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The Relationship of Appearance of the Surface of Ovarian Follicles to Atresia and In Vitro Maturation of Oocytes in Cattle. presented by

Randall William Grimes

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The Relationship of Appearance of the Surface of Ovarian Follicles to Atresia and *In Vitro* Maturation of Oocytes in Cattle

by

Randall William Grimes

A Thesis

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ABSTRACT

THE RELATIONSHIP OF APPEARANCE OF THE SURFACE OF OVARIAN FOLLICLES TO ATRESIA AND *In Vitro* MATURATION OF OOCYTES IN CATTLE

By

Randall William Grimes

This study investigated whether atresia of ovarian follicles and *in vitro* maturational capacity of oocytes could be predicted from appearance of the surface of bovine follicles. Follicles (n=284) were classified as clear, intermediate or opaque depending on appearance of their surface. Opaque follicles were atretic more often than were clear or intermediate follicles. However, selection of atretic or non-atretic follicles based only on surface appearance was unreliable. Relative to non-atretic, atretic follicles had higher concentrations of progesterone, lower concentrations of estradiol and reduced capacity to bind FSH.

Oocytes (n=73) from clear and opaque follicles were cultured *in vitro*. Mature oocytes were defined as those that extruded a polar body by 30 h in culture. Incidence of maturation of oocytes from clear follicles (54%) was twice ($p<.01$) that of opaque follicles (24%). Maturation of oocytes was greater (61%; $p<.05$) when progesterone in FF was greater than estradiol than when the opposite was true (25%).

In conclusion, surface appearance of a follicle does not accurately predict whether a follicle is atretic. However, surface appearance can be used to improve the likelihood of selecting oocytes that will mature *in vitro*.

This Thesis is dedicated to my

Parents and Grandparents:

Barbara Northup

Jay Northup

Martha Iona Grimes

Paul Grimes, Sr.

Maryann Grimes

Robert Grimes

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List of abbreviations

CL	corpora lutea
CPM	counts per minute
Ci	Curie
CAMP	cyclic-adenosine-mono-phosphate
DNA	deoxy-ribose nucleic acid
E	estradiol
FCS	fetal calf serum
FSH	follicle-stimulating hormone
FF	follicular fluid
GV	germinal vesicle
GVBD	germinal vesicle break-down
GnRH	gonadotropin releasing-hormone
HCG	human-chorionic gonadotropin
hFSH	human-follicle stimulating hormone
IU	international-unit(s)
$\log_e$	natural logrithm (logrithm to base e)
LH	luteinizing hormone
mOsm	milliosmole(s)
mmole	millimole
N	normal
n	number of samples in a test group
OMI	oocyte maturation inhibitor
oFSH	ovine-follicle stimulating hormone
oLH	ovine-luteinizing hormone
PBS	phosphate buffered saline
P	progesterone
RIA	radioimmunoassay
SEM	standard error of the mean
T	testosterone
TC199	tissue culture medium #199

INTRODUCTION

The objective of this research was to determine if appearance of the surface of ovarian follicles in cattle could be used, a) to predict whether ovarian follicles were atretic, and b) to predict whether these follicles contained oocytes capable of meiotic maturation *in vitro*.

The ability to predict whether a follicle is atretic by a quick, reliable method of examining the surface of follicles would be valuable. For example, if it is possible to determine atresia of follicles *in situ* by laparotomy and laprascorpy or in ovaries from a slaughter house, then the likelihood of obtaining viable oocytes for *in vitro* fertilization would be improved. In addition, the laborious and time-consuming efforts required to determine if a follicle is atretic by examination of the follicular wall (Ingram, 1962) or by concentration of steroids in follicular fluid (Byskov, 1978; Moor *et al*, 1978; Bomsel-Helmreich *et al*, 1979; Carson *et al*, 1981; Merz *et al*, 1981; McNatty *et al*, 1979a, b, c, 1982; Ireland and Roche, 1982; Bellin and Ax, 1984) could be avoided.

The ability to predict whether an oocyte is capable of maturation *in vitro* by appearance of a follicular surface would is also be valuable since only mature oocytes are

capable of fertilization (Donahue, 1972; Fleming and Saacke, 1972). Oocytes from an economically valuable dam could be selected by appearance of a follicular surface, via laparotomy, matured and fertilized *in vitro* and then transferred to a surrogate mother. The animal's reproductive potential would, thus, be more fully utilized without the use of exogenous hormones required for superovulation. Viable oocytes capable of maturation could be selected from slaughtered animals at a local abattoir and utilized to test the capacity of fresh or frozen bull semen to fertilize oocytes *in vitro*. Efficiency of studies investigating fertilization or embryology would be improved since only oocytes capable of maturation would be utilized.

In order to reach the first objective, follicles were classified as clear, intermediate or opaque depending on appearance of their surface. Atresia was determined by histological evaluation of the follicular wall. Relationship of appearance of a follicular surface and atresia to concentration of steroids in follicular fluid and binding of gonadotropins by granulosal cells was also examined.

In Experiment 2, oocytes from clear and opaque follicles were cultured *in vitro* for 30 h to determine if they were capable of meiotic maturation. Relationship between concentration of steroids in follicular fluid and maturation of oocytes was also examined.

I will conclude from this research that appearance of the surface of bovine ovarian follicles does not accurately indicate whether a follicle is atretic but does indicate whether an oocyte is capable of maturation *in vitro*.

REVIEW OF THE LITERATURE

1. Follicular atresia

A. Definition of atresia

Although many ovarian follicles grow during a reproductive cycle of mammals only a species specific number of follicles ovulate; the remainer are lost through atresia (Farookhi, 1981). Atresia is a process during which an ovarian follicle degenerates (loses its cellular integrity) and the oocyte is removed from the ovarian population by means other than ovulation (Byskov, 1979). Follicular atresia is not unique to mammals but occurs in most, if not all, vertebrates (Saidapur, 1978). The underlying cause for atresia is unknown. However, a number of factors such as age, stage of the reproductive cycle, pregnancy and hormonal status (Rajakoski, 1960; Ingram, 1962), inhibin (Dejong and Sharpe, 1976; Schwartz, 1977), an ovarian GnRH-like molecule (Ingram, 1962), gonadotropin binding inhibitors (Darga and Reichert, 1978) and products (inhibitors) of other follicles (Byskov, 1978; Zeleznik, 1982) influence the incidence of atresia. Clearly, the ultimate fate of all follicles, except the few destined for ovulation, is atresia (DiZerega

- et al, 1981). Atresia results in abnormal morphology of follicular cells and changes in concentration of steroids in follicular fluid and number of gonadotropin receptors in granulosal cells.

B. Morphological atresia at the cellular level

During atresia, granulosal cells lining the antrum slough off into the follicular fluid, the basement membrane disappears, the granulosal layer becomes disoriented and cells of the theca interna hypertrophy (Marion et al, 1968; Priedkalns et al, 1968; Hay et al, 1976). The extent to which these cell changes occur indicates the degree of atresia within a follicle.

Atresia of the granulosal layer

In many species, appearance of pyknotic nuclei in the granulosal layer is one of the first morphological signs of atresia (Koering et al, 1982). During pyknosis cells and nuclei shrink. The cellular matrix may condense and mitochondria swell (Hay et al, 1976). Pyknotic nuclei stain darkly. Pyknosis of 1% of granulosal cells in a follicle is considered normal; whereas pyknosis of 5% or greater generally indicates atresia (Bomsel-Helmreich et al, 1979; Brailly et al, 1981).

In addition to pyknosis, meiotic divisions of granulosal cells of atretic follicles are reduced (Ingram, 1962). As atresia progresses the granulosal layer becomes thinner and dead cells are removed by the phagocytic activity of granulosal cells (Hay et al, 1976; Byskov, 1978), invading leukocytes, or macrophages (Byskov, 1978). During atresia, turgidity of the follicle can not be sustained (Byskov, 1978). Antral follicles may become compressed by the pressure of surrounding tissue and the antrum obliterated by invading connective tissue during final stages of atresia (McKay, 1961; Koering, 1969; Byskov, 1978). During later stages of atresia, after a follicle is compressed, the basement membrane folds and increases in thickness (Byskov, 1978). Occassionally, large follicles continue to expand and form follicular cysts (Ingram, 1962; Nalbandov, 1976).

Atresia in the thecal layer

During atresia the theca interna hypertrophies and the enlarged thecal cells grow into the granulosal layer. In some follicles, this process results in formation of a small group of interstitial-like cells, whereas in others luteinization occurs (Ingram, 1962; Koering, 1969). The theca also may degenerate and disappear altogether (Koering, 1969).

Since the theca is vascular, a deficient thecal layer in large follicles may limit supplies of nutrients going to the vascular granulosal cells and result in atresia (Hisaw, 1947). The capillary network in the theca of follicles in sheep is significantly reduced in atretic follicles as compared to non-atretic ones (Hay et al, 1976) and blood flow is greater to a dominant follicle than other follicles in the monkey (Zeleznik et al, 1981). These observations indicate that blood flow varies to individual follicles. This may result in preferential delivery of gonadotropins and other nutrients to certain follicles while other follicles with a reduced supply of blood, nutrients and gonadotropins may undergo atresia.

Atresia of oocytes

During atresia oocytes may become necrotic, shrink, lyse or leave the ovary through the surface epithelium (Byskov, 1978). In some cases, follicular atresia results in premature chromosomal maturation of oocytes (Byskov, 1979).

Overall, atresia may start in the granulosal layer, thecal layer or oocyte. Morpholoigical changes of follicular cells can indicate whether a follicle is atretic. However, follicular cells may slough off during removal of follicular fluid (Bellin and Ax, 1984), during collection of tissue or the fixation process. This may lead

to a reduction in number of pyknotic cells which could result in misclassification of degree of atresia. Because almost all follicles, including dominant follicles contain pyknotic cells (Bomsel-Helmreich et al, 1979; Brailly et al, 1981; Koering et al, 1982) and distribution of pyknosis is not uniform within a follicle (Koering et al, 1982), pyknosis may not always be a reliable indicator of atresia. In addition, classification of follicular atresia is subjective (Marion et al, 1968). Follicular function (see section C) and morphology of the follicular wall should be examined when determining extent of follicular atresia.

C. Role of atresia in cellular function

Steroidogenesis

The fate of atretic follicles is "partly to disappear completely, partly to persist to form part of the endocrine tissue of the ovary" (Williams, 1956).

Ovaries are a major source of serum estradiol, progesterone, and androgens (Kanchev, 1976). Follicles undergoing atresia retain their ability to produce progestins and androgens but may lose their ability to aromatize these to estradiol (Byskov, 1978; Moor et al, 1978; Richards, 1980; Terranova, 1981; Ireland and Roche, 1982). As a result, concentrations of progesterone and androgens in follicular fluid of atretic follicles will

be elevated relative to levels of estradiol (Byskov, 1978; Moor et al, 1978; Bomsel-Helmreich et al, 1979; Carson et al, 1981; Merz et al, 1981; McNatty et al, 1979a, b, c, 1982; Ireland and Roche, 1982; Bellin and Ax, 1984).

Because concentration of steroids in follicular fluid can reflect steroidogenic capability of follicular cells (Byskov, 1979), it may be a more precise indicator of follicular function (McNatty et al, 1979a; Brailly et al, 1981) than histological indices such as pyknosis or morphological appearance of granulosal and thecal cells.

One problem with using concentration of steroids in follicular fluid as an indicator of endocrine function is that changes in endocrine function due to atresia are similar to those following the gonadotropin surge in ovulatory follicles (Moor et al, 1978; Bomsel-Helmreich et al, 1979; Carson et al, 1979; Merz et al, 1981; McNatty et al, 1979a, b, c, 1982; Ireland and Roche, 1982, 1983b; Bellin and Ax, 1984). The relationship between histological atresia and steroidogenic capacity of a follicle needs to be examined in more detail.

Specific binding of gonadotropins by granulosal layer

If there is a loss of gonadotropin stimulus or gonadotropin receptors, a follicle may become atretic (Peters et al, 1975). Specific binding of FSH and HCG, an LH-like molecule, by granulosal cells is lower in atretic

follicles than healthy ones (Channing and Kammerman, 1973; Carson et al, 1979; Ireland and Roche, 1982, 1983a). Specifically, binding of FSH and HCG to granulosal cells is two-fold lower in atretic than non-atretic follicles in cattle (Ireland and Roche, 1982). Therefore, low specific binding of FSH and HCG (LH) by granulosal cells may indicate that a follicle is atretic. However, specific binding of gonadotropins by granulosal cells is low not only in atretic follicles but also in ovulatory follicles following a preovulatory gonadotropin surge (Peluso et al, 1977; Ireland and Roche, 1982, 1983a, b), and in newly developing antral follicles (Channing and Kammerman, 1973; Kammerman and Ross, 1975; Ireland and Roche, 1983b). Thus, level of gonadotropin receptors in granulosal cells may not always be a good indicator of follicular atresia unless coupled with histological and endocrine evaluations.

D. Appearance of the follicle as an indicator of atresia

It is impossible to distinguish morphologically between 'healthy-looking' follicles, which are committed to undergo atresia and those which will terminate their growth phase by ovulating an egg (Byskov, 1979).

Physical characteristics of a follicle may make it possible to determine whether a follicle is atretic. Follicular fluid of strongly atretic follicles appears milky due to cell debris, while follicular fluid of non-atretic

follicles is clear (Bjersing, 1967). Non-atretic murine follicles dissected free of the ovary appear translucent while atretic ones are more opaque and pink in color (Wilkinson et al, 1979). Following excision of follicles from an ovary and removal of stromal tissue from each follicle, the degree of translucency of the wall of a follicle, vascularization and integrity of the membrane granulosa provide an indication of atresia in ovarian follicles (Moor et al, 1978). Follicles in sheep, classified as atretic according to Moor et al, (1978) exhibit reduced binding of hFSH and HCG by the granulosal layer compared with non-atretic follicles (Carson et al, 1979). In another study, using the scheme of Moor et al (1978), concentration of estradiol in follicular fluid of cattle was lower in atretic follicles than non-atretic ones (Kruip and Dieleman, 1982).

In the cow, non-ovulatory follicles marked with India ink, appear to become progressively covered with tissue. This gives the surface of the follicle an opaque appearance before it disappears completely below the surface of the ovary (personal communication with Dr. James Ireland, Departments of Animal Science and Physiology, Michigan State University, East Lansing). The surface of some follicles in the cow appear pinkish in color similar to that noted in atretic follicles in the mouse (Wilkinson et al, 1979). I hypothesized that the degree of opaqueness of the surface of

bovine ovarian follicles may indicate if a follicle is histologically or functionally atretic.

2. In vitro maturation of bovine oocytes

A. Meiotic maturation of oocytes

Meiotic maturation of the female gamete commences during fetal life and usually becomes arrested shortly after birth (Erickson, 1966; Thibault et al, 1976; Tsafriri, 1978). In the cow, the immature ovum remains in diplotene of the first meiotic prophase until stimulated to mature by a preovulatory surge of luteinizing hormone (LH) (Donahue, 1972; Thibault et al, 1975). Oocytes can remain in this arrested state of meiosis without degenerating for more than 40 years in humans (Tsafriri, 1978). Although an oocyte is meiotically inactive, it continues to grow and maintains metabolic and synthetic activity (Tsafriri, 1978). The oocyte is maintained in an arrested state of meiotic development through interactions with follicular cells. Follicular cells (granulosal and thecal) apparently produce maturation inhibiting substances (Tsafriri and Channing, 1975; Tsafriri et al, 1982), retain nutrients necessary for oocyte development (Tsafriri, 1978; Crosby et al, 1981), and exert a direct, dynamic control on the oocyte through gap junctions (Sheridan, 1971; Gilula et al, 1972; Azarnia et al, 1974). Luteinizing hormone (LH) disrupts 60-80% of the

gap junctions between the oocyte and follicular cells (Moor and Osborn, 1983), through which inhibitors produced by the follicular cells may pass. LH probably reduces the inhibitory effects of follicular cells in maturation of oocytes so that maturation of an oocyte is now possible. Removal of oocytes from their follicular surroundings into a simple culture medium results in spontaneous maturation (Pincus and Enzmann, 1935; Edwards, 1965; Foote and Thibault, 1969) probably because the inhibitory effects of the follicular cells are removed. When follicular cells are added to cultures of porcine oocytes, maturation of oocytes is prevented (Tsafriri and Channing, 1975). In addition, ovulation of a mature bovine oocyte occurs 24-28 hours after a preovulatory LH surge (Hafez *et al*, 1963; Christenson *et al*, 1974) while maturation *in vitro* requires a similar amount of time, 24-30 hours (Edwards, 1965; Sreenan, 1970; Thibault, 1972; Shea *et al*, 1976). These three facts indicate that resumption of meiosis *in vitro* is a result of removal of the oocyte from inhibitory effects of follicular cells.

Once meiosis is initiated, *in vivo* or *in vitro*, it proceeds sequentially through distinct maturational phases: germinal vesicle breakdown (GVBD), metaphase I, anaphase I, telophase I and polar body extrusion and is then arrested again in metaphase II until sperm penetration of the ovum occurs (Donahue, 1972; Fleming and Saacke, 1972). Oocytes extruding the first polar body and proceeding to the

metaphase II stage are capable of being penetrated by spermatozoa and are considered mature and viable (healthy) in most studies.

B. Oocyte culture conditions

Many oocytes remain in the germinal vesicle (GV) stage or fail to complete maturation to metaphase II when cultured in vitro. These oocytes are neither meiotically mature nor viable and thus are not able to be fertilized (Donahue, 1972). Culture conditions can also affect the proportion of oocytes that mature in vitro (Fukui et al, 1982).

Bovine oocytes develop best under culture conditions of pH 7.0 to 7.3 (Shea et al, 1976), medium osmolality of 285 mOsm which is similar to plasma and follicular fluid (Edwards, 1975), a low oxygen atmosphere (5% O_2; Trounsen et al, 1977) and a temperature of 37 to 39°C (Lenz et al, 1983). These conditions were utilized in the present study. The addition of gonadotropins was necessary in culture of oocytes in the sheep and pig (Jaigello et al, 1975; Meincke and Meincke-Tillman, 1979). Interestingly, requirement of gonadotropins can be overcome in the culture of oocytes in sheep by addition of fetal calf serum. No requirement for gonadotropins in culture was demonstrated in pigs (Hillensjo and Channing, 1980) or cattle (Rush, 1973; Fukui et al, 1982). Gonadotropins can act directly on cumulus cells by changing their metabolic pathways towards

pyruvate and lactate production (Thibault et al, 1975; Hillensjo et al, 1976), increasing production of progesterone (Nicosia and Michail, 1975) which may be stimulatory towards maturation of oocytes (Robertson and Baker, 1969), enhancing synthesis of glycoproteins by the golgi apparatus (Moricard, 1968) and by breaking of gap junctions between the oocyte and cumulus cells through which an inhibitor of oocyte maturation (Channing et al, 1982) may pass. This suggests that the stimulatory effect of gonadotropins may be due to synthesis of nutrients missing in some media or, alternately, removal of inhibition caused by adhering follicular cells (cumulus).

In the cow (Foote and Thibault, 1969), rat (Tsafriri et al, 1972), pig (McGaughey, 1977) mouse (Tyler et al, 1980) and sheep (Jagiello et al, 1975), addition of estradiol and progesterone has no effect on oocyte maturation. In other reports, steroids improve the proportion of oocytes maturing in culture in cattle (Robertson and Baker, 1969; Fukui et al, 1982), rabbits (Bae and Foote, 1975) and whole follicles in sheep (Moor and Trounson, 1977). In one case, addition of progesterone, testosterone or androstenedione to mouse ovarian fragments prevented maturation of oocytes (Tyler et al, 1980). When oocytes were placed in a steroid-free medium, however, meiotic maturation continued. Fukui et al (1982) determined that addition of steroids, just as with addition of gonadotropins, may only be necessary when the medium is deficient. My study utilized a medium

supplemented with fetal calf serum (Heat treated, charcoal extracted) and devoid of added gonadotropins and steroids.

C. Acquisition of meiotic competence: Effect of follicular size, steroids in follicular fluid and atresia

Since the preovulatory gonadotropin surge induces marked changes in follicular steroidogenesis (Linder et al, 1974; Eiler and Nalbandov, 1977) prior to ovulation, steroids have historically been added to cultures of oocytes in an attempt to enhance resumption of meiosis. Tsafriri (1978) and Channing et al (1983) suggest that exposure of mammalian oocytes to high levels of steroids prior to ovulation or culture may be responsible for acquisition of maturational competence.

Concentrations of estradiol and progesterone increase in follicular fluid of ovulatory follicles in cattle as these follicles increase in size (England et al, 1981; Henderson et al, 1982; Ireland and Roche, 1982). Perhaps, capacity for resumption of meiosis after a preovulatory LH surge is related to a marked increase in concentration of estradiol or progesterone within an ovulatory follicle. If so, there should be a relationship between intrafollicular concentration of estradiol and progesterone, diameter of a follicle (an indicator of development) and ability of an oocyte to mature in vitro. In support of

this hypothesis, human oocytes capable of maturation *in vitro* come from follicles with high levels of estradiol and progesterone in follicular fluid while those oocytes not developing meiotically come from follicles with lower concentrations of these steroids (McNatty *et al*, 1979b; Channing *et al*, 1983). In pigs (Tsafriri and Channing, 1975), oocytes from small follicles (1-2mm in diameter) have a lower rate of maturation than those from medium (3-5mm) or large follicles (6-12mm). Rates of maturation were 15%, 55%, and 80%, respectively. Also, oocyte maturation inhibitor activity (OMI) is greater in small follicles and least in large follicles of the pig (Channing *et al*, 1982).

In the hamster, only fully grown oocytes from larger follicles are capable of maturation *in vitro* (Iwamatsu and Yanagimachi, 1975). In the adult mouse, oocytes from preantral follicles (100-150um in diameter) do not mature in culture while those from antral follicles (150-600um) resumed meiosis upon release from the follicle (Erickson and Sorensen, 1974). Interestingly, no relationship exists between follicular diameter and ability of oocytes to mature *in vitro* in the cow (Thibault *et al*, 1976; Leibfried and First, 1979; Fukui and Sakuma, 1980). This may be because oocytes in cattle develop the ability to mature independently of development of the follicle. Perhaps, all antral follicles in cattle contain competent oocytes, capable of meiotic maturation if not inhibited. Why all

oocytes do not mature *in vitro* will be examined in the discussion section of Experiment 2.

Because of the paucity of information regarding the relationship among follicular histology, function and oocyte development in cattle, I examined the association between follicular size, concentration of steroids in follicular fluid and ability of oocytes to mature *in vitro*. I also attempted to determine whether appearance of the surface of an ovarian follicle was related to ability of an oocyte to mature *in vitro*.

Experiment 1

The Relationship between Appearance of the Surface of Bovine Ovarian Follicles and Atresia

Experiment I

A. Materials and Methods

The objective of this experiment was two fold: first, to determine if appearance of the surface of an ovarian follicle could be used as an indicator of histological atresia, and second, to examine the relationship between histological atresia and follicular function.

Ovaries (n=222) were collected in pairs from non-pregnant cows at a local abattoir and placed on ice within 30 min of slaughter. All ovaries were processed within 6 h of collection. One or two follicles from each ovary were placed into one of 24 categories consisting of estimated stage of an estrous cycle (I to IV), follicular size (5.0-13.0, 13.0-25.0 mm in diameter) and class of surface appearance (clear, intermediate, opaque). Number of follicles in each of the 24 categories ranged from 10 to 15. Total number of follicles equaled 284.

Determination of estimated stage of an estrous cycle

Stage of an estrous cycle for each pair of ovaries was estimated based on gross appearance of a corpus luteum (Ireland et al, 1980; see Appendix I).

Determination of surface appearance of a follicle

During atresia, the surface of a follicle becomes opaque (Wilkinson et al, 1979) and translucency of the surface is lost (Moor et al, 1978). These changes may be due to de-differentiation of the theca externa into stromal cells (Marion et al, 1968) or migration of connective tissue (stroma) over the normally translucent (clear) surface of a follicle as occurs in a newly ovulated follicle (Bloom and Fawcett, 1975). As explained in Figure 1, a follicle was classified as clear (Figure 2; panel A) if less than 30% of its surface was opaque, intermediate (Figure 2; panel B) if 30-70% of its surface was opaque or opaque (Figure 2; panel C) if greater than 70% of its surface was opaque.

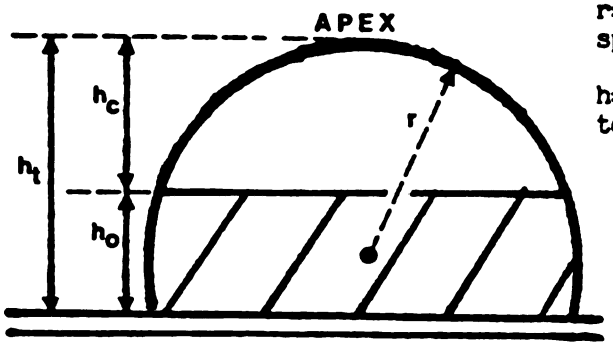
Collection of follicular fluid

Follicular fluid (FF) from clear, intermediate and opaque follicles greater or equal to 5mm in diameter was aspirated and volume recorded. Follicular fluid was then centrifuged at 1000xg for 5 min at $5^{o}C$ to remove any debris

and frozen with dry ice and methanol. The FF was stored at -20°C until concentrations of estradiol, progesterone and testosterone were determined by radioimmunoassay previously validated in the Animal Reproduction Laboratory at Michigan State University.

Determination of histological atresia

A tissue section approximately 4 mm^2 was removed from each follicle with a scapel and placed directly into a modified Karnovsky's fixative (Appendix II). Each piece of tissue was dehydrated and mounted on a slide. Following impregnation with liquid paraffin, each piece of tissue was oriented in a paraffin block so that a transverse section would extend through the ovarian surface, stromal, thecal and granulosal layers. The tissue was sectioned serially. Number of sections varied from 50 to more than 500 per sample depending on size of each piece of tissue. Three out of every ten adjacent slices were then mounted to obtain a representative image of the whole sample. Degree and type of histological atresia was determined by Dr. Pierre Matton, Departmenté de Biologie, Faculté des Sciences, Universite de Sherbrooke (Québec), Canada. Based on degree of atresia, each follicle was assigned to one of four classes of atresia (Table 1). Examples of the four classes of atresia are presented in Figure 3 (plates 1 to 4).



-opaque tissue.

-clear, non-opaque tissue.

r=radius of a follicle or sphere.

h=height of cap from base to apex:

h_t=height of visible surface of a follicle,

h_c=height of clear surface,

h_o=height of opaque surface

Figure 1: Determination of degree of opaqueness of a follicular surface.

The surface area of a cap on a sphere (SAC) is equal to $2\pi rh$ (Anon., 1980). Since a follicle is generally spherical (Rondel, 1970), SAC of the clear portion or cap is $2\pi rh_c$ and SAC of the visible portion of the follicle is $2\pi rh_t$. Therefore, degree of clearness of a follicle (%) is $2\pi rh_c/2\pi rh_t$) x 100% or (h_c/h_t) x 100%.

Degree of opaqueness (%) of a follicle thus equals: 100% - (h_c/h_t x 100%). Clear follicles were arbitrarily classified as less than 30% opaque, opaque follicles were greater than 70% opaque, and the intermediate group contained all follicles 30 to 70% opaque (see Figure 2; panels A to C).

Figure 2

Examples of clear, intermediate and opaque follicles[1,2]

Plate A: This clear follicle protrudes from the surface of the ovary and only a small amount of the periphery is covered by an opaque tissue.

Plate B: This intermediate follicle protrudes from the surface of the ovary. Approximately half of the visible surface is covered by an opaque tissue.

Plate C: This opaque follicle does not protrude from the surface of the ovary. The visible surface is entirely covered by an opaque tissue.

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[1]Surface area within the inner circle is clear. Outer circle indicates boundary between follicular and ovarian tissues. Area between inner and outer circles is opaque.

[2]See Figure 1 for explanation of how opaqueness of follicles is determined. Clear follicles (Panel A) are less than 30% opaque, opaque follicles (Panel C) are greater than 70% opaque and the intermediate group (Panel B) contains all follicles 30 to 70% opaque.

Figure 2 (Continued)

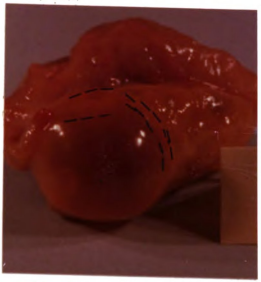

Plate A: a clear follicle.

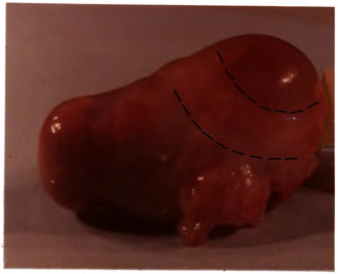

Plate B: an intermediate follicle.

Plate C: an opaque follicle (entire surface is opaque).

Table 1

Characteristics of four classes of histological atresia of bovine follicles[a]

Rating	Description
I	Non-atretic:
	-granulosa has at least 7 layers of cells.
	-few pycnotic or fragmented cells.
	-basal membrane usually present.
	-granulosal layer usually thicker than thecal layer.
	-thecal layer contains glandular and fibroblastic cells and are oriented parallel to the basement membrane.
II	Atretic:
	-only one to six layers of granulosa and layers are disorganized.
	-pycnotic or fragmented cells are abundant.
	-basal layer and basement membrane are usually absent.
	-theca interna is twice or triple the size of the granulosal layer.
	-cells of the theca are rounded and densley arranged

Table 1 (Continued)

III	Strongly Atretic:
	-granulosa is almost completely absent.
	-there are only a few squamous cells scattered along the theca.
	-theca varies in thickness but cells are loosely arranged and disorganized.
	-basement membrane is absent.
IV	Luteinized Atresia:
	-granulosa is one or two cells thick.
	-granulosal cells are elongated rather than round, hypertrophied, have a basophilic cytoplasm and lie on the theca which is thin and disorganized.

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[a]Histological assessment of atresia was performed by Dr. Pierre Matton, Departmenté de Biologie, Faculté des Sciences, Universite de Sherbrooke (Québec), Canada.

[b]Pictures representative of each rating index are shown in Figure 3.

Figure 3

Examples of four classes of histological atresia of bovine follicles[1].

Key for all plates: a=granulosal layer; b=thecal layer (interna and externa combined); c=ovarian stromal tissue (cortex); d=capillary; e=antrum, containing follicular fluid.

Plate 1: Non-atretic follicle. An example of a non-atretic follicle (see Table 1 for explanation). Thecal and granulosal layers are normal.

Plate 2: Atretic follicle. Granulosal and thecal layers are thin and disorganized.

Plate 3: Strongly atretic follicle. Granulosal layer is almost completely absent. Thecal cells are loosely arranged and disorganized.

Plate 4: Luteinized atretic follicle. Similar to Plate 3, except cells are luteinized.

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[1]Plates representative of each class of follicular atresia in this study are compliments of Dr. Pierre Matton, Departmenté de Biologie, Faculté des Sciences, Universite de Sherbrooke (Québec), Canada.

Figure 3 (Continued)

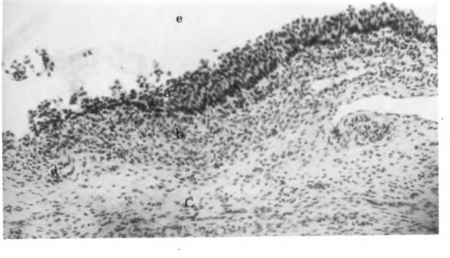


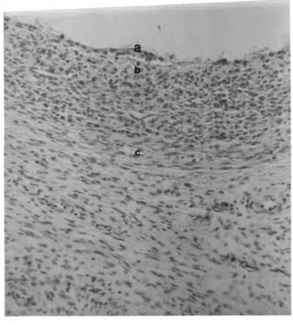
Plate 1

Plate 2

Figure 3 (Continued)

Plate 3

Plate 4



Binding of gonadotropins to granulosa cells

Granulosal cells were scraped free of the follicle wall with a wire spatula and a phosphate buffered saline solution containing 20% glycerol (PBS-glycerol) was used to aspirate the cells from each follicle. The granulosal cell-glycerol solution was frozen with dry ice and methanol and stored at -20°C until specific binding of FSH and HCG (LH) was determined. Saturation analysis was used to determine capacity of granulosal cells to bind gonadotropins (Spicer et al, 1981).

Iodination of FSH and HCG

Ovine FSH (10ug, Siaram-oFSH-1390; 110x NIH-FSH-S10) and HCG (5ug; CR-119, 11600 IU/mg) were iodinated at room temperature (Spicer et al, 1981; Tonetta et al, 1984). A Bio-Gel P-60 (Bio Rad Laboratories, Richmond, CA) column was used to separate radioactive hormone from radioactive iodine not bound to hormone. FSH was further purified with a Con-A-sepharose column (Patritti-Laborde et al, 1979). Specific activity of each radioactive hormone was estimated based on percentage of incorporation of radioactive iodine into the total mass of the hormone. Specific activity of ^{125}I-oFSH and ^{125}I-HCG ranged from 22 to 29 CPM/pg and 45 to 75 CPM/pg, respectively. Active fraction, defined as the maximum percentage of radioactive hormone that could be specifically bound in the presence of excess receptor, varied from 25-35% for ^{125}I-oFSH and 20-40% for ^{125}I-HCG.

Radioreceptor assay

After thawing, granulosal cells were washed twice in ice-cold phosphate buffered saline and centrifuged at 2200xg for 15 min at 5°C after each wash. The final pellet was resuspended in 1 ml of a solution of phosphate buffered saline containing 0.1% bovine serum albumin (Sigma Chemical Company, Fairlawn, New Jersey). A 100 ul aliquot was then assayed in duplicate for concentration of DNA (Burton, 1956) or incubated for 18-24 h at room temperature with 300,000 CPM of ^{125}I-oFSH or ^{125}I-HCG to estimate number of receptor sites for FSH or LH (See Ireland and Roche, 1982). Non-specific binding was determined by addition of a 100 fold excess of nonradioactive FSH (Abbott oFSH-S90390) or LH (NIH-LH-B10) to a parallel set of tubes. Specific binding was determined by subtraction of nonspecific binding from total binding values. All data were expresed as CPM of ^{125}I-oFSH or ^{125}I-HCG specifically bound per ug DNA.

Radioimmunoassay (RIA) of follicular fluid

Tritiated [1,2,5,7-^{3}H] estradiol (100 Ci/mmole), [1,2,6,7-^{3}H] progesterone (100 Ci/mmole) and [1,2,6,7-^{3}H] testosterone (85 Ci/mmole) were purchased from New England Nuclear (Boston, Mass.).

Antiserum produced in the rabbit against estradiol (rabbit-MSU#74) cross reacts less than 4% with other

estrogens and less than 0.02% with other ovarian steroids (Oxender et al, 1977). Antiserum against progesterone (rabbit-MSU#74) cross reacts 10% with 20-∝-hydroxypregn-4-ene-2-one, 4.2% with 17-∝-hydroxyprogesterone, 3.5% with testosterone, 2.5% with androstenedione and less than 0.4% with other ovarian steroids (Convey et al, 1977). Antiserum against testosterone (rabbit-MSU#74) cross reacts 60% against dihydrotestosterone, 1.7% against androstenedione and less than 0.14% against other ovarian steroids (Kiser et al, 1978).

Before RIA, samples of FF were diluted 1:100 for estradiol and progesterone and 1:50 for testosterone assays. Diluted samples of FF were assayed directly without extraction (Ireland and Roche, 1982). Standard curves ranged from 0-200.0 pg/tube for estradiol, 0-2.0 ng/tube for progesterone and 0-1.0 ng/tube for testosterone.

Fifty to one hundred microliters of diluted FF was added to duplicate sample tubes. Two hundred microliters of tritiated estradiol (5,000 CPM), progesterone (10,000 CPM) or testosterone (5,000 CPM) was then added to all total binding, background, standard curve and sample tubes. Two hundred microliters of antiserum to estradiol (1/40,000 dilution), progesterone (1/2,000 dilution) or testosterone (1/10,000 dilution) was finally added to all except 3 background tubes in each assay. After incubation, 500 ul of dextran-coated charcoal (0.5% in PBS) was added to each tube, mixed and immediately centrifuged at 3000xg

for 15 min at 5°C. Supernatant was poured into scintillation vials, 500 ul Safety Solve (Research Products International, Mt. Prospect, Ill.) added, contents mixed and counted for 2 min in an Isocap 300 scintillation counter (Searle Analytic Company).

B. Statistical Analysis

When proportions were examined, they were arranged into contingency tables and tested for independence using the Chi-square statistic (Gill, 1978). If significant ($p<.1$), main effects were evaluated using the Bonferroni-Chi-square test of goodness of fit (Gill, 1978).

If concentration of steroids in follicular fluid or specific binding data were not normally distributed or were heterogenous in variance, data were corrected by natural log ($\log_e$) transformation prior to analysis. Note that arithmetic rather than transformed means were reported in tables even though analysis had been performed with transformed values. Hormone and binding data were arranged into contingency tables and tested for independence using the Fisher Analysis of Variance test (Gill, 1978). If significant ($p<.1$), individual mean comparisons were performed with the Bonferroni-t-test (*a priori* contrasts; Gill, 1978) or Dunnett's test (*a posteriori* contrasts; Gill, 1978).

Three specific questions will be addressed in the results section for Experiment 1:

1. Can appearance (opaqueness) of the surface of an ovarian follicle be used as an indicator of histological atresia?

2. What is the relationship between appearance of a follicular surface and concentration of steroids in follicular fluid and binding of gonadotropins by granulosal cells?

3. What is the relationship between histological atresia and concentration of steroids in follicular fluid and binding of gonadotropins by granulosa?

Specific statistical analyses performed for each question have been incorporated into the results section.

C. Results of Experiment 1

1. Can appearance of the surface of an ovarian follicle be used as an indicator of histological atresia?

In order to examine the relationship between degree of opaqueness of a follicle and histological atresia, a 3 x 3 x 2 x 4 contingency table was created consisting of surface appearances of follicles (clear, intermediate, opaque), classes of atresia (non-atretic, atretic, strongly atretic), categories of follicular size (5.0-13.0, 13.0-25.0 mm in diameter) and estimated stages (I to IV) of an estrous

cycle. Luteinized follicles (atresia class IV) were excluded (n=16). These follicles were not undergoing degenerative atresia as defined by Byskov (1979) but were luteinized and cystic at time of collection (Ingram, 1962; Nalbandov, 1976).

The proportion of non-atretic, atretic and strongly atretic follicles within each class of surface appearance did not differ ($p>.1$) with stage of an estrous cycle or category of follicular size. In addition, proportions of non-atretic, atretic and strongly atretic follicles did not differ ($p>.1$) between clear and intermediate classes. Therefore, data were pooled to form a 2 x 3 contingency table (Table 2) consisting of 2 classes of surface appearance (opaque, non-opaque) and 3 classes of atresia. The proportion of non-atretic, atretic and strongly atretic follicles differed ($p<.01$) between categories of surface appearance. In the non-opaque category, approximately twice as many follicles were classified histologically as non-atretic or atretic than strongly atretic. Whereas, in the opaque category, more follicles were classified as strongly atretic than atretic or non-atretic.

Can opaqueness of a follicle be used as an accurate indicator of atresia? In order to examine this question, a 2 x 2 contingency table (Table 3) consisting of 2 classes of surface appearance (opaque, non-opaque) and 2 classes of atresia (non-atretic, atretic) was formed from the above data. A diagnostic test (see Appendix IV; Youden,

Table 2

Proportion of atretic follicles within each category of surface appearance

Classes of Atresia[1]	Surface appearance of a follicle[2] Non-opaque	Opaque	Total
Non-atretic	61(40%)[3]	32(29%)	93
Atretic	57(37%)	30(27%)	87
Strongly Atretic	36(23%)	49(44%)	85
Total	154 (100%)	111 (100%)	

[1]Refer to Table 3 for explanation.

[2]Refer to figures 1 and 2 for explanation.

[3]Parentheses represent percentage of follicles in each atresia class. Proportions of non-atretic, atretic and strongly atretic follicles differed ($p<.01$) between opaque and non-opaque classes.

Table 3

How well does opaqueness of a follicular surface indicate atresia?[1]

	Atretic follicle[2]	Non-atretic
Opaque	79(46%)[4]	32(34%)
Non-opaque[3]	93(54%)	61(66%)
Total	172	93

[1]See Appendix IV for explanation of statistical analysis.

[2]Atretic and strongly atretic follicles were pooled into an 'atretic' class.

[3]Clear and intermediate follicles were pooled into a 'non-opaque' class.

[4]Parentheses indicate percentage of column total in each category. The Index (see Appendix IV)=11.5±6.2%. The index rates how well atretic or non-atretic follicles can be selected by appearance of their follicular surfaces. This test indicated that reliability was low.

1950) was utilized to test how accurately appearance of the surface of a follicle could be used to identify histological atresia in a follicle. The rating index for this diagnostic test (see Table 3; Appendix IV) was 11.5 ± 6.2%. An index rating of zero results if equal proportion of atretic follicles occur in both opaque and non-opaque classes and 100% if all non-opaque follicles are non-atretic and all opaque follicles are atretic. An index rating of 11.5% indicates that degree of opaqueness of the surface of a follicle was not an accurate method of predicting whether a follicle was histologically atretic.

2. <u>What is the relationship between appearance of a follicular surface and concentration of steroids in follicular fluid and binding of gonadotropins by granulosal cells</u>?

A 3 x 2 x 4 contingency table was created consisting of classes of surface appearance (clear, intermediate, opaque), classes of follicular size (5.0-13.0, 13.0-25.0 mm in diameter) and estimated stages of an estrous cycle (I to IV). Concentration of steroids in FF and binding of gonadotropins by the granulosa within each surface appearance category did not differ ($p>.1$) with stage of an estrous cycle or follicular size so data were pooled to form Table 4. After pooling, concentration of testosterone in FF and binding of gonadotropins by granulosal cells did not

Table 4

Concentration of steroids in follicular fluid and binding of gonadotropins by granulosal cells of opaque and non-opaque follicles

	Surface appearance of a follicle[1]			
	Clear	Intermediate	Opaque	Mean
Estradiol (mean±SEM; ng/ml)	$93\pm22_a$ (n=81)[2]	$155\pm42_b$ (78)	$137\pm26_b$ (103)	129±17 (262)
Progesterone (ng/ml)	$143\pm17_a$ (83)	$233\pm58_b$ (79)	$183\pm25_b$ (103)	185±21 (265)
Testosterone (ng/ml)	$17\pm3_a$ (79)	$15\pm2_a$ (77)	$14\pm2_a$ (116)	15±1 (258)
FSH binding (CPM/ug DNA)	$1072\pm96_a$ (86)	$953\pm78_a$ (79)	$1115\pm83_a$ (116)	1056±50 (281)
HCG binding (CPM/ug DNA)	$557\pm54_a$ (86)	$748\pm90_a$ (79)	$630\pm61_a$ (116)	641±40 (281)

[1]Refer to figures 1 and 2 for explanation.

[2]Parentheses indicate number of follicles in each category.

Values within a row not sharing a common subscript differed at ($p<.1$), but not at ($p<.05$).

differ between clear, intermediate or opaque classes. Concentrations of estradiol and progesterone here lower ($p<.1$) in clear follicles than intermediate or opaque ones.

3. What is the relationship between histological signs of atresia and concentration of steroids in follicular fluid and binding of gonadotropins by granulosal cells?

In order to examine the relationship between atresia determined histologically and concentration of steroids in FF and capacity of granulosal cells to bind gonadotropins, a 4 x 2 x 4 contingency table was created. This table consisted of classes of atresia (non-atretic, atretic, stongly atretic, luteinized), categories of follicular size (5.0-13.0, 13.0-25.0 mm in diameter) and stages of an estrous cycle (I to IV). Concentration of steroids in FF and binding of gonadotropins by granulosa did not differ ($p>.1$) with stage of an estrous cycle or follicular size so data were pooled to form Table 5. Concentration of steroids in FF and binding of gonadotropins by granulosal cells did not differ between non-atretic and atretic classes of follicles (Table 5). Concentration of estradiol in FF and binding of ^{125}I-oFSH by granulosa in non-atretic and atretic follicles were higher ($p<.05$) and concentration of progesterone lower ($p<.05$) than in strongly atretic and luteinized follicles. Concentration of steroids in FF and binding of ^{125}I-oFSH by granulosal cells differed ($p<.05$) by

Table 5

Concentration of steroids in follicular fluid and binding of gonadotropines by granulosa of follicles in four classes of atresia

	Non-atretic	Atretic	Strongly Atretic	Luteinized
Estradiol (mean±SEM; ng/ml)	$142\pm21_a$[2] (n=85)[3]	$163\pm49_a$ (77)	$105\pm21_b$ (85)	$13\pm12_c$ (13)
Progesterone (ng/ml)	$105\pm14_a$ (87)	$111\pm12_a$ (77)	$225\pm29_b$ (85)	$882\pm303_c$ (13)
Testosterone (ng/ml)	$16\pm2_a$ (83)	$16\pm3_a$ (75)	$14\pm2_a$ (84)	$6\pm2_b$ (13)
FSH binding (CPM/ug DNA)	$1259\pm86_a$ (93)	$1034\pm92_{ab}$ (86)	$983\pm89_b$ (83)	$385\pm117_c$ (16)
HCG binding (CPM/ug DNA)	$693\pm63_a$ (93)	$634\pm67_a$ (86)	$630\pm88_a$ (83)	$486\pm104_a$ (16)

[1]Refer to Table 3 for explanation.

[2]All samples transformed prior to statistical analysis. Values within a row not sharing a common subscript differed ($p<.05$).

[3]Parentheses indicate number of follicles in each category.

2 to 8-fold between strongly atretic and luteinized follicles. Binding of ^{125}I-HCG did not differ ($p>.1$) between classes of atresia.

In order to further quantify the relationship between concentrations of estradiol and progesterone in FF and atresia, follicles were divided into 3 groups (I-III) depending on ratio of concentration of estradiol to progesterone in FF (Table 6). Proportion of non-atretic to atretic and strongly atretic follicles in group I (E:P = .001 to .1) differed ($p<.001$) from groups II(E:P = .1 - 1.0) and III (E:P = 1 to 20). Four-times as many of the follicles in group II (39%) or III (47%) were non-atretic as in group I (11%). This suggests that ratio of estradiol to progesterone in follicular fluid may be useful in the selection of non-atretic follicles.

D. Discussion of Results--Experiment 1

I hypothesized that degree of opaqueness of a follicular surface would indicate whether a follicle is atretic. Results of Experiment 1 indicated that the diagnostic value of selecting atretic or non-atretic follicles by degree of opaqueness of the follicular surface was poor. Concentration of steroids in FF but not binding of gonadotropins by granulosal cells, two indicators of follicular function, differed with degree of opaqueness of a follicular surface.

Table 6

Relationship of atresia to ratio of concentrations of estradiol (E) to progesterone (P)

	I	II	III	
Atresia class[2]	E:P[1]=.001-.1	.1-1.0	1-20	Total
Non-atretic	7 (11%)[3]	35 (39%)	43 (47%)	85
Atretic	21 (33%)	34 (37%)	22 (24%)	77
Strongly Atretic	36 (56%)	22 (24%)	27 (29%)	85
Total	64	91	92	247

[1]Concentration of estradiol divided by progesterone.

[2]See Table 1 for explanation.

[3]Parentheses indicate percent of total within a column. Proportions of non-atretic, atretic and strongly atretic follicles in group I differed from groups II and III $p<.001$). Groups II and III did not differ ($p>.1$) from each other.

Differences in binding of gonadotropins by granulosal cells (Carson et al, 1979) and concentration of estradiol in FF (Kruip and Dielman, 1982) were evident between follicles classified as atretic versus non-atretic by their appearance using methods of Moor et al, (1978). Moor et al (1978) examined excised follicles, dissected free of stromal tissue. It should be noted that the present study examined intact, non-excised follicles where stroma was not dissected free of follicles.

Although degree of opaqueness of the surface of a bovine follicle did not accurately indicate whether a follicle was atretic, concentration of steroids in FF which represent steroidogenic capacity of the follicular cells (England et al, 1981; Hillier et al, 1981) and granulosal binding of FSH were related to histological signs of atresia. Reduced binding of FSH by granulosal cells and a shift from estradiol to progesterone production in strongly atretic follicles, as evident by FF concentrations, are consistent with reports by Channing and Kammerman (1973), Moor et al (1978), Bomsel-Helmreich et al (1979), Carson et al (1979), Merz et al (1981), McNatty et al (1979a, b, c, 1982), Ireland and Roche (1982, 1983a, b) and Bellin and Ax (1984). A reduction in binding of HCG (LH) atresia was not observed in the present study. Since binding of gonadotropins by granulosa and concentration of steroids in FF did not differ between (histologically) non-atretic follicles and follicles in beginning stages of atresia, it

was possible that some atretic follicles were functionally similar to non-atretic ones. Thus, as previously suggested by McNatty et al (1979a), Brailly et al (1981) and Ireland and Roche (1983a), steroid levels in follicular fluid and ratio of these levels may be a better indicator of follicular function than histological condition of the follicular cells.

In conclusion, selection of atretic or non-atretic follicles by appearance of the surface of bovine ovarian follicles is unreliable. However, ratio of estradiol to progesterone in FF, as well as actual concentrations, may be useful in determining if a follicle is atretic.

Experiment 2

The Relationship between Appearance of the Surface of Bovine Ovarian Follicles and Ability of Oocytes to Mature *In Vitro*.

Experiment 2

The Relationship between Appearance of the Surface of Bovine Ovarian Follicles and Ability of Oocytes to Mature In Vitro

A. Materials and Methods

The objective of this second experiment was two fold: first, to determine if degree of opaqueness of the surface of bovine ovarian follicles could be used to predict capacity of oocytes to mature *in vitro*; and, second, to examine the relationship between concentration of steroids in follicular fluid and capacity of oocytes to mature *in vitro*.

Bovine ovaries (n=74) were collected in pairs from non-pregnant cows at a local abattoir and placed on ice within 30 min of slaughter. All ovaries were processed within 6 h of collection. One follicle from each ovary (n=73) was placed into one of 16 categories consisting of 4 estimated stages of an estrous cycle (I-V; see Appendix I), 2 categories of follicular size (5.0-13.0, 13.0-25.0 mm in diameter) and 2 classes of surface appearance (clear, opaque; see Figure 2). Number of follicles (oocytes) in each of the 16 categories ranged from 4 to 6.

Collection of oocytes

Follicular fluid from each follicle was aspirated, deposited onto a sterile glass plate, and examined under a stereomicroscope (magnification = 20 x) for presence of an occyte. If an occyte was not located, the wall of the follicle was scraped with a wire spatula, phosphate-buffered-saline injected and an attempt made to locate the oocyte in the washings. Follicular fluid was frozen until concentrations of estradiol, progesterone and testosterone were determined as specified in Experiment 1.

Incubation of oocyte and evaluation of meiotic maturity

Following collection, oocytes were washed with a modified T.C. 199 medium (Appendix III) and evaluated for extrusion of a polar body. Oocytes were then deposited individually into a chamber of a tissue culture slide (Lab-Tek; Miles Laboratories Inc., Napierville, Ill.) containing 500 ul of culture medium. Oocytes were cultured for 30 h in humidified air at 37-39^{o}C.

After culture, oocytes were evaluated under a dissecting microscope (magnification = 50-70x). Oocytes which had extruded a polar body by 30 h in culture were classified as mature while all others were classified as immature. No attempt was made to determine stage of meiosis in immature oocytes.

B. Statistical Analysis

Statistical analyses of results were performed as explained for Experiment 1.

Two specific questions will be addressed in the results section for Experiment 2:

1. Can appearance of the surface of bovine ovarian follicles be used to predict whether an oocyte will mature *in vitro*?

2. What is the relationship between concentration of steroids in follicular fluid and maturation of bovine oocytes *in vitro*?

Specific statistical analysis performed for each question have been incorporated into the results section.

C. Results of Experiment 2

1. Can appearance of the surface of bovine ovarian follicle be used to predict whether an oocyte will mature *in vitro*?

Atretic follicles in ewes (Moor *et al*, 1978) and mice (Wilkinson *et al*, 1979) lose their translucency and become opaque. In addition, during atresia the oocyte may become necrotic, shrink, lyse or be resorbed by the follicle (Byskov, 1978) so that maturation of the oocyte may be impossible. I hypothesized that clear follicles, which were

expected to be non-atretic would yield oocytes with a higher incidence of maturation to metaphase II *in vitro* than opaque follicles which were expected to be atretic.

To test this hypothesis, incidence of oocyte maturation was determined for clear and opaque follicles. Proportions of oocytes maturing within categories of surface appearance did not differ by stage of an estrous cycle or follicular size so data were pooled Table 7. Twice as many ($p<.01$) oocytes from clear follicles matured as from opaque follicles. This indicated that appearance of the surface of a bovine follicle was a good indicator of capability of an oocyte to mature *in vitro*.

2. What is the relationship between concentration of steroids in follicular fluid and maturation of oocytes in vitro?

Concentrations of estradiol and progesterone in follicular fluid of humans are higher in follicles yielding oocytes that mature *in vitro* than follicles yielding oocytes that do not (Channing *et al*, 1983) . In support, Robertson and Baker (1969) in cattle and McGaughey (1977) in pigs found that addition of progesterone to culture of oocytes had a stimulatory effect. In contrast, addition of estradiol to culture inhibited maturation of oocytes.

Table 7

Incidence of maturation to metaphase II of oocytes removed from clear versus opaque follicles

	Appearance of the surface of a follicle[1]	
	Clear	Opaque
Number of mature oocytes[2]	19/35(54%)[3]	9/38(24%)

[1] See figures 1 and 2 for explanation.

[2] Oocytes were classified as mature if they extruded a polar body by 30 h in culture

[3] Percent maturation in parenthesis. Proportion of oocytes that matured differed ($p<.01$) between clear and opaque follicles.

I hypothesized that concentration of steroids in follicular fluid of cattle would relate to incidence of oocyte maturation *in vitro*. To test this hypothesis, a 2 x 2 contingency table for each steroid (estradiol, progesterone, testosterone) consisting of classes of surface appearance (clear, opaque) and stages of maturation of oocytes (mature, immature) was created (Table 8).

Overall, opaque follicles had a 3-fold greater ($p<.05$) concentration of estradiol in follicular fluid than clear follicles. Concentrations of testosterone and progesterone in FF did not differ between clear and opaque groups. However, clear and opaque follicles which yielded oocytes capable of maturation had a 2-fold greater ($p<.05$) concentration of progesterone in FF than those yielding immature oocytes. High concentrations of progesterone and low concentrations of estradiol in FF, similar to those found in strongly atretic or luteinized follicles (see Table 5), appeared to be related to capability of bovine oocytes to mature *in vitro*.

To further examine the relationship between concentrations of estradiol and progesterone in FF and maturation of oocytes *in vitro*, follicles were divided into 3 groups (I-III) based on the ratio of concentrations of estradiol to progesterone (E:P) in FF (Table 9). Proportions of oocytes maturing *in vitro* in group I (E:P=.001 to .1) was

Table 8

Concentrations (ng/ml) of steroids in follicular fluid of clear and opaque follicles.

	Appearance of follicle surface[1]								
	Clear			Opaque			Mean		
Oocyte:	Mature[2]	Immature[2]	Mean	Mature	Immature	Mean	Mature	Immature	Mean
Estradiol (mean±SEM)	$66\pm16_a$[3]	$66\pm33_a$	$66\pm17_a$	$351\pm202_b$	$179\pm74_b$	$216\pm36_b$	$151\pm63_{ab}$	$138\pm50_{ab}$	$143\pm39_{ab}$
Progesterone	$192\pm29_a$	$115\pm23_b$	$157\pm20_{ab}$	$228\pm73_a$	$130\pm31_b$	$153\pm29_{ab}$	$204\pm30_a$	$125\pm21_b$	$155\pm18_{ab}$
Testosterone	$30\pm6_a$	$35\pm4_a$	$33\pm4_a$	$39\pm13_a$	$32\pm4_a$	$34\pm4_a$	$33\pm6_a$	$33\pm3_a$	$33\pm3_a$
n	(19)	(16)	(35)	(8)	(29)	(37)	(28)	(45)	(73)

1 See figures 1 and 2 for explanation.
2 See legend of Table 7 for explanation
3 All values transformed prior to statistical analysis. Values in a row not sharing a common subscript differed ($p<.05$).

n=number of follicles in each group.

Table 9

Relationship of incidence of oocyte maturation, concentration of progesterone (P) and estradiol (E) in follicular fluid and ratio of E to P.

	Ratio of E to P:[1]		
	.001 - .1	.1 - 1.0	1.0 - 20
Number of Oocytes that Maturated in vitro.	$11/18_a$[3] (61%)	$10/30_{ab}$(33%)	$6/24_b$ (25%)
Estradiol (mean ± SEM;ng/ml)	$5\pm1_a$	$56\pm10_b$	$356\pm104_c$
Progesterone (ng/ml)	$271\pm48_a$	$112\pm10_b$	$95\pm14_b$

[1]Concentration of estradiol divided by progesterone.

[2]See legend of Table 7 for explanation.

[3]Values within a row not sharing a common subscript differed ($p<.05$). In addition, number of oocytes that matured in vitro tended to differ ($p<.1$) between group I (.001 - .1) and group II (.1-1.0).

greater ($p<.05$) than in group III (E:P=1-20) and was also greater ($p<.1$) than group II (E:P=.1-1.0).

These data indicated that oocytes capable of *in vitro* maturation come from follicles with high concentrations of progesterone and low concentrations of estradiol in FF.

D. Discussion of Results--Experiment 2

Oocytes are arrested in prophase of meiosis I, because of interactions with follicular cells. These cells produce an oocyte maturation inhibiting substance (OMI; Tsafriri and Channing, 1975; Tsafriri *et al*, 1982) and CAMP (Beers and Dekel, 1981) which may enter the oocyte through gap junctions. The preovulatory gonadotropin surge (LH) disrupts 60-80% of these gap junctions (Moor and Osborn, 1983) isolating the oocyte from these inhibitory substances and allowing the oocyte to mature. Removal of an oocyte from the follicle also results in maturation *in vitro*. This procedure could also result in removal of inhibitors produced by the follicular cells. Cholera endotoxin, which irreversibly activates andenylate cyclase, inhibits maturation of cumulus-enclosed oocytes but not denuded ones (Beers and Dekel, 1981). This observation suggests that oocytes lack adenylate cyclase and receive inhibition by CAMP (or substances stimulated by CAMP) via gap junctions. As expected, LH removes the inhibition of cholera toxin on cumulus-enclosed oocytes (Dekel and Beers, 1978). Thus, one

reason for an incidence of maturation less than 100% by oocytes in this study may be due to continued inhibition of oocyte maturation by adhering follicular cells. This seems reasonable since Leibfied and First (1979) have demonstrated a relationship between condition of adhering follicular cells and ability of oocytes to mature in vitro.

The preovulatory gonadotropin surge induces a marked reduction in concentration of estradiol in preovulatory follicles and a several-fold increase in concentration of progesterone (Linder et al, 1974; Eiler and Nalbandov, 1977; Staigmiller and England, 1982; Dielman et al, 1983; Ireland and Roche, 1982, 1983a) concurrent with maturation of an oocyte (Ainsworth et al, 1980; Dielman et al, 1983). Similar changes in concentration of steroids in follicular fluid occur during atresia (Moor et al, 1978; Bomsel-Helmreich et al, 1979; Carson et al, 1979; Merz et al, 1981; McNatty et al, 1979a, b, c, 1982; Ireland and Roche, 1982; 1983b, Bellin and Ax, 1984). Oocytes sometimes mature prematurely within atretic follicles (Byskov, 1979) due to changes in these steroids and a reduction in oocyte maturation inhibitor (Channing et al, 1983) due to degeneration of the follicular cells. Addition of estradiol to culture media inhibits maturation of cow, rabbit (Robertson and Baker, 1969) and pig (McGaughey, 1977) oocytes while addition of progesterone to these cultures overcomes this inhibition. Following an injection of HCG (Mori et al, 1983) in rats, injection of an antiserum to

progesterone markedly decreases the proportion of oocytes from follicles greater than 250um in diameter undergoing germinal vesicle breakdown. Injection of progesterone partially reverses the inhibitory actions of the progesterone antiserum while injection of progesterone with an antiserum to estrone completely reverses the inhibitory effects of the progesterone antiserum. These studies suggest that high levels of estrogens in follicular fluid, as found during development of a preovulatory follicle, may inhibit maturation of oocytes. Thus, high intrafollicular levels of estradiol may prevent premature maturation of oocytes *in vivo*. Reduction in concentration of estradiol and an increase in concentration of progesterone in follicular fluid, as occurs in atretic follicles or following the preovulatory gonadotropin surge, may provide an oocyte with the capability to mature *in vivo* or *in vitro*. The potential inhibitory effect of estradiol on maturation of oocytes is supported by the results of Experiment 2. Although it was not determined whether a follicle was preovulatory or atretic in Experiment 2, proportion of oocytes maturing *in vitro* in a group of follicles with high concentrations of progesterone and low estradiol (see Table 8), was over twice ($p<.05$) that of a group with low progesterone and high estradiol.

In contrast, human follicles yielding oocytes capable of maturation have high levels of both estradiol and progesterone in follicular fluid (McNatty *et al*, 1979b;

Channing et al, 1983). Perhaps, estradiol has no inhibitory effect on maturational ability of oocytes in humans as it may in cattle. Alternately, inhibition of oocyte maturation in humans by estradiol could be overridden by the effects of progesterone.

Clear follicles contained a lower overall mean concentration of estradiol in follicular fluid and a higher proportion of oocytes maturing in vitro than opaque follicles. Mean concentration of progesterone did not differ between classes of surface appearance. Further studies are needed to determine if differences in proportion of oocytes maturing in vitro between clear and opaque classes were due primarily to differences in concentration of steroids in follicular fluid between these 2 classes of surface appearance or if others factors, such as concentration of an oocyte maturation inhibitor (Channing et al, 1982) were also involved.

Proportion of oocytes maturing in vitro did not differ with stage of an estrous cycle or follicular size. These results agreed with Thibault et al (1976), Leibfried and First (1977) and Fukui and Sakuma (1980). Oocytes in cattle may be capable of in vitro maturation by the time they are 5 mm in diameter since proportion of oocytes maturing in vitro did not differ by follicular size in this study. One would expect elevated concentrations of estradiol in larger follicles and during the follicular phase (data not shown), which could be inhibitory towards maturation of oocytes in

cattle (Robertson and Baker, 1969), would be related to a lower incidence of maturation than in small follicles and during later stages of the luteal phase in cattle when estradiol levels in follicular fluid may be lower. Since follicular size or stage of an estrous cycle did not affect in vitro maturation of oocytes, I speculate that mean concentration of estradiol or oocyte maturation inhibitor (OMI) in smaller follicles may be sufficient to inhibit subsequent maturation of oocytes until intrafollicular levels of progesterone override the inhibitory effects of estradiol or OMI.

Why don't all oocytes mature in vitro? Osborn and Moor (1983) suggest that sequential changes in estradiol and progesterone stimulate the synthesis of RNA and proteins, including actin, which are necessary for the subsequent maturation of oocytes. If levels of steroids or patterns of secretion of steroids in follicular fluid are insufficient for proper stimulation of oocytes, oocytes may not be capable of maturation even when inhibitory effects of the follicular cells are removed. The addition of steroids and nutrients to culture media may overcome insufficiency of the follicular environment in some cases and may explain the variation in results among investigators.

In summary, maturation of bovine oocytes was maximal when collected from clear follicles. In addition, maturation was related to high concentrations of progesterone in follicular fluid and low estradiol. I

speculate that these hormones may be involved in the control of maturation of bovine oocytes, possibly by regulation of RNA and protein synthesis or uptake of CAMP, OMI and/or required nutrients.

Summary and Conclusions

Results of these experiments indicated that:

1. Even though non-opaque follicles (clear and intermediate) were twice as likely to be non-atretic as atretic or strongly atretic, selection of atretic or non-atretic follicles by appearance of their follicular surface was not accurate.

2. Concentrations of estradiol and progesterone in follicular fluid and their ratio of these hormones and binding of gonadotropins by granulosal cells may indicate if a follicle is atretic.

3. The relationship between follicular function (concentration of steroids in follicular fluid and binding of gonadotropins by granulosal cells) and histological signs of atresia is not strong under certain conditions and needs to be further examined.

4. Proportion of oocytes that matured to metaphase II *in vitro* was greater in clear than opaque follicles.

5. Concentration of estradiol in follicular fluid, which may inhibit maturation of oocytes, was higher in opaque than clear follicles. Concentration of progesterone, which may be stimulatory toward ability of an oocyte to mature, did not differ between clear and opaque follicles

but was higher in both clear and opaque follicles which yielded an oocyte capable of maturation *in vitro*.

I conclude that prediction of whether a bovine follicle is atretic or non-atretic based only on appearance of the surface of a follicle, is not reliable. However, appearance of a follicular surface and concentration of steroids in follicular fluid can be used to predict whether bovine oocytes are likely to mature *in vitro*.

Appendices

Appendix I

Changes in characteristics of corpora lutea during the bovine estrous cycle[a]

Estimated days of an estrous cycle	Stage of an estrous cycle	Appearance of the corpus luteum (CL)
1-4	I	Point of rupture of ovulatory follicle is apparent and not grown over, surface of CL red.
5-10	II	Body of CL is red to brown, vasculature around periphery of surface of CL, point of rupture of ovulatory follicle covered over with stromal tissue.
11-17	III	Body of CL tan to orange, no red or brown color remaining, vasculature apparent over apex of surface of CL.
18-20	IV	Body of CL is yellow, vasculature is absent from surface of CL.

[a]Ireland et al (1980).

Appendix II

Modified Karnovsky's fixative[a]

Ingredients:

1g paraformaldehyde (Fisher Scientific Co., Fairlawn, N.J.)
10ml double distilled-$h_2 0$ at 70°C
2.5ml of 25% glutaraldehyde (Sigma Chemical Co., St. Louis, MO)

0.2M phosphate buffer (Ingredients: Sigma Chemical Co.)

(1.92g NaH_2PO_4 + 11.92g Na_2HPO_4. Bring up to 500 ml with ddH_2O. Add 0.5ml of 1:100 thimersol-ddH_2O. Adjust pH to 7.4).

Dissolve the paraformaldehyde and H_2O by continuous stirring. Clear with 1-2 drops of 1N NaOH. Cool to room temperature. Add the glutaraldehyde and then enough phosphate buffer to make a total volume of 25 ml.

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[a]Formula compliments of Kay Brabec, University of Michigan, Ann Arbor.

Appendix III

Basic Contents of Medium for Culture of Oocytes

Ingredients[a]	Amount	Source
TC 199[b]	72 ml	GIBCO laboratories Grand Island, N.Y.
$NaHCO_3$	2.38 mg/ml	Mallinckrodt, Inc. St. Louis, MO
D-Glucose	6.2 mg/ml	Mallinckrodt, Inc. St. Louis, MO
Sodium Pyruvate	40 ug/ml	Sigma Chemical Co. St. Louis, MO.
Phenol Red	3.6 ug/ml	Sigma Chemical Co. St. Louis, MO.
Fetal Calf Serum[c]	10 ml	GIBCO Laboratories Grand Island, N.Y.
Penicillin	50 I.U./ml	GIBCO Laboratories Grand Island, N.Y.
Streptomycin	50 ug/ml	GIBCO Laboratories Grand Island, N.Y.
D.D. H_2O[d]	Approx. 18 ml	

[a]Ingredients to make 100 ml of a modified TC 199 Medium (Fulka et al, 1982; Brackett, 1983).

[b]Medium 199 with Hanks salts and L-glutamine.

[c]Fetal calf serum was heated for 30 min at 56°C and then charcoal extracted while stirring for 20 min at 37°C. Fetal calf serum was substituted for calf serum protein.

[d]Double-Distilled H_2O to bring total volume up to 100 ml.

Appendix IV

Rating of diagnostic tests from 2 x 2 contingency tables[a]

A diagnostic test for a specific disease may give false negatives (failure to detect disease that exists) and false positives (results that indicate disease in non-diseased individuals, i.e., one of the control group). This index rates the overall performance of a diagnostic test in avoiding both kinds of false results.

In Experiment 1, I wanted to know how well appearance of the surface of ovarian follicles could be used to predict whether a follicle was atretic. Therefore, Youden's (1950) rating system for diagnostic test was used to evaluate reliability of predicting atresia based on surface appearance of a follicle. In this test, atresia was substituted for diseased individuals. Opaque follicles were expected to be atretic while non-opaque follicles were expected to be non-atretic.

Let A = classification given by the test (i=1=positive or opaque; i=2=negative or non-opaque).

Let B = incidence of a specific disease (j=1=diseased or atretic; j=2=control or non-atretic).

Appendix IV (Continued)

	$B_1(D)$	$B_2(C)$
$A_1(+)$	y_{11}	y_{12}
$A_2(-)$	y_{21}	y_{22}
Totals	$y_{.1}$	$y_{.2}$

Correctly minus incorrectly-classified atretic (A) individuals: $(y_{11}-y_{21}/y_{.1})=y_A$.

Correctly minus incorrectly-classified non-atretic (N) individuals: $(y_{22}-y_{12}/y_{.2})=y_N$.

Index=$J=(y_A+y_N)/2$; $0 \leq J \leq 1$; Zero is achieved if the test gives the same proportion of positives for both atretic and non-atretic groups. Unity is achieved only if the test gives no false results of either kind.

The index has standard error = $SE_J=[(y_{11}y_{21}/y_{.1}^3)+(y_{12}y_{22}/y_{.2}^3)]^{1/2}$.

Results of Test:

$y_A=(79-93/172)=-8.1\%$

$y_N=(61-32/93)=31.2\%$

Index=$(y_A+y_N)/2=(-8.1+31.2)/2=11.6\%$

$SE_J=[(79 \times 93)/172^3 + (32 \times 61)/93^3)]^{1/2}=6.2\%$

Therefore, Index rating = $11.6 \pm 6.2\%$. The index indicates that selection of atretic or non-atretic follicles by appearance of their follicular surface was not accurate.

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[a]Youden (1950).

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