanted region of the uterus were analysed. The results of early gestation studies are explained below.

3.3.3 Early pregnancy loss (Day-6^{th} of pregnancy) in PCOS mice

Since it is known that steroid hormones and ovarian function are vital for the establishment of pregnancy, systemic steroid (Testosterone, progesterone, and estradiol) levels and ovarian histology were analyzed on day 6 of pregnancy. As shown in Table 3.1**,** the serum testosterone content in the PCOS animals was significantly elevated than that in the control group (**P<0.01), which can be correlated with hyperandrogenaemia in PCOS. Additionally, a decline in progesterone levels was observed in PCOS mice (***P<0.001) whereas estradiol content remain unaltered in both groups on day 6 of pregnancy. In the histology profile of the ovary, the untreated (control) group showed normal ovarian morphology (Matured, tertiary and Graafian follicles & corpora lutea). PCOS animals demonstrated reduced mature follicles, fewer corpus luteum and multiple large peripheral cysts as compared to control group (Figure 3.5). In addition, PCOS is associated with hyperinsulinemia, hyperglycaemia, and an increase in body weight. Thus, insulin, random blood glucose levels, and body weight have been monitored. The enhanced insulin (**P<0.01) and blood glucose levels (*P<0.05) were observed in the letrozole-treated animals with no difference in the body weight during the early gestation period (Day 1 to 6 of pregnancy) (Figure 3.6).

Further, to study the early pregnancy complications, reproductive performance, number, and histology of implanted region of the uterus were analyzed. It was observed that the number of

pregnant females was reduced in the letrozole-induced PCOS mouse model Table 3.2. Also, a fewer number of embryo implants were observed in PCOS animals compared to control animals (**P<0.01). Figure 3.7a demonstrates the pictorial representation of implanted embryos in the uterus of both groups. Histology of implanted region of the uterus demonstrates the embryo has attached to the antimesometrial uterine lumen epithelium (marked by black arrows) and is surrounded by developing decidual cells in the control group. However, the PCOS group exhibited an accumulation of erythrocytes (marked by a black arrow) caused by a gain in vascular permeability (Figure 3.7b).

Table 3.1. Serum hormone levels on the day 6th of pregnancy.

	Control	**PCOS**
Testosterone (ng/ml)	0.7020 ± 0.04903	1.240 ± 0.1435 **
Progesterone (ng/ml)	58.25 ± 4.029	16.00 ± 1.826 ***
Estradiol (pg/ml)	73.50 ± 5.377	83.00 ± 4.655 ^{ns}

The values are represented as Mean ± SEM. N=6 per group. **P<0.01, ***P<0.001, ns-not significant as compared to Control.

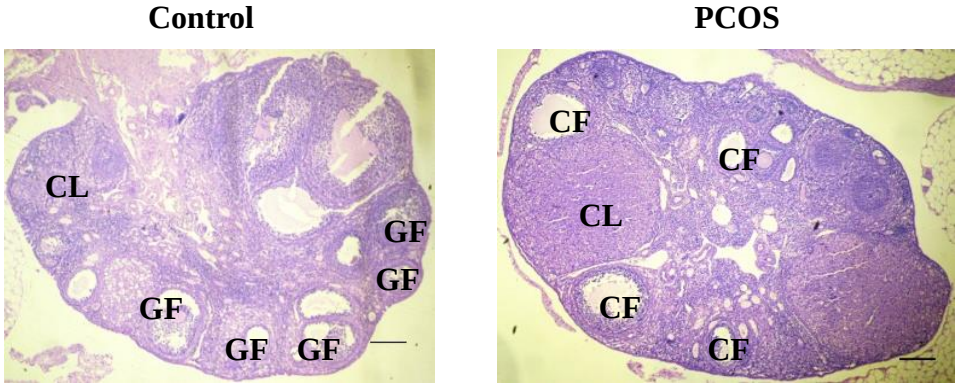


Figure 3.5. Effect of letrozole on the ovarian histology, Hematoxylin, and eosin-stained sections of the ovary on the day 6th of pregnancy. Corpus luteum (CL), Cystic follicle (CF), Graafian follicle (GF), magnification 4X.

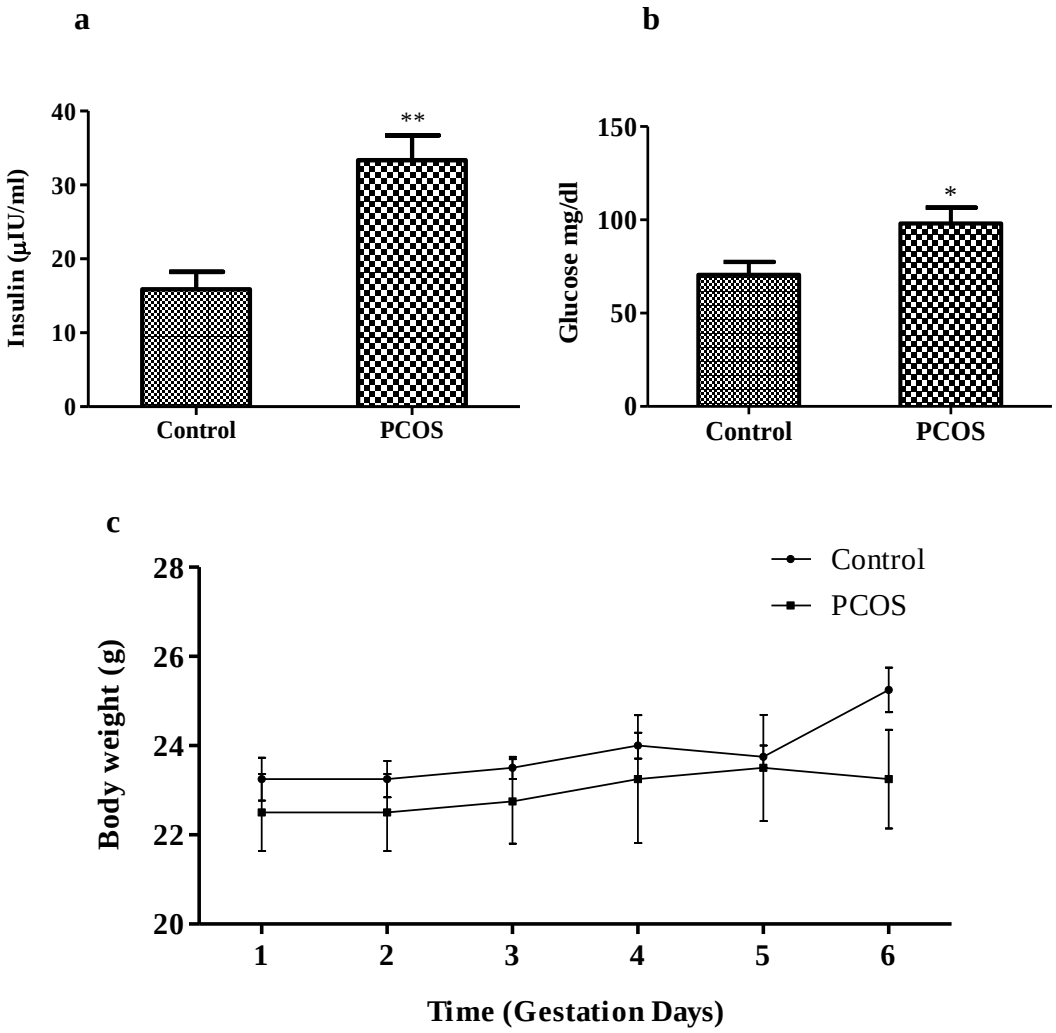
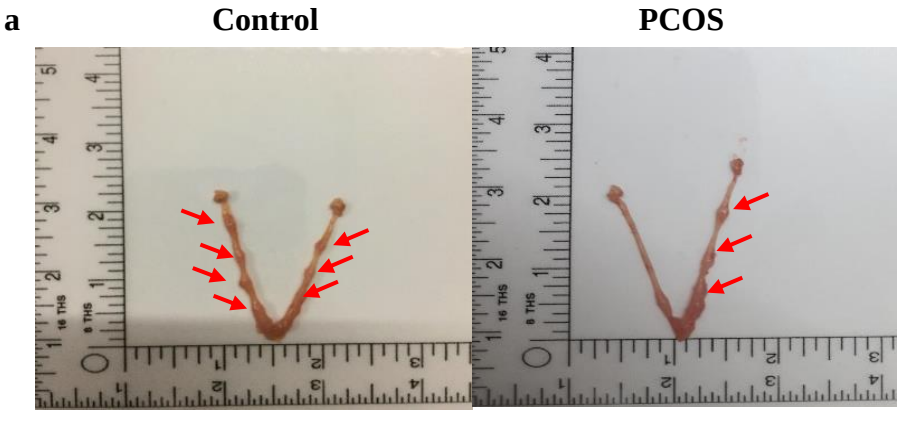


Figure 3.6. Effect of letrozole on the serum **a.** Insulin levels **b.** Random blood glucose and **c.** Changes in body weight during the early gestation period. All the values are represented as mean ± SEM; N = 6 per group; *P<0.05, **P<0.01, ns not significant as compared to the control group.



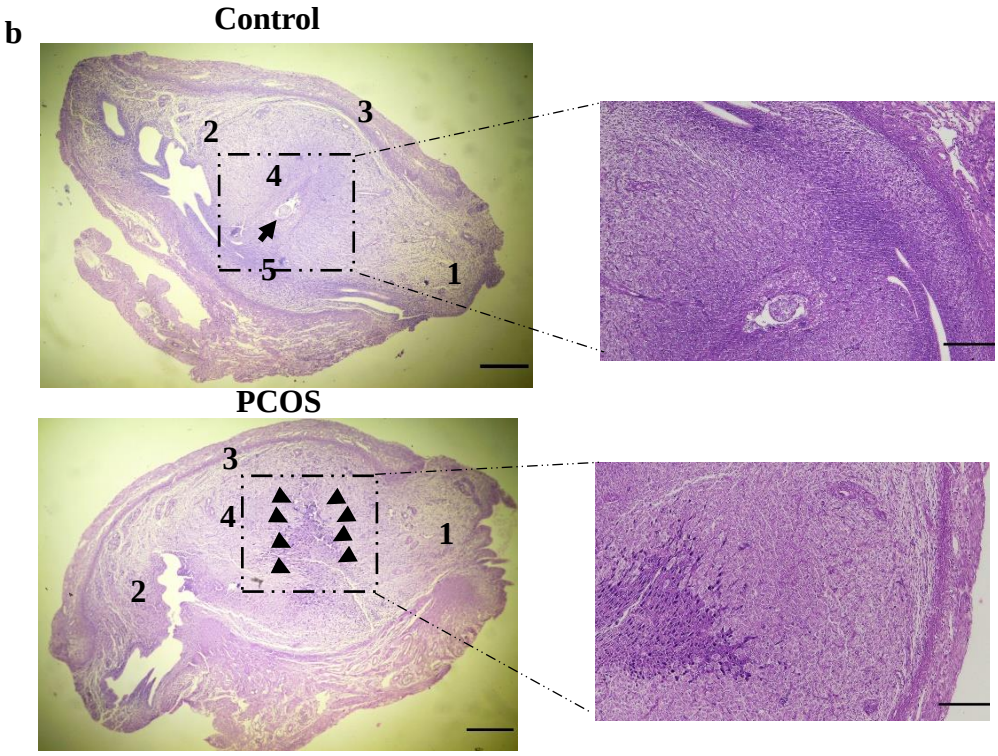


Figure 3.7. Effect of letrozole on the embryo implanted region of the uterus. **a.** Pictorial representation of a number of implanted sites; Arrows indicate the implanting embryo. **b.** Hematoxylin and eosin-stained sections of the embryo implanted region of the uterus; in the control group, the black arrow indicates embryo and in the PCOS group, black arrows indicate accumulation of erythrocytes. 1. Mesometrium. 2. Mesometrial endometrium. 3. Myometrium. 4. Anti-mesometrial decidua. 5. Embryo. Scale bar = 100μm.

Table 3.2. Reproductive performances for female fertility.

	Control	**PCOS**
Females (n)	14	14
Mated females (n)	13	12
Pregnant females (n)	12	8
Not pregnant females (n)	1	4
Time required for conception (in days)	3.769 ± 0.2809	3.308 ± 0.3279 ^{ns}
Mating index* (%)	92.85714 (~93)	85.7248 (~86)
Fertility index** (%)	85.71429 (~86)	57.14286 (~57)
Total number of implants	9.000 ± 0.3162	5.800 ± 1.020 *

The values are represented as Mean ± SEM. *P<0.05, ns = not significant as compared to the control group. *Mating index = Mated females/Total females kept for mating × 100. **Fertility index = Pregnant females/Total females kept for mating females × 100.

Till now the results signify the effect of letrozole on female reproductive dysfunction and early pregnancy loss. Also, abnormal ovarian structure and steroid hormone production was observed on the day-6th of pregnancy. As the ovary synthesizes steroids, the observed changes might be originated from the molecular changes in the ovary. Therefore, in the next part of the study, key regulators of ovarian steroid production have been investigated.

3.3.4 Altered ovarian gonadotropin receptors on the day 6th of pregnancy in PCOS mice

Luteinizing and follicle-stimulating hormone (Gonadotropins) act on the ovaries by their receptors, luteinizing hormone receptors (LHR), and follicle-stimulating hormone receptor (FSHR), respectively. Hence, gonadotropin receptor transcript levels were studied in the ovary using quantitative real-time PCR. Transcriptional upregulation of *Lhr* (*P<0.05) was observed in the PCOS group compared to the control group with no difference in *Fshr* in both groups (Figure 3.8).

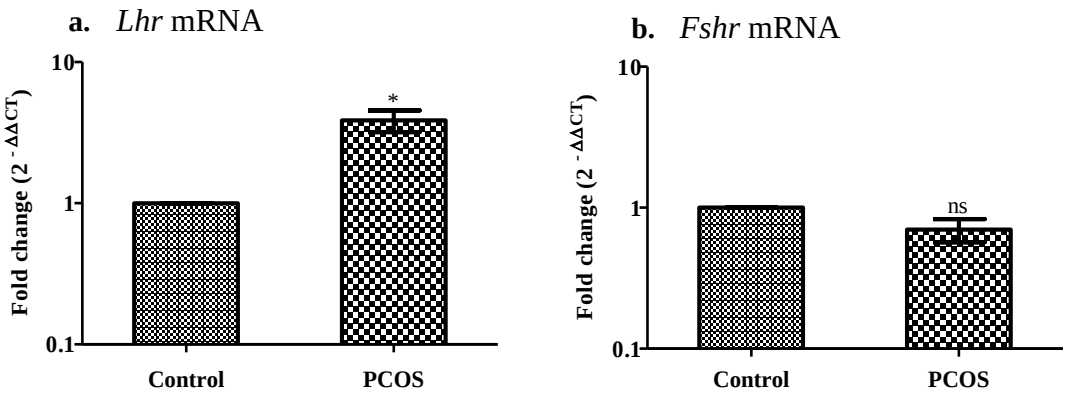


Figure 3.8. Gonadotropin receptors in the ovary. Values are mean fold changes in gene expression, **a.** Luteinizing hormone receptor **b.** Follicle-stimulating hormone receptor in the letrozole-induced PCOS mice model. Error bars represent SEM; N=6 per group. *P<0.05, ns-not significant as compared to the control group.

3.3.5 Impairment of ovarian steroidogenesis on the day 6th of pregnancy in PCOS mice

The binding of the LH and FSH to their receptors results in the stimulation of signalling pathway to enhance steroid synthesis in the ovaries. The key gene encoding proteins of

steroidogenesis, Steroidogenic Acute Regulatory Protein (*Star*), cytochrome P450-17α-hydroxylase/C17, 20-lyase (*Cyp17a1*), and cytochrome P450 aromatase (*Cyp19a1*) were evaluated in the ovaries. When analysed for gene expression, *Star* (**P<0.01) and *Cyp17a1* (*P<0.05) were found remarkably high in letrozole-treated animals when compared with control animals. On contrary, *Cyp19a1* (**P<0.01) was downregulated in the PCOS ovary compared to the control group (Figure 3.9).

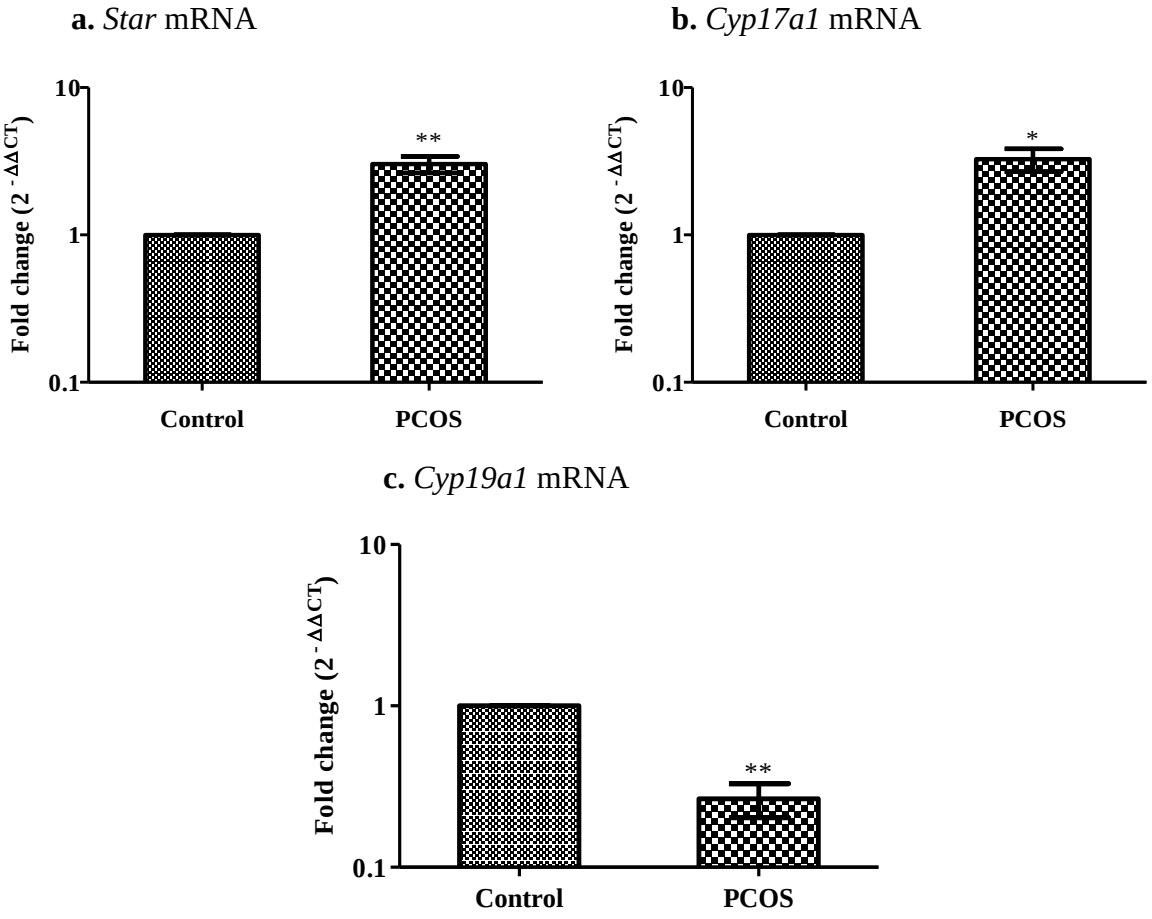
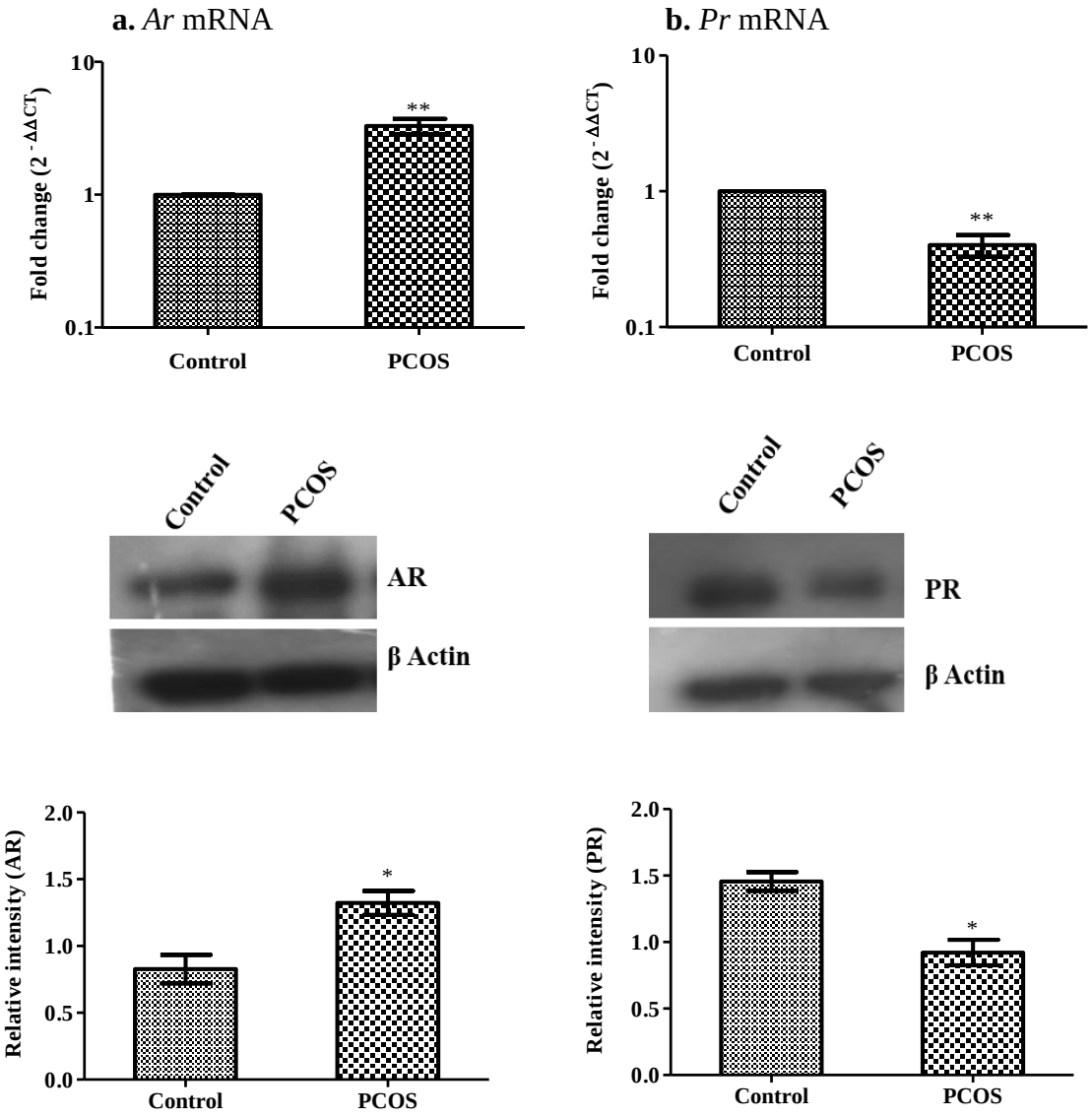


Figure 3.9. Key mediators of steroidogenesis in the ovary. Values are mean fold changes in gene expression, **a.** Steroidogenic Acute Regulatory Protein **b.** Cytochrome P450 17α-hydroxylase/C17, 20-lyase **c.** Cytochrome P450 aromatase in the letrozole-induced PCOS mice model. Error bars represent SEM; N=6 per group. *P<0.05, **P<0.01 as compared to the control group.

3.3.6 Modulation of ovarian steroid receptors on the day 6th of pregnancy in PCOS mice

Steroids like androgen, progesterone, and estrogen mediate their autocrine effect through their receptors, progesterone receptor (*Pgr*), androgen receptor (*Ar*) and estrogen receptors α

& β (*Esr-1* & *2*) respectively. The mRNA expression pattern of *Ar* (**P<0.01) was significantly elevated in the letrozole-treated animals as compared to untreated animals. On the contrary, transcriptional downregulation of *Pgr* (**P<0.01) was observed. These modulations were correlated with the difference in protein expression. Further, gene expression of *Esr1* (*P<0.05), and *Esr2* (**P<0.01) was reduced in PCOS compared to the control ovary while protein expression of estrogen receptors did not show any difference in both groups (Figure 3.10).



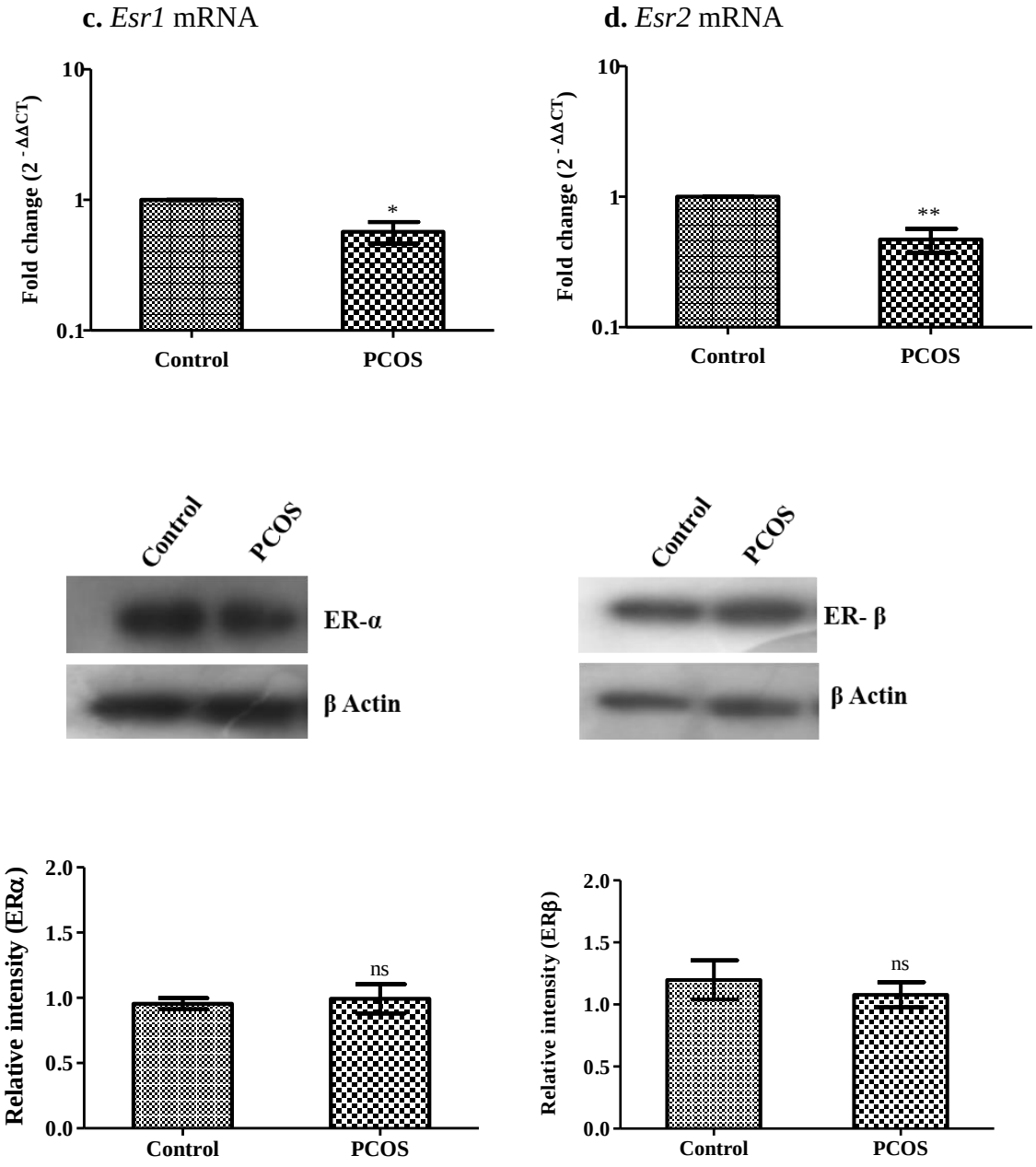
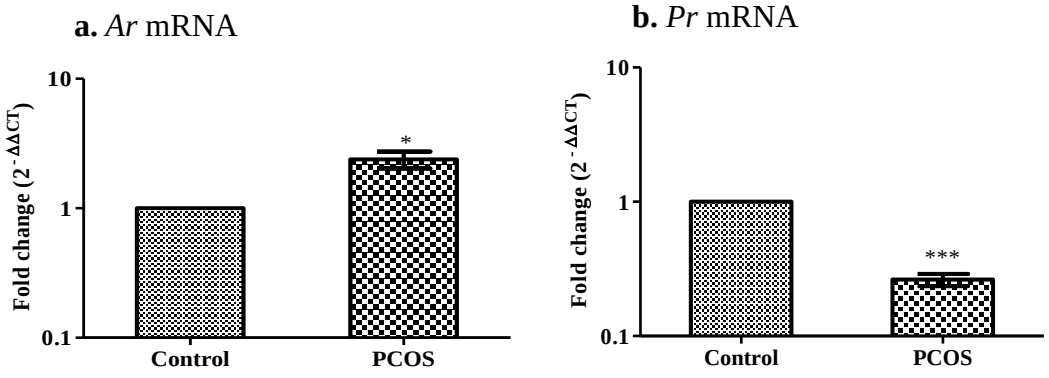


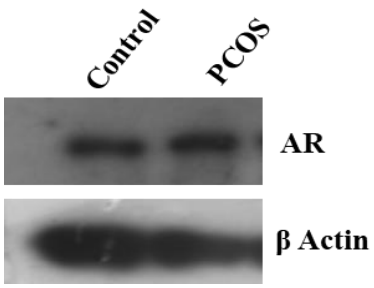
Figure 3.10. Steroid hormone receptors in the ovary. The upper graph represents values of mean fold changes in gene expression and the lower graph represents relative intensities of bands obtained on western blots **a.** Androgen receptor **b.** Progesterone receptor **c.** Estrogen receptor-α **d.** Estrogen receptor-β in the letrozole-induced PCOS mice model. Error bars represent SEM; N=6 per group. * P<0.05, **P<0.01, ns-non significant as compared to the control group.

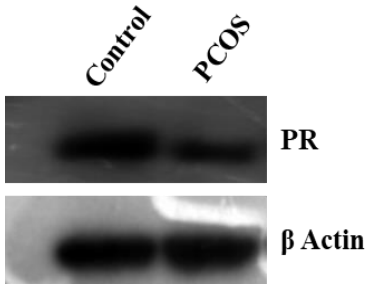
The observed changes in the transcript level of ovarian steroid mediators could be co-related with abnormal hormone profiles and their aberrant folliculogenesis in the PCOS animals. Further, steroid hormones exert their effect through their receptors and regulate the uterine downstream pathways in the establishment of pregnancy. Thus, the complex mechanistic signalling of early pregnancy was investigated in a letrozole-induced PCOS mouse model. The plan of work is given in the figure 3.1 and 3.2 and the results are explained below.

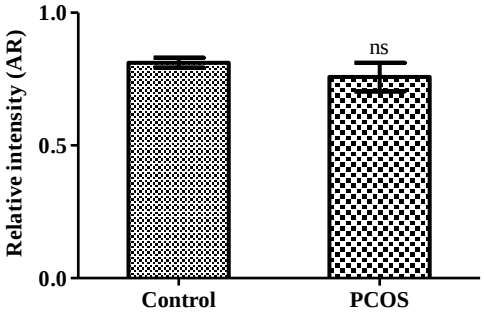
3.3.7 Altered steroid hormone receptor in the embryo implanted region of the uterus in PCOS mice

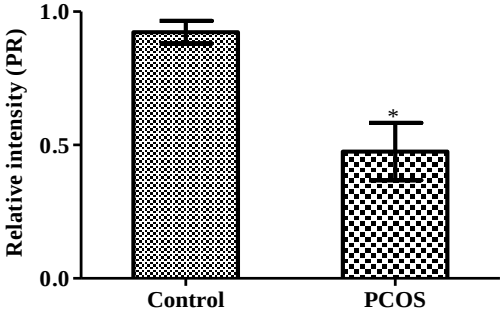
To accomplish a sequential event of pregnancy, the endometrium requires to undergo steroid-dependent changes. Steroids like estrogen, progesterone, and testosterone mediate their effect through their receptors like estrogen receptors α & β (*Esr-1* & *2*), progesterone receptor (*Pgr*), and androgen receptor (*Ar*), respectively. Hence, in the implanted region of the uterus, transcript and protein expression of steroid receptor were done. Both, gene and protein expression of *Pgr* were downregulated (***P<0.001). The transcript level of *Esr1* (**P<0.01), and *Esr2* (***P<0.001) was decreased in PCOS animals while their protein expression was found to be unchanged. On the contrary, mRNA levels of *Ar* (* P<0.05) were found markedly high in the implanted site of the uterus in PCOS animals with no difference in protein expression when compared with control tissues (Figure 3.11).





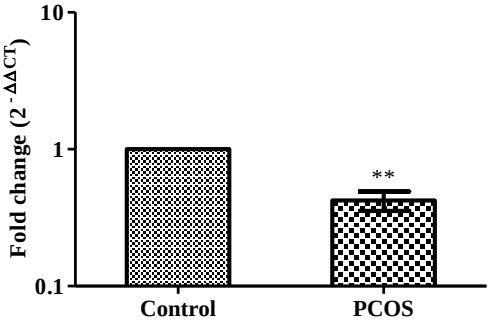


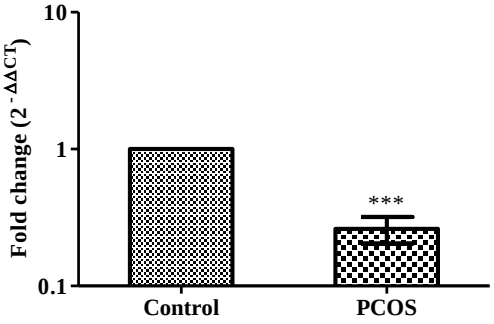


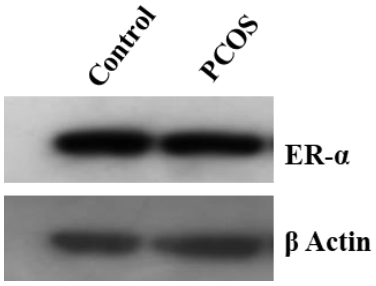


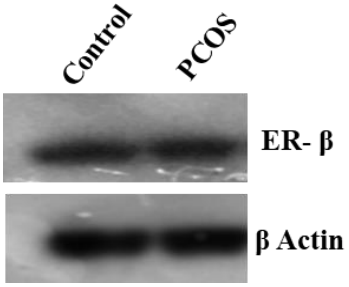
c. *Esr1* mRNA

d. *Esr2* mRNA









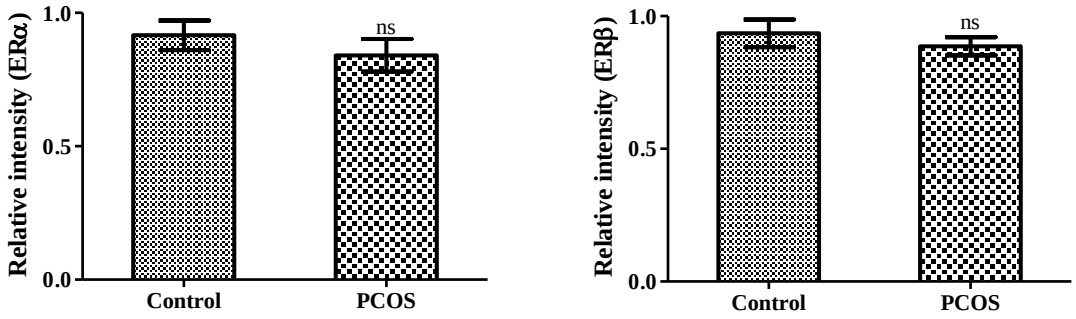


Figure 3.11. Steroid hormone receptors in the embryo implanted region of the uterus. The upper graph represents values of mean fold changes in gene expression and the lower graph represents relative intensities of bands obtained on western blots **a.** Androgen receptor **b.** Progesterone receptor **c.** Estrogen receptor-α **d.** Estrogen receptor-β in the letrozole-induced PCOS mice model. Error bars represent SEM; N=6 per group. * P<0.05, **P<0.01, ***P<0.001, ns-non significant as compared to the control group.

3.3.8 Impairment of feto-maternal interaction in the embryo-implanted region of the uterus in PCOS mice

Subsequently, integrin, α4β1 (*Itga4, Itgb1*) are known to involve in embryo-endometrium interaction in the establishment of a healthy pregnancy. Hence, the transcript levels of these markers were evaluated in the implanted region of the uterus. When analysed for gene expression, *Itga4* and *Itgb1* (***P<0.001) declined in the PCOS group compared to the control group (Figure 3.12).

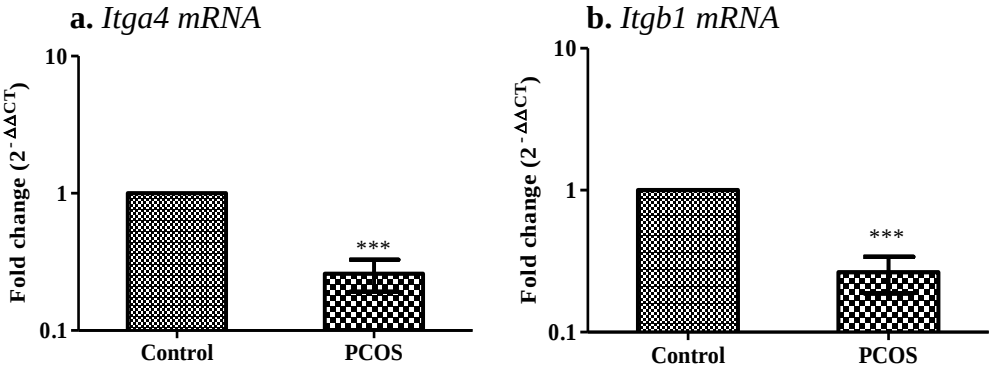


Figure 3.12. Integrin gene expression in the embryo implanted region of the uterus. Values are mean fold changes in gene expression, **a.** Integrin- α4 **b.** Integrin- β1 in the letrozole

induced PCOS mice model. Error bars represent SEM; N=6 per group. ***P<0.001 as compared to the control group.

3.3.9 Modulation in decidualization of embryo implanted region of the uterus in PCOS mice

Transcription factors, such as homeobox genes (*Hox10a)* are known to involve in the decidualization of uterine stroma to complete the early stage of pregnancy. Hence, the transcript levels of this marker were evaluated in the implanted site of the uterus in the letrozole-induced PCOS mouse model. When analyzed for gene expression, *Hox10a* (**P<0.01) showed a decline in the implanted region of the uterus in the PCOS group compared to the control group (Figure 3.13).

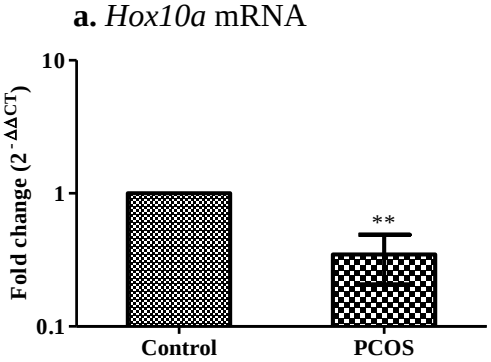


Figure 3.13. Transcription factor gene expression in the embryo implanted region of the uterus. Values are mean fold changes in gene expression, **a.** Homeobox-10A in the letrozole-induced PCOS mice model. Error bars represent SEM; N=6 per group. **P<0.01 as compared to the control group.

3.3.10 Imbalance in the expression of matrix metalloproteinases and their endogenous inhibitor in the embryo implanted region of the uterus in PCOS mice

Invasiveness of the embryo to receptive endometrium required extensive degradation and remodelling of the extracellular matrix (ECM). Matrix metalloproteinases (*Mmp2* & *9*) are responsible for the breakdown of ECM during the implantation process. The activity of MMPs is firmly regulated by their endogenous inhibitors, TIMPs (Tissue inhibitors of MMPs. When analyzed for the transcript levels and activity of MMPs, gene expression (**P<0.01) and activity (*P<0.05) of MMP-9 were decreased in the implanted region of the uterus in PCOS animals compared to the control group. However, the activity of MMP-2 did not show any change in both groups. Gene expression of *Timp1* (**P<0.01) and *Timp3*

(***P<0.001) were reduced in the embryo implanted region of the uterus in the PCOS group compared to the control group (Figure 3.14).

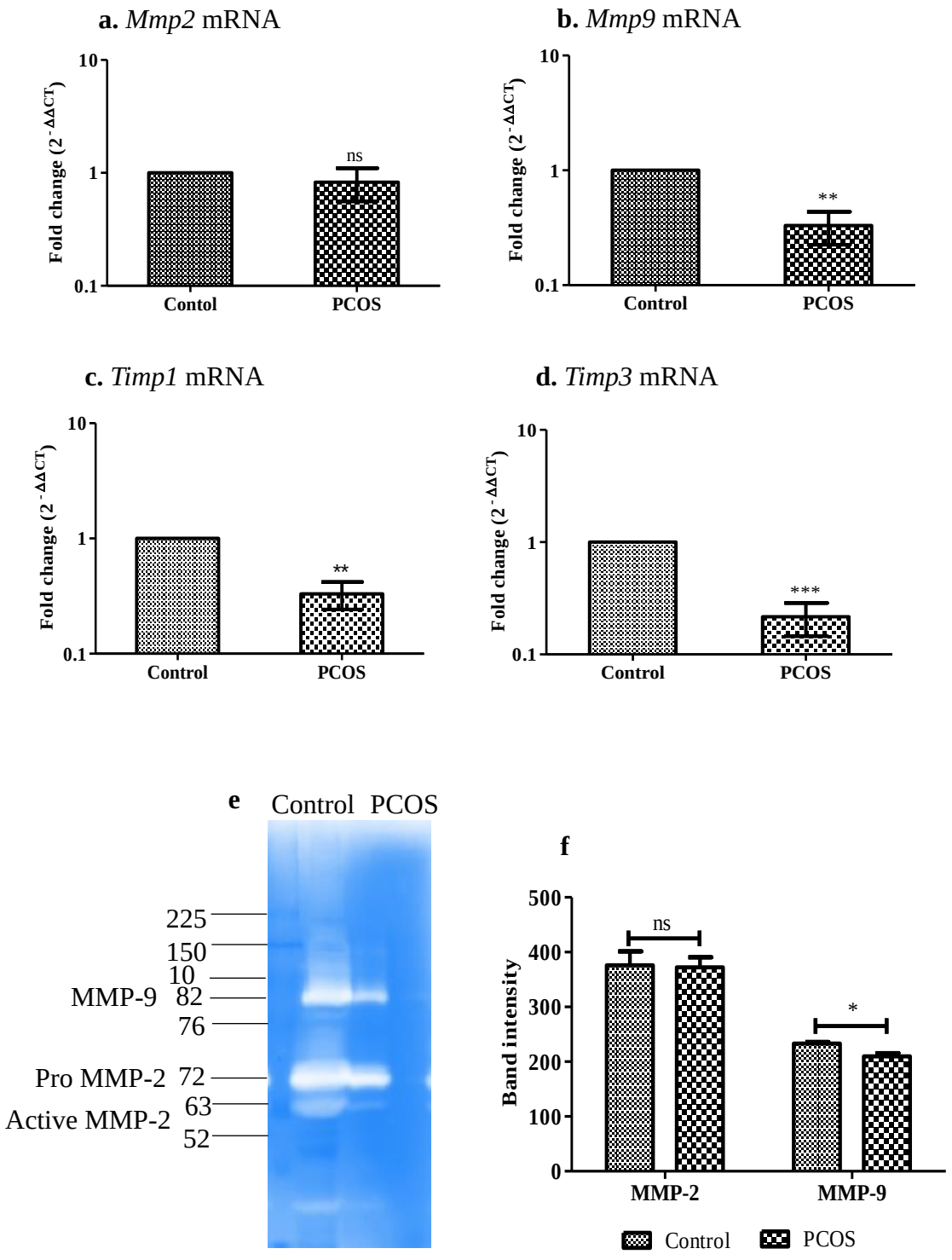


Figure 3.14. Matrix metalloproteinases and their inhibitors in the embryo-implanted region of the uterus. Values are mean fold changes in gene expression, **a.** MMP-2 **b.** MMP-9 **c.** TIMP-1 **d.** TIMP-3; Error bars represent SEM **P<0.01, ***P<0.001 as compared to the

control group **e.** Gelatin gel zymograms showing pro-MMP2, active MMP2, and MMP9 activity (arrows) in the letrozole-induced PCOS mice model **f.** Quantification of total (Pro and active) MMP9 and MMP2 by computer-based densitometry analysis; Error bars represent mean ± SEM; N=6 per group. *P<0.05 and ns-non-significant as compared to the control group.

3.3.11 Disruption of the LIF-STAT3 signalling pathway in the embryo implanted region of the uterus in PCOS mice

A tightly controlled rhythm between uterine maturation and embryonic development is necessary for a successful pregnancy. This function is mediated through the cytokine on their receptors, mainly by the LIF-STAT pathway. When analyzed for key mediators of the pathway, the transcript level of glycoprotein 130 (*Gp130*) (**P<0.01) and Signal transducer and activator of transcription 3 (*Stat3*) (***P<0.001) were declined in the PCOS animal as compared to control group; However, no difference was observed in leukemia inhibitory factor (*Lif*) and leukemia inhibitory factor receptor levels (*Lifr*) (Figure 3.15).

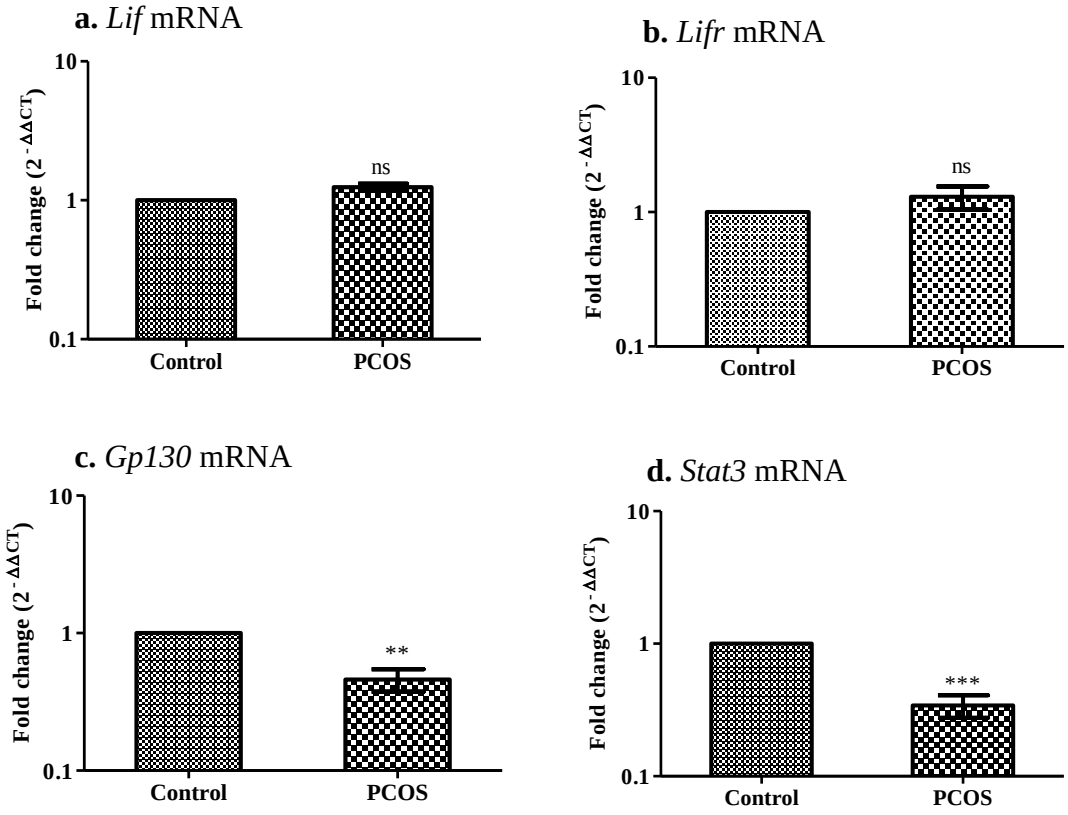


Figure 3.15. Key mediators of LIF-STAT3-related genes in the embryo implanted region of the uterus. Values are mean fold changes in gene expression, **a.** Leukemia inhibitory factor **b.** Leukemia inhibitory factor receptor **c.** Glycoprotein 130 **d.** Signal transducer and activator of transcription 3 in the letrozole-induced PCOS mice model. Error bars represent SEM; N=6 per group. **P<0.01, ***P<0.001, ns-non-significant as compared to control group.

Overall, the results demonstrated that disturbed ovarian steroidogenesis could further affect the steroid-responsive pathways during early gestation events in the letrozole-induced PCOS mouse model; which further has an impact on abnormal fetal outcomes.

3.4 Discussion

In PCOS pathology, pregnancy has been implicated with several complications that have translated as poor fetal growth and loss (Zhang et al., 2020). Along the same lines, the current study also exhibited retarded fetal growth and a lesser number of pups were born to PCOS animals. In this context, early gestational events are found to be the important window that directs proper fetal growth. Currently, a great deal has been brought out in the domain of assisted reproductive technology (ART) and its approach could assist numerous infertile couples to have their babies. However, a major issue with this approach is the low implantation rate after several transfers of good-quality of embryos (Somigliana et al., 2018). Also, it was examined that alterations in oocytes and embryos could be responsible for abortive outcomes in PCOS patients who are undergoing assisted reproduction (Qiao & Feng, 2011b).

Still, it could not be concluded whether the reduced pregnancy rates seen are due to abnormal embryos which fail to implant or whether there are some modifications in the endometrium which do not allow implantation (Shang et al., 2012b). Hence, it is possible that abnormalities in embryos and uterine independently or in coordination may lead to a reduced pregnancy rate in women with PCOS. However, there are not sufficient pieces of evidence in the context of molecular alterations in the embryo-containing uterine microenvironment. Thereby, this study emphasizes the molecular players that are important for the maintenance of embryo-uterine transmission for proper embryo development. Further, targeting them for therapeutic interventions could help us in the management of early pregnancy complications linked to PCOS pathology. Hence, the present study attempted to decode the complex mechanistic signalling of early pregnancy in a letrozole-induced PCOS mouse model.

In a previous lab study, it was reported that oral administration of letrozole (0.5 mg/kg body weight) daily for 21 days exhibits reproductive and metabolic alteration signs similar to the human PCOS condition (Dey et al., 2022). In the current experiment, letrozole-induced female Balb/c mice showed elevated testosterone levels during early pregnancy. It could be correlated with the ability of an aromatase inhibitor (Letrozole) in the development of PCOS in rodents; leading to low conversion of androgens to estrogens, followed by an excessive cumulation of androgens in the ovary (Kafali et al., 2004). In addition, low levels of progesterone were observed, which can be associated with the disruption of corpus luteum formation in PCOS animals. Similar observations were noted wherein PCOS patients are not able to form corpus luteum which causes a low level of progesterone, thus resulting in abnormal ovulation (Huang et al., 2016). Interestingly, circulating estrogen did not change in both groups, which is in accordance with studies reported in the letrozole-induced mouse model (Kauffman et al., 2015). Additionally, PCOS animals show elevated insulin and glucose levels. Similar observations have been reported in previous lab studies (Dey et al., 2022; Desai et al., 2012; Maharjan et al., 2010a). The consequence of hyperinsulinemia and insulin resistance in PCOS pathology is very well documented. The excess insulin is known to arrest the proliferation of granulosa cells and subsequent follicle growth (Goodarzi et al., 2011) which further, promotes the hypersecretion of ovarian androgen by modulation of steroidogenesis (Diamanti-Kandarakis & Dunaif, 2012).

The altered hormone profile in PCOS animals influences the ovarian structure, when examined for ovarian histology, the number of peripheral cysts was observed, which is one of the characteristic features of PCOS (Fox et al., 1991). The alteration in steroid hormone levels could result from the hyperactivation of steroidogenesis in the PCOS ovaries. When analysed for the steroidogenic modulators, the overexpression of luteinizing hormone receptors (*Lhr*), steroidogenic acute regulatory protein (*Star*), and cytochrome P450-17α-hydroxylase/C17, 20-lyase (*Cyp17a1*) was observed. However, cytochrome P450 aromatase (*Cyp19a1*) expression was reduced in the ovary. These results indicated that ovarian dysfunction in PCOS is a cumulative result of excess androgen with a poor progestogenic response. It is very well known from previous lab studies and other reports that hyper steroidogenic activity in the ovaries is liked to disturb intra-ovarian function that synchronizes the steroid hormone secretion in the ovary (Maharjan et al., 2010; Dey et al., 2022; Balen, 2004). Further, the autocrine role of steroid hormones in ovarian function has been reported (Drummond, 2006). The findings noted that the intraovarian progesterone and androgen receptors help in follicle

maturation and successful ovulation (Lydon et al., 1995; Drummond, 2006). Thus, the overexpression of androgen receptors and decreased progesterone receptors in the ovary could extensively affect ovarian follicle maturation, as seen in our histopathological analysis. This evidence points out the continuation of ovarian dysfunction during the early gestation period in PCOS animals.

Moreover, reports have shown that hyperandrogenism and low progesterone content in women with PCOS have a lower probability of childbirth, decreased pregnancy rates, and higher miscarriage rates (Elenis et al., 2021; Su et al., 2017; Gaggiotti-Marre et al., 2019). Indistinguishable, results were noticed in our study, the number of pregnant females and the implanting embryos in the uterus were significantly reduced in the PCOS animals. Altogether indicate that an altered hormone production could modulate the growth and survival of a fetus in PCOS pathology. It is interesting to note that the above-cited reports have not clearly defined the molecular interplay of the reduced pregnancy outcomes seen in PCOS infertility. Hence, the present study attempts to further narrow down molecular deficits of pregnancy loss the in PCOS phenotype.

In the embryo-implanted region of the uterus, steroid hormones mediate their effect through their receptors in the establishment of pregnancy. When examined for the expression of steroid hormone receptors in the implanted region of the uterus on the day 6^{th} of pregnancy, PCOS animals exhibited declined PGR expression. Progesterone signalling is known to have an inhibitory effect on the E/ESR signalling pathway in stromal cells of the endometrium for the establishment of pregnancy (Wetendorf & DeMayo, 2014). Thus, it can be noted that in our experimental model, the decline in P/PGR signalling did not show an inhibitory effect on estrogen, resulting in no difference in the estrogen-responsive gene (leukemia inhibitory factor) has been observed. Furthermore, it was reported that in ovariectomized mice, the progesterone signalling inhibited androgen receptor expression, whereas estrogen greatly enhanced AR abundance in the stroma of uteri during early pregnancy (Li et al., 2014). In this direction, the current study revealed that altered P/PGR signalling in PCOS animals did not have a prohibited influence on AR expression, as the overexpression of AR was observed in the implanted region of the uterus. Hence, the possibility of the observed changes in the steroid receptor expression could be due to the reduced progesterone signals in letrozole-treated animals, leading to the dysregulation of downstream targets in the embryo implanted region of the uterus during the early pregnancy window.

Further, ovarian steroid hormones have been known to regulate the cell adhesion molecules during the attachment phase of the embryo (Merviel et al., 2001). In this direction, the study reported that when ovariectomized rats were treated with sex steroids to mimic the hormonal patterns of early pregnancy, their findings have shown that the administration of testosterone resulted in a marked decrease in the expression of integrin αvβ3 in the uterus. Furthermore, the upregulated αvβ3 integrin expression was observed in the uterus following progesterone restoration suggesting that progesterone is accepted to be responsible for the observed increase in αvβ3 integrin level (Mokhtar et al., 2018). In addition to this, *in vitro* study demonstrated that when estrogen was administrated at a higher concentration, a decrease in the expression of integrin β3 & β1 was observed in Ishikawa cells. However, when the cells were treated with progesterone, the repressive effect of estrogen on integrin β3 & β1 was abolished (Chen et al., 2016). In our study, letrozole-treated animals exhibited a decrease in gene expression of integrin β1 and α4 in the implanted region of the uterus. Hence, PCOS animals having low systemic progesterone content can lead to a decline in the expression of adhesive markers in the implanted region of the uterus. Apart from steroidogenic control, LIF and LIFR are known to enhance the expression of integrin αvβ3 and αvβ5 on the plasma membrane surface of endometrial cells, which play a role in the attachment of the blastocyst to the uterus (Chung et al., 2016). Results from the current study exhibited no difference in the gene expression of LIF & its receptor in PCOS animals. This suggests that the declined expression of integrins was not mediated via LIF & LIFR. Hence, we can conclude that progesterone could be one of the contributory reasons causing the reduced integrin expression, which could result in impaired embryo-uterine attachment during early pregnancy in the letrozole-induced PCOS animals.

Blastocyst attachment with the uterine epithelium is followed by the decidualization of the stromal cells. In mice, the blastocyst is the stimulus for decidualization (Deng et al., 2019), and the homeobox transcription factors are known to regulate this process (Du & Taylor, 2016). In addition, progesterone is influential in decidualization during implantation in the mouse uterus (Lim et al., 1999). Also, it was observed that progesterone and its receptor signalling upregulate the HOX10a expression while the upregulation has been inhibited by the progesterone signalling blocker in the isolated human endometrial stromal cells (Taylor et al., 1998). When examined for the expression of the HOX10a, reduced gene expression of the HOX10a was observed in the implanted region of the uterus in the PCOS animals compared to that of control animals. Based on our results, and the above-cited reports, it is evident that

low serum progesterone concentration in the PCOS group could not induce the signals that are required for decidualization. As a consequence of this aberrant early embryonic-uterine communication may alter the pregnancy outcomes in the PCOS phenotype, as observed by the poor fetal outcome.

Matrix metalloproteases (MMPs) and their inhibitors (TIMPs) have a significant role in tissue remodelling, and homeostasis of the MMPs & TIMPs is thought to be crucial during normal early gestation (Curry & Osteen, 2001). The importance of these proteases has been described in one of the studies whereas in women with an imbalance in the serum levels of MMPs and TIMPs has been associated with spontaneously terminated pregnancy in the first trimester (Nissi et al., 2013). There are reports indicating that proteases and TIMPs in the uterus have been controlled by the action of estrogen wherein female ovariectomized mice when treated with estradiol or in combination with progesterone, the uterine gene expression and activity of TIMP-1 and MMP-9 were increased in estrogen alone or in combination with progesterone, whereas estrogen receptor antagonist blocked this effect. In contrast, it was reported that MMP-2 and TIMP-3 expression was not changed in the uterus by steroidal treatment (Zhang et al., 2007; Nothnick et al., 2004). Altogether, evidence suggests the estrogenic regulation of MMP and TIMPs expression in the uterus. However, in the present study, PCOS animals did not exhibit any change in estradiol content as compared to the control animals. Also, results revealed that the expression of MMP-9, TIMP-1 & 3 was significantly decreased in the letrozole-treated animals. Hence, the imbalance in the expression of the proteases and their inhibitors may be attributed to improper blastocyst invasiveness during early gestation in PCOS.

Furthermore, proper growth and development of the implanting embryo is a fundamental event in early pregnancy, and the leukemia inhibitory factor (LIF) is one of the regulators of this process (Salleh & Giribabu, 2014). In this direction, binding of LIF to LIFR recruits Glycoprotein130 (gp130, common signalling receptor for IL-6 family cytokines) to form a functional complex leading to activation of the downstream signal transduction pathway such as signal transducer and activators of transcription (STAT). When analyzed for the key markers of the LIF signalling pathway, letrozole-treated animals did not show any significant changes in the mRNA levels of LIF and LIFR in the embryo-implanted region of the uterus compared to that of control animals. Alternatively, the expression of gp130 and STAT3 significantly declined in the PCOS animals. Emerging evidence suggests that steroid

hormones are described to play a critical role in the management of LIF, LIFR, and gp130 expression in the uterus throughout the implantation period (Salleh & Giribabu, 2014). This is supported by the observation wherein exogenous administration of estrogen can induce LIF and LIFR expression in the endometrium of ovariectomized mice (Chen et al., 2000; Ni et al., 2002). Also, a high level of gp130 mRNA levels was detected in the uterus of ovariectomized mice treated with both estrogen and progesterone (Ni et al., 2002). In addition, uterine conditional ablation of STAT3 leads to the disruption of PR-mediated pathways and declined PR protein expression *in utero*, suggesting that STAT3 has a pivotal role in PR-dependent pathways during implantation in mice (Lee et al., 2013). In this study, disrupted LIF-STAT signalling was observed in the PCOS animals. However, it couldn't be confirmed whether the unbalanced LIF signalling could be because of altered progesterone signals or whether declined STAT3 does not activate the PR-mediated pathways in the embryo-implanted region of the uterus.

Based on all of the above molecular deficits in the PCOS pregnant uteus, it was speculated that the changes observed might be originated from the modification of the histological architecture structure of the embryo implanted region of the uterus on the day 6^{th} of pregnancy. Moreover, healthy growing implanted embryos were found in the untreated animals, whereas in the letrozole-treated animals, the appearance of vascular permeability was observed. These inherent changes in the structure area of the uterus could be implicated in endometrial dysfunction in pregnant PCOS mice.

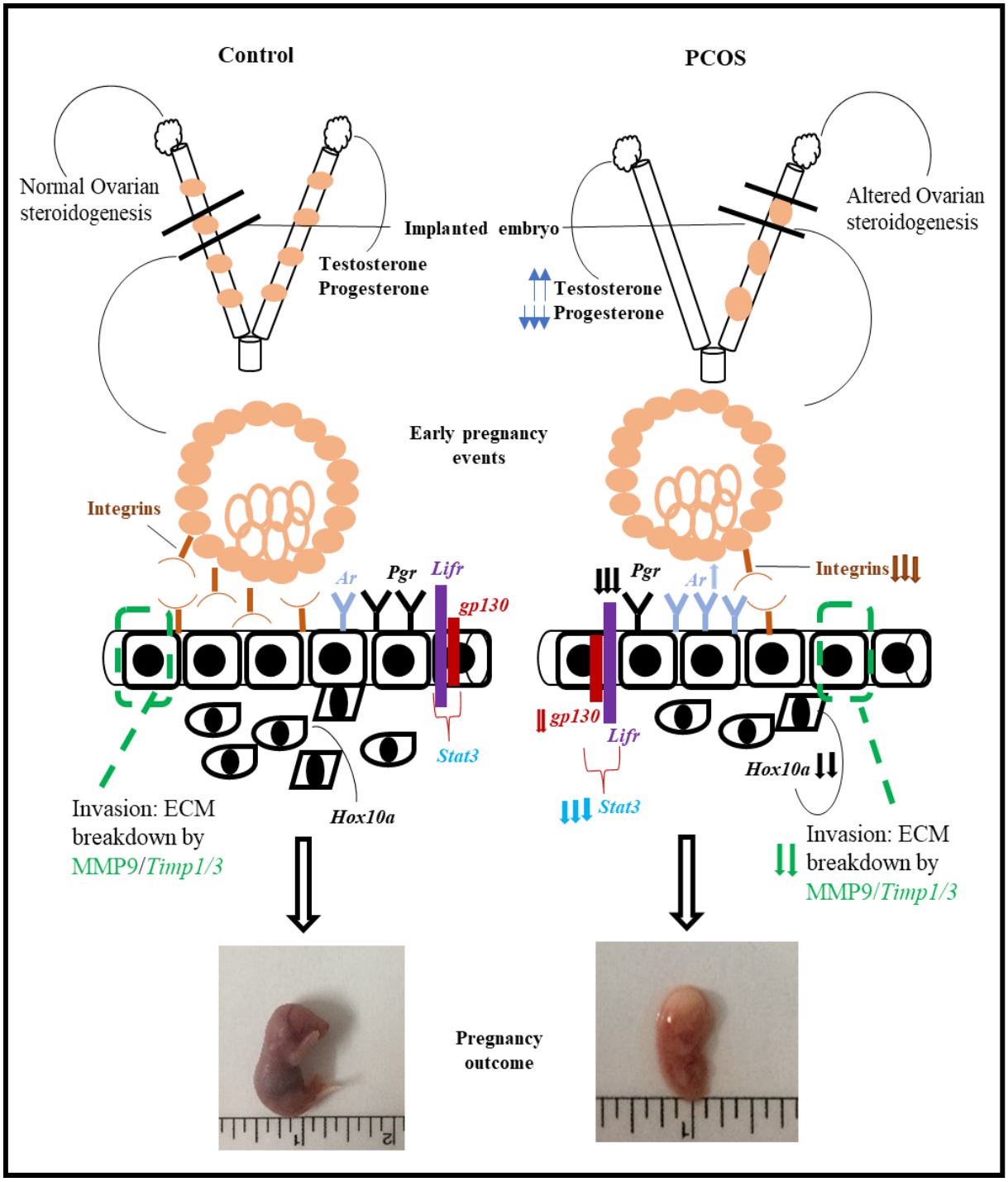


Figure 3.16. Diagrammatic summary of the current study. *Ar* androgen receptor, *Pgr* progesterone receptor, *Hox10a* homeobox transcription factor 10a, MMP Matrix metalloproteinase, *Timp* tissue inhibitor of metalloproteinase, *Lifr* leukemia inhibitory factor receptor, *Gp130* glycoprotein 130, *Stat3* signal transducer and activator of transcription 3.

3.5 Conclusion

A significant strength of this research was to explore the probable mechanism by which PCOS may alter the embryonic-uterine microenvironment thereby preventing the establishment of a healthy pregnancy (**Figure 3.16**). The effect of the letrozole on the embryo-implanted region of the uterus suggests that the majority of the molecular alterations were due to the aberrant PR expression and its signalling, which further dysregulates the expression of the key markers that are involved in the uterine-embryonic cross-talk during the early gestation period. This could be the reason for the early pregnancy complications/early fetal loss associated with PCOS, as evidence of the poor fertility index and reduced number of implanted embryos were observed in the PCOS animals. ***Thus, the current study gives insight into the regulation of intracrine molecules to improve uterine-embryonic functions and potential medicinal targets to expand the conceptive outcome of the PCOS patient.***