A Role for Androgens in HCG-Induced Ovulation

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A ROLE FOR ANDROGENS IN hCG-INDUCED OVULATION

by

David E. Stude

A Thesis Submitted to the Faculty of the Graduate School of Loyola University of Chicago in Partial Fulfillment of the Requirements for the Degree of Master of Science

November

1979
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ACKNOWLEDGMENTS

I am indebted to my academic advisor and director of the reviewing committee, Dr. John Peluso, Ph.D., for his encouragement, patience, skillful assistance, and friendship the past couple of years while completing my graduate requirements. I am also grateful to other members of the thesis committee, Dr. G. Lopez, Ph.D., and Dr. A. Rotermund, Ph.D., for their time and patience in evaluating my candidacy for the Master of Science degree.

I would like to acknowledge special thanks to my wife and best friend, Mary, primarily for her loving and consistent support, and also for her excellent secretarial skills, without which I may not have completed this in time. Also, special thanks to my parents and friends for their understanding and encouragement when it counted most.

As in the past, I thank God I have had this opportunity and am in a position to serve and help others. With him, all is possible, and the possible worthwhile.
VITA

The author, David Edward Stude, is the son of Edward Andrew Stude and Dolores Jean (Kroeger) Stude. He was born September 6, 1955, at Delnor Hospital in St. Charles, Illinois.

He completed his elementary education at St. Mary's Grammar School in Elgin, Illinois, and his secondary education at St. Edward Central Catholic High School, also in Elgin, where he graduated in June of 1973.

In September, 1973, he began his undergraduate studies at St. John's University, and in May, 1977, received the degree of Bachelor of Science with a major in Biology. While attending St. John's University, he served as a resident hall counselor, co-president of the S.J.U. Judo Team, and elected to represent the student body on the Committee for the Revision of Athletic Policy at the University.

In September, 1977, he was granted a graduate teaching assistantship in Biology at Loyola University of Chicago. On June 9, 1979, he was married to Mary Ann (Stock) Stude in Minneapolis, Minnesota. In May, 1980, he was awarded the Master of Science degree in Biology.
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ABSTRACT

The effects of the antiandrogen Flutamide (FL) on human chorionic gonadotropin (hCG) induced ovulation was studied. hCG, given 75 h after pregnant mare's serum gonadotropin (PMSG), induced ovulation in 77% of the rats with an ovulation rate of 9.9 ± 1.3 ova/ovulating rat. In the non-ovulatory follicles, the cumulus oophorus was dispersed and the ova had resumed meiosis. FL treatment, given 3 h prior to hCG, reduced the ovulation rate by 50% (p < 0.05) and prevented cumulus dispersion and ova maturation (p < 0.05). In a subsequent study, 16 PMSG-primed rats were pretreated with FL and autopsied 8 h after hCG. One ovary was then assayed for steroid content, while the other ovary was used for the histochemical localization of Δ5 - 3B-hydroxysteroid dehydrogenase (3B-HSD). In the ovaries of control and FL-treated rats, hCG reduced estradiol content, did not alter testosterone content, and increased progesterone content (p < 0.05). However, FL blocked the hCG-induced appearance of 3B-HSD activity in the granulosa cells of many preovulatory follicles which could result in a local decrease in progesterone. Thus, androgens, presumably synthesized in the theca interna in response to hCG, may play an important role in the process of follicular rupture and ova maturation. The mechanism by which androgens affect these
functions appears in part to be related to the regulation of 3B-HSD activity.
INTRODUCTION

A finite number of primordial germ cells (ova) enter as the mammalian ovary develops prenatally. The ova soon become associated with flattened epithelial cells, forming the primordial follicles and remain in a quiescent, non-proliferating state. Follicle growth begins when the primordial follicle enters the proliferating pool and is characterized by the formation of a granulosa cell layer, successive granulosa cell mitosis, and the formation of a theca layer which becomes organized from surrounding stroma cells. Once the follicle begins to grow and differentiate it is destined to either ovulate or become atretic. Only a small percentage of follicles develop into mature, preovulatory follicles, also called Graafian follicles. These preovulatory follicles, at a critical time, possess the capacity to ovulate in response to the surge of gonadotropic hormones secreted from the anterior pituitary gland.

The overall importance of androgens in reproductive physiology has been documented (Jones, 1978), but only recently has a role for androgens in the process of ovulation (follicular rupture) been postulated (Mori et al., 1977). In their study, rabbit antiserum to test-
Oxysterone injected intravenously (i.v.) along with hCG reduced the number of tubal ova released in intact or hypophysectomized immature rats primed with PMSG. In addition, they found that testosterone restored ovulation rate when administered along with antiserum to progesterone. Furthermore, it was demonstrated that androgens are capable of increasing progesterone production in granulosa cells in vitro (Hillier et al., 1977; Lucky et al., 1977; Schomberg et al., 1978; Haney and Schomberg, 1978).

These studies suggest a role for androgens in the process of follicular rupture and led to a detailed investigation in order to more clearly define "A Role for Androgens in hCG-Induced Ovulation."
DEVELOPMENT OF THE PREOVULATORY FOLLICLE

Factors involved in the initiation of follicular growth are not well understood but do not seem to involve gonadotropic hormones (Nakano et al., 1975). Once the follicle enters the proliferating pool and has grown to four or more layers of granulosa cells, Follicle-stimulating hormone (FSH) appears to be essential for proper development (Ryle, 1972; Goldenberg et al., 1972). Following the formation of a layer of theca cells, both FSH and LH (Luteinizing hormone) are necessary for normal follicular proliferation and differentiation (Goldenberg et al., 1973; Nakano et al., 1975; Schwartz, 1974).

Estrogen is essential for the early stages of follicular growth (Harman et al., 1975) and subsequent differentiation of granulosa cells (Rao et al., 1978). Furthermore it has recently been demonstrated that estrogen can increase the content of its own receptor and increase the responsiveness of follicles to FSH (Richards, 1975; 1978).

Following the early stages of follicular growth which seems to be largely dependent on the presence of estrogen (Harman et al., 1975), FSH appears to be most responsible for the succeeding stages of follicular growth (Goldenberg et al., 1972; Ireland and Richards, 1978). In the presence of estrogen, FSH increases the content of FSH receptors.
within the granulosa cell layer but does not increase the number of FSH receptors/granulosa cell (Richards et al., 1976). FSH also increases the LH receptor content of granulosa cells by increasing the number of LH receptors/cell (Richards, 1978). In addition, FSH tends to promote antrum formation in the final stages of follicular maturation (Goldenberg et al., 1972).

The responsiveness of follicles to gonadotropic hormones, therefore, depends upon the development of receptor sites. Granulosa cells of most follicles possess FSH receptors (Richards et al., 1976) but only granulosa cells of mature, preovulatory follicles possess FSH and LH receptors (Kammerman and Ross, 1975; Richards et al., 1976; Peluso et al., 1976, 1977).

Follicular growth and maturation leads to the development of mature, preovulatory follicles. These follicle(s) now possess a sensitive gonadotropin receptor population and morphological features making them capable of ovulating in response to the surge of gonadotropic hormones.
It has been demonstrated previously that gonadotropic hormones, released from the anterior pituitary gland, play an essential role in follicular growth, maturation, and ovulation. Most investigators believe that LH is the primary hormone involved in promoting ovulatory responses (Espey, 1978b), provided the species under consideration is sufficiently primed with FSH in the initial stages of follicular growth and differentiation. Previous investigations have demonstrated the ability of LH to independently stimulate ovulation of mature, preovulatory follicles (Schwartz, 1974; Schwartz et al., 1975), but the possibility exists that LH and FSH function together to promote ovulation.

After the release of gonadotropins from the adenohypophysis into the circulation, both FSH and LH bind to specific receptors in ovarian tissue. LH binds to receptors in the theca and granulosa cell layers while FSH binds only to receptors located in the granulosa layer in large, preovulatory follicles (Richards et al., 1976). After gonadotropins bind to their receptors, the initial effect appears to be an increase in adenylate cyclase activity (Lamprecht et al., 1973). Adenylate cyclase activation promotes the formation of intracellular cAMP, resulting in an increase in the concentration of this nucleotide in ovarian tissue (Kolena and Channing, 1972; Nilsson et al.,
1975; Zor et al., 1972). Ovarian cAMP content increases shortly after the LH surge, suggesting a causal relationship (Nilsson et al., 1974). The surge of gonadotropic hormones at proestrus may not affect small follicles at all, but may induce ovulation in those preovulatory follicles which possess both LH and FSH receptors and are therefore most responsive. Thus, the gonadotropin surge stimulates an increase in intracellular cAMP which may subsequently be involved in the regulation of three primary events associated with the ovulatory process: 1) the resumption of meiosis within the oocyte (preovulatory oocyte maturation), 2) steroidogenesis within the preovulatory follicle, and 3) follicular rupture (Rondell, 1974; Lipner and Greep, 1971; Hamberger et al., 1978; Bullock and Kappauf, 1973; Zor et al., 1972; Tsafiri, 1978; Bjersing, 1978).
OOCYTE MATURATION

Once the primordial germ cells enter the fetal ovary, meiosis begins and progresses from leptotene to diplotene of the first meiotic division. Shortly after, meiosis becomes arrested in the prophase or dictyate stage ("first meiotic arrest") (Tsafriri, 1978). A few hours prior to follicular rupture, there is a resumption of oocyte meiosis within mature, preovulatory follicles, and this is accompanied morphologically by breakdown of the oocyte nucleus, the germinal vesicle. This process of preovulatory oocyte maturation begins at the dictyate stage of the first meiotic division and progresses to metaphase of the second meiotic division, ending in the "second meiotic arrest" and preparing the oocyte for fertilization after follicular rupture has occurred (Tsafriri, 1978).

A. Gonadotropins and Oocyte Maturation: In Vivo Studies

The surge of gonadotropic hormones on proestrus is necessary for the resumption of meiosis within the oocyte, an event preceding follicular rupture and coinciding with behavioral estrus in the rat (Odor, 1955; Tsafriri and Kracier, 1972). In addition, after the injection of gonadotropic hormones, germinal vesicle breakdown (GVBD) occurred after two to three hours in the rat (Vermeiden and Zeilmaker, 1974) and hamster (Iwamatsu and Yanagimachi, 1975), and after eighteen hours in the pig.
as well as follicular rupture, was prevented when the LH surge was blocked by sodium pentobarbitone (Ayalon et al., 1972). Similar results were obtained when the LH surge was neutralized by the administration of an antiserum to the B-subunit of LH (Tsafriri et al., 1976a) or hypophysectomy (Vermeiden and Zeilmaker, 1974).

During the preovulatory gonadotropin surge, FSH, as well as LH, is secreted (Butcher et al., 1974a). However, there is much debate about which hormone is responsible for the resumption of meiosis within the oocyte, if indeed a specific role for each hormone in the process exists. The possibility exists that both gonadotropins act synergistically to initiate ova maturation (Tsafriri, 1978).

Purified FSH is capable of inducing ovulation on its own (Nuti et al., 1974; Schwartz et al., 1975), but some regard it as merely "a pharmacological effect" (Tsafriri, 1978). LH appears to be the most important gonadotropic hormone in the induction of oocyte maturation in vivo (Tsafriri et al., 1976a). More work is needed, however to understand completely the role of gonadotropins in the process of preovulatory oocyte maturation and other related events. In vivo studies provide a valuable tool that aid in our understanding of causal relationships, yet the variability that exists when these types of investigations are employed could make results difficult to
interpret or fully comprehend. Conversely, our understanding of the events involved in the process of ovulation can be clarified by examining data from studies performed in vitro, where variability can be better controlled and specific roles can be assigned more easily.

B. Oocyte Maturation: In Vitro Studies

It was demonstrated that cumulus cell-free (denuded) rabbit oocytes were capable of resuming meiosis spontaneously in vitro, in the absence of gonadotropic stimulation (Pincus and Enzmann, 1935), and this has since been confirmed in other mammalian species (Schuetz, 1974; Channing and Tsafriri, 1977; Neal and Baker, 1973). Recent studies indicate that the ability of the oocyte to mature upon release from the follicle is acquired only at late stages of follicular maturation. In adult (Erickson and Sorensen, 1974) and immature mice (Sorensen and Wassarman, 1976), pigs (Tsafriri and Channing, 1975a) and hamsters (Iwamatsu and Yanagimachi, 1975), the ability of the oocyte to resume meiosis in vitro and to reach metaphase II is possible only if the ova is fully grown and contained in medium or large antral follicles (Neal and Baker, 1973, 1974, 1975).

When rat oocytes are cultured within preovulatory follicles, oocyte maturation is dependent on stimulation by gonadotropic hormones (Tsafriri et al., 1971; Thibault
et al., 1975). Large Graafian follicles, explanted at proestrus from adult cycling rats, contain oocytes which will spontaneously resume meiosis only if the follicle is removed after the endogenous LH surge (Ayalon et al., 1972). Similar observations have been made using immature rats primed with PMSG (Hillensjo et al., 1974; Hillensjo, 1976). Administration of LH, hCG, immunochemically pure FSH, or prostaglandin E2 (PGE2) can induce resumption of meiosis in the rat oocyte of follicles explanted on the day of proestrus, as well as simultaneously increasing cAMP content (Tsafriri et al., 1971, 1972, 1973; Hillensjo, 1976).

Which gonadotropic hormone is most important in the resumption of meiosis within follicle-enclosed oocytes in vitro may be species specific, but in all mammals both FSH and LH play a key regulatory role. LH has previously been shown to be capable of inducing germinal vesicle breakdown (GVB) and completion of at least the first meiotic division in rat oocytes (Tsafriri et al., 1971; Tsafriri et al., 1973). Other investigations, however, clearly indicate that FSH is more effective in inducing preovulatory oocyte maturation in follicle-enclosed oocytes in vitro. Neal and Baker (1975) showed that FSH was more effective than LH in inducing ova maturation in twenty-seven day-old albinoid mice primed with PMSG, regardless of the dose administered. In this study, the FSH preparation was examined for purity and did not contain LH-like activity in quan-
tities sufficient to induce ova maturation. Similarly, Tsafriri et al. (1976a) have demonstrated FSH stimulated ova maturation (GVB) in oocytes from three month old rats in vitro. In this study, antiserum to the B-subunit of LH was used simultaneously with FSH, thus suggesting the FSH alone was capable of inducing ova maturation. Kaplan et al. (1978) demonstrated that in adult proestrus rats, LH accelerated the process of ova maturation in vitro, as measured by GVB within the cumulus oocyte complex. This could mean that other factors, such as FSH, could be responsible primarily for the induction of oocyte maturation (Neal and Baker, 1975; Tsafriri et al., 1976a) while LH could accelerate the process as well as promote steroid synthesis, preparing the follicle for rupture (Neal and Baker, 1975; Smith and Tenney, 1979; Lipner and Greep, 1971). Thus, the control of the induction of ova maturation is a complex event and cannot be interpreted as being the sole responsibility of FSH at this time.

C. Role of Steroids in Oocyte Maturation.

The role of steroids in the preovulatory maturation of oocytes has been investigated. Estrogen administration appeared to stimulate and complete the first meiotic division in denuded oocytes (Smith and Tenney, 1979), but this has not been confirmed. Using estrone antiserum
in vivo, Mori et al. (1979) demonstrated that an LH-induced production of estrogen results in a local inhibitory effect on the resumption of meiosis within oocytes from twenty-two day old rats primed with PMSG.

Progesterone may mediate the action of LH on oocyte maturation, since it is known that the preovulatory surge of gonadotropins results in an increase in ovarian progesterone content, and that progesterone accelerates the process of ova maturation in rabbit oocytes (Bae and Foote, 1975). Most evidence indicates, however, that progesterone and other steroids are not involved in the induction of oocyte maturation. Addition of progesterone or estrogen to culture media did not induce the maturation of follicle-enclosed rat oocytes (Tsafriri et al., 1972). Also, LH-induced oocyte maturation was not affected when steroidogenesis was inhibited (Lieberman et al., 1976).

It appears, therefore, that the effect of gonadotropins on oocyte maturation is not mediated through steroid synthesis. Steroids, though, could be involved in preparing the oocyte for the preovulatory resumption of meiosis, since the mammalian oocyte is exposed to high levels of steroids prior to the gonadotropin surge (Edwards, 1974; McNatty et al., 1975; Chang et al., 1976).

D. The Cumulus Oophorus and Oocyte Maturation

The role of the surrounding cumulus cells in the
preovulatory maturation of oocytes is controversial. Initially, it was believed that the cumulus cells provided a beneficial effect for the maturing oocyte in the human (Kennedy and Donahue, 1969) and in the mouse (Cross and Brinster, 1970). This effect has been recently shown to be related to the specific energy requirements of the denuded oocyte maturing in vitro (Donahue and Stern, 1968). When pyruvate is added to the culture medium containing denuded oocytes of the mouse (Biggers et al., 1967), rat (Niwa and Chang, 1975), hamster (Iwamatsu and Yanagimachi, 1975), and pig (McGaughey, 1976), maturation progressed readily and was indistinguishable from maturation of oocytes enclosed in the cumulus mass.

The cumulus oophorus may actually have an inhibitory effect on the resumption of oocyte meiosis in the rat (Dekel and Beers, 1978). Chang in 1955 first demonstrated that rabbit follicular fluid (FFL) had an inhibitory effect on ova maturation and these observations were later confirmed in the pig (Tsafriri and Channing, 1975b) and in the hamster (Gwatkin and Anderson, 1976). Investigations involving the co-culture of liberated oocytes along with follicular constituents have recently been performed. Tsafriri and Channing (1975b) have demonstrated and inhibitory substance from granulosa cells, eventually secreted into follicular fluid, and capable of arresting meiosis in porcine oocytes in the dictyate stage. It
was further suggested that gonadotropins were capable of overriding its inhibitory influence, and the mode of action of this inhibitor could be mediated by cAMP (Ayalon et al., 1972; Dekel and Beers, 1978). Recently, an inhibitor of oocyte maturation isolated from porcine follicular fluid (FFL) has been partially characterized (Tsafriri et al., 1976b). Its inhibitory action could not be destroyed by heating to 60°C or repeated freezing and thawing, could not be extracted with charcoal, and had a molecular weight of about 2,000 daltons.

This inhibitor of ova maturation, partially purified and characterized in the pig (Tsafriri et al., 1976b) and in the cow (Gwatkin and Anderson, 1976), does not appear to be species specific since FFL from the pig interferes with ova maturation in the mouse (Gwatkin and Anderson, 1976). The addition of ovine LH to culture medium partially reversed the effect of FLL inhibitor (Tsafriri et al., 1976a, 1976b; Gwatkin and Anderson, 1976), supporting the hypothesis that FFL inhibitor and LH are both involved in the control of preovulatory oocyte maturation.

Regarding the mode of action of gonadotropins on ova maturation, LH could be responsible for a loss of communication (contact) between the oocyte and the surrounding cumulus cells. Cumulus cells and the oocyte are joined by gap junctions during follicular growth and
maturation (Zamboni, 1974; Dekel et al., 1976; Peluso et al., 1979) and LH terminates this junctional complex (Dekel et al., 1978). Preceding the surge of gonadotropic hormones, the resumption of oocyte meiosis could be inhibited by the presence of cAMP (Bauminger et al., 1978; Dekel et al., 1978, 1979; Gwatkin and Anderson, 1976), since cAMP and cyclic nucleotide phosphodiesterase inhibitors were both shown to inhibit oocyte maturation (Dekel and Beers, 1978). cAMP could be produced by the oocyte maturation inhibitory factor described by Tsafriri and Channing (1975b) and Tsafriri et al. (1976b), or could be responsible for the production of this factor. LH, then, could be responsible for terminating communicative channels between the cumulus cells and the oocyte, resulting in removal of an inhibitor and the subsequent resumption of meiotic processes.

It appears, therefore, that ova maturation is under the influence of several stimulatory and inhibitory factors and that some of their actions may be mediated through secondary messengers such as cAMP. In the presence of gonadotropins just prior to follicular rupture, the oocyte is capable of resuming meiosis, possibly as a result of previous exposure to steroid hormones. The gonadotropin surge, therefore, appears to be responsible for overcoming all inhibitory influence(s) on ova maturation, allowing the events necessary for ovulation to proceed normally.
and preparing the ovum for rupture and eventual fertilization.
PREOVULATORY STEROID SYNTHESIS

In addition to stimulating preovulatory oocyte maturation, the gonadotropin surge stimulates the synthesis and secretion of steroid hormones within the preovulatory follicle as well as the morphological alterations which eventually lead to rupture of the follicle. Evidence suggests that the rate of progesterone, 17β-estradiol, and testosterone synthesis and/or secretion markedly changes during the preovulatory period. Steroid synthesis is a dynamic event occurring only within follicles that have developed appropriate receptor populations.

Throughout the cycle of mammalian species, there is a characteristic pattern of gonadotropin secretion and a corresponding pattern of ovarian steroid hormone production and secretion (Butcher et al., 1974b; Hamberger et al., 1978). Prior to follicular rupture on the afternoon of proestrus, there is a significant increase in serum LH and FSH concentrations, and both of these hormones bind to specific receptors on specific cell types in the ovary, resulting in characteristic preovulatory steroid hormone levels. LH can bind to receptors on both theca and granulosa cells of large preovulatory follicles (Channing and Kammerman, 1974). At the level of the theca interna, LH stimulates the formation of androgen, primarily androstenedione (Bjersing, 1967). These androgens are then transferred across the basement membrane of the follicle.
to the granulosa cells where androstenedione is converted
to testosterone, and testosterone is converted to estro-
gen (Bjersing, 1967; Armstrong and Papkoff, 1976). When
LH binds to granulosa cell receptors, progesterone pro-
duction is stimulated (Hamberger et al., 1978). This
effect could be mediated by prostaglandin E\textsubscript{2}, since the
addition of PGE\textsubscript{2} \textit{in vitro} stimulates progesterone pro-
duction by human granulosa cells (Henderson and McNatty,
1975). FSH can bind only to granulosa cells of pre-
ovulatory follicles (Zelenik et al., 1974; Richards et al.,
1976; Peluso et al., 1976), and after binding, sti-
mulates the aromatization of androgens. According to the
modified two-cell-type theory for preovulatory estrogen
production, thecal cells can also aromatize androgens
to estrogen (Bjersing, 1967). Thus, in the presence of
stimulatory concentrations of gonadotropins, thecal and
granulosa cells work synergistically to promote the pre-
ovulatory formation of steroid hormones.

It has been suggested that preovulatory steroido-
genesis, primarily progesterone synthesis, is important
in the process of ovulation, specifically follicular
rupture (Takahashi et al., 1974; Kendle et al., 1975).
This was based on the observation that cyanoketone inter-
fers with progesterone synthesis and can inhibit ovu-
lation (Lipner and Greep, 1971). In addition, intrafolli-
cular injection of a progesterone antiserum was found
to inhibit ovulation (Swanson and Lipner, 1977). A def-
inite conclusion concerning the role of steroids and the
disruption of the follicle wall has not been reached, but
it has been demonstrated that progesterone (and LH and
cAMP as well) can increase the distensibility of large
follicular strips, supporting a positive role for pro-
gesterone in the process of ovulation (Rondell, 1974).
Progesterone has been found to increase 2 h after the LH
surge in preovulatory hamster follicles, most of it pro-
duced and released from the surrounding interstitial tis-
sue (Greenwald, 1974).

The preovulatory production of steroids also in-
fluences the production of histamine by tissue mast cells
and basophils, and the activation and/or elaboration of
proteolytic enzymes by fibroblasts within the theca in-
terna (Lipner and Greep, 1971), which are changes asso-
ciated with follicular rupture.

An important enzyme, 3B-Hydroxysteroid Dehydrogenase
(3B-HSD) is necessary for the biosynthesis of ovarian
The localization of this enzyme within specific cell types
is therefore of importance in the ovulatory process.
3B-HSD activity has been identified in cells of the theca
interna of sexually mature rats but was not present in
granulosa cells unless the animals were in estrus, the
time corresponding to maximal estrogen secretion (Bjer-
sing, 1967; Stoklosowa and Szoltys, 1978). It has also
been found that 3B-HSD activity appears increased in granulosa cells in mature rats in which ovulation is blocked with sodium pentobarbital (Prabhu and Weisz, 1970). Also in mature rats, 3B-HSD activity within granulosa cells increases steadily as ovulation approaches, reaching peak activity at proestrus (Pupkin et al., 1966).

Preovulatory steroid synthesis is necessary for the induction and continuation of ovulatory processes. All steroid hormones may play a vital role(s) in these events and their presence appear to be controlled, at least in part, through the activity of 3B-HSD.
FOLLICULAR RUPTURE

The extrusion of the ovum in the culminating event in the process of ovulation and is dependent on sequential biochemical and morphological events that occur only within ovulatory follicle(s).

Recent evidence indicates that there is no increase in intrafollicular pressure that could be responsible for the forcible extrusion of the ovum at the time rupture occurs. This data has been generated from work performed using the rat (Blandau and Rumery, 1963; Rondell, 1970) and the rabbit (Espey and Lipner, 1963; Rondell, 1964). As mentioned previously, the changes most responsible for rupture include an increase in distensibility, a reduction in breaking strength immediately prior to rupture (Rondell, 1964), and thinning of the follicle wall which occurs as the follicle swells following plasma transudation (Espey, 1967, 1976, 1978b).

Preovulatory follicles require gonadotrophic hormones (LH and FSH) in order to rupture (Greenwald, 1978) and the timing of the gonadotropic surge affects eventual ovum fertilization and successful implantation (Butcher, et al., 1974). Thus, in the presence of a preovulatory surge of LH/FSH the follicle undergoes a sequence of changes that culminate in rupture. Since rupture occurs approximately 8-10 h following the LH/FSH surge in the rat (Schwartz, 1974), these are changes that are rapidly manifested.
Morphological changes that occur within large pre-ovulatory follicles of the rabbit, just prior to follicular rupture, have been extensively studied. They are induced by the preovulatory surge of gonadotropic hormones. Surface cells within the germinal epithelium increase in size and many show large, round, dense cytoplasmic bodies, located predominantly in the apical region of the follicular wall. It has been suggested that these bodies are lytic and/or secretory in nature and are associated with the degeneration of the collagen matrix. Surface cells near the apex appear collapsed and may be shed just before the follicle ruptures. Edema also occurs within the theca externa and is responsible for degeneration of cells found in this layer. The collagen framework of the tunica albuginea dissociates and fragments proceeding from the outermost to innermost regions of the follicle. The collagen-rich tunica albuginea is the region that will distend first as rupture approaches and lytic substances from the surface epithelium play a key role in this process. In addition, there is a decrease in the number of gap junctions, resembling those found in the granulosa layer, and a small increase in vascularization and edema within the thecal layer. This edema accounts for the preovulatory swelling of the follicle that occurs as a result of an increase in capillary circulation within the thecal layer in the rat (Bassett, 1943), rabbit (Burr and Davies, 1951),
and the mouse (Byskov, 1969). Granulosa cells also begin
to dissociate and thin out in the apical region. There
is an increase in the number of granulosa cell projections
from the granulosa cells which penetrate and fragment the
basement membrane. The number of gap junctions present be-
tween the granulosa cells progressively decreases as rup-
ture approaches (Espey and Stuts, 1972; Merk et al., 1973;
Albertini and Anderson, 1974). Three to five h prior to
follicular rupture in the rat, the macula pellucida or
stigma forms at the apex of the follicle and is the place
where the ovum will eventually be extruded (Blandau, 1955;
Bjersing and Cajander, 1974a, 1974b, 1974c, 1974d, 1974e,
1974f).

Collagen has been identified in many mammalian spe-
cies (Rendell, 1970; Espey, 1976; Espey, 1978b) and
collagenolytic activity, possessing properties of a true
collegenase, has been identified in follicles of the rat
(Okazaki, et al., 1973; Morales et al., 1975). In addition,
collegenase-like enzymes injected into the antra of mature
follicles of the rabbit stimulate swelling, stigma forma-
tion, and ovulation (Espey, 1967, 1974). Thus, many of
the changes that occur within the preovulatory follicle
prior to rupture appear to be events that promote the
assimilation and/or secretion of an ovulatory enzyme,
which may be a collagenase, responsible for the degrada-
tion of the collagen framework of the follicle wall. In
the rabbit 8 h after mating, there is progressive degeneration of the area where the stigma will develop, largely as a result of collagen fiber dissociation (Espey, 1967, 1976).

The distensibility of the follicle wall increases as rupture approaches (Espey, 1967, 1978b; Rondell, 1970, 1974). Distensibility can be induced in large follicular strips with LH or cAMP. Distensibility and steroid release can also be blocked with the addition of cyanoketone, an inhibitor of the action of 3B-Hydroxysteroid Dehydrogenase, and the cyanoketone block of distensibility can be overcome in both cases with progesterone administration (Rondell, 1970).

The role of contractile cells in the ovulatory process is controversial. The presence of these cells in the apex of the preovulatory hamster follicle has been observed (Pendergrass and Talbot, 1979), but their functional significance is not clear. Although some investigators have proposed a role for ovarian contractility in ovulation resulting from smooth muscle activity (Okamura et al., 1972; Bjersing and Cajander, 1974b, 1974c), there is still insufficient evidence to support it (Espey, 1978b).

Follicular rupture is a dynamic event that requires major morphological changes throughout the follicle wall. The integrity of the wall is upset as rupture nears, vas-
cularization increases, and the follicle swells above the surface of the ovary. Just moments prior to rupture, as a result of structural changes within the follicle wall, the most apical portion of the follicle distends, the tissue loses its integrity and the ovum, surrounding cumulus cells, and antral fluid are slowly expelled from the follicle (Espey, 1978b).
PRESENTATION OF THE PROBLEM

The overall importance of androgens in reproductive physiology has been documented (Jones, 1978), but only recently has a role for androgens in the process of ovulation (follicular rupture) been postulated (Mori et al., 1977). In their study, rabbit antiserum to testosterone injected intravenously (i.v.) along with hCG reduced the number of tubal ova released in intact or hypophysectomized immature rats primed with PMSG. In addition, they found that testosterone restored ovulation rate when administered along with antiserum to progesterone. Also in recent studies, testosterone receptors have been identified in the cytoplasm of granulosa cells and evidence for their nuclear translocation was demonstrated (Schrieber et al., 1976; Schrieber and Ross, 1976). Furthermore, it was demonstrated that androgens are capable of increasing progesterone production in granulosa cells in vitro (Hil-lier et al., 1977; Lucky et al., 1977; Schomberg et al., 1978; Haney and Schomberg, 1978).

Therefore, the purpose of this study was to further investigate the role of testosterone in ovulation. Four major parameters were examined, namely, the effect of testosterone on preovulatory oocyte maturation, ovulation rate, whole ovarian steroid hormone concentration, and 3B-HSD activity.
EXPERIMENT I

Thirty one, 24 day-old female rats were injected intraperitoneally (i.p.) with 5 I.U. PMSG and 72 h later given a subcutaneous injection of ethanol-saline vehicle or 5 mg of Flutamide (L-L-L-trifluoro-2-methyl-4'nitro-m-propiorotoluidide). The Flutamide was dissolved in ethanol then diluted with equal volumes of saline. Three h later, groups of 6-10 rats were given intraperitoneal injections of either 0.1 ml of saline or 1 I.U. hCG dissolved in 0.1 ml of saline. All animals were killed 24 h later by decapitation and the oocytes were flushed from the oviducts and counted. The ovaries were then weighed and fixed in Bouin's solution. The ovaries were embedded in paraffin, serial sectioned at 10 u and stained with hematoxylin and eosin. All non-ovulatory follicles of at least 600 u in diameter were counted and classified as either non-atretic or in Stage I of atresia. A follicle was considered in Stage I if pyknotic nuclei were present in the antrum. In addition, non-ovulatory follicles were considered to have undergone preovulatory changes if the cumulus oophorus cells were dispersed and the oocyte had undergone germinal vesicle breakdown.
EXPERIMENT II

Sixteen, 24 day-old female rats were injected with 5 I.U. of PMSG intraperitoneally, and 72 h later given a subcutaneous (s.c.) injection of an ethanol-saline vehicle or 5 mg Flutamide. Three h later the animals were given intraperitoneal injections of 0.1 ml of saline or 1 I.U. of hCG dissolved in 0.1 ml saline. All rats were killed 8 h later by decapitation, the ovaries were excised, trimmed of fat, weighed, placed in pre-labelled vials containing O.C.T. compound (embedding media for frozen tissue) and immediately frozen by placing vials in an ethanol-dry ice bath. One ovary from each animal was subsequently assayed for progesterone, estradiol and testosterone content, while the remaining ovary was used for the histochemical localization of 3B-hydroxysteroid dehydrogenase activity.
STEROID RADIOIMMUNOASSAY

The ovaries were homogenized in 1.0 ml of PBS-gelatin with a ground glass homogenizer and by sonification. A 500 ul aliquot was extracted with 10.0 ml of anhydrous ethyl ether. The ether extract was evaporated to dryness and the steroids were resuspended in 1.0 ml of PBS-gelatin. The efficiency of extraction was estimated by the addition of $^3$H-estradiol, $^3$H-testosterone, or $^3$H-progesterone (New England Nuclear) to a series of ovarian homogenates prior to ether extraction. The extraction efficiency for estradiol and progesterone was 93% and for testosterone, 91%. As a result, none of the values were corrected for extraction efficiency.

Anti-progesterone-11-BSA, GDN #337; 1:2, 500, anti-estradiol-6-BSA, GDN #244 1:20,000, and anti-testosterone-11-BSA, GDN #5250, 1:40,000, were provided through the courtesy of Dr. G.D. Niswender of Colorado State University. Specificity of the estradiol anti-serum had been determined by Campbell et al. (1977), for progesterone anti-serum by Gibori et al., 1977, and for testosterone anti-serum by Dr. J. Bruni (personal communication). Methods used for separation of bound from free steroids by charcoal-dextran and for scintillation counting were previously described by Campbell et al. (1977). Estradiol was assayed in duplicate in 50 and 100 ul aliquots, progesterone was assayed in duplicate in 20 and 50 ul
aliquots, and testosterone in duplicate in 50 and 100 ul aliquots. Estradiol samples were corrected for distilled water blanks. Progesterone and testosterone levels in the water blanks were below the sensitivity of the assay. The hormone assays were performed at Michigan State University by Dr. R.W. Steger.

Localization of $\Delta^5$-3-B Hydroxysteroid Dehydrogenase.

Frozen sections were cut on a cryostat at 10 u., placed on slides, and kept frozen. Endogenous steroid substrates were extracted from the tissue section by exposure to cold acetone for 30 minutes. The sections were then rinsed twice in 0.2M Tris buffer (4°C, pH 7.4) and incubated for 60 minutes at 37°C in a solution containing 20 ml of 0.2M Tris buffer (pH 7.4), 10 mg Nitro-blue tetrazolium, 20 mg NAD and 2 mg of either pregnenolone or dehydroepiandrosterone. After incubation the sections were fixed in phosphate buffered formalin (pH 7.0), rinsed in distilled water and mounted in glycerol-gelatin. To insure that all endogenous substrate was removed by acetone, control sections were incubated in a solution without substrate. An NADH diaphorase substrate medium containing 20 ml of 0.2M Tris buffer (pH 7.4), 10 mg Nitro-blue tetrazolium, and 20 mg of NADH was used as control. Control and experimental incubation solutions were prepared at the same time and under identical conditions.
3B-HSD activity was detected by the presence of a blue-purple stain.

STATISTICAL ANALYSIS

The data from these studies were statistically analyzed by using either chi-square, Student's "t" test, or an analysis of variance.
CHAPTER III

RESULTS

EXPERIMENT I

Rats, treated only with either Flutamide or ethanol-saline vehicle 72 h after PMSG, did not ovulate. In these animals, 100% of the non-ovulatory follicles of at least 600 μ in diameter were in Stage I of atresia, the cumulus mass was not dispersed and the oocyte possessed an intact germinal vesicle membrane. In the PMSG–hCG treated rats, seven of nine rats ovulated at a rate of 9.9 ± 1.6 ova/ovulating rat. In 68 ± 15% of the non-ovulatory follicles greater than or equal to 600 μ in diameter, the cumulus cells were dispersed and the oocyte had resumed meiosis (p < 0.05). In rats given Flutamide 3 h prior to hCG treatment, the ovulation rate and the percentage of non-ovulatory follicles in which the cumulus cells were dispersed and the oocyte resumed meiosis were reduced (p < 0.05) (Figs. 1 & 2).
EXPERIMENT II

Ovarian progesterone, estradiol and testosterone content in rats treated with an ethanol-saline vehicle 72 h after PMSG averaged 181 ± 31, 26.4 ± 5.3 and 23.1 ± 7.9 ng/mg ovary respectively (values expressed as pg/mg ovary in Fig. 3). Flutamide treatment did not alter these values (p < 0.05) (Fig. 3). In the ovaries of either control or Flutamide treated rats, 3B-HSD activity was demonstrated in the thecal layers of 100% of the preovulatory follicles (Fig. 4A). 3B-HSD activity was also observed in the granulosa cell layers of 11-25% of the preovulatory follicles examined when pregnenolone was used as a substrate (Fig. 5A). When dehydroepiandrosterone was used as a substrate, 14-36% of the preovulatory follicles possessed 3B-HSD activity (Fig. 5B).

Treatment with 1 I.U. of hCG in both ethanol-saline or Flutamide treated rats increased the ovarian progesterone content (p < 0.05), decreased the ovarian estradiol content (p < 0.05) and did not affect the ovarian testosterone concentration (Fig. 3). In the ethanol-saline treated group, hCG increased the percentage of preovulatory follicles with 3B-HSD activity in the granulosa cell layers to 60% (p < 0.05) (Fig. 4B). However, pretreatment with Flutamide inhibited the hCG-induced appearance of 3B-HSD activity in the granulosa cell layers with only 30% of the follicles possessing 3B-HSD activity when
pregnenolone was used as a substrate (Fig. 4C). There was no change in the distribution or the percentage of follicles with 3B-HSD activity when dehydroepiandrosterone was used as a substrate.

3B-HSD activity was also demonstrated in the thecal cell layer and in the interstitial tissue using either pregnenolone or dehydroepiandrosterone as a substrate. 3B-HSD activity was present in all treatment-groups (Figs. 5A & 5B).
Figure 1. The effect of Flutamide on hCG-induced ovulation in PMSG-primed immature rats.
Figure 2. The effect of Flutamide on hCG-induced changes in the cumulus oophorus and oocyte of non-ovulatory follicles of PMSG-primed immature rats.

- 100
- 50
- 0

% Ova in Follicles ≥600µ in Diameter

0.1 U. hCG

- SALINE
- FLUTAMIDE

1 U. hCG

G.V. & C.C.
Intact

G.VB & C.C.
Dispersed

36
Figure 3. The effect of Flutamide on hCG-induced changes in ovarian progesterone, 17β-estradiol and androgen content in PMSG-primed immature rats.
Figure 4. Histochemical localization of 3B-HSD activity using pregnenolone as a substrate. 

A) A preovulatory follicle of a control rat. Note the 3B-HSD activity which is localized in the thecal cell layers only. 

B) A preovulatory follicle from a sal/hCG treated rat. 3B-HSD activity is localized in both the thecal and granulosa cell layers. 

C) A preovulatory follicle from a Flutamide-hCG treated rat. In this follicle 3B-HSD activity is only found in the thecal layers. ( X 200 ).
Figure 5. Histochemical localization in the ovary of PMSG-primed rats utilizing A) pregnenolone and B) dehydroepiandrosterone as substrates. Notice the intense activity of 3β-HSD in the interstitium regardless of substrate. (X 40).
CHAPTER IV

DISCUSSION

The results of this investigation show that Flutamide (FL) administration inhibits the hCG-induced germinal vesicle breakdown (GVB) and cumulus cell dispersion within preovulatory follicles. These changes are preovulatory changes that occur in follicles destined to ovulate (Tsafriri, 1978). FL treatment did not alter the percentage of animals ovulating, but, in those animals that did ovulate, significantly suppressed the number of tubal ova flushed from the oviducts (Fig. 1). In a previous study, Hagino and Goldzieker (1975) showed that the administration of testosterone propionate (TP) to rats on days 28-30, decreased the percentage of animals ovulating and the number of tubal ova produced. However, this effect could have been due to a central feedback effect, since TP was found to suppress the synthesis and release of FSH and LH. In 28-30 day old animals, partial maturation of the hypothalamic-pituitary-ovarian axis may have occurred to confound data interpretation. TP treatment inhibited the response of the ovary to hCG, suggesting a direct target-organ effect. Since TP cannot be metabolized rapidly to other active forms (Hagino and Goldzieker; 1975), this effect could be pharmacological.

Administration of hCG results in an increase in ovarian progesterone content, and suppresses 17B-estradiol concentration. In addition, the percentage of pre-
ovulatory follicles that had 3β-HSD activity within their granulosa layers was increased. When animals were pre-treated with FL and then autopsied 8 h after hCG, ovarian steroid hormone content was not altered. However, the appearance of 3β-HSD activity within the granulosa layers was reduced.

3β-HSD activity has been consistently demonstrated in cells of the theca interna (Bjersing, 1977) and various investigators have reported 3β-HSD activity in granulosa cells (Prabhu and Weisz, 1970). It was also shown by Pupkin et al. (1966) and Prabhu and Weisz (1970) that 3β-HSD activity within granulosa cells increases steadily as ovulation approaches, reaching peak activity at proestrus. With a FL-related decrease in 3β-HSD activity within the granulosa cells, a decrease in the concentration of this steroid would be expected. However, an increase in ovarian progesterone content is observed that may be due to progesterone synthesis within the interstitial tissue, since 3β-HSD activity was consistently high in the intestitium regardless of treatment or substrate used (Fig. 5). There may be a local decrease in progesterone within those preovulatory follicles that did not ovulate in the presence of FL. No change in whole ovarian testosterone concentration is not surprising since FL competes with the binding of testosterone and 5 alpha dehydrotestosterone at the receptor site (Hellman et al., 1977; Neri et al., 1972; Mainwaring et al., 1974; Liao et al.,
It appears then that testosterone is being produced in normal physiological amounts but that its effects (on progesterone synthesis?) are significantly suppressed.

The importance of androgens in the ovulatory process has recently been gaining attention. Lucky et al. (1977) showed that progesterone production by isolated rat granulosa cells from preantral follicles was enhanced upon the addition of androgens. This was confirmed using estrogen-primed hypophysectomized immature rats by Hillier et al. (1977). This effect of testosterone on progesterone production was suppressed in the presence of two antiandrogens, cyproterone acetate (CPA) or SCH 16423. Armstrong and Dorrington (1976) showed that androgens increase progesterone production by rat granulosa cells taken from antral follicles and demonstrated synergism between androgens and FSH in vitro. In addition, 5-alpha DHT was found to be more effective than testosterone in increasing progesterone secretion. This was confirmed with porcine granulosa cells in vitro by Haney and Schomberg (1978). Schomberg et al. (1978) also found that by using intra-ovarian silastic implants containing Flutamide, a potent antiandrogen, progesterone secretion by porcine granulosa cells in vitro is reduced by 50%. These studies suggest the possibility that androgens influence ovulation by regulating the secretion of progesterone, which is thought to be involved in an intraovarian mechanism.
of ovulation.

In conclusion, hCG induces ovulation by promoting preovulatory oocyte maturation, GVB, cumulus cell dispersion, 3β-HSD activity within the granulosa cells, and progesterone synthesis. Although FL pretreatment did not alter the concentration of progesterone, the ovulatory response to hCG was blocked. The ability of FL to block hCG-induced responses could be accounted for by two possible modes of action: 1) hCG could stimulate the production of androgen in the theca interna which could then be involved in the ovulatory process; and/or 2) pretreatment with FL could alter the responsiveness of the preovulatory follicle to hCG.

The first possibility appears straightforward: hCG binds to theca cells to produce androgen, which in turn stimulates the production of progesterone, considered the "ovulatory hormone" by most investigators. However, androgens are also important in the process of follicular growth and maturation (Louvet et al., 1975). In addition, androgens appear to be involved in maintaining the integrity and responsiveness of follicles to hCG, for treatment with CPA, an antiandrogen, significantly increases the presence of acid phosphatase, a lysosomal enzyme associated with follicular degradation (Peluso et al., 1979). The possibility remains, therefore, that the removal of the action of testosterone by treatment with
FL removes an agent that would normally maintain the integrity and thus the responsiveness of the follicle to the surge of gonadotropic hormones. This is supported by the finding that androgen priming increases $^{125}$I-hCG uptake in the ovaries of adult intact and hypophysectomized rats (Mizejewski, 1976). Further investigation is necessary in order to determine whether or not one or both of the proposed mechanisms accounts for the inhibitory effects of FL on the ovulatory process.
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APPROVAL SHEET

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The final copies have been examined by the director of the thesis and the signature which appears below verifies that fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

Date: 2-16-80

[Signature]
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