# CHAPTER 5

EFFECT OF ALOE VERA GEL ON REPRODUCTIVE

# PARAMETERS IN PCOS MODEL

5.1 Introduction

An important stage of female life cycle is pregnancy. Initial event for conception involves fertilization. Fertilization involves the fusion of a matured female gamete called ovum with a male gamete called sperm to form a zygote. The zygote undergoes cell differentiation and structural changes to form a blastocyst, which further gets implanted into the uterine wall (Wassarman 1999).
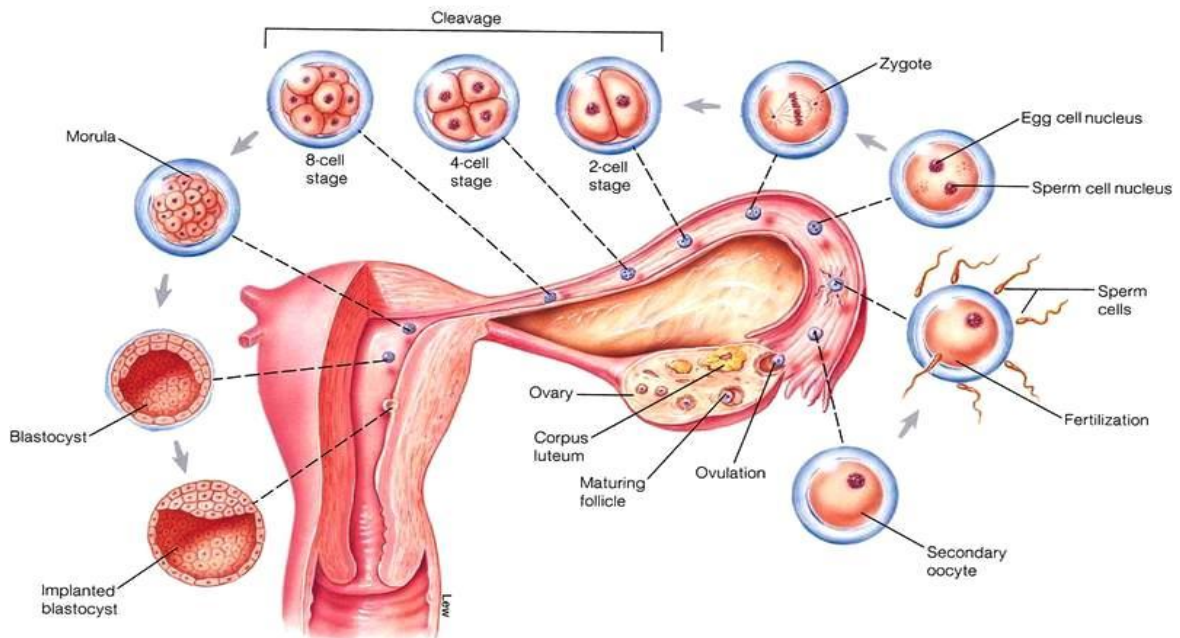
Figure 5.1 Process of fertilization

The blastocyst cells are totipotent in nature i.e. they have the ability to differentiate into any cell type within the developing embryo. Trophoblast cells play a crucial role in the implantation process and establishment of pregnancy, as they are the first cells to reach the maternal surface by invasion (Meseguer et al. 2001) and produce a number of biomolecules like hormones (eg. Human chorionic gonadotropin) , cytokines(Tumor necrosis factor-α (TNFα), interleukins, adhering proteins like cadherins, growth factors (Insulin like growth factor-1 (IGF-1)) and many other factors, including implantation enzymes such as Cathepsin D, alkaline phosphatase, Matrix Metalloproteinases (MMPs). These factors facilitates communication with the maternal tract and thereby promotes implantation and ongoing embryonic development through paracrine and/or autocrine

interactions (Dimitriadis et al. 2005)**.** Successful implantation requires a receptive endometrium, a functional embryo at the blastocyst developmental stage and a synchronized dialog between maternal and embryonic tissues (Diedrich et al. 2007). The implantation of the blastocyst in the uterine endometrium can be divided into three phases: apposition, adhesion and embedding in the endometrium.
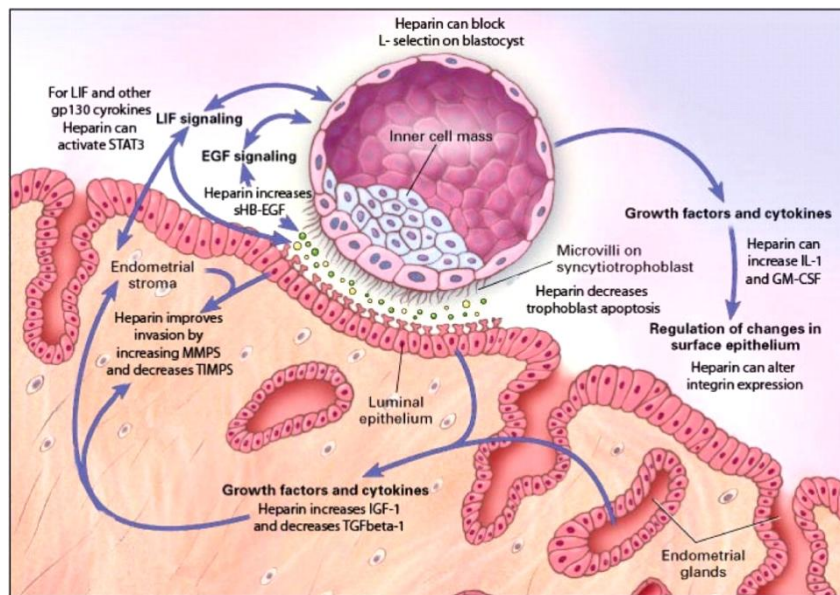
Figure 5.2 Implantation of the blastocyst during implantation window

Implantation window is a precise period and plays a crucial role in the successful pregnancy. The entire process takes place under tight regulation (Cha et al. 2012). During implantation, there is breakdown of complex proteins and polysaccharides, which is mainly mediated by lysosomal enzymes namely Cathepsin-D and Alkaline phosphatase (Moulton et al. 1978). Cathepsin-D is one of those molecule, with the help of which the trophoblast invades the maternal endometrium and makes contact with the maternal blood supply (Salamonsen 1999). These proteolytic enzymes of the lysosomes of uterine luminal epithelial provide epithelial cell autolytic activity (Elangovan and Moulton 1980). Alkaline phosphatase (ALP) also plays an important role in implantation process. It contributes to uterine receptivity, implantation, decidualization, and defense against bacterial endotoxin in rodent model (Lei et al. 2013). Recent study demonstrated that alkaline phosphatase (ALP) isozyme may have a unique therapeutic potential to minimize

the LPS- or Gram-negative bacteria-induced pregnancy complications in women (Lei et al. 2015).

During implantation, the endometrium undergoes pronounced structural and functional changes induced by the ovarian steroids namely estrogen and progesterone that prepares the endometrium to be receptive to the invading embryo (Ramathal et al. 2010). These steroid hormones are also essential components for the normal progression of a pregnancy and the survival of the fetus in both humans and rodents during gestation.(Albrecht and Pepe 1990). Specifically, progesterone signaling is absolutely necessary for successful pregnancy. Loss of progesterone during pregnancy is associated with the termination of pregnancy (Spencer and Bazer 2002). Progesterone signaling functions in an inhibitory manner to the estrogen signaling pathway (Hsueh et al. 1975). The antagonistic relationship between the two hormone pathways provides an important regulatory pattern necessary for implantation (Wetendorf and DeMayo 2014).

These ovarian steroid hormones that play crucial role in implantation and its production is termed as steroidogenesis. Steroidogenesis is a tightly regulated process wherein first rate limiting step involves a protein- Steroidogenic Acute Regulatory protein (StAR). It initiates the process of steroidogenesis by transporting cholesterol from the outer to the inner mitochondrial membranes of the cell (Clark and Stocco 2014). However, higher expression of StAR can lead to alteration in steroidogenesis that contributes to imbalance in ovarian steroidal mileu (Anuka et al. 2013). Positive stimulation of StAR expression leads to progesterone production, wherein its expression is positively regulated by trophic hormones such as FSH, LH, insulin, and insulin-like growth factor I (IGF-I) in granulosa cells (Sekar et al. 2000). Insulin acts in synergy with LH to elevate intracellular concentration of cAMP, which activates StAR and potentiates steroidogenic activity (Rojas et al. 2014). The regulation of steroidogenic protein and enzymes are under the influence of steroid hormones and gonadotropin feedback mechanism (Planas et al. 2000)**.**

Apart from ovary, placenta is also capable of producing sufficient amounts of steroid hormones (i.e. glucocorticoids, mineralocorticoids, progestins, androgens and estrogens) independently and hence can contribute to the steroidal pool (Fowden et al. 2015). Steroidogenic substrates flow from mother to fetus through placenta; which permits an array of metabolic pathways that can maintain hormonal milieu of pregnancy(Fowden and Forhead 2004). Important steroid, estrogens play an important role in pregnancy and fetal development. Also, it has major role in stimulation of muscles in the uterus to maintain pregnancy (Bondesson et al. 2015).
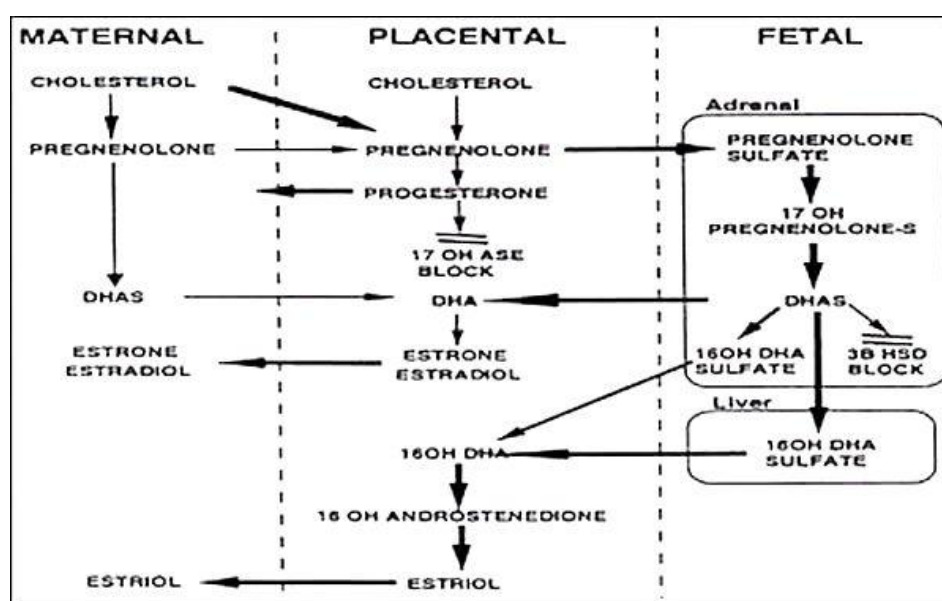
Figure 5.3 Steroidogenesis in feto-placental unit during pregnancy

The human placenta utilizes fetal and maternal adrenal derived C19 androgens, mainly dehydroepiandrosterone sulfate (DHEA-S). The principal steroid products of ACTH-stimulated fetal adrenal cells were dehydroisoandrosterone sulfate, pregnenolone, pregnenolone sulfate, and 17α-hydroxypregnenolone. In placental tissue, sulfate group is cleaved by steroid sulfatase (STS). The other unconjugated steroids are converted by the activity of 3β-Hydroxysteroid dehydrogenase into androstenedione and testosterone subsequently. The inter-conversions of androstenedione to testosterone and estrone to estradiol are catalyzed by 17β- Hydroxysteroid dehydrogenase. The C19 androgens are aromatized to estrone and estradiol respectively by P_{450} aromatase (Henderson and

Swanston 1978). Placental estrogen production appears to be mostly dependent on the amount of substrate provided by the fetal adrenal gland, utero-placental blood flow, and placental trophoblast mass. Estrogen stimulates the uptake of high-density lipoprotein cholesterol substrate (Albrecht 1980) and $P_{450}scc$ expression in the corpus luteum of rats (Goldring et al. 1987) and rabbits (Keyes et al. 1990), thereby promoting progesterone production. Hence, any steroid alteration during pregnancy could affect fetal life and its survival.

Reduced levels of important steroids like estrogen and progesterone could probably lead to early pregnancy loss (EPL), defined clinically as first trimester miscarriage in PCOS females. It occurs in 30 to 50% of PCOS women compared to 10 to 15% of normal women (Jakubowicz et al. 2002; Kjerulff et al. 2011). In addition to altered steroid status, deregulated expression of uterine receptivity markers was found in the endometrium of PCOS women such as the expressions of αvβ3-integrin, HOXA-10, HOXA-11 and insulin-like growth factor binding protein (IGFBP-I) was found to be decreased in PCOS women (Cermik et al. 2003). Moreover, these PCOS women also demonstrated over expression of androgen receptor and impaired regulation of estrogen receptor (ERs), when compared to normal women (Gregory et al. 2002).

Thus, alterations in the above factors may lead to pregnancy-related complications during late gestation (such as pre-eclampsia, preterm labour and recurrent miscarriage). Moreover, the incidence rate between PCOS and recurrent miscarriages remains high and various etiologies have been proposed in this regard (Chakraborty et al. 2013). Gonadotropin abnormalities with characteristic increased GnRH pulse amplitude and frequency have been recognized as a factor to cause an elevation in LH:FSH ratio and contribute to hyper-androgenism (Banaszewska et al. 2003), which could be a risk factor for spontaneous abortions and increased early pregnancy loss (Gürbüz et al. 2004).

In pregnant women with PCOS, androgen levels are significantly higher compared with non-PCOS controls (Sir-Petermann et al. 2002). During pregnancy, PCOS women demonstrated an abnormal placental steroidogenic function (Escobar-Morreale et al. 2011)

and closely related to the high incidence of microscopic alterations in early trophoblast invasion and placentation (Palomba et al. 2012). High androgen levels could also affect neonatal weight, impairing the maternal energy homeostasis changes and the nutrient transport through the placenta and/or with a direct effect on fetal growth (Sir-Petermann et al. 2005). Apart from high androgens, low estrogen in PCOS females is known to have an effect on oocyte and embryo quality, endometrial receptivity and development of the embryo (Bestwick et al. 2012). An additional modulatory factor affecting uterine estrogen levels is hyperinsulinemia. Elevated insulin contributes to the hyperandrogenism by both increasing serum concentrations of ovarian androgens and decreasing the levels of circulating sex hormone binding globulin (SHBG) (Kavanagh et al. 2013). In PCOS women, maternal hyperinsulinemia during pregnancy induces excessive placental human chorionic gonadotropin (hCG) secretion leading to fetal ovarian hyperplasia and hyperandrogenism (Dumesic et al. 2014). Hence, both hyperinsulinemia and hyperandrogenism can affect fetal development (Peters et al. 2013) and alter "*in utero*" condition during pregnancy (Gluckman et al. 2008). In human cytotrophoblasts, insulin has been shown to inhibit aromatase and stimulate 3β-HSD activities (Maliqueo et al. 2012). Thereby, pregnant PCOS patients with high insulin level have significantly increased androgen content due to high expression of 3β-hydroxysteroid dehydrogenase (3β-HSD1) and leading to lower levels of P_{450} aromatase, which mainly disturbs the steroidal milieu during gestation (Maliqueo et al. 2012).

Elevated steroid hormone levels are seen in women with polycystic ovaries, suggesting that an alteration in the metabolism of the steroid hormones occurs in PCOS condition (Greisen et al. 2001). Phase I and II pathways of biotransformation plays a major role in homeostasis of molecules like steroids. Some of enzymes belonging to the cytochrome P_{450} and glutathione S-transferase families are implicated in this process (Hayashi et al. 1991). Phase I metabolism involves an initial oxidation, reduction or dealkylation of the substrate by cytochrome P_{450} monooxygenases. This step is often needed to provide a molecule with hydroxyl or amino groups, which are essential for phase II reactions. In Phase II reactions, generally addition of the hydrophilic moieties takes place, thereby making a steroid more water soluble and less biologically active (You 2004). Conjugation of sex steroids via

glucuronidation [catalyzed by UDP-glucuronosyltransferases (UDPGT)] (Fisher et al. 2001) and sulfation [catalyzed by sulfotransferases (GST)] (Medeiros et al. 2004) are the major pathways for estrogen and androgen clearance in humans (Starlard-Davenport et al. 2008). In rodents, cytochrome P_{450} breaks down some of the steroid hormones and removes them from the blood circulation. Any alteration in this process may also contribute to several hormone related dysfunction (Guillemette et al. 2004).

Thereby, several complimentary therapies have been studied for management of PCOS and regain their fertility. Several traditional Chinese medicines (TCM) and ayurvedic medicines have been reported that helps in ovulation and reduced pregnancy complications (Lyttleton 2013; Dayani Siriwardene et al. 2010). Several medicinal plants and various phyto-components isolated from plant extract that act as good insulin receptor sensitizers, decrease hyperandrogenic condition. Researchers have implicated that targets of phytocomponents could be steroid receptors, steroid metabolizing enzymes and proteins involved in implantations (Nagarathna et al. 2014; Pérez et al. 2007)**.** These modulatory effects might help in treatment of ovarian dysfunction and restoration of fertility (Kage et al. 2009; Yakubu et al. 2009; Kaido et al. 1997) . Many indigenous plants have been reported which are used in traditional herbal remedies during pregnancy and childbirth.

With above context, it is evident from the data of previous chapter that *Aloe vera* gel significantly modulated the ovarian steroidogenesis and its structure-function in non-pregnant stage in letrozole indued PCOS rodent model. Hence, it would be interesting to investigate the efficacy of *Aloe vera* gel as a pre-conceptive fertility agent and promote the sustenance of pregnancy till term in letrozole induced PCOS rats. Thereby, present study was undertaken to analyze the reproductive performance, implantation enzymes, expression of key proteins involved in ovarian and placental steroidogenesis along with steroids status when *Aloe vera* gel treatment was given to PCOS rodent model prior to conception.
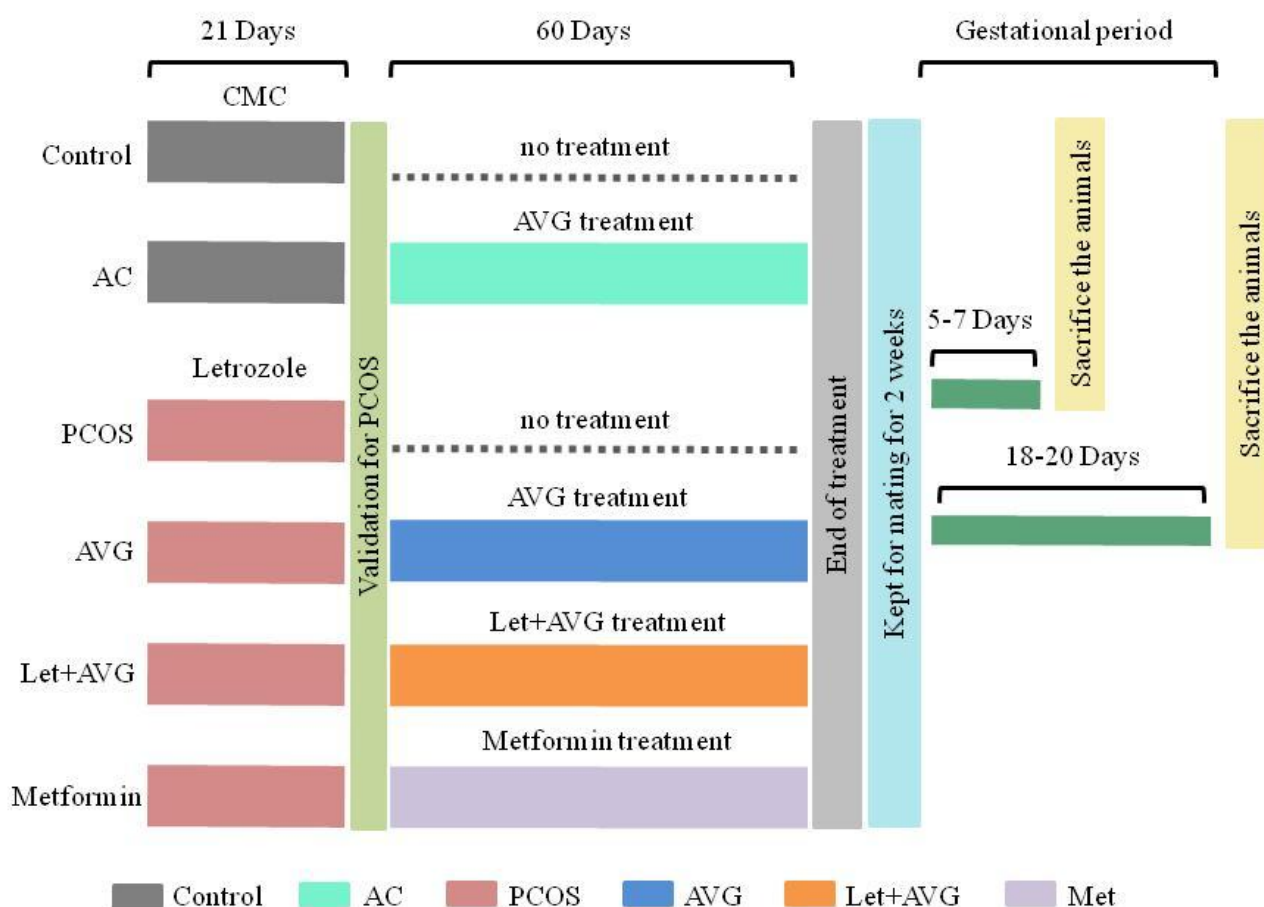
5.2 MATERIALS AND METHODS
5.2.1 ALOE VERA GEL EXTRACTION
Fresh Aloe vera gel was used for the study. The protocol has been discussed in material and methods section.

5.2.2 PLAN OF WORK

Adult virgin Charles foster female rats (2-3 months; 200 ± 15 g) were used for the study

Control   : 1% Carboxy methyl cellulose (CMC) for 21 days

AC   : Control animals treated with 10 mg dry weight of *Aloe vera* gel for 60 days

PCOS   : 0.5 mg of letrozole per kg body weight for 21 days

AVG   : PCOS animals treated with 10 mg dry weight of *Aloe vera* gel for 60 days

Let+AVG : PCOS animals treated with 0.5 mg/kg letrozole along with 10 mg dry weight of *Aloe vera* gel for 60 days

Met   : PCOS animals treated with 100mg metformin per kg body weight

All treatments were given daily by oral gavages

n = 6-8 for each group

5.2.3 ANIMALS TREATMENT

Adult Charles foster female rats (weight 150–200 g) were used for the study. All rats were housed in cages and maintained in ambient temperature of 25±1°C and 45.5% relative humidity, with a photoperiod cycle of 12 h:12 h (light and dark) with food and free access of water. All experimental protocols were approved by the institutional animal ethical committee under CPCSEA guideline. Animals were initially divided into 2 groups wherein 1st group received 1% CMC (carboxymethylcellulose) and served as vehicle control. The other groups of animals were treated orally with letrozole daily for 21 days (0.5 mg/kg body weight). Letrozole treated animals demonstrated insulin resistance, disturbed estrus cyclicity and were considered as PCOS (group 3). One set of animals were treated with AVG (10mg/dry weight) for 2 months after the induction of PCOS (Group 4). Next group of animals, wherein letrozole treatment continued along with AVG was considered as Let + Aloe group (Group 5). Separate group of animals received 100 mg/kg body weight of metformin (standard insulin sensitizing drug) and served as a positive control group (Group 6). In addition to this, untreated animals receiving AVG were designated to be herbal control (Group2) during the course of experiments.

Parameters such as Serum glutamate pyruvate transaminase (SGPT) and serum Creatinine levels were analyzed to check the toxicity of *Aloe vera*.

After *Aloe vera* gel treatment, rats of all groups in late diestrus to early proestrus stage of estrus cycle were allowed to mate with male rats. The date of copulation was determined on the basis of vaginal smear. Presence of sperm on the next day confirmed the pregnancy and considered as the first day of pregnancy. Animals of all groups were divided into further two sets wherein one set of animals were sacrificed at initial gestation period: 5th – 8th day (Implantation window) and another set of animals were sacrificed at late gestation period: 18th – 20th day.

Initially, animals were sacrificed in the implantation window and reproductive parameters were assayed. Excised implanted part of uterine wall was used for estimation of enzymes that play an important role in implantation: Alkaline phosphatase and Cathepsin D. Also, ovaries and liver tissues of the animals were excised and levels of various enzymes involved in steroid biosynthesis as well as their metabolism were evaluated respectively.

The protocols for the assays have been mentioned earlier in material and methods section. In order to study the modulations occurring in late gestation period i.e., 18th -20th day, blood samples were collected from all groups of animals and later, sacrificed by cervical dislocation. Fetal and placental weights were checked in all groups of animals. Further, ovarian and placental tissues were processed for estimation of steroidogenic enzyme activities- 3β Hydroxy steroid dehydrogenase (3β-HSD) and 17β Hydroxy steroid dehydrogenase (17β-HSD) along with fertility index. Fertility parameters including numbers of live pups, litter size and weight, placental weight, resorbed fetus, post implantation loss was calculated. Also, metabolic enzymes of phase I metabolism-17β Hydroxy steroid oxidoreductase (17β-HSOR) and cytochrome P_{450} along with enzymes of Phase II metabolism -UDP-glucuronosyltransferase (UDPGT) and glutathione S-transferase (GST) activities were checked in liver tissues in all groups of animals. Steroid hormones: Testosterone, Estradiol, Progesterone and Insulin levels were checked in the serum by ELISA.

Total RNA were extracted from ovarian and placental tissue by TRIZOL method and studied for gene expression of StAR, Aromatase, Androgen receptor (AR), Insulin receptor (IR), Luteinizing hormone receptor (LHR), Follicle stimulating hormone receptor (FSHR) by Reverse transcription Polymerase Chain Reaction (RT-PCR) method and were normalized using internal control-GAPDH.

In addition to this, ovarian and placental tissues were excised and kept in lysis buffer and stored at -80°C. Later, tissues were processed for western blot analysis to check the key protein expression of StAR, 3β-HSD, Aromatase and Androgen receptor (AR) as they play important role in steroidogenesis.

All the methods discussed above are explained in materials and methods.

5.3 RESULTS

In our earlier objective, efficacy of *Aloe* vera gel (AVG) in PCO condition during non-pregnant stage has been discussed. This fact could be further strengthened by understanding the role of AVG in altered physiological stage like pregnancy. Hence, aim was to evaluate efficacy of AVG when used as pre-conceptive agent in PCOS rodent model and study its effect in pregnant stage especially during implantation and at term. In

this experiment, letrozole induced PCOS rats was treated with AVG (10mg/daily/orally) for 60 days and were further allowed to mate with male rats. Along with this treatment regime, an additional group was considered wherein PCO rats continued to receive letrozole along with AVG till end of the experiments to understand the protective effect of Aloe vera gel.

After AVG treatment, the animals were sacrificed at 2 different stages of gestation period: - 5th -8th day and 18th -20th day and further various experiments were performed to check efficacy of AVG on reproductive performance.

5.3.1. 5th – 8th Day Parameters

In order to study the efficacy of AVG on implantation, animals were sacrificed between 5th – 8th day, wherein vaginal plug formation and presence of sperm was considered as 0 day of pregnancy. Parameters like total number of implantation and resorption sites on uterine wall were checked in all groups.
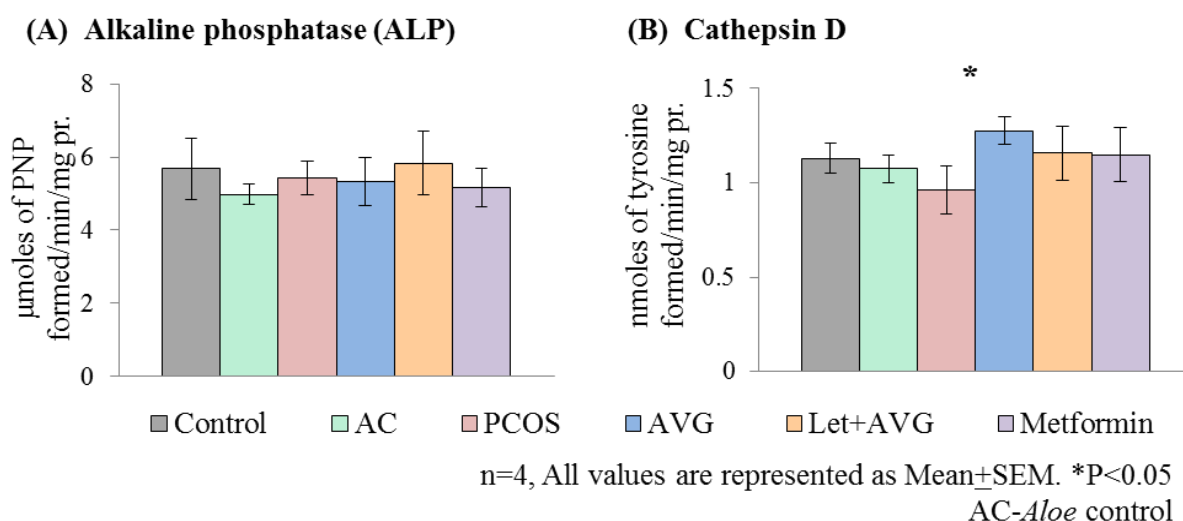
Further, implanted areas was excised from the uterine wall and assayed for key implantation enzyme alkaline phosphatase (ALP). This enzyme didn't show any significant change amongst all group of animals studied as shown in Figure 5.3.1A. Also, Cathepsin D plays a key role in early implantation. Reports showed that PCOS females with higher miscarriage rates exhibit low cathepsin D levels in their endometrium (Borro et al., 2007). In our study, PCOS rats exhibited no significant change in Cathepsin D level in all groups studied (Figure 5.3.1B).

PCOS women with high insulin levels are more likely to exhibit low fertilization rates even after IVF and their embryos are unable to implant (Cano et al. 1997). Hence, the status of implantation in all groups of animals were assessed, wherein letrozole induced PCOS rats exhibited lesser number of implantations on uterine wall as compared to control group where normal implantations were observed. AVG treated PCOS rats showed an improvement in implantation as evident by increased number of live pups as compared to PCOS group. Those animals treated with letrozole along with AVG (let + *Aloe*) also demonstrated reversion to normalcy as compared to resorption that were observed in metformin treated PCOS rats (Figure 5.3.2 and Table 5.3.1)

Implantation of the embryo in the maternal endometrium is the first step leading to

placentation and ultimately ensures that the conception is provided with an adequate blood supply (Ford et al. 1979). Current study has demonstrated that AVG treatment before conception improved implantation status and reduced resorption.

Figure 5.3.1 Effect of *Aloe vera* gel on Uterine implantation enzymes activity in letrozole induced PCOS rat model

(A) Alkaline phosphatase (ALP)   **(B) Cathepsin D**

n=4, All values are represented as Mean±SEM. *P<0.05
AC-*Aloe* control

C=Control; AC= Aloe control; P=PCOS; AVG= PCOS treated with AVG;

Let+AVG= PCOS treated with AVG +Letrozole; Metformin= PCOS treated with Metformin

Table 5.3.1 Effect of *Aloe vera* gel on reproductive performance in letrozole induced PCOS rat at early gestation

| Groups	No. of Implantation sites	Time required for conception
Control	9±0.66	3 cycles
AC	8±0.65	4 cycles
PCOS	2.3±0.71***	7 cycles
AVG	9.6±0.66@@@	4 cycles
Let+AVG	6.1±0.65@@	5 cycles
Metformin	5.6±0.66@@	6 cycles

N=4, The values are represented as Mean±SEM
***p<0.001 as compared to Control group, @@@ p<0.001, @@p<0.01 as compared to PCOS group
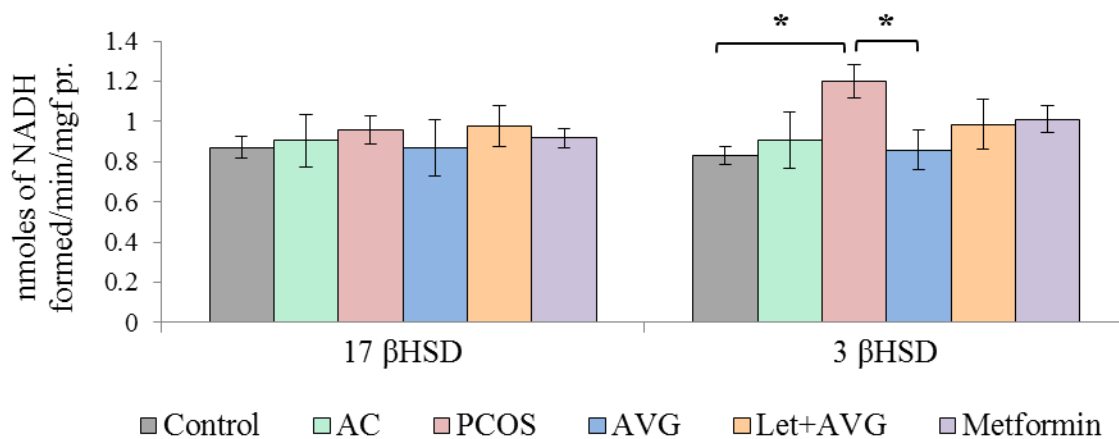C=Control; AC= Aloe control; P=PCOS; AVG= PCOS treated with AVG;
Let+AVG= PCOS treated with AVG +Letrozole; Metformin= PCOS treated with Metformin

Figure 5.3.2 Effect of *Aloe vera* gel on implantation in letrozole induced PCOS rat at early gestation

Control   Aloe Control   PCOS

Aloe   Let+Aloe   Metformin
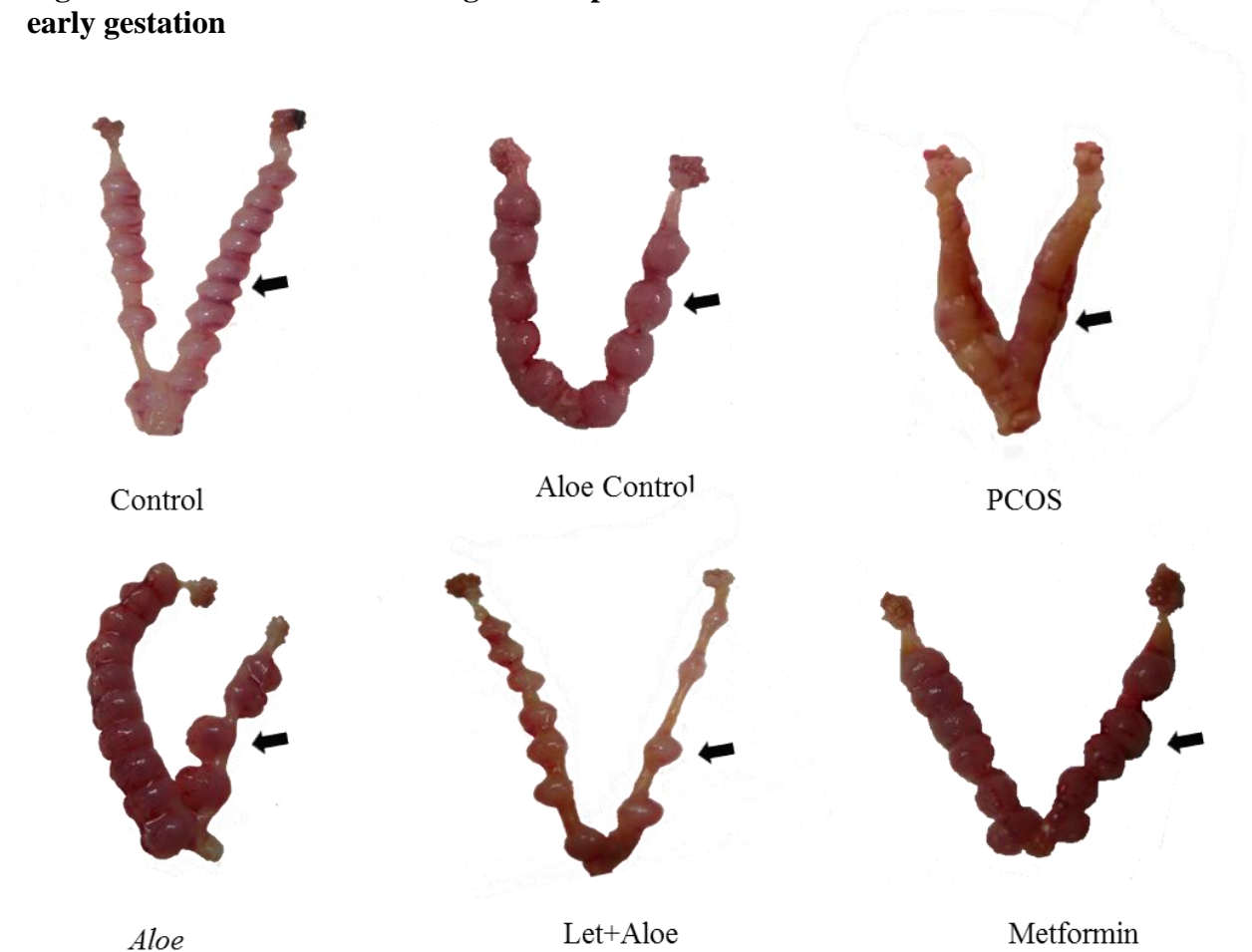
During initial gestation period, maternal steroid status also plays a major role in the regulation of blastocyst implantation and fetal development. Reports suggest that high systemic insulin levels increases ovarian androgen production and alters steroidogenic enzyme activity (Makieva et al. 2014). Hence, estimation of key enzymes (3β-HSD and 17β-HSD) involved in ovarian steroidogenesis were assayed, wherein an increase in the 3β-HSD enzyme activity was observed in PCOS group whereas AVG exhibited significant reduction (*$p<0.05$). However, Let+AVG (Group 5) and metformin (Group 6) showed no significant change in both enzymes activities (Figure 5.3.3).

Figure 5.3.3 Effect of *Aloe vera* gel on ovarian steroidogenic enzymes activity in letrozole induced PCOS rat at early gestation
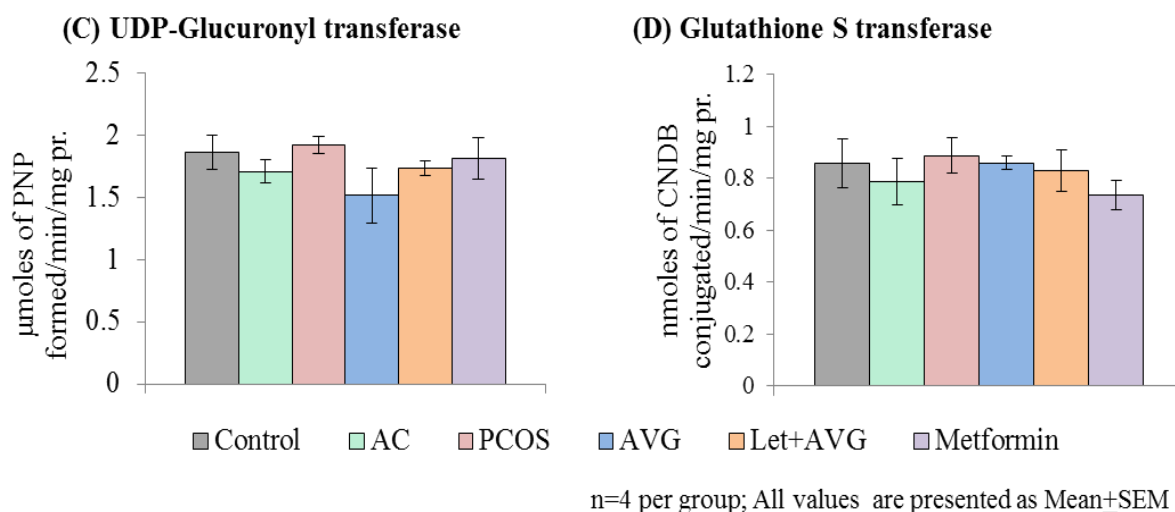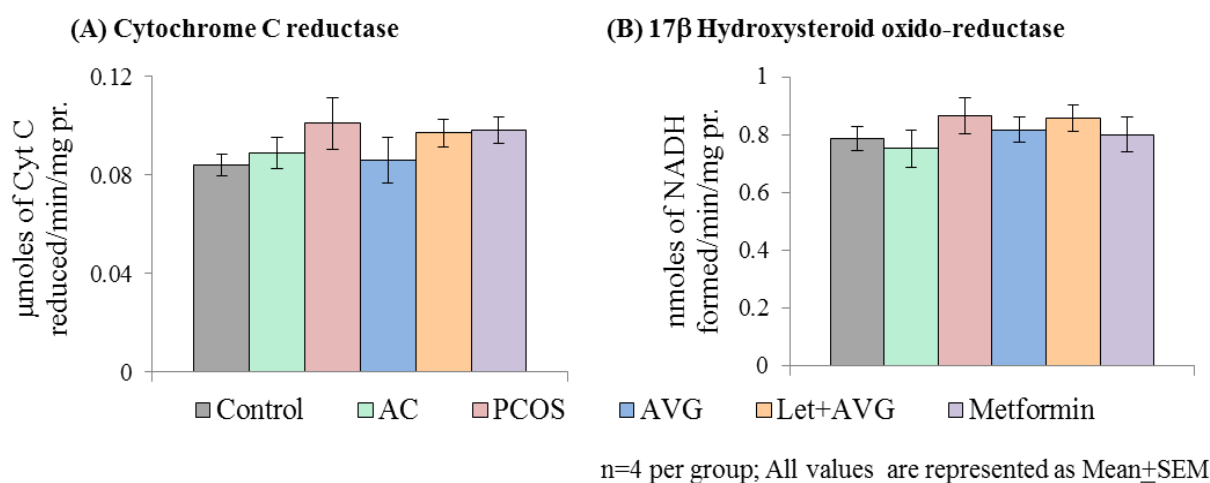

n=4 per group; All values are represented as Mean±SEM; *P<0.05

C=Control; AC= Aloe control; P=PCOS; AVG= PCOS treated with AVG;
Let+AVG= PCOS treated with AVG +Letrozole; Metformin= PCOS treated with Metformin

Pregnancy significantly decreases the specific activity of steroid-metabolizing microsomal enzymes in female rat liver (Kim 1995; Kavanagh et al. 2013; Czekaj et al. 2000). Reports also suggest that use of steroids like androstenedione may increase the risk of drug interactions by inducing CYP3A and other hepatic cytochrome P450 activities (Flynn et al. 2005). Thereby, we estimated liver metabolizing enzymes belonging to phase I: cytochrome C reductase and 17β hydroxysteroid oxido reductase along with phase II metabolising enzymes: UDP glucuronosyltransferase (UDPGT) enzyme activities wherein no significant change in all enzymes of all groups of animals as exhibited in figure 5.3.4 A,B, C and D.

Figure 5.3.4 Effect of *Aloe vera* gel on Liver steroid metabolizing enzymes in letrozole induced PCOS rat at early gestation

(A) Cytochrome C reductase

(B) 17β Hydroxysteroid oxido-reductase

n=4 per group; All values are represented as Mean+SEM

(C) UDP-Glucuronyl transferase

(D) Glutathione S transferase

n=4 per group; All values are presented as Mean+SEM

C=Control; AC= Aloe control; P=PCOS; AVG= PCOS treated with AVG; Let+AVG= PCOS treated with AVG +Letrozole; Metformin= PCOS treated with Metformin

5.3.2. 18th – 20th Day Parameters

Clinical studies have demonstrated that infants born to mothers with PCOS were more frequently delivered prematurely (Roos et al. 2011), and the normal gestational age of neonates was significantly lower in PCOS as compared to non-PCOS women (Palomba et al., 2014). Hence, it is important to evaluate the important biomarkers of pregnancy at term.

Thereby, various biochemical changes were observed in late gestation period i.e.

18th - 20th day wherein resorptions and retarded fetal growth were observed in PCOS rats as compared to live fetuses in control group (Figure 5.3.5). AVG treated PCOS rats demonstrated an increase in litter size and improved percent fertility growth as compared to PCOS group (Table 5.3.2). AVG group demonstrated a protective effect against letrozole and helped to improve fertility index during gestation period as compared to PCOS group. However, metformin group demonstrated lesser number of developed fetuses along with few resorptions. Herbal control showed similar results as vehicle control in all parameters studied; thereby results will be compared to vehicle control.

Table 5.3.2 Effect of *Aloe vera* gel on fertility parameters in letrozole induced PCOS rats at late gestation period

Groups	No. of rats kept for mating	No. of rats conceived	Total No. of resorption sites	Post implantation loss	No. of Live Pups (In gms)	Pups weight (gms)	Placental Weight (mgs)
Control	12	12	1±0.28	0.8 %	8±0.91	4.5±0.57	478±0.02
AC	12	11	1±0.26	1.5%	7.7±0.47	3.88±0.37	594±0.03 @@@
PCOS	12	8	42±4 ***	69.3 %	2±0.57 ***	2.03±0.15 **	270±0.01 ***
AVG	12	12	1.0±0.2 @@@	3 %	6.7±0.47 @@	4.43±0.35 @@	426±0.03 @@
Let+AVG	12	10	9±0.75 @@@	14%	5.2±0.57 @	3.9±0.46 @	387±0.04 @
Metformin	12	7	22.7±4.6 @@	36.2 %	5.25±0.47	2.96±0.34	275±0.02

N=4, The values represented as Mean±SEM.
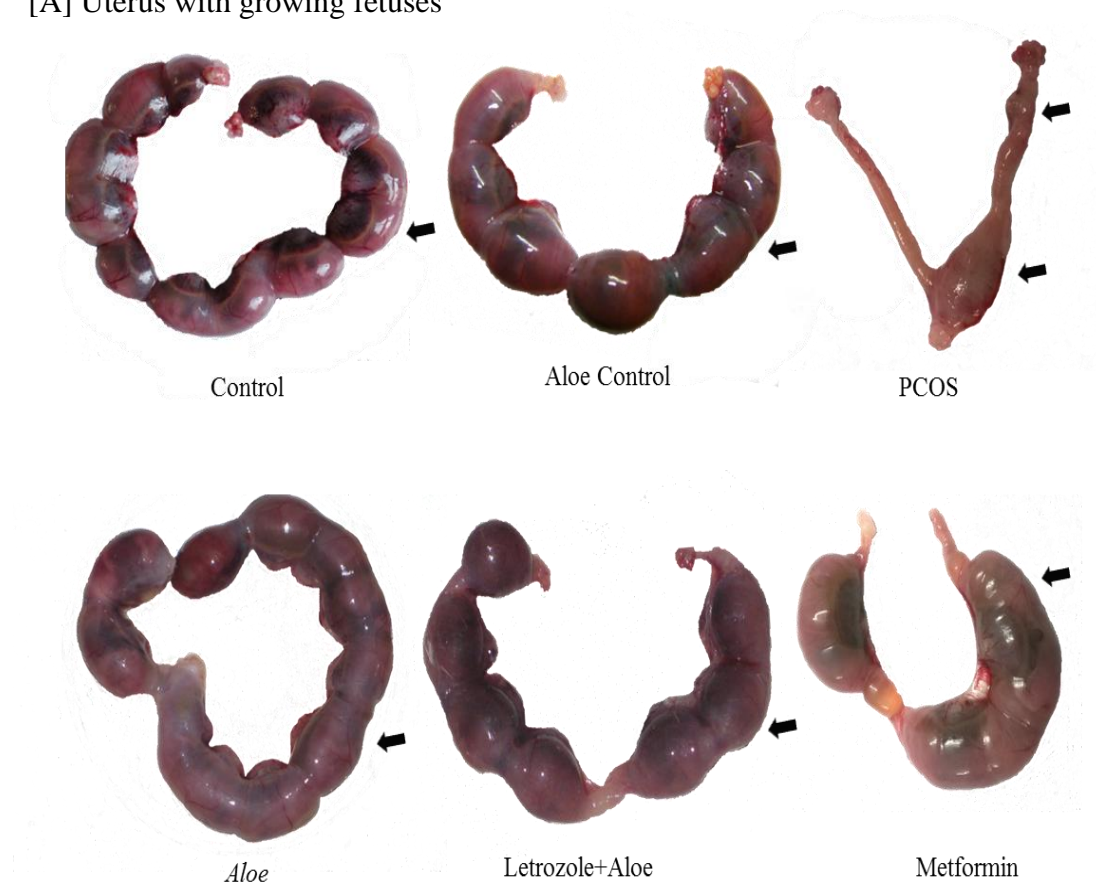***p<0.001, **p<0.01 and *p<0.05 as compared to Control group,
@@@p<0.0.001, @@p<0.01, @p<0.05 as compared to PCOS group.
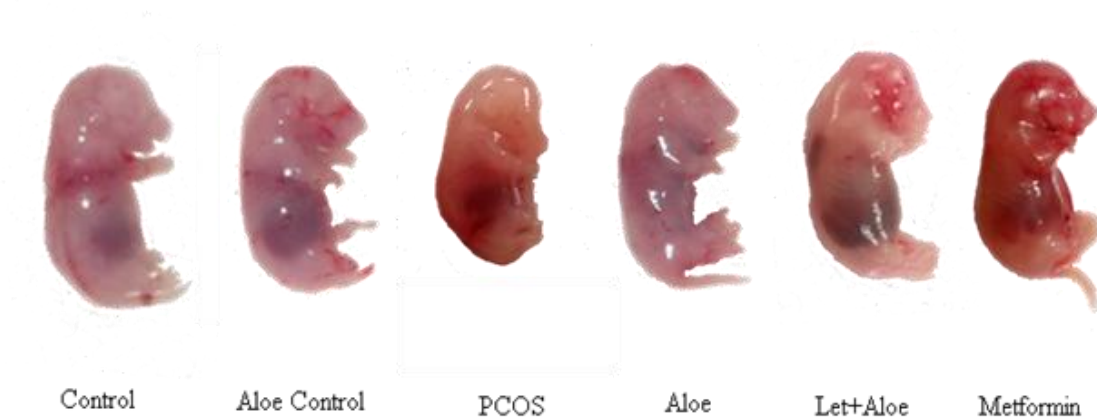C=Control; AC= Aloe control; P=PCOS; AVG= PCOS treated with AVG;
Let+AVG= PCOS treated with AVG +Letrozole; Metformin= PCOS treated with Metformin

Figure 5.3.5 Effect of *Aloe vera* gel on fetal development in letrozole induced PCOS rats at late gestation period

[A] Uterus with growing fetuses

Control   Aloe Control   PCOS

Aloe   Letrozole+Aloe   Metformin

[B] Fetuses

Control   Aloe Control   PCOS   Aloe   Let+Aloe   Metformin

Steroidogenesis plays a major role in maintaining ovarian function both in non-pregnant as well as pregnant stage. One of published data suggests that AVG has modulatory effect on ovarian steroidogenesis by altering the steroidogenic enzyme activities (Maharjan et al. 2010). Hence, effect of AVG on ovarian steroidogenic enzyme activity during late gestation period was assayed.

The effect of *Aloe vera gel* treatment in PCOS rats revealed that the ovarian and placental -3β hydroxy steroid dehydrogenase (3β HSD) and 17β hydroxy steroid dehydrogenase (17β HSD) activities were significantly altered (@@$p<0.01$) at 18th - 20th day (Figure 5.3.6 A and B). PCOS control animals demonstrated high activity of 3β HSD in both ovary and placenta as compared to control group (**$p<0.01$), whereas AVG treated group demonstrated modulation of the steroid enzyme activities towards normalcy. Let + AVG (Group 5) and metformin groups demonstrated improved 3β HSD enzyme activity in ovary (@$p<0.05$) whereas no significant change was observed in placenta as compared to control group.

Figure 5.3.6 Effect of *Aloe vera* gel on steroidogenic enzymes activity in letrozole induced PCOS rats at late gestation
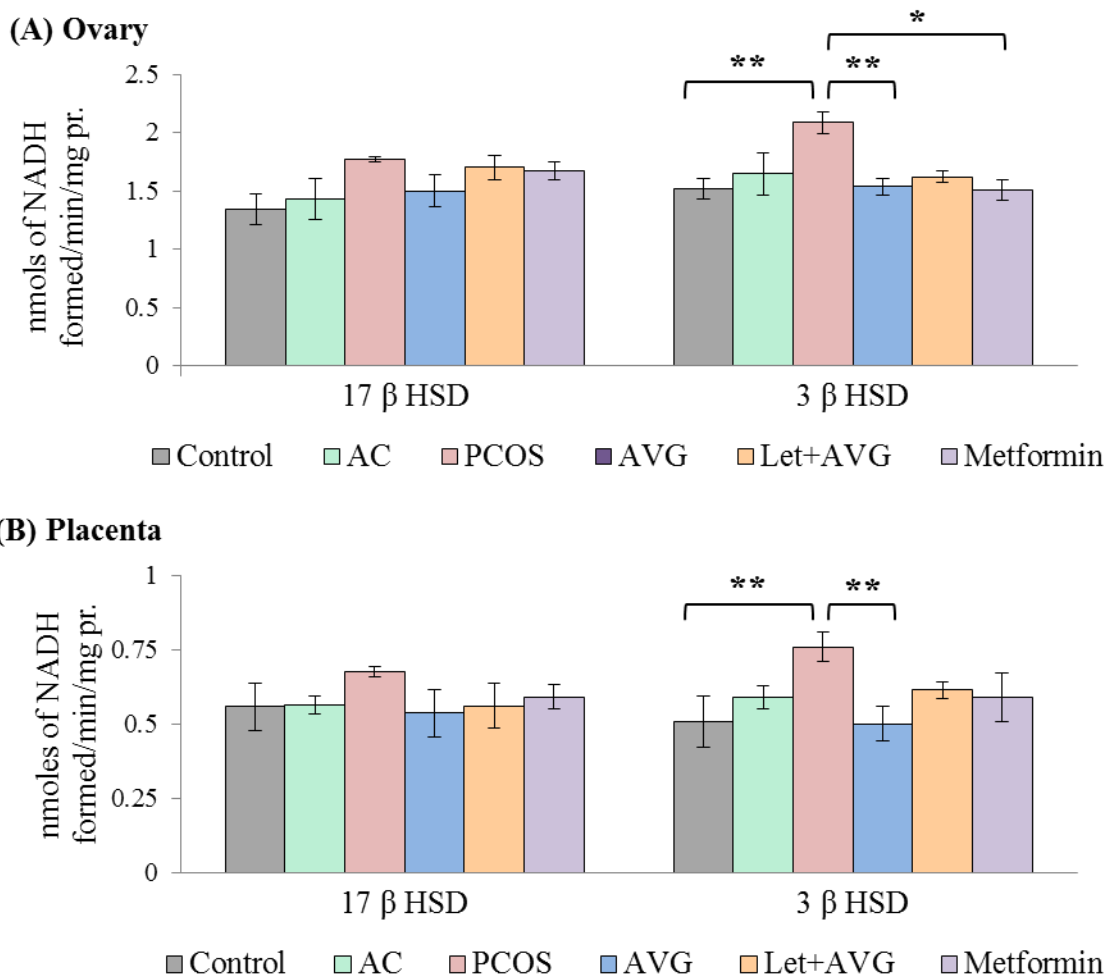
n=4 per group; All values are presented as Mean±SEM; *P<0.05; **P<0.01.

C=Control; AC= Aloe control; P=PCOS; AVG= PCOS treated with AVG; Let+AVG= PCOS treated with AVG +Letrozole; Metformin= PCOS treated with Metformin
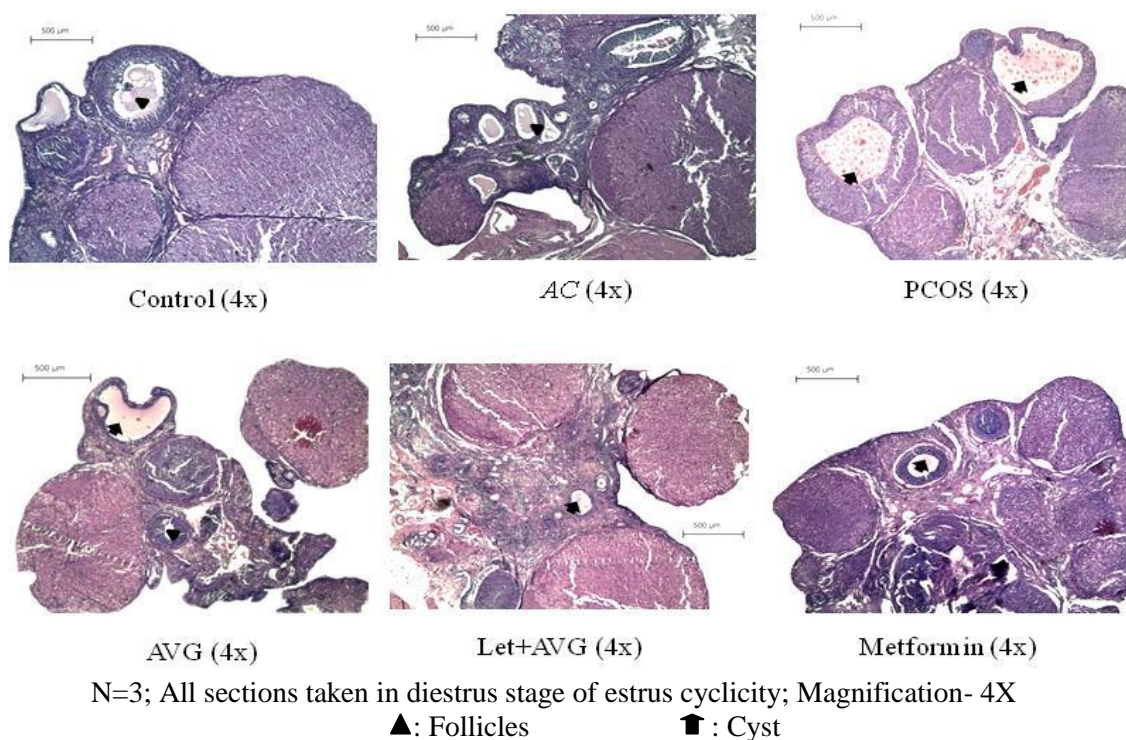
Fundamental alterations could be revealed by changes in the tissue structure. Hence, ovarian and placental histology was studied in all groups of animals. Histological sections of PCOS rat ovary exhibited presence of multiple peripheral cysts as compared to control group which showed the presence of healthy growing follicles. AVG treatment in PCOS rats regained normal follicular development. Let + AVG (Group 5) animals also demonstrated partial reversion of PCO phenotype as comparable to control group (Figure 5.3.7) It is interesting to note that AVG treatment caused significant decrease in atretic

follicles and reverted back ovarian structure - function to normalcy as compared to PCOS animals whereas no change was observed in placental structure in all groups of animals (Figure 5.3.8).

Table 5.3.3 demonstrates the hormonal profile of all the groups of animals, wherein serum insulin levels was significantly higher in untreated PCOS rats (***$p < 0.001$) as compared to control group, whereas AVG treated PCOS rats exhibited significantly reduced insulin levels ($^{@@}p < 0.01$). Apart from AVG group, metformin group also represented a decrease in insulin levels which was comparable to control group ($^{@}p < 0.05$). HOMA-IR index also has been evaluated to indicate insulin resistance condition. The PCOS rats demonstrated an increased insulin resistance condition (HOMA IR- 4.2) whereas AVG treatment reduced the resistance in all group as similar to control group (HOMA IR <3).

Steroid hormones like Testosterone, progesterone and estradiol also have been evaluated, wherein PCOS rats demonstrated significant high level of testosterone (**$p < 0.01$) whereas significant decreased level of progesterone (**$p < 0.01$) and estradiol (***$p < 0.001$). AVG treated PCOS rats exhibited significant reduction in testosterone level and improved progesterone and estradiol levels as comparable to control group. Also, Let+AVG (Group 5) demonstrated reduction in testosterone level as compared to PCOS rats (*$p < 0.05$) whereas no significant change was observed in metformin group.

Figure 5.3.7 Effect of *Aloe vera* gel on Ovarian structure at late gestation period in letrozole induced PCOS rats

N=3; All sections taken in diestrus stage of estrus cyclicity; Magnification- 4X
▲: Follicles   ⬆ : Cyst

Figure 5.3.8 Effect of *Aloe vera* gel on Placental structure at late gestation period in letrozole induced PCOS rats
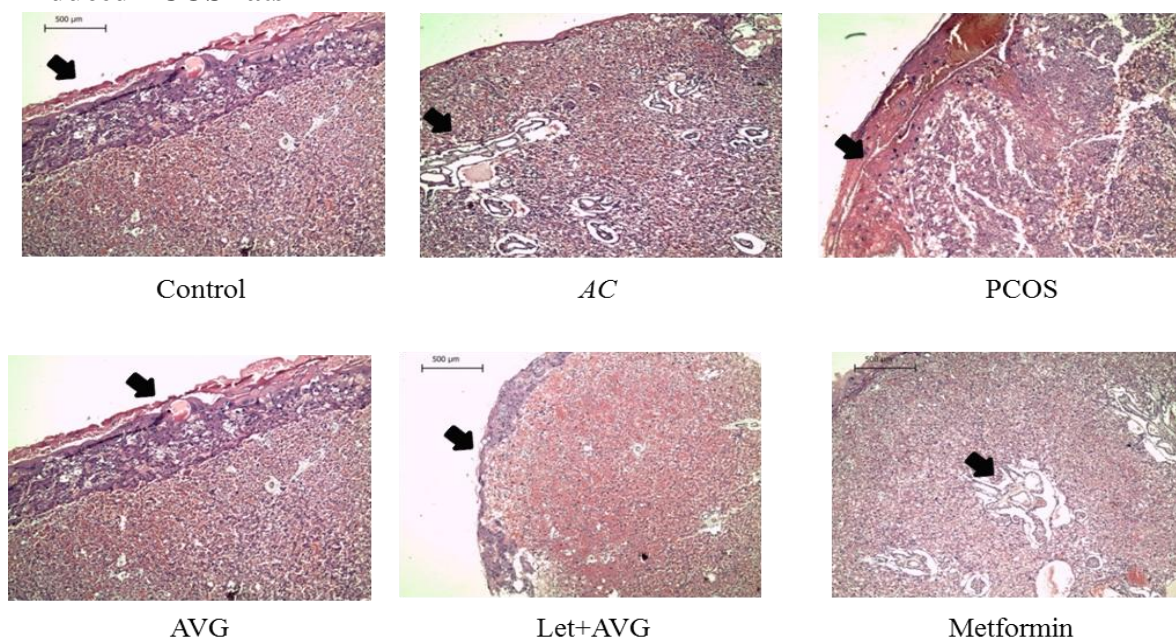
N=3; Magnification- 4X; C=Control; AC= *Aloe* control; P=PCOS; AVG= PCOS treated with AVG;
Let+AVG= PCOS treated with AVG+Letrozole; Metformin= PCOS treated with Metformin

Table 5.3.3 Effect of *Aloe vera* gel on hormonal profile late gestation period in letrozole induced PCOS rats

	Testosterone (ng/ml)	Progesterone (ng/ml)	Estradiol (pg/ml)	Insulin (µIU/ml)	HOMA-IR
Control	1.185+.22	26.5+2.0	10.8+0.8	8.66+2.18	1.63+.044
AC	0.845+.52	29.0+2.1	7.3+0.3	12.33+1.74	2.25+0.28
PCOS	3.47+0.61**	13.13+3.3**	1.16+0.3***	20+2.08***	4.69+0.36***
AVG	0.685+0.29@@@	40+4.61@@@	5.8+2.1@	9.5+1.32@@	1.89+0.24@@@
Let+AVG	1.46+0.47	25.33+4.1@	3.4+1.2	16+0.57	3.0+0.17@@
Metformin	0.94+0.33	23.0+3.2@	1.5+0.2	12+0.57@	2.23+0.26@@@

N=4, Mean+SEM.
**p<0.01 , ***p<0.001 as compared to PCOS group.
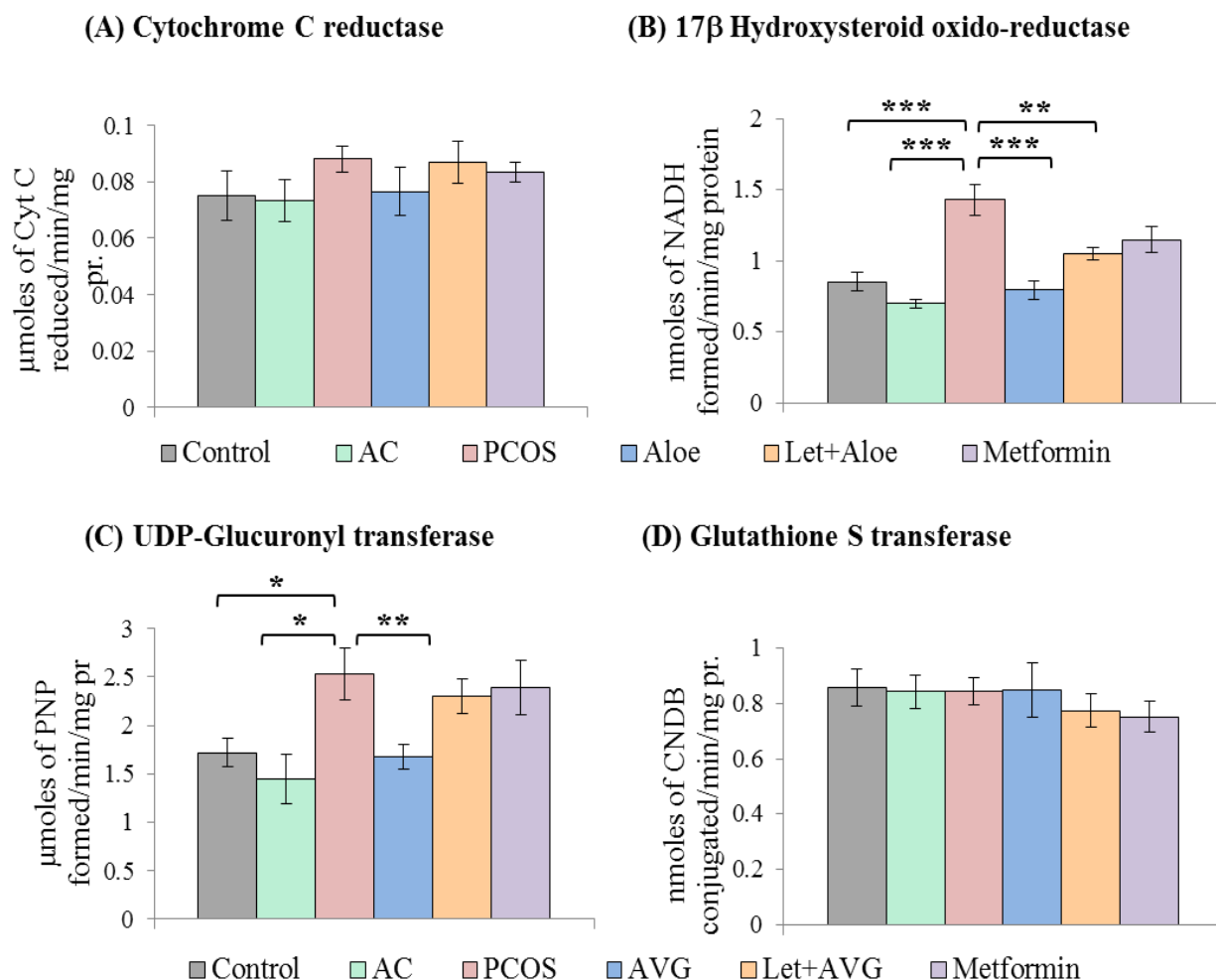@@@ p<0.001, @@ p<0.01, @ p<0.05 as compared to PCOS group
C=Control; AC= Aloe control; P=PCOS; AVG= PCOS treated with AVG; Let+AVG= PCOS treated with AVG +Letrozole; Metformin= PCOS treated with Metformin

An altered hormone profile as seen above could be due to the reflection of altered biotransformation. Thereby, Phase I and Phase II enzymes were also evaluated. The cytochrome P450 oxidoreductase (Cyt C) enzyme activity of phase I reaction exhibited no significant increase in letrozole induced PCOS rats as compared to control group. Also, rest of groups exhibited no change in enzyme activity (Figure 5.3.9 A, B, C and D). 17β Hydroxysteroid reductase enzyme activity (phase I) reaction demonstrated a significant reduction in its activity in AVG treated (@@p<0.01) and Let+ AVG (Group 5) which could be compared to the control group (@@p<0.01); however, non-significant change was observed in metformin group. The liver steroid metabolizing enzyme UDP-Glucoronyl transferase (UDPGT) (Figure 3.18) exhibited a significant increase in its activity in PCOS rats as compared to control group at 18th -20th day of gestational period (*P<0.05) whose activity was reduced upon AVG treatment (@@p<0.01). The effect of AVG on liver steroid metabolizing enzyme UDP-Glucoronyl transferase (UDPGT) demonstrated significant decreased enzyme activity as compared to PCOS rats at term. Let + AVG

(Group 5) and metformin group exhibited non-significant change in UDP-Glucoronyl transferase (UDPGT) activity as compared to PCOS group.

Figure 5.3.9 Effect of *Aloe vera* gel on Liver steroid metabolizing enzymes in letrozole induced PCOS rat at late gestation

(A) Cytochrome C reductase

(B) 17β Hydroxysteroid oxido-reductase

(C) UDP-Glucuronyl transferase

(D) Glutathione S transferase

n=4 per group, All values are represented as Mean+SEM. *P<0.05; **P<0.01; ***P<0.001
C=Control; AC= Aloe control; P=PCOS; AVG= PCOS treated with AVG;
Let+AVG= PCOS treated with AVG +Letrozole; Metformin= PCOS treated with Metformin
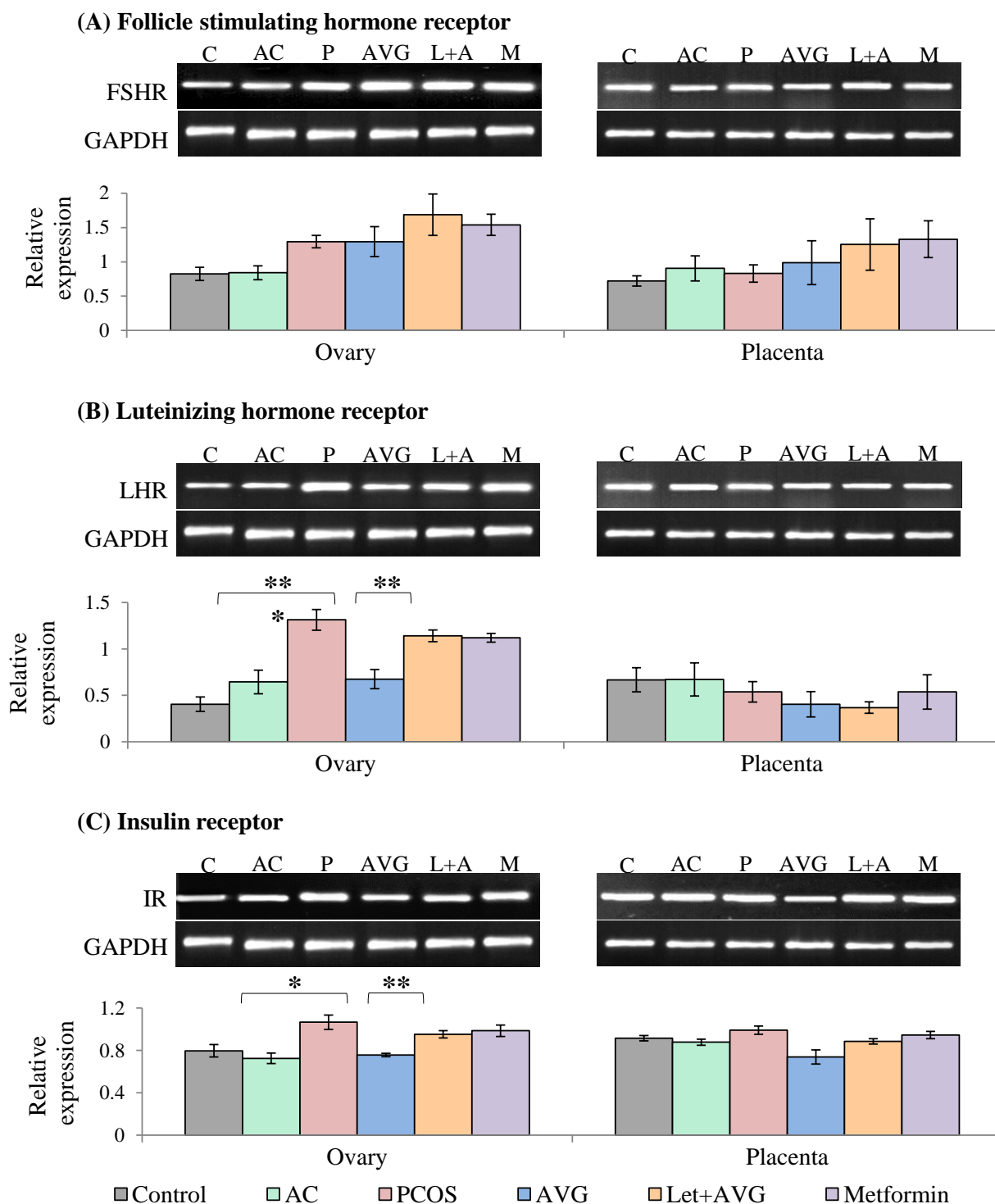
All the above biochemical parameters studied exhibited steroid alterations mainly at the biosynthetic level. Therefore, to understand changes that occurred on AVG treatment, molecular level studies were attempted. In this context, gene expression studies of key steroid regulatory proteins that play an important role in ovarian and placental structure-function were evaluated. Letrozole induced PCOS rats exhibited significant increase in key steroid regulatory protein – Steroidogenic acute regulatory (StAR) in ovary (***$p<0.001$) whereas non-significant change was seen in placenta. AVG treated PCOS rats exhibited reduction in StAR expression in ovary. A significant increase in expression of key receptors namely Androgen receptor (AR) and Luteinizing hormone receptor (LHR) was observed in PCOS rats as compared to control rats (**$p<0.01$). AVG treatment significantly reduced the gene expression of these receptors in PCOS rats (@$p<0.05$, **$p<0.01$) (Figure 5.3.10). Both Let+AVG treated group (Group 5) and metformin groups demonstrated non-significant change in StAR and receptor genes expression as compared to PCOS group.

PCO phenotype is associated with hyperinsulinemia and insulin resistance (Anderson et al. 2013). Hence, gene expression of insulin receptor (IR) in ovary and placenta, wherein PCO positive rats exhibited significant increase in ovarian IR gene expression as compared to control group (*$p<0.05$). However, no significant change was observed in placenta. These results can be correlated with high serum insulin levels along with elevated HOMA-IR in PCOS as compared to control. This increased serum insulin and gene expression levels reverted back to normalcy after AVG treatment as compared to PCOS group (@@$p<0.01$). Let+ AVG (Group 5) and metformin groups did not show any significant change in expression level as compared to control group.

In addition to this, gene expression of aromatase was studied, as it plays an important role in estrogen biosynthesis in both ovary and placental tissues. PCOS positive rats demonstrated significant decrease in gene expression of aromatase as compared to controls (***$p<0.001$). AVG treatment significantly increased the expression of aromatase in ovary (@@$p<001$) as well as in placenta (@$p<0.05$). Let+AVG (Group 5) demonstrated significant modulation of aromatase gene expression in ovary as compared to PCOS group (@$p<0.05$) whereas no significant change was observed in metformin group in both the

reproductive tissues (Figure 5.3.10).

A summary of the relative protein expression of key proteins involved in steroidogenesis is presented in Figure 5.3.11. Expression of StAR protein in placenta was significantly elevated (*$p<0.05$) with no significant change in ovary of PCOS rats while AVG treatment caused a reduction in its expression (@$p<0.05$). The placental StAR protein demonstrated a reduced expression in Let+ AVG (Group 5) and metformin as compared to PCOS animals (@$p<0.05$). Ovarian androgen receptor protein was significantly reduced in AVG treated PCOS animals (@$p<0.05$) and Let+AVG (Group 5) animals as compared to PCOS positive animals (*$p<0.05$) where AR expression was high but no significant change was observed in the protein expression of AR in placental tissues amongst all groups of animals.

Figure: 5.3.10 Effect of *Aloe vera* gel on important steroidogenesis regulating genes in letrozole induced PCOS rats

(A) Follicle stimulating hormone receptor

(B) Luteinizing hormone receptor

(C) Insulin receptor

□ Control　□ AC　□ PCOS　□ AVG　□ Let+AVG　□ Metformin

Relative expression = Expression of Target gene/Expression of GAPDH

n=3-4 per group; All values are presented as Mean ±SEM; *P<0.05; **P<0.01; ***P<0.001

(D) Steroid acute regulatory protein (StAR)
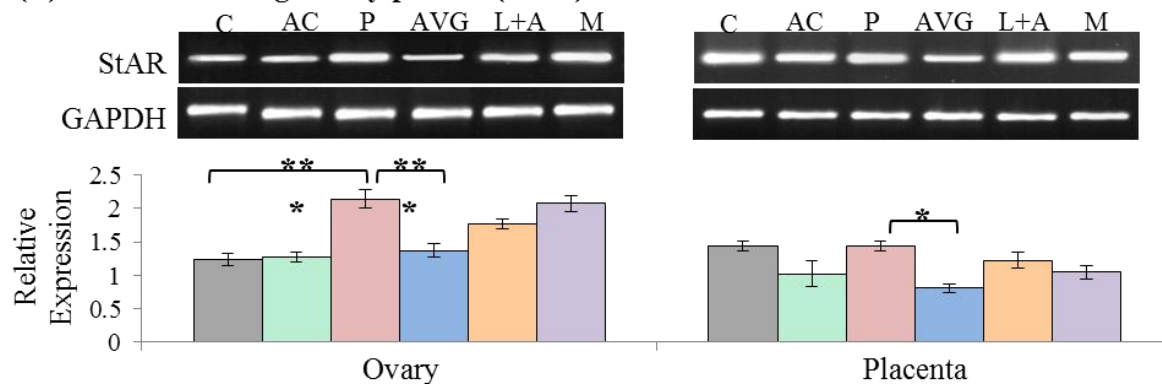
(E) Aromatase
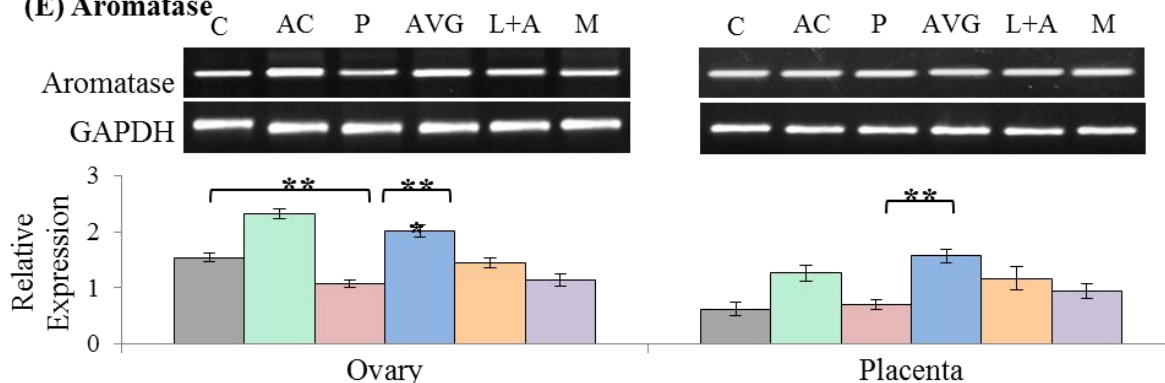
(F) Androgen receptor
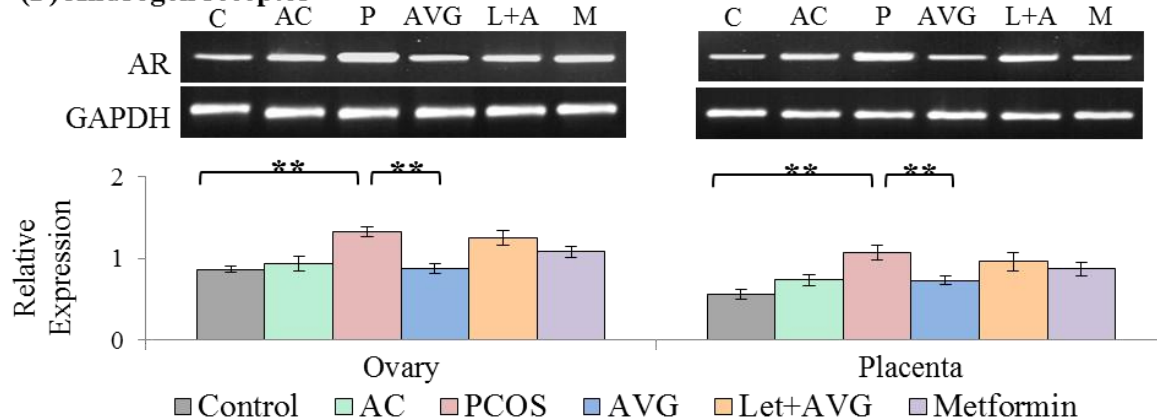
Relative expression = Expression of Target gene/Expression of GAPDH
n=3-4 per group; All values are presented as Mean $\pm$SEM; *P<0.05; **P<0.01; ***P<0.001
C=Control; AC= Aloe control; P=PCOS; AVG= PCOS treated with AVG; Let+AVG= PCOS treated with AVG +Letrozole; Metformin= PCOS treated with Metformin

Figure: 5. 3.11 Effect of *Aloe vera* gel on the expression of Steroid regulating protein in letrozole induced PCOS rats

(A) Steroid acute regulatory protein (StAR)
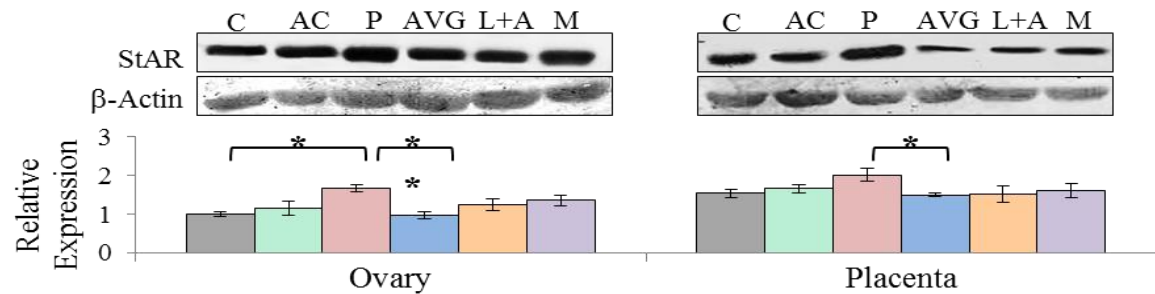
(B) 3β Hydroxysteroid dehydrogenase
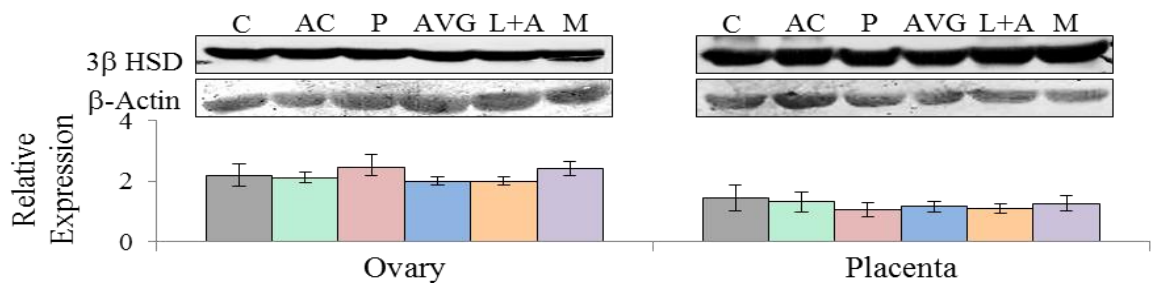
(C) Aromatase
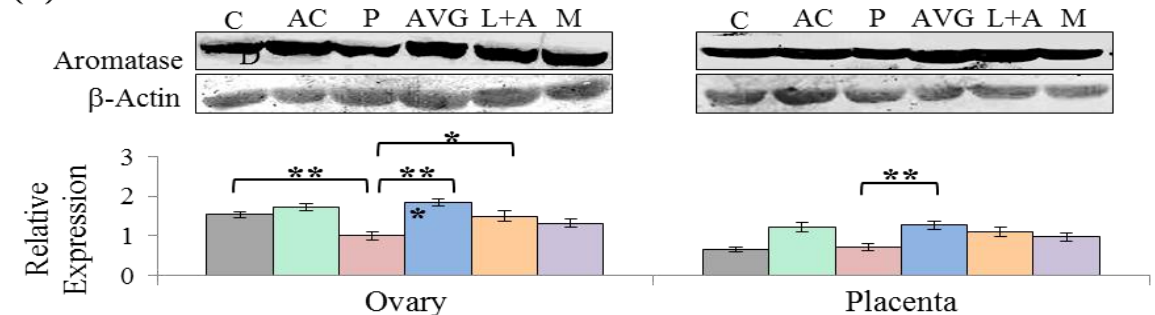
(D) Androgen receptor
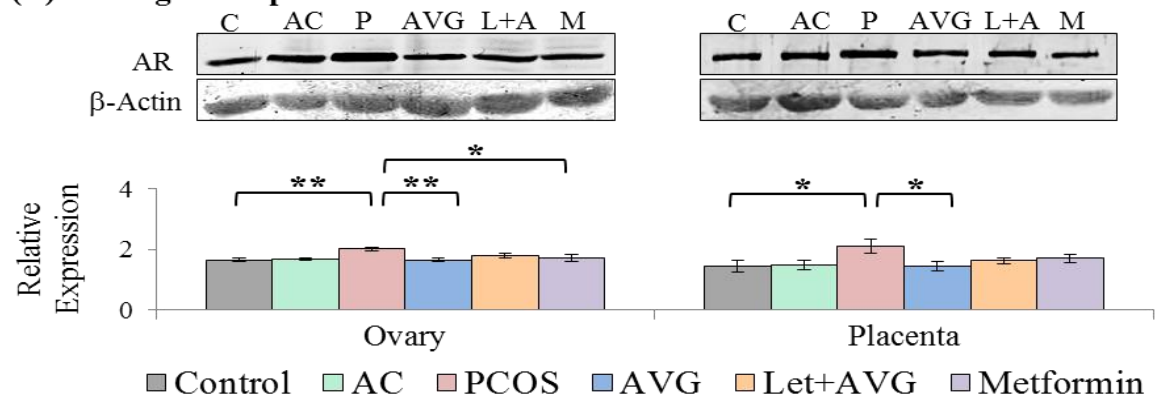
Relative expression = Expression of Target gene/Expression of β-Actin

n=3-4 per group; All values are presented as Mean ±SEM; *P<0.05; **P<0.01; ***P<0.001

During the course of experiments, toxicity parameters like Serum Glutamate Pyruvate Transaminase (SGPT) and creatinine was evaluated. AVG treated groups exhibited non-significant change in the liver toxicity parameters. In addition to this, Letrozole treated PCOS group as well as metformin treated animals showed no change in the above parameters upon treatment (Figure 5. 3.12).

Figure 5.3.12 Effect of *Aloe vera* gel on Serum Toxicity markers in letrozole induced PCOS rats

(A) Serum glutamate pyruvate transaminase   (B) Creatinine
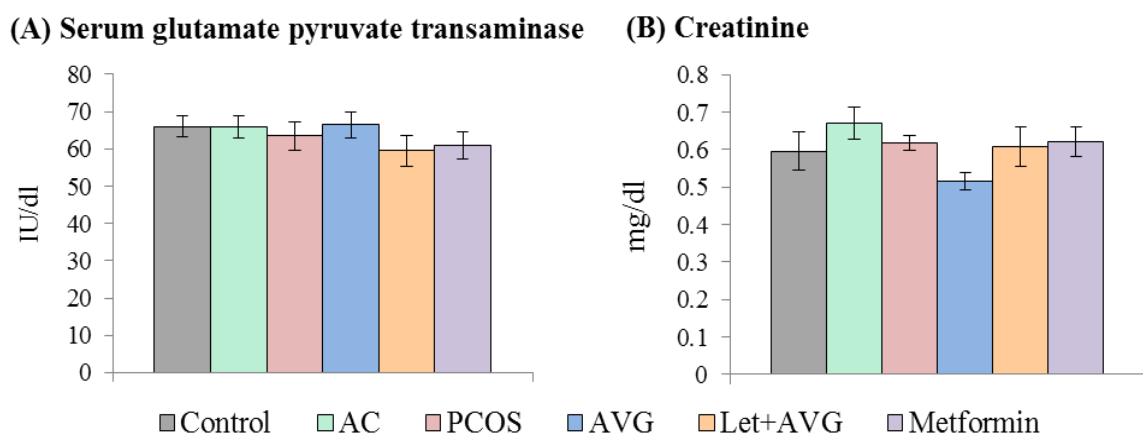
n=4 per group, All values are represented as Mean±SEM. *P<0.05; **P<0.01; ***P<0.001

5.4 DISCUSSION

In current study, PCOS rat model exhibited an increase in ovarian androgens and high insulin levels that led to hyperandrogenism, and hyperinsulinemia which are hallmarks of PCOS. PCOS rats exhibited lesser numbers of implantations and altered steroidogenesis in implantation window of gestation period. This may be due to the fact that hyperinsulinemia is also believed to play a key role in implantation failure due to its antagonistic effect on glycodelin which is an adhesive glycoprotein necessary for implantation in the endometrium, and hence results in increased rate of miscarriages in women with PCOS (Jakubowicz et al. 2004). This could be one of the reasons of implantation failure observed in our study in case of PCOS phenotype although there were no significant change observed in the implantation enzymes- Cathepsin-D and alkaline phosphatase (ALP). During implantation, the endometrium becomes receptive for a limited

period of time under the influence of steroid hormones. Progesterone plays a key role in establishing uterine receptivity by blocking the proliferative effect of estrogen on uterine epithelial cells and inducing genes that allow the endometrium to respond to the attachment of embyo (Halasz et al. 2012). Implantation failure could be attributed to the low levels of progesterone observed in PCOS rats. However, further studies at molecular level needs to be done to confirm the above fact.

Gestation period is also a crucial period for the fetal growth and development, wherein PCOS rats in current study demonstrated lesser number of live pups and presence of retarded fetal growth that may be due to high androgenic uterine microenvironment that inhibit factors involved in embryo implantation and their development (Sir-Petermann et al. 2002;(Carlsen et al. 2006). Also, PCOS endometrium over expresses androgen receptors and fails to down regulate estrogen receptor-α in the window of implantation (Apparao et al. 2002). Hence, dysregulation of steroid receptor expression and disturbed steroid hormone status also plays a crucial role in implantation as these may contribute to the lower pregnancy rates seen in PCOS women (Gregory et al. 2002). AVG treatment decreased testosterone levels and improved progesterone levels that were useful in increasing uterine receptivity and fetal growth in PCOS rats during gestation. It has been suggested that maternal excess testosterone reduces fetal growth, placental weight and birth weight via impaired placental function (Sathishkumar et al. 2011). Hormone profile in this study clearly demonstrates that *Alo*e has potential to sensitize the insulin receptor and reduce insulin level in PCO condition; thereby reverting insulin resistant state to sensitive status indicated by improved HOMA-IR change.

In the current study, the alteration in hormones could be correlated with changes observed in steroidogenic enzyme activities, wherein PCO rat demonstrated the altered enzyme activities of ovarian and placental steroidogenic enzymes such as 3β hydroxy steroid Dehydrogenase (3β HSD) and 17β hydroxy steroid dehydrogenase (17β HSD) during early as well as late gestation period (Doi et al. 2006). Also, high insulin levels have direct effect on ovarian steroidogenesis and stimulate thecal androgen production (Diamanti-Kandarakis and Dunaif 2012). These key steroidogenic enzymes activities were significantly decreased in both ovarian and placental levels in Aloe treated PCOS rats. The

result of altered activity could be correlated with serum steroid hormones level that regained normalcy after AVG treatment.

Increased insulin levels in PCOS rats directly stimulate ovarian Luteinizing Hormone receptor (LHR) gene expression leading to thecal androgens flux- Testosterone, DHEA, androsteindione rather than aromatization into estrogens in granulosa cells. This might be due to high 3β-HSD dehydrogenase enzyme activity, which is one of the key enzymes involved in ovarian androgen production. Additionally, LH pulse amplitude increases in women with PCO phenotype (Nestler 1997) and insulin specifically augment pituitary release of luteinizing hormone in various "in-vitro" studies (Adashi et al. 1981). Hence, a potential mechanism wherein insulin could enhance ovarian androgen production is by altering LH release. The elevated levels of insulin regain normalcy after AVG treatment in PCOS rats. This may be due to the hypoglycemic effect of AVG attributed by several phyto-components. AVG reduces the hyperinsulinemic condition as well as hyper androgenic condition by modulating the steroidogenic enzyme activities in the ovary of letrozole induced PCOS rats.

Disturbed steroidogenesis was observed as a result of altered enzyme activity which may due to change in expression profile of StAR in both tissues studied. High expression of StAR was observed in PCOS group which might be mainly because of synergistic effect of high LH and insulin levels that increase StAR expression by co-binding to the StAR promoter region (Sekar et al. 2000). High insulin levels also augmented LH stimulated cAMP levels that further affect StAR expression as cAMP dependent kinase A is known to be a key regulator of StAR expression. In addition to this, ovarian and placental protein content of StAR was evaluated, wherein placenta exhibited significant change in PCOS rat but no change was observed in ovary. This may be due to the fact that during the mid late gestation period of pregnancy, placenta takes up charge of major steroid production for fetal development (Maliqueo et al. 2013). Apart from altered steroidogenesis, PCOS rat also exhibited high gene expression of steroid receptor- Androgen receptor (AR) that plays a major role in high androgen production in PCO phenotype in ovary and placenta (Zurvarra et al. 2009).

Present study confirms the above fact that protein expression wherein high

expression of AR protein was observed in PCOS rats as compared to control. However, AVG treatment reduced the expression of androgen receptor in PCOS rats which could be compared with that of control. This may be due to the presence of flavonoids present in the gel, which are known to possess anti androgen effect by directly inhibiting the expression of androgen receptor (Xing et al. 2001). Under normal conditions, maternal androgens or fetal adrenal androgens are rapidly converted to estrogens by the activity of the placental enzyme aromatase. In PCOS condition, the activity of this enzyme is inhibited as the bio-availability of androgens is increased. Also, high Insulin has been shown to inhibit aromatase activity in cytotrophoblasts and stimulate 3β-hydroxysteroid dehydrogenase activity (Nestler et al. 1987). In the current study, AVG treatment decreased aromatase gene expression in ovary as well as placenta in PCO condition. The gene expression study of aromatase could also be correlated with the total estradiol content.

As function is altered, it is plausible that structural alterations do occur. Hence, histological study was performed. Studies revealed that PCO rats demonstrated the presence of multiple fluid filled peripheral cysts in the ovary as indicated by Kafali et al (2004). PCOS rats treated with AVG revealed normal follicular growth and reversal to normal cyclicity. The restoration in the ovarian structure and function can be attributed to the presence of several phyto-components that lead to modulation in the HPO axis. This modulation helped in maturation of follicles and release matured ova during ovulation. The normal follicular growth is necessary for formation and release of matured ova. Only healthy matured ova will get successfully fertilized and implanted. Apart from ovary, placenta acts as an important structural component of pregnancy. It is a mediator for both mother and fetal steroid exchange during gestation period. Reports suggest that high testosterone levels may affect placental development and function by modulating amino acid transporters (Sathishkumar et al. 2011) or by regulating the expression of enzymes and androgen/ estrogen receptors, as demonstrated in human placentas (Glueck et al. 2002). Structure and function of placenta was examined, wherein mild changes were observed in structure of placenta of PCOS rat.

The altered steroid status during pregnancy leads to disturbance in steroid

metabolism (Flynn et al. 2005). In current study, PCOS rats with high androgen levels demonstrated increased levels of both liver steroid metabolizing enzyme activities during late gestation period. The CYP1A1 (Cytochrome P450 1subfamily A polypeptide 1 gene encodes phase I cytochrome P450 enzyme, involved in metabolism of estrogens. In this regards, women who carry polymorphic variants in this gene confers higher CYP1A1 activity and may be at higher risk of PCOS (Wang et al. 2009). AVG treated PCOS rats exhibited modulatory effects on both phases I and II steroid metabolizing enzymes activities wherein they showed reduced activities of both these enzymes during pregnant stage. This could be due to the phyto-components present in AVG which are reported to possess modulatory effect on liver metabolizing enzymes (Misawa et al. 2012; Guo et al. 2010).

5.5 CONCLUSION

In this chapter, study was focused to investigate the efficacy of *Aloe vera gel* (AVG) as preconceptive agent in letrozole induced PCOS rats. Current study demonstrated that PCOS positive rats failed to implant during early gestation period. At late gestation, conceived PCOS rats demonstrated increased post implantation loss, wherein it exhibited resorbed fetus with retarded growth. This could be attributed to abnormal hormonal status revealed by altered ovarian structure-function. These alterations could be overcome by treatment with AVG (10mg dry weight/60 days daily orally) before conception. It helped to restore back the ovarian structure – function to normalcy leading to improved fertility index as compared to PCOS rats. AVG treatment improved ovarian and placental steroidogenesis by modulating key protein expression leading to normalised steroid mileu resulting in successful pregnancy. These observed changes could be attributed to Phyto-nutrient rich AVG that could act at various targets in the reproductive organs in the PCOS rats before conception. This study principally emphasized that AVG can act as preconceptive agent which can successfully induce structural-functional changes leading to successful term pregnancy.

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