CHAPTER 1

INTRODUCTION

1. INTRODUCTION

Current era has evidenced an increase in female infertility. Several reports have suggested that female infertility would pose a threat in upcoming decades. In this context, the present thesis has focused to search new alternative therapeutic options for management of female infertility.

1.1 FEMALE REPRODUCTIVE SYSTEM

The female reproductive system consists of a pair of ovaries, fallopian tubes, uterus and vagina (Figure 1.1). These organs are involved in the production and transportation of gametes under the influence of sex hormones. It also facilitates the fertilization of ovum by sperm and supports the development of foetus during pregnancy.

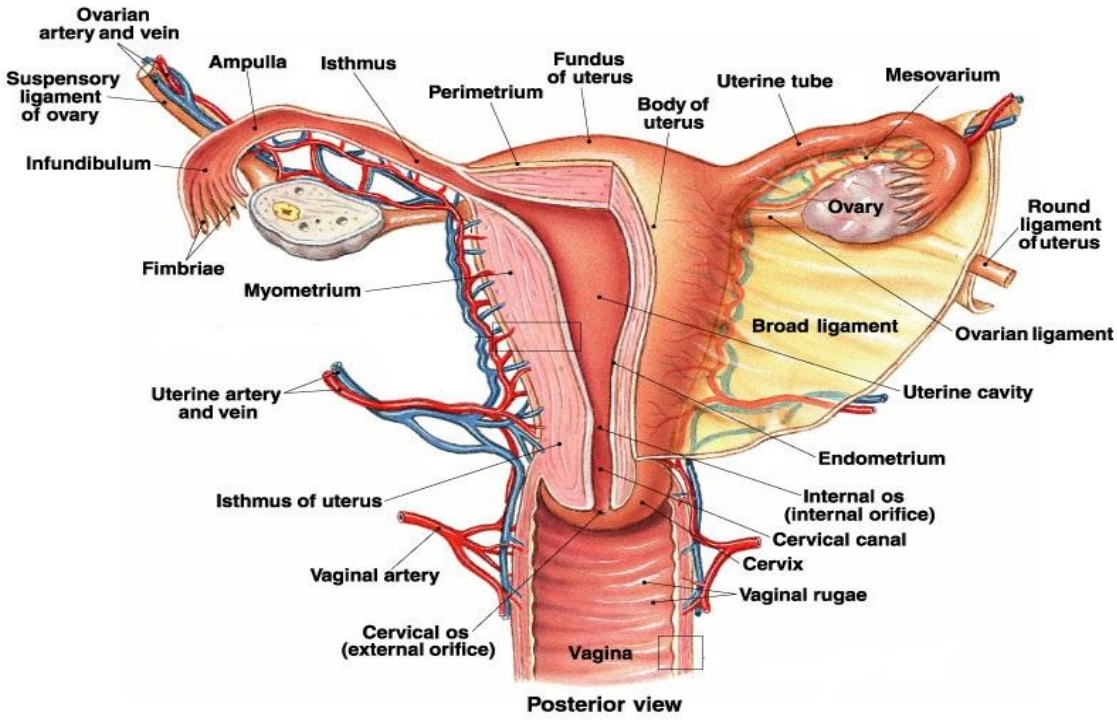


Figure 1.1 Systematic diagram of female reproductive system

A pair of ovary is located in the pelvic region whose primary function is to nurture and prepare oocytes (eggs) for the process of ovulation. These processes of ovulation and fertilization are controlled largely by cells of the ovaries that produce and secrete hormones namely estrogen, testosterone and progesterone under the influence of higher

regions of brain. Hence, they act as both gonad as well as endocrine glands. These hormones are essential for female sexual development, sustenance and maintenance of pregnancy. An organ that plays a vital role in pregnancy is uterus. The uterus is the hollow pear-shaped organ located in the lower centre of women's pelvis. The uterus is divided into two parts: the cervix, which is the lower part that opens into the vagina, and the main body of the uterus, called the corpus. The corpus can easily expand to hold a developing foetus.

The main function of the uterus is to receive the embryo; implant it on the uterine wall and deliver the foetus at-term. Structurally, uterus has two regions- 1) Endometrium and 2) Myometrium. Endometrium is the layer where the implantation takes place. This layer experiences morphologic and functional changes that are closely associated with the cyclic release of sexual hormones during the menstrual cycle. Myometrium is the muscular wall of the uterus. This layer lies between the endometrium and internal layer of glands lining the uterus. The behaviour of the myometrium is determined mainly by the relative amounts of the ovarian hormones- oestrogen and progesterone. In follicular phase of menstrual cycle, estrogen acts on its receptor present in the endometrium to proliferate the cellular and glandular structure. Thereby, follicular phase of menstrual cycle is called proliferative phase. During early pregnancy, proliferation of endometrial tissues is supported by the dramatic increase in cellular proliferation, neovascularization, and blood flow. The endometrial growth and regression are synchronized with the ovarian function through the changes in circulating and/or local levels of estrogen and progesterone, thereby ensuring that the uterine environment will be favourable for embryonic development and placentation.

1.1.1 DEVELOPMENT AND MATURATION OF OVARIAN FOLLICLE

In females, the total number of eggs to be produced is initially arrested at the diplotene stage of the meiosis-I in early foetal life until puberty. Upon onset of puberty, luteinizing hormone (LH) surge stimulates the resumption of meiosis I. The production of one egg cell via oogenesis normally occurs only once a month till menopause is attained.

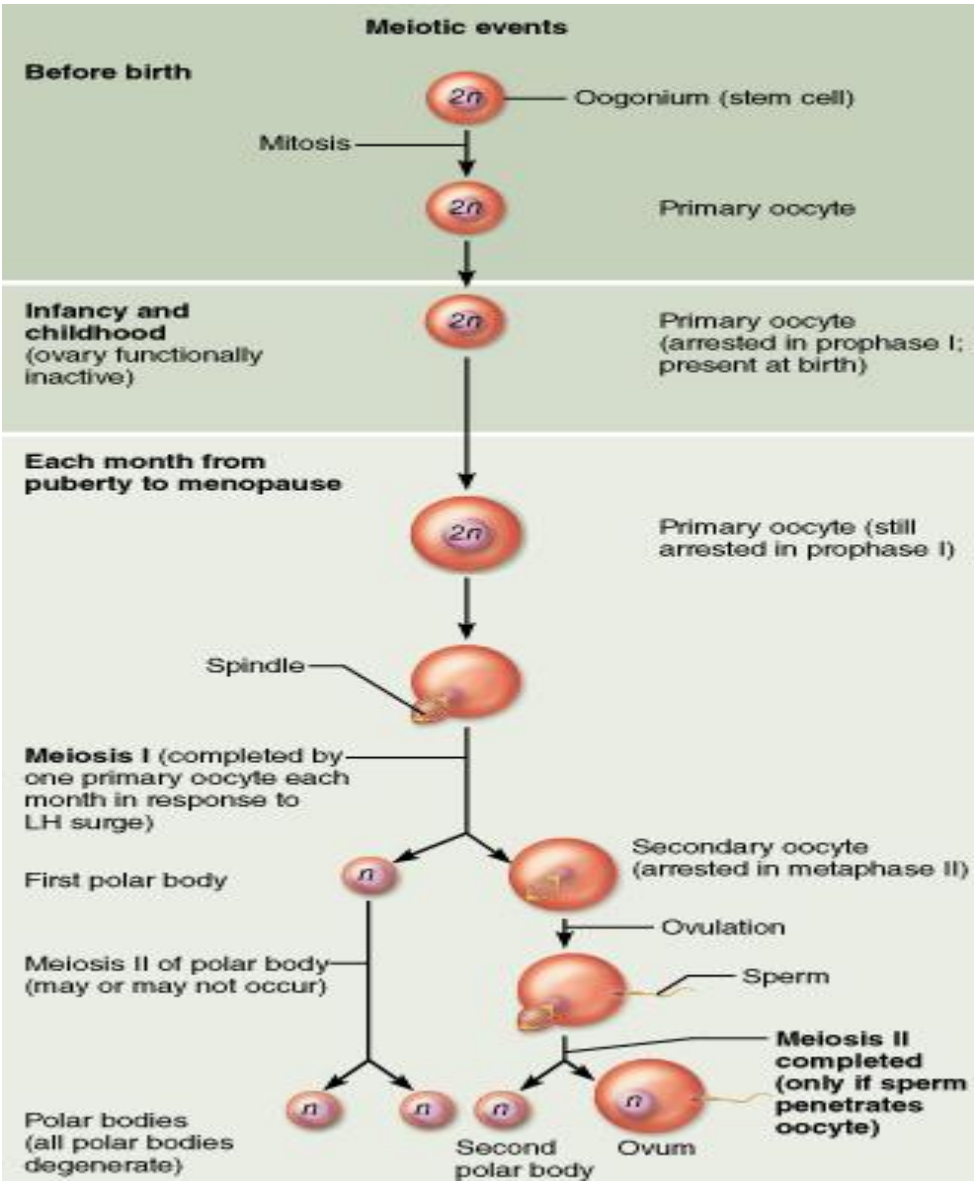


Figure 1.2 Process of oogenesis

At birth, the number of follicles are around 50,000 to 5,00,000. Gradually these immature follicles decrease in number as age of female increases till the menopause phenomena where the cessation of reproductive function takes place (Johnson et al., 2004). During maturation, immure egg cells transform into matured enlarged follicles and migrate towards the outer surface of the ovary. The cells lining the follicles multiply to form a layer known as zona granulose and a cavity forms within this zone.

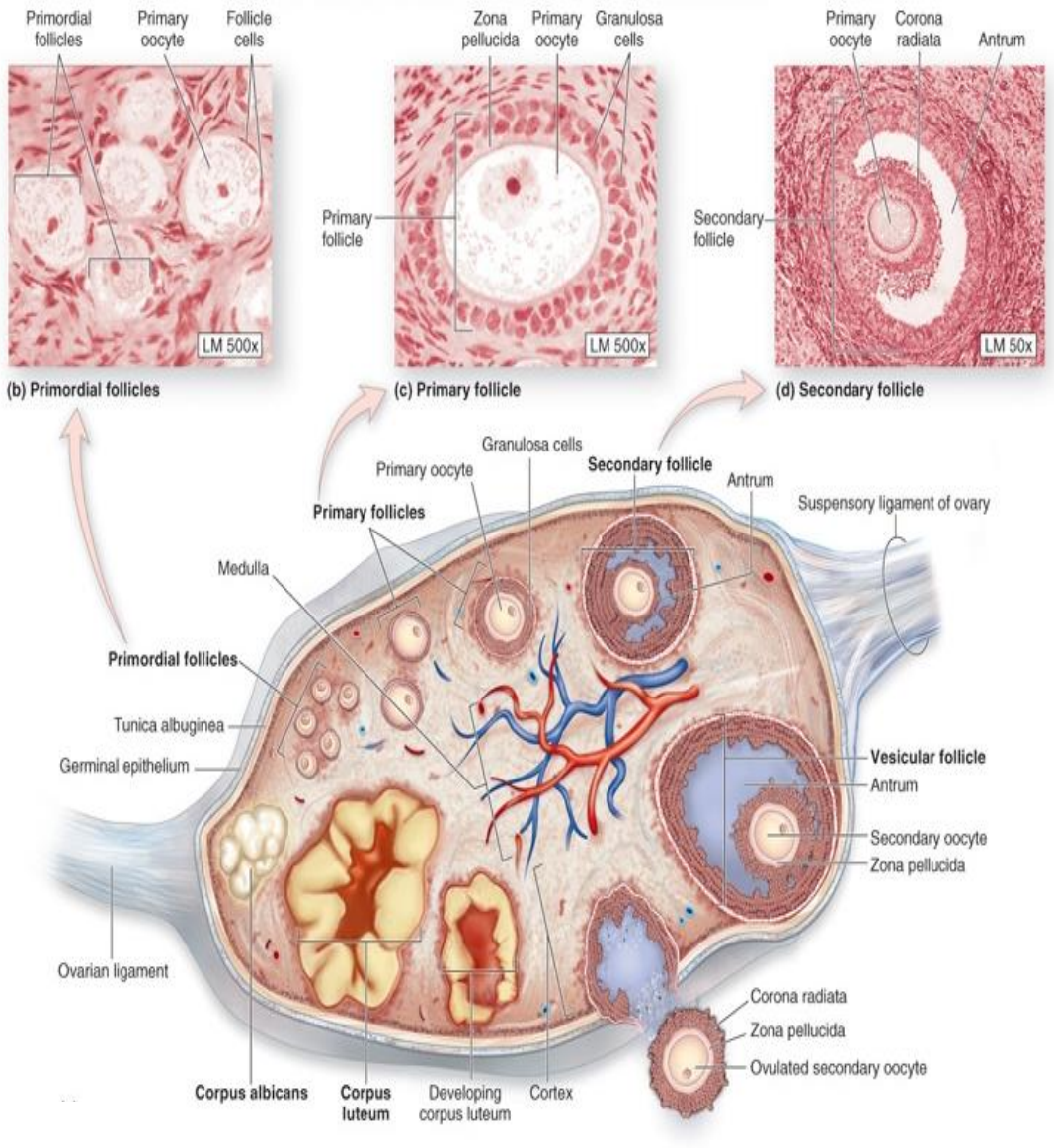


Figure 1.3 Folliculogenesis

The stromal and interstitial cells that surround the follicles arrange themselves concentrically to form a theca (an enclosing sheath) around the zona granulosa. One or sometimes more of the follicles are selected for further growth and maturation known as Graffian follicles. These follicles may reach 30 mm (about 1.2 inches) in diameter before they rupture. Follicular maturation and development is a complex process of interrelated intra- and extra ovarian events that ultimately lead to ovulation of a mature oocyte and transformation of the ruptured follicle into corpus luteum.

The primordial follicle consists of an immature oocyte arrested in the prolonged resting phase in oogenesis. These oocytes remain in immature state because of oocyte maturation inhibitor (OMI) secreted by granulosa cells. The cumulus cells may be intimately involved in the action of OMI to arrest the oocyte as well as the resumption of meiosis during the Luteinizing hormone (LH) surge that leads to initiate ovum maturation process upon onset of puberty. The compartments of the follicle that change most dramatically during follicular maturation are the cells lining of the follicle- the granulosa and theca cells (Messinis, 2006)**.** All the above events are tightly regulated by brain forming H-P-O axis.

1.2 REGULATION BY HYPOTHALAMUS-PITUITARY-OVARIAN (HPO) AXIS

Hypothalamic–pituitary-ovarian axis plays a very important role in development and regulation of female reproductive system, wherein Gonadotropin Releasing Hormone (GnRH) is released from hypothalamus-central region of brain. This GnRH further stimulates anterior pituitary for release of gonadotropins: Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH). These gonadotropins stimulates the growing follicle for the release of principal hormones- estradiol (E2), progesterone, inhibin; which are responsible for regulating reproductive cycle as well as reproduction. The levels of each of these hormones are regulated by a complex feedback loop – activities where periphery stimulates GnRH secretion from the hypothalamus.

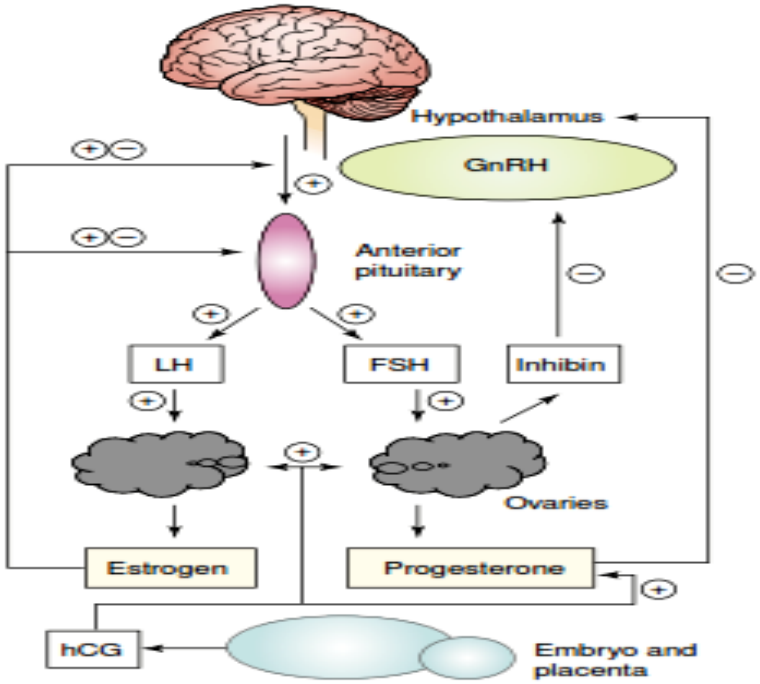


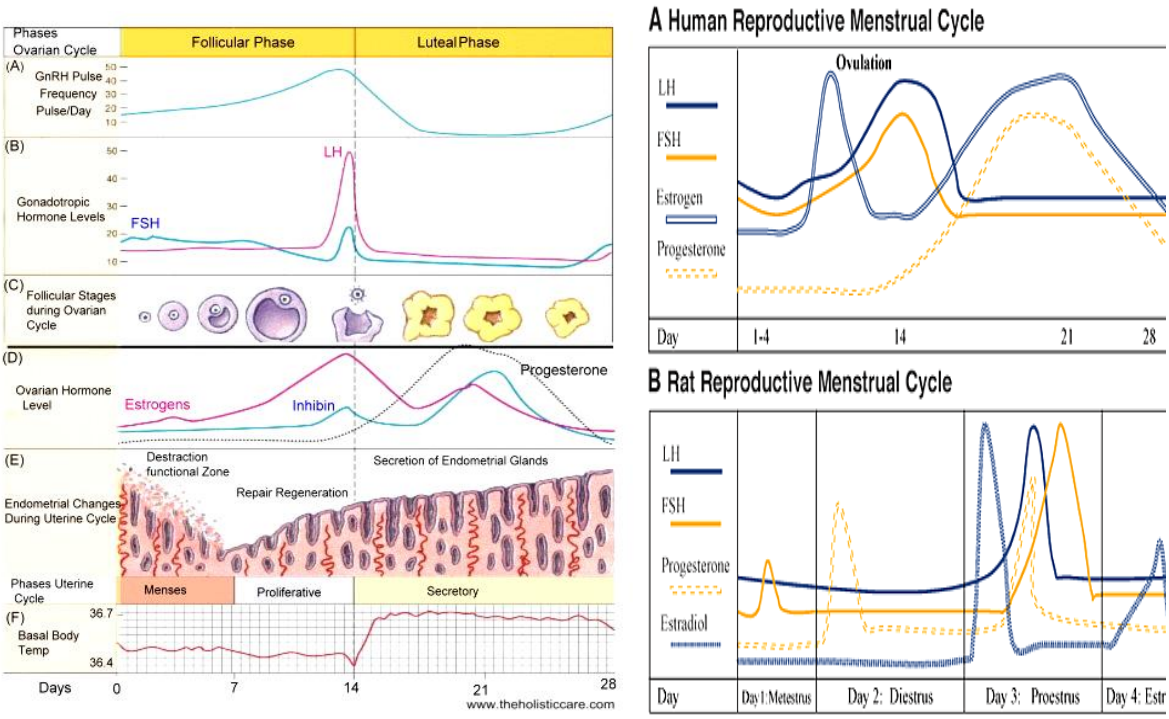
Figure 1.4 Regulation of ovarian function by Hypothalamus-Pituitary-ovarian (HPO) axis.

Estrogen (E2) is produced by the ovarian follicles during proliferative phase wherein increase in levels of estrogen initiates LH in positive feedback regulation. Further, estrogen level continues to rise to the threshold level and causes LH surge. At LH surge ovulation occurs, which results in a decline in the estrogen levels due to the feedback mechanism and further leads to establishment of the corpus luteum. Finally, regression of the corpus luteum occurs indicating decline in estrogen and progesterone release from ovary. The decrease in levels of these ovarian hormones elicits a negative effect on the secretion of LH. Thus, hormonal regulation plays a crucial role in regulation of female reproductive cycle by both positive and negative feedback mechanism.

1.2.1 REPRODUCTIVE CYCLE AND ITS REGULATION BY HPO AXIS

The menstrual cycle is tightly controlled by endocrine, autocrine and paracrine factors. These factors regulate the ovarian follicular development, ovulation, luteinisation, luteolysis, and remodelling of the endometrium. The menstrual cycle in humans is characterised by high variability in cycle length (26–35 days), 5-day menses and a fertile

phase from 5 days before to the day of ovulation whereas in rat, the reproductive cycle completes around 4-5 days and is called Estrus cycle**.** It can be divided into four stages: proestrus, estrus, meta-estrus and diestrus.



(a) **(b)**

Figure 1.5 (a) Hormonal regulation of Human menstrual cycle ;(b) Comparison between Human and rat menstrual cycle.

Estrus cycle in rodents is similar to human menstrual cycle wherein matured ovum is release from the follicles. Estrus cycle can be divided into main four stages: Proestrus, Estrus, Meta-estrus and Diestrus as explained below:

- Proestrus: In this phase, one or several follicles in the ovaries are in the initial phase of growth. Under the influence of estrogen, the lining in the uterus (endometrium) starts to develop. This stage is around 18-20 hours and vagina shows the presence of epithelial cells.
- Estrus ('Heat'): During estrus phase, ovarian follicles start maturing and estrogen secretions exert their biggest influence under the regulation of gonadotropic hormones. Ovulation is spontaneous and occurs about 10 hours after the beginning of estrus. This phase lasts for 5-8 hours and the vaginal smear exhibits irregular shaped cells.

- Meta-estrus: During this phase, the estrogen stimulation subsides and the corpus luteum starts to form. The uterine lining is under the influence of progesterone and becomes secretory. During this phase, the corpus lutea grows to a maximal volume, achieved within 24 hours of ovulation. Here, smear of vagina shows presence of irregular shaped cells along with leucocytes.
- Diestrus: Diestrus is characterized by the secretion of progesterone from the corpus luteum. This phase lasts for about 24-48 hours and the vagina shows the presence of only leucocytes.

In regularly cycling women, concentration of FSH is maintained throughout the follicular phase and slowly declines as process advances from Days 5 to 13 (Ginther et al., 2005). The ovulatory cycle is characterised by an increase in mean FSH concentration, followed by an increase in mean LH concentration during the late luteal phase. Lower LH pulse frequency during the mid-to late follicular phase and a reduced response to estradiol leads to induction of the surge (Park and Jordan, 2002). Pulsatile release of GnRH, FSH and LH play essential role in regulation of female reproduction. LH pulse frequency is regulated by ovarian hormones- estradiol and progesterone.

Progestin is secreted by the luteinized follicles of the ovary and they stimulate the release of proteolytic enzymes from the theca cells and ultimately prepares for ovulation. During the luteal phase, progestins induce swelling and secretion of the endometrium. After ovulation, the corpus luteum secretes progesterone & helps to prepare the uterine lining for pregnancy. In the presence of a GnRH pulse, the pituitary and ovarian hormones exert mutual control over the circulating levels of one another. The complex interactions between pituitary and ovarian hormones involve forward control, positive feedback, and negative feedback mechanisms, and serve to sustain a self-perpetuating monthly cycle (Oakley et al., 2009). These effects of gonadotropins on ovaries lead to formation of steroids by process called steroidogenesis.

1.3 STEROID HORMONES PRODUCED BY STEROIDOGENESIS PROCESS

Steroidogenesis is the process wherein various forms of steroids are synthesized by transformation of other steroids in specialized cells of specific tissues. Examples are

formation of the ovarian and placental progestogens and estrogens, which regulate reproductive function and secondary sex characteristics in the female.

Steroid hormones are the derivatives of cholesterol, and are synthesized by a variety of tissues, most prominently by the adrenal gland and gonads. Biosynthesis of these hormones requires a battery of oxidative enzymes located in both mitochondria and endoplasmic reticulum. The rate-limiting step in this process is the transport of free cholesterol from the cytoplasm into mitochondria. Transportation of free cholesterol is done by activation of a regulatory protein namely Steroidogenic Acute Regultaory protein (StAR), a novel mitochondrial cholesterol transporter that transports cholesterol to inner mitochondrial membrane. Following which, it is converted to pregnenolone by P_{450} side-chain cleavage (P_{450}scc) (Jefcoate et al., 1992; Stocco, 2001). After this rate-limiting step, subsequent biosynthetic steps proceed with the flow of substrates through the enzyme systems located in the endoplasmic reticulum and mitochondria. Steroid hormones are hydrophobic molecules that can penetrate biological membranes, and flow into the blood stream after their synthesis. An increase in the blood levels of steroid hormones is dependent on the continual synthesis and secretion of steroids.

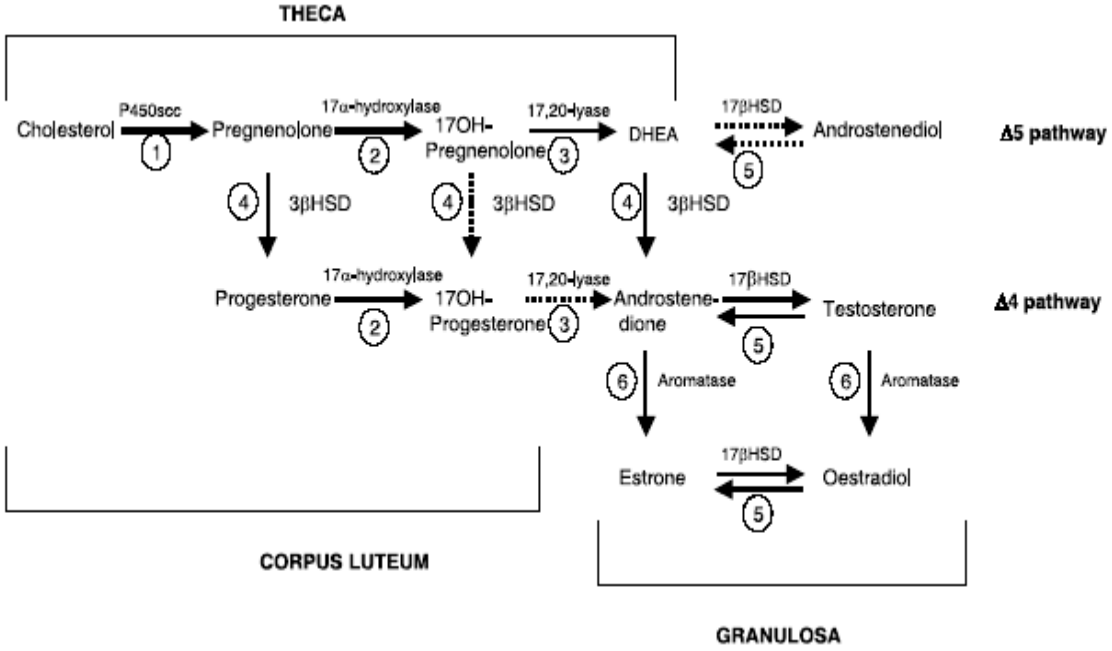


Figure 1.6: Steroidogenesis process in the ovary

Steroid hormone output is mainly regulated by events that ultimately affect steroid production through four parameters or processes:

(1) Steroidogenic enzyme level, determined by transcription, stability and translation of the mRNAs encoding the enzymes.

(2) Steroidogenic enzyme activity, determined by the conditions of the intracellular milieu, cofactor availability, or the posttranslational modification of the enzymes.

(3) Substrate availability, generally determined by cholesterol mobilization and transport to the mitochondrial P_{450}scc which catalyses the first step in the pathways of steroid biosynthesis.

(4) Tissue growth, determined by cell division and multiplication, as in the corpus luteum formation.

These above events are controlled by the expression of key proteins involved in steroidogenesis. Ovarian steroidogenesis is postulated to occur in two cells leading to two-cell/two-gonadotropin theory (Ben-Chetrit et al., 1996). For estrogen biosynthesis to occur, synthesis of androgens from cholesterol occurs in the luteinizing hormone (LH) stimulated theca cell compartment, as CYP17 activity is predominantly limited to the theca cells (Magoffin, 2005).These androgens then diffuse into the vascular, CYP17 deficient granulosa cell compartment. Under follicle stimulating hormone (FSH) stimulation, these androgens undergo aromatization to estrogens via aromatase (CYP19) activity (Dorrington and Armstrong, 2013). To undergo above reactions, several enzymes are needed which are explained as follows:

1.3.1 KEY PROTEINS IN PATHWAY OF STEROIDOGENESIS

1.3.1.1 STEROIDOGENIC ACUTE REGULATORY PROTEIN (STAR)

The candidate protein Steroidogenic acute regulatory protein (StAR), was first described by Orme-Johnson and colleagues as an ACTH-induced 30-kDa phosphoprotein in hormone-treated rat and mouse adrenocortical cells and as an LH-induced protein in rat corpus luteum cells and mouse Leydig cells (Miller, 2007). StAR is expressed in the adrenal cortex, ovarian theca, granulosa, and ovarian corpora lutea cells, fetal mouse giant trophoblast cells, fetal and adult testes, ovaries, and adrenals, adrenal tumors, and

testicular Leydig cells (Kiriakidou et al., 1996). StAR is directed to the mitochondria via its N terminus and, presumably utilizing C-terminal sequences, produces alterations in the outer mitochondrial membrane that result in the transfer of cholesterol from the outer to the inner membrane. This transfer of cholesterol is specific. This hypothesis is pertinent to the situation in steroidogenic mitochondria because the outer mitochondrial membrane is cholesterol rich, whereas the inner membrane is relatively devoid of cholesterol (Kallen et al., 1998). In addition to the signals generated through membrane receptors, StAR expression are up-regulated by LH, LH with insulin, Human chorionic gonadotropin (HcG), (PMSG), FSH, intracellular cAMP-inducing agents, IGF-I, growth hormone whereas down regulated by prostaglandin F2alpha, interferon gamma, TGF beta 1, TNF alpha, interleukin 1β GnRH agonist. Following the transfer into inner mitochondria, P450scc catalyses the initial reaction of steroidogenesis.

1.3.1.2 CHOLESTEROL SIDE-CHAIN CLEAVAGE ENZYME (P_{450} SCC)

Conversion of cholesterol to pregnenolone is rate-limiting step in the synthesis of all steroid hormones. This conversion entails three steps- 1) 20-hydroxylation, 2) 22-hydroxylation, and 3) cleavage of the C20-C22 bond to produce pregnenolone and isocaproic acid. These three steps are mediated by a single mitochondrial cytochrome termed P_{450}scc [cholesterol, reduced-adrenal-ferrodoxin:oxygen oxidoreductase (side-chain-cleavage)] (Katagiri and Moriya, 1976). P_{450}scc functions as the terminal oxidase in an electron transport chain consisting of adrenodoxin reductase, a flavoprotein accepting electrons from NADPH, and adrenodoxin, an iron-sulfur protein mediating electron transfer from adrenodoxin reductase to the P_{450} (Rosenfeld et al., 1983).

1.3.1.3 17A-HYDROXYLASE/17, 20 LYASE

Once pregnenolone is formed, CYP17 (P_{450}c17) catalyzes two mixed function oxidase reactions utilizing cytochrome P_{450} oxidoreductase and the microsomal electron transfer system (Fig. 2B). The two reactions catalysed by P_{450}c17 are the 17-hydroxylation of the C21 steroids, pregnenolone (Δ^5 steroid) or progesterone (Δ^4 steroid), followed by the cleavage of the C17–20 bond to produce the C19 steroids, Dehydroepiandrosterone

(DHEA) or Androstenedione, respectively. In the ovary, P_{450}c17 is expressed in theca interna cells (Sasano et al., 1989). Thus, theca interna cells can synthesize androgens, but granulosa cells which produce estrogens are dependent on androgen precursor supply from theca interna (Adashi et al., 1981). This process is called the two cell hypothesis of follicular estrogen production (Adashi et al., 1981). In ovary, CYP17 expression is restricted to theca cells that are the site of androgen production (Tamura et al., 1995). The general consensus is that granulosa cells and luteal cells do not express CYP17 (Conley et al., 1995). A recent report, however, suggests that human luteinized granulosa cells in culture do express CYP17 (Moran et al., 2004). In the adrenal glands of human, bovine, macaque, and guinea pig, CYP17 is expressed in the zona reticularis and the zona fasciculata but not in the zona glomerulosa, which is the site of aldosterone synthesis (Crivello and Gill, 1983). Direct stimulation of ovarian androgen secretion by insulin, possibly through stimulatory effects on the 17α-hydroxy- lase/17,20-lyase and P_{450}scc enzymes activity upon direct stimulation of LH secretion (Miura et al., 1996).

1.3.1.4 HYDROXYSTEROID DEHYDROGENASE/ISOMERISE

3β-HSD/isomerases are membrane-bound enzymes that are distributed to both mitochondrial and microsomal membranes depending upon the type of cell in which they are expressed. It has largest number of isoforms (six) and variedly distributed in tissues like ovary, uterus, adrenal, brain region-hypothalamus, pituitary of mouse. These isoforms are highly homologous in their sequence, but fall into two distinct functional groups. Mouse 3β-HSD I, III, and VI function are classic dehydrogenase/isomerases, whereas mouse 3β-HSD IV and V function are exclusively 3-ketosteroid reductases and are not involved in active steroid hormone biosynthesis (Clarke et al., 1993).Human 3β-HSD I is expressed in placenta, skin, and breast tissue, whereas 3β-HSD II is expressed in adrenal gland, ovary, and testis (Shimard et al., 1996). Studies on the regulation of 3β-HSD mRNA in mouse Leydig cells in culture have shown that these cells exhibit high constitutive expression of 3β-HSD (Payne et al., 1991). Treatment with cAMP results in a marked decrease in the expression of 3β-HSD mRNA, which could be reversed in the presence of aminoglutethimide, an inhibitor of cholesterol metabolism. It was

demonstrated that endogenously produced testosterone negatively regulates the expression of cAMP-induced $P_{450}17\alpha$ and 3β-HSD (Payne et al., 1991).

1.3.1.5 17 BETA- HYDROXYSTEROID DEHYDROGENASES

Like 3β-HSDs, the 17β-HSDs play essential roles in steroidogenesis. Estradiol production in the ovary is dependent on the action of 17β-HSD. In ovarian granulosa cells of developing follicles, 17β-HSD1 converts estrone to estradiol. Upon ovulation, follicles luteinize and transform into corpora lutea and continue to secrete estradiol at high concentrations although, upon luteinisation 17β-HSD1 expression declines precipitously in the ovary. These enzymes catalyse the final step in the biosynthesis of active gonadal steroid hormones, estradiol, and testosterone. The 17β HSDs differ in tissue distribution, catalytic preferences, substrate specificity, subcellular localization, and mechanisms of regulation. Among the many different forms of 17β-HSDs, three forms participate in the final step of biosynthesis of active steroid hormones in gonads, type 1, 3, and 7. Human 17β-HSD1 has substrate specificity for estrogens, whereas the rodent enzyme can utilize both estrogens and androgens. 17β-HSD1 is abundantly expressed in the granulosa cells of developing follicles and in human, but not in rodent placenta. Immunoreactive 17βHSD1 protein has been confirmed in the syncytiotrophoblast of human placenta and in granulosa cells of human ovary. Human 17β-HSD1 predominantly catalyses the conversion of estrone to estradiol, mouse and rat 17β-HSD1 also efficiently convert androstenedione to testosterone (Puranen et al., 1997). Mouse 17β-HSD7 mRNA is highly expressed in the mouse corpus luteum during the second half of pregnancy. Expression is most abundant around E14.5 when the ovaries are filled with corpora lutea (Nokelainen et al., 2000). 17β HSD3 converts androstenedione, a weak androgen, to testosterone, a potent androgen. 17HSD3 prefers NADPH as a cofactor, and its primary activity is reductive. 17β HSD3 is exclusively expressed in testes (Andersson et al., 1996). Its expression is restricted to the adult Leydig cell population and thus serves as a specific marker for Leydig cell development (O'Shaughnessy et al., 2000). 17β HSD7 is the major 17β HSD expressed in the ovaries of pregnant mice and rats, and the expression pattern parallels estradiol secretion from the

corpus luteum. During the luteal phase of the rodent ovarian cycle, estradiol is secreted mainly from the corpus luteum, where 17β-HSD7, not 17β-HSD1, is responsible for the final step in estradiol synthesis (Peltoketo et al., 1999).

1.3.1.6 P_{450} AROMATASE

After the conversion of DHEA to androstenedione by 17β-HSD in the thecal compartment, it is then transferred to granulose cells where it converts into estrogen (E2) by key enzyme- aromatase. This aromatase enzyme encoded by the CYP19a1 gene, which contains an unusually large regulatory region. In most mammals, aromatase expression is under the control of two distinct promoters a gonad- and a brain-specific promoter. Therefore, the coordinated and cell-specific expression of the aromatase (CYP19a1) gene in the ovary plays a key role in the normal progress of the menstrual/estrous cycle. In rodents, aromatase is restricted to the gonads, brain and corpus luteum during the second half of pregnancy (Swinney et al., 1993). In the ovaries of sexually mature animals, aromatase expression is limited to the follicle and the corpus luteum wherein cAMP is the main intracellular messenger mediating FSH stimulation of aromatase expression (Weniger and Zeis, 1988). The increase in intracellular cAMP levels induced by FSH leads to the activation the cAMP-dependent protein kinase A (PKA). P450arom catalyzes the conversion of testosterone into the 17β estradiol. In the ovary P450arom is expressed in granulosa cells which are the major site of estrogen production in females (Noble et al., 1997). However, this enzyme is widely expressed in many tissues besides gonads, e.g. adipocytes, breast, central nervous system, skin and placenta (Simpson and Fernandez, 1992). The gene encoding P450arom is the longest amongst steroidogenic P450 genes. This gene is also unique among P450 genes in having alternative promoters that are utilized in a tissue-specific manner (Simpson and Fernandez, 1992). CYP19 (P450arom) catalyzes the conversion of the C19 androgens, androstenedione and testosterone, to the C18 estrogens, estrone and estradiol, respectively. The expression of CYP19 is widely distributed. The major sites in humans (Simpson, 2002) and rats (Swinney et al., 1993) are in the pre-ovulatory follicle, the corpus luteum of ovulatory humans. Luteal cell function is greatly affected by locally

produced estradiol, which stimulates both progesterone biosynthesis and luteal cell hypertrophy (Gibori et al., 2013). In newly formed corpora lutea, aromatase remains expressed at low levels, which starts to increase between days 6 and 8 of pregnancy (Krasnow et al., 1990) in rat models.

1.4 REGULATION OF STEROIDOGENESIS

The studies, summarized above, indicate that these changes result from major changes in the levels of the steroidogenic enzymes during the development of the ovarian follicle. The rise in estradiol levels prior to ovulation is dependent on LH/FSH stimulation during final stages of pre-ovulatory follicle development (Richards, 2013). As noted above, estradiol biosynthesis by P_{450}arom in granulosa cells is dependent on androgen precursor from the theca interna. Thus, to increase estradiol biosynthesis, the following changes would be expected to occur in the follicle: a rise in theca cell enzymes responsible for androgen biosynthesis, e.g. P_{450}scc and P_{450}c17, and a rise in granulose cell P_{450}arom. Studies on rats showed that gonadotropin stimulation greatly enhances P_{450}scc levels in thecal and interstitial tissue within 24 h, while granulosa cells remain without detectable P_{450}scc (Gal and Orly, 2014). *"In vitro"* studies with human and rat granulosa cells showed that gonadotropin stimulation greatly increases P_{450}arom mRNA, protein level and activity (Hickey et al., 2012). The increase in blood progesterone levels after ovulation, results from two concomitant processes: growth of the corpus luteum, and an increase in steroidogenic enzymes. Progesterone biosynthesis is dependent on two enzymes P_{450}scc, and 3β-HSD. Expression of P_{450}scc in rat granulosa cells is initiated with a delay after expression in theca cells, and takes place close to the time of LH surge (Goldring et al., 1987). After ovulation, the theca and granulosa cells become the progenitors of small and large luteal cells of the corpus luteum, while this cell lineage continues to be reflected in the functioning of the two types of luteal cells (Meduri et al., 1992). The ovulatory surge of LH is associated with the disappearance of P_{450}c17 expression in bovine (Rodgers et al., 1986) and rat (Sakaki et al., 1992), but not in human corpora lutea.

In the corpus luteum, the total amount of P_{450}scc can increase about 100-fold over the

levels found in the ovary. This is accompanied by increases in the levels of the electron carriers' adrenodoxin reductase and adrenodoxin (Lin et al., 1993). In contrast, the level of the microsomal P_{450} reductase shows no significant change (Rodgers, 1990). The change in the levels of these enzymes is probably directly initiated by gonadotropins which have been shown to also enhance their expression in granulosa cells in culture, (Bao and Garverick, 1998). These steroids produced serve as autocrine regulator of steroidogenesis.

1.5 ROLE OF STEROID HORMONES

Steroid hormones are mainly produced in the gonads. The steroid hormones in these tissues are produced either by *in situ* synthesis from cholesterol or by enzyme catalysed conversion of DHEA or androstenedione excreted to the circulation from the adrenal cortex.

The concentrations of steroid hormones in blood fluctuate with a specific periodicity, or in response to physiological or pathological changes, to regulate diverse processes in the body. In mammals there are three endocrine organs that specialize in steroid hormone production: adrenal cortex, ovary, and testis. During pregnancy, placenta develops as an additional major source of steroid hormones. These steroid hormones output depends on the rate of steroid metabolism, i.e. biosynthesis and catabolism. They are divided into two groups; androgens and estrogens. Androgens are testosterone, androstenedione, Dihydroepiandrostenedione (DHEA) and 5 -dihydrotestosterone (DHT) and Estrogens are 17β-estradiol (estradiol), estrone and estriol. The tissue-selective expression and activity of 5α reductase and aromatase regulates the production of androgens and estrogens in steroidogenesis (Risbridger et al., 2010).

Steroid hormones play a crucial role in the differentiation, development, growth, and physiological function of most vertebrate tissues mainly in the growth and differentiation of reproductive tissues and in the maintenance of fertility. Produced *de novo* from cholesterol, progestins, androgens and oestrogens are synthesised by the ovary. The two-cell, two gonadotrophin model describes the role of theca and granulosa cells in the production of steroids, highlighting the cooperation between the two cell types, which is

necessary for oestrogen production. Produced *de novo* from cholesterol, progestins, androgens and oestrogens are synthesised by the ovary in a sequential manner, with each serving as a substrate for the subsequent steroid in the pathway. Every steroid produced have a regulatory role.

1.5.1 PROGESTERONE

Progesterone is also one of the most important endogenous steroid hormones which play an important role in regulation of follicular growth, pregnancy and embryogenesis. Progesterone majorly produced by corpus luteum during luteal phase of menstrual cycle and placenta during pregnancy. If conception occurs, the placenta will take over the secretion of progesterone; therefore the mother cannot ovulate again. At first, the source is the corpus luteum that has been "rescued" by the presence of human chorionic gonadotropin (hCG) from the conceptus. But, after the 8th week of pregnancy, production of progesterone shifts to the placenta which utilizes maternal cholesterol as the initial substrate, and most of the produced progesterone enters the maternal circulation, but some is picked up by the fetal circulation and used as substrate for fetal corticosteroids (Spencer and Bazer, 2002). If conception does not occur, decreasing secretion of progesterone will allow the hypothalamus to restart secretion of GnRH. This hormone levels mainly control the uterine cycle causing higher expression of secretary glands leading to the secretory phase after ovulation, and menstruation when conception does not occur (Kunz and Leyendecker, 2002). If conception takes place then under the influence of progesterone, the endometrium (uterine lining) changes to prepare for potential implantation of an embryo to establish a pregnancy and increase blood flow and uterine secretions and reducing the contractility of the smooth muscle in the uterus.

1.5.2 ANDROGEN

Androgens, primarily androstenedione and testosterone, are produced by theca cells in response to LH Androgens act via receptors (AR) localised to granulose cells, stromal cells (Gervásio et al., 2014) human theca cells (Gervásio et al., 2014). One of the most important roles played by androgens in the ovary is the synthesis of oestrogen. Androgens serve as substrate for P450aromatase, which mediates the conversion to

oestrogens (Yoshimoto and Guengerich, 2014). Apart from effects on growth, androgens have been shown to enhance the follicle stimulating hormone (FSH)-mediated differentiation of granulosa cells, as indicated by an increase in progesterone and oestradiol production (Grzesiak et al., 2014) and to play roles in oocyte maturation. Androgens, primarily testosterone, are produced by theca cells in response to LH. Androgens target granulosa cells via their receptors (AR), where they initiate three possible actions depending on the developmental status of the follicle. In the early stages of folliculogenesis, androgen promotes the follicle stimulating hormone (FSH)-mediated differentiation of granulosa cells by amplifying cAMP-mediated post-receptor signalling. During late preovulatory development AR decline and androgens are metabolised as opposed to exerting direct effects folliculogenesis (Gervásio et al., 2014). Exogenous androgens exert both inhibitory and stimulatory effects at different developmental stages throughout the process of follicular development wherein stimulated FSH-mediated differentiation of bovine granulosa cells, indicated by an increase in aromatase activity and estrogen production (Hamel et al., 2005). This may be due to the increased postreceptor cAMP response (Hillier et al., 1997). Androgens are also required as a substrate for the FSH-dependent process of estrogen biosynthesis. Both FSH (Markstrom et al., 2002) and Estrogen (Drummond and Findlay, 1999) are known to act as follicle survival factors, with continued follicular growth from the late preantral/early antral stage dependent upon FSH stimulation and E2 production.

1.5.3 ESTROGEN

Estrogens have three main molecular forms: estrone (E1), estradiol (E2) and estriol (E3). E2 has much higher biological potency than E1 and E3, and E2 is thus the physiologically most significant form of estrogen. The cellular effects of estrogens are mediated by estrogen receptors (ERs), which have been recognized in two forms: ERα and ERβ. As members of the nuclear receptor superfamily, ERα and ERβ have structural similarities wherein the N-terminal or A/B domain, the DNA-binding or C domain and the ligand-binding or D/E/F domain. In human, the identities between ERα and ERβ in their N-terminal, DNA-binding and ligand-binding domains are 30%, 96% and 53%, respectively. Also, ERα and ERβ are present in various tissues and play different roles in

mediating the estrogen effect (Leska et al., 2015). It is well established that estrogens are synthesized in the ovary are released into blood circulation and are mostly bound to sex hormone binding globulin (SHBG) and albumin, from which they are released and enter the target cells by passive diffusion for the action.

Oestrogen is also responsible for facilitating the differentiation of granulosa cells including the induction of receptor systems for FSH, LH and prolactin, which can influence post-receptor mechanisms through estrogen receptors (ERβ) (Drummond and Findlay, 1999). During postnatal development, the mRNA expression of ERβ increases in synergy with the proliferation of GC in the rat ovary whereas ERα mRNA levels in contrast; remain stable after its initial induction (Drummond and Findlay, 1999), indicative of a more widespread expression profile as highlighted by protein localisation studies in the ovary (Saunders et al., 2001). In conjunction with LH and FSH, E2 stimulates cAMP accumulation and increases the number of cAMP binding sites in granulose cells (Richards, 1980). Further, steroid receptors also regulate steroidogenesis.

1.6 Role of Steroid Receptors

The steroid hormones regulate transcriptional events through nuclear receptors. The localisation of the steroid receptors within the ovary is depicted in figure 1.7 given. These receptors form a part of a nuclear receptor superfamily, all of which contain common structural elements (Wu et al., 2013). Progesterone act through its receptor (PR), and plays key roles various biological processed such as reproduction, ovulation, implantation and the maintenance of pregnancy (Akison and Robker, 2012; Wetendorf and DeMayo, 2014). The expression of PR is induced by oestrogen in most target tissues and declines in response to progesterone. In mature preovulatory follicles, luteinising hormone (LH) induces PR expression by GC. PR is clearly required for ovulation and the development of corpora lutea.

Androgens target granulose cells through their receptors (AR) wherein they initiate three processes depending on the developmental status of the follicle. In the early stages of folliculogenesis, androgen promotes the follicle stimulating hormone (FSH)-mediated differentiation of granulose cells by amplifying cAMP-mediated post-receptor signalling.

During late preovulatory development AR decline and androgens are metabolised as opposed to exerting direct effects folliculogenesis. The androgen receptor is also expressed in neuroendocrine and musculoskeletal tissues and in the male urogenital system (Koochekpour, 2010). Ligand-activated androgen receptor forms a homodimer that binds to an androgen response element in the gene promoter and alters the transcriptional rate by recruitment of co modulators.

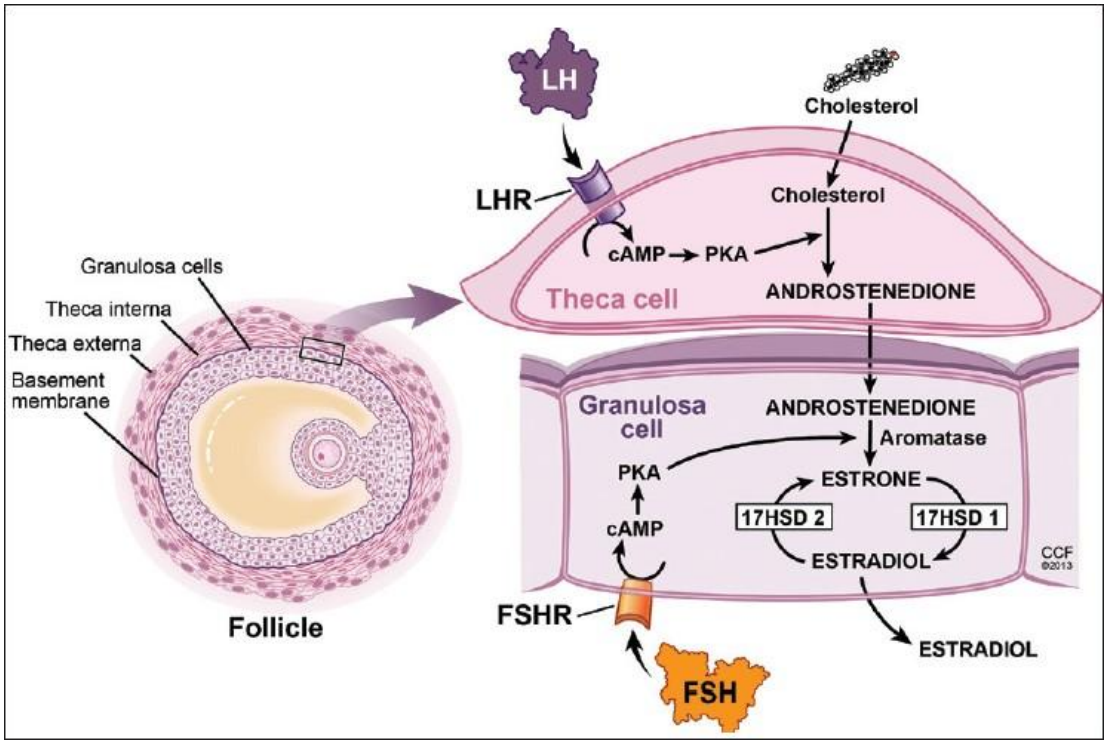


Figure 1.7: Two cell theory of estrogen production

Table 1.1 Tissue specific expressions of estrogen receptors

(Nilsson and Gustafsson, 2011)

	ER α	ERβ
Expression in tissues	breast, liver, bone, brain, urogenital tract and in the cardiovascular system	breast, bone, urogenital tract, gastrointestinal tract, lung, cardiovascular system and in the brain

Estrogenic effects are mediated by estrogen receptor α (ER α) and estrogen receptor β (ER β). The two estrogen receptors are encoded by different genes and have separate expression patterns. Estrogens have also been found to exert rapid responses, which have been suggested to be mediated by membrane-bound receptors (Lokuge et al., 2010).

All above mentioned factors play an important role in different aspects of reproduction and one of which is fertilization.

1.7 FERTILIZATION

Once mature ovum gets fertilized with sperm and form zygote formation occurs. Fertilization is a complicated multi-step event, involving maturation and development of the spermatozoa and eggs, followed by sperm migration into the oviduct, and ending with sperm-egg interaction and then fusion. The zygote undergoes cell differentiation and structural changes to form a blastocyst, which further gets implanted into the uterine wall (Wassarman, 1999).

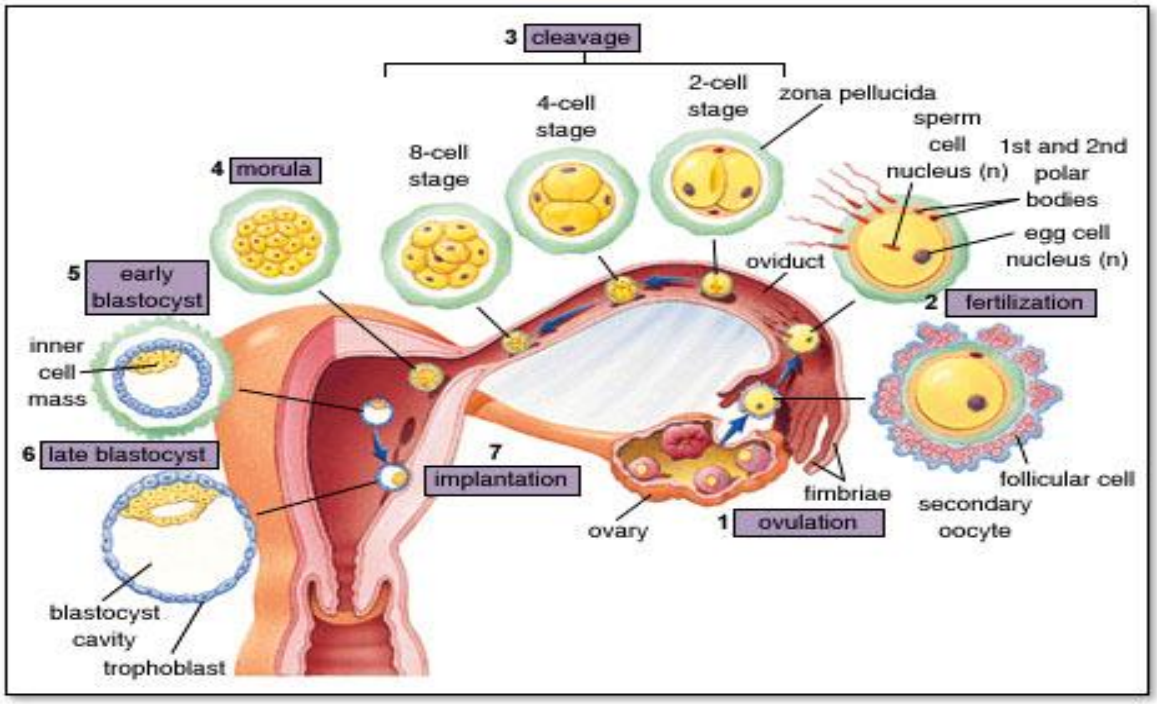


Figure1.8 Figure shows the process of Fertilization, cleavage, and the formation of a blastocyst

Implantation results from the action of trophoblast cells that develop over the surface of the blastocyst. These cells secrete proteolytic enzymes that digest and liquefy the adjacent cells of the uterine endometrium (Stewart et al., 1992) to implant growing mass of cells. 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).

In pregnancy, the placenta forms large quantities of human chorionic gonadotropin, estrogens, progesterone, and human chorionic somato-mammotropin are important for a normal pregnancy (Myatt, 2002).

As discussed above, female reproduction has complex events biochemically and endocrinologically. If any disturbance takes place in this steroid status or at HPO axis, it may lead to various reproductive disorders and cause infertility.

1.8 CAUSES OF FEMALE INFERTILITY

Approximately 20% of female infertility results from tubal disease. This disease can be suspected by a history of pelvic infection disease (PID), tubal or pelvic surgery, or ectopic pregnancy. Apart, abnormalities include congenital malformations, such as a septum or bicornuate uterus, leiomyomas, intrauterine adhesions (Asherman's syndrome), and endometrial polyps also, contribute to infertility. Uterine abnormalities more commonly affect pregnancy outcome than fertility.

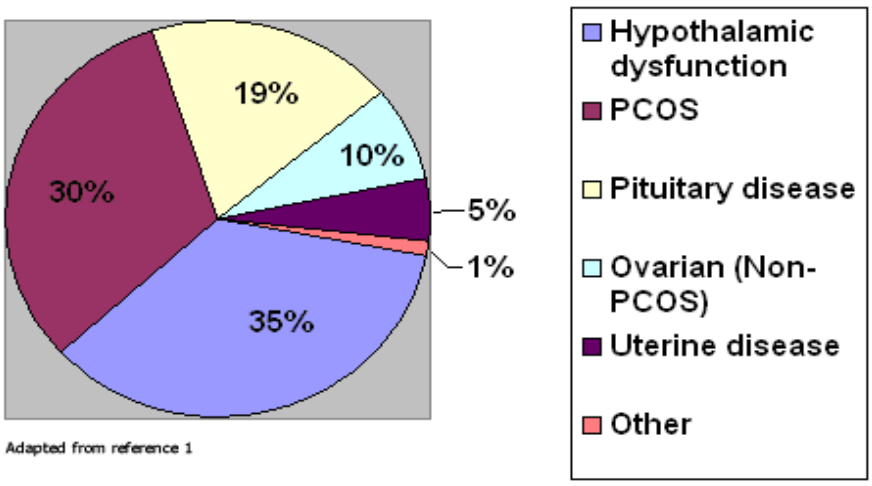


Figure 1.9: Various causes of Female infertility

Also, there is a higher incidence of endometriosis in infertile women (25%– 48%) as compared with all women in the reproductive age group (3%–10%) (Speroff et al.,2005). Such alterations can affect ovum maturation, fertilization, or implantation.

Above diseases mentioned could be several factors-internal and external factors-physiological and environmental factors. Major physiological factor that attribute to ovarian dysfunction is through H-P-O axis.

1.9 FACTORS AFFECTING HPO AXIS

The endocrine system is a system of glands that secrete hormones and further circulate within the body via the blood stream. It is instrumental in regulating metabolism, mood, growth, development, tissue function and sexual reproduction. Reproduction is under the governance of hypothalamus-pituitary-ovarian axis. Thereby, any defect in HPO axis may lead to several defects thrpugh axis. Hypothalamic amenorrhea is a condition wherein a change in the normal pattern of episodic secretion of the GnRH pulse generator occurs but with failure of ovulation resulting in amenorrhea. A hypothalamic amenorrhea (HA) is the absence of menstrual cycles for more than 6 months which is diagnosis of exclusion.

It is known that various other endocrine axis crosstalk with ovarian axis. Any defect in other axis could contribute defect in ovarian axis. The prevalence of different types of menstrual abnormalities was reported to be about 50% in hyperthyroid women (Koutras, 1997). Hypothyroid women have a decreased clearance of androstenedione and estrone and exhibit an increase in peripheral aromatization. Although LH/FSH levels are normal, blunted or delayed LH response to GnRH has been reported in some hypothyroid women. This serum prolectin (PRL) would increase in hypothalamic TRH secretion. At ovarian level, ovulatory dysfunction is observed in approximately 15% of all infertile couples and 40% of all infertile women. Ovulatory dysfunction is associated with infertility and is usually manifested either by oligomenorrhea, defined as eight or fewer menstrual periods per year, or amenorrhea, defined as the absence of menstrual bleeding. This lack of menstrual periods may be classified as primary or secondary. Primary amenorrhea refers to absence of menses by the age of 14 years in the absence of secondary sexual

characteristics or by the age of 16 years in the presence of secondary sexual characteristics. Secondary amenorrhea applies to women who have not menstruated for at least 6 months but who have previously menstruated. Furthermore, it is estimated that about 10% of ‘‘normal’’ menstrual cycles of 35 days or less are actually anovulatory.

There are several pathology where defects are contributed by either by hypothalamus/pituitary/ ovary or all. One such condition is polycystic ovarian syndrome (PCOS).

1.10 POLYCYSTIC OVARIAN SYNDROME (PCOS)

Polycystic ovary syndrome (PCOS) is one of the most common (15–20%) endocrine disorders in women belonging to reproductive age. It is a new epidemic involving metabolic complication associated with ovarian dysfunction as well as reproductive disorder (Valenzuela and Stone, 2014). Earlier, in 1935, Stein and Leventhal (Stein and Leventhal, 1935) described several women presenting with oligo/amenorrhoea combined with the presence of bilateral polycystic ovaries (PCO) established during surgery. Three of these seven women had obesity, while five showed signs of hirsutism. Only one woman was both obese and showed hirsutism. These findings imply that not all the features which are believed to be associated with PCOS needs to be present to characterize as PCOS (Rotterdam and ASRM-Sponsored, 2004). Thereby, criteria defined are indicated below in table.

The polycystic ovary syndrome (PCOS) is a hyperandrogenic disorder associated with chronic oligo-anovulation and polycystic ovarian morphology. It is chiefly associated with dyslipidemia and hyperglycemia along with compensatory hyperinsulinaemia, which as recognized as a major factor responsible for altered androgen production and metabolism (Escobar-Morreale et al., 2005).

1990 NIH diagnostic criteria (both 1 and 2)*	2003 Rotterdam diagnostic criteria (two out of three)*
1. Chronic anovulation	1. Oligo- or anovulation
2. Clinical and/or biochemical hyperandrogenism	2. Clinical and/or biochemical hyperandrogenism
	3. Polycystic ovaries

*Exclusion of other aetiologies essential for diagnosis with both the 1990 and 2003 diagnostic criteria.

Rotterdam criteria for PCOS

Most women with PCOS are overweight or obese have enhanced androgen secretion leading to impairment in metabolism and reproductive functions with developing PCOS phenotype. It is often associated with psychological impairments, including depression, other mood disorders and metabolic derangements.

1.10.1 PREVALENCE OF PCOS (WORLDWIDE AND INDIA)

Irrespective to geographic locations, a rapidly increasing prevalence of polycystic ovarian syndrome has been reported worldwide (Wijeyaratne et al., 2013). The majority of reports on the prevalence of PCOS have been based on studies carried out in Europe and the United States (Conway et al., 2014; Okoroh et al., 2012) . The prevalence of metabolic syndrome in the US population was determined as 6.7% in the twenties, increasing to 43.5% in the sixties (Johnstone et al., 2012). Aging increases the visceral fat deposition, which worsens the glucose and lipid metabolism. In southern Asia, various studies also have been studied wherein Kumarapeli et al. (2008) shown that community-based women in the Gampaha District of Sri Lanka appear to be predominantly reproductive rather than androgenic phenotype of PCOS.

The epidemic increase in prevalence of obesity and diabetes mellitus in most countries including China and India can be attributed to the westernization and urbanization. In addition, evidence suggests a pathogenic role of obesity in the development of PCOS and related infertility. In Indian context, prevalence of PCOS is quite high wherein, recent study showed that girls in north India with normal body weight of about 24% had a BMI $\geq$ 23 Kg/m^2, and none was morbidly obese. In contrast, 30-38% subjects were obese in

other studies. In spite of lower prevalence of obesity, waist-hip ratio was abnormal in 44% of PCOS cases, highlighting that Indians have more central obesity, even at lower BMI. A significant number of these girls had pre-hypertension; both these factors increasing their long term cardiovascular disease risk (Birdsall et al., 1997). In India, prevalence of PCOS is around 13.56% in Thiruvananthapuram. Nidhi et al. (2011) found that the prevalence of PCOS in Indian adolescents is around 9.13% in Andhra Pradesh, South India. (Balaji et al., 2015; Varghese et al., 2015). Also, PCOS patients from southern India demonstrated high BMI and dyslipidemia rather than the glucose impairment (Varghese et al., 2015).

1.10.2 PATHOPHYSIOLOGY OF PCOS

Pathophysiology of PCOS is complex wherein genetic and environmental contributors to hormonal disturbances combine with other factors. This includes obesity, ovarian dysfunction and hypothalamic- pituitary abnormalities that contribute to the aetiology of PCOS (Usadi and Legro, 2012). Several authors have explained different factors that contribute to PCOS pathology. One amongst them is Gonadotropin Releasing Hormone (GnRH).

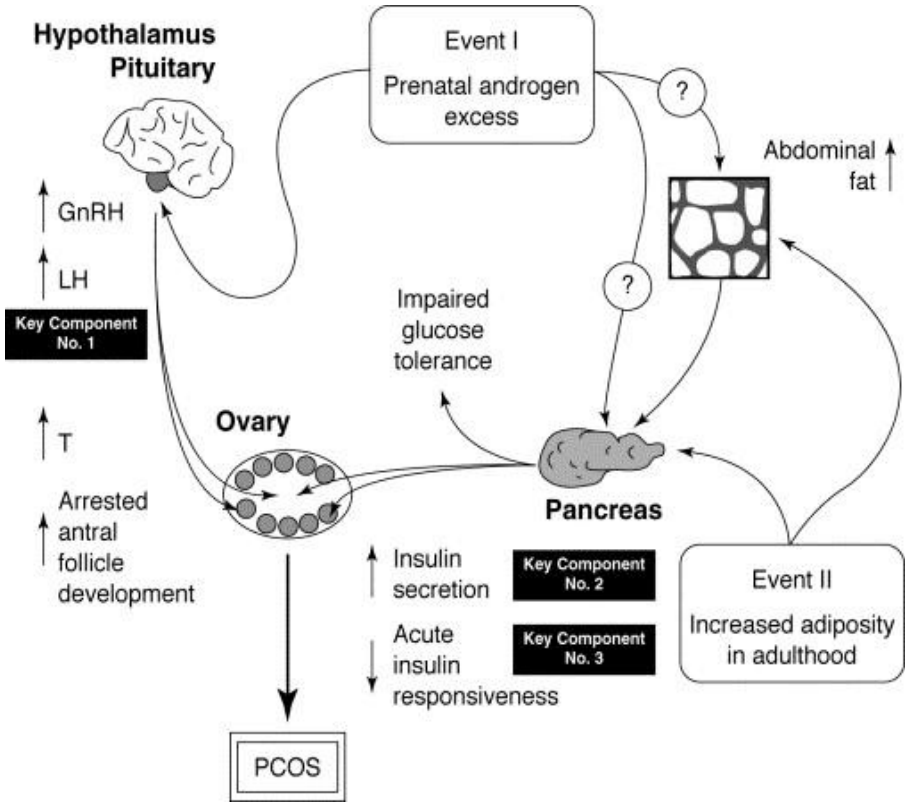


Figure 1.10 Pathophysiology of Polycystic ovarian syndrome

Gonadotropin-releasing hormone (GnRH) from the hypothalamus controls the secretion of LH and FSH from anterior pituitary in pulses. Elevated levels of LH are characteristic of PCOS, caused by accelerated frequency and/or higher amplitude of GnRH pulses leading to augmentation of LH secretory burst mass and more disorderly LH release (Barontini et al., 2001). Follicle-stimulating hormone (FSH), however, is secreted with a normal or suppressed pattern in PCOS, leading to an increased LH: FSH ratio (Banaszewska et al., 2003). The gonadotropin secretory abnormalities in PCOS are probably the result of increased GnRH pulsatility of unknown primary cause (Barontini et al. 2001). Women with PCOS also demonstrate decreased sensitivity to the feedback effects of gonadal steroids on GnRH secretion (Tosi et al., 2012). The first study that demonstrated that there exists an increase in LH secretion in PCOS was conducted by Franks et al. (1985) .

These alterations of HP axis would reflect on ovarian steroidogenesis. PCOS is mainly caused by an abnormality in ovarian and adrenal androgen biosynthesis specifically by an

enzyme- localized to the 17α-lyase reaction, catalysed by a single enzyme, CYP17 (Rosenfield et al., 1994). Increased *"in vivo"* flux through CYP17 has been suggested from results of 17α-hydroxyprogesterone measurements in PCOS women challenged with a GnRH agonist or human hCG after GnRH agonist induced pituitary suppression. Because the relative ratio of 17α-hydroxylase to lyase activity of CYP17 represents a key control locus for androgen biosynthesis, a shift in this ratio could account for aberrations in ovarian and adrenal androgen production. Therefore, it is interesting that cleavage of phosphate from serine residues on CYP17 by alkaline phosphatase treatment reduces lyase activity without affecting 17 α hydroxylase activity (Apter et al., 1994). An important enzyme for androgen production- 17α Hydroxylase (CYP17) expression in PCO theca cells is persistently elevated compared to normal ovaries (Li et al., 2013). Wickenheisser et al. (2000) also have shown that PCOS follicle demonstrated high levels of LH, steroidogenesis acute regulatory protein (StAR), CYP17 and CYP11A mRNA expression than size-matched control follicles. This has been interpreted as hyper stimulation of the thecal cells with possible premature luteinisation. Role of posttranslational modification of CYP17 influences androgen synthesis. Reports suggest that ovarian theca cells attained patients with PCOS convert steroid precursors into testosterone (T) more efficiently than normal theca cells. The rate of conversion of pregnenolone (P) and dehydroepiandrosterone (DHEA) into T was markedly increased in PCOS theca cells. Hence, high androgens level leading to hyperandrogenic condition which is well established contributor to PCOS aetiology (Nelson et al., 1999).

In PCOS, multiple factors such as ovarian hyperandrogenism, hyperinsulinemia and altered intra-ovarian paracrine signalling can disrupt follicle growth. The consequent follicular arrest in PCOS is accompanied by menstrual irregularity, anovulatory subfertility and the accumulation of small antral follicles within the periphery of the ovary, giving it a polycystic morphology. Follicular arrest in PCOS develops when granulosa cells (Li et al., 2014) in antral follicles normally begin to express aromatase (at a size of 7 mm), as excess intraovarian 5α-reduced androgens inhibit granulosa cell aromatase activity (Yang et al., 2015). The frequent occurrence of associated hyperinsulinemia in PCOS further exacerbates ovarian follicular arrest by stimulation of

17α-hydroxylase activity in theca cells. Hyperinsulinemia also amplifies luteinizing hormone (LH)-stimulated and insulin-like growth factor 1 (IGF-1)-stimulated androgen production (Sindelka et al., 2015). Hyperinsulinemia elevates serum free testosterone levels through decreased hepatic sex hormone-binding globulin production, and enhances serum IGF-1 bio-activity through suppression of IGF-binding protein production. In addition, high anti-Müllerian hormone (AMH) concentrations in women with PCOS play an integral role in causing anovulation due to its inhibitory influence on the actions of follicle-stimulating hormone, which normally promotes follicular development from the small antral to the ovulatory stage (Homburg and Crawford, 2014).

As evident from the literature, there are several extra-ovarian factors which can interact with androgens leading to complicate the pathology further (Diamanti-Kandarakis and Dunaif, 2012; Doi et al., 2006; Nelson et al., 1999). PCOS is a common and well-defined clinical model of insulin resistance and pre-diabetic state. Insulin sensitivity is decreased by an average of 35% to 40% in women with PCOS, as compared to matched controls (Stepto et al., 2013). Thus, most women with PCOS are insulin resistant and develop compensatory hyperinsulinemia, which seems to play a critical role in the syndrome's pathogenesis (Baptiste et al., 2010). Insulin resistance is a condition where insulin receptor is no longer sensitive to initiate cascade of insulin signalling pathway and lead to hyperinsulinemia in PCOS females (Diamanti-Kandarakis and Dunaif, 2012). In addition, inhibition of lipolysis (stimulated by insulin) decreased in PCOS women as compared to controls from obese PCOS women (Ciaraldi et al., 1992). These findings were also observed in PCOS women presenting incidence of obesity, glucose intolerance, or increased waist-to-hip ratio (Lim et al., 2012). Moreover, muscles biopsied from PCOS obese women exhibited impaired insulin-stimulated samples association of IRS-1 with PI-3K, concomitant with a decrease in glucose transport "*in vivo"* (Dunaif et al., 2001). Akt is an important mediator of insulin-stimulated GLUT4 translocation and glucose transport. This seems to be dependent on the phosphorylation of AS160 (Akt substrate) [important for membrane trafficking of GLUT 4] at several sites by Akt (Sano et al., 2003). In addition, skeletal muscle of obese PCOS women, demonstrated decreased insulin action on peripheral glucose metabolism which is associated with impaired insulin

signalling at the level of Akt and AS160 (Akt substrate) (Højlund et al., 2008). This leads to decrease in insulin-mediated phosphorylation of AS160 and of Akt (at Thr308 and Ser473) (Makker et al., 2012). Most women with PCOS have increased circulating levels of free fatty acids (FFAs), which have been shown to cause insulin resistance *in vivo* (Morin-Papunen et al., 2000). Interestingly, FFA metabolites (e.g. diacylglycerols and ceramides) that accumulate in cells have been postulated to activate intracellular serine/threonine kinases (Tanti et al., 2004) and protein phosphatases, such as protein phosphatase 2 (PP2A) (Cazzolli et al., 2001). In addition, serine phosphorylation of P_{450}c17 have been shown to increase its 17,20-lyase activity (Dunaif et al., 1995). Therefore, FFAs may be act as key factor for the serine phosphorylation that induces an increase in P450c17 activity that leads to defect in the insulin signalling pathways causing insulin resistance (Figure 1.11).

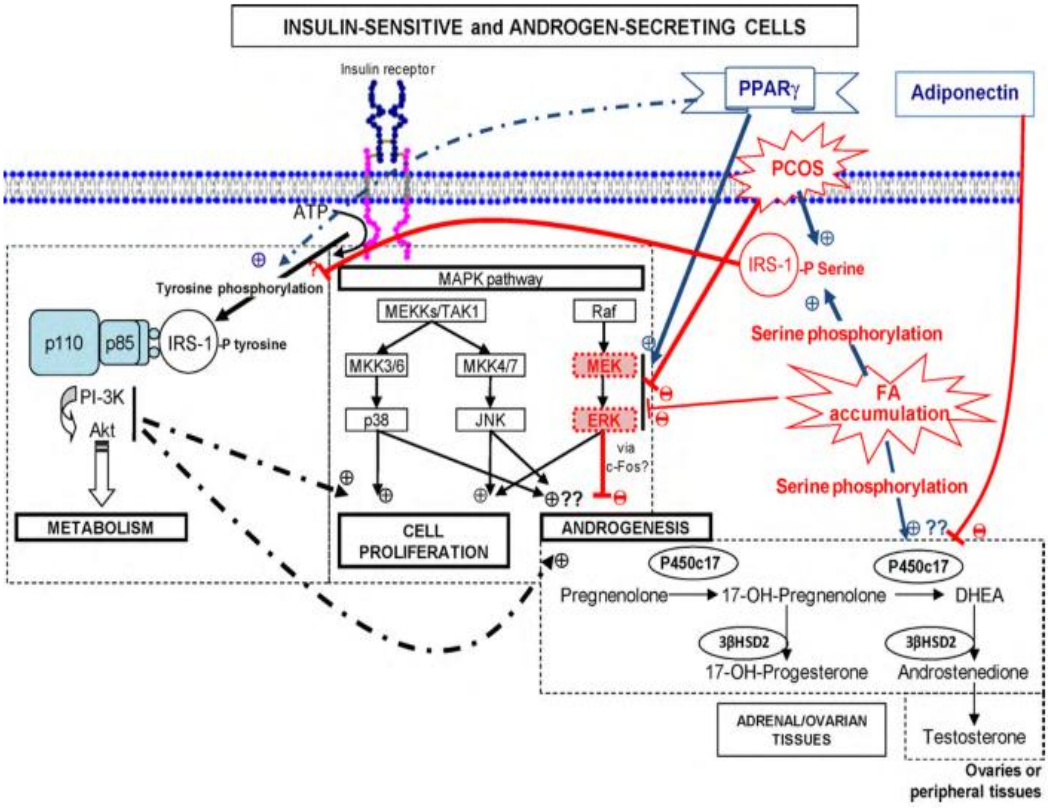


Figure1.11: Proposed cellular mechanisms involved in insulin-stimulated androgen biosynthesis in case of PCOS (Baptiste et al., 2010)

Further, Wu et al. (2003) found that PCO luteinized granulosa cells have a selective increase in insulin activation of its mitogenic pathway, via the MAP kinase pathway. This is contradicted to resistance seen in the metabolic pathway of insulin action. The same group also found, in cultured porcine thecal cells, that dexamethasone induces resistance to insulin-mediated glucose transport with increased testosterone production and expression of P_{450}c17 (Qu et al., 2009).

In addition, SHBG is sex hormone binding globulin protein that control levels of steroids. Insulin can decrease hepatic SHBG production (Kavanagh et al., 2013), explaining the frequently observed inverse correlation between peripheral insulin and SHBG levels (Wickham III et al., 2011). PCOS females also suffered more severe hyperandrogenemia, which is higher serum free testosterone and lower serum SHBG concentrations suggesting that SHBG has been noted to correlate inversely with insulin sensitivity (Apridonidze et al., 2005). Recent studies demonstrate that presence of (TAAAA)n long allele repeat on SHBG gene might be an important predictor for hyperandrogenemic clinical presentation of PCOS (Ferk et al., 2007; Wickham III et al., 2011). Further decrease in SHBG content contributes to elevated circulating testosterone and which impairs ovarian follicle development. Clinical hyperandrogenism primarily includes hirsutism, acne and male pattern alopecia (Azziz et al., 2009). Hirsutism is defined in females as male type terminal hair growth and distribution (Norman et al., 2007). PCOS is a common cause of hirsutism occurring in approximately 60% of cases; however this varies with race and degree of obesity (Azziz et al., 2009). Typically, women with PCOS have increased levels of serum testosterone and luteinizing hormone (LH), and decreased levels of sex hormone-binding globulin (SHBG) is well correlated with hyperinsulinemia and obesity (Nestler, 2000).

Literature suggests that the prevalence of overweight and obesity in the PCOS population is above 50% in the United States (Qu et al., 2009) and between 30% and 50% in Europe (Svendsen et al., 2008) and in India respectively. Obesity by itself is associated with insulin resistance and compensatory hyperinsulinemia, which is followed by accumulation of intra-abdominal fat. Indeed, the visceral fat metabolism is more active than the subcutaneous one (Carpentier, 2008). Intra-abdominal fat tissue is more sensitive

to lipolysis and releases more FFAs in the circulation, and produces several cytokines (i.e. tumor necrosis factor-[TNF], IL-6, leptin, resistin), which involved in insulin resistance (Carpentier, 2008). As compared to non-obese PCOS women, obese women with PCOS have more menstrual irregularities and uterine dysfunctional bleeding, as well as an increased prevalence of infertility, which were also associated with an abdominal distribution of fat (Gambineri et al., 2012). Accumulation of visceral fat leads to insulin resistance, hyperglycemia, dyslipidemia, hypertension, prothrombotic and proinflammatory states that are also relatively common in PCOS patients (NIH, 1998). Adipose tissue exerts many of these influences through paracrine and endocrine effects which are mediated by the increased or reduced secretion of molecules such as leptin, adiponectin, tumor necrosis factor a (TNFa), interleukin 6 (IL-6) and plasminogen activator inhibitor-1 that have been found to be altered in women with PCOS (Azziz et al., 2006). The role of adipocytes on steroid hormonal metabolism in PCOS is also important. Adipocytes from centrally located adipose tissue can convert Δ-4 androstenedione to testosterone, a strong androgen, via 17β hydroxydehydrogenase enzyme. These androgens further can increase in central adiposity. In turn, visceral adipocytes are able to convert inactive cortisone to metabolically active cortisol, which therefore enhances insulin resistance. The increased cortisol and testosterone then by a feedback mechanism thereby lead to an increase in the degree of central obesity (Ahima and Flier, 2000). This further, enhances the production of androgen. Also, hyperandrogenism in human confirms the vicious cycle leading to obesity. Apart from, peripheral factors; brain exerts its effect in amplification of PCOS induced obesity by affecting feeding centre (Sepilian et al., 2006).

Apart from extra ovarian factors, intra ovarian factors also play important role in ovarian regulation. One of them being Anti-Müllerian hormone (AMH) is produced predominantly in the ovarian granulosa cells of pre-antral and antral follicles (Freeman et al., 2012). It has been proposed as a marker of ovarian dysfunction by disrupting folliculogenesis through diminishing follicular sensitivity to follicle stimulating hormone (FSH) and inhibiting follicle recruitment and growth (Dalal and Mishra, 2012). Recent studies suggest that an increased AMH concentrations in PCO, may be correlated to

follicular development or AMH production increased by growing follicles (Catteau-Jonard and Dewailly, 2011). Recent data have shown that overproduction of antimullerian factor (AMH) from granulose cells in PCOS (Nardo et al., 2009) could be implicated in hyperandrogemism, since a positive correlation has been found between AMH, Testoserone and androstenedione in PCOS. (Pigny et al., 2003). In PCO condition, hyperandrogenism accelerates pre-antral and antral follicular growth in the ovary, and increased LH resulting in premature luteinization causing follicular through mechanism of driving increased AMH levels. AMH levels may be related to the severity of PCOS with higher concentrations seen in women with features including polycystic ovaries (PCO), anovulation, hyperandrogenism and IR (Pierre et al., 2013).

With the above understanding of PCOS etiology, researchers are still postulate that this form of infertility is still incomplete. Thereby, various attempts have done by creating animal models and mimicking human phenotype to study this area of research.

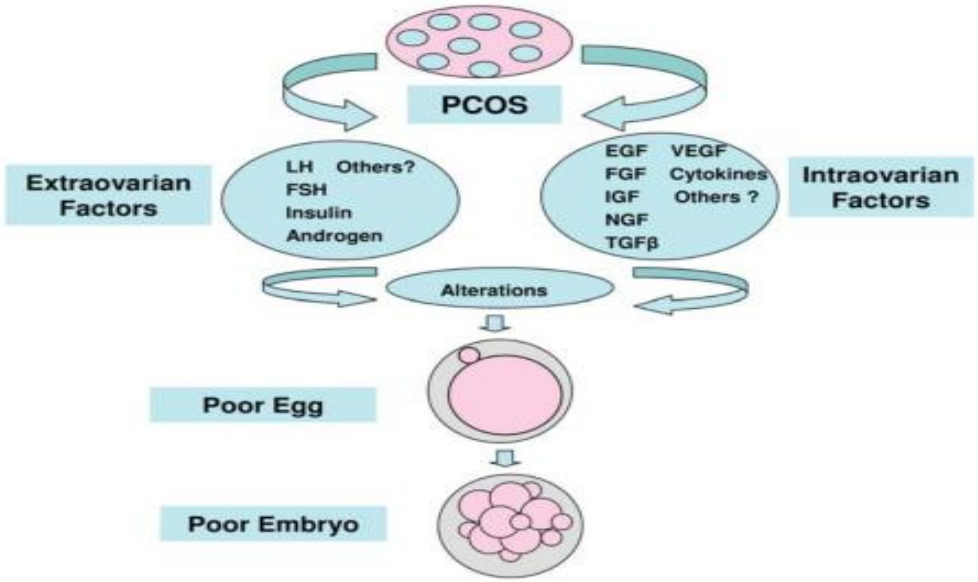


Figure 1.12: Intra-and extra-ovarian factors associated with the PCOS pathology that negatively affect oocyte and subsequent embryo quality

1.10.3 ANIMAL MODELS FOR POLYCYSTIC OVARIAN SYNDROME (PCOS)

The morphological criteria for a diagnosis of polycystic ovaries is based on ultrasonographic data where patients exhibit ovarian enlargement, a thickened outer

tunica albuginea, more than 12 follicles per ovary with a diameter of 2 to 10 mm, and an increased density and area of stroma (Manneras et al., 2007). Human PCOS ovaries also exhibit an increase in the numbers of growing preantral and antral follicles and an arrest in mid-antral follicle growth, which leads to antrum expansion, increased granulose cell degeneration, and development of cystic follicles with thin granulosa cell walls (Chang et al., 2010). Hence ideally, animal models of human conditions, such as PCOS, should replicate many or most clinical characteristics of that disorder. Since the 1960s, a range of animal models, including rodents, sheep, and non-human primates, have been used to study the origins and pathology of this condition (Singh, 2005; West et al., 2001). However, a range of characteristics similar to those seen in women with PCOS have been described in distinct animal models. Prenatal exposure of sheep and non-human primates to androgens has provided models that show striking similarities to women with PCOS (Abbott et al., 1998; Recabarren et al., 2008). Rodent models provide a versatile tool for deciphering the precise biological mechanism(s) associated with the development of PCOS. Among the numerous advantages of using rats and mice over other animal species used as *"in vivo"* models include their stable genetic backgrounds, ease of handling and maintenance, shorter reproductive lifespan and generation times, short estrous cycles, feasibility of genetic manipulations, and affordability. Moreover, use of these models would provide holistic understanding of pathology.

1.10.3.1 ANDROGEN INDUCED PCOS RAT MODEL

A direct role for androgen receptor (AR)-mediated effects in the ovary and female reproductive functions has been recently confirmed by findings from AR knockout mouse models, where a loss of AR actions lead to sub fertility, predominantly due to defective gonadotropin regulation, follicular development, and ovulation(Walters et al., 2008; Walters et al., 2010). Defect in AR gene can be associated with different diseases such as, androgen insensitivity syndrome (AIS), Kennedy's disease, and infertility. Thereby, recent studies exhibited that, with hep of molecular biological analyses lead to a more detailed view on the AR mechanism of action and on the specifics of the physiological role of androgens. Kerkhofs et al. (2009) has shown the development of

AR knockout and knock-in mouse models and correlated them with various human diseases.

1.10.3.1.1 TESTOSTERONE AND ANDROSTENEDIONE

The exposure of rodent fetuses to testosterone propionate (TP) by intra-amniotic administration induced anovulation in 64% of rats (Fels and Bosch, 1971)(Fels and Bosch, 1971). Also, studies have shown that prenatal treatment of mice with TP (Keisler et al., 1991) or rats with TP (Huffman and Hendricks, 1981) had no effect on cyclicity or ovarian function, inferred by the presence of follicles at various stages and corpora lutea. A detailed study by Wu et al. (2010) showed that prenatal treatment of rats with TP on Days 16 and 19 of gestation resulted in irregular estrous cycles and an ovarian phenotype of increased numbers of preantral and antral follicles but a decrease in preovulatory follicle and corpus lutea populations. Treated rats also exhibited an increase in Testosterone, estradiol (E2), progesterone (P), and LH serum levels and an increase in the frequency of LH pulse secretion (Wu et al., 2010). The variation in the findings of the presence of disrupted cyclicity and anovulation appears to be due to the degree of transplacental transfer of the administered steroid into the fetus (Fels and Bosch, 1971). These model descriptions lack detailed analysis of metabolic disturbances, and defining androgen-regulated mechanisms can be difficult to interpret as steroid effects may be induced by either the AR or estrogen receptors (ER).

1.10.3.1.2 DIHYDROTESTOSTERONE (DHT)

In postnatal DHT induced rat model, 21-day-old (prepubertal) rats treated with 90-day continuous-release pellet containing DHT and collected 11–13 weeks later displayed irregular estrous cycles and ovarian features similar to human PCOS, including increased numbers of large atretic follicles and follicular cysts with a thickened theca interna cell layer and thin granulosa cell layer and fewer corpora lutea than controls (Manneras et al., 2007). However, unlike human PCOS ovaries, ovary weight was reduced. At the estrous stage, plasma T and E2 levels were unaltered, but P was significantly decreased, indicating anovulation. DHT-treated rats also showed many metabolic features also present in human PCOS that includes increased body weight, body fat, and abdominal

fat; enlarged adipocytes; elevated leptin and cholesterol levels; and insulin resistance (Manneras et al., 2007; Johansson J et al., 2010).

Rats and mice prenatally exposed to DHT exhibited increased T and LH serum levels, replicating the human PCOS traits of androgen and LH hypersecretion (Sullivan et al., 2004). Rats that were exposed also exhibited an increase in the frequency of LH pulse secretion and elevated serum E2 and P levels (Wu et al., 2010) thereby suggesting excessive androgens may disrupt negative steroidal feedback signaling to the hypothalamus. In addition to reproductive axis abnormalities, prenatally androgenised mice (treated with DHT on Days 16–18 of gestation) exhibit metabolic alterations with impaired glucose tolerance but normal insulin sensitivity and increased adipocyte size, indicating altered adipocyte function; however, body and fat mass were unchanged (Roland et al., 2010). PCOS rat model exhibits metabolic alterations with impaired glucose tolerance but normal insulin sensitivity and increased adipocyte size, indicating altered adipocyte function; however, body and fat mass were unchanged.

1.10.3.1.3 DEHYDROEPIANDROSTERONE (DHEA)

The observation that dihydrotestosterone (DHEA) levels are increased in women with PCOS (Mahesh and Greenblatt, 1962) led to the development of a PCOS animal model using postnatal DHEA treatment (22- to 23-day-old rats treated with DHEA for 36 days) as the inducer of polycystic ovaries (Roy et al., 1962). The DHEA rodent model exhibits some features of the human PCOS condition, such as acyclicity, abnormal maturation of ovarian follicles, and anovulation (Ward et al., 1978). Postnatal treatment of mice (Sander et al., 2006; Familiari et al., 1985) and rats (Lee et al., 1991; Anderson et al., 1992) with DHEA dose for 20 consecutive days resulted in all or most females exhibiting follicular cysts with a thin granulosa cell layer and anovulation. Ovaries exhibited an increase in fat and stroma tissues and increased numbers of atretic follicles (Ward et al., 1978), hyperandrogenism, and altered ovarian steroidogenesis with elevated serum levels of androgens estrogens, progesteronr, and prostaglandin (Lee et al., 199; Familiari et al., 1985). Limited data are available on whether DHEA treatment induced the metabolic disturbances associated with PCOS. However, DHEA treatment of mice did not affect

body weight, but did increase serum fasting insulin levels without affecting fasting glucose levels (Sander et al., 2006). Prenatal exposure can lead to vaginal fusion, and although tried with different doses of androgens to minimize this effect, that is a significant limitation of this model for evaluation of fertility, which is a key feature of PCOS.

Overall, prenatal and postnatal exposure to various androgens can induce both reproductive and metabolic deficits similar to those exhibited in PCOS women. Prenatal exposure can lead to vaginal fusion, and although researchers have varied doses of androgens to minimize this effect (Sullivan and Moenter, 2004), This is a significant limitation of this model for evaluation of fertility, which is a key feature of PCOS.

1.10.3.2 ESTRADIOL BENZOATE AND ESTRADIOL VALERATE

Apart from androgen, estrogen induced PCOS rats also have been developed wherein adult rats, postnatal treated with estradiol benzoate (EB) on Day 1 (After single dose), displayed acyclicity, anovulation and ovarian atrophy (Baravalle et al., 2006). Hormone differences were observed which included a significant increase in both FSH and serum prolactin levels (Baravalle et al., 2006). Young cycling adult rats exposed to E2 for 8 weeks via a subcutaneous continuous release implant (McCarthy and Nrawer, 1990) or a single injection of estradiol valerate (EV) exhibited acyclicity, anovulation, and polycystic ovaries. This contained an increased number of atretic follicles and cysts with a thin granulosa cell layer and an abnormally thickened theca layer (McCarthy and Nrawer, 1990; Brawer et al., 1986). However, EV treatment decreased ovary weight and failed to provoke LH hypersecretion (Brawer et al., 1978; Grosser et al., 2000), hyperandrogenism, obesity, and changes in glucose and insulin concentrations, which differs significantly from human PCOS, but rats did exhibit hypertension and an increase in inguinal fat depot weight (Stener Victorin et al., 2005). E2 exposure resulted in ovarian morphological feature of anovulation and polycystic oavaries similar to PCOS women (Manneras et al., 2007). However, these models are limited by lack of endocrine and metabolic features associated with human PCOS.

1.10.3.3 ANTIPROGESTINS

The antiprogestin RU486 is a synthetic steroid with a high affinity for progesterone (and glucocorticoid) receptors with potent antagonistic but no agonistic activity (Baulieu, 1991). Rodents treated with RU486, hence lacking progesterone action; thus showed numerous endocrine and ovarian morphological features similar to those of human PCOS. Administration of RU486 to adult cycling female rats for 4–9 days resulted in acyclicity, polycystic ovaries (Sanchez-Criado et al., 1993) and anovulation (Zhou et al., 2008). Similar to human PCOS, serum LH, T, and E2 levels were significantly increased (Ruiz et al., 1996; Lakhani et al., 2006). In respect to metabolic abnormalities associated with human PCOS, RU486 treatment did not alter body weight or insulin sensitivity (Lakhani et al., 2006).

1.10.3.4 AROMATASE INHIBITORS

Polycystic ovaries can be induced by androgen exposure including not only exogenous androgens but also as a result of secondary endogenous androgen excess (Pache et al., 1993). The latter includes the rat PCOS model induced by letrozole, a nonsteroidal aromatase inhibitor, which blocks the conversion of androgens to estrogen (Kafali et al., 2004). Letrozole treatment of adult rats for at least 21 consecutive days induced acyclicity or irregular estrous cycles and anovulation (Kafali et al., 2004). These rat ovaries exhibiting many large follicular cysts and either reduced numbers or no corpora lutea. Ovaries exhibited increased follicle atresia and multiple cysts with thin granulose cell layers and thickened theca cell layers (Kafali et al., 2004). Endocrine disruptions included elevated levels of LH, FSH, and T, reflecting the accumulation of endogenous ovarian androgen secretion due to a block in aromatase activity. In contrast, the decreased progesterone secretion was observed which is consistent with the observed anovulation. However, an elevation glucose, cholesterol, and triglyceride levels in female rats was observed when treated orally with letrozole (Sasikala et al., 2009). The letrozole-induced PCOS rodent models induced many features of human PCOS, although further work is required to confirm the metabolic disruptions present before this model can be confirmed as a valid and useful model for the metabolic features of PCOS. Furthermore, the

reduction in E2 observed (Hauffman , 2015; Mannnaras, 2007).

Elaborate literature survey suggests that letrozole induced PCOS rat model mimicked most of metabolic and reproductive characteristic of PCOS. Hence, present study opted for letrozole induced rat model.

1.10.4 CURRENT THERAPIES FOR PCOS

Polycystic ovarian syndrome characterized by oligo-anovulation, clinical or biochemical hyperandrogenism and peripheral atretic cysts (Franks, 1995; Fauser, 2004). Insulin resistance (IR) accompanied by compensatory hyperinsulinaemia constitutes another major biochemical feature of PCOS (Dunaif, 1997; Jakubowicz et al., 2001).

Pharmacologic Agents for PCOS

Drug	Place in Therapy	Dosage	Adverse Effects
Oral contraceptives	Androgen excess, contraception, regulation of cycle, protection of endometrium	One tablet po daily	Breast tenderness, weight gain, fluid retention, increased risk of thromboembolism
Clomiphene	Ovulation induction	50 to 150 mg po daily days 5 to 9 of cycle	Hot flashes, nausea, headache, blurred vision, multiple gestation
Gonadotropins	Ovulation induction	Dosage and duration dependent on product and patient response	Abdominal pain, nausea, breast tenderness, injection site reaction, multiple pregnancy, ovarian hyperstimulation
Metformin (Glucophage, Glucophage XR)	Hyperinsulinemia, anovulation, androgen excess	500 mg po bid to 850 mg po tid	Nausea and vomiting, diarrhea, anorexia, metallic taste, lactic acidosis (rare)
Spironolactone (Aldactone)	Hirsutism, acne	100 to 200 mg po daily	Intermenstrual bleeding, hyperkalemia, hypotension
Pioglitazone (Actos) Rosiglitazone (Avandia)	Hyperinsulinemia, anovulation, androgen excess	30 to 45 mg po daily 4 mg po daily to bid	Weight gain, edema, increased LDL cholesterol with rosiglitazone

Table 1.2: Current therapies available for management of PCOS

Hence, metformin – insulin sensitizer either alone or in combination with clomiphine citrate is widely used for ovulation induction in women with PCOS (Gkueck et al., 2003). Apart from this treatment, several oral contraceptives (OCs) and lifestyle modifications are advised to minimize relative PCOS symptoms. OCs reduces androgen levels and thus causes improvement in menstrual cycles in adolescents with PCOS wherein lifestyle modification has been to be effective in reduction of obese condition and the restoration of ovulation. Metformin, which has peripheral insulin-sensitizing effects, has shown

several beneficial effects in women with PCOS (Glueck et al., 2003). These clinical manifestations bring some side effects wherein apart from gastrointestinal problems like bloating, vitamin B12 deficiency and lactic acidosis (Hermann, 1979). Hence, current research looked into ancient wisdom of Ayurveda in search of an effective alternative modality and number of herbs that could act at the level of hypothalamic-pituitary-axis. Various herbs are reported in Ayurveda which aid in bleeding and clotting mechanisms along with potentially corrects the lipid and carbohydrate metabolism. Several herbal formulation have been developed which helps to improve PCO phenotype via directly acts on hypothalamo-pituitary-ovary-uterine axis and thereby regularize the menstrual cycles (Sasikala et al., 2010).

1.11 MEDICINAL PLANTS

Complementary medicinal approaches have become common for the management of such metabolic syndromes (Shashikala et al., 2009). According to Ayurveda, many formulations have been reported which help to restore the ovulation and minimize the PCO phenotypes (Shukla et al., 2009). Some principal herbal ingredients of formulations, such as *S. indica*, *S. racemosa*, *C. rotundus*, *T. cordifolia*, and *A. vera*, are known to possess various beneficial activities. *S. indica* has a stimulatory effect on the ovarian tissue, which may produce an estrogen-like activity that enhances repair of the endometrium and stops bleeding. It is found to be effective in menorrhagia and dysmenorrhea (Sharma *et al*., 2010). Ethanolic extract of *Boerhaavia diffusa* is found to halt intrauterine contraceptive device-induced bleeding in monkeys (Lami *et al.*, 2000). Shashikala et al. (2010) has shown comparative study of Ashokarishtham and clomiphere citrate wherein, Ashokarishtham treated PCOS rats demonstrated improved reduction in oxidative stress induced parameters as compared to clomiphere citrate treated group.

1.11.1 ALOE VERA

Aloe barbadensis Miller (*Aloe vera* Linne), commonly referred to as *Aloe vera*, is one of approximately 420 species of *Aloe* belonging to the lily family (family Liliaceae, tribe *Aloineae*) that originated in South Africa, but have been indigenous to dry sub-tropical and tropical climates, including the southern United States (Reynolds *et al*., 1999).

Recently, only a few species of *Aloe* are of commercial importance (Eshun *et al.*, 2004). The *Aloe vera* plant has been used in folk medicine for over 2000 years, and *Aloe vera* has remained an important component in the traditional medicine of many contemporary cultures, such as China, India, the West Indies, and Japan (Lee *et al*., 2009).

Aloe vera is succulent plants which is xerophytes, and adapted to living in areas of low water availability and are characterized by possessing a large water storage tissue. The main feature of the *Aloe vera* plant is its high water content, ranging from 99–99.5% (Hamman, 2008). The remaining 0.5–1.0% solid material is reported to contain over 75 different potentially active compounds, including water- and fat-soluble vitamins, minerals, enzymes, simple and complex polysaccharides, phenolic compounds, and organic acids. In compositional studies on the structural components of the *Aloe vera* plant leaf portions, the rind was found to compose 20–30% and the pulp 70–80% of the whole leaf weight. On a dry weight basis, the percentages of the rind and pulp represented as lipids (2.7% and 4.2%) and that as proteins (6.3% and 7.3%) only accounted for a minor fraction (Femenia *et al*., 1999). However, the non-starch polysaccharides and lignin represent bulk of each leaf fraction and was found to be 62.3% and 57.6% of the dry weight of the rind and pulp, respectively. *Aloe vera* gel polysaccharides consist of linear chains of glucose and mannose molecules, of which more mannose present than glucose, thereby the molecules are referred to as polymannans (Ni *et al.*, 2004). These are linear chains range in size from a few to several thousand molecules (Hutter *et al*., 1996). The major polysaccharide, Acemannan, is composed of one or more polymers of various chain lengths with molecular weights ranging from 30–40 kDa or greater, and consisting of repeating units of glucose and mannose in a 1:3 ratio (Femenia *et al*., 1999; Chow *et al*., 2005).

In Western societies, especially in the U.S., *Aloe vera* has been grown mainly to supply the latex component of the leaf to the pharmaceutical industry spp (Lee *et al*., 2000). However, over the last decade, *Aloe vera* spp. has gained popularity as a therapeutic botanical and, consequently, a large industry has developed (Reynolds *et al.*, 1999). Many investigators have endeavoured to establish the active principles in *Aloe vera* gel. It has been used for many centuries for its curative and therapeutic properties and although

over 75 active ingredients from the inner gel have been identified, therapeutic effects have not been correlated well with each individual component (Habeeb *et al*., 2007). Many of the medicinal effects of *Aloe* leaf extracts have been attributed to the polysaccharides found in the inner leaf parenchymatous tissue (Ni *et al*., 2004) but it is believed that these biological activities should be assigned to a synergistic action of the compounds contained therein rather than a single chemical substance (Mahboubi *et al*., 2014).The *Aloe* parenchyma tissue or pulp has been shown to contain proteins, lipids, amino acids, vitamins, enzymes, inorganic compounds and small organic compounds in addition to the different carbohydrates (Hamman *et al*., 2008).

In South Africa, the most widely distributed *Aloe* species are *Aloe greatheadii var. davyana* (Asphodelaceae) and *Aloe ferox* Mill. (Asphodelaceae). *Aloe. greatheadii* grows wild in the northern parts of South Africa, whereas *A. ferox* grows wild primarily in the Eastern and Western Cape provinces. The *A. ferox* contains various combinations of glucose and galactose as main monosaccharides, while the *A. barbadensis* yields only mannose (O'brien and Van wyk, 2011). Various extracts of these *Aloe* species are been used traditionally for the treatment of arthritis, skin cancer, burns, eczema, psoriasis, digestive problems, high blood pressure and diabetes (Hossain *et al*., 2013). As different *Aloe* species would have varying phytochemical contents due to inter-species variation, and varying climate and soil conditions, direct correlation of biological activity would be inaccurate.

Many beneficial effects of this plant have been attributed to the polysaccharides present in the pulp. The clear pulp which is also known as gel is widely used in various medical, cosmetic and neutraceutical applications (Ni *et al*. 2004). Studies by Hu *et al.* (2005) noted that higher anti oxidative activities present in its rind. *Aloe vera* has been used externally to treat various skin conditions such as cuts, burns and eczema (Serrano 2006). These *Aloe* species are currently listed in the pharmacopoeia of many countries in form of main *Aloe*, extract and powder (Park and Jo 2006).

1.11.1.1 CLINICAL EFFICACY AND MECHANISM OF ACTION

1.11.1.1.1 BURN WOUND HEALING EFFECT

Due to presence of various phyto-components, Aloe is used for several efficacies. *Aloe* spp. is known as "the healing plant". *Aloe vera* has been used for traditional medical purposes in several cultures (Grace *et al*., 2008; Eshun *et al*., 2004). *In vitro*, extract components of *Aloe vera* stimulate the proliferation of several cell types. Many studies have shown that treatment with whole *Aloe vera* gel, extracts resulted in faster healing of wounds (Tarameshloo *et al*., 2012; Liu *et al*., 2006) which is manifested by an increase in rate of contraction of wound area Subramanian *et al (2006)* also confirmed this effect of *Aloe vera* on increasing wound contraction and collagen synthesis. This property is attributed to the mannose-6-phosphate known to be present in *Aloe vera* gel (Subramanian *et al.*, 2006). Polysaccharide from *Aloe* promotes both the proliferation of fibroblasts and the production of hyaluronic acid and hydroxyproline in fibroblasts that plays an important role in the extracellular matrix remodeling during wound healing (Liu *et al.*, 2010). Acemannan, a polysaccharide also the significantly increases periodontal ligament cell proliferation, up regulation of growth/differentiation factor 5, type I collagen and alkaline phosphatase activity in Primary human periodontal ligament cells (Chantarawaratit *et al*., 2013). In a clinical study, to check the efficacy of *Aloe vera* gel compared with 1% silver sulfadiazine cream as a burn dressing for the treatment of superficial and partial thickness burns, healing of burn wounds were remarkably early than those patients treated with 1% silver sulfadiazine (Shahzad and Ahmed, 2013). Polysaccharides isolated from *Aloe vera* induces Matrix Mellatopeptidase (MMP)-3 and Metallopeptidase inhibitor (TIMP)-2 gene expression during the skin wound repair of rat which directly helps to regulate the wound healing activity of *Aloe vera* gel (Tabanden *et al*., 2014).

1.11.1.1.1.2 IMMUNOMODULATORY EFFECT

Aloe vera gel has strong immunomodulatory activity wherein it down regulates Lippolysaccharides (LPS) -induced inflammatory cytokine production and expression of NLRP3 (NACHT, LRR and PYD domains-containing protein inflammasome in human macrophages (Budai *et al.*, 2013). *Aloe vera* could inhibit the inflammatory process following burn injury, as characterized by the reduction of leukocyte adhesion, as well as

those pro-inflammatory cytokines (Duansak *et al.,* 2003). Liu *et al*., (2012) has shown that *Aloe* polysaccharides pre-treatment can attenuate the cerebral ischemia and reperfusion injury in severe traumatic-haemorrhagic rats first entering high altitude through inhibiting systemic inflammatory response and leukocyte aggregation and lipid peroxidation in the brain. Administration of *Aloe vera* has been universally demonstrated to result in marked increase in phagocytic and proliferative activity of the reticulo-endothelial system (Im *et al*., 2005). *Aloe vera* directly inhibits the cyclooxygenase pathway and reduces prostaglandin E2 production (Picchietti *et al*., 2013) which plays an important role in inflammation. *Aloe* also contains anthraquinones and chromone in inner gel that possess strong anti-inflammatory effects as shown in murine macrophages (Park *et al.* 2009a). This report suggests that *Aloe* as whole has anthraquinones (*Aloin*), chromone (*Aloesin*) components, and *Aloe gel* have pharmacological activity to alleviate inflammatory responses in Inflammatory bowel disease (IBD) (Langmead *et al.,* 2004). Recent clinical study has evaluated the therapeutic effect of *Aloe vera* gel wherein 2% oral gel is not only effective in decreasing the recurrent aphthous stomatitis patients' pain score and wound size but also decreases the wound healing period (Babaee *et al.,* 2014).

1.11.1.1.1.3 INTESTINAL ABSORPTION

Aloe has been used for drug absorption enhancement for drugs with low bioavailability (Carein *et al*. 2013). *Lactobacillus brevis* strains were isolated from naturally fermented *Aloe vera* gel which inhibited the growth of many harmful enteropathogens without restraining most of normal commensals in the gut and hence named POAL (Probiotics Originating from *Aloe* Leaf) strains and these exhibited discriminative resistance to a wide range of antibiotics (Kang *et al.,* 2014). The compound namely Aloin present in gel is metabolized by the colonic flora to reactive aloe-emodin which is responsible for the purgative activity. *Aloe* emodin from isolated from *Aloe vera* inhibits colon cancer cell migration/angiogenesis by down regulating Matrix Metalloproteinase (MMP)-2/9, ras homolog family member B (RhoB) and Vascular Endothelial Growth Factor (VEGF) via reduced DNA binding activity of NF-κB (Nuclear Factor kappa-light-chain-enhancer of activated B cells) (Suboj *et al*., 2012). *Aloe vera* gel supercritical CO2 extract (AVGE)

has been shown to contain five phytosterols present in gel, which able to reduce visceral fat accumulation, and influence the metabolism of glucose and lipids in animal model experiments wherein it reduced large-sized intestinal polyps and ameliorated reduction in plasma High Molecular Weight (HMW) adiponectin levels in Adenomatous polyposis coli (APC) gene-deficient Min (multiple intestinal neoplasia) mice fed high fed diet. Further, an "*In vitro"* study has shown that *A. vera* gel and whole leaf extract was able to significantly reduce the transepithelial electrical resistance of the Caco-2 cell monolayers and thereby showed the ability to open tight junctions between adjacent cells. Hence, *A. vera* gel and whole leaf extract solutions significantly enhanced the transport of insulin across the Caco-2 cell monolayers (Chen *et al*., 2009).

1.11.1.1.1.4 ANTI-DIABETIC EFFECT

The clinical studies have suggest that *Aloe vera* gel may be a safe anti-hyperglycemic and anti-hypercholesterolemic agent for hyperlipidemic type 2 diabetic patients without any significant effects on the other blood lipid levels and liver/kidney function (Huseini *et al*., 2012). "*In vivo"* and "*in vitro"* studies strongly demonstrate that the water soluble fraction of *Aloe* spp. possesses glucose-lowering activities and some of its component (s) modulates GLUT-4 mRNA expression (Kumar *et al*., 2011). In randomized controlled trial, *Aloe vera* gel complex reduced body weight, body fat mass (BFM), and insulin resistance in obese pre-diabetes and early non-treated diabetic patients (Choi *et al.,* 2013). Further, in pilot study of two *Aloe* products in patients with pre-diabetes over an 8-week period, tend revert the impaired fasting glucose and impaired glucose tolerance observed in conditions of pre-diabetes/metabolic syndrome (Devraj *et al.,* 2012). Study discusses the efficacy of *Aloe* emodin-8-O-glycoside (AEG) isolated from *Aloe vera* gel in glucose transport wherein AEG enhances glucose transport by modulating the proximal and distal markers involved in glucose uptake and its transformation into (Anand *et al*., 2010). Tanaka *et al*. (2006) reported reductions in both fasting and random blood glucose levels of db/db diabetic mice chronically treated with the same phytosterols from *Aloe vera* gel. Jain *et al.,* (2010) has shown that *Aloe vera* gel has significant anti-diabetic and cardio protective activity wherein it significantly reduced the

oxidative stress in Streptozocin induced diabetic rats and improves anti-oxidant status. *Aloe vera* gel also helps to improve the metabolism wherein recent report suggest that it helps to improve metabolic condition in obese prediabetes and early non-treated diabetic patients through reducing body weight, body fat mass (BFM), fasting blood glucose (FBG), fasting serum insulin, and Homeostasis Model of Assessment - Insulin Resistance (HOMA-IR) in obese individuals (Choi *et al*., 2013). Shin *et al*., (2011) shown that dietary *Aloe* formula also reduces obesity-induced glucose tolerance not only by suppressing inflammatory responses but also by inducing anti-inflammatory cytokines in the White adipose tissue (WAT) and liver, both of which are important peripheral tissues affecting insulin resistance. *Aloe vera* also has shown improvement in the function of isolated Rat Pancreatic islets wherein it increased survival of the islet cells, modulated mitochondrial activity, and insulin levels at the same time as reducing production of ROS (Rahimifard *et al*., 2014).

1.11.1.1.1.5 ANTIOXIDANT EFFECT

Aloe vera contains substantial amounts of antioxidants including- tocopherol (vitamin E), carotenoids, ascorbic acid (vitamin C), flavonoids and tannins (Hanmman, 2008), and it has been suggested that antioxidant action may be an important property of plant medicines used in treatment of various diseases. Shilve *at al.,* (2014) Topical *A. saponaria* treatment has shown anti-nociceptive and anti-inflammatory effects in a UVB-induced sunburn model through its antioxidant components present in gel. *Aloe* gel contain good scavenge property wherein it able to scavenge the free radicals, DPPH•, ABTS (+•) and NO in a concentration dependent manner in "*in vitro*" study of radio protective efficacy of *Aloe vera* gel (Saini *et al*., 2011). Administration of the ethanolic extract of *Aloe vera* gel on tissue antioxidants have been attributed to reduction in blood glucose level in diabetic rats, which helps to prevents excessive formation of free radicals through various biochemical pathways and also reduces the potential glycation of the enzymes (Rajasekaran *et al.,* 2005). The *In vitro* and *In vivo* antioxidant potentials of a polysaccharide isolated from *Aloe vera* gel were investigated. Enzymatic extracts were prepared from *Aloe vera* gel containing ten digestive enzymes including five

carbohydrases and five proteases and *Aloe* Polysaccharides (APS). This extract showed a protective effect against AAPH-induced oxidative stress and cell death in Kidney epithelia cells (Vero cells) as well as in the *in vivo* zebrafish (Kang *et al.,* 2014). One study determined the total phenolic content of *Aloe vera* leaf skin (AVLS) extracts and a significant correlation was established between the total phenolic content and the antioxidant capacity (Kammoun *et al.,* 2011). The methanol extracts of leaf skins and flowers of *Aloe vera* also screened for their antioxidant and antimycoplasmic activities wherein *"in vitro"* antioxidant activities of both extracts exhibited antioxidant activity, being the leaf skin extract the most active fraction (Lopaz *et al*., 2013).

1.11.1.1.1.6 HEPATO-PROTECTIVE EFFECT

Isolated phytosterol namely lophenol (Lo) and cycloartanol (Cy) administrations induced down regulation of fatty acid synthesis and a tendency for up-regulation of fatty acid oxidation in the liver which favour for the reduction in intra-abdominal fat and improvement of hyperlipidemia and in ddition to SREBP1/PPARα ratio was decreased and metabolicsyndrome- related disorders were improved and liver steatosis in aloe-sterol-treated ZDF rats (Misawa *et al*., 2012). *Aloe* formulas suppresses obesity-induced inflammatory responses by reducing levels of the proinflammatory cytokines, PPARγ/LXRα, and 11β-HSD1, and by enhancing anti-inflammatory cytokines in WAT and liver, both of which are important peripheral tissues for insulin response. The beneficial effects of aloe formula with respect to obesity-induced insulin resistance and hepatic steatosis have been associated with its action on PPARγ/LXRα (Shin *et al*., 2011). Saito et al. shown that *Aloe vera* gel extract (AVGE) prevent the ethanol induced fatty liver by suppressing mRNA expression of lipogenic genes in liver. Desai *et al* (2012) have shown that *Aloe vera* gel reduced level of LCAT activity and increase HMG-CoA activity along with increase in plasma HDL-C levels in PCOS rat model. The combination of probiotic Lactobacillus rhamnosus GG (LGG) and *Aloe vera* gel have a therapeutic potential to decrease cholesterol levels and the risk of cardiovascular diseases (Kumar *et al*., 2013).

1.11.1.1.1.7 ANTI-CANCER ACTIVITY

Aloin (AL), being a natural compound and the main ingredient of *Aloe*, has been documented for its remarkable potential therapeutic options in cancer wherein it showed chemo preventive effects against 1,2-dimethylhydrazine-induced preneoplastic lesions in the colon of Wistar rats (Hamiza *et al*., 2014). Aloin treatment could inhibit the secretion of Vascular endothelial growth factor (VEGF) by cancer cells. VEGF is one of the most important pro-angiogenic cytokines known and well characterized inducers in tumor neovascularization and significantly inhibited in vitro VEGF-induced angiogenic response of human endothelial cells, to inhibit proliferation and migration of endothelial cells (Pan *et al*., 2013). Aloe-emodin (AE), a natural compound that found to have diverse biological activities one of them is anticancer functions (Lin *et al*., 2009; Muto *et al.*, 2007). AE (1,8-dihydroxy-3-hydroxymethyl-9,10- anthracenedione) is a herbal anthracenedione derivative from *Aloe vera* leaves. Recent reports have shown that AE possesses antiproliferation effects on some types of cancer cells, such as lung, squamous, glioma, and neuroectodermal cancer cells (Lin *et al*., 2011; Masaldan *et al.,* 2012). The inhibitory effect of AE on the activity and gene expression of N-acetyl transferase, which plays an initial role in the metabolism of aryl amine carcinogens, was found in human malignant melanoma cells (Lin *et al*., 2005). Recently, Lin *et al*. (2006) found that AE-induced apoptosis in T24 human bladder. Aloin (AL), derived from *Aloe barbadensis* Miller leaves, has been shown to possess anti-cancer potential activities wherein it inhibit tumor angiogenesis and growth via blocking STAT3 activation, with the potential of a drug candidate for cancer therapy (Pan *et al.,* 2013). Anthraquinone derivatives such as emodin like natural (emodin, rhein, and aloin) and synthetic (AQ2S) anthraquinones have recently been shown to protect in models of beta amyloid β (Aβ) and tau aggregation-induced cell death through anti-aggregation properties, and/or enhancing the phosphatidylinositol-3-kinase (PI3K)/AKT survival mechanism and AQ2S is a new neuroprotective compound and a novel caspase inhibitor (Jackson *et al*., 2013).

1.11.1.1.1.8 ANTI-MICROBIAL ACTIVITY

A. barbadensis has been described as an antibacterial agent. The *Aloe* protein of 14 kDa from the *Aloe vera* leaf gel was isolated and the purified *Aloe* protein exhibited a potent

anti-fungal activity against *Candida paraprilosis, Candida krusei and Candida albicans* (Das *et al*., 2011). *A. barbadensis* has anthraquinones as an active compound, which is structural analogue of tetracycline. The anthraquinones acts like tetracycline that inhibits bacterial protein synthesis by blocking the ribosomal A (where the aminoacylated t-RNA enters) site. Therefore, the bacteria cannot grow in the media containing *A. barbadensis* extract. Pandey *et al*., (2010) have established the susceptibility of Gram-positive (Gram +ve) and Gram-negative (Gram +ve) bacteria to an extract of the inner gel of the plant *Aloe barbadensis* Miller or *Aloe vera* (L.) Burma. f. (Ferro *et al.,* 2003; Habeeb *et al*., 2007). Polysaccharides of *Aloe vera* gel have been attributed direct bacterial activity through the stimulation of phagocytic leucocytes to destroy bacteria (Lawless *et al.,* 2000, Pugh *et al*., 2001). The presence of pyrocatechol in *Aloe vera*, reported by Kametani *et al.,* (2007) and Cowan *et al.,* (1999) is a hydroxylated phenol, known to be toxic to micro-organisms. Recent study demonstrated that the *Aloe vera* inner gel expresses antibacterial properties against both susceptible and resistant Helicobacter pylori strains and impact on the antimicrobial resistance phenomenon of H. pylori, proposing the *A. vera* inner gel as a novel effective natural agent for combination with antibiotics for the treatment of H. pylori gastric infection (Cellini *et al.,* 2014).

It is seen from the literature that *A. vera* is a very important plant for its large number of medicinal properties as well as medicinally important chemicals like amino acid, anthraquinone, enzyme, hormone, sterol, and vitamin (Hamman et al., 2008) which attributes to different efficiencies of the plant. *Aloe* has been analyzed for toxicity during clinical trials and considered to be safe. But certain reports suggested that higher dose could modulate digestive tract. Dosed water studies in mice revealed no acute toxicity of the leaf pulp at 500 mg/kg (Shah *et al*., 1989). At higher doses, however, a decrease of central nervous system activity was observed. During sub-chronic ninety-day studies, increased mortality, decreased red blood cell count, and significant sperm damage were noted, in addition to decreased central nervous system activity (Shah *et al*., 1989). Hence, upper limit of dose of *Aloe vera* gel or *Aloe vera* extract plays a very crucial for the treatment of various diseases.

From whole plant, *Aloe vera* latex is a laxative regulated as a drug by the FDA and is also

used as a bitter flavoring additive by the food industry; *Aloe vera* gel is primarily a topical agent for skin wounds and irritations but is also taken internally for the treatment of gastric ulcers and diabetes; and the whole leaf extract, which combines both the gel and latex, is popular as a dietary supplement for various systemic ailments and is promoted as a potential anti-cancer, anti-AIDS, and anti-diabetic agent.

Thus, it is quite promising as a multipurpose medicinal agent so further experiments are needed to isolate and to find out the mechanism of the bioactive chemicals whose identification need to be done by using modern instruments like HPLC, HPTLC and NMR and extend clinical trials on the road to generate novel drugs. Food and Drug Administration of USA has already approved the developmental study of *Aloe vera* in the treatment of Cancer and AIDS. Thereby, controlled studies are required to prove the effectiveness of *Aloe vera* under the various conditions.

In view of above context, *Aloe vera* as multifaceted plant that exploited for various biological properties as discussed earlier. But no such report in literature suggesting role of *Aloe vera* gel for reproduction. Hence, it was interesting to explore the potentiality of the plant for the fertility.

2. REFERENCES

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