Section 1 Oocyte Recruitment

Chapter 1

Physiology of Ovulation

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1.1 The Follicle

At the time of menstruation there are numerous antral follicles in the ovaries measuring 4–8 mm in diameter. These follicles have developed from primordial follicles through gonadotrophin-independent and gonadotrophin-sensitive phases of growth. It is difficult to determine how long this process takes in vivo, but evidence from tissue transplantation studies suggests that it is longer than three months. These antral follicles are gonadotrophin-dependent and will not grow further without gonadotrophin stimulation.

The rate of gonadotrophin-dependent follicle growth can vary from cycle to cycle and woman to woman. When the lead follicle reaches 12 mm in diameter, generally about day 9 or 10 of the menstrual cycle, it then grows at an average of 2 mm in diameter each day. This growth is associated with rapidly increasing oestradiol concentrations. Oestradiol stimulates endometrial growth and exerts negative feedback on the pituitary to reduce follicle stimulating hormone (FSH) secretion.

Follicles more than 12 mm in diameter will already be developing LH receptors on granulosa cells and luteinicing hormone (LH) will maintain follicle growth and function in the presence of declining FSH concentrations. Growth of smaller follicles will not progress when FSH concentrations decline. This mechanism is responsible for follicular selection to promote the selection of a single dominant follicle.

Clinical Correlation

Using the developing LH receptors on granulosa cells as a marker of the ability of a follicle to respond to the LH surge: follicles of 12 mm diameter may ovulate, and follicles reaching 14 mm or more generally will ovulate in response to an LH surge.

1.1.1 Follicular Formation

The primordial follicles represent the quiescent gamete stores within the ovary. They consist of an oocyte surrounded by a flattened layer of somatic pre-granulosa cells that reside within the ovarian cortex. They are formed before birth and peak in number in the mid-gestation fetal ovary [1].

The fetal gonad starts to develop around the fourth week of gestation. Extraembryonic primordial germ cells from the yolk sac enter the embryo along the hindgut and migrate to the developing ovary. They proliferate extensively, and by ten weeks of gestation there are

around 250,000 germ cells in the ovaries. These germ cells differentiate into oogonia and undergo further mitosis within germ cell clusters. These become oocytes that enter meiosis, which arrests at prophase I. The germ cell clusters break down and there is a marked loss of oocytes. The remaining oocytes associate with a single layer of somatic cells to form primordial follicles. The first follicles in the fetal ovary can be detected from around 16 weeks of gestation.

Although oogonial stem cells in adult ovaries have been described in women, there is no compelling evidence for clinically significant follicle formation in postnatal life. All the follicles that will be ovulated are formed in fetal life and can stay quiescent for over 50 years [2].

Clinical Correlation

New follicles cannot be made in women with a depleted ovarian reserve as a consequence of genetics, age or iatrogenic insult.

1.1.2 Follicular Activation and Growth

From mid-gestation and throughout postnatal and reproductive life some primordial follicles will activate and start growing. Once follicular growth begins the follicle has one of two fates: ovulation or atresia. At birth there are approximately 1 million follicles within the ovaries and by the time of menarche half of these will have activated and have undergone atresia. Overall, very few follicles will ovulate: just over 400 in an uninterrupted reproductive lifespan.

The first stage of follicular growth is the transition of a primordial follicle into a primary follicle. This is characterized by enlargement, then proliferation of the somatic cells, and an increase in the size of the oocyte. When the follicle has acquired three to six layers of granulosa cells, some stromal cells near the basal lamina become aligned parallel to each other around the primary follicle. These fibroblast-like cells change into epithelioid-like cells, capable of steroidogenesis, and stratify into the theca cell layers. At this stage the follicles develop an independent blood supply. This is the preantral stage of follicular development [3].

The appearance of the antral cavity starts with the development of small fluid-filled spaces, within the granulosa cell layer, that coalesce to form the antrum. This is the secondary follicle and from this point onwards there is a specific group of granulosa cells that surround the oocyte. These granulosa cells form the cumulus cells that have cytoplasmic connections to the oocyte. There appears to be a stratification of the rest of the granulosa cells, as those nearest the basement membrane become more columnar in shape. At this stage the follicles measure 180–250 μm in diameter.

Through the accumulation of fluid in the antral cavity and the proliferation of granulosa and theca cell layers, the follicles continue to grow until they measure between 2 and 5 mm in diameter. This process takes at least three months in vivo, and these small antral follicles can be found at all stages of the ovarian cycle. The oocyte enlarges over this time from 30 μm to 120 μm in diameter as RNA is accumulated and protein is synthesized. In addition, at this stage the zona pellucida, a thick glycoprotein surrounding the oocyte, becomes fully developed (Figure 1.1).

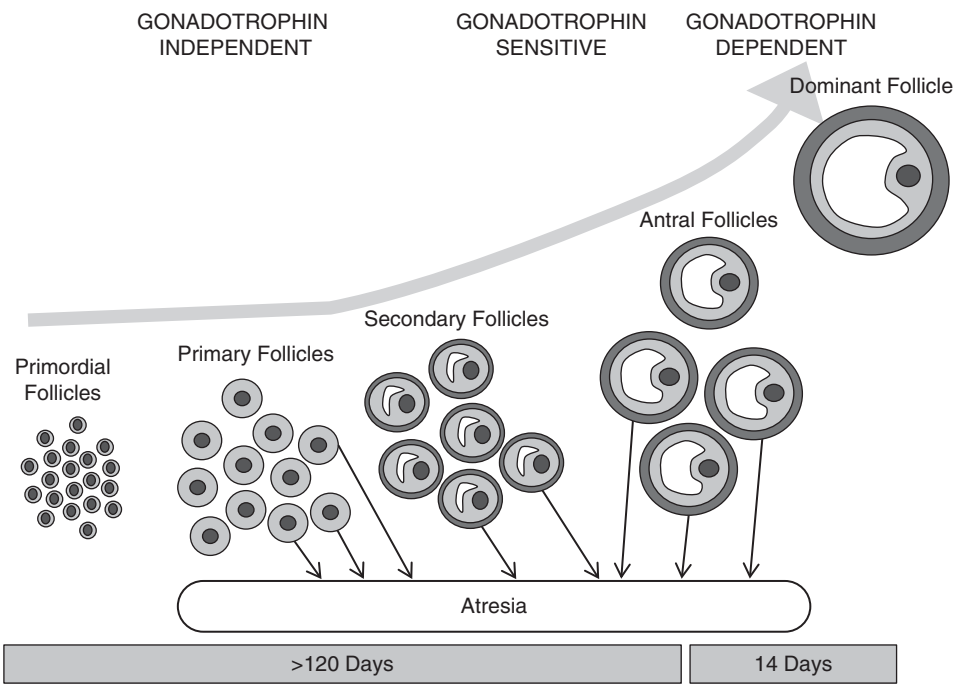

Figure 1.1 Illustration of follicular activation and growth in women. After activation the fate of primordial follicles is either atresia or ovulation. Over more than three months the follicles develop through gonadotrophin-independent, sensitive and dependent stages. The final gonadotrophin-dependent stage is only around two weeks in length and a fraction of the time follicles are growing

Clinical Correlation

Small antral follicles identified in the early follicular phase by ultrasonography have been growing for at least three months.

1.1.3 Regulation of Follicular Activation and Growth

Each day up to 30 primordial follicles are recruited into the pool of growing follicles. It is not entirely certain what causes this activation, but it is clear that it is pituitary independent. Follicular recruitment, and growth to the antral phase, occurs in the absence of gonadotrophins and is seen in fetal life, prepubertally, during pregnancy and while breast feeding, as well as when on hormonal contraception.

Follicular activation is controlled by autocrine/paracrine mechanisms. Whether it is induced by a stimulator or the loss of an inhibitor is uncertain; however, primordial follicles undergo rapid recruitment when removed from the ovary and cultured in vitro, and the chance of activation is increased when there is a reduction in the density of neighbouring follicles. These observations support the theory that activation is by removal of local inhibition [4].

The nature of the inhibitor(s), however, is uncertain. In rodents there is evidence for a role of anti-Müllerian hormone (AMH) in the initiation of follicular activation, but in larger mono-ovulatory animals AMH seems to slow down the growth of follicles rather than

activate them. Certainly there is a role for PTEN, which suppresses the PI3K-AKT-mTORC1 pathway. Inactivation of PTEN promotes activation, but how this is regulated in vivo is not known [5].

Once activated, follicular growth is regulated by multiple local factors. The rate of growth depends on the balance between factors that promote and factors that inhibit growth. In vitro it appears that some inhibitory ones are lost and follicular growth is faster. Multiple intraovarian molecules and pathways have been shown to regulate the initial stages of follicular growth, and these include the insulin growth factor (IGF) and transforming growth factor (TGF) β families as well as many other growth factors produced by ovarian somatic cells, stromal cells, vascular and neural cells or the oocyte. The complex interplay between local factors involved in the activation and initial growth of the follicle remains inadequately understood [3,4].

Clinical Correlation

As yet we don't have the capacity to manipulate the pathways involved in the initiation of follicular activation and growth in vivo, and mimicking them in vitro remains experimental.

1.1.4 Function of Small Growing Follicles

These small growing follicles are not steroidogenically active, but they do synthesize and secrete autocrine, paracrine and endocrine molecules. These molecules are generally local regulators of follicular development and growth. AMH is one of these molecules that is of particular importance. While the physiological role of AMH remains unclear, it has particular utility as a biomarker of ovarian reserve.

AMH is made in the granulosa cells of growing preantral and small antral follicles. As this growth is not dependent on gonadotrophins AMH will generally remain constant throughout the menstrual cycle and still be made when gonadotrophins are suppressed. Quantification of AMH in the serum correlates with the number of small growing follicles and can be used as a biomarker of ovarian reserve, ovarian morphology and capacity to respond to exogenous stimulation [6].

Small growing antral follicles also secrete inhibin B. This will feedback to lower FSH concentrations in the early follicular phase and it is the reason why FSH increases, despite no alteration in oestradiol concentrations, when women age. It also functions as a biomarker of ovarian reserve and is more consistent than measurement of FSH. However, it does vary across the menstrual cycle, and as it is made of two subunits, which can contribute to other molecules, it is more challenging to measure. Clinically it is inferior to AMH as a marker of ovarian reserve and is not routinely used [7].

Clinical Correlation

AMH is not a marker of the primordial follicle pool. A treatment, such as some chemotherapy regimes, that depletes the number of growing follicles but not the primordial pool would involve a decline and then recovery of AMH.

1.1.5 Gonadotrophin Sensitivity

When the follicles reach around 3–5 mm in size they become dependent on gonadotrophins for their further growth and development. However, both LH and FSH receptors can be

detected on follicles much smaller than this. That means that there is a stage where follicles are sensitive to LH and FSH but not dependent on them for their survival. It is likely however that gonadotrophins can promote growth in the smaller follicles before gonadotrophin dependence. There is a role for gonadotrophins in the growth and development of follicles before they become entirely dependent on them for survival [3].

Clinical Correlation

In prolonged gonadotrophin-releasing hormone (GnRH)-induced down-regulation, or in women without a functioning pituitary, gonadotrophins are required for a longer duration to stimulate follicular growth.

1.1.6 Gonadotrophin dependence

The small antral follicles present on the ovary at the time of menstruation express both functional FSH and LH receptors. These follicles require gonadotrophin stimulation to grow further. During the preceding luteal phase progesterone, oestrogen and inhibin A cause negative feedback to lower gonadotrophins. This prevents follicular growth beyond the small antral phase and eventual atresia of a cohort of follicles. The fall in steroids and inhibin A at menstruation increases gonadotrophins and stimulates the growth of a cohort of follicles that enter the gonadotrophin-dependent phase of growth [8].

Clinical Correlation

The antral follicle count (AFC) gives an indication of the maximal potential response to ovarian stimulation with exogenous FSH.

1.1.7 Gonadotrophin Regulation of Follicular Growth

The size of the follicle destined for ovulation increases greatly during the follicular phase of the ovarian cycle by cellular multiplication and accumulation of follicular fluid. This process requires trophic stimulation by FSH. The diameter of the preovulatory follicle increases from 6.9±0.5 mm in the early follicular phase to 18.8±0.5 mm in the late follicular phase. During this period, the mean number of granulosa cells increases from approximately 2–5 million in the early follicular phase to 50–100 million at the time of ovulation. This growth occurs during the follicular phase of the ovarian cycle and is completed within 15 days.

There is a direct correlation between the size of the follicle and its blood supply. The preovulatory follicle becomes a highly vascular structure because of active endothelial cell proliferation in the theca cell layers. The granulosa cells remain separated from the theca cells by a basement membrane and remain avascular. The granulosa cells obtain their nutrients by diffusion, and the thickness of the granulosa cell layer in the dominant follicle is, therefore, limited to a maximum of seven cells [9].

FSH binds to its cell surface receptor on granulosa cells and activates adenylyl cyclase. As well as stimulating cell growth, FSH induces proteins involved in steroidogenesis, such as aromatase (CYP19). This means that there is a marked increase in steroidogenic capacity

as the follicle matures. It has become increasingly clear that the ovarian cellular responses to gonadotrophins can be modified by factors that are produced and have actions within the follicle. Many of the disparate actions of gonadotrophins on the follicle are transduced by multiple regulatory factors in a local paracrine fashion. The local regulators that have generated the most interest are steroids, the IGFs and the inhibin/activin family of proteins. It is clear that the development of the vasculature around the growing follicle involved angiogenesis and that vascular endothelial growth factor (VEGF) is the primary angiogenic factor in this process [10].

Clinical Correlation

A dominant follicle will have a clear vascular network visible on colour Doppler assessment, but this is all in the theca cell layer, as the granulosa cells have no direct contact with the microvasculature.

1.1.8 Follicular Steroidogenesis

The granulosa cells and the theca cells of the antral follicle are steroidogenic in nature. Studies using labelled precursors revealed that the isolated granulosa cells were capable of producing oestrogens only when precursor hormones were present. They could not synthesize the androgens that are the immediate precursors of oestrogens in the steroidogenic pathway. In contrast, isolated theca cells produced progesterone and androgens. Localization of steroidogenic enzymes within the follicle has demonstrated that theca cells express 17α-hydroxylase (CYP17), the enzyme responsible for androgen synthesis. They do not express aromatase (CYP19), the enzyme responsible for aromatization of androgens to oestrogens. In contrast, granulosa cells express CYP19 but do not have CYP17 activity. Follicular oestrogen biosynthesis therefore requires the co-operation between granulosa cells and their thecal neighbours. LH stimulates the formation of androgens in the theca cells and FSH stimulates the conversion of androgens into oestrogens in the granulosa cells (Figure 1.2) [11].

As well as acting on multiple tissues including the uterus, hypothalamus and pituitary, ovarian steroids also act on the follicle itself. Oestrogen receptors are found on granulosa cells and they may augment FSH action. Some women with hypogonadotrophic hypogonadism have normal FSH concentrations but very low LH and oestradiol concentrations. These women have multicystic ovaries, but follicular growth tends not to progress beyond 8–9 mm despite normal FSH concentrations.

Androgen receptors are also present on granulosa cells. High androgen concentrations can cause atresia of larger antral follicles, pause the growth of small antral follicles and potentially promote the growth of preantral follicles [12]. The net effect of increased androgens is a build up of paused small antral follicles and the development of a polycystic ovary [13].

Gonadotrophin-induced steroidogenesis is modified by other local and endocrine factors. Insulin, IGFs, inhibin, activin and other members of the TGFβ superfamily, as well as disparate growth factors and cytokines, all can affect ovarian steroidogenic function. Some of these are regulated by gonadotrophins and others are not. The paracrine regulation of

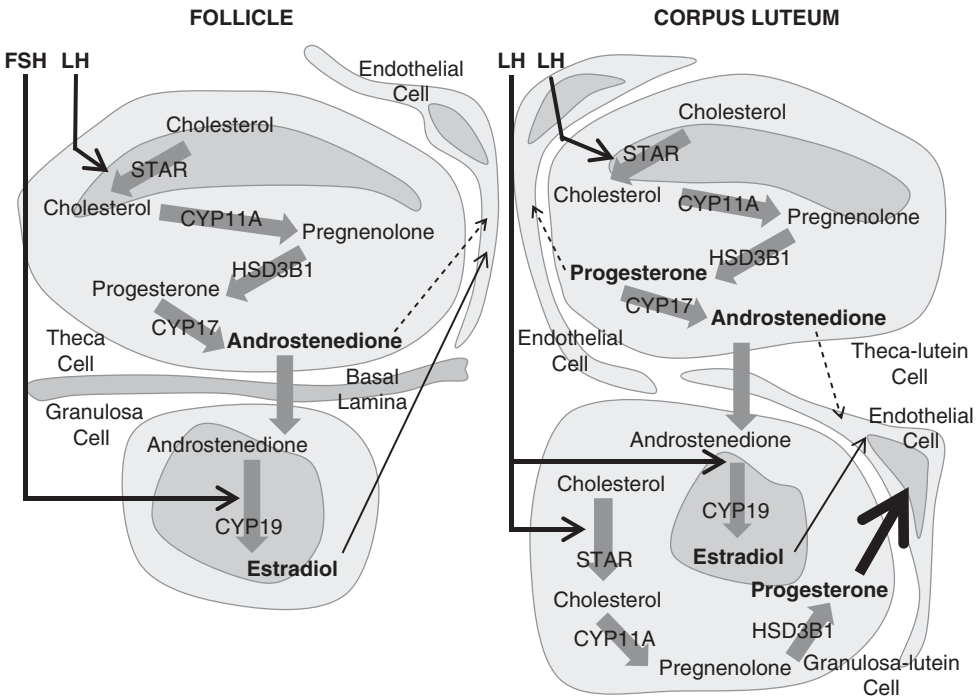

Figure 1.2 Illustration of steroidogenesis in the follicle and corpus luteum. In the follicle oestradiol synthesis required both LH and FSH and theca cells and granulosa cells. In the corpus luteum both the theca-lutein cells and granulosa-lutein cells can secrete progesterone. The theca-lutein cells continue to secrete androgen and the granulosa-lutein cells continue to secrete oestrogen, but only LH is required for luteal steroidogenesis

follicular function remains complex and not fully understood. Manipulation in vitro however remains controversial, although there are studies on the effect of androgens and insulin modification through metformin as adjuvant therapy [14].

Clinical Correlation

There is a local role for oestrogen as well as FSH in the growth and development of a dominant follicle. A polycystic ovary is a biomarker of increased intraovarian androgen concentrations.

1.1.9 Negative Feedback on Gonadotrophins

As follicles grow and mature under the influence of FSH, oestrogen secretion, regulated by both FSH and LH, increases. The FSH will stimulate the growth of small antral follicles at various stages of development within the ovary. The increasing oestradiol concentrations feedback on the hypothalamus and pituitary to decrease FSH secretion (Figure 1.3). As follicle survival is dependent on endocrine trophic support, the fall in FSH concentrations will cause atresia of these growing follicles (Figure 1.1). However, larger follicles are less sensitive to the decline in FSH concentrations.

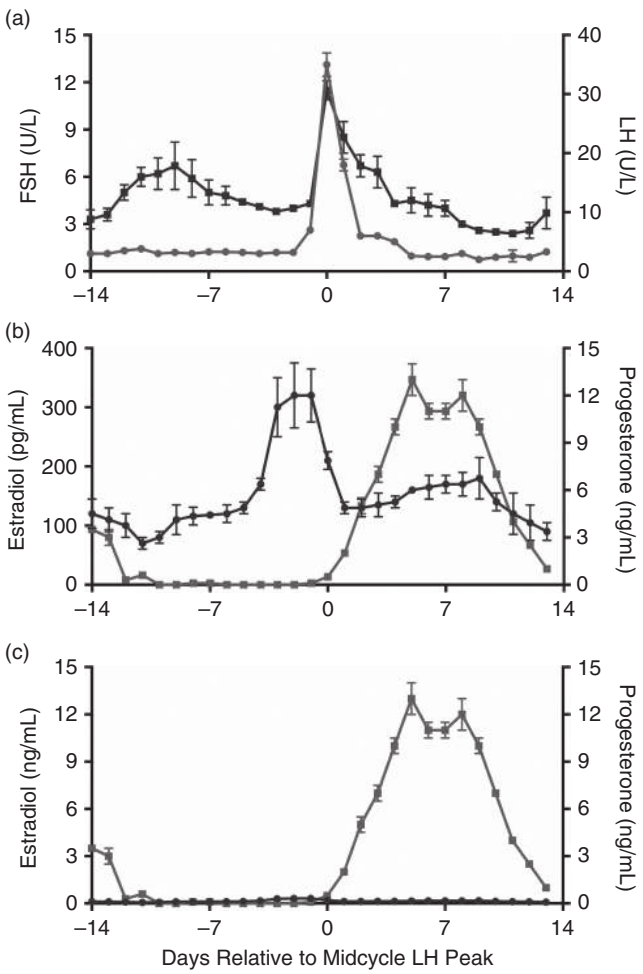

Figure 1.3 Hormone profile of the menstrual cycle of women. (a) Concentrations of FSH and LH. (b) Concentrations of oestradiol and progesterone. (c) Concentrations of oestradiol and progesterone plotted on the same scale highlighting the dominance of progesterone. Data reproduced from [25] and adapted from [27].

Clinical Correlation

When exogenous FSH is used in the absence of down-regulation, endogenous FSH is still present and subject to negative feedback so that total FSH will fall while exogenous FSH concentrations are maintained. Exogenous FSH therefore does not need to be reduced to ensure unifollicular development in gonadotrophin ovulation induction.

1.1.10 Follicular Selection

During the late follicular maturation of the granulosa cells, FSH induces the expression of LH receptors that are also coupled to adenylyl cyclase. Consequently, in the preovulatory

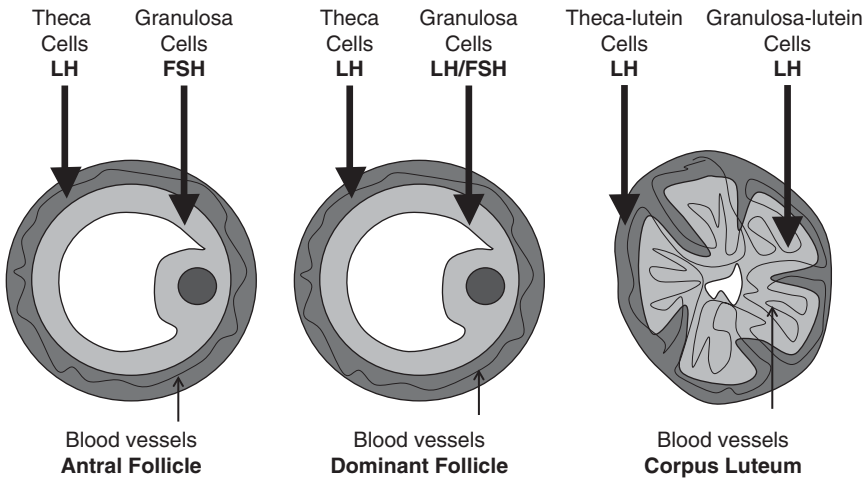

Figure 1.4 Changing requirements for gonadotrophins as the follicle matures. In the dominant follicle both LH and FSH regulate granulosa cell function. In the granulosa cells of smaller antral follicles FSH regulates granulosa cell function while after ovulation LH regulates granulosa-lutein cell function. After ovulation the avascular granulosa cell layer becomes highly vascular

follicle, LH can regulate both androgen synthesis (in theca cells) and aromatization of androgen (in granulosa cells). This increases the responsiveness of the follicle in the face of FSH levels that are declining. This FSH-dependent maturation of granulosa cells during follicular development is associated with increases of messenger ribonucleic acids (mRNAs) for the LH receptor and steroidogenic enzymes (Figure 1.4).

Unlike subordinate follicles, the dominant follicle continues to grow despite low FSH levels because it acquires the capacity to (1) express LH receptor in its granulosa cells and (2) produce large amounts of growth factors such as IGF1 and oestradiol which (a) allow the dominant follicle to respond to LH in addition to FSH and (b) provide higher sensitivity to FSH and LH, respectively. The role of the IGF system in the dominant follicle can be seen during in vitro exposure of granulosa cells to IGF-1 that induces secretion of oestrogen, which in turn stimulates proliferation of granulosa cells, expansion of the antrum and formation of gap junctions [8,11].

In addition, at this stage of development, the granulosa cells of the follicle have matured to acquire the capacity to respond to the ovulatory LH surge. When the dominant follicle is fully mature, the secreted oestrogen has prepared the endometrium for pregnancy. The oocyte is now ready for release and therefore the next process to be considered is ovulation.

Clinical Correlation

In hypogonadotrophic hypogonadism, where there are low LH concentrations, dropping exogenous FSH can result in atresia of dominant follicles, as the LH might not be enough to maintain their growth.

1.2 Ovulation

Increasing oestradiol secretion from the dominant follicle promotes a switch to positive feedback at the hypothalamus and pituitary that results in the LH surge. At this stage serum oestradiol

concentrations are usually 740–1250 pmol/l and the dominant follicle will generally measure between 17 mm and 23 mm in diameter. The LH surge usually occurs on day 14 of a normal menstrual cycle and it induces the three separate components involved in the ovulatory process.

One component of ovulation is reactivation of oocyte maturation where the oocyte, which has been maintained in the diplotene stage of prophase, progresses to the metaphase of the second meiotic division. Another component is luteinization of the granulosa cells where they develop the enzymatic machinery to synthesize progesterone. The third and final component of the ovulatory response is follicular rupture, which is an acute inflammatory process involving the breakdown of the apical follicle wall.

Clinical Correlation

Even when follicular rupture is not required, the LH surge is needed to initiate oocyte maturation as well as the transition to progesterone secretion, which promotes endometrial receptivity.

1.2.1 The LH Surge

The gonadotroph cells in the anterior pituitary gland respond to GnRH pulses from the hypothalamus by secreting FSH and LH. While GnRH facilitates FSH synthesis and secretion in a constant manner, its effects on LH are different. LH is sequestered into granules within the gonadotrophs and only some granules release their content with each GnRH pulse, giving characteristic LH pulses. Most LH remains stored in granules for release during the LH surge.

High circulating oestradiol has effects on the hypothalamus. It stimulates a GnRH surge that markedly increases both FSH and LH secretion (Figure 1.3). Although a surge of GnRH can cause an LH surge, there is a direct effect of oestrogens on gonadotrophin secretion in the absence of any alteration in GnRH concentrations. The negative feedback induced by oestrogen during follicular growth and the positive feedback of oestrogen at the time of ovulation are seen in women with hypogonadotrophic hypogonadism treated with pulsatile GnRH, where pulses of GnRH are consistent. Thus, women, deficient in GnRH, on exogenous fixed pulsatile GnRH concentrations will have an ovulatory LH surge. This suggests that GnRH is needed to facilitate the oestradiol-induced LH surge but that a surge of GnRH is not obligatory [15].

The evidence for functional oestradiol receptors on GnRH neurons isn't very convincing and there must be other oestrogen-responsive neural networks that regulate GnRH secretion. It is now clear that kisspeptin neurons stimulate GnRH secretion from GnRH neurons, and this is facilitated by neurokinin B (NKB). Treatment with kisspeptin will stimulate GnRH secretion while NKB antagonism will suppress GnRH secretion. Unlike GnRH administration, which produces a relatively normal LH surge, a kisspeptin injection produces an attenuated LH surge with a reduction in amount and duration of LH bioavailability [16].

The concentration of LH increases one hundred fold and is maintained during the surge that lasts up to 48 hours. LH itself has a very short half-life, of around 20 minutes, and an injection of LH is cleared too quickly to mimic the effects of an LH surge. However, hCG, which acts through the LH receptor and has the same action as LH, has a half-life of more