IMAGE ANALYSIS OF DOMINANT OVARIAN FOLLICLES AND OVARIAN FOLLICULAR DEVELOPMENT DURING CONTINUOUS AND CONVENTIONAL ORAL CONTRACEPTIVE DOSING SCHEMES

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ABSTRACT

The objective of this research was to assess ultrasound image attributes of human dominant ovarian follicles in the final stages of development during natural and oral contraceptive (OC) cycles, as well as characterize ovarian follicular and endometrial development during and after continuous versus conventional dosing schemes. We utilized sophisticated computer algorithms to elucidate an association between image attributes and physiologic status of follicles in their final stage of development. We used transvaginal ultrasonography to quantify changes in the numbers and diameters of ovarian follicles and changes in endometrial thickness and pattern during and following discontinuation of two different regimens of OC. Developmental changes in ovarian follicles and corpora lutea were correlated with serum estradiol-17β and progesterone, respectively to provide a comprehensive approach to examining ovarian and uterine function.

We reported for the first time that follicles which develop during natural and OC cycles have similar image attributes, which provides preliminary evidence that image attributes of human follicles are associated with physiologic status during the growth phase. Further research should be performed to elucidate the exact correlation between image attributes during all stages of follicular development throughout the menstrual cycle, prediction of dysfunctional follicular development (i.e., hemorrhagic anovulatory follicles) and the effects of different OC formulations on follicle development. Once the association between image attributes and various scenarios of follicular development are determined, a computer program could be developed to assess follicular health with a

single ultrasound examination, obviating many ethical constraints that currently prevent large scale progress in ovarian follicular research.

We further documented that continuous OC administration schemes provide greater follicular suppression than conventional dosing schemes. No dominant follicles developed during three consecutive 28 day cycles of continuous OC use, whereas eight dominant follicles developed during the same time period of conventional OC use. We interpreted these findings to mean that continuous OC dosing schemes provide a more effective contraceptive with a decreased risk of "escape" ovulation compared to conventional dosing schemes. Most follicles ovulated in the immediate cycle following discontinuation of OC. We suggest that the delay to fertility following cessation of OC is not due to anovulation but other yet, unknown, biological factors.

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DEDICATION

For my husband Evan, my parents Wendy and Rob and my sister Tania.
Thank you for believing in me when I didn't believe in myself.

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LIST OF ABBREVIATIONS

AUC = area under the curve

BK = bradykinin

BMI = body mass index

cAMP = cyclic adenosine monophasphate

EE = ethinyl estradiol

ECM = extracellular matrix

FSH = follicle stimulating hormone

Gn = gonadotropin

HAF = hemorrhagic anovulatory follicle

HFI = hormone free interval

HI = histamine

MHz = megahertz

IL = interleukin

LH = luteinizing hormone

LNG = levonogestrel

LT = leukotriene

MCP = monocyte chemotactic protein

mg = milligram

mm = millimeter

mm Hg= millimeters of mercury

MMP = matrix metalloproteinase

ng = nanogram

NGM = norgestimate

NO = nitric oxide

NPV = numerical pixel value

OC = oral contraceptive

P = progesterone

PA = plaminogen activator

PAF = platelet activating factor

pg = picogram

PG = prostaglandin

PH = pixel heterogeneity

RAP = Roger Allen Pierson

RLB = Rebecca Lynn Birtch

SEM = standard error of the mean

VEGF = vascular endothelial growth factor

μm = micrometer

μg = microgram

Chapter 1

GENERAL INTRODUCTION

1.1 Human Ovarian Follicular Dynamics

The study of reproductive medicine and biology, arguably, began in 1672 when the Dutch anatomist, Regnier de Graaf (1641-1673), discovered the ovarian follicle and ovulation[1]. de Graaf, mistakenly defined the fluid-filled follicle as the oocyte and was corrected in 1827 by Karl Ernst von Baer, when von Baer described the follicle-enclosed "ovulum", which later became known as the "Graafian Follicle"[2]. The form and function of the ovaries and uterus have been under examination since de Graaf's documentation of ovarian follicles. However, modern investigations involve novel techniques, such as the biomicroscope; a union between ultrasonography and microscopy. We have come a long way to understanding the intricacies of uterine and ovarian physiologic function and anatomy through these investigations, but we are far from completely understanding the physiologic function and microscopic anatomy of the reproductive organs and how their form and function affect women's reproductive health.

Introduction of the first Food and Drug Administration approved oral contraceptive (OC), Enovid™, in the early 1960's, heralded a contraceptive revolution for women. Prior to the introduction of Enovid™, women had fewer, less efficacious contraceptive choices. Only three major changes have been made to OC formulations in

the last 40 years; these changes include: (i) a decrease in the concentration of synthetic hormones contained in each pill, (ii) a switch from manestrol to ethinyl estradiol (EE) and (iii) the introduction of new, different progestin formulations. We do not yet fully understand the exact physiologic mechanisms of action associated with the suppression of follicular growth and endometrial development during OC administration.

The research in this thesis focuses on the ultrasound image attributes of dominant ovarian follicles that develop during natural and OC cycles as well as ovarian follicular dynamics and endometrial development during and following discontinuation of continuous and conventional OC dosing schemes. This review examines the current data pertaining to ovarian folliculogenesis during natural cycles and OC administration. Furthermore, the review briefly examines the principles of ultrasound imaging and image analysis techniques.

1.1.1 Oogenesis and Primordial Follicle Development

The formation, development and maturation of an ovum, or oogenesis, occurs completely during fetal life; thus, a female is born with her complete complement of oocytes and is incapable of producing more during her reproductive life-span[3]. Oogenesis begins during the third week of gestation when primordial germ cells migrate from the yolk sac entoderm to the genital ridge under direction of unknown cellular mechanisms and chemotaxic factors[4]. Arrival of the primordial germ cells at the primordial gonadal structure is associated with the differentiation and maturation of the embryonic ovary and redefinition of the primordial germ cells to oogonia[4]. Interestingly, gonadal development will not occur in the absence of germ cells[4]. Oogonia division continues until the twentieth week of gestation, when approximately 7 million oogonia are present; thereafter, oogonia

enter meiosis in response to factors produced by the rete ovarii[3,5,6]. Once oogonia enter meiosis, they are redefined as primary oocytes and remain arrested in the diplotene stage of meiotic prophase I (32 μm in diameter) until stimulated to resume meiosis during the ovulatory process of a woman's ovarian cycle[4,7].

Follicular development begins during the sixteenth week of gestation when spindle-shaped pre-granulosa cells envelope the primary oocyte in a single layer[7]. An outer layer of collagen, known as the basal lamina, forms around the pre-granulosa cell layer[7]. The entire primitive structure is defined as the primordial follicle. Although some primordial follicles will begin growing immediately, the majority remain in a resting state until they either degenerate, or enter the growing phase[6]. The population of arrested primordial follicles constitutes the ovarian follicular reserve or resting pool of follicles (approximately 2.7×10^5 to 4.7×10^5 follicles at menarche) that provides a woman with her complete follicle complement for her entire reproductive life[3].

1.1.2 The Population of Pre-antral Follicles

The population of non-growing follicles is comprised of four types of follicles: (i) primordial follicles (35.0 μm in diameter) are composed of a primary oocyte surrounded by flattened granulosa cells, (ii) intermediary follicles (38.0 μm in diameter) are composed of a primary oocyte surrounded by flattened and cuboidal granulosa cells, (iii) primary follicles (46.0 μm in diameter) are composed of a primary oocyte and a single layer of cuboidal granulosa cells and (iv) secondary follicles (77.0 μm in diameter) are composed of a primary oocyte surrounded by more than one layer of cuboidal granulosa cells[7,8]. The majority of follicles residing in the non-growing pool are primordial and intermediary follicles[10]. True follicular growth does not occur until the germinal vesicle reaches 19 μm

in diameter during the secondary follicle stage of development[9]. Approximately 1000 follicles are lost from the resting pool each month until the age of 35 after which the rate of loss increases such that by menopause 100 to 1000 resting follicles remain within the ovaries[11-14]. Follicular growth and development from the primordial follicle to ovulation is illustrated in Figure 1.1. Morphologic changes associated with each stage of development are summarized in Table 1.1

1.1.3 Initiation of Follicular Growth

The exact mechanisms and factors responsible for initiation of follicular development are poorly understood; however, the process is considered to be multi-phasic and regulated by biological molecules that act on the oocyte and/or granulosa cells[6]. It is unclear if initiation of follicular growth is due to the release from inhibitory factors or stimulation by activating factors. Available evidence suggests that a balance between inhibiting and activating factors produced by the pre-antral follicles and/or factors present in the ovarian microenvironment are responsible for initiating follicular growth.

A number of factors have been implicated in initiating follicular growth, including; epidermal growth factor, insulin-like growth factor, transforming growth factor, and fibroblast growth factor as well as factors which stimulate cyclic adenosine monophosphate (cAMP) production including vasoactive intestinal peptides, pituitary adenylate cyclase activating peptide and neurotropins such as nerve growth factor brain-derived neurotrophic factor and neutrophin-4[6,15]. Conversely, Anti-Müllerian hormone and somatostain are suggested to inhibit the initiation of follicular growth[6]. The rate at which resting follicles are stimulated to grow is reported to be directly proportional to the number

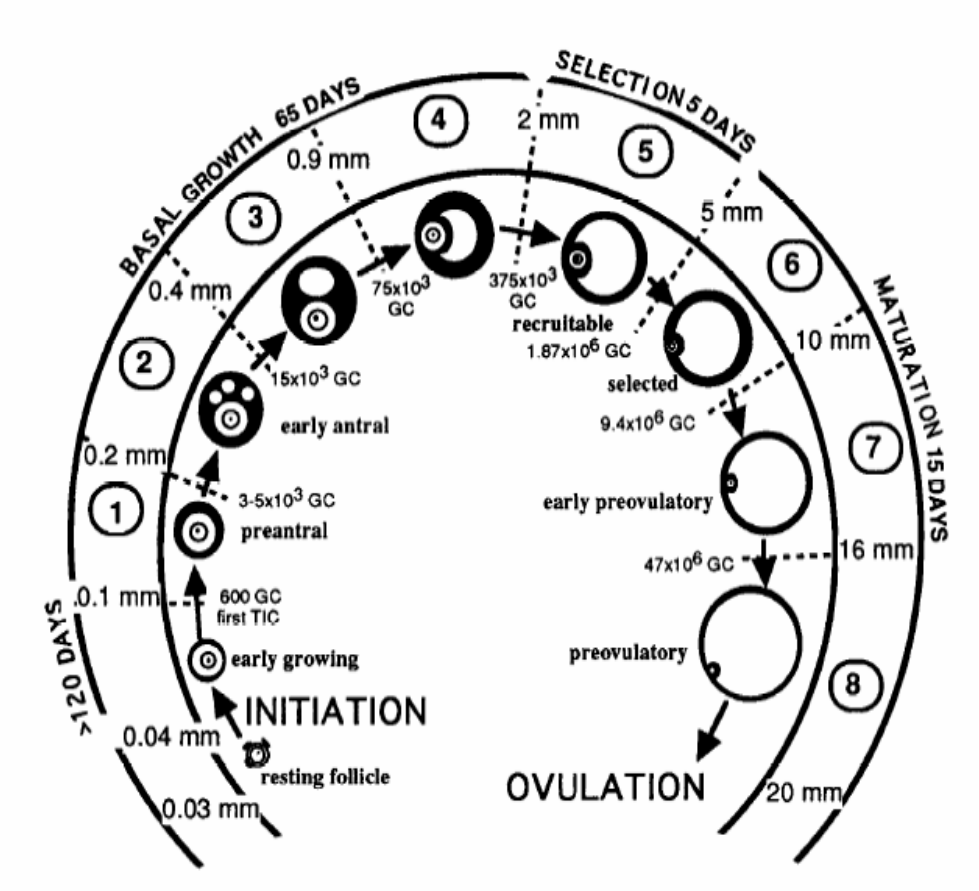

Figure 1.1: Classification of follicles in the human ovary (From Gougeon, 1996)

Table 1.1: Morphological changes associated with each stage of follicular development (From Gougeon, 1996)

Stage of Development	Mean Follicle Diameter	Morphological Characteristics
Primordial Follicle	35 μm	• Single layer of flattened granulosa cells
Intermediary Follicle	38 μm	• Single layer of flattened and cuboidal granulosa cells
Primary Follicle	46 μm	• Single layer of cuboidal granulosa cells zona pellucida
Secondary Follicle	77 μm	• >1 layer of cuboidal granulosa cells • FSH, estrogen and androgen receptors are expressed in granulosa cells • LH receptors expressed on theca interna • Theca interna and externa begin to differentiate • Differentiation of follicular vascular and lymphatic circulatory systems
Class 1	0.1 - 0.2 mm	• No antrum • Two-cell-two gonadotropin hormone synthesis occurs
Class 2	0.2 - 0.4 mm	• Cumulus oophorus develops • Antrum development commences • Call Exner bodies form in granulosa cells
Class 3	0.4 - 0.9 mm	• Small antrum
Class 4	0.9 - 2.0 mm	• Medium antrum
Class 5	2.0 - 5.0 mm	• Medium antrum • Recruitment occurs
Class 6	5.0 - 10.0 mm	• Medium antrum • Selection occurs • Aromatase activity detected in granulosa cells
Class 7	10 - 16 mm	• Large antrum
Class 8	>16 mm	• Ovulation

of follicles remaining in the resting pool and appears to be affected by testosterone, age, nutrition, activity of the thymus gland and opioid peptides[16,17].

1.1.4 Preantral Growth Phase or Basal Follicular Growth

Follicles begin to enter the preantral growth phase during fetal development and do not stop entering this phase until menopause[6,7]. The preantral growth phase begins with the progression of a primordial follicle (35.0 μm) into a primary follicle (46.0 μm) through the differentiation of spindle-shaped granulosa cells into cuboidal cells. During this transition a mucopolysaccharide layer is synthesized and secreted by the granulosa cells and/ or primary oocyte to form a zona pellucida around the oocyte[18] (Table 1.1). Gap junctions traverse the zona pellucida between the granulosa cell layer and the oocyte to form a communication system between the cell types[4]. Adherent and gap junctions also develop between adjacent granulosa cells forming a functional synctium that is essential to maintaining communication within the avascular granulosa cell layer[6].

Primary follicles are redefined as secondary follicles (≤120 μm) when at least two layers of granulosa cells surround the primary oocyte and the ovarian stroma cells align parallel next to the basal lamina[10] (Table 1.1). Granulosa cells, of secondary follicles, express follicle stimulating hormone (FSH), estrogen and androgen receptors; however, these receptors are likely not fully functional[4,19]. The surrounding stromal tissue differentiates into two theca layers, the inner theca interna and the outer theca externa, as the secondary follicle increases in diameter. The theca interna, in secondary follicles, is composed of fibroblast-precursor cells containing luteinizing hormone (LH) receptors and a blood supply that contains one or two arterioles which terminate in a wreath-like fashion of capillaries adjacent to the basal lamina of the granulosa cells[20,21]. The theca externa, in

secondary follicles, is composed of undifferentiated theca cells[4,22]. The secondary follicle may continue growing or undergo atresia:, with the latter occurring in most cases (>99%)[23].

1.1.5 Tonic Growth Phase

The tonic growth phase is associated with a 15-fold increase in diameter through the conversion of a Class 1 (pre-antral) follicle (0.12-0.20 mm in diameter) into a Class 5 follicle (2.0-5.0 mm in diameter)[4]. Follicular growth and development during this phase are largely due to antral cavity formation, beginning during Class 2, and proliferation of granulosa and theca cells. Antral cavity formation sustains follicular growth and development by providing a unique hormone micro-environment to the follicle and oocyte[16]. The antral fluid is composed of protein-bound and free sex steroids, proteins, proteoglycans and electrolytes[24]. The complete growth trajectory of a follicle from Class 1 to Class 8 is diagrammed in Figure 1.2.

Entry into the tonic growth phase is marked by the transition of a secondary follicle into a Class 1 follicle[4, 25]. The transition into Class 1 occurs when at least one fibroblast-like stromal cell of the theca interna differentiates into an epithelioid cell[26]. By Class 1 of follicular development, the follicle is capable of producing estrogen via the two-cell two gonadotropin relationship between theca and granulosa cells (Table 1.1). Theca cells are stimulated by LH to produce androgens which cross the basal lamina into granulosa cells, where the androgens are aromatized by enzymes to estrogens and subsequently released into the follicular fluid, the intrafollicular space or the systemic circulation[4,10].

It takes approximately 25 days for Class 1 or pre-antral (0.12-0.2 mm in diameter) follicles to develop into Class 2 or early-antral follicles (0.2-0.4 mm in diameter; Figure 1.2).

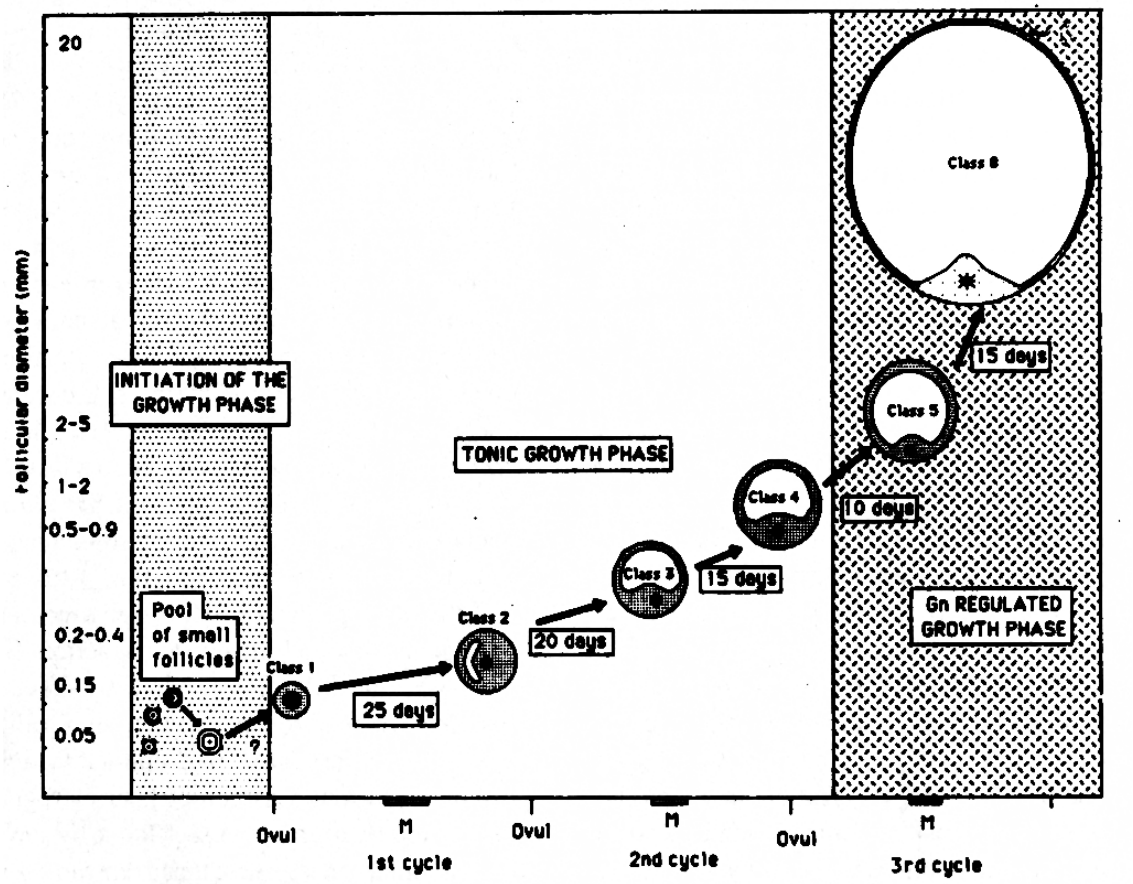

Figure 1.2: Complete growth trajectory: conversion of a Class 1 follicle into a Class 8 follicle. Gn, Gonadotropin. (From Adashi *et al.*, 1996).

If Class 1 follicular development began during the luteal phase than entry of the follicle into Class 2 would generally occur between days 11 and 14 of the late follicular phase of the following cycle[4,25]. Gougeon suggested that increased levels of FSH during the late follicular phase are likely responsible for a peak in the population of Class 2 follicles[10]. Class 2 is marked by differentiation of granulosa cells and the formation of small antral cavities (0.04 mm in diameter) which eventually coalesce to form a single large antral cavity[10] (Table 1.1). Follicle stimulating hormone is suggested to induce antrum formation[10,27,28]. Antrum formation subsequently results in the formation of the cumulus oophorus which is a stalk-like projection of granulosa cells surrounding the oocyte that extends into the center of the antral cavity[22]. Call Exner Bodies develop in tandem with the antrum, however the role of these structures is unknown.

Class 2 follicles proceed to Class 3 (0.4-0.9 mm in diameter) during the end of the luteal phase[4,25] (Figure 1.2). Transition into Class 3 is marked by an increase in follicle diameter due to an increase in the mitotic index of granulosa cells[25]. Approximately 15 days later, during the follicular phase of the following cycle, Class 3 follicles convert to Class 4 follicles (0.9-2.0 mm in diameter)[4,25] (Figure 1.2). Class 4 follicles convert to Class 5 follicles (2.0-5.0 mm in diameter) ten days later during the late luteal phase[4,25] (Figure 1.2). Transformation from Class 1 to Class 5 takes approximately two cycles in total.

The role of gonadotropins in early follicular development remains controversial[29]. For example, Hodgen (1989) suggests that follicular development from primordial follicle to primary follicle is gonadotropin independent and subsequent development to a secondary follicle requires gonadotropins, whereas Fauser (1997) and Hillier (1994) suggest follicular growth to Class 2 occurs in the absence of gonadotropins. Continual growth and

development of follicles to Class 5 is observed throughout life, including during conditions when endogenous gonadotropins are significantly decreased such as: pre-puberty, pregnancy, during OC use, and in women without gonadotropin secretion due to hypothalamic-pituitary failure or hypophysectomy, suggesting that follicles <2 mm in diameter require only basal levels of gonadotropins for growth and development[10,20,21,30-35]. Regardless of when follicles become dependent on gonadotropins, basal levels are required to sustain growth and development to Class 5. Circulating levels of FSH are correlated to follicle quality of Class 5 follicles, therefore, it is suggested that follicles become dependent on cyclic changes when they reach Class 5 and remain dependent on cyclic gonadotropin changes until ovulation during Class 8[7,10].

1.1.6 Exponential Growth Phase

During the exponential growth phase the follicle progresses from Class 5 to Class 8 (Figure 1.2). This phase takes approximately 20 days, with each stage lasting five days[25]. The predominant events of the exponential growth phase include, but are not limited to, recruitment and selection of a dominant follicle followed by ovulation of a pre-ovulatory follicle[7].

1.1.7 Recruitment

The term 'recruitment' is used throughout literature to define two separate events. "Initial recruitment" refers to the entry of primordial follicles into the growing phase and occurs continuously throughout reproductive life beginning during fetal development and continuing to menopause. "Cyclic recruitment" refers to the process whereby a small cohort of antral follicles is rescued from atresia via FSH-induced growth. Although both

definitions outline a process of recruitment, for the purposes of this review the term recruitment refers to "cyclic recruitment".

The demise of the corpus luteum during the late luteal phase results in remarkable changes within the ovary including, but not limited to, a decrease in progesterone, estradiol and inhibin A allowing for an increase in FSH approximately 12 days after the preceding midcycle LH surge[10,36,37]. Follicle stimulating hormone must increase 10 to 30% above basal levels in order to rescue a cohort of 1 to 15 Class 5 follicles, at similar but not identical stages of development, from atresia[10,26,38-41] (Table 1.1). Although all follicles are suspected to have equal potential for maturation, only the follicles at advanced stages of development during the intercycle gonadotropin rise are recruited for further follicular development[22]. Prolonged increases of FSH above threshold or extremely high levels of FSH result in higher than average numbers of follicles being recruited, such as during hyperstimulation protocols of IVF treatment[22]. The FSH threshold varies between individuals, but appears to remain relatively constant within individuals.

The recruited cohort is comprised of follicles that left the resting pool, by chance, at approximately similar times several months earlier[22]. The best knowledge, to date, suggests that the continual entry of primordial follicles into the growth phase ensures a cohort of follicles are developmentally capable of responding to the intercycle increase in FSH[10]; however, the recent finding of follicular waves suggests that two to three cohorts of follicles are recruited at separate times during a single ovarian cycle[42,43]. Recruitment of a cohort of follicles is preceded by an increase in FSH, suggesting that FSH plays a large role in follicle recruitment[42]. Further research is needed to determine the exact physiologic mechanisms and biologic factors responsible for follicle recruitment in humans.

The largest follicles present at the beginning of the follicular phase possess the greatest number of FSH receptors, which in turn provides these follicles with a low FSH threshold[38]. Large follicles have a competitive advantage over smaller follicles because large follicles can respond to lower levels of FSH earlier than smaller follicles. Responses to increasing FSH include an increase in granulosa cell mitosis and glycosaminoglycan production (increases the volume of the antral cavity), induction of gene expression of granulosa cell FSH receptors and nonsteroidal growth factors including the insulin like growth factor system, fibroblast growth factor, transforming growth factor-β, activin and the aromatase enzyme complex; however, this complex is not fully engaged until follicles exceed 10 mm in diameter[10,44-46].

1.1.8 Selection

Selection is the process by which one follicle from the recruited cohort is physiologically "selected" to attain "dominance" and continue development to ovulation, with the remaining non-selected follicles of the cohort undergoing atresia. The process of selection ensures the species specific ovulatory quota of follicles develop[47]. Although humans develop two to three follicular waves per ovarian cycle, only the wave that initiates development during the late luteal phase typically produces one pre-ovulatory sized follicle resulting in subsequent ovulation[42,43].

Selection of the pre-ovulatory follicle from the cohort of recruited follicles occurs between days 5 to 7 of the menstrual cycle[42]. However, the diameter at which selection occurs is debatable; Gougeon (1989) suggested follicles are selected between 5.5 to 8.2 mm in diameter (Class 6), Chikazawa *et al.* (1986) reported follicles are selected at 4.7 ± 0.2 mm in diameter (Class 5); whereas, Macklon and Fauser, (1999) Pache *et al.*, (1990) van

Santbrink *et al.,* (1995) and Baerwald *et al.* (2003) suggested follicles are selected at approximately 10 mm in diameter (Class 6)[7,29,43,46,48,49] (Table 1.1). Follicle diameter at the beginning of the follicular phase ranges from 4 to 8 mm in diameter and few morphological differences are observed between follicles <8 mm. Taken together, this data is interpreted to mean that follicle selection does not occur before follicles reach 8 mm in diameter[50].

At the time of selection, the selected follicle can be differentiated from the other healthy follicles of the cohort by it its larger size, higher mitotic index, detectable amounts of FSH and an appreciable amount of estrogen in the follicular fluid[50,51]. Gougeon and Lefevre suggested that the largest follicle present at the beginning of the follicular phase is selected for dominance, because the selected follicle typically has the highest mitotic index of all follicles within the cohort, thus preventing smaller follicles from making up the growth delay[50]. Once selected the follicle is defined as a "dominant" follicle and can be differentiated from the other follicles due to its larger size, morphology, increased secretion of estrogen and inhibin and complete induction of aromatase[46,49,52-55]. The mechanisms responsible for selection of a single dominant follicle have not been fully elucidated; however, researchers suggest that decreasing levels of FSH in concert with increasing levels of estrogen are the primary regulators of selection[56,57].

1.1.9 Maturation of the Dominant Follicle Destined to Ovulate

Once selected the dominant follicle continues increasing in size through accumulation of follicular fluid and proliferation of granulosa and theca cells until it reaches Class 7 (8-16 mm in diameter) followed by Class 8 (18-20 mm in diameter)[10] (Figure 1.1). The dominant follicle undergoes its most profound transformation during the

last 10-15 days of development[7]. The final two weeks of follicular growth are associated with a 12 mm increase in diameter[7]. To put this into perspective, the follicle takes more than 330 days to reach 6 mm in diameter from initiation of growth but takes 10 days to increase its size by 12 mm in diameter.

Soon after selection, the dominant follicle transforms from an androgen producing structure to an estrogen producing structure; therefore, the androgen:estrogen ratio of the follicular fluid decreases due to increased aromatase expression and subsequent estrogen production[10]. Systemic estrogen levels begin increasing approximately five days before the mid-cycle gonadotropin surge[42,58,59]. Ultrasonographic visualization of the "dominant" follicle coincides with the initial increase in plasma estradiol concentrations[49]. The majority of systemic estrogen present during the mid-to-late follicular phase is produced by the dominant follicle as demonstrated by dramatically elevated levels of estrogen in the venous effluent of the ovary containing the dominant follicle, and a significant correlation between increasing concentrations of serum estradiol and growth of the dominant follicle[49,58]. The increasing levels of estrogen synergistically act with FSH to further stimulate granulosa cell proliferation and stimulate the effects cAMP in an autocrine fashion[44,60].

Continued dominant follicle development is maintained by autocrine and paracrine growth factors that stimulate increased vasculature of the dominant follicle and FSH responsiveness, which allows the follicle to sustain growth and development in the face of waning levels of FSH[61]. Further growth is supported via the expression of LH receptors during the mid-to-late follicular phase, at a time when LH concentrations begin to increase[62,63]. Luteinizing hormone receptors are predominantly expressed on the mural

granulosa cells adjacent to the basal lamina, placing them in close proximity to the vascular supply of the theca interna[63]. By day nine, the vasculature of the theca interna within the dominant follicle is two times that of any other follicle providing preferential delivery of gonadotropins to the newly formed LH receptors of the mural granulosa cells[64]. Binding of FSH and LH to their respective receptors produces similar cellular responses since both receptors are coupled to a cAMP signaling system[65]. Thus, increasing LH concentrations during the mid-follicular phase sustains dominant follicle growth and development by stimulating similar cellular pathways to that of FSH binding to FSH receptors. Furthermore, FSH and LH receptors have an additive affect when the receptors are not saturated, thus providing continued gonadotropin support in the face of declining FSH and increasing LH levels[59]. Zeleznik suggests the decreased dependency of the dominant follicle on FSH may be the result of the acquisition of LH receptors on granulosa cells[59].

The dominant follicle is suggested to cause atresia of the subordinate follicles by one of two mechanism: (i) the dominant follicle secretes a factor that directly inhibits subordinate follicular growth and development or (ii) the dominant follicle indirectly causes atresia of the subordinate follicles though negative feedback mechanisms[66]. The dominant follicle produces increasing levels of estrogen and inhibins which suppresses FSH release from the anterior pituitary thus withdrawing the gonadotropin support needed to sustain follicular development of subordinate follicles[36,67]. The lack of gonadotropin support results in atresia of all but the dominant follicle[61].

Dominant follicle growth is associated with granulosa cell mitosis and antrum fluid accumulation[26]. The theca layer undergoes increased vascularization and hypertrophy until ovulation[7]. During Class 8 the basement membrane becomes less visible, mitosis of the

granulosa cells dramatically declines and the granulosa cells and their nuclei increase in diameter[52]. Upon reaching Class 8, the follicle is considered mature and will ovulate if stimulated by a gonadotropin surge; if a gonadotropin surge does not occur than the follicle regresses.

1.1.10 Ovulation

Ovulation begins at the time of the gonadotropin surge and involves local alterations in and around the 15 to 28 mm graafian follicle until the follicle ruptures releasing the fertilizable oocyte[10,42,43,52,68]. The complex process of ovulation takes approximately 36 to 38 hours in humans and involves biochemical, morphological and physiological changes, all of which have not been completely elucidated[68].

The mid-cycle estradiol peak initiates organization of the granulosa cells, followed by disorganization of the granulosa layer during preluteinization of the follicle[7,52]. The onset of the mid-cycle gonadotropin surge stimulates the oocyte (which is arrested in dictyate stage of prophase I) to complete its first meiotic division and become arrested in prophase II as a secondary oocyte[26]. An LH threshold must be reached and maintained for 14 to 27 hours for full maturation of the oocyte to occur[69]. Oocyte Maturation Inhibitor is suggested to inhibit oocyte maturation before the mid-cycle gonadotropin surge. The mid-cycle gonadotropin surge suppresses the action of oocyte maturation inhibitor, allowing meiosis to proceed to prophase II[71]. The LH surge is terminated by negative feedback of progesterone and 17β-hydroxyprogesterone on the hypothalamo-pituitary-ovarian axis[10,70].

The midcycle gonadotropin surge also suppresses granulosa cell mitosis, stimulates large lacunae formation within the granulosa cells, stimulates a decrease in estrogen and

androgen concentrations in follicular fluid and stimulates granulosa cell lutienization as well as synthesis of prostaglandin E_2 and $F_{2\alpha}$ and other eicosanoids[16,72]. The exact role of prostaglandin E_2 and $F_{2\alpha}$ is unknown[71]. Further, the LH surge activates a number of intraovarian regulatory systems (Figure 1.3) that act synergistically to induce vascular changes and degrade the extracellular matrix through the dissociation of fibroblasts and collagen of the theca externa and tunica albuginea on the apex of the follicle[68,68]. The vascular changes stabilize or increase the liquid pressure within the follicle to avoid collapse of the follicle when the follicle is leaking fluid followed by rupture[73].

Few morphological changes occur in the follicle wall until a few hours before ovulation[74]. Hours before ovulation blood vessels from the theca interna invade the, once avascular, granulose layer. Extra-vasiated blood cells are observed within the extracellular spaces around the fibroblasts[75]. Follicular fluid volume increases immediately before ovulation without any significant changes in pressure[70]. Minutes before follicular rupture, a stigma forms in the apical region of the follicle wall due to thinning of the collagen layers and degradation of the connective tissue elements. Immediately before follicle rupture, the surface epithelium becomes necrotic and sloughs off the apex of the follicle[76].

Ovulation is often likened to an inflammatory event due to the presence and involvement of inflammatory cells. The LH surge stimulates the production of interleukin-1 and chemokines which attract leukocytes, neutrophils and macrophages to work together to aid in the degradation of the extra cellular matrix[68].

Two theories of ovulation - the pressure theory and the smooth muscle theory - predominated during the first half of the 20th century. The pressure theory states an increase in intrafollicular pressure due to vasodilatation and ruptured blood vessels,

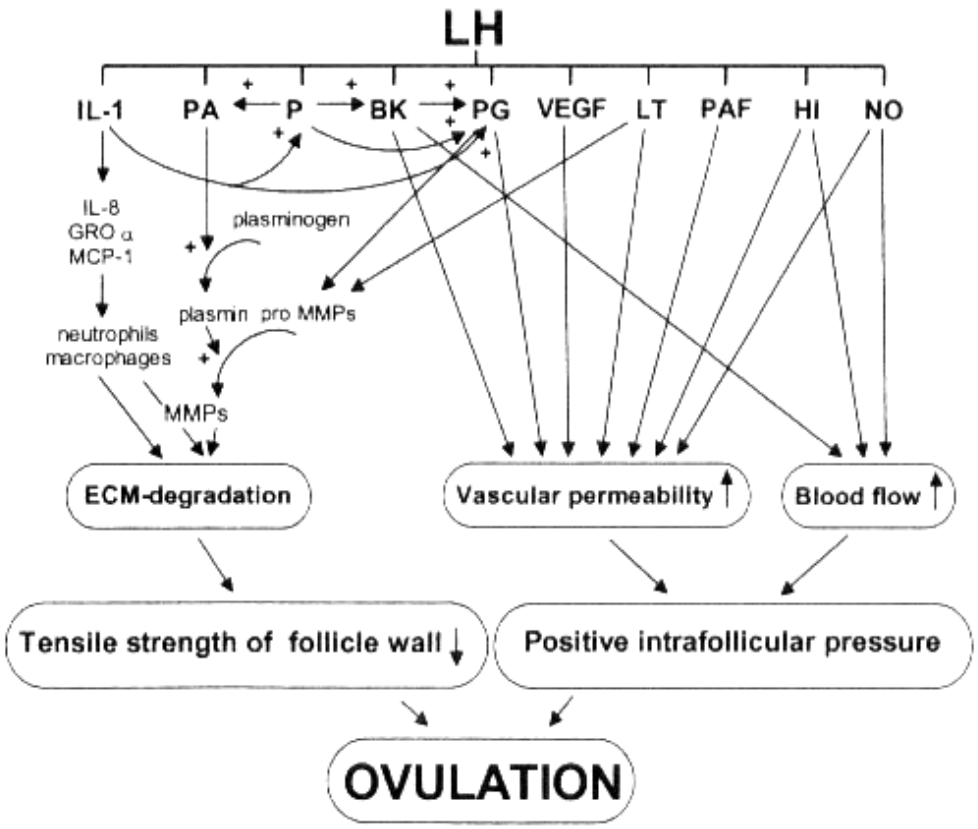

Figure 1.3: Overview of LH-induced pathways in the ovulatory cascade. LH induces synthesis of mediators that promote either degradation of the extracellular matrix (ECM) and/or vascular changes (permeability increase, blood flow increase). These changes lead to a decreased tensile strength and a positive intrafollicular pressure. *BK,* Bradkinin; GRO, growth regulating onocogene; *HI,* histamine; *IL*, interleukin; *LT* , leukotriene; *MCP*, monocyte chemotactic protein; *MMP*, matrix metalloproteinase; *NO*, nitric oxide; *P*, progesterone: P*A*, plasminogen activator; *PAF*, platelet activating factor; *PG*, prostaglandin; *VEGF*, vascular endothelial growth factor. (from Branstrom, 2004).

increases in intrafollicular osmotic pressure and increases in follicular fluid volume cause follicular rupture[77-79]. However, researchers have disproved the pressure theory by reporting that intrafollicular pressure does not increase during the few hours leading to ovulation but remains between 15 - 20 mm Hg[76,80].

The smooth muscle theory is defined as a contraction in smooth muscle contained in the follicular wall, inducing ovulation[74]. In the early 1900's, researchers reported the presence of smooth muscle cells in the follicular wall, however the muscle could not be chemically or electrically stimulated to induce ovulation[81,82]. In 1919, Corner suggested the "smooth muscle cells" of the follicle wall were actually long spindle-shaped fibroblasts enveloped in collagenous fibrils[83]. Technological advances in polarization microscopy supported this report when Claesson revealed that the birefringence characteristic of muscle cells was not present in follicle walls but birefringence typical of connective tissue was[84]. To date, there is no convincing evidence to suggest ovarian contractions are essential to ovulation. However, rhythmic ovarian contractions are occasionally observed near the time of ovulation, suggesting smooth muscle contractions may play a role in ovulation[74].

The proteolytic enzyme theory is the currently accepted theory for the mechanism of ovulation[74]. This theory states that a small portion of the follicular wall, known as the apex, is digested by enzymes until the distensibility of the wall decreases to the point that it can no longer sustain the constant intrafollicular pressure of 15 to 20 mm Hg, resulting in rupture of the follicular wall to release the mature oocyte. Progesterone, FSH and LH induce synthesis of mediators and activate proteolytic enzymes in an orderly sequence to digest the collagen within the follicle wall and to decrease the distensibility of the apex

(Figure 1.3)[85]. Granulosa and theca interna cells produce plasminogen activator in response to the mid-cycle gonadotropin surge. Plaminogen activator activates plasminogen to plasmin, in the follicular fluid. Plasmin subsequently generates collagenases which digest collagen at the apex of the follicular wall[70]. The changes that occur at the ovulatory site have been described as a pathophysiological process because the dense collagen layer of the tunica albuginea and theca externa is disrupted[86]. Once the stigma of the follicular wall ruptures, the follicular fluid and oocyte evacuate the antral cavity.

1.1.11 Duration of Human Folliculogenesis

Follicular development from primordial follicle to ovulation takes approximately 12 menstrual cycles[26] (Figure 1.1). Initially, follicular development is a slow process. It is estimated that a primordial follicle takes > 150 days to develop into a primary follicle and >120 days for the transition from the primary to secondary stage of follicle development[26]. The speed at which follicles progress through each stage of development increases dramatically once the secondary follicle develops. It takes 65 days for a secondary follicle to progress to Class 5 , five more days are needed for selection to occur, followed by 15 days of maturation to the pre-ovulatory sized follicle[10]. Gougeon suggested that follicular development from Class 1 (pre-antral) to Class 8 (pre-ovulatory) takes approximately 85 days[10] (Figure 1.2).

1.1.12 Summary

The knowledge of the basic biology and physiologic processes underlying reproductive function, in women, has grown exponentially since de Graaf defined the ovarian follicle and oocyte[87]. However, we do not yet completely understand the

complexity, and intricacies of the ovary and ovarian follicle. New dynamic, interdisciplinary research techniques are providing researchers the tools to uncover the mysteries of reproductive organs in women. For example, follicular wave development was recently documented in humans[42,43]. Further research is needed to determine the physiologic and endocrine processes responsible for initiation and maintenance of waves. New research findings regarding the biology and physiologic mechanisms associated with reproductive functioning will allow us to develop safer, more efficacious hormonal contraception and infertility treatments.

1.2 Ultrasonographic Imaging of the Ovaries and Uterus

1.2.1 Overview of Ultrasound Imaging

The introduction of gray-scale ultrasonography has revolutionized how clinicians and researchers examine ovarian follicular development. Before the introduction of ultrasonography, tissue samples were examined using histological techniques that are time consuming and result in destruction of the tissue as well as only providing information about a single point in time. Further, histological studies are ethically difficult in live humans. Ultrasound provides a direct, non-invasive and atraumatic tool to observe the dramatic morphological changes that occur within the reproductive organs without interfering with the physiological processes of these organs while minimizing discomfort to the individual[88,89]. Further ultrasound can be used to detect minute differences in morphology and physiologic state or reproductive organs thus eliminating the need for surgery[90].

Ultrasonography is based on the ability of tissue structures to reflect high frequency sound waves[91,92]. The ultrasound machine is composed of a transducer, a computer processor and a monitor screen. The transducer contains piezoelectric crystals that expand and contract to produce a high frequency sound wave when stimulated by an electrical current. The sound wave propagates into the tissue until it is reflected back towards the transducer after collision with a tissue interface. The degree of wave reflection is associated with tissue density and is represented by shades of gray ranging from black to white. Dense structures (e.g. cervix) reflect the majority of sound waves and therefore appear white or hyperechoic whereas fluid-filled structures appear black or hypoechoic because they propagate sound waves[93]. Waves which are not reflected back to the transducer are scattered, refracted or absorbed into the tissue[91]. The reflected sound wave returns to the piezoelectric crystal within the transducer causing the piezoelectric crystal to compress followed by the subsequent generation of an electrical signal. The electric signal is processed for display in the receiver and then stored as binary digits or "bits" in a digital scan converter.

Ultrasound images are produced when the stored information from the digital scan converter is converted to an electrical signal. The electrical signal causes an electron gun to expel an electron beam onto a phosphor screen resulting in the emission of light. The horizontal movement of the electron beam forms raster lines. Vertical scanning lines are subsequently produced when the electron beam of the raster line turns on and off[91]. The intersection of a vertical scanning line with a raster line produces a pixel. Each pixel represents a discrete tissue reflector. Pixel brightness is displayed as one of 256 shades of grey (black = 0 and white = 255) and is related to the amplitude of the reflected signal or tissue density[91]. Currently, ultrasound machines usually display images of 480 x 640 pixels.

Each ultrasound image is created by the processing and displaying of numerous sound beams which have been focused to increase lateral resolution and optimize viewing[92].

The primary reason for performing transvaginal ultrasound is to monitor ovarian follicular maturation and ovulation during natural menstrual cycles and controlled ovarian hypserstimulation and ovulation induction during infertility treatments[93]. Current infertility investigations utilize transvaginal ultrasonography to track ovarian follicular development and ovulation, aid in invasive ultrasound guided procedures, detect early pregnancy, examine pathologies and assess the uterus and ovaries for anomalies such as cysts, tumors, fibroids and endometriomas[90]. The uterus and ovaries are easily examined using B-mode (brightness mode) transvaginal ultrasound, particularly due to the close proximity of the organs to the transducer during ultrasound examination.

1.2.2 Ultrasonographic Characteristics of the Ovaries

The ovaries are visualized as a pair of almond shaped structures (3 cm x 2 cm x 1 cm) that lie on either side of the uterus[93]. They are typically located posterior to the broad ligament, anterior to the iliac vessels and ureters and inferior to the uterine tube[93]. The ovaries are secured in the peritoneal space by the ovarian and suspensory ligaments[93]. Each ovary is supplied with blood by an ovarian branch of the adnexal branch of the uterine artery[93]. Ovaries have a coarse low-level echo pattern which is interspersed with anechoic areas representing ovarian follicles, cysts or corpora lutea[93]. Mature ovarian follicles measure approximately 20 to 24 mm in diameter[93]. Ovarian follicles appear as round anechoic spheres surrounded by a thin hyperechoic follicle wall[93].

Morphological changes associated with impending ovulation can easily be detected using two-dimensional transvaginal ultrasonography. The cumulus-oophorus expands and can be observed in up to 80% of follicles 12 to 24 hours before ovulation[89]. The apex of the follicle wall thins and the deep internal portion of the follicle wall thickens beginning approximately three hours before ovulation[88]. The follicle wall becomes less echoic (i.e., darker) as the tissue layer expands and becomes loosely organized and vascularization increases in preparation for ovulation[88]. The increased vascularization can be visualized using Doppler ultrasonography[93]. The follicle flattens out or becomes irregularly shaped in association with expansion and crenelation of the follicle wall[88]. Approximately 15 to 20 minutes before ovulation a stigma forms at the follicular apex followed by the rupture of the follicle and the rapid release of approximately 50% of the follicular fluid within 15 seconds[89,3]. Complete evacuation of follicular fluid ranges from six seconds to >18 minutes in follicles that completely expel their follicular fluid[89]. The point of follicular rupture can be observed for up to a week following ovulation[90].

Corpora lutea are observed in the ovarian location where the follicle was formerly observed immediately following follicle collapse using transvaginal ultrasonography[89,94,95]. The wall of the corpus luteum becomes highly vascularized and thickens and folds during lutienization[94]. The corpus luteum is typically described as having mid-range echogenicity[96]; the outer surface (i.e., luteal wall) has a more echogenic echotexture than the relatively hypoechogenic central portion which is either cystic (i.e., hypoechoic) or solid (i.e., hyperechoic) in structure[94]. Approximately 60% of women hemorrhage into the developing corpus luteum following ovulation resulting in the formation of a corpus hemorrhagicum[97]. If pregnancy does not occur, the corpus luteum begins to regress two to five days before the following menstrual period begins[94]. Regression of the corpus luteum

involves a decrease in cell size and degenerative changes in the nuclei of luteinized cells as well as fatty degeneration[94]. The vascular supply decreases and fibrosis is observed[94]. The cells of the corpus luteum ultimately degenerate into a amorphous hyaline mass, held together by strands of connective tissue; this structure is defined as a corpus albicans[94]. Complete regression of the corpus albicans occurs over the following few menstrual cycles[94]

Ovulatory failure can result in the formation of ovarian cysts, luteinized unruptured follicles and hemorrhagic anovulatory follicles[90]. Anovulatory follicles posses characteristic echotexture that can be used to assess follicular health. Anovulatory follicles generally have thin highly echoic (i.e., bright), clearly demarcated follicular walls of uniform thickness with an echotexture that is characteristic of a cohesive tissue layer[88]. However, echoic structures protruding into the antral cavity as well as free floating in the antral cavity are commonly observed[90]. The increased gray-scale values of the follicular wall may be associated with decreases in vascularity[88]. The cumulus-oocyte complex is not evident in anovulatory follicles[88]. Anovulatory follicles do not form a stigma nor do they change shape[88]. These follicles generally develop past normal ovulatory diameter (i.e., >25 mm) remain static for one to several days and then regress without lutienization[90]. Luteinized unruptured follicles generally have thick follicular walls with hazy indistinct borders between the antrum/wall interface. These follicle walls have similar echotexture to luteinized tissue[90]. Luteinized unruptured follicles typically regress in a similar time period to normal corpus luteum regression[90]. Hemorrhagic anovulatory follicles are characterized by hemorrhage into the antral cavity followed by the formation of fibrin network within the antrum[90].

1.2.3 Ultrasonographic Characteristics of the Uterus

The uterus is a pear-shaped, hollow, muscular organ with thick walls and an average length of 7.5 cm, a depth of 2.5 cm and a width of 5 cm at its widest point[93]. The uterus lies posterior to the urinary bladder and anterior to the rectum and has a homogenous echotexture with echoes of medium intensity[90,93]. The uterine cavity appears as a transverse slit in sagittal section and a triangle in transverse section with the opposing layers of the endometrium appearing as an echogenic line in the central uterine cavity[93]. The echotexture of the endometrium varies throughout the menstrual cycle and is related to the circulating concentrations of estrogen and progesterone[90]. The echotexture of the myometrium does not change during the menstrual cycle[90].

1.2.4 Computer Assisted Image Analysis

Currently, researchers are developing new techniques to evaluate ultrasound image attributes to elucidate the physiologic status of ovarian follicles and corpora lutea with a single non-invasive ultrasound exam[98]. The development of non-invasive techniques will result in immediate improvement in the clinical management of infertility patients[98].

Each ultrasound image is composed of thousands of discrete picture elements or pixels[99]. The human eye can perceive smooth transitions in shades of gray, but can only distinguish between 18 to 20 shades of gray, therefore, most of the gray-scale information contained within an ultrasound image can not be evaluated by the human eye[100]. Ultrasound image analysis overcomes subjective human evaluation of ultrasound images by using a series of processing steps and complex computer algorithms to quantify echotextural characteristics within the image[98]. The image attributes can then be correlated

to physiologic status of ovarian structures. The validity of the image analysis technique has been verified through correlation of ultrasound image attributes with histologic attributes in animal models[101-103]. The ultimate goal of image analysis techniques is to provide clinicians and researchers with a tool to assess the physiology underlying ovarian follicular growth and development with a single ultrasound exam[99].

Three image attributes are typically quantified during analysis. These include, but are not limited to: (i) numerical pixel value (NPV) defined as the mean pixel gray-scale value of the sampled pixels, (ii) pixel heterogeneity (PH) defined as the standard deviation of the mean gray-scale values of the sampled pixels, and (iii) the area under the curve (AUC) defined as the total sum of the gray-scale values within the sampled area. Spot metering, linear and time-series analyses, and regional surface analyses are techniques utilized to sample ultrasound image attributes of ovarian structures[98,99]. Caution must be taken when performing all analyses to avoid placing sampling lines on image artifacts (e.g., enhanced through transmission, shadowing, specular echoes, refraction and beam width artifacts) that may falsely increase or decrease image attribute values resulting in inaccurate interpretation of the physiologic status of the ovarian structure.

1.2.5 Spot Metering

The simplest imaging technique utilizes a small circle to isolate and quantify pixels in an area of interest[98, 99]. The computer program determines the precise gray-scale value of the pixels within the sample area and provides output in the form of image attributes such as NPV, PH and AUC. This technique is used to compare different areas within the same follicle or the same area of the follicle at different time points[98, 99]. For example portions of the follicle wall or corpus luteum may be quantified to evaluate characteristics of follicular

growth and regression, impending ovulation, atresia and luteal development and regression[98,99,101-105].

1.2.6 Line and Time Series Analysis

A computer-generated line (one or more pixels wide) is drawn across a desired region of the follicle or luteal structure[98,99]. The intensity of each pixel within the line are displayed in a graphical format comparing distance versus pixel value[98,99]. This technique is commonly used to observe changes in the follicle wall of a single follicle or many follicles over time[101,106]. To increase the precision and accuracy of analysis image attributes of many lines at different locations of the follicle wall can be averaged to obtain a single image attribute.

Time series analysis of the same follicle may be done by concatenating individual linear pixel intensity graphs into a single image[98,99]. The composite image undergoes shading algorithms to provide a three-dimensional graph which compares follicle diameter, pixel intensity and time[98]. Evaluation of the surface features can provide clues regarding the physiologic health of the follicle. Echotexture of the follicular wall and antrum can be assessed as the follicle progresses through different stages of growth and regression[99].

1.2.7 Region Analysis

Region analysis is the most promising technique to evaluate the physiological status of a follicle[98]. This technique involves overlaying a pixel-by-pixel mesh onto a selected region of the follicle to produce a three-dimensional image[98,99]. A computer-generated skin can be placed on top of the mesh framework to provide a topographical image. The image can be further enhanced by applying shading algorithms to the topographical image.

Shading highlights detail and enhances perception of surface features such as texture or the portion of the image comprised of echo intensities of interest. Region analysis may develop into a clinical tool to instantly assess ultrasound image attributes associated with the health and viability of a follicle.

1.3 Ovarian Follicular Development during Oral Contraceptive Use

1.3.1 The Introduction of Oral Contraceptives

Contraceptive choices radically changed for women in 1957, when the Food and Drug Administration approved the first menstrual regulator, Enovid™, which contained synthetic estrogen (150 μg mestranol) and synthetic progestin (9.85 mg norethynodrel)[107]. Enovid™ was initially introduced as a menstrual regulator to navigate around secular and religious tenets against the use of birth control. Interestingly, Enovid's™ packaging initially carried a warning that it induced contraceptive properties including, but not limited to, inhibition of ovulation[108]. Soon after Enovid™ was introduced, the number of women experiencing menstrual irregularities and disorders dramatically increased, likely due to the fact that the only legal method of obtaining birth control in many American States was under the guise of menstrual irregularities[108]. In 1960, the Food and Drug Administration approved Enovid™ as an oral contraceptive. The effects Enovid™ had on ovarian function were not completely known. However, it was hypothesized that administration of synthetic progestin created a pseudo-pregnancy that prevented ovulation while the estrogen component provided cycle control to reduce unscheduled bleeding.

1.3.2 Characteristics of Oral Contraceptives

Prior to 1960, non-steroidal contraceptives were the only available contraception to prevent pregnancy. The introduction of Enovid™, revolutionized contraceptive use by providing women with more contraceptive choices. Today, OC are the most commonly used, reversible contraceptive in the world[109]. Combined OC have evolved to include different progestins with lower androgenic activity and mestranol has been replaced with EE; thus, virtually all currently used OC combine ≤35 μg EE with a synthetic progestin[110]. The progestin component of combined OC is thought to directly affect ovarian function, suppress the mid-cycle LH surge and subsequent ovulation, decrease the permeability of the cervical mucus and endometrial receptivity for embryo implantation, and decrease tubal and uterine motility causing a delay in gamete transport[111]. The estrogen component is postulated to directly affect the ovary, suppress FSH release, inhibit ovulation and provide endometrial stability leading to predictable bleeding patterns. The extent of pituitary-ovarian suppression appears to be related to the dose of EE[111]. For example, OC containing ≥30 μg EE suppress follicular development better than an OC containing 20 μg EE. Further research is required to determine the exact mechanisms of action that estrogen and progestin have on the reproductive organs during OC use. Progestin-only OC are also available as a contraceptive choice. However, progestin-only OC have lower efficacy than combined OC so they are typically used for special cases, particularly in lactating women or women with sensitivity to EE.

Conventional OC administration consists of a 28 day cycle comprised of 21 daily active pills followed by a 7 day hormone free interval (HFI). This 28-day administration scheme was implemented to mimic the physiological event of monthly menstruation and

provides only the illusion of natural menstrual cyclicity. The purpose of inducing a menstrual bleed during OC use was to increase the acceptability of OC even though it has no reported physiologic benefit. Currently, researchers are examining the efficacy, acceptability and physiologic effects of replacing the HFI with seven daily active pills, in a continuous dosing scheme[112-132].

Oral contraceptives are generally prescribed in one of three dosing fashions: (i) monophasic, wherein each active pill contains the same concentration of steroids; (ii) biphasic and (iii) triphasic, changes in estrogen concentration are dependent on the OC type wherein the concentration of progestin increases by one (biphasic), or two (triphasic), increments as the cycle progresses from Day 1 to 28.

1.3.3 Follicular Development during Oral Contraceptive Use

Twenty-one studies were reviewed to determine the effect of administering 20 to 40 μg of exogenous EE in combination with various progestins on the hypothalamo-pituitary-ovarian axis[112,133-148,149,152-154]. Follicular development ≥10 mm in diameter was observed in all 17 studies, which utilized ultrasound to examine follicular diameter during OC use[112,133-148]. Follicular development ≥ 10 mm increases the chances of "escape" ovulation during OC use, since follicles are physiologically selected at 10 mm for preferential growth and ovulation during natural cycles[149]. The high incidence of dominant follicle development during compliant OC use, in conjunction with the report of OC failure rates ranging from 16-40% in subgroups of women attending family planning clinics, suggests that the current conventional OC dosing schemes do not completely suppress follicular development and should be improved to decrease the current "real-life" failure rates[150,151]. Furthermore, 15 of the 21 studies reviewed reported follicular

development to ostensibly ovulatory diameter[133,134,136,137,139-144,147,148,152-154]. Eight of the 21 studies observed ovulation during compliant OC use[135,139,142,144,146,148,153,154]. No pregnancies were reported; however, only 3 of the 8 studies reported pregnancy rates. Follicles which develop to pre-ovulatory diameter during OC use likely do not ovulate due to suppression of the mid-cycle gonadotropin surge or the inability of the follicle to respond to LH due to the effects of progesterone on the population or sensitivity of LH receptors[133]. The variable ovulatory ability of follicles during compliant OC use can not yet be explained. Further studies examining changes in gonadotropins, in the presence of an ovulatory-sized follicle, as well as morphological changes of pre-ovulatory sized follicles may help to elucidate some of the mechanisms associated with ovulation during compliant OC use.

Most dominant follicles which developed to pre-ovulatory diameter during compliant OC use regressed[133,134,139-141,143,144,148]. However, some follicles develop to pre-ovulatory diameter in association with pre-ovulatory levels of estrogen[133,146]. Further, follicles which develop to ovulatory diameter have similar ultrasound image attributes and are ultrasonographically indistinguishable from comparable natural-cycle follicles[155,156]. Similarities in ultrasound image attributes between natural cycle follicles and follicles which develop under the suppressive effect of OC can be interpreted to mean that follicles which develop during OC use have similar physiologic status to natural cycle follicles; hence, ovulations can occur resulting in pregnancy and OC failure.

1.3.4 Follicular Development during the Hormone Free Interval

Conventional OC regimens provide a seven day HFI to induce a withdrawal bleed during each 28-day cycle and decrease exposure to exogenous hormones. Numerous studies have reported dominant follicle development during the HFI[133,140,147,156-158] with a

number of these follicles continuing development to pre-ovulatory diameter[133,140,158]. Over 85% of dominant follicle development is initiated during the HFI[133] and ovulation of up to 50% of pre-ovulatory sized follicles has been reported[158]. Ultra low dose OC (≤20 μg EE) appear to increase the risk of dominant follicle development during the HFI[133,147].

The seven day absence of exogenous hormones allows pituitary-ovarian activity to recover to levels observed during the early follicular phase of the natural menstrual cycle, particularly during administration of ultra-low dose OC[133,145,156,157,159-164]. Follicle-stimulating hormone often rises above the threshold for ovarian stimulation during the HFI, allowing for gonadotropin-dependent follicular growth[133,156,160,164]. Initiation of active dosing pills following the HFI causes a decrease in FSH regardless of the presence of a dominant follicle[111]. If a dominant follicle is not present after initiation of active dosing pills, then folliculogenesis is completely suppressed. If a dominant follicle is present, then follicle growth usually continues after initiation of the OC strip due to the decreased dependence of dominant follicles on gonadotropins[160,164]. In the presence of an ovulatory sized follicle contraceptive efficacy is dependent on inhibition of the pre-ovulatory LH surge and the secondary contraceptive benefits of OC, which include, decreased permeability of the cervical mucous to suppress the entry of spermatozoa into the uterus, decreased endometrial receptivity for embryo implantation, and tubal and uterine motility causing a delay in gamete transport[109].

Decreasing the duration of the HFI or replacing some hormone-free days with administration of EE suppresses follicular development better than conventional dosing schemes[126,142,145,147,165]. For example, decreasing the length of the HFI to four or five days during ultra low dose (≤20 μg EE) OC administration decreases ovarian activity and

estradiol levels better than conventional dosing schemes[145,147,165]. Similarly, replacing the last five inert pills during the HFI with pills containing 10 μg EE reduces follicular growth compared to the conventional OC dosing scheme[126,142]. Taken together, these findings are interpreted to mean that shortening or omitting the HFI would prevent a rebound in gonadotropins and increase contraceptive efficacy by decreasing the risk of dominant follicle development and subsequent contraceptive failure.

1.3.5 Follicular Development after Prolongation of the Hormone Free Interval

Prolonging the HFI through missed OC doses increases the risk of "escape" ovulation compared to doses missed during mid-cycle[157,161-163,166,167]. Prolongation of the HFI provides more than seven days of unsuppressed follicular development. The longer the HFI is extended past seven days, the greater the risk of pre-ovulatory follicular development. For example, estradiol levels continue increasing if the HFI is extended to 11 days[120,157]. In addition, mean estradiol concentrations continue to increase, or remain static after reinitiation of OC following a ten day HFI, compared to a decrease in estradiol levels once OC are initiated after the seven day HFI[157]. It is suggested that extending the HFI past seven days increases the number of dominant follicles (≥10 mm in diameter) that develop, as reflected by the higher estradiol levels following a ten day HFI[157]. Prolongation of the HFI likely increases the risk of "escape" ovulation and subsequent conception due to increased dominant follicle development in combination with reduced secondary contraceptive benefits.

Ovulation was observed in five of the ten studies which examined follicular development following prolongation of the HFI, however, no pregnancies were reported[120,162,163,168,169]. Follicles which develop to ≥18 mm in diameter, after the extension

of the HFI, are probably capable of ovulation because these follicles can be induced to ovulate when stimulated with human Chorionic Gonadotropin[170].

1.3.6 Follicular Development after Missed Doses

The risk of "escape" ovulation after missing doses of OC is largely dependent on the number of consecutive doses missed, the stage of the cycle where the OC were missed and the type of progestin contained in the OC. Pituitary-ovarian suppression increases with the number of consecutive OC taken with maximum suppression typically occurring at the end of a 21-day OC cycle, although seven consecutive days of OC administration can adequately suppress ovarian activity[168]. Differences in the type of progestins and their associated half-life can affect the number of consecutive doses that may be missed before the risk of "escape" ovulation increases. Oral contraceptives comprised of progestins with longer half-lives appear to better maintain contraceptive properties following a higher number of consecutive missed doses than progestins with shorter half lives. For example, an OC containing levonogestrel, which has a relatively long half life of 29 hours, maintains contraceptive efficacy for four days of consecutive missed doses, compared to an OC containing desogestrel, which has a half life of 16 hours, and can maintain contraceptive efficacy for only two consecutive missed doses[166-169,171-174] .

Differences between "escape" ovulation rates following missed dosing may also be due to individual variation in the effect the OC has on the hypothalamo-pituitary-ovarian negative feedback system. It is important to note that the small sample size of the above studies may not have included individuals with greater susceptibility to "escape" ovulation; therefore, large-scale studies would be required to estimate the incidence of "escape" ovulation in association with the timing and number of missed OC doses. Up to 74% of

women report omitting ≥ 1 OC during each cycle[175-178]. The high omission rate in combination with OC failure following missed doses, particularly missed doses that prolong the HFI suggest that a more efficacious OC should be developed that is capable of maintaining high efficacy following missed OC doses.

1.3.7 Follicular Development during "Sunday Start" Initiation Schemes

"Sunday Start" initiation schemes prolong the initiation of OC to the first Sunday during menses, compared to immediate start initiation schemes which administer OC on Day 1 of menses. "Sunday Start" schemes were designed to increase user acceptability by suppressing menstruation during weekends when most sexual activity is presumed to occur. Physiologically dominant follicles (≥ 10 mm) are commonly observed during the first seven days of the natural menstrual cycle[149]. Utilization of "Sunday Start" regimens can increase the chances of OC failure because follicles can attain dominance during the HFI and continue developing to pre-ovulatory diameter once active OC are re-initiated, up to one week after the onset of menses[133,144,156]. The risk of follicle development, ovulation and pregnancy in women using "Sunday Start" regimens has not been thoroughly examined.

1.3.8 Follicular Development during Three Different Regimens of Oral Contraceptive use

Schlaff *et al.,* are the only group to date to use ultrasonography to examine ovarian follicular and endometrial development during continuous dosing schemes[126]. This study examined ovarian follicular development ≥8 mm in diameter with transvaginal ultrasonography during the use of three different OC dosing schemes: (i) 21 days of 20 μg

EE + 100 μg levonorgestrel followed by seven days of inert pills, (ii) 20 μg EE + 150 μg desogestrel followed by two days of placebo then 10 μg EE for five days, (iii) 28 days of 20 μg EE + 150 μg desogestrel for two 28 day cycles. The results of the study were interpreted to mean, that continuous OC use provided greatest follicular suppression, in both the number and size, of follicles developing ≥ 8 mm in diameter. The conventional group provided the least amount of suppression, in both follicle number and size, and the conventional dosing scheme with estrogen added to the HFI provided intermediate suppression of follicle number and size. The findings of this study further reinforce the idea of shortening or omitting the HFI to suppress follicular development past the physiological stage of selection.

1.3.9 Inhibiting Menstruation

Many clinicians, researchers and anthropologists propose that monthly menstruation is unnecessary and actually increases health risks to women[179]. Thomas and Ellertson suggest that monthly menstruation is not the historical norm[132]. For example, primitive women menstruated 66% less than the typical modern-day woman. The increase in menstrual frequency in modern women is suggested to be due to evolutionary progression resulting in an earlier onset of menarche, later age of first birth, fewer pregnancies, shorter periods of lactation and a later onset of menopause in modern women compared to primitive women[119]. Eaton *et al.*, reported that late menarche, high parity, early age at first birth and early age at menopause are protective characteristics for breast and gynecologic cancers[180]. It would then follow that suppressing menstruation would also provide women with protection from these cancers[180].

Inhibiting menstruation reduces undesirable menstrual-related symptoms which are reported to be the most common gynecologic complaint in American women aged 18 to 50 years[181]. Undesirable menstrual symptoms include dysmenorrhea, headache, migraine, asthma, premenstrual syndrome, acne and anemia[131]. Thirty one percent of American women report spending approximately 10 days annually in bed due to menstrual morbidity[181]. Menstrual morbidity also affects society as a whole. Menstrual disorders decrease productivity by 25%, and are estimated to cost eight percent of the total wage bill in the United States[132]. Thomas and Ellertson adequately summarized the status of menstrual related disturbances in today's modern industrialized society when they stated, "there can be no other disease or condition that affects so many people on such a regular basis with consequences at both the societal and individual level, which is not prioritized in some way by health professionals or policy makers"[132].

1.3.10 Women's Attitudes towards Inhibiting Menstruation

Most women in industrialized nations prefer to have a menstrual period with a frequency of every three months or not at all[116,182,183]. Women's attitudes toward inhibiting menstruation are culture-dependent. Glasier *et al.* reported women's acceptability to inhibiting menstruation varied depending on their country of residence[183]. For example, 6% of women living in Hong Kong compared to 37% of Scottish women prefer to be amenorrheic[183]. Whereas 15-41% of women from Germany, Denmark and Australia prefer to inhibit menstruation[116,125,184]. Common findings between the studies reviewed were: (i) that most women do not like menstruation, but (ii) less than half of women would actively inhibit menstruation. Menstrual related adverse events were the most commonly reported reason for disliking menstruation. The reported adverse events associated with

menstruation included: inconvenience, menstrual related symptoms, fatigue, acne, premenstrual syndrome, headaches and migraines[130,183]. Women who preferred monthly menstrual cycles falsely believed that monthly menstruation was "natural" and ensured them that they remained fertile but were not pregnant[125,183]. Women who have experienced amenorrhea, during continuous OC dosing, reported fewer menstrual complaints, better hygiene, higher quality of life and less blood loss[122,124,182].

Ten percent to 65% of women stated optimal bleeding frequency would be every three months if they could use OC to control their menstrual frequency[116,125,183,184]. Curiously, the majority of women who actually participated in studies designed to evaluate continuous OC dosing schemes on ovarian function and patient acceptability preferred to continue with the continuous regimen after study completion[112,123]. It appears that women are initially reluctant to initiate continuous dosing schemes but once initiated they prefer continuous OC administration to conventional dosing schemes.

1.3.11 Non-contraceptive Benefits of Oral Contraceptives

Oral contraceptives were initially marketed for the treatment of menstrual disturbances. Ironically, this therapeutic use does not appear in Food and Drug Administration approved labeling for currently marketed OC[185]. Oral contraceptives are commonly prescribed to provide beneficial health benefits and to treat a variety of gynecologic disorders in addition to preventing pregnancy. The health benefits of OC include: (i) providing 99% effective contraception, (ii) treating a number of gynecologic symptoms and (iii) preventing some gynecologic and other medical conditions[186].

Oral contraceptives protect women from primary and secondary dysmenorrhea and menorrhageia, menstrual cycle irregularities, iron deficient anemia, ectopic pregnancy, perimenstrual migraines, menses-related epilepsy and pregnancy related complications[109,185]. Oral contraceptives appear to aid in the prevention of osteopenia and may be used to manage a number of gynecologic disorders including dysfunctional uterine bleeding, persistent anovulation, premature ovarian failure, functional ovarian cysts, and pelvic pain[109,185].

Long term OC use decreases the risk of developing uterine fibroids, endometriosis, recurrent ovarian cysts and acute Pelvic Inflammatory Disease by 50-70%[109]. Long-term OC use also helps to protect women from iron deficient anemia, benign breast lumps, toxic shock syndrome, premenstrual syndrome, breast cancer, endometrial cancer and ovarian cancer, acne and hirsuitism[187].

A number of OC protective benefits increase with increasing duration of OC use. Results from a recent large-scale study which examined the risk of reproductive cancers in OC users were interpreted to mean, that a history of OC use significantly reduced the risk of gynecologic cancers with the risk decreasing with increased duration of OC use[188]. For example, the risk of ovarian cancer decreases by 40% after four years of OC use, 54% after eight years and 60% after 12 years[189]. Similarly, the risk of endometrial adenocarcinoma decreases by 56% after four years of OC use, 67% after eight years and 72% after 12 years[190]. The protective benefits of OC against ovarian and endometrial cancer can persist for up to 20 years after discontinuation of OC use[191-194].

1.3.12 Return to Fertility following Discontinuation of Oral Contraceptives

Oral contraceptives are one of the only forms of contraception that inhibit ovulation by physiologically suppressing the hormonal cues required for follicular development and ovulation. Furthermore, OC are the contraceptive of choice for women in most industrialized nations[109]. Therefore, it is essential to determine any residual effects OC may have on reproductive function once they are discontinued. Differences in study design and population demographics among studies make it difficult to compare the return to fertility following discontinuation of OC. However, it is apparent that previous OC use temporarily delays conception, but does not permanently affect fertility. A two to three month delay in conception following discontinuation of OC is commonly observed compared to discontinuation of non-hormonal contraception (i.e., intrauterine device, diaphragm, condom)[195-204]. In contrast, a single study conducted in Malaysian women reported no difference between conception rates of women discontinuing hormonal or non-hormonal contraception[205].

Researchers report that approximately half of all women (39%-56%) conceive within three months of discontinuing OC compared to almost two-thirds (54% - 65%) for previous users of intrauterine devices, diaphragms, condoms and "other" contraceptive methods[200,205,206]. Furthermore, conception rates are similar two years after discontinuation of OC compared to discontinuation of non-hormonal contraceptives (90-99% versus 98%, respectively)[200,201,203,205]. Conception rates following discontinuation of contraceptives, both hormonal and non-hormonal, should be compared to conception rates during natural cycles to determine the true residual effect contraceptives have on the return to fertility. For example, during natural cycles approximately 57% of women conceive within one

month of unprotected intercourse, 72% within six months, 85% within one year and 93% within two years[207]. It appears that conception rates return to normal expected values within two years of discontinuing contraception.

The delay in the return to fertility following discontinuation of OC is due to both biological and behavioral factors. Post-pill anovulation or dysfunctional ovulation following discontinuation of OC may explain the delay in fertility[198, 208]; however, this is unlikely because approximately 98% of women ovulate within three months following discontinuation of OC[110]. The concentration of EE can effect the median time to conception following discontinuation of OC. For example OC with EE doses ≥50 μg increase the median time to conception following discontinuation of OC by one month compared to OC containing < 50 μg EE[195]. However, this factor is likely not responsible for the delay in fertility since OC containing ≥50 μg EE accounted for <2% of retail prescriptions written for OC in 1998 in the United States[110]. Another mechanism responsible for the delay in fertility following discontinuation of OC may be due to continued hypothalamic suppression for a short period of time following discontinuation of OC.

A women's parity can affect the delay in fertility following discontinuation of OC because nulliparous women took slightly longer to conceive than multiparous women[197,198,201,203,208,209]; however, this difference was reversed in Asian women[200,205]. Oral contraceptives are often prescribed for non-contraceptive reasons (i.e., cycle regularity). Therefore, a higher proportion of women with infertility issues may use OC compared to non-hormonal contraceptives resulting in a falsely inflated interval of infertility following discontinuation of OC. Conception rates decline as age increases in both men and women,

thus an older couple would likely take longer to conceive than a younger couple with similar health characteristics[198,208].

Behavioral factors can also affect conception rates. For example, the timing and frequency of intercourse, recall errors in determining when contraception was discontinued and "rounding up" while reporting the time at which conception occurred. Accidental pregnancies that occur during non-hormonal contraceptive use are more likely to be reported as planned which would artificially decrease the time to conception in non-hormonal contraceptive users. Women who discontinue OC may take longer to conceive because they are more likely to refrain from attempting to conceive during the few months following contraceptive use to decrease the risk of residual effects of OC on the developing fetus. It appears that the delay in fertility is likely due to a combination of both biological and behavioral factors. Further research is needed to determine the exact mechanisms of action responsible for the delay in fertility following discontinuation of OC.

A number of researchers have reported conception rates follow an oscillatory pattern following discontinuation of OC with increases in conception rates occurring at approximately three to four month intervals[199,200,202,209]. Janerich *et al.* suggest three different explanations for the oscillations in conception rates: (i) OC acts as a synchronizing agent resulting in previously unrecognized natural oscillatory cycles approximately four menstrual cycles in length, (ii) oscillations are caused by the transition of the reproductive organs from a suppressed state of functioning to an unsuppressed state, (iii) intrauterine mortality following OC use results in an infertile period[202]. It is possible that the oscillations may be due to recall errors whereby women preferentially rounded their preconception intervals at one-quarter and one-half year[199-201, 203]. Closer scrutiny of Pardthaisong and Gray's data

suggests that they did not observe true "peaks" in conception rates but simply increases in conception rates three and six months following discontinuation of OC[200]. Harlap and Baras reported conception rates do not follow an oscillatory pattern[203]. These contradictory findings suggest that further research is needed to determine if conception rates exhibit an oscillatory pattern following discontinuation of OC use.

1.3.13 Summary

Oral contraceptives have radically changed the way women approach contraception. Initially OC were introduced to regulate menstrual disturbances and provide women with relief from menstrual related adverse events. It appears that attempts to decrease the exposure to exogenous hormones results in inferior follicular suppression, particularly during the HFI or following missed doses. It is unknown at this time as to why some follicles ovulate during compliant OC use while others regress. Much research has examined various aspects of OC composition and function; however, we are far from completely understanding the exact mechanisms responsible for ovarian and uterine suppression during OC use.

Most women desire changes to their menstrual patterns whether it is a decrease in menstrual related side effects or changes to bleeding frequency. Continuous OC use is associated with high acceptability and effectiveness while providing women with the desired changes in menstrual patterns. Ovarian follicular development and the associated endocrine profiles have not yet been critically examined during continuous OC use. Further research needs to examine endometrial development and ovarian follicular growth, regression and ovulation during both conventional and continuous OC dosing schemes to develop a more effective, better accepted OC. Furthermore, the information could be

used to develop new contraceptive technologies which could provide more contraceptive choices for women.

It has been well established that discontinuation of OC results in a two to three month lag in fertility compared to non-hormonal contraceptives. Many hypotheses to explain the delay in fertility following discontinuation of OC have been elucidated; however, an acceptable mechanism of action to explain the decrease in fertility has not yet been suggested. A better understanding of the exact effect OC administration has on the reproductive organs would likely aid in the explanation of the delay in fertility following discontinuation of OC.

Chapter 2

GENERAL OBJECTIVES AND HYPOTHESES

The general objectives of the studies contained in this thesis were:

1. to assess ultrasonographic image attributes of dominant ovarian follicles during the final stages of development prior to ovulation in natural cycles and prior to peak estradiol concentrations for dominant anovulatory follicles during OC cycles;
2. to characterize the development and ovulation of ovarian follicles and related endocrine changes, during and following discontinuation of the suppressive effects of continuous and conventional OC dosing schemes; and
3. to elucidate the mechanisms of action responsible for the delay in fertility following discontinuation of OC.

The research hypotheses tested were:

1. Image attributes of follicles from natural cycles differ quantitatively from follicles that developed under the suppressive effects of OC.
2. Continuous OC dosing schemes better suppress follicular development than conventional OC dosing schemes.
3. A longer time interval to ovulation occurs following discontinuation of OC compared to the first day of menses to ovulation in natural cycles.

Chapter 3

ULTRASOUND IMAGE ATTRIBUTES OF HUMAN OVARIAN DOMINANT FOLLICLES DURING NATURAL AND ORAL CONTRACEPTIVE CYCLES

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3.1 Abstract

***Objective*:** Computer-assisted analyses were used to examine ultrasound image attributes of human dominant ovarian follicles that developed during natural and oral contraceptive cycles. We hypothesized that image attributes of natural cycle follicles would differ quantitatively from those in oral contraceptive cycles and that oral contraceptive cycle follicles would possess image attributes indicative of atresia.

***Methods*:** Dominant ovarian follicles of 18 clinically normal women were compared using image analysis techniques for the seven days before ovulation during a natural cycle (n=9) or the seven days before peak estradiol in women using oral contraception (n=11) in a retrospective study. Follicles were analyzed using region and line techniques designed to compare the image attributes of numerical pixel value, pixel heterogeneity and area under the curve.

***Results*:** Numerical pixel value was higher in oral contraceptive cycle follicles with region analysis and tended to be higher with line analysis ($p=0.005$ and $p=0.06$, respectively). No differences were observed in area under the curve and pixel heterogeneity measured with either technique, between natural and oral contraceptive cycle follicles.

***Conclusions*:** The increased numerical pixel value of oral contraceptive cycle follicles and lack of differences in pixel heterogeneity and area under the curve values between natural cycle and oral contraceptive cycle follicles did not support the hypothesis that oral contraceptive cycle follicles would show ultrasonographically detectable signs of atresia. Image attributes observed in oral contraceptive cycle follicles were not clearly indicative of

atresia nor were they large enough to preclude preovulatory physiologic status in oral contraceptive cycle follicles.

3.2 Introduction

Diagnostic gray-scale ultrasonography has revolutionized the study of ovarian biology in animals and humans because it allows researchers and clinicians to assess the development of individual follicles in a direct, non-invasive, and atraumatic manner without interruption or distortion of ovarian function[1]. Prior to the introduction of ultrasonography, histological slices of ovarian tissue were used to elucidate ovarian follicular development; however, histologic investigation only provides information about a single time point and does not permit assessment of follicular function over time. Further, histology cannot be used for time-series studies in humans.

Animal models have been developed to elucidate the basic mechanisms of ovarian function in humans and to overcome ethical objections of some aspects of research in humans. To date, however, no appropriate animal models are available to elucidate the physiological effects of oral contraceptives (OC) on human ovarian function due to species specific differences in the metabolism of the exogenous estrogen and progestins used to control reproductive function. Non-invasive techniques do not yet exist that will allow for the determination of the physiological status of individual ovarian follicles with a single observation. However, new technologies involving computer assisted image analysis to elucidate a follicle's physiologic status show promise[1,2].

Quantitative changes in ultrasound image echotexture as indicators of physiological function of ovarian structures have been described in domestic animal models[3-8]. The validity of the image analysis technique has been verified through correlation of ultrasound

image attributes with histologic attributes[3,4,8]. Similar studies in humans are objectionable ethically; however, information generated in animal studies may be applied to human imaging based studies[3-6,9,10]. The overall goal of this line of research in our laboratory is to elucidate physiologic status of dominant follicles with non-invasive ultrasonography in humans. Imaging-based techniques that could be used to determine follicular health would obviate the ethical and logistical limitations associated with ovarian function research in women.

It has recently been reported that women grow follicles in two or three follicular waves during each natural menstrual cycle[11,12]. The pattern of folliculogenesis is similar in women to those observed in several species of domestic animals (bovine, equine, caprine and ovine)[10,13-17]. Follicular waves, in humans, are characterized by an increase in the number of follicles ≥ 5 mm in diameter, occurring in association with the growth of at least two follicles to ≥ 6 mm in diameter[11]. Growth of all follicles in the cohort continues until one follicle is physiologically selected as the dominant follicle. The dominant follicle continues its development to pre-ovulatory diameter while the remaining follicles in the cohort undergo atresia. The dominant follicle will ovulate if the appropriate hormone signals (i.e., mid-cycle luteinizing hormone surge) are provided. If the hormone signals which trigger ovulation are not provided, the dominant follicle enters a static phase and remains approximately the same diameter until it enters the regressing phase, when it decreases in diameter until it is no longer detectable.

Dominant follicles develop in women during adherent use of OC, with most dominant follicles initiating growth during the hormone free interval[18-22]. Ovulatory follicles that develop during natural menstrual cycles are presumed to be healthy because

they usually ovulate. It is not known whether dominant follicles that arise during OC use have the same physiologic status or ovulatory capacity as natural preovulatory follicles.

The primary objective of the present study was to assess ultrasonographic image attributes of dominant ovarian follicles during the final stages of development in natural menstrual cycles and OC cycles. We hypothesized that image attributes of follicles from natural cycles will quantitatively differ from OC cycle follicles and that the image attributes of OC cycle follicles would be consistent with those indicative of atresia.

3.3 Materials and Methods

This study was a retrospective, observational study designed to evaluate and compare ultrasound image attributes of dominant ovarian follicles ($\geq$ 10 mm in diameter) during natural and OC cycles. Images of twenty (n=20) dominant follicles were analyzed. The images were obtained in two previous studies designed to characterize ovarian follicular wave dynamics during natural cycles[12,13] and OC cycles[22]. Both study protocols were initially approved by the University of Saskatchewan Ethical Review Board. The inclusion and exclusion criteria of both studies were similar. Participants were assessed, by history and physical examination, to be healthy women of reproductive age (28.0 $\pm$ 0.14 years, natural cycles; 24.5 $\pm$ 0.02 years, OC cycles; mean $\pm$ SEM). All women in both studies had a history of normal menstrual cycles and did not use OC for a minimum of three months before participating. In the OC trial, one of three different OC formulations were administered to thirty six women for three consecutive 28-day cycles; (i) 20 μg ethinyl estradiol (21)/100 μg levonogestrel (21) (n=7), (ii) 30 μg ethinyl estradiol (21)/150 μg desogestrel (21) (n=2), or (iii) 35 μg ethinyl estradiol (21)/180 μg norgestimate (7)/215 μg norgestimate (7)/250 μg norgestimate (7) (n=2). Nine women grew 11 dominant follicles

during compliant OC use. Two women grew dominant follicles during two separate cycles of OC-use; therefore, images from eleven dominant, ostensibly, ovulatory follicles were analyzed in the present study[22]. Images of nine dominant follicles from nine volunteers were randomly selected from natural cycle data to act as controls[11,12]. Blood samples were drawn every third day for women in the natural cycle trial and every second day once a follicle reached $\geq$ 14mm diameter for women in the OC cycle trial.

A high resolution ultrasound instrument equipped with 5-9 MHz multi-frequency intravaginal convex array transducer (ATL Ultramark HDI 5000, Advanced Technologies Laboratories, Bothwell, WA, USA) was used. Settings of the ultrasound instrument that affect image attributes (beam focus, overall time-gain, near-field and far-field gain) were standardized to the same predetermined standards for both studies. Each image was digitally acquired and transferred into a customized computer database during the ultrasound examinations. Images of the dominant follicle acquired in largest cross-sectional diameter with the fewest image artifacts were selected for image analysis. All image analyses were performed by the same individual (RLB). Blinding was not possible because each image contained identifying study information.

Image attributes of dominant follicles were analyzed using a graphics workstation equipped with a customized software (SYNERGYNE 2©, Saskatoon, SK, Canada) integrating complex algorithms designed for ultrasonographic image analysis[1,2]. Each image was analyzed with two different techniques: (i) region analysis, and (ii) line analysis designed to quantify gray-scale values of selected regions[1,2]. Three image attributes were quantified: (i) numerical pixel value (NPV) defined as the mean pixel gray-scale value of the sampled pixels, (ii) pixel heterogeneity (PH) defined as the standard deviation of the mean

gray-scale values of the sampled pixels, and (iii) area under the curve (AUC) defined as the total sum of the gray-scale values within the sampled region.

Briefly, region analyses involve overlaying a computer-generated grid onto a selected area of the 2-dimensional image of a follicle to generate a 3-dimensional framework representing processed pixel intensities. Placing a computer-generated opaque "film" or blanket over the wire framework representing pixel values comprising the ultrasound image yields a 3-dimensional contoured surface. This technique allows discriminatory examination of the surfaces and rapid visual assessment of ultrasonographic attributes associated with follicle health (state of viability or atresia)[2]. Region analyses were used to measure NPV, PH and AUC within a region of the follicle wall. A one-pixel-wide line was used to outline and isolate the largest continuous portion of follicular wall extending from the peripheral antrum to the stroma with the fewest image artifacts. The antrum-wall interface was defined as the last pixel along the line in which a sequential rise in gray-scale occurred[45].

Line analysis places a line across a specified section of the image of the follicle to produce a graph of the pixel intensities along the line displayed. The graph depicts the amplitude of the echoes located along the line[3]. Line analysis was used to measure NPV, PH and AUC of a single line across the follicle wall from the antrum-wall interface to the ovarian stroma using two straight three-pixel wide lines. The two linear measurements were taken between the 4 and 9 o'clock positions of the follicle image.

Ovulation was defined as the disappearance of a follicle greater than 15 mm in diameter identified the previous day and confirmed with visualization of a corpus luteum[12,13,24,25]. Ovulation was used as a reference point (Day 0) to standardize the data for

natural cycle follicles. No follicles ovulated during OC cycles; therefore, OC cycle follicles were standardized to the day of peak estradiol concentration (Day 0) which was also defined as the last day of the growth phase. The day of peak estradiol concentration for each individual was determined by reviewing the estradiol profiles for each woman in the OC trial[22].

Ultrasound images were available for each of the seven days leading to ovulation for women in the natural cycle study and approximately every second day for seven days leading to peak estradiol levels for women participating in the OC trial. Data were standardized by aligning data from Day 0 as defined for each group. Image attributes on each of the seven days (Days -1 to -7) leading to ovulation during the natural cycle were compared to the corresponding days (Day -1 to -7) leading to peak estradiol concentrations during the OC cycle. Data were inadequate for statistical comparison of image attributes among the three different OC formulations, therefore data were combined.

Image attributes were compared using repeated measures analysis of variance (PROC MIXED, SAS/STAT, *v*8) for main effects of time and follicle type. Significance was set at $p<0.05$. Results are expressed as the mean ± SEM.

3.4 Results

Peak estradiol-17β concentrations for OC cycle follicles were 170.1 ± 30.4 pg/ml (range, 35.1 – 364.5 pg/ml)[22]. Mean follicle diameter of OC cycle follicles at peak estradiol concentrations was 20.3 ± 2.0 mm. Mean estradiol concentrations for natural cycle follicles on Day –1 were 190.8 ± 19.6 pg/ml (range 20.0 - 340 pg/ml)[11]. Mean follicle diameter of natural cycle follicles on Day –1 was 21.1 ± 1.1 mm. Mean growth profiles for

natural and OC cycle follicles are displayed in Figure 3.1. Ultrasonographic images of a natural cycle follicle one day before ovulation and an OC cycle follicle on the day of peak estradiol are shown (Figure 3.2, a and b, respectively).

Numerical pixel values were higher ($p=0.005$) for OC cycle follicles versus natural cycle follicles using region analyses and tended to be higher ($p=0.06$) using line analysis for the seven days prior to Day 0. Oral contraceptive cycle follicles had visually similar NPV for both region and line analyses; however, the values were initially high and then decreased to a nadir at Day -3 (region analysis) or Day -4 (line analysis) after which they increased until Day -1 and subsequently decreased on Day 0 (Figure 3.3 a, b). Numerical pixel values for natural cycle follicles progressively increased from Day -7 (Region analysis; 31.64 ± 4.26, Line analysis; 41.66 ± 10.49) to Day 0 using line analysis (64.03 ± 6.37; $p=0.01$) and tended to increase (43.80 ± 5.30; $p=0.08$) with region analysis (Figure 3.3 a, b).

Pixel heterogeneity values were not different between natural and OC cycle follicles for either region or line analyses ($p=0.113$ and $p=0.515$, respectively). No changes were observed over the seven days prior to Day 0.

Area under the curve evaluated using line analyses tended to be higher ($p<0.09$) in OC cycle follicles; however, no differences were observed ($p=0.23$) in follicle type using region analyses. Values for AUC for both natural and OC cycles increased as the interval to Day 0 decreased.

Nadirs in image attributes for the values representing AUC and PH were observed on Day -4 (Figure 3.3 c-f).

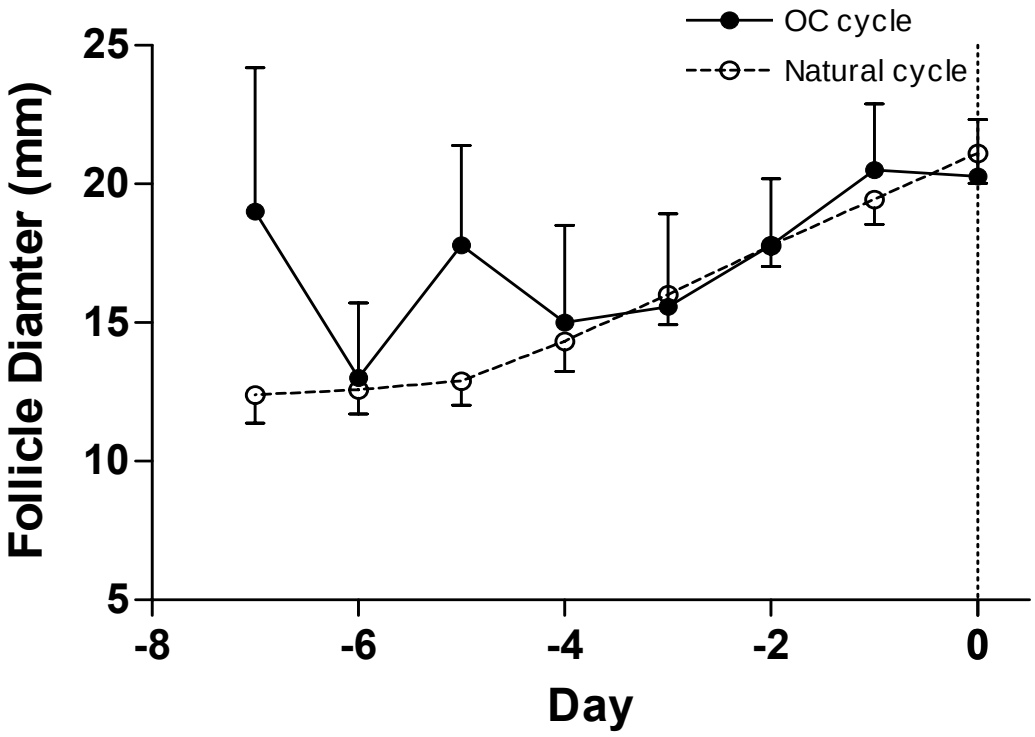

Figure 3.1: Growth profiles of natural and OC cycle follicles

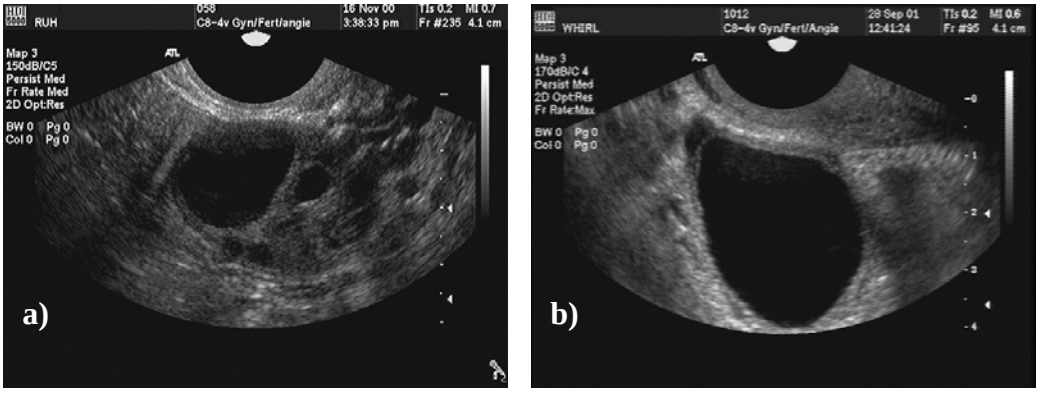

Figure 3.2: Ultrasonographic images of a natural cycle and OC cycle follicles. (a) Natural cycle follicle on Day 0, (b) OC cycle follicle on Day 0.

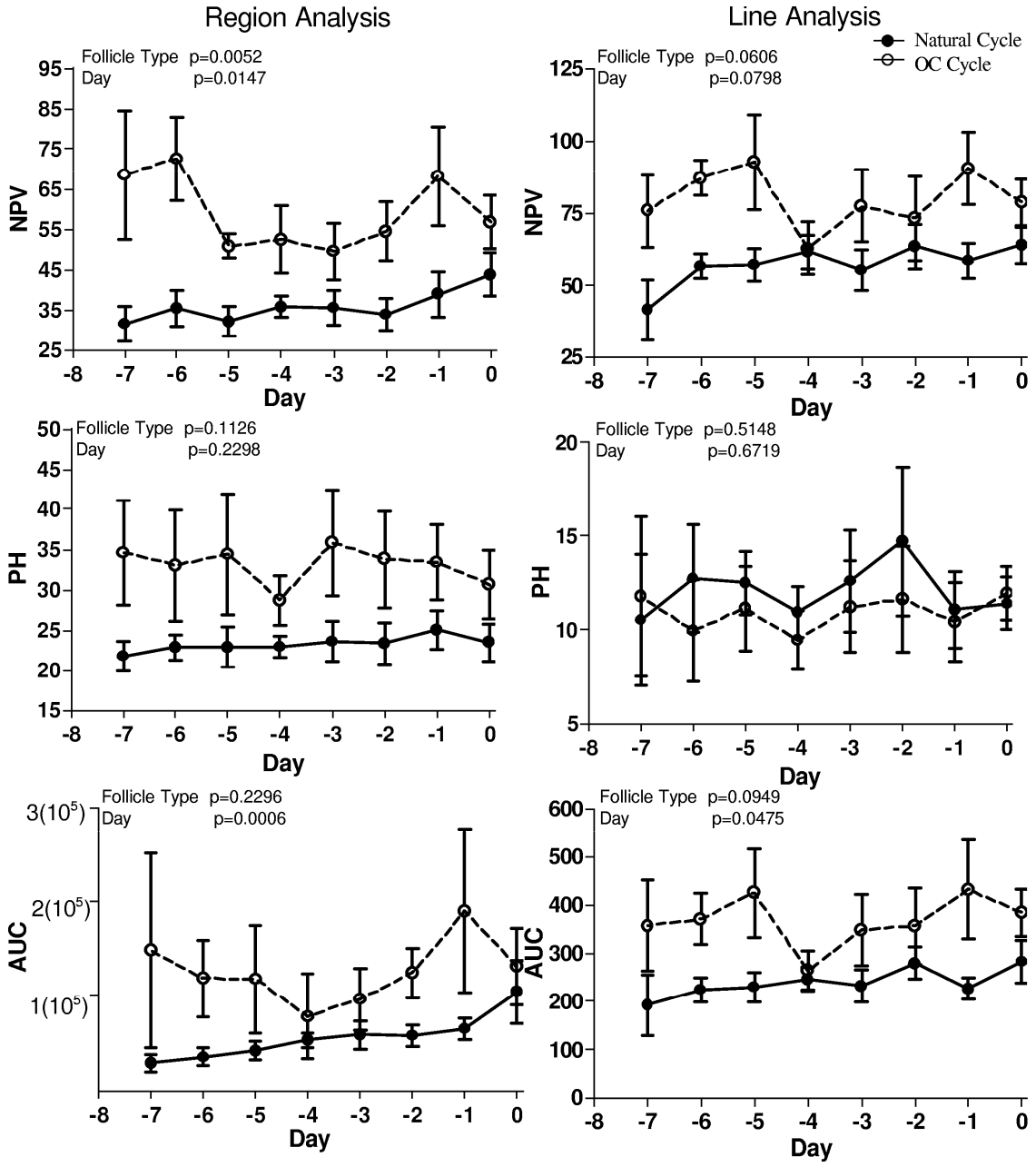

Figure 3.3: Graphical representation of NPV, PH and AUC. Mean ± SEM (a, b) numerical pixel value (c,d) pixel heterogeneity and (e, f) area under the curve obtained by region analysis (a, c, e) and line analysis (b, d, f) of natural cycle and OC-cycle follicles.

3.5 Discussion

The study of ovarian follicular dynamics in women taking hormonal contraceptives is a relatively new research area with profound clinical relevance. Follicular development in up to 50% of new OC cycles and up to a 25% ovulation rate have been reported[18]. Most follicular development observed in OC cycles is initiated during the hormone free interval of the OC cycle[22]. The objective of this study was to assess the indicators of follicular physiological status in women by comparing ultrasound image attributes of human dominant ovarian follicles leading to ovulation in natural cycles or peak estradiol concentrations in OC cycles. Numerical pixel values representing the relative brightness of the elements comprising the ultrasound image were the only image attributes that differed between natural and OC cycle follicles. Morphologic changes indicative of atresia in animal models are typically represented by high values of NPV[4,6]. These morphologic changes include but are not limited to: (i) an increase in the number of multivesicular bodies, (ii) lipid droplets and vacuoles within granulosa cells, (iii) dilation of the smooth endoplasmic reticulum and golgi apparatus and (iv) thecal cell hypertrophy/degeneration; which, when taken together, appear to affect the tissue properties enough to change the image attributes[25,26]. Although NPV were greater in OC cycle follicles, the increased values were not high enough to indicate poor physiologic status as described in studies using animal models[4,6]. The hypotheses that image attributes of natural and OC cycle would quantitatively differ and that OC cycle follicles would possess image attributes characteristic of atresia were only partially supported.

An interesting common observation in all image attributes of OC follicles that were evaluated fell to a nadir at Day -4, suggesting that an important physiological event occurs

approximately four days before peak estradiol concentrations were attained. The image attributes of the values reflected in NPV and AUC in both natural and OC cycle follicles tended to increase from Day -4 to Day 0. This observation may be interpreted to mean that the follicles in both natural and OC cycles have similar physiologic status and undergo similar changes during the final stages of development. Classically described, ovulatory changes include thickening of the follicle wall, development of lipid inclusion granulosa and theca cells, hypertrophy of theca cells and increased vascularization of the theca layer[27,28]. Taken together, these developmental changes may be reflected as increases in AUC as the follicle wall thickens and increases in NPV as the follicle wall develops to fulfill its biological functions. Further research is needed to determine the physiologic changes responsible for changes in image attributes.

No OC cycle follicles analyzed in the present study ovulated. However, we suggest that follicles which develop during compliant OC use may differ in ovulatory potential because ovulation has been documented in up to 25% of follicles which develop to ostensibly ovulatory diameter during compliant OC use[18]. Differences in OC formulations (i.e. estradiol concentrations and progestin type and associated activity) may have different physiological effects on follicular development and subsequent ovulatory ability, and individual responses to the different estrogen levels and the various progestins used in OC formulations may affect ovulatory ability differently. The authors were unable to test this hypothesis directly in the present study. Variations in image attributes of OC cycle follicles may be due to differences in physiologic status and ovulatory ability. Further research to determine the physiological mechanisms responsible for differences in ovulatory ability of OC cycle follicles needs to be undertaken."

Studies in domestic animals have demonstrated that ultrasound image attributes of ovarian structures are related to their physiologic function[3,4,8]. The present study provides rationale for exploring the hypothesis that the same is true for humans. In order to have a complete understanding of the relationship between image attributes and follicular development in humans, we must assess all stages of follicular development throughout the menstrual cycle, prediction of dysfunctional follicular development (i.e., hemorrhagic anovulatory follicles) and the effects of different OC formulations on follicle development. Once the association between image attributes and various conditions of follicular development are determined, follicular status could be assessed with a single ultrasound examination, obviating many ethical constraints that currently prevent progress in human ovarian follicular research.

Ovarian follicles provide a unique endocrine environment fundamental to normal oocyte development and competence. Abnormal follicular development can lead to incompetent oocytes; therefore it follows that image attributes of the follicle wall may be related to oocyte competence. Currently, image attributes only provide us with information about the physiologic status of the follicular wall. Image analyses do not provide information about the health and viability of the oocyte; however, this area is under active investigation[7].

The primary objective of this research was to evaluate and compare ultrasound image attributes of dominant follicles that develop during natural cycles and OC cycles. We were not able to examine the effect of different OC formulations on image attributes due to constraints imposed by the study design. It is hoped that future research will explore the effects of different OC formulations on the image attributes of dominant

follicles. Research to evaluate physiologic status may well hinge upon follicular response to administration of ovulation inducing doses of recombinant human chorionic gonadotropin in women who develop dominant follicles during OC cycles in concert with development of an animal model to test the biological effects of varying doses of ethinyl estradiol or different progestins on follicle and oocyte status.

In conclusion, similarities in image attributes between natural and OC cycle follicles provides preliminary evidence that ultrasound image attributes of human follicles are associated with physiologic status during the growth phase and that follicles which develop during OC cycles have image attributes similar to those of natural cycle follicles. Only one of the three image attributes studied differed between natural and OC cycle follicles. The changes observed in OC cycle follicles were not clearly indicative of atresia nor were they large enough to preclude preovulatory physiologic status in OC cycle follicles.

3.6 Acknowledgments

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Chapter 4

OVARIAN FOLLCIULAR DYNAMICS DURING AND AFTER CONTINUOUS VERSUS CONVENTIONAL DOSING SCHEMES

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4.1 Abstract

Objective: Characterize ovarian follicular and endometrial development during conventional versus continuous oral contraceptive (OC) dosing regimens to explore follicular development during the hormone free interval and examine follicular development following OC discontinuation**.**

Methods: Transvaginal ultrasonography and blood sampling were done to ascertain ovarian function.

Results: Fewer follicles >4 mm developed during continuous versus conventional OC use ($p=0.006$). No dominant follicles developed during continuous OC use versus eight dominant follicles (16.1 ± 3.3 mm) during the conventional OC regimen. Two of eight (25%) dominant follicles ovulated. All dominant follicles began development during the HFI. Following discontinuation of OC ovulation took approximately five days longer when compared to natural cycles.

Conclusion: Continuous OC regimens more effectively prevent dominant follicle development and breakthrough ovulation. The slight delay in time to ovulation following OC discontinuation and natural cycles could be attributed to suppression of follicle wave activity.

4.2 Introduction

Since their introduction in the late 1950's, oral contraceptives (OC) have been traditionally prescribed in schemes consisting of 28 day cycles comprised of 21 daily active pills followed by a seven day hormone free interval. The conventional dosing scheme was

developed to mimic the physiologic event of monthly menstruation in nonpregnant women and to provide the illusion of natural menstrual cyclicity. The hormone free interval is reported to have no physiologic benefit, but was initially included to increase user acceptability[1]. Oral contraceptive withdrawal symptoms associated with the hormone free interval include: bleeding, pain, breast tenderness, bloating, swelling, migraines, premenstrual syndrome, acne, back pain, dysmenorrhea, anemia, increased use of analgesics and a higher incidence of follicular cyst development[2,3].

Twenty-eight years ago, Loudon *et al.* reported that three months of continuous OC use suppressed monthly menstruation, was accepted by women and prevented pregnancy in all study volunteers[4]; yet no changes in OC regimens were introduced[5]. Since then, the efficacy and acceptability of continuous dosing schemes have been examined[6-17]; however, the physiologic effect of continuous OC use on follicular and endometrial development has been examined in only two studies[5,18].

Ultrasonography allows assessment of ovarian follicular development at key points, such as during the hormone free interval, and may be combined with hormone assays and image analysis techniques to determine the physiologic status of a follicle[19-21]. The loss of endocrine suppression during the hormone free interval is suggested to provide an environment conducive to follicular development due to increases in endogenous follicle stimulating hormone (FSH)[2,22]. Dominant follicles have been observed during adherent OC use[2,23-27] and a proportion of these follicles continue development to pre-ovulatory diameter[2,23,25,27-29]. It has been shown that over 85% of dominant follicle development is initiated during the hormone free interval[2] and ovulation of up to 50% of pre-ovulatory sized follicles, depending on the OC formulation, has been reported[27].

Ultrasonography has been used to assess the ovarian activity during continuous OC use in only one known study. Schlaff *et al.* compared follicular development during two consecutive 28-day cycles of either: (i) 20 μg ethinyl estradiol (EE)/100 μg levonorgestrel for 21 days followed by a seven day hormone free interval, (ii) 20 μg EE/150 μg desogestrel followed by two days of placebo then 10 μg EE for five days, or (iii) 28 days of 20 μg EE /150 μg desogestrel[18]. Weekly ultrasound examinations in combination with endocrine profiles were used to monitor follicular development ≥8 mm in diameter. Dosing schemes with a seven day hormone free interval provided the least follicular suppression[18]. The proportion of follicles which manifested dominance and the number of "escape" ovulations during the three dosing schemes, were not reported. An intensive ultrasound examination schedule in combination with endocrine profiles has not yet been used to examine ovarian follicular and endometrial development during continuous OC dosing schemes.

Since their introduction, OC have become the most popular reversible contraception chosen by women residing in industrialized nations[30]. Therefore, it is essential to determine if OC have residual effects on reproductive function following their discontinuation when fertility is desired. Fertility is temporarily reduced for approximately two to three months following discontinuation of conventional OC compared to discontinuation of non-hormonal contraceptives (i.e., intrauterine device, diaphragm, or condom)[31-40]. Approximately half of all women (39% - 56%) conceive within three months of discontinuing conventionally administered OC[36,41,42] which is slightly lower than for women using other methods of contraception (e.g., intrauterine device, diaphragm and

"other" contraceptive methods; 54% - 65%)[41]. Follicular kinetics following discontinuation of continuous OC dosing schemes apparently have not yet been examined.

The objectives of the present study were to characterize ovarian follicular and endometrial development during and following discontinuation of conventional versus continuous regimens of two different OC formulations using high-resolution transvaginal ultrasonography. We hypothesized that the continuous OC dosing scheme would better suppress ovarian follicular development during the first three cycles of use and that the interval from discontinuation of OC to ovulation would be longer than that from menstruation to ovulation in natural cycles.

4.3 Materials and Methods

The present study was a randomized, single center trial. Informed consent was obtained from all volunteers before performing any study procedures. The study protocol was approved by the University of Saskatchewan Biomedical Ethics Review Board. Thirty-six women between the ages of 18 and 35 (24.4 ± 0.7 years and BMI 24.3 ± 0.9; mean ± SEM) were enrolled. Volunteers were assessed to be healthy women by history and physical examination. Information on body mass index, gravidity, parity, and previous OC use were collected from each woman. Women were excluded if they smoked, were pregnant or lactated within 6 months of enrolling in the study, had used OC in the previous two months, had a history of irregular menstrual cycles, were using contraindicated medications, or were planning surgery during the study period.

Each woman received one of two different monophasic OC formulations in a continuous or conventional dosing scheme for three 28-day cycles. The four study groups were:

(i) 30 μg EE /150 μg levonogestrel (LNG) (21 days and 7 day HFI; n=8);

(ii) 30 μg EE/150 μg LNG (28 days; n=9);

(iii) 35 μg EE/250 μg norgestimate (NGM; 21 days and 7 day HFI; n=8);

(iv) 35 μg EE /250 μg NGM (28 days; n=11).

Oral contraceptives were initiated on the first day of menses during Cycle 1. The conventional regimen consisted of 21 days of daily active pills followed by a seven day hormone free interval. The continuous regimen consisted of 28 daily active pills with no hormone free interval. Women did not take OC during the fourth and final cycle of the study.

Ovarian follicular and endometrial development were monitored throughout the study with high-resolution transvaginal ultrasonography (ATL HDI 5000, Advanced Technologies Laboratories, Bothwell, WA, USA) as described[19]. Approximately 90% of the examinations were performed by one individual (RLB). A second investigator (RAP) performed examinations when the primary ultrasonographer was unavailable. Diameters of all follicles ≥ 2mm, endometrial thickness and echotextural pattern (i.e., M, A, B, C or D) were recorded during each ultrasound examination[19,20,43].

The scanning schedule was designed to capture key physiological processes of follicular and endometrial development and was based on previous studies which characterized follicular wave dynamics during natural and OC cycles[2,19,20]. Monitoring began on Cycle 1 day 7 and continued every seven days for the first 21 days of Cycle 1.

After Cycle 1 Day 21, an every third day ultrasound scanning schedule was followed until Cycle 3 Day 28. If a follicle grew ≥14 mm in diameter during Cycles 1 to 3, daily blood sampling and ultrasound examinations were performed until the physiologic status of the follicle was determined [i.e., ovulation, regression or haemorrhagic anovulatory follicle (HAF)]. Ovulation was defined as the disappearance of a follicle ≥15 mm in diameter detected ultrasonographically the previous day followed by the subsequent visualization of a corpus luteum[44,45].

Ultrasound examinations for Cycle 4 began on Cycle 4 Day 2 and continued every second day. Daily ultrasound examinations commenced when a follicle reached ≥16 mm in diameter and continued until the physiologic fate of the follicle was determined. Blood samples were taken during Cycle 4 when a follicle first reached 18 ± 1 mm and 20 ± 1 mm, and were assayed for estradiol-17β. A final ultrasound examination and blood sample were completed six to nine days post-ovulation to image the corpus luteum and correlate corpus luteum morphology to serum progesterone levels[43].

Data from 18 women who participated in a previous study designed to characterize ovarian follicular wave dynamics during natural cycles were selected randomly and used as historical controls for comparison of Cycle 4 endpoints[19,20]. Inclusion and exclusion criteria and ultrasound imaging techniques for the natural cycle study were similar to the present study. Cycle 4 Day 1 was defined as the first day of menses during natural cycles or the first day following cessation of OC for follicles developing after discontinuation of OC. The first day following discontinuation of OC was chosen as the comparator for Cycle 4 Day 1 because the high incidence of unscheduled bleeding at the time of OC

discontinuation made it difficult to accurately determine Day 1 of menses in women discontinuing OC.

Volunteers were assigned a diary card for all four cycles to record OC initiation, missed pills, concomitant medication used during each cycle, menstrual patterns and adverse events. Diary cards were reviewed with each volunteer at the conclusion of each cycle. Oral contraceptive use was reviewed during each visit to minimize missed doses.

The growth profiles of all follicles which grew ≥4 mm were retrospectively determined as described and graphed for each dosing scheme[2,19,20,44,45]. The day of selection was defined as the day prior to the manifest of dominance. The day dominance was manifest was defined as the day the largest follicle of the cohort surpassed all other follicles by ≥2 mm in diameter. Pre-ovulatory diameter was defined as diameter the day before ovulation was observed.

Repeated measures analyses of variance were used to compare follicle diameter, endometrial thickness and pattern between regimens during Cycles 1 to 3 (PROX MIXED, SAS/STAT software, 2004). Mann-Whitney U tests were used to examine differences in bleeding patterns during Cycles 1 to 3 and to compare Cycle 4 endpoints between continuous and conventional regimens (SPSS Version 12, 2004). Kruskal-Wallis tests were used to compare estradiol-17β concentrations of follicles 18 ± 1 mm and endpoints during Cycle 4, among OC study groups and the reference cycles (SPSS Version 12, 2004). Significance was set at $p<0.05$. Results are expressed as the mean ± SEM.

4.4 Results

Data for all endpoints were analyzed initially for differences between OC formulations within a dosing scheme as well as differences between the continuous and conventional dosing schemes. No statistical differences were observed within dosing schemes; therefore, data were combined.

Cycles 1-3

Overall, fewer follicles developed during continuous OC use than conventional OC use ($p<0.001$). Follicle number, regardless of size, decreased as time progressed from Cycles 1 to 3 for both continuous and conventional regimens ($p<0.001$). More follicles >4 mm in diameter were observed during conventional OC use compared to continuous OC use ($p=0.006$). The number of follicles >4 mm during conventional OC use increased during the hormone free interval of Cycles 1 through 3 ($p<0.001$; Figure 4.1). Representative follicular growth profiles from continuous and conventional regimens are shown (Figure 4.2). Little to no follicular development was observed during continuous dosing schemes whereas follicular development was initiated during the hormone free interval of conventional dosing schemes.

More dominant follicles (≥10 mm) developed during conventional OC than continuous OC use (8 versus 0, respectively; $p=0.01$). All dominant follicles began growing during the hormone free interval. Five dominant follicles developed during the use of 30 μg EE (21)/150 μg LNG (21) and three dominant follicles development during the use of 35 μg EE (21)/250 μg NGM (21). One dominant follicle from each conventionally administered OC ovulated (25%; Figure 4.3). The follicle which ovulated in the 30 μg EE (21)/150 μg LNG (21) group had no detectable estradiol output for the No

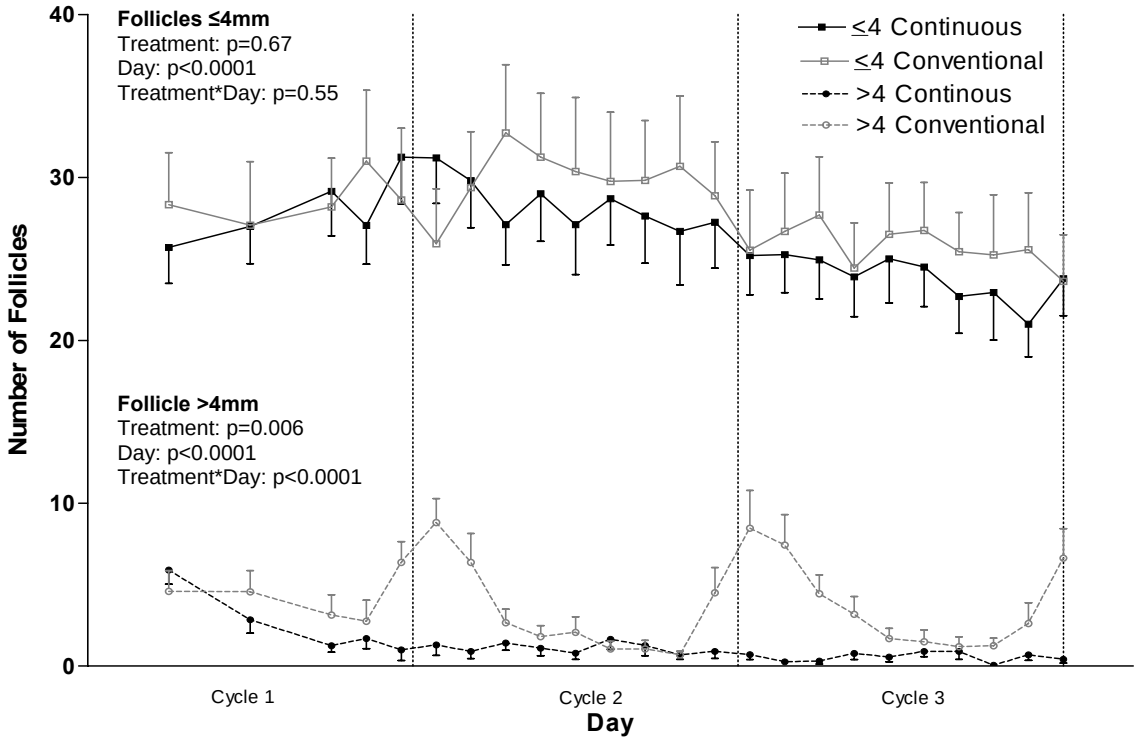

Figure 4.1: Follicle numbers for follicles ≤4 mm, and > 4 mm in diameter during continuous and conventional OC use Cycles 1 to 3.

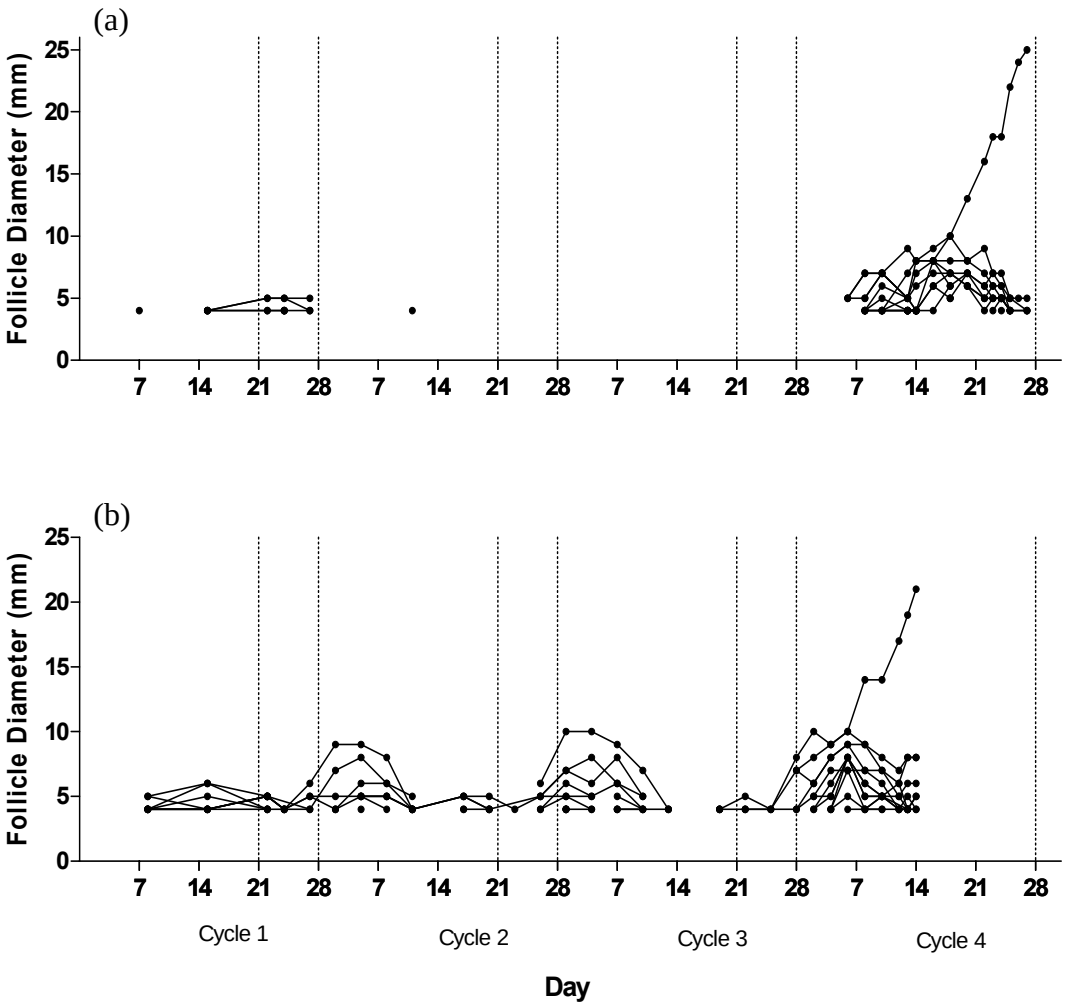

Figure 4.2: Representative follicular growth profiles during (a) continuous and (b) conventional OC use.

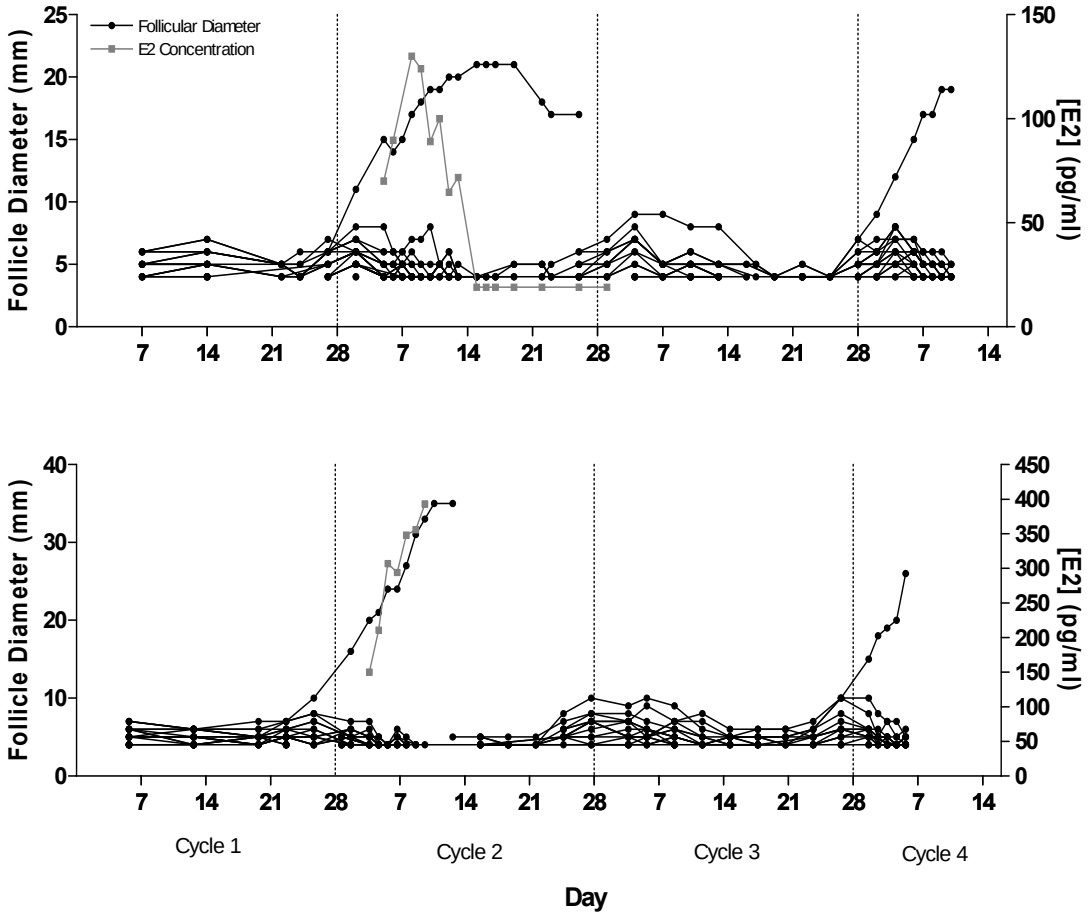

Figure 4.3: Growth profiles and serum estradiol concentrations of follicles that ovulated during adherent conventional OC use.

seven days prior to its ovulation. A corpus luteum was visualized ultrasonographically and the serum progesterone level observed on Day 6 post-ovulation was 0.79 ng/ml. The follicle which ovulated in the 35 μg EE (21)/250 μg NGM (21) group exhibited clinically normal serum estradiol concentrations for the pre-ovulatory period. The progesterone level observed on Day 8 post-ovulation was 3.5 ng/ml.

Endometrial thickness for the continuous OC groups remained stable from Cycles 1 to 3 (Figure 4.4). Decreases in endometrial thickness attributed to menstrual bleeding were observed during the hormone free interval of the conventional OC groups (Figure 4.4). No differences were observed between continuous and conventional dosing schemes for endometrial thickness ($p=0.57$) or endometrial pattern ($p=0.71$).

Women using continuous OC experienced more total days of flow and spotting ($p<0.05$) than women using conventional OC. No differences were observed between continuous and conventional regimens during Cycle 1 in the number of days of spotting, light flow or heavy flow ($p>0.46$). Women using continuous OC experienced more days of spotting in Cycle 2 and 3 ($p<0.006$), more days light flow in Cycle 2 ($p=0.03$) and tended to have more days of light flow in Cycle 3 ($p=0.09$). Women using continuous OC had fewer days of heavy flow in Cycle 2 ($p=0.01$) and tended to have fewer days of heavy flow in Cycle 3 ($p=0.08$). Differences were not observed in the number of days of spotting, light flow or heavy flow between the two OC formulations within a dosing scheme ($p=0.10$).

Cycle Four

One woman was excluded from analysis of Cycle 4 endpoints because she did not experience menses, or an ovulatory event, by Cycle 4 Day 63 (12 standard deviations from

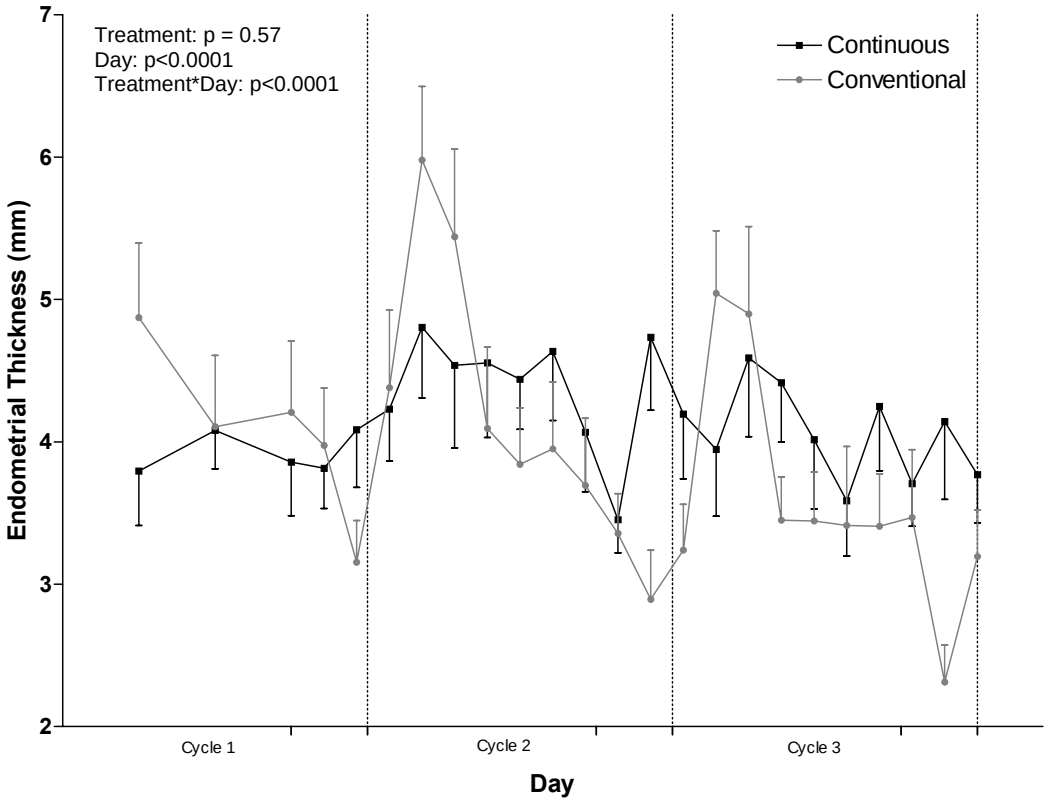

Figure 4.4: Endometrial thickness during Cycles 1 to 3 during continuous and conventional OC use.

the mean). Therefore, 35 women were included in the analysis of Cycle 4 endpoints following discontinuation of conventional OC (n=15) and continuous OC (n=20).

Cycle 4 endpoints are shown in Table 4.1. Selection took approximately three days longer ($p<0.001$) and dominance was manifest approximately two days later ($p < 0.001$) following discontinuation of OC than for natural cycles. Irrespective of their groups it took approximately five days longer for follicles to ovulate once OC were discontinued compared to natural cycle follicles ($p<0.001$).

In the conventional dosing group, 14 of the 15 women (93%) grew pre-ovulatory sized follicles (Table 4.1). Twelve of 14 follicles (86%) ovulated and the remaining two follicles (14%) formed a HAF. The woman who did not develop a pre-ovulatory follicle during Cycle 4 had developed a dominant follicle during the hormone free interval which reached pre-ovulatory diameter mid-cycle, persisted and regressed. In the continuous dosing group, all women developed a pre-ovulatory sized follicle (100%; Table 4.1). Thirteen of the 20 women (65%) developed a single pre-ovulatory follicle which ovulated and four women (20%) grew two pre-ovulatory sized follicles. Three of the four women with two dominant follicles had one follicle that ovulated and one follicle that regressed; the fourth woman formed a HAF and the remaining follicle regressed. One woman (5%) grew three pre-ovulatory sized follicles during Cycle 4; two of which ovulated and one regressed. The remaining two women developed a HAF.

Serum estradiol concentrations at a follicle diameter of 18 ± 1 mm were analyzed from 15 of the 17 women (88%) following continuous OC, 11 of the 12 women (92%) following conventional OC and 14 of the 18 women (78%) from natural cycles. Two women in the continuous OC group and one woman in the conventional OC group were

Table 4.1: Selection of the dominant and subordinate follicles (mean ± SEM), day to ovulation (mean ± SEM), estradiol concentrations, and follicle growth rates in natural cycles and in women following discontinuation of conventional and continuous OC regimens.

	Continuous OC	**Conventional OC**	**Natural Cycles**
Day of selection	13.1 ± 0.7[a]	13.0 ± 1.1[a]	8.6 ± 0.5[b]
Day dominance was manifest	12.1 ± 0.3[a]	12.7 ± 0.6[a]	9.6 ± 0.5[b]
Days to ovulation from Day 1 menses (natural) or discontinuation of OC	20.3 ± 0.7[a]	20.5 ± 1.0[a]	15.1 ± 0.5[b]
Days to ovulation from dominance	7.1 ± 0.5[a]	7.5 ± 0.5[a]	6.5 ± 0.3[a]
Dominant follicle diameter (mm)	14.2 ± 0.7[a]	14.0 ± 1.1[a]	12.3 ± 0.4[a]
Ovulatory diameter (mm)	21.4 ± 0.8[a]	21.2 ± 0.8[a]	21.8 ± 0.7[a]
# females developing pre-ovulatory (>14 mm) sized follicles (%)	20/20 (100)[a]	14/15 (93)[a]	15/15 (100)[a]
Follicle Growth Rate (mm/day)	1.1[a]	1.1[a]	1.4[b]
Estradiol concentration* (pg/ml)	172.5 ± 22.0[a]	178.3 ± 23.2[a]	105.6 ± 2.7[b]
Progesterone concentrations 6-9 days post ovulation (ng/ml)	12.3 ± 12.5[a]	16.8 ± 19.0[a]	--

*Serum estradiol concentrations were measured when follicles were 18 ± 1 mm in OC cycles and 18 ± 2 mm in natural cycles

[a,b] Within rows values with no common superscripts are different

Significance indicated at $p<0.05$

unable to provide a blood samples when the dominant follicle was 18 ± 1 mm and a stratified blood sampling schedule during the natural cycle study prevented the determination of serum estradiol concentrations at the comparable follicular diameter for four preovulatory follicles. Serum estradiol concentrations at 18 mm are shown in Table 4.1. No differences in estradiol concentrations were observed between OC formulations within a dosing scheme ($p>0.26$), or following discontinuation of continuous versus conventional dosing schemes ($p=0.74$); however, women discontinuing OC in the present study exhibited higher ($p<0.03$) estradiol concentrations than observed in the natural cycle reference cycles.

Blood samples taken six to nine days following ovulation were obtained from 15 of the 17 women (88%) in the continuous OC group and ten of the 12 women (83%) in the conventional OC group. Two women from each OC dosing scheme were unable to provide a blood sample. Serum progesterone levels are listed in Table 4.1. In the continuous OC group, nine of 15 women (60%) had clinically normal progesterone levels (i.e., 6-24 ng/ml); five of the 15 women (33%) had progesterone levels <6 ng/ml and the remaining woman (7%) had progesterone levels >24 ng/ml. In the conventional OC group, six of the ten women (60%) had clinically normal levels of progesterone; two (20%) had progesterone levels <6 ng/ml and two (20%) had progesterone levels >24 ng/ml. Progesterone levels did not differ between OC formulations within a dosing scheme ($p>0.60$) or between dosing schemes ($p=0.22$).

4.5 Discussion

Our hypothesis that a continuous OC regimen would suppress follicular development more effectively than conventional OC dosing was supported. The number

of follicles ≤4 mm in diameter did not differ between dosing schemes; however, continuous OC dosing suppressed the development of follicles >4 mm and did not permit dominant follicle development. The number of follicles, regardless of size, decreased slightly as the duration of OC use increased suggesting that the suppressive effects of OC on follicular populations increases with prolonged use. We suggest that a decrease in the number of developing follicles may be attributed to recruitment of fewer follicles into the growing pool due to increased hypothalamo-pituitary-ovarian axis suppression with each subsequent cycle of OC administration. Further research needs to examine the idea that increased duration of OC use is correlated to progressively increased suppression of follicular development, particularly during the hormone free interval.

All dominant follicles that developed during conventional OC use initiated their growth during the hormone free interval, consistent with a previous report that 86% of dominant follicles initiated their development during the hormone free interval[2]. A new follicular wave is initiated by a nadir in circulating FSH levels followed by two to three days of increase[19]. Fauser *et al.* have demonstrated that the lowest FSH concentrations of the HFI are from Day 1 to 3 and are followed by five days of increasing FSH levels[22]. Thus, we suggest that follicular development in OC cycles is manifest by this mechanism. Initiation of the next pill strip following the hormone free interval results in decreased FSH concentrations regardless of the presence of a dominant follicle[46]. However, it is clear that the decreased dependence of the dominant follicle on gonadotropins allows some follicles to continue developing to preovulatory diameter and to ovulate if the proper hormonal cues are present, while subordinate follicles undergo atresia[2,27,47-53]. Conventional dosing schemes appeared to provide cyclic increases in FSH during each hormone free interval resulting in follicular growth >4 mm, whereas continuous dosing schemes maintained

suppression of gonadotropins to basal levels resulting in suppression of most follicular growth >4 mm.

It was interesting that the number of follicles ≤4 mm in diameter did not differ between continuous and conventional OC regimens. Follicles ≤4 mm require basal levels of gonadotropins to sustain growth but are not yet fully responsive to cyclic changes in gonadotropins[54]. The total number of follicles ≤4 mm in diameter did not increase during the hormone free interval of conventional OC use [22]. We suggest that OC do not affect the basal level of gonadotropins required to allow follicles into the recruitable pool, but do suppress endogenous cyclic increases in gonadotropins. An environment with basal levels of gonadotropins would permit follicular development ≤4 mm in diameter but would suppress follicular development >4 mm, consistent with our observations.

Our hypothesis that follicles would take longer to ovulate following discontinuation of OC than following initiation of menses during natural cycles was supported. It took longer for selection, dominance to become manifest and ovulation, irrespective of the OC dosing scheme. It has been well established that follicles which develop during natural cycles are recruited and begin to grow during the late luteal phase[54]. The longer interval to selection, dominance and ovulation in follicles following cessation of OC may be due to follicular recruitment occurring before the first day of menses during natural cycles and after the comparable time point in follicles following discontinuation of OC. It is also possible that OC synchronize follicular wave development such that follicles are at an earlier stage of development and take longer to attain selection, dominance and ovulation than natural cycle follicles. The difference between the times of selection, dominance and ovulation was only a few days and could not explain the two to three month lag in fertility

following discontinuation of OC. We interpreted these data to mean that other mechanisms are responsible for the delay in fertility.

The serum estradiol levels were higher in women in the OC groups compared to women in natural cycles. However, the estradiol concentration from follicles developing during natural cycles and following OC cessation were both well within the clinically normal range, suggesting that follicles from both groups were physiologically similar. Further research is needed to determine if the elevated level of estrogen following discontinuation of OC negatively effects follicular development and oocyte competence. In a previous study, follicles which developed during natural and OC cycles had similar ultrasound image attributes which was interpreted to mean that follicles which developed during different hormonal conditions had similar physiologic function[21]. Further support for this hypothesis is by the observations of follicle development and ovulation in women during compliant use of several types of currently prescribed OC[27]. Similarities in the size of dominant follicles at selection, dominance and ovulation, as well as similarities in the interval from the manifestation of dominance to ovulation between natural cycle follicles and follicles which develop following discontinuation of OC further supports the hypothesis of similar physiologic function between these follicle types.

Clinically abnormal progesterone concentrations were observed following ovulation in approximately 40% of women following discontinuation of OC. Thus, reduced fertility following discontinuation of OC may be hypothesized to be due to luteal dysfunction following ovulation. This is only one possible explanation for the delay in fertility and further investigations are required before a cause and effect relationship could be established.

Factors other than anovulation in the cycle following OC cessation are likely responsible for the two to three month lag in the return to fertility. It is possible that previous OC use has negative residual effects on oocyte growth and development resulting in reduced oocyte competence. Novel image analysis techniques are currently under development to determine the physiologic status of a follicle and oocyte competence with a single ultrasound examination[21,55-57]. For example, in a recent study, ultrasound image attributes of subordinate follicles in cattle were correlated to oocyte competence[57]. Although image analysis is in an early stage of development, it demonstrates promise as a clinical tool to evaluate oocyte competence and physiologic status of a follicle in a non-invasive manner[56].

We expected to observe marked differences in endometrial thickness and endometrial pattern between continuous and conventional dosing schemes, particularly during the hormone free interval. However, both dosing schemes appeared to suppress endometrial development similarly. Each cycle of conventional dosing was typically associated with four to seven days of menstrual bleeding. We propose that similar endometrial patterns between dosing schemes was due to the high incidence of unscheduled bleeding observed during continuous dosing schemes. It has been suggested that cycle control during continuous regimens increases with prolonged use[5,8,17]; further research is needed to determine if increasing the duration of continuous dosing schemes would result in markedly different patterns of endometrial development and unscheduled bleeding episodes with these OC formulations.

In conclusion, continuous dosing schemes with 30 and 35 μg EE OC completely suppressed dominant follicle development better than the commonly used conventional

dosing scheme. All follicular development was initiated during the hormone free interval. Continuous OC dosing did not allow development of dominant follicles, thus reducing or eliminating the possibility of break through ovulation. We suggest that follicles which develop following discontinuation of OC have similar physiologic status to natural cycle follicles due to similarities in follicular diameter and the interval from dominance to ovulation. The brief delay in ovulation following discontinuation of OC can not explain lower conception rates following cessation of OC.

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Chapter 5

GENERAL DISCUSSION

Men and women have been preventing pregnancy and controlling their fertility through the use of many different forms of contraception for thousands of years[210]. By the late 1800's, many types of contraceptives were available in Canada, including but not limited to, abstinence, vaginal sponges, withdrawal, condoms, diaphragms, douching, prolonged lactation, the rhythm method, sterilization and abortion[210]. These methods of contraception have improved greatly since their introduction, and are still used currently by many Canadians.

It was not until the early 1960's that oral contraceptives (OC) were introduced to the Canadian market; however, birth control was not legalized until 1969[210]. Secular and religious stigmas against contraception were highly prevalent during the late nineteenth and early twentieth century. Many of these stigmas presently continue to influence the large-scale dissemination of information about different contraceptive choices such that the majority of Canadian women are unaware of most of their contraceptive options[211]. Canadians are interested in learning about new contraceptive choices; however, stigmas and personal embarrassment associated with sexual relations and contraception prevent many individuals from becoming informed.

The demand to manipulate human ovarian follicular development is on the rise. The number of women desiring contraception to inhibit pregnancy is increasing[212]. Ironically, the number of women requiring artificial reproductive technologies is also increasing[213]. A better understanding of the physiologic mechanisms responsible for the growth, development and ovulation of ovarian follicles will allow us to understand how different hormones, factors and proteins can alter reproductive function, and this information would allow us to develop more acceptable, efficacious contraceptive techniques or new artificial reproductive technologies to improve pregnancy rates during infertility treatment.

The studies contained in this thesis evaluated and compared the ultrasound image attributes of dominant ovarian follicles during natural and OC cycles; as well as, characterized changes in follicular and uterine development during the use and following discontinuation of two different OC dosing schemes. The findings of these studies provide the rationale for further examination and development of image analysis techniques as a clinical diagnostic tool and the development of more efficacious OC dosing schemes.

5.1 Follicular Development during Continuous and Conventional Oral Contraceptive Use

Administration of exogenous, synthetic estrogen in combination with different progestins causes atresia of follicles during the initial cycle of use, followed by suppression of follicular growth in subsequent cycles. An OC primary mechanism of action is to prevent follicular growth and ovulation in 99.9% of users when taken exactly as directed[109]. However, "real-life" failure rates are reported to be up to 80 times higher (3-8%) than rates

reported for perfect use[109,214-216]. The decrease in OC efficacy during "real-life" use is suggested to be due to many factors including: user non-adherence, not reading and/or misunderstanding product information, administration of OC with concomitant medication, gastrointestinal upset, high body mass index and inter/individual and intra-individual metabolism of the exogenous hormones.

User non-adherence accounts for approximately 20% of unintended pregnancies in the United States[217]. Poor OC adherence includes missing doses, taking doses in the improper order, sporadic use, not using back up contraception when indicated and initiating a new package early or late[217]. Up to 74% of women report missing ≥ 1 OC per 28-day cycle[175,176,178]. User non-adherence contributes to increased OC failure rates, bleeding irregularities and a three-fold increase in the risk of unintended pregnancy[178,214,215]. Furthermore, problems caused by non-adherence typically result in many women discontinuing OC and failing to use a substitute contraceptive, or adopting a less effective method[177]. Non-adherence is particularly prevalent during the transition from one pill package to the next, an occurrence that takes place 13 times per year with conventional dosing schemes[218]. The use of continuous dosing decreases the number of transitions per year which may lead to increased user adherence and a decreased risk of pill failure. We suggest that an OC ought to be developed that has the ability to accommodate "real-life" use, while maintaining an efficacy rate of 99.9%.

Follicular development during OC use is largely attributed to uninhibited gonadotropin suppression during the hormone free interval, such that by the end of the hormone free interval levels of FSH increase above the threshold for gonadotropin dependent follicular growth[160]. Reinitiation of the next pill strip results in decreases in FSH

and subsequent atresia of most follicles; however, if a dominant follicle is present it may continue development to pre-ovulatory diameter and ovulation[133,135,139,142,144,146,148,158], if the proper hormonal cues are present[219]. We used high-resolution transvaginal ultrasonography to serially track and compare ovarian follicular development during the administration of continuous versus conventional dosing schemes to determine if replacing the hormone free interval with active dosing pills would better suppress follicular development than conventional dosing schemes, and thus provide a more efficacious OC (Chapter 4). Continuous OC dosing schemes suppressed follicular development >4 mm better than conventional dosing schemes and completely prevented dominance from being manifest (Chapter 4). Conversely, conventional dosing schemes allowed eight follicles to manifest dominance and two dominant follicles to subsequently ovulate (Chapter 4). All dominant follicles initiated growth during the hormone free interval (Chapter 4). We interpreted this data to mean, that continuous dosing schemes provide a more effective contraceptive than conventional dosing, by completely suppressing follicles from attaining dominance and ultimately preventing ovulation and subsequent OC failure.

Follicles ≤ 4mm are not fully responsive to gonadotropins[9,57]. Therefore, increased levels of gonadotropins associated with missed doses would likely have little effect on follicles ≤4 mm. We observed very little follicular development >4 mm during continuous OC use compared to conventional use (Chapter 4). We interpreted this data to mean that more consecutive doses of OC could be missed before follicular growth was initiated during continuous OC dosing schemes compared to conventional dosing schemes. Further research needs to examine the number of consecutive doses that can be omitted during continuous dosing schemes before follicular development >10 mm is observed.

A new model of follicular dynamics in women suggests that women develop two to three follicular waves of follicles ≥4 mm during each menstrual cycle[56,149]. Waves of small antral (1-3 mm) follicles in bovine ovaries have been observed in association with circulating increases in endogenous FSH[220]. Furthermore, the dominant follicle can be identified retrospectively at 1 mm in diameter in bovine ovaries[220]. Since cattle are commonly used as human models it follows that follicular waves of small antral follicles may occur in humans[98]. Currently, we do not know if OC suppress waves of follicular development. Further studies should examine if OC suppress follicular waves, particularly waves of small antral follicles, as these follicles are generally not fully responsive to cyclic increases in gonadotropins. Residual effects of previous OC use on follicular wave development should also be examined. A further understanding of wave dynamics in humans may aid in the development of more efficacious OC that could target specific stages of follicular growth to prevent ovulation. A contraceptive of this nature would decrease a women's exposure to exogenous hormones by only administering hormones at key physiological time points, instead of throughout the entire ovarian cycle.

We have documented that ultrasound image attributes of dominant follicles during natural and OC cycles are similar during the mid-to-late growth phase (Chapter 3). Area under the curve, pixel heterogeneity and numerical pixel value were evaluated in the study. The only difference observed between the three image attributes was increased values of numerical pixel value for OC cycle follicles versus natural cycle follicles. Although numerical pixel value were greater in OC cycle follicles, the increased values were not high enough to indicate poor physiologic status as described in studies using animal models[101,221,222] . Furthermore, follicles which develop to pre-ovulatory diameter during adherent OC use have markedly similar growth profiles and serum estradiol-17β levels to

natural cycle follicles at similar stages of development[133,156]. Taken together, we interpret this data to mean that follicles that develop under the suppressive effects of OC have similar physiologic function and ovulatory capacity as natural cycle follicles at similar stages of development. Currently, we do not know why some pre-ovulatory sized follicles ovulate and others do not during OC use. Further research should examine if luteinizing hormone secretion differs between women who do, and do not ovulate, during OC use. Examining an association between luteinizing hormone secretion and ovulation would enhance our understanding of the physiologic mechanisms of action associated with OC use and aid in the development of an OC that could prevent ovulation in the entire population of women using OC.

The health and viability of oocytes which develop during OC use has not yet been examined. However, we suggest that oocytes which develop and ovulate during OC use have similar health and viability to natural cycle oocytes because pregnancies have been reported during OC use[223,224]. Additional research should examine the health and viability of oocytes that develop during compliant OC use. An appropriate animal model would need to be developed to examine the effects of exogenous synthetic estrogen and progestin on oocyte competence due to ethical constraints preventing research of this nature on human oocytes. Results of a recent study performed in cattle, were interpreted to mean that image attributes of the follicle wall are correlated to oocyte competence[222]. Further development of image analysis techniques could lead to a clinical tool that is capable of determining oocyte health and viability in a non-invasive manner during stimulation cycles for infertility treatment. A clinical tool of this nature would have the potential to decrease the costs associated with infertility treatment and increase pregnancy rates by ensuring only competent oocytes were utilized during clinical treatment.

5.2 Menstrual Regulation during Continuous and Conventional Oral Contraceptive Use

Menstruation is simply a by-product of ovarian function. OC were initially developed as menstrual regulators, and as such, had to induce menstruation once every 28-day cycle. Currently, most women believe that monthly menstruation is necessary to maintain reproductive health and that menstruation during OC use is due to the same physiologic mechanisms that induce menstruation during natural, spontaneous cycles[123,125]. Furthermore, women falsely believe that monthly menstruation during OC use confirms they are not pregnant and remain fertile[123,125].

Clinicians, researchers and anthropologists would argue that the current state of monthly menstruation is unnecessary to maintain reproductive health[132,185]. For example, primitive women menstruated 66% less than the typical modern-day woman. The increase in menstrual frequency in modern women is suggested to be due to evolutionary progression resulting in an earlier onset of menarche, later age of first birth, fewer pregnancies, shorter periods of lactation and a later onset of menopause compared to primitive women[119]. The increased frequency of menstruation in modern day women and the associated large fluctuations in hormones, is thought to be associated with the development of many illnesses, including menstrual morbidity, endometriosis, anemia, uterine fibroids and cancers of the reproductive organs[184]. It is suggested that the constant level of exogenous hormones provided by continuous OC dosing schemes and the associated state of amenorrhea is a healthier alternative to monthly menstruation because it would result in a decrease of many illnesses associated with hormone fluctuations and menstruation.

Women's attitudes towards inhibiting menstruation are age and culture dependent[116,183]. The percentage of women desiring amenorrhea increases with increasing age[116]. Most women (81%) of Western or Western-oriented cultures desire a change to the duration or frequency of menses[116,123,125,182], with approximately 40% of women preferring not to menstruate[116,182,183]. Conversely, the majority (42- 71%) of African and Asian women would prefer to menstruate on a monthly basis, with only approximately 18% of these women preferring to inhibit menstruation[183]. The most common reasons for wanting to suppress menstruation include: convenience, decreased menstrual related complaints, better hygiene, higher quality of life and less blood loss[182]; whereas, the desire to menstruate on a monthly basis appeared to be associated with strong cultural beliefs. For example, most Nigerian women (81%) stated that monthly menstruation rids the body of bad blood[183].

Cycle control, defined as the occurrence of spotting or breakthrough bleeding is one of the key factors contributing to the acceptability of a contraceptive method[225]. Poor cycle control is highly correlated to discontinuation of OC[226]. Studies designed to compare bleeding characteristics of women using continuous versus conventional dosing schemes reported higher dropout rates in the continuous dosing group due to a higher incidence of unscheduled bleeding[112,113,122]. Interestingly, most women who have administered OC in a continuous manner report preferring the inconvenience of unscheduled bleeding to monthly scheduled withdrawal bleeding[112,113,115,122,123,227].

We reported that women who used conventional dosing schemes experienced fewer days of unscheduled bleeding than women using continuous dosing schemes, during the first three cycles of OC use (Chapter 4). Cycle control during continuous OC use is

reported to increase with increased duration of use, such that breakthrough bleeding is comparable between continuous and conventional dosing schemes containing ethinyl estradiol (EE) and levonorgestrel by approximately 9 months of use[112,113,124]. We did not observe a decrease in unscheduled bleeding as the duration of OC use increased; however, the study design only permitted observation during the initial three cycles of OC use. Further investigations should examine the effects of prolonged continuous OC use on bleeding patterns and health.

We did not observe a difference in cycle control during continuous or conventional dosing between levonogestrel and norgestimate (Chapter 4). Cycle control is related to the half-life of the progestin; generally, the longer the half-life the greater the cycle control[110]. We suggest that similarities in the half-life (15 hours) of levonorgestrel and norgestimate are responsible for the similarities we observed in cycle control during both continuous and conventional OC dosing schemes (Chapter 4)[110]. The dose of EE does not appear to effect cycle control during continuous OC dosing schemes[112,124] (Chapter 4); however, few studies are available to compare the effects of the dose of EE on cycle control during continuous OC use. It appears that continuous administration of exogenous EE and progestin during continuous OC dosing schemes maintains continual endometrial suppression and good cycle control which ultimately over rides the need for high doses of EE.

The duration of continuous OC use and the type of progestin administered appears to effect the time required to induce amenorrhea. Progestins are reported to directly exert their effects at the uterus; therefore, differences in bioavailability, serum half-life and relative binding affinity can alter the progestin's effect on the uterus[228]. The histologic

appearance of the endometrium during progesterone administration resembles that of an early pregnant uterus; the epithelium is thin and atrophic without mitosis, few blood vessels are present, the stroma is thick and decidualized, and the glands are poorly developed[228].

Comparison of studies which examined cycle control during continuous OC use with different OC formulation containing the same dose of EE but different progestins reveal that desogestrel and gestodene provide the greatest cycle control of all progestins tested. Spotting and break through bleeding occurred less frequently with desogestrel and gestodene compared to levonorgestrel. Furthermore, amenorrhea was induced in 81% of women within two consecutive 28 day cycles of 30 μg EE / 75 μg gestodene and 52-75% of women were amenorrheic within two consecutive 28 day cycles of 30 μg EE / 150 μg desogestrel[115,117]. Only 42% of women were amenorrheic within 12 consecutive 28 day cycles of 30 μg EE/ 150 μg levonorgestrel[112,227]. Furthermore, we did not observe amenorrhea during three 28-day cycles of continuous OC dosing of 30 μg EE/ 150 μg levonorgestrel or 35 μg EE/ 250 μg norgestimate (Chapter 4). Superior cycle control with gestodene and desogestrel compared to levonorgestrel and norgestimate is likely due to gestodene's and desogestrel's higher affinity for progesterone receptors, a lower affinity for androgen receptors, a higher selectivity of action, and a higher potency at the level of the endometrium[228].

5.3 Return to Fertility following Discontinuation of Oral Contraceptives

Oral contraceptives are the reversible contraceptive of choice of women in industrialized nations[109]. Approximately half of Canadian women (49%) use OC as their method of contraception[211]. Due to the high popularity of OC it is essential to determine if

OC have residual effects on ovarian function following their discontinuation. It has been established that women who discontinue OC take two to three months longer to conceive than women who discontinue other types of contraceptive therapies (i.e., intrauterine device, diaphragm, or condom)[195-204]. Few studies have examined the physiologic mechanisms responsible for the delay in fertility following discontinuation of OC.

We used highly sophisticated transvaginal ultrasonography to serially monitor ovarian follicular development following discontinuation of both continuous and conventional dosing schemes (Chapter 4). Most women ovulated following discontinuation of OC, suggesting that the two to three month delay in the return to fertility is not due to anovulation (Chapter 4). Furthermore, we suggest that follicles which develop following discontinuation of OC have similar physiologic function to follicles which develop during spontaneous natural cycles due to similarities in follicle diameter at selection, dominance and ovulation as well as the time to ovulation once the follicle manifest dominance (Chapter 4). Pre-ovulatory follicles that develop following discontinuation of OC are suggested to have equal potential to natural cycles to create a viable conception and pregnancy.

Interestingly, follicles which developed following discontinuation of OC took slightly longer to be selected, manifest dominance and ovulate, compared to follicles which develop during spontaneous natural cycles (Chapter 4). We suggest that this delay is due to wave synchronization of follicles during OC administration. During spontaneous natural cycles a cohort of follicles is recruited for growth and development during the late luteal phase, such that gonadotropin dependent follicles are commonly observed on Day 1 menstruation. The delay to ovulation following discontinuation of OC may be due to

follicular recruitment occurring before the first day of menses during natural cycles and after Day 1 in follicles following discontinuation of OC (Chapter 4). The delay in ovulation was not great enough to explain the two to three month delay in fertility. Further research is needed to determine the exact physiologic mechanisms associated with the delay in fertility.

5.4 Development of Novel Methods of Contraception

The once daily administration scheme of OC has a number of disadvantages, such as fluctuations of hormone plasma levels which can increase the incidence of hormone related side effects and increase non-adherence rates[229]. Many women regard the adherence required by daily OC to be a drawback to the use of OC and would prefer a contraceptive with a longer dosing interval[229]. Women report that the ideal contraceptive would be: efficacious, safe, easy to use, have few side effects and provide excellent cycle control[230]. A weekly transdermal patch containing norelgestromin and EE; (Ortho Evra: Ortho-McNeil, Raritan, NJ), and a monthly vaginal ring containing etonogestrel and EE (NuvaRing; Organon, West Orange, NJ) have been introduced within the past 5 years in response to women's requests[230].

The contraceptive transdermal patch delivers 20 μg EE and 150 μg norelgestromin to the bloodstream every 24 hours through a 20 cm^2 transdermal patch[231]. The patch is applied once a week for three consecutive weeks followed by one patch-free week[158]. Transdermal contraceptive patch use is reported to have higher adherence rates and greater cycle control while maintaining or increasing contraceptive efficacy compared to OC[158,232,233] .

The vaginal ring delivers 15 μg EE and 120 μg etonogestrel per day in a continuous manner such that daily peaks in serum concentrations are only observed at the beginning of each cycle compared to daily peaks observed during OC use[230]. A cycle consists of wearing the ring vaginally for three consecutive weeks followed by removal of the ring for one week[230]; however, the vaginal ring maintains its efficacy for up to seven weeks of continuous use[234,235].

New contraceptive methods such as the transdermal patch and vaginal ring are only the beginning of new contraceptive choices. Both administration schemes provide patient acceptability equal to or greater than OC administration, suggesting that women are open to utilizing new, novel administration schemes as long as the schemes are easier to use, provide equal or greater efficacy, have few side-effects and maintain cycle control equal to or greater than OC. Furthermore, bioavailability of the exogenous hormones is increased with these administration schemes because gastrointestinal metabolism and the first pass effect are avoided resulting in decreased doses required to maintain ovarian suppression and thus exposure to exogenous hormones is decreased[229].

Modern day women are interested in new, novel contraceptive therapies; however, many modern women do not completely understand their own reproductive function, nor do they realize the number of contraceptive choices available to them[211]. Health care professionals must ensure women are informed about their own reproductive function, contraceptive methods and how each contraceptive method effects reproductive functioning. "Real life" OC use appears to decrease contraceptive efficacy. The studies in this thesis are interpreted to mean that follicles that develop during OC use have similar physiologic function to natural cycle follicles, such that follicles that develop during OC

use are capable of ovulating resulting in subsequent pregnancy and pill-failure, if the secondary contraceptive characteristics of OC are not maintained. New contraceptive methods ought to be developed to maintain extremely low failure rates during "real life" use. Continuous OC dosing schemes, vaginal rings and transdermal patches all appear to be superior methods of contraception with respect to cycle control, ease of use, efficacy and associated side-effects compared to conventional dosing schemes. Finally the return to fertility following discontinuation of OC does not appear to be due to lack of anovulation. Further investigations should examine the physiologic mechanisms associated with the delay in fertility following cessation of OC.

5.5 Overall Conclusions

The following hypothesis was not supported.

1) Taken together, there was inadequate support for the hypothesis that ultrasound image attributes of human ovarian dominant follicles from natural cycles quantitatively differed from follicles which developed under the suppressive effects of OC.

The following hypotheses were supported:

2) Continuous OC dosing schemes better suppress follicular development than conventional OC dosing schemes due to greater hypothalamic-pituitary-ovarian axis suppression.
3) Follicles take longer to ovulate following discontinuation of OC compared to natural cycle follicles.

5.6 General References

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