1999

Oocyte morphology and estrogen concentrations following a reduction in progesterone in beef cattle

Robert A. Taft Jr.
West Virginia University

OOCYTE MORPHOLOGY AND ESTROGEN CONCENTRATIONS FOLLOWING A REDUCTION IN PROGESTERONE IN BEEF CATTLE

Robert A. Taft Jr.

Dissertation

Submitted to the Graduate Faculty

of the College of Agriculture and Forestry at West Virginia University

in partial fulfillment of the requirements for the Degree of

Doctor of Philosophy

in Reproductive Physiology

Dr. Keith Inskeep
Dr. Robert Dailey
Dr. Mark Gibson
Dr. John Killefer
Dr. Edwin Townsend

Division of Animal and Veterinary Sciences

Morgantown West Virginia

1999

Keywords: ovary, oocyte, follicle, estrogen, cattle

ABSTRACT

Oocyte Morphology and Estrogen Concentrations Following a Reduction in Progesterone in Beef Cattle

Robert A. Taft Jr.

Low dosages of progestogens promote persistent follicles, high systemic estrogen and low fertility. The objectives of this study were to determine effects of a reduction in progesterone on (1) morphology of oocytes and intrafollicular concentrations of estradiol. Cows on low progesterone (n=12) received used intravaginal progesterone inserts on d 4 after estrus and prostaglandin (PG) $F_{2\alpha}$ (25 mg, i.m.) on d 6. Control animals (n=12) received saline on d6. The oocyte and follicular fluid were recovered from the largest follicle on d 8 or d 10.

Serum estradiol was lower during d 4-6 but greater ($P < .01$) during d 7-10 in cows treated with progesterone inserts and $PGF_{2\alpha}$ while the largest follicle was larger in treated cows on day 10 only (14 vs. 12 mm; $P < .05$). Intrafollicular concentrations of estrogen were greater in treated than in control cows (990 ± 87 vs 191 ± 106; $P < .01$). Progesterone in follicular fluid (mean = 42 ng/ml) did not differ. Oocytes were observed in oocyte nuclear stage I in the control group on d 8. All other oocytes were in nuclear stage II. In addition, the degree of clumping of mitochondria, the percentage of intact cumulus cell processes and percentage of normally shaped mitochondria was greater in oocytes from d 8 control cows than in all other groups.

Changes in concentrations of estradiol and oocyte morphology typically associated with the preovulatory period had occurred within 2 d after a reduction in progesterone, even when low peripheral concentrations of progesterone were maintained. These earliest stages of oocyte maturation occurred in response to a reduction in progesterone. Similar changes in oocyte morphology were observed in control animals by d 10 of the estrous cycle, probably representing the onset of atresia.

ACKNOWLEDGEMENTS

I would like to thank my mentor, Dr. Keith Inskeep, for his guidance, wisdom and support that have helped me complete my program and grow as a scientist. I would also like to thank the other members of my committee Drs. Robert Dailey, Mark Gibson, John Killefer and Edwin Townsend for their insight and assistance. I would especially like to thank Dr. Killefer for allowing me the opportunity to work in his lab.

Sincere appreciation is also extended to Brian Sayre our former Postdoctoral Fellow and Diana Keller for their assistance and friendship. I would also like to thank all the graduate students I have worked with who have helped to provide an atmosphere conducive to learning and whose help made it possible to complete this experiment and others. A special acknowledgement is due to Diane Berry whose technical help made it possible for us to use electron microscopy to examine oocytes from this experiment.

Finally, I would like to thank my wife, Conni and my daughters Emily and Jessica for their love, patience and support.

TABLE OF CONTENTS

LIST OF FIGURES

Figure

LIST OF TABLES

INTRODUCTION

This review will focus on ways in which the endocrine environment may affect follicular function and oocyte maturation in the cow. Excellent reviews of follicular development in cattle (Fortune, 1994; Campbell et al., 1995; Bevers et al., 1996; Ginther et al., 1996) and of oocyte maturation in mammals (Wickramasinghe and Albertini, 1993; Eppig, 1996; Taieb et al., 1997) have been published in the last five years. Those reviews give a broader treatment of these topics than will be presented here.

Two to three periods of follicular development are observed during the estrous cycle of cows of *Bos Taurus* breeding. In each period, the number of cells increases in a cohort of follicles, in response to an increase in follicle stimulating hormone (FSH). Increasing amounts of inhibin and estrogen are produced as follicles grow and these inhibit the secretion of FSH from the anterior pituitary.

Follicular granulosal cells acquire luteinizing hormone (LH) receptors in response to FSH. Having receptors for LH purportedly allows granulosal cells to survive when FSH declines by shifting their dependence upon gonadotrophic support from FSH to LH (reviewed by Campbell et al., 1995). While granulosal cells of all follicles could acquire LH receptors, they do not develop in all follicles at the same time. Follicles that lack sufficient LH receptors become atretic as concentrations of FSH decline. Selection is the term that denotes the process whereby follicles that acquire LH receptors continue growth and those that do not acquire LH receptors undergo atresia. In cattle, only one follicle from each period of growth normally remains after this selection process. During the luteal phase, this follicle persists for a few days. Because recruitment and growth of other follicles are suppressed during that period, the single large follicle has been described as "dominant" before it undergoes atresia. Demise of the dominant follicle reduces negative

feedback from estradiol and inhibin and allows secretion of FSH to increase and recruit another group of follicles. The repetition of this pattern of follicular development has been termed a follicular wave.

The increase in the frequency of pulses of LH that occurs after concentrations of progesterone decline during luteolysis breaks the cycle of follicular growth and demise and supports the continued growth of the truly dominant follicle. In the absence of progesterone, estrogen produced by this follicle triggers ovulation by stimulating the release of a surge of LH.

Controlling follicular growth and ovulation is essential to the development of effective regimens for synchronization of estrus. Because most of the estrous cycle consists of the luteal phase, approaches to shorten or lengthen the luteal phase often have been tried. The luteal phase can be shortened using exogenous prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), but this approach cannot synchronize estrus at the beginning of the luteal phase or during periods of anestrus. Progestogens can be used to extend the luteal phase, as even low (equivalent to <2.0 ng/ml of progesterone) peripheral concentrations of progestogen are capable of preventing the LH surge. The degree of estrous synchrony has been variable, but often is tight following withdrawal of progestogen, making this a desirable approach. However, fertility following breeding at the synchronized estrus after progestogen withdrawal has been unacceptably low in most reports (Patterson, 1990).

During treatment with low dosages of exogenous progestogen, increases occurred in both the frequency of pulses of LH and in peripheral concentrations of estrogen. During this time the largest follicle continued to increase in size and was said to “persist”

on the ovary as its lifespan was prolonged (reviewed by Kinder et al., 1996). Oocytes in follicles that developed during treatment with low doses of progesterone prematurely completed meiosis (Revah and Butler, 1996). When these follicles were allowed to ovulate, oocytes were fertilized at a normal rate, but most of the resulting zygotes recovered on d 6 of pregnancy had not developed beyond the 16 cell stage (Ahmad et al., 1995).

Changes in follicular function or in the timing of preovulatory events may be the cause of low pregnancy rates. When the persistent follicle was ablated and another follicle ovulated pregnancy rates were not decreased in cows, so the effect appeared to be confined to the oocyte in the persistent follicle (Fike et al., 1997). However, Johnson et al. (1996) reported that persistence of follicles in ewes resulted in a decrease in fertility rather than in prolificacy and suggested that the effect was systemic in that species. Transfer of embryos to cows previously treated with low dosages of progesterone produced pregnancy rates equal to control animals, demonstrating that the effect of the persistent follicle is not on the uterus (Wehrman et al., 1997), but effects on the oviduct have not been excluded.

It can be concluded that the function of large follicles is altered in response to increased stimulation by LH, resulting in the ovulation of an oocyte that has matured prematurely and may not be viable after fertilization. However, the ways in which follicular and oocyte function are changed so that embryonic development is impaired are not known. Understanding how increased stimulation by LH affects the function of large follicles and oocytes may help to explain why pregnancy rates are low following treatment with low dosages of progesterone.

REVIEW OF LITERATURE

Control of the Growth and Function of Large Follicles by Gonadotropins

Large follicles present on the ovaries of cattle have two possible fates, ovulation or atresia. The fate of a large follicle is determined by the endocrine environment in which the follicle develops. In non-manipulated heifers, a 5-mm follicle has a 20% chance of becoming dominant (Bodensteiner et al., 1996). Furthermore, many follicles respond to FSH (Bo et al., 1994) and before selection, produce similar quantities of estrogen, regardless of whether they will become dominant or not (Ginther et al., 1996). Therefore, it is unlikely that the fate of a follicle is predetermined, but rather it is dependent upon the environment in which the follicle develops and the time it begins to grow. In fact, Gastal et al. (1999) have demonstrated that subordinate follicles can become dominant after removal of the dominant follicle.

Follicle Stimulating Hormone

Receptors for FSH are found exclusively on granulosal cells (Xu et al., 1995; Evans and Fortune, 1997). FSH influences cellular function by binding to a transmembrane receptor connected to a cyclic adenosine mono-phosphate (cAMP) dependent pathway (Funkenstein et al., 1984) that activates protein kinase A (Lavoie et al., 1999). FSH is required for the recruitment and growth of small follicles (<5 mm) and supports steroidogenesis in small follicles.

The increasing secretion of inhibin and estrogen by large follicles results in decreasing concentrations of FSH (Ireland and Roche, 1987; Badinga et al., 1992), which alter follicular function before the onset of atresia (Mihm et al., 1997). Acquisition of receptors for LH on granulosal cells does not eliminate dependence of large follicles

upon FSH. Acute decreases in FSH result in the atresia of large follicles (Turzillo and Fortune, 1993), so although large follicles may require less FSH, FSH is required to support their growth.

Administration of FSH before and during selection results in the continued growth of multiple follicles with several becoming co-dominant (Revah and Butler, 1996). Expression of aromatase and 3β hydroxy-steroid dehydrogenase (3βHSD) were not affected by FSH. However, FSH did increase the expression of steroidogenic acute regulatory (StAR) protein and side chain cleavage enzyme (SCC; Xu et al., 1995; Tian et al., 1995), thereby increasing substrate availability. The effects of FSH on transcription are mediated by cAMP response elements found in the promoters of genes involved in steroidogenesis (Carlone et al., 1997; Sugawara et al., 1997). It is not known how transcription of a portion of the genes encoding steroidogenic enzymes is enhanced selectively. Transcription of certain genes may be facilitated by altered transcription of A kinase anchoring proteins by FSH. These proteins target the actions of protein kinase A (PKA) to specific intracellular locations (reviewed by Scott, 1997).

FSH influences steroidogenesis indirectly by increasing the production of IGF-I, decreasing IGF binding-protein secretion (Resnick et al., 1998) or altering the forms of inhibin being produced. As FSH declines during selection, the bioavailability of IGF-I decreases, as does the production of the 34-kDA form of inhibin. Increases in FSH delay changes in availability of IGF-I and in isoforms of inhibin and support follicular growth (Mihm et al., 1997). Inhibin and IGF-I may mediate the effects of FSH by acting directly on granulosal cells to promote follicular growth. However, the effects of FSH on granulosal cells at the molecular level are not well understood.

Luteinizing Hormone

Crucial to large follicles is the development of receptors for LH on granulosal cells in response to stimulation by FSH (Ratoosh and Richards, 1985; Tian et al., 1995; Xu et al., 1995, Evans and Fortune, 1997). While some FSH is necessary to support continued growth and function of large follicles, LH becomes their primary support. During the luteal phase, increased sensitivity to LH allows large follicles to continue to grow despite the declining or low rate of secretion of LH (Bao et al., 1997). However, dominant follicles are sensitive to changes in the frequency of pulses of LH, the duration of their dominance being shortened by treatments which decrease pulse frequency (Anderson and Day, 1994) and lengthened by treatments that increase pulse frequency (Stock and Fortune, 1993).

During the estrous cycle, the tonic secretion of LH is increased after luteolysis and before the LH surge. This period is referred to as the follicular phase and in cattle is associated with the growth of a single large follicle with the capacity to synthesize estrogen. The follicle continues to grow, producing more estrogen until elevated concentrations of estrogen trigger a surge of LH that initiates ovulation (see reviews by Fortune, 1994; Campbell et al., 1995 and Ginther et al., 1996). In granulosal cells, expression of side chain cleavage enzyme (SCC) and 3βHSD increased following the initiation of luteal regression, but expression of aromatase was not increased. In thecal cells, expression of SCC and 3β-HSD increased by 24 h. However, expression of 17α-hydroxylase was increased by 12 h and the subsequent increase in androgen production was reflected in increased concentrations of estrogen in follicular fluid. Therefore,

androgen synthesis, not aromatization of androgens, is the limiting step in estrogen synthesis during the follicular phase (Xu et al., 1995; Tian et al., 1995).

The role of LH in follicular growth has been examined by altering the frequency and amplitude of pulses of LH. Follicles undergo the same changes when luteolysis is induced by exogenous $PGF_{2\alpha}$ as they do when luteolysis is initiated naturally (Tian et al., 1995). However, the time from initiation of luteolysis to estrus varies during the estrous cycle. Estrus occurs sooner if luteolysis is initiated during the dominance phase than if luteolysis is initiated early in the development of a wave. The larger number of cells and increased number of receptors per cell in large dominant follicles may allow them to respond more robustly and more quickly than smaller follicles. However, the concentration of progesterone at the time luteolysis is initiated is also important (Deaver et al., 1986) and it is difficult to be sure whether progesterone or follicular status is the most important determinant of intervals to onset of estrus, the LH surge, and ovulation.

Treatment with progesterone has long been used to synchronize estrus (Christian and Casida, 1948). In developing regimens for synchronization of estrus, low dosages of progestogens were selected based on their ability to inhibit behavioral estrus and ovulation, but fertility at the synchronized estrus often has been disappointing (Odde, 1990). Low pregnancy rates are probably the result of changes in hormone secretion and follicular function occurring as a result of maintaining low concentrations of progesterone. As suggested by Ulberg et al. (1951), maintaining low (1-2 ng/ml) concentrations of progesterone increased the frequency of pulses of LH (Ireland and Roche, 1982), which may induce changes in follicular function that result in reduced fertility.

Changes in follicular growth or function during treatment with low dosages of progesterone were first observed by Ulberg et al. (1951) who reported unusually large follicles and indications of increased estrogen production during treatment with progesterone. Sirois and Fortune (1990) also observed increased concentrations of peripheral estrogen and a prolonged period of follicular dominance when low concentrations of progesterone were maintained. The lengthened dominance phase was thought to be the result of the increased frequency of pulses of LH during treatment with progesterone (Sirois and Fortune, 1990). Taft et al. (1996) demonstrated that dominant follicles were maintained when the frequency of pulses of LH was increased during a normal luteal phase. In that study, concentrations of peripheral estrogen were not increased. However, Glencross et al. (1987) observed increases in peripheral estrogen following injection of a GnRH analog at a frequency greater than that used for LH by Taft et al. (1996). Nonetheless, the following observations lead to the suggestion that follicular function can be altered as a result of an intraovarian effect of progesterone or the CL: 1) concentrations of estrogen increased before LH at luteolysis (Bergfeld et al., 1996), 2) progesterone can inhibit estrogen synthesis in vitro, 3) granulosal cells have progesterone receptors (Rae et al., 1998), and 4) estrogen increased after luteolysis even when the rise in LH was suppressed in the cow and ewe (Fogwell et al., 1978; Gust et al., 1984).

Just as increased secretion of LH prolongs the growth of large follicles, decreasing LH hastens their demise. Early workers observed a dose effect of progesterone, as follicles did not become as large when larger dosages of progesterone were used. Anderson and Day (1994) and Manikkam and Rajamahendran (1997)

demonstrated that an acute increase in progestogen resulted in the demise of large follicles previously maintained during treatment with a lower dosage, an observation supported by other workers. Not surprisingly, increases in the concentration of progesterone are associated with decreases in the frequency of pulse of LH (Jolly, 1994b; Bergfeldt et al., 1996; Bao et al., 1997).

Effects of LH at the Cellular Level

Receptors for LH are found on thecal cells of growing follicles. Granulosal cells from follicles less than 8 mm in diameter usually lack LH receptors, while granulosal cells from follicles greater than 8 mm in diameter usually have LH receptors (Tian et al., 1997). The number of LH receptors per cell in both the thecal and granulosal layers increases with size of the follicle (Ireland and Roche, 1983). However, only the dominant follicle in a cohort acquires enough LH receptors to continue growing. Therefore, the ability of the dominant follicle to respond to LH can increase even when the concentration or pulse frequency of LH does not change. However, a frequency of pulses of LH that maintained dominant follicles in heifers and dry cows during the luteal phase (Taft et al., 1996) was not sufficient to maintain dominance in lactating beef cows (Cole, 1997).

LH receptors are transmembrane receptors similar to those for other protein hormones. The second messenger system used by LH receptors has traditionally been thought to be cAMP, which activates protein kinase A (PKA), in turn phosphorylating various proteins to cause the end effects of LH stimulation. That granulosal and thecal cells have multiple isoforms of the regulatory and catalytic subunits of PKA (Ratoosh and Richards, 1985), multiple protein kinase A anchoring proteins (Hunzicker- Dunn et al., 1998) and multiple phosphodiesterases (Carr et al., 1993; Furger et al., 1996)

demonstrates the complexity of cellular regulation. Evidence also exists that LH can activate other second messenger pathways, resulting in production of phospholipase C and inositol triphosphate, release of calcium (Davis et al., 1986; Dimino et al., 1987) and activation of protein kinase C (Gudermann et al., 1992; Morris and Richards, 1993). The consequences of activation of multiple second messenger pathways by LH are not known.

Oocyte Maturation

Oocytes are arrested in prophase of the first meiotic division throughout adult life until near the time of ovulation. However, they are not inactive, only prevented from resuming meiosis. Arrested oocytes transcribe RNA, produce proteins (Fair, 1997), coordinate the organization of the follicle (Li and Mather, 1997) and secrete substances capable of altering follicular steroidogenesis (Lanuza et al., 1998). Communication between the oocyte and surrounding cumulus cells is bi-directional as substances from the granulosa can affect oocyte function and act as the source of signals to the oocyte for maintenance or release of meiotic arrest. Therefore, the function of the follicle and its interaction with the oocyte may influence whether or not a viable embryo will be produced after mating. Some aspects of the interplay between the oocyte and the follicle were reviewed by Driancourt and Thuel (1998).

Maintenance of Meiotic Arrest

Morphologically, the oocyte of a large growing follicle is characterized by a spherical nucleus located in the periphery of the oocyte and a compact, dense, fibrillar nucleolus (Hyttel et al., 1987a,b; Assey et al., 1994a,b). Chromosomes are not condensed, allowing for transcription. Mitochondria are in the periphery of the oocyte, are often pleomorphic in shape and are in close contact with endoplasmic reticulum. Lipid droplets are distributed throughout the cytoplasm. The perivitelline space is not readily apparent, but intimate points of contact occur between the oocyte and cumulus cell processes. These processes extend through the zona pellucida to the oolemma where gap junctions form between the bulbous termini of the processes and the oolemma (Zamboni et al., 1972). Microvilli are present at the surface of the oocyte and in some cases wrap around the endings of the cumulus cell process (Flemming and Saacke, 1972; Hyttel et al., 1987; Assey et al., 1994a,b).

Cumulus cell processes allow communication between the oocyte and the cumulus cells (Anderson and Albertini, 1976). Although the cytoplasms do not commingle, the presence of gap junctions and pinocytotic vesicles between the process endings and the oolemma are evidence that material is passed between these cells. Experiments using dyes and other markers have demonstrated the coupling and passage of materials from cumulus cells directly to the oocyte (Gilula et al., 1978; Moore et al., 1980; 1983). Maintenance of cell to cell communication is essential for oocyte growth (Eppig, 1979). Disruption of cumulus-oocyte communication leads to resumption of meiosis in oocytes competent to undergo nuclear maturation (Dekel and Beers, 1978; De Smedt and Szollosi, 1991). Conversely, inhibiting the breakdown of cell to cell

communication prevents resumption of meiosis in vitro (Wert and Larsen, 1989). Some of the compounds passed between cumulus and oocyte are involved in oocyte maturation.

cAMP

In cattle, cAMP of granulosal origin is an inhibitor of oocyte maturation that may be responsible for maintaining meiotic arrest. Injection of dbcAMP, phosphodiesterase inhibitors, or stimulators of adenylate cyclase into oocytes prevents resumption of meiosis in cattle (Wiersman et al., 1998; Aktas et al., 1995 a,b) and rats (Richards et al., 1998). Granulosal cAMP passes through the cumulus cell processes to the oocyte, explaining why disruption of communication between the cumulus cells and oocyte leads to the resumption of meiosis. The effect of cAMP is mediated by PKA, because injection of the catalytic subunit of PKA in the presence of high concentrations of cAMP prevents GVBD (Aktas et al., 1995). It appears that PKA maintains the activation of a phosphatase, preventing the activation of cell cycle control proteins (Matten et al., 1994).

Understanding the actions of PKA is complicated by the existence of two isoforms of PKA. Type I PKA is found in the oocyte and prevents GVBD, while type II is found in the granulosa and promotes cumulus expansion and GVBD (Downs and Hunzicker Dunn, 1995). The distribution of the type I and type II enzymes seems to be controlled by the presence of A kinase anchoring proteins (AKAPs). Thirty-six AKAPs have been identified in the rat, illustrating the complex regulation of cAMP-dependent signaling. Controlling the localization of A kinases within a cell is one way in which ligand specific responses can occur following stimulation of common second messenger pathways (reviewed by Scott, 1997). Little is known about the role of AKAPs in oocyte function but FSH, a more potent stimulator of meiosis in vitro than LH (Van Tol, 1996),

selectively induces an AKAP specific for the type II enzyme (Carr et al., 1993). The conclusion that FSH has greater potency might be presumptuous. Oocytes for in vitro studies usually are collected from small to medium size follicles, and cumulus cells from these follicles do not have receptors for LH (Van Tol et al., 1996).

Calcium

Unlike cAMP, calcium regulates pathways stimulating oocyte maturation (reviewed by Homa et al., 1995). Blocking phophoinositol-dependent increases in intracellular calcium blocks progression of meiosis (Homa, 1991; Kaufman and Homa, 1993). Conversely, injecting oocytes with inositol triphosphate (IP3) can partially overcome the cAMP-mediated block to meiosis in bovine oocytes (Homa et al., 1993). The observation that bovine oocytes have receptors for IP3 further supports this line of reasoning (Yue et al., 1995). Mechanical disruption of contact between cumulus cells and the oocyte results in release of calcium and spontaneous resumption of meiosis (McConnell et al., 1995). Once mobilized, calcium can activate PKC and calmodulin-dependent protein kinase (CAM-II), both of which have been shown to regulate maturation promoting factor (MPF) activity (Gabrielli et al., 1993; Coskun and Lin, 1995).

Treatment of bovine cumulus oocyte complexes in vitro with LH resulted in oscillating release of calcium (Zuelke et al., 1991; Mattioli et al., 1998). The calcium came from the cumulus cells and was associated with suppressed adenylate cyclase activity and a reduction in concentrations of cAMP (Preston et al., 1987). This appears contradictory, because LH is typically thought to stimulate adenylate cyclase activity. However, LH can use either cAMP or calcium as a second messenger (Davis et al., 1981; Sadighian et al., 1989). Interestingly, the activation of both second messenger pathways

is mediated by one receptor (Gudermann et al., 1992). Thus, calcium influences the timing of the resumption of meiosis by reducing the synthesis of cAMP as well as by directly stimulating kinases that interact with proteins that control the cell cycle.

Resumption of Meiosis

Oocyte maturation can occur spontaneously or in response to stimulation by LH and FSH (Pincus and Enzman, 1935; Lonergan, 1994). Therefore, initiation of oocyte maturation is probably controlled by several different pathways that intersect at a common point that regulates components of the cell cycle. Gap junctions between cumulus cell processes play an important role in stimulated and spontaneous oocyte maturation as they facilitate the passage of inhibitory and stimulatory compounds to the oocyte. However, not all regulators of meiosis have to pass through gap junctions. Examples of such compounds include (1) meiosis activating sterol (MAS), (2) a protein produced by thecal cells that affects cumulus cells and (3) a protein in follicular fluid that inhibits oocyte maturation (Sirard and First, 1988; Richard and Sirard et al., 1996,1997; Mottlik et al., 1996; Byskov et al., 1997).

Morphological Changes

In vivo and in vitro, disruption of cell-cell contact between the cumulus cells and oocyte precedes the resumption of meiosis. Gap junctions between cumulus cell processes and the oocyte disappear and the processes begin to degenerate. Consequently, the nucleus becomes eccentrically located, and the nuclear membrane becomes irregular. The nucleolus becomes vacuolated during this time, possibly representing an increase in transcription before protein synthesis supporting oocyte maturation. Mitochondria are

clustered near the nucleus in close association with endoplasmic reticulum. Cortical granules move to the periphery of the oocyte and the perivitelline space becomes apparent. Chromosomes begin to condense and the nuclear membrane or germinal vesicle folds back on itself, forming invaginations before breaking down as meiosis proceeds (Flemming and Saacke, 1972; Hyttel et al., 1987b). Breakdown of the germinal vesicle allows mixing of cytoplasmic and nuclear components, a process important for the completion of meiosis. During this time, a bipolar spindle forms and separation of pairs of chromosomes soon follows. One set of chromosomes is then extruded from the cell with a small amount of cytoplasm to form the first polar body. After completion of the first meiotic division, the second one begins without reformation of the nuclear membrane, replication of DNA or decondensation of chromosomes. However, the second meiotic division is arrested during metaphase until fertilization (reviewed by Wickramasinghe and Albertini, 1993; Albertini, 1992).

The timing of the resumption of meiosis is crucial to fertility. Maturation begins within 24 h after the initiation of luteolysis in the cow. However, breakdown of the germinal vesicle and completion of the first meiotic division generally do not occur until after the LH surge (Assey et al., 1994a,b). Following superovulation, the time between initiation of luteolysis and the LH surge is decreased, and some of the oocytes ovulated are incapable of completing meiosis (Sirard et al., 1989; Kastrop et al., 1991). Decreasing the time between the initiation of luteolysis and the surge of LH may not allow oocytes enough time to synthesize the proteins needed to complete meiosis (reviewed by Greve et al., 1995). Consequently, some oocytes could be deficient in proteins needed for the cell cycle to advance. Conversely, delaying the LH surge results

in the ovulation of oocytes that do not develop after fertilization, and appear to be degenerated (Wise et al., 1994). Revah and Butler (1996) reported that meiosis was resumed prematurely in oocytes collected on day 13 following maintenance of low concentrations of progesterone on d 7-13 of the estrous cycle. Alteration in the timing of meiotic events may lead to the ovulation of an aged oocyte incapable of developing after fertilization, thus accounting for the low pregnancy rates in these animals. However, it is not known when maturation begins in these oocytes, or if maturation proceeds normally.

Modulators of Oocyte Maturation

Gonadotropins

The LH surge is thought to be the normal preovulatory trigger for oocyte maturation to begin in mammals (reviewed by Eppig, 1993). However, in the cow, changes in oocyte ultrastructure are observed during the period between the initiation of luteolysis and the LH surge, a period during which the secretion of LH increases. Dosages of gonadotropin ¼ to ½ of that required for ovulation stimulate oocyte maturation in rabbits (Pincus and Enzman, 1935). Oocytes maturing in this manner are capable of normal development if removed near the time ovulation would have occurred (Clewe et al., 1958). In the rat, dosages of LH incapable of causing ovulation also stimulate oocyte maturation. Advancing the time oocyte maturation by as little as eight hours with this treatment resulted in low fertility (Mattheij et al., 1993; Dekel et al., 1995). Therefore, abnormal patterns of secretion of LH can influence oocyte function independently of ovulation.

Cumulus-enclosed oocytes resume meiosis spontaneously in vitro, but do so more quickly after exposure to LH and FSH (De Smedt and Szollosi, 1991; Dominko and First,

1997). Because oocytes do not have receptors for LH or FSH, communication between cumulus cells and the oocyte is essential to transfer this signal (Fagbohun et al., 1991; Van Tol et al., 1994). Similarly, meiotic arrest maintained by treatment with cAMP or hypoxanthine (rats) can be overcome by the treatment of cumulus-enclosed oocytes with LH or FSH but not by treatment of denuded oocytes with these compounds. Thus, cumulus produced stimulators of oocyte maturation must mediate the effects of gonadotropins (De Smedt and Szollosi, 1991; Dominko and First, 1997). The stimulatory effects of LH and FSH may be due to activation of a second messenger pathway involving IP3 and calcium. Calcium and IP3 are increased in cumulus cells following treatment with LH, and both are transported to the oocyte where they activate proteins involved in control of the cell cycle.

Stimulation by gonadotropins can decrease the number of gap junctions between cumulus cells and the oocyte. Treatment with gonadotropins may increase cAMP in cumulus cells but abolishing gap junctions blocks transport of cAMP from cumulus cells to the oocyte, allowing meiosis to proceed (Dekel, 1978). In rats, cumulus-oocyte coupling was decreased by 55% within 1h and 80% within 2h of culture with LH (Sherizly et al., 1988). Treatment with LH and FSH disrupts cell-cell communication through phosphorylation of gap junctions, rendering them inactive, and through decreases in the transcription of Connexin 43, an essential component of gap junctions (Granot and Dekel, 1994, 1998) identified in bovine cumulus oocyte complexes (Sutovsky et al., 1993; Wrenzycki et al., 1996).

Growth Factors

The growth factors IGF-I and EGF are produced in the ovary and can promote oocyte maturation (Levesque et al., 1995; Lorenzo et al., 1994, 1995). They are thought to act in concert with gonadotropins to promote resumption of meiosis. Similar to LH and FSH, either of these growth factors can reduce the function of gap junctions. Oocytes have receptors for IGF-I and EGF, indicating the potential for a direct effect on the oocyte. In fact, IGF-I and EGF hastened maturation and improved viability of embryos from denuded oocytes in some (Sirotkin et al., 1998; Lonergan et al., 1996), but not all (Lorenzo et al., 1994) studies. The ability of EGF and IGF I to activate transcription through the estrogen receptor via a MAP-kinase-dependent pathway may have important effects on oocyte maturation (Bunone et al., 1996; reviewed by Smith, 1998).

Steroids

In mammals, steroids (estrogens, progesterone and androgens) have been implicated as influencing oocyte maturation and can inhibit as well as initiate meiosis, but are not required for completion of meiosis (Sirotkin et al., 1992). However, alterations in steroidogenesis alter post fertilization development (Yoshimura et al., 1988). Osborne and Moor (1983) concluded that the correct sequence and balance of steroids was essential for normal protein synthesis by ovine oocytes. Inclusion of estradiol in maturation media *in vitro* often improved the proportion of oocytes maturing and their subsequent developmental potential (Sirotkin et al., 1992). Disruption of steroidogenesis *in vivo* altered protein synthesis by oocytes, but effects were more severe when the ratio of estrogen to progesterone was altered than when steroidogenesis was

completely blocked (Osborn and Moor, 1983; Osborn et al., 1986; Nagai et al., 1993; Zelinski-Wooten et al., 1989). The identification of estrogen receptors in oocytes from mice and humans and the observation that incubation of oocytes with estrogen results in oscillations of calcium, established that oocytes can respond directly to estrogen (Tesarik and Mendoza, 1995). Progesterone, which is the trigger for oocyte maturation in Xenopus, can act similarly in bovine oocytes (Sirotkin et al., 1992).

Prolonged exposure of oocytes to preovulatory concentrations of estrogen lowered fertility and increased the frequency of embryonic anomalies in rats in which the follicular phase was prolonged by aging or by injection of sodium pentobarbital (Butcher et al., 1979). During treatment of cows with low doses of progesterone, peripheral estrogen was elevated and the oocyte was exposed to higher concentrations of estrogen for longer times (Ahmad et al., 1995). However, Revah et al. (1996) observed elevated peripheral concentrations of estrogen early during treatment, but did not observe elevated concentrations of estrogen in the follicular fluid of superstimulated follicles maintained by treatment with low progesterone and collected on d 13, yet oocytes from these follicles had completed meiosis prematurely. Deleterious effects of treatment of cows with low dosages of progesterone on oocyte function could be due to direct effects of estrogen or to changes in LH secretion.

Cell Cycle Regulation of Meiosis

During meiosis in oocytes, two blocks to progression of the cell cycle occur, the first during prophase of the first meiotic division and the second during metaphase of the second meiotic division. Therefore, understanding control of the cell cycle is important for understanding how oocyte maturation is regulated. Fortunately the mechanisms

controlling mitosis and meiosis appear to be very similar and are highly conserved across species. However, the factors that trigger or inhibit progression of the cell cycle vary with species.

Importantly, the oocyte is dependent on stored mRNA during maturation and early embryogenesis. A prolonged follicular phase may lower fertility by prematurely exhausting the supply of mRNA needed for the production of proteins essential for control of the cell cycle in the oocyte. For example, MPF activity in MII oocytes can be maintained for only 30 h in vitro (Wu et al., 1997). In vivo, maintenance of oocytes at MII might occur during treatment with low dosages of progestogen, disrupting spindle structure and causing shortages of key cell cycle proteins during embryogenesis.

Maturation Promoting Factor

The mechanisms controlling cell cycle progression seem to converge at one point, the regulation of maturation, mitosis or M phase Promoting Factor (MPF; see reviews by Eppig, 1993; Wickramasinghe and Albertini, 1993; Taieb et al., 1997). In meiosis as well as mitosis, MPF seems to be central in controlling cell cycle progression. MPF is a dimer of the cell division control (Cdc) 2 gene product and a cyclin. Cdc2 is a 34 KDa protein, which when activated, has serine/threonine kinase activity. Structurally, Cdc2 is similar to cAMP-dependent protein kinase and has been modeled based on the structure of that protein. Points of interest on Cdc2 include the cyclin binding site and phosphorylation sites at threonine 161, threonine 14, and tyrosine 15, all of which are important regulatory sites. The binding of cyclin facilitates phosphorylation of these residues (Marcote et al., 1993).

Conformational changes induced by cyclin binding facilitate phosphorylation of threonine 161 of Cdc2. Phosphorylation of threonine 161 stabilizes the Cdc-2: cyclin b dimer, maintaining the structure of the catalytic site. An analogous protein (threonine-197) in cAMP-dependent protein kinase is crucial for recognition of substrates and inhibitors. This may explain why threonine-161, like threonine-197, is essential for proper catalytic activity. The other two phosphorylation sites are inhibitory and require dephosphorylation for enzyme activity. The major site of regulation by dephosphorylation is tyrosine 15 and activity of the phosphatase that dephosphorylates this residue is regulated by cAMP. Decreasing cAMP activates the phosphatase that activates MPF. Phosphorylation of tyrosine 15 occurs only after binding of cyclin and inhibits enzyme function by altering the positioning of sites involved in peptide recognition (Marcote et al., 1993; Novak and Tyson, 1993).

Role of MPF

During oocyte maturation, MPF seems to be an essential regulatory protein controlling resumption of the cell cycle during meiosis. A requirement for MPF has been documented for several processes required for oocyte maturation; among these are GVBD, chromosome condensation, changes in spindle organization and maintenance of metaphase II arrest (reviewed by Parrish et al., 1992; Eppig, 1993; Wickramasinghe and Albertini, 1993).

Germinal Vesicle Breakdown

Key to disassembly of the nuclear membrane is the phosphorylation and subsequent depolymerization of lamins, a major polypeptide component of the nuclear

membrane (Gerace and Blobel, 1980; Whytock et al., 1990). The injection of Xenopus MPF into oocytes from several species results in GVBD, demonstrating the importance of MPF in GVBD and the conservation of mechanisms to regulate the cell cycle (Hashimoto and Kishimoto, 1988). In Xenopus oocyte extracts, nuclear lamin C is phosphorylated by MPF, accompanied by depolymerization/disassembly of lamin filaments in the nuclear membrane, thus MPF acts directly on components of the nuclear membrane to promote GVBD (Ward et al., 1990).

Chromosome Condensation

During mitosis and meiosis, condensation of chromosomes is essential if the cell cycle is to continue. The phosphorylation of histones and other proteins facilitates chromosome condensation (Bradley et al., 1974). Because MPF phosphorylates histones (Langan et al., 1989), it is quite likely that MPF plays a key role in inducing chromosome condensation.

Changes in spindle organization

Changes in the organization and length of microtubules are essential during cell division, as microtubules are needed for chromosome segregation. In cell free Xenopus systems, MPF maintains spindle length, supporting the idea that MPF influences spindle organization (Verde et al., 1990). MPF can be co-localized with centrosomes and microtubules (Ookata et al., 1993) as can cyclin B, one of the subunits of MPF. The binding of cyclin to centrosomes may target centrosomes and microtubule associated proteins as substrates for MPF kinase activity. MPF phosphorylates proteins such the

kinesin-related motor proteins. Preventing phosphorylation of these proteins prevents formation of bipolar spindles (Blangy et al., 1995).

Metaphase II arrest

When the first meiotic division has been completed, the oocyte immediately begins the second meiotic division. However, the cell cycle is again arrested, but this time during metaphase instead of prophase. As long as MPF activity is maintained at metaphase concentrations, meiosis will be arrested (Huchon et al., 1993). Normally, MPF activity is maintained by cystostatic factor (CSF) which stabilizes MPF and prevents the degradation of cyclin, thereby arresting the cell cycle by preventing the reformation of the nuclear envelope and decondensation of chromosomes (Masui and Markert, 1971; Sagata et al., 1989). The product of the Mos gene, a serine/threonine protein kinase (Yew et al., 1992), is able to mimic the effects of CSF in Xenopus by maintaining cell cycle arrest. Blocking synthesis of the product of the Mos gene results in failure of meiosis to continue as chromosomes decondense and the nuclear lamina reforms (O'Keefe et al., 1989). Therefore, Mos may be CSF, or a component of CSF (Sagata et al., 1989) or may phosphorylate another protein that prevents the inactivation of MPF.

Regulation of MPF

The regulation of MPF activity is accomplished through three mechanisms; 1) cyclin degradation, 2) phosphorylation / dephosphorylation of inhibitory sites on Cdc2, and 3) phosphorylation/dephosphorylation of stimulatory sites (Marcote et al., 1993; Novak and Tyson, 1993). These different mechanisms form a complex control system linking second messenger systems to proteins that control the cell cycle.

Cyclin Availability

Cyclin binding is necessary for MPF activation. Cyclin B is synthesized by bovine oocytes before the resumption of meiosis, is not present in immature oocytes, and when injected causes otherwise immature oocytes to mature (Levesque and Sirard, 1996). The binding of cyclin to cdc2 alters the structure of cdc2, facilitating its phosphorylation. Conversely, the degradation of cyclin results in the inactivation of MPF. Surprisingly, MPF initiates a cascade that ultimately results in the degradation of cyclin (Lorca et al., 1991). How the lag between MPF activation and cyclin degradation is controlled is not known.

Control of cyclin degradation plays an important role during the second meiotic division. CSF stabilizes the Cdc2:cyclin complex, preventing the ubiquitination of cyclin, thus leading to the metaphase II arrest. Following fertilization, cyclin is degraded and CSF is inactivated, as is MPF, permitting the cell cycle and meiosis to continue (Lorca et al., 1993).

Phosphorylation/Dephosphorylation of Inhibitory Sites

At least three enzymes (wee1, myt1, cdc25) control the phosphorylation state of the inhibitory phosphorylation sites at threonine 14 and tyrosine 15. Phosphorylation of these sites by wee1 and/or Myt1 inactivates MPF. Before the onset of maturation in mouse oocytes, Cdc2 is phosphorylated in a way consistent with tyrosine 15 phosphorylation. Thus, phosphorylation at this site may prevent premature entry into meiosis (Choi et al., 1991). In *Sacchromyces pombe*, mutation of wee1 hastened entry into mitosis, while overexpression delayed entry into mitosis. Antagonistic to the actions

of wee1 and myt1 is cdc25, a phosphatase that dephosphorylates threonine 14 and tyrosine 15, leading to activation of MPF. Preventing the activation of cdc25 by mutation in drosophila (Ripoll et al. 1992) or by antibodies to cdc25 in HeLa cells arrests the cell cycle (Sadhu et al. 1990; Galaktionov and Beach, 1991).

Phosphorylation/Dephosphorylation of an Activating Site

Regulation of phosphorylation of threonine161 also can control the activation of MPF. The enzymes CAK (cdc2 activating kinase) and INH (a form of phosphatase 2A) are thought to play antagonistic roles in controlling the phosphorylation state of threonine 161. INH was first identified in Xenopus oocytes and postulated to inhibit the activation of MPF (Cyert and Kirshner, 1988), because it can dephosphorylate threonine 161 (Solomon et al., 1990; Lee et al., 1991). In bovine and porcine oocytes incubation with okadaic acid, an inhibitor of phosphatase, especially phosphatase 2A (PPA2) activates MPF (Levesque and Sirard, 1995). However, based on work in Xenopus, PPA2 may inactivate MPF by stimulating the activity of a tyrosine kinase that phosphorylates tyrosine 15 (Rime et al., 1995). In addition, PPA2 may block a step in the pathway leading to CAK activation, thereby preventing the phosphorylation of threonine 161 (Lee et al., 1991).

STATEMENT OF PROBLEM

Maintenance of low (1-2 ng/ml) concentrations of peripheral progesterone in the cow altered follicular and oocyte function. The dominance phase of follicular growth is prolonged and peripheral concentrations of estrogen are increased in response to increased secretion of LH. When progesterone is withdrawn, the dominant follicle maintained during treatment with progesterone ovulates, and while fertilization rate is normal, pregnancy rate is low. Bovine oocytes collected at the end of treatment with low dosages of progesterone or other progestogens, but before withdrawal of progestogen, have completed the first meiotic division. Therefore, by the time fertilization occurs, oocytes have been arrested at metaphase II for an abnormally long time. The resulting embryos die between the two and 16 cell stages (Ahmad et al., 1995), perhaps due to the premature activation of the oocyte. Because germinal vesicle breakdown and extrusion of the polar body occur by the end of treatment with progestogen, changes that result in the initiation of meiosis must occur earlier. However, follicular function and oocyte morphology have not been examined during the early stages of treatment with low dosages of progesterone. The present study was conducted to test the null hypothesis that reducing progesterone on day 6 of the estrous cycle does not alter the maturation state of oocytes in follicles of the first wave of development within 2 or 4 days.

OOCYTE MORPHOLOGY AND CONCENTRATIONS OF ESTROGEN FOLLOWING A REDUCTION OF PROGESTERONE IN BEEF COWS

INTRODUCTION

Low dosages of progesterone or progestogens have been used since the late 1940s to synchronize estrus (Christian and Casida, 1948), but pregnancy rates following breeding at the synchronized estrus have been highly variable and often low (Odde, 1990). Maintaining low peripheral concentrations of progesterone increased the frequency of pulses of LH to a rate greater than that normally observed during the luteal phase (Cupp et al., 1992; Bergfeld et al., 1996; Taft et al., 1996). These changes in secretion of LH, after the initiation of luteolysis or a reduction in progesterone, prolonged the growth of the largest follicle and resulted in increased peripheral concentrations of estradiol (Stock and Fortune, 1993; NE-161, 1996).

Estradiol typically increases before estrus, but prolonged exposure to increased concentrations of estradiol may reduce fertility. Butcher and Pope (1979) demonstrated in rats that prolonged exposure to preovulatory concentrations of estradiol decreased the number of normal embryos on d 4 and the number of implantation sites on d 11. Similarly in cattle, peripheral concentrations of estradiol were elevated for six days before estrus (d 15) in cows maintained from d 6 to 13 on a low dosage of progesterone, but were increased for only two days in control animals given $PGF_{2\alpha}$ on d 6 that were in estrus on d 9 (Ahmad et al., 1995). Sixteen of 18 embryos recovered from treated cows on d 6 after breeding had only 2 to fewer than 16 cells whereas 16/18 control embryos had reached the morula stage. Ahmad et al., (1995) proposed that changes in the oocyte before ovulation reduced embryo survival and development by d 6. Revah and Butler (1996) observed condensed chromatin and germinal vesicle breakdown in oocytes after 9 d of treatment with a low dosage of progesterone. Mihm et al. (1994, 1999) observed

that when follicular dominance was maintained for 10 d, oocytes underwent germinal vesicle breakdown and extrusion of the first polar body by the end of treatment and pregnancy rates were reduced. Thus, oocytes resumed meiosis prematurely during treatment with low dosages of progesterone.

Premature maturation of oocytes can be induced in rats and rabbits by treatment with dosages of LH that are insufficient to cause ovulation (Dekel et al., 1995; Mattheij et al., 1993). Increased release of LH during treatment with low dosages of progesterone may have a similar effect. Although the LH surge normally triggers resumption of meiosis, the development of irregularities in the nuclear membrane and a redistribution of mitochondria, changes thought to precede germinal vesicle breakdown and the resumption of meiosis, were observed in oocytes collected 24 h after initiation of luteolysis (Hyttel et al., 1987; Assey et al., 1994 a,b). Oocytes in those studies were collected well before the LH surge, but during a period when secretion of LH and estrogen increased. Therefore, changes in follicular function and oocyte morphology began soon after reductions in circulating concentrations of progesterone.

Mihm et al. (1999) reduced progesterone concentrations by inducing luteolysis after 2 d of follicular dominance. Low peripheral concentrations of progesterone were maintained by treatment with progestogen and oocytes were collected from follicles 2 d later. After 4 d of dominance the morphology of oocytes was similar to that described for oocytes collected 24 h after the initiation of luteolysis (Hyttel et al., 1987; Assey et al., 1994a,b). However, Mihm et al. (1999) collected oocytes 18 h after removal of progestogen, so it could not be determined whether changes in oocyte morphology occurred before or after withdrawal of exogenous progestogen. It is unknown whether

changes in oocyte morphology occur soon after the initiation of luteolysis during treatment with a low dosage of progesterone.

The objectives of this study were to determine 1) if concentrations of estradiol in follicular fluid were increased during the early stages of treatment with a low dosage of progesterone following regression of the CL and 2) if oocytes collected from these follicles differed from oocytes from follicles of the same age from cows with functional CL. In previous studies (Mihm et al., 1994, 1999; Revah and Butler, 1996), oocytes from control cows were collected at an earlier stage of follicular growth than oocytes from treated animals, thus effects of time and treatment could not be separated. Collection of oocytes from control and treated cows on the same days of the estrous cycle allowed examination for effects of time and treatment.

MATERIALS AND METHODS

Experimental Design

Crossbred beef cows (N=24) previously observed to have normal estrous cycles were used for this experiment. Cows had ad libitum access to hay and water and were observed twice daily for signs of estrus. At estrus, (day 0) each cow was assigned at random to one of four experimental groups. A 2 x 2 factorial design was used with two expected concentrations of progesterone (normal, greater than 2 ng/ml and low, 1-2 ng/ml) and collection of follicles on 2 d (d 8 and 10). These days for were chosen to facilitate comparisons of first wave follicles during a normal luteal phase and during maintenance of low progesterone for 2 or 4 d. Controlled Internal Drug Releasing Devices -Bovine (CIDR-B, InterAg, Division of DEC International, Hamilton, New

Zealand) containing progesterone, previously used for seven days, were inserted on d 4 after estrus to provide exogenous progesterone in the low progesterone groups (LP-d 8, LP- d 10). These cows were given two intramuscular injections of prostaglandin $F_{2\alpha}$ (25 mg, Lutalyse, Pharmacia and Upjohn Inc., Kalamazoo, MI) 12 h apart on d 6 to regress the CL. Animals in the control groups (Cont-d 8, Cont-d 10) had a blank CIDR inserted on d 4 and received two injections of 3 ml of saline (intramuscularly) 12 h apart on d 6. CIDRs were removed at the time of ovariectomy on either d 8 or 10 of the cycle (2 or 4 d of low progesterone).

Ultrasonography

Follicular development was monitored by transrectal ultrasonography using an Aloka 500 (Aloka Ltd., Japan) ultrasound machine equipped with a 7.5 MHz linear array transducer. Ultrasonography was performed daily beginning on d 4 and continued until ovariectomy. The relative locations and diameters of all follicles greater than 5 mm in diameter were recorded each day.

Transvaginal Ovariectomy and Oocyte Collection

Ovaries were removed using the technique described by Casida (1959). Briefly, 10 ml of 2% lidocaine were administered as an epidural block, a supravaginal incision was made, the ovary containing the largest follicle was retracted into the vagina and the ovarian pedicle was severed using an ecrasure. Following hemi-ovariectomy, follicular fluid from the largest follicle was aspirated and the oocyte was retrieved. Follicular fluid was frozen at –80° C until assay, and oocytes were fixed in 1% glutaraldehyde in .1M phosphate buffer.

Blood Sampling and Hormone Assays

Jugular blood samples were collected by venipuncture every day at the time of ultrasonography, beginning on d 4 and continuing until the time of ovariectomy. Samples were stored overnight at 4 °C then centrifuged for 20 minutes at 1800 x g. Serum was removed and stored at –20 °C until assayed for progesterone (Sheffel, et al., 1982) and estrogen (Rozell and Keisler, 1990; Tortonese et al., 1990). Assay sensitivity was 20 pg per tube for progesterone and .5 pg per tube for estrogen. Intra-assay coefficients of variation were 7% and 9% and inter-assay coefficients of variation were 8% and 12% for progesterone and estrogen, respectively. Samples of follicular fluid were diluted in assay buffer before being assayed (1:100 for progesterone and 1:10,000 for estrogen).

Electron Microscopy and Oocyte Evaluation

Oocytes were embedded individually in Epon and stained with lead acetate and propidium iodide. Ultrathin sections (700 nm) were cut on a Leica Ultracut microtome by an experienced technician. Ultrastructural evaluations and photography were performed on a Jeol JEM 1220 transmission electron microscope (Jeol, Tokyo, Japan).

Cumulus and oocyte morphology were evaluated blindly and independently by two people. Scores of the two readers were in such close agreement that no statistical evaluation of reader differences was made. The degree of cumulus expansion, degree of clustering of mitochondria and shape of the nucleus were evaluated separately on a scale of 0, 1 or 2. A score of 0 represented no expansion of the cumulus, no clustering of mitochondria or that the nucleus was round. A score of 1 represented some expansion of cumulus cells, some mitochondria were in clusters or that slight to moderate irregularity

in the nuclear membrane were present. A score of 2 indicated that the cumulus had expanded, that mitochondria were found in large clusters or that the shape of the nucleus was highly irregular. The ends of cumulus cell processes were classified as intact, degenerating/separating (appeared to be superficial and separating from oolema or contained vacuoles) or degenerated/separated (no apparent contact with oolema). The morphology of mitochondria was evaluated by counting 100 mitochondria and determining the frequency of normally shaped mitochondria, hooded mitochondria or mitochondria with other shapes. The computer program Optimas (Bioscan Inc., Edwards WA) was used to evaluate the circularity of nuclei and the percentages of cytoplasmic area occupied by mitochondria and lipid. Nuclear morphology was classified according to Assey et al. (1994a). Oocytes in nucleus stage I (ONI) have a spherical nucleus located in the periphery of the oocyte and represent immature, meiotically arrested oocytes. Oocytes in nucleus stage II (ONII) were characterized by irregularity of the nuclear envelope thought to represent activation of the oocyte nucleus. The oocyte nucleus breakdown stage (ONBD) was identified by breakdown of the nuclear envelope and the presence of condensed chromatin. The metaphase I (MI) stage is characterized by the presence of chromosomes in the ooplasm. The final stage, metaphase II (MII), is characterized by the presence of the first polar body.

Analyses of Data

Twenty-four cows were assigned to this experiment. Data were not collected from one cow in which the dominant first wave follicle began to regress prior to ovariectomy. During the recovery, processing and sectioning of oocytes, seven were lost or damaged so that no data relative to cumulus and oocyte morphology were available from these

animals. Size of the largest follicle and concentrations of peripheral hormones were examined using the PROC MIXED procedure of SAS (SAS, 1988) with treatment, day of the estrous cycle and the treatment by day interaction included in the model. Cow within treatment was used as the error term in this analysis. Data on cumulus cell and oocyte morphology were analyzed as a 2 x 2 factorial design using the GLM procedure of SAS with treatment, day of ovariectomy and their interaction included in the model. The percentages of cumulus cell processes that were intact, degenerating or degenerated were analyzed following arcsine transformation. When interactions were detected, differences among individual treatments were determined by the test of least significant difference (SAS, 1988). The proportions of oocytes in the ONI or ONII stage were examined using Lancaster's Chi-square analysis.

RESULTS

Peripheral Concentrations of Progesterone and Estrogen and Follicular Dynamics

Initiation of luteolysis during treatment with a low dosage of progesterone created changes in the patterns of peripheral progesterone (Fig. 1) that altered the pattern of secretion of estradiol. Although low dosages of progesterone did not increase peripheral progesterone during d 4 to 6, peripheral concentrations of estradiol declined after insertion of a CIDR on d 4 and remained lower ($P < .05$) than in controls until after d 6. After luteal regression was initiated on d 6 of the estrous cycle, peripheral concentrations of progesterone declined to less than 2 ng/ml and remained low in all treated cows, as expected (Fig. 1). Twenty four hours after initiation of luteal regression (d 7), as progesterone declined, peripheral concentrations of estradiol increased and tended ($P < .06$) to be higher in treated than in control cows. During the period 48 to 96 h (d 8 to 10)

after initiation of luteal regression, peripheral concentrations of estrogen were greater ($P < .01$) in treated than in control cows (Fig. 2). Concentrations of progesterone in treated cows declined to 1 to 1.5 ng/ml during this period, but increased to >2 ng/ml in controls. Diameter of the largest follicle did not differ between treatments on d 8 but by d 10 diameter of the largest follicle was greater ($P < .05$) in treated than in control cows (Fig. 3). Growth of the largest follicle appeared to plateau by d 8 in control but not in treated cows (Fig. 3).

Concentrations of Steroids in Follicular Fluid

Intrafollicular concentrations of progesterone did not differ with treatment, day of ovariectomy or their interaction. Furthermore, the ratio of estradiol to progesterone was > 1 in all follicles, indicating that all were estrogen active and should not have been atretic. Concentrations of estradiol in follicular fluid were several fold greater ($P < .0001$) in treated than in control cows on both d 8 and d 10 and no treatment by day interaction was detected (Fig. 4).

Characteristics of Cumulus Cells and Oocytes

Nuclear Morphology of Oocytes

Oocytes from treated animals on d 8 and 10 and control animals on d 10 were more advanced in their development as evidenced by a day by treatment interaction for stage of the nucleus and for degree of irregularity of the nuclear membrane (Table 1). Two of three oocytes collected from control animals on d 8 were at the ONI (Hyttel et al., 1987; Assey et al, 1994a,b); whereas, all oocytes in the other groups were at ONII (Fig. 5, a-d). A greater degree of irregularity in the shape of the nucleus in oocytes observed when control d 8 oocytes were compared to oocytes in all other groups ($P < .05$; Table 1).

In some oocytes collected on d 10 in both groups the nuclear membrane was highly irregular, folding back on itself and forming invaginations (Fig. 6d). While germinal vesicle breakdown was not observed, the typical bilayer structure of the membrane was not present in some areas at which the nuclear membrane was highly convoluted (Fig. 6e).

Cumulus Cells

The degree of cumulus expansion varied with day ($P < .05$) being greater on d 10 than on d 8 (score of 1.5 vs 1.0). No treatment or day by treatment interaction could be detected statistically. A significant treatment by day interaction was detected ($P< .05$) in the proportion of intact cumulus cell process endings due to the high proportion in control cows on d 8 relative to the other groups(Table 1). The majority of cumulus cell processes appeared to be in close contact with the oocyte in the control d 8 cows. In all other groups many cumulus cell processes appeared to be retracting, more superficial and degenerating as evidenced by the presence of vacuoles, lysosomes and in some cases, breakdown of the cell membrane.

Cytoplasmic Morphology

Although no differences were detected in the percent of the cytoplasm occupied by lipid or by mitochondria, the distribution of mitochondria changed from peripheral and diffuse in control cows on d 8 to more cortical and clustered in all other groups ($P<.05$; Table 1). In addition, mitochondrial morphology differed between oocytes from control cows on d 8 and all other groups ($P<.05$) with the percentage of normal mitochondria being greatest in control cows on d 8. The percentage of hooded

mitochondria was greater in oocytes from treated animals on d 8 and in both groups on d 10 than in control cows on d 8 (Table 1). Hooded mitochondria are a form of pleomorphic mitochondria and are a distinctive feature of oocytes from ruminants, found in very few other species or tissues (Flemming and Saacke, 1970; Stephens and Bills, 1965; Assey et al., 1994a,b; Fig. 6c).

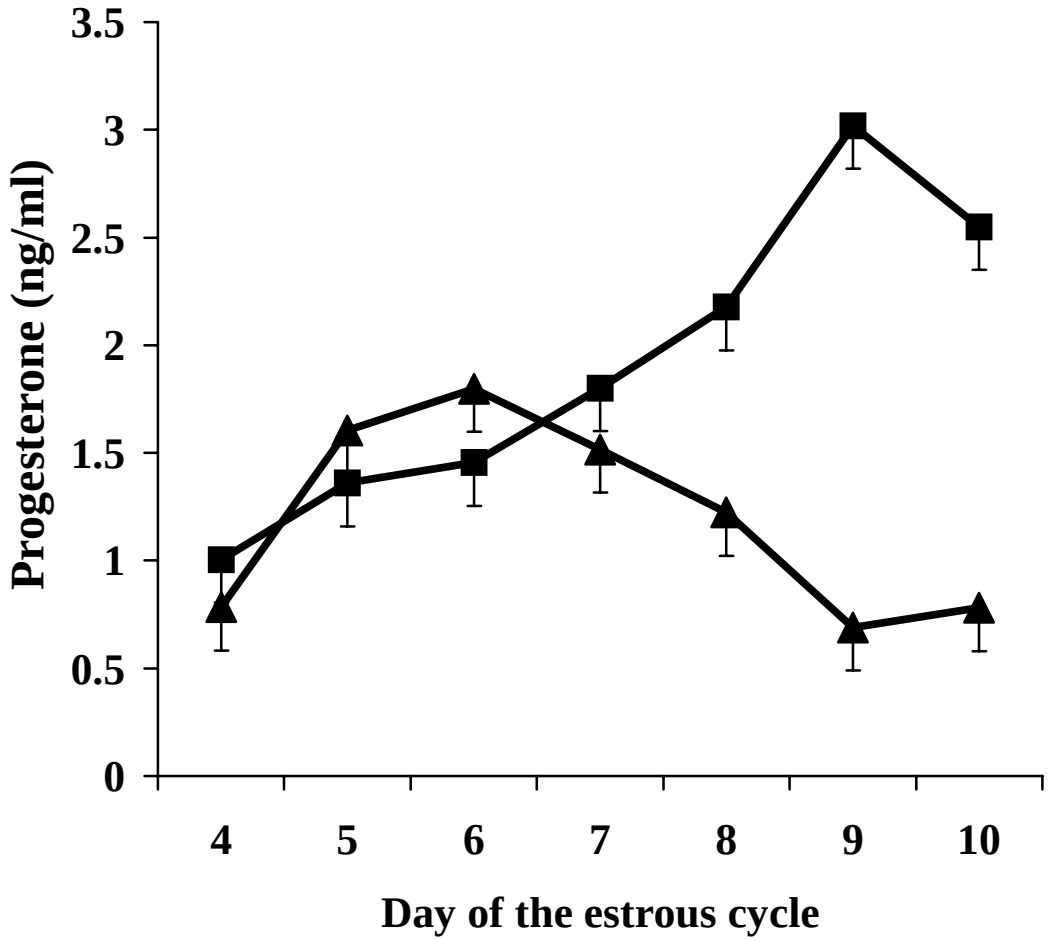


Fig. 1. Patterns of concentrations of progesterone in serum of cows treated with low dosages of progesterone (triangles) and in control cows (squares) from d 4 until ovariectomy on d 8 or 10 of the estrous cycle.

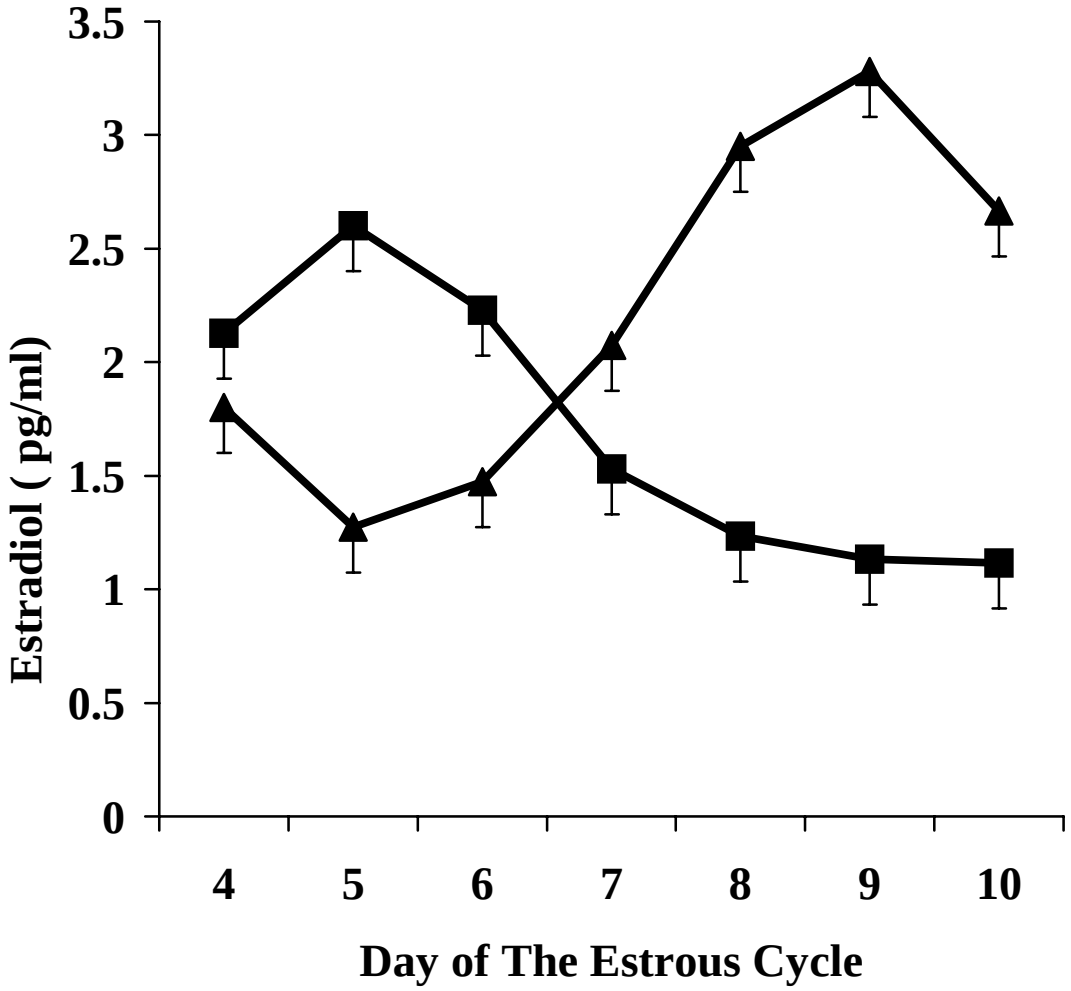


Fig. 2. Patterns of concentrations of estradiol in serum of cows treated with low dosages of progesterone (triangles) and in control cows (squares) from d 4 until ovariectomy on d 8 or 10 of the estrous cycle.

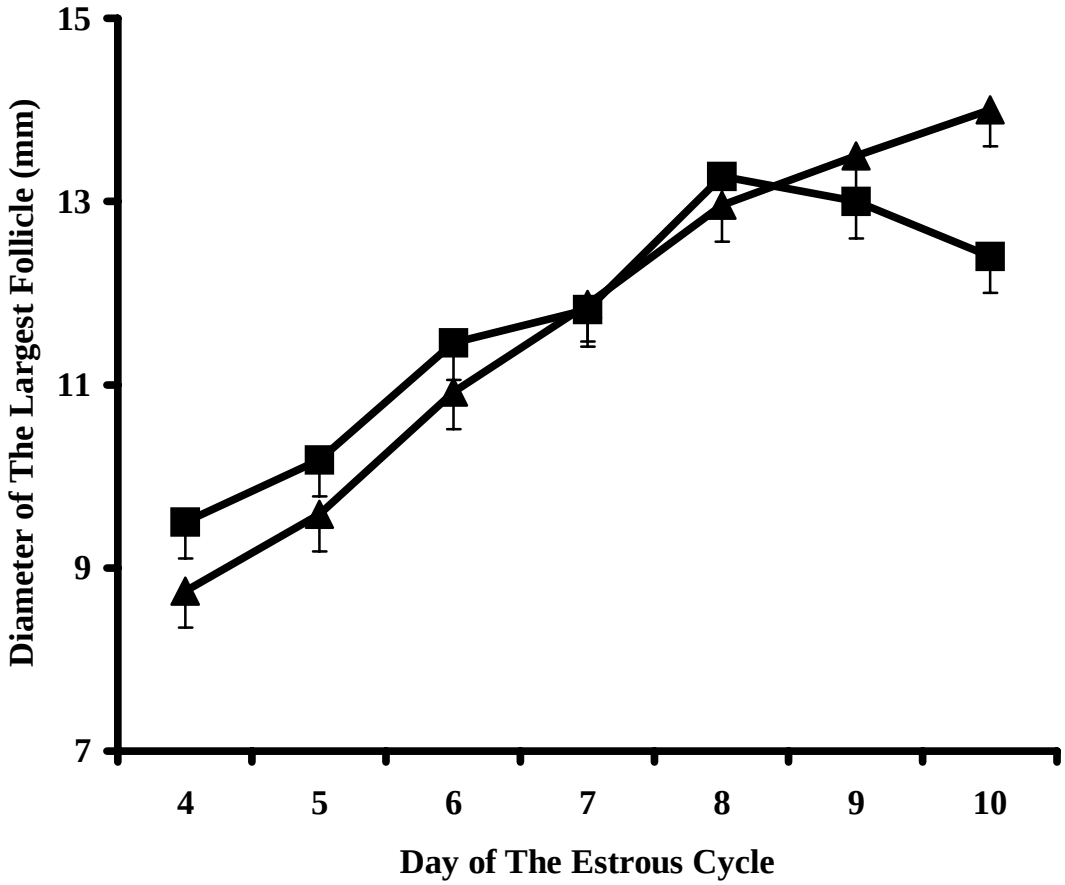


Fig. 3. Diameter of the largest follicle in cows treated with low dosages of progesterone (triangles) and in control cows (squares) from d 4 through d 8 or 10 of the estrous cycle.

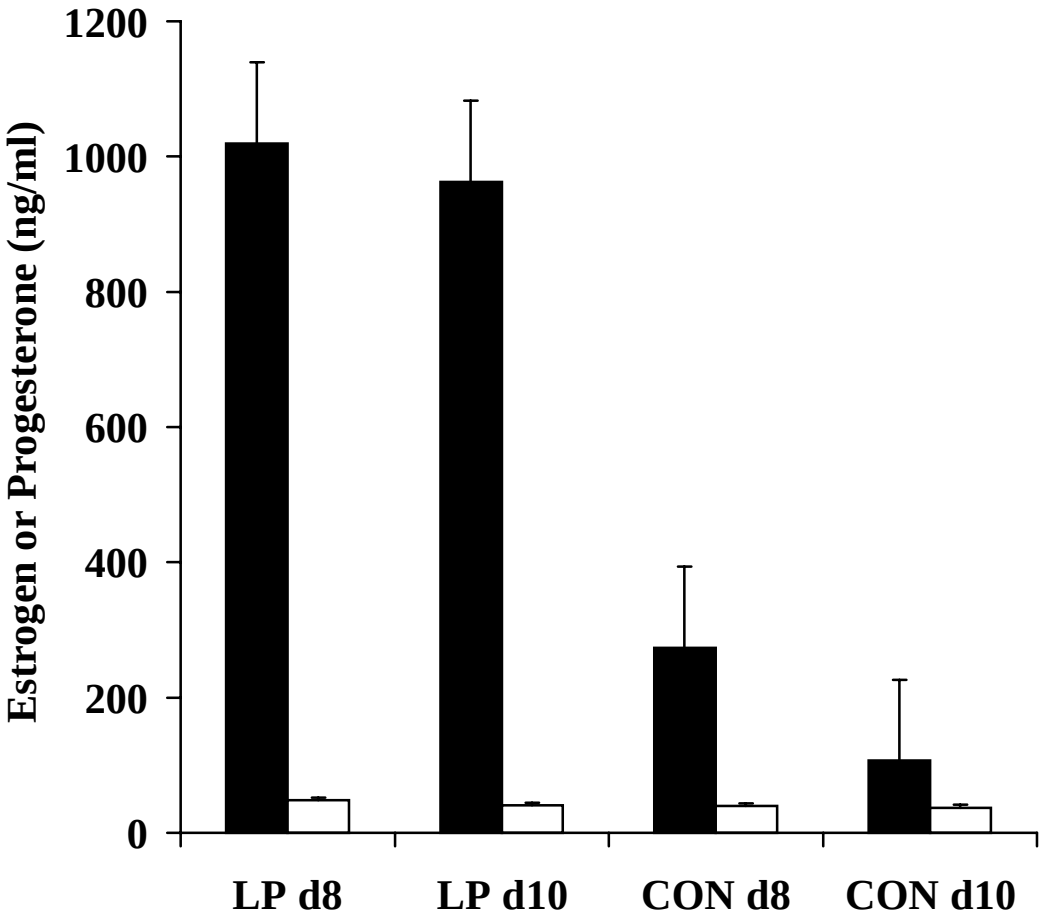


Fig. 4. Concentrations of estradiol (black bar) and progesterone (striped bar) in follicular fluid collected on d 8 or d 10 after estrus in cows treated with a low dosage after d 6 of progesterone or in control cows.

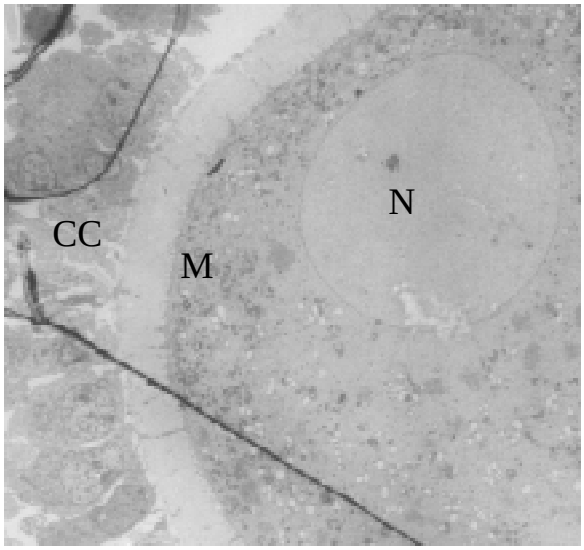
Table1. Summary of data for characteristics of cumulus cells and oocytes

	Control		Low Progesterone	
Variables	**Day 8**	**Day 10**	**Day 8**	**Day 10**
Cumulus Cell				
Expansion[c]	0.50 ± 0.35	1.80 ± 0.27	1.50 ± 0.35	1.70 ± 0.27
Cumulus Cell Processes				
Intact (%)	73. ± 13[a]	16. ± 9.87[b]	30 ±13[b]	23 ± 10[b]
Degenerating (%)	19 ± 11	31 ± 8	45 ± 9	41 ± 7
Degenerated (%)	4 ± 16	52 ± 11	25 ±13	36 ± 10
Nucleus				
Stage I	2/3 [a]	0/5 [b]	0/3 [b]	0/5 [b]
Stage II	1/3 [a]	5/5 [b]	3/3 [b]	5/5 [b]
Irregularity of nucleus[c]	0.33 ± 0.33[a]	1.50 ± 0.28[b]	1.33 ± 0.33 [b]	1.60 ± 0.25 [b]
Circularity	16 ± 3	19 ± 3	21 ± 3	25 ± 3
Mitochondria				
%area	6.2 ± 1.8	5.7 ± 1.5	7.6 ± 1.6	5.4 ± 1.4
Clustering[c]	0.3 ± 0.3[a]	1.8 ± 0.2[b]	1.7 ± 0.3[b]	1.4 ± 0.2[b]
Shape				
Normal (%)	73 ± 6[a]	48.20 ± 5[b]	46.00 ± 6[b]	50 ± 5[b]
Hooded (%)	25 ± 6[a]	49 ± 5[b]	51 ± 6[b]	46 ± 5[b]
Other (%)	1 ±1	1 ± 1	3 ± 1	4 ± 1
Lipid (%area)	25 ± 6	26 ± 5	29 ± 6	35 ± 4

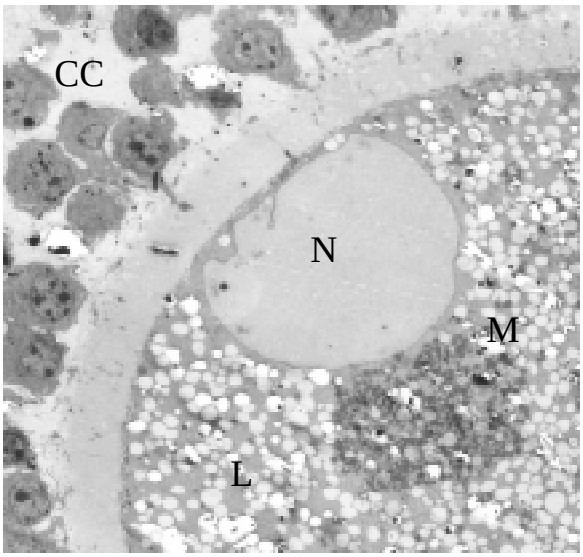
a,b Values (mean ± SEM) with different superscripts in same row differ ($P < .05$).

[c]Variable was evaluated on a scale of 0, 1 or 2; see text for details.

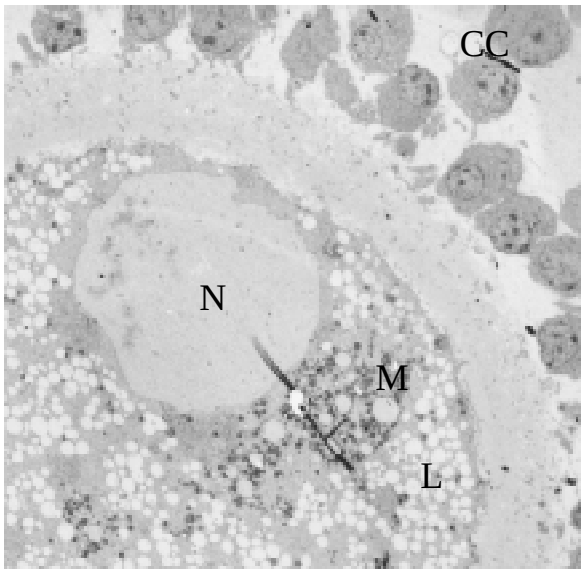
Fig. 5 Representative micrographs- CC, cumulus cells; N nucleus; ZP, zona pelucida; L, Lipid; M, mitochondria (1,100 X). Cows were treated either with a low dose of progesterone and $PF_{2\alpha}$ to produce low peripheral concentrations of progesterone or received no treatment (control). (a) Oocyte recovered on d 8 from control cow, note round nucleus and peripheral distribution of mitochondria. Notice irregular shape of the nucleus, clustering of mitochondria and expansion of cumulus cells, in oocytes from (b) a treated cow on d 8 (c) a control cow on d 10 or (d) a treated cow on d 10.



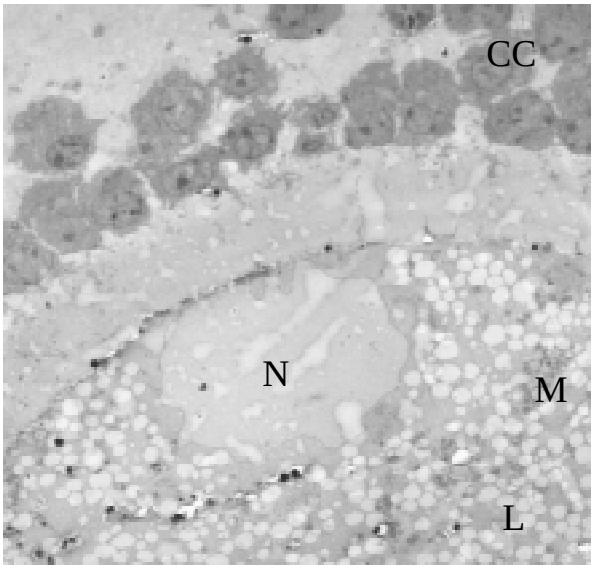
A



B

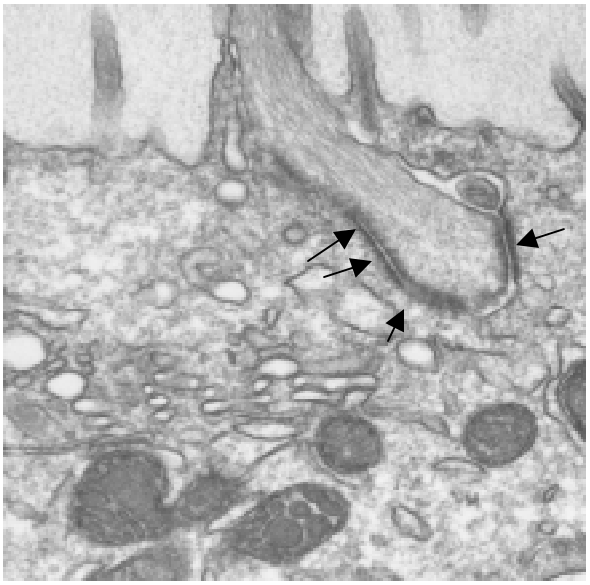


C

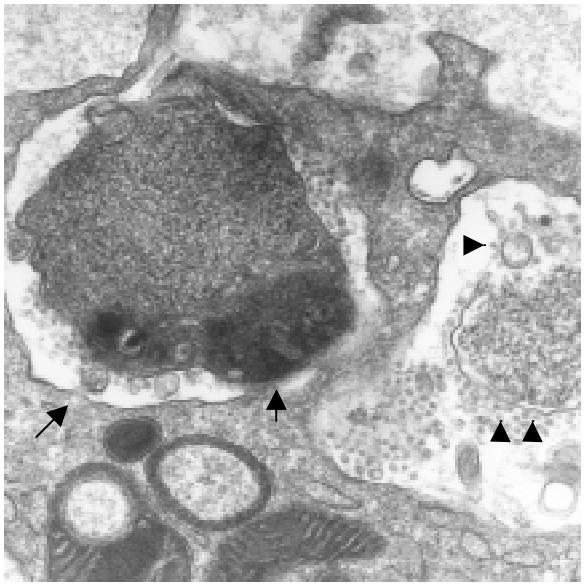


D

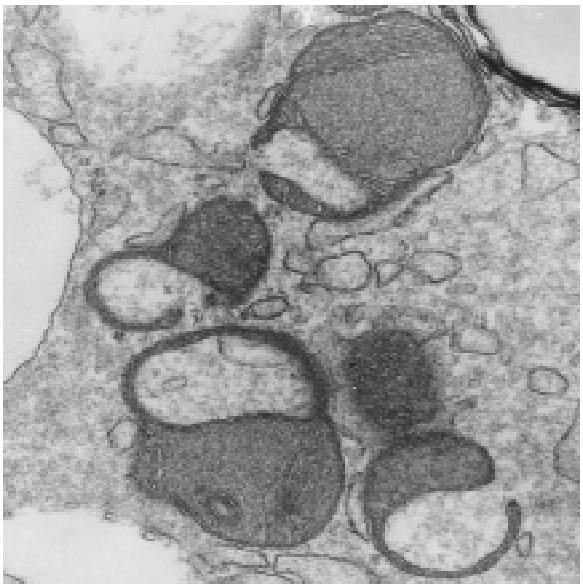
Fig. 6. Representative micrographs (a) Intact endings of cumulus cell processes in control animal on d 8 (arrows; 33,000X). (b) Degenerated endings of cumulus cell processes lacking contact with oolema (arrows) and on the right (arrowheads), appearing to have broken down completely (35,000X) in oocyte from treated cow on d 10. (c) Hooded mitochondria (27,500X) in oocyte from treated cow on d 8. (d) Invagination in nuclear envelope (21,000X) in oocyte from control cow on d 10. (e) Section of nuclear membrane that appears to be breaking down in oocyte from control cow on d 10, note intact section on left (arrows) and disrupted section on right (arrowheads; 41,250X).



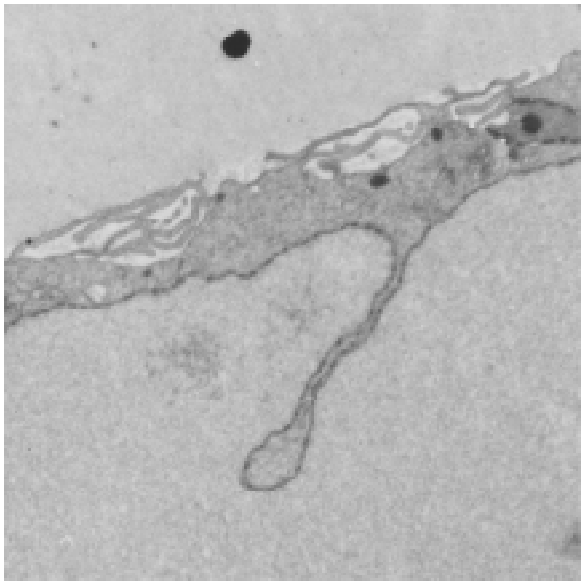
A



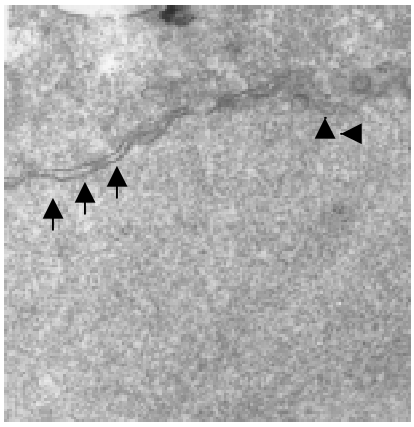
B



C



D



E

DISCUSSION

Follicular function and oocyte morphology were altered in this study within 48 h after a reduction in progesterone to a sustained low concentration. Similar changes were observed previously as a result of maintaining low progesterone (Ahmad et al., 1995; Revah and Butler, 1996; NE-161, 1996; Taft et al., 1996) for 7d or more. However, comparisons to follicles of the same age were not made (Mihm et al., 1994, 1999; Revah and Butler, 1996, Revah et al. 1997). In contrast, oocytes in this study were collected shortly after peripheral concentrations of progesterone were reduced and comparisons were made to follicles of a similar age.

Both peripheral and intrafollicular concentrations of estrogen increased after d 6 in treated cows, but size of the largest follicle was not greater than in control cows until d 10. In previous studies, a reduction, or maintenance of low concentrations of progesterone, resulted in an increase in the frequency of pulses of LH in peripheral circulation (Roche and Ireland, 1982; Stock and Fortune, 1993; NE-161, 1996; Bergfeld et al., 1997). Expression of aromatase in granulosal cells was not increased after a reduction in progesterone (Tian et al., 1995). Instead, thecal expression of side chain cleavage enzyme (SCC), 3β hydroxysteroid deydrogenase (3βHSD), and 17 α hydroxylase increased, making more androstenedione available for aromatization by granulosal cells (Tian et al., 1995; Xu et al., 1995). Thus, increased expression of steroidogenic enzymes in the thecal layer, as a response to increased frequency of pulse of LH, could account for the increase in estradiol in cows with low concentrations of progerone.

Alternatively, increased synthesis of estradiol in the present study may have resulted from reduction of the direct inhibitory effect of progesterone or the CL. After withdrawal of progestogens, Bergfeld et al. (1997) reported that peripheral concentrations of estrogen increased before changes in secretion of LH were detected. Gust et al. (1984) found that peripheral concentrations of estrogen increased within 4 h after the initiation of luteal regression in ewes, even when changes in secretion of LH were blocked by systemic infusion with dopamine. Fogwell et al. (1978) prevented increased secretion of LH in cows after luteal regression and observed that concentrations of estrogen were increased. In cows, with normal concentrations of progesterone (Taft et al., 1996) mimicked the pattern of secretion of LH (1 exogenous pulse every 2 h) observed during maintenance of low peripheral concentrations of progesterone. Peripheral concentrations of estrogen were not increased by LH in this case, although GnRH at a greater frequency (1 exogenous pulse per h) did increase estrogen secretion in heifers during the luteal phase (Glencross et al., 1987).

Intrafollicular concentrations of estradiol in treated cows were increased to approximately 1µg/ml, a value similar to that reported for follicular fluid from preovulatory follicles (Ireland and Roche, 1982). In both cases, peripheral concentrations of progesterone were reduced and the frequency of pulses of LH was increased as were peripheral and intrafollicular concentrations of estrogen (Cupp et al., 1992; Bergfeld et al, 1996; Taft et al., 1996; Revah and Butler, 1996; Bigelow and Fortune, 1998). In the absence of exogenous progesterone, these changes in follicular function culminate in the LH surge and ovulation, but during treatment with low progesterone the surge of LH and ovulation are delayed until after withdrawal of progesterone.

The idea that similar changes in follicular function occur after luteolysis regardless of whether low concentrations of exogenous progesterone are present was supported by the changes in oocyte morphology observed in this study. Oocytes from control animals on d 8, had a round nucleus, mitochondria were dispersed and located in the periphery, cumulus cell processes were in contact with the oocyte, and cumulus cells had not expanded (Hyttel et al., 1987; Assey et al., 1994a,b). In oocytes collected on d 8 and 10 during treatment with low dosages of progesterone, the nucleus was irregular in shape and had an irregular nuclear membrane. Mitochondria were found in clusters and were located more centrally. Endings of cumulus cell processes appeared to be retracting and degenerating and cumulus cells were partially expanded. The morphology of the oocytes was similar to that of oocytes in other studies collected 24 h after initiation of luteal regression, but well before the LH surge (Hyttel et al., 1987, Assey et al., 1994a,b).

Hyttel (1987) and Assey (1994a,b) concluded that changes in oocyte morphology following luteolysis indicated that activation and "prematuration" of the oocyte had occurred. These steps preceded germinal vesicle breakdown and resumption of meiosis and were thought to represent changes in the oocyte that were necessary for transcription of ribosomal RNA and synthesis of proteins required for germinal vesicle breakdown and condensation of chromatin (Sirard et al., 1989; Kastrop et al., 1991; Fissore et al., 1996; Levesque and Sirard, 1996). Altering the timing of these processes likely would reduce the subsequent developmental capacity of the oocyte (Greve et al., 1995).

Although the kinetics of oocyte maturation have not been evaluated, it is apparent that treatment with a low dosage of progesterone extended the interval between the prematuration and subsequent developmental processes in this study and in others (Mihm

et al., 1994, 1999; Revah and Butler, 1996). Lengthening the interval between initiation of oocyte maturation and activation of the embryonic genome at the 8 to 16 cell stage of embryonic development (Frei et al., 1989) may have exhausted stores of mRNA upon which oocytes and embryos depend. Consequently, oocytes were unable to make proteins needed for development, resulting in death of embryos. Revah et al. 1997 observed that maturation promoting factor kinase activity was reduced and that chromatin was condensed and scattered in oocytes collected after low progesterone had been maintained for 7 d. Those oocytes may have been unable to continue to synthesize proteins needed to control the cell cycle and maintain spindle structure when the time between initiation of maturation and ovulation was extended. Therefore, intrafollicular aging may account for the reduced developmental capacity of oocytes from follicles in which growth is prolonged by maintaining low concentrations of progesterone.

If oocytes from large follicles normally undergo prematuration following luteolysis and can complete meiosis before exogenous progesterone is withdrawn, when does oocyte maturation begin? In general, the LH surge is presumed to initiate oocyte maturation. However, in rats and rabbits, dosages of LH that were insufficient to cause ovulation stimulated oocytes to resume meiosis (Pincus and Enzmann, 1935; Mattheij et al., 1994; Dekel et al., 1995). The increased secretion of LH following a reduction in progesterone may initiate oocyte maturation. Prematuration may represent the earliest stages of oocyte maturation rather than a separate process.

The mechanisms by which LH could promote meiosis include effects on cumulus cells to reduce the function and number of gap junctions between cumulus cells and oocytes (Sherizly et al., 1988), blocking the flow of a meiosis inhibiting factor (Dekel et

al., 1978,1991) or stimulating the thecal production of sterols that promote meiosis (Richard and Sirard, 1993, Mottlik et al., 1996). However, Mihm et al. (1999) did not detect changes in secretion of LH, but observed that oocytes had undergone prematuration when collected after four days of dominance, the last two of which occurred during maintenance of low progesterone. Oocytes in the study by Mihm et al. (1999) were collected 18 h after progestogen withdrawal and were not compared to oocytes from cows in which progestogen treatment had been continued; prematuration may have occurred as the result of ending treatment rather than as a result of treatment.

Aside from the possible adverse effects of extending the time between the initiation of meiosis and subsequent developmental events, prolonged exposure to elevated concentrations of estrogen may have an adverse impact on the oocyte. Receptors for estrogen are found on oocytes (Wu et al., 1992, 1993) and in rats, prolonged exposure of oocytes to elevated concentrations of estrogen within the follicle reduced the percentage of normal embryos after mating (Butcher and Pope, 1979). Prolonged exposure to increased concentrations of estrogen may have similar adverse effects in cattle (Breuel et al., 1993; Wehrman et al., 1993; Ahmad et al., 1995). Alternatively, in cattle, increased estrogen may be only a marker for effects of increased LH or other changes in follicular function that initiate oocyte maturation during the cascade of preovulatory events. Ablation of estrogen and replacement therapy have not been tested in this species.

Oocytes collected on d 10 from control cows were similar in morphology to oocytes from treated animals indicating that prematuration occurred not only as a consequence of treatment with low dosages of progesterone but as a consequence of

aging of the follicle in animals with normal progesterone. Growth of the largest follicle in control cows plateaued by d 10, but follicles were estrogen-active and based on the ratio of estrogen to progesterone were not atretic (Ireland and Roche, 1983). However, an appreciable amount of apoptosis occurs before the ability of a follicle to produce estradiol declines (Jolly et al, 1997a,b). Jolly (1994) observed that signs of apoptosis could be detected in 80% of follicles with a ratio of estrogen to progesterone greater than one. Therefore, changes in oocytes were probably a sign that atresia was beginning and that communication between the cumulus cells and oocyte had broken down as evidenced by the decrease in intact endings of cumulus cell processes. Ovulation of an aged oocyte may occur in this situation as oocytes from atretic follicles can resume meiosis and some atretic follicles can ovulate. However, Moor and Trounson (1977) observed that oocytes recovered from the atretic follicles of ewes had difficulty in progressing to metaphase II. It is not known if bovine oocytes have similar defects.

Reduced pregnancy rates following treatment with $PGF_{2\alpha}$ on d 10 (Momont and Seguin, 1984; Watts and Fuquay, 1985) may be the result of ovulation of aged oocytes from early atretic follicles. Growth of the first wave follicle usually plateaus near d 10 and the next cohort of follicles is recruited near this time as well (Pierson and Ginther, 1987; Sirois and Fortune, 1988). Hence, initiation of luteolysis on d 10 may result in the ovulation of first wave follicles that contain oocytes that have begun to mature prematurely. Changes in oocyte morphology such as irregularities in the nuclear membrane and alterations in distribution of organelles may be early markers for atresia as cell-cell communication is impaired and the flow of inhibitors of meiosis to the oocyte is blocked. In support of this idea, Hagemann et al. (1999), reported that oocytes from

follicles whose growth had plateaued did not develop as well as oocytes from growing follicles following in vitro fertilization.

Because oocytes are capable of secreting substances that can alter follicular function, an interesting possibility is that changes in oocyte function as atresia begins could alter follicular function, hastening atresia. Factors originating in the oocyte have been implicated in such follicular functions as luteinization (El –Fouly et al., 1970), steroidogenesis and expression of gonadotrophin receptors (Vanderhyden et al., 1998; Eppig, 1993). Other authors have observed no effects of oocytes on these same processes (Channing and Tsafriri, 1977). It would be interesting to relate changes in oocyte morphology to other indices of atresia and to determine if oocytes affect the process of atresia.

One common feature of oocytes from all groups was the presence of pleomorphic mitochondria. While many were hooded, other shapes including dumbbell and cloverleaf shapes were noted, but vacuolated mitochondria as described in compact bovine morulae (Crosier et al., 1999) were not observed. Hooded mitochondria have been described previously as common in bovine oocytes (Senger and Saacke, 1970; Fleming and Saacke, 1972; Hyttel et al., 1987; Assey et al., 1994a,b). Fleming and Saacke proposed that the hood served to increase surface area and facilitated transport from the endoplasmic reticulum to hooded mitochondria. They proposed that hooded mitochondria were a unique feature of ruminant oocytes as they were found also in oocytes from goats and sheep. However, they have been observed in opossum oocytes, in normal rat liver and in rat liver following induction of vitamin B-12 deficiency with hydroxycobalamin (Stephens and Bills, 1965; Tandler et al., 1991). Induction of vitamin B-12 deficiency

results in the production of metabolic intermediates that interfere with mitochondrial metabolism. During this deficiency, the number of mitochondria is increased and hooded mitochondria become prevalent, but the link between form and function is unknown.

The observation that changes in the shape of mitochondria occur in a variety of species including turkeys, mice, pigs, cows, sheep and goats may be more important than the actual shape assumed (Senger and Saacke, 1970; Cran and Moor, 1980; Assey et al., 1994a,b; Carlson et al., 1996). Changes in mitochondrial shape may indicate changes in oocyte function that are similar in the various species; what differs is the way mitochondria respond. Given the role of mitochondria in metabolism, changes in the shape and distribution of mitochondria are likely the result of changes in metabolism within oocytes. Changes in mitochondrial morphology were observed in this study at the same time cumulus cell processes began to breakdown and the distribution of mitochondria became more clustered often in association with lipid droplets. These changes may represent a shift in oocyte metabolism from a dependence on the cumulus cells to a dependence on internal stores of energy and nutrients. Further studies into oocyte mitochondrial function may yield interesting information about oocyte metabolism.

In conclusion, changes in concentrations of estradiol and oocyte morphology typically associated with the preovulatory period occurred on d 8 two days after a reduction in progesterone, although low peripheral concentrations of progesterone were maintained. It appeared that these earliest stages of oocyte maturation occurred in response to a reduction in progesterone. Similar changes in oocyte morphology were observed in control animals on d 10 of the estrous cycle. Thus, while changes in oocyte

morphology in the treated group on d 8 were the result of changes in progesterone and/or secretion of LH or estradiol, changes in control animals on d 10 probably represent the onset of atresia.

BIBLIOGRAPHY

Ahmad N, Schrick FN, Butcher RL, Inskeep EK. Effect of persistent follicles on early embryonic losses in beef cows. Biol Reprod 1995; 52(5): 1129-35.

Aktas H, Wheeler MB, First NL, Leibfried-Rutledge ML. Maintenance of meiotic arrest by increasing [cAMP]i may have physiological relevance in bovine oocytes. J Reprod Fertil 1995a; 105(2): 237-45.

Aktas H, Wheeler MB, Rosenkrans CF Jr, First NL, Leibfried-Rutledge ML. Maintenance of bovine oocytes in prophase of meiosis I by high [cAMP]i. J Reprod Fertil. 1995b; 105(2): 227-35.

Albertini DF, Carabatsos MJ. Comparative aspects of meiotic cell cycle control in mammals. J Mol Med. 1998; 76(12): 795-9.

Albertini DF. Regulation of meiotic maturation in the mammalian oocyte: interplay between exogenous cues and the microtubule cytoskeleton. Bioessays. 1992; 14(2): 97-103.

Anderson E, Albertini DF. Gap junctions between the oocyte and companion follicle cells in the mammalian ovary. J Cell Biol. 1976; 71(2): 680-6.

Anderson LH, Day ML. Acute progesterone administration regresses persistent dominant follicles and improves fertility of cattle in which estrus was synchronized with melengestrol acetate. J Anim Sci. 1994; 72(11): 2955-61.

Assey RJ, Hyttel P, Greve T, Purwantara B. Oocyte morphology in dominant and subordinate follicles. Mol Reprod Dev. 1994; 37(3): 335-44.

Assey RJ, Hyttel P, Kanuya N. Oocyte structure in dominant and subordinate follicles in zebu cattle (Bos indicus). Embryol (Berl). 1994 ; 190(5): 461-8.

Assey RJ, Hyttel P, Roche JF, Boland M. Oocyte structure and follicular steroid concentrations in superovulated versus unstimulated heifers. Mol Reprod Dev. 1994; 39(1): 8-16.

Badinga L, Driancourt MA, Savio JD, Wolfenson D, Drost M, De La Sota RL,Thatcher WW. Endocrine and ovarian responses associated with the first-wave dominant follicle in cattle. Biol Reprod. 1992; 47(5):871-83.

Bao B, Garverick HA, Smith GW, Smith MF, Salfen BE, Youngquist RS. Expression of messenger ribonucleic acid (mRNA) encoding 3beta-hydroxysteroid dehydrogenase delta4,delta5 isomerase (3beta-HSD) during recruitment and

selection of bovine ovarian follicles: identification of dominant follicles by expression of 3beta-HSD mRNA within the granulosa cell layer. Biol Reprod. 1997; 56(6):1466-73.

Bergfeld EG, Kojima FN, Cupp AS, Wehrman ME, Peters KE, Mariscal V, Sanchez T, Kinder JE. Changing dose of progesterone results in sudden changes in frequency of luteinizing hormone pulses and secretion of 17 beta-estradiol in bovine females. Biol Reprod. 1996; 54(3):546-53.

Bevers MM, Dieleman S, van den Hurk R, Izadyar F. Regulation and modulation of oocyte maturation in the bovine. Theriogenology 1997; 47:13-22.

Bigelow KL, Fortune JE. Characteristics of prolonged dominant versus control follicles: follicle cell numbers, steroidogenic capabilities, and messenger ribonucleic acid for steroidogenic enzymes. Biol Reprod. 1998; 58(5): 1241-9.

Blangy A, Lane HA, d'Herin P, Harper M, Kress M, Nigg E. Phosphorylation by p34cdc2 regulates spindle association of human Eg5, a kinesin-related motor essential for bipolar spindle formation in vivo. Cell. 1995 29; 83(7): 1159-69.

Bo GA, Adams, G.P., Pierson, R.A., Tribulo, H.E.,Caccia, M., Mapletoft, R.J. Follicular wave dynamics after estradiol-17 beta treatment of heifers with or without a progestogen implant. Theriogenology. 1994; 41 (8)

Bodensteiner KJ, Wiltbank MC, Bergfelt DR, Ginther OJ. Alterations in follicular estradiol and gonadotropin receptors during development of bovine antral follicles. Theriogenology 1996; 45:499-512.

Bradley DM, Goldin HH, Claybrook JR. Histone analysis in Volvox. FEBS Lett. 1974; 41(2):219-22.

Breuel KF, Lewis PE, Schrick FN, Lishman AW, Inskeep EK, Butcher RL. Factors affecting fertility in the postpartum cow: role of the oocyte and follicle in conception rate. Biol Reprod. 1993; 48:655-661.

Bunone G, Briand PA, Miksicek RJ, Picard D. Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. EMBO J. 1996; 15(9):2174-83.

Butcher RL, Pope RS. Role of estrogen during prolonged estrous cycles of the rat on subsequent embryonic death or development. Biol Reprod. 1979; 21(2): 491-5.

Byskov AG, Yding Andersen C, Hossaini A, Guoliang X. Cumulus cells of oocyte-cumulus complexes secrete a meiosis-activating substance when stimulated with FSH. Mol Reprod Dev. 1997; 46(3): 296-305.

Campbell BK, Scaramuzzi RJ, Webb R. Control of antral follicle development and selection in sheep and cattle. J Reprod Fertil Suppl. 1995; 49:335-50.

Carlone DL, Richards JS. Evidence that functional interactions of CREB and SF-1 mediate hormone regulated expression of the aromatase gene in granulosa cells and constitutive expression in R2C cells. J Steroid Biochem Mol Biol. 1997; 61(3-6): 223-31.

Carlson JL, Bakst MR, Ottinger MA. Developmental stages of primary oocytes in turkeys. Poult Sci. 1996; 75(12): 1569-78.

Carr DW, DeManno DA, Atwood A, Hunzicker-Dunn M, Scott JD. Follicle-stimulating hormone regulation of A-kinase anchoring proteins in granulosa cells. J Biol Chem. 1993; 268(28): 20729-32.

Casida LE. Research techniques in physiology of reproduction in the female. In: Chapman AB (ed.), Techniques and Procedures in Animal Production Research. Albany, NY: American Society of Animal Production. 1959: 106-121.

Channing CP and Tsafriri A. Lack of an inhibitory influence of oocytes upon luteinization of porcine granulosa cells in culture. J Reprod Fertil. 1977; 50(1): 103-5.

Choi T, Aoki F, Mori M, Yamashita M, Nagahama Y, Kohmoto K. Activation of p34cdc2 protein kinase activity in meiotic and mitotic cell cycles in mouse oocytes and embryos. Development. 1991; 113(3): 789-95.

Christian RE, Casida LE. The effect of progesterone in altering the estrus cycle of the cow. J. Anim. Sci 1948; 7:540 (abstract).

Cooperative Regional Research Project, NE-161. Relationship of fertility to patterns of ovarian follicular development and associated hormonal profiles indairy cows and heifers. 1996; J Anim Sci 74:1943.

Coskun S, Lin YC. Mechanism of action of epidermal growth factor-induced porcine oocyte maturation. Mol Reprod Dev. 1995; 42(3): 311-7.

Cran DG, Moor RM, Hay MF. Fine structure of the sheep oocyte during antral follicle development. J Reprod Fertil. 1980; 59(1): 125-32.

Crosier AE, Farin PW, Alexander JE, Farin CE. Ultrastructural morphology of bovine compact morula produced in vivo or in vitro. Biol Reprod. 1999; 60 (supplement 1):192 (abstract 318).

Cupp A, Garcia-Winder M, Zumudio A, Mariscal V, Wehrman M, Kojima N, Peters K, Bergfeld E, Hernandez P, Sanchez T, Kittock R, Kinder J. Two concentrations of

progesterone (P4) in circulation have a differential effect on pattern of ovarian follicular development in the cow. Biol Reprod 1992; 45 (supplement 1) 106 (abstract 222).

Cyert MS, Kirschner MW. Regulation of MPF activity in vitro. Cell. 1988; 53(2):185-95.

Davis JS, Farese RV, Marsh JM. Stimulation of phospholipid labeling and steroidogenesis by luteinizing hormone in isolated bovine luteal cells. Endocrinology. 1981; 109(2): 469-75.

Davis JS, Weakland LL, West LA, Farese RV. Luteinizing hormone stimulates the formation of inositol trisphosphate and cyclic AMP in rat granulosa cells. Evidence for phospholipase C generated second messengers in the action of luteinizing hormone. Biochem J. 1986; 238(2): 597-604.

De Smedt V, Szollosi D. Cytochalasin D treatment induces meiotic resumption in follicular sheep oocytes. Mol Reprod Dev. 1991; 29(2): 163-71.

Deaver DR, Stilley NJ, Dailey RA, Inskeep EK, Lewis PE. Deaver DR, Stilley NJ, Dailey RA, Inskeep EK, Lewis PE. Concentrations of ovarian and pituitary hormones following prostaglandin F2 alpha-induced luteal regression in ewes varies with day of the estrous cycle at treatment. J Anim Sci. 1986; 62(2): 422-7.

Dekel N, Ayalon D, Lewysohn O, Nevo N, Kaplan-Kraicer R, Shalgi R. Experimental extension of the time interval between oocyte maturation and ovulation: effect on fertilization and first cleavage. Fertil Steril. 1995; 64(5): 1023-8.

Dekel N, Beers WH. Rat oocyte maturation in vitro: relief of cyclic AMP inhibition by gonadotropins. Proc Natl Acad Sci U S A. 1978; 75(9): 4369-73.

Dominko T, First NL. Timing of meiotic progression in bovine oocytes and its effect on early embryo development. Mol Reprod Dev. 1997; 47(4): 456-67.

Dimino MJ, Snitzer J, Brown KM. Inositol phosphates accumulation in ovarian granulosa after stimulation by luteinizing hormone. Biol Reprod. 1987; 37(5): 1129-34.

Downs SM, Hunzicker-Dunn M. Differential regulation of oocyte maturation and cumulus expansion in the mouse oocyte-cumulus cell complex by site-selective analogs of cyclic adenosine monophosphate. Dev Biol. 1995; 172(1): 72-85.

Driancourt MA, Thuel B. Control of oocyte growth and maturation by follicular cells and molecules present in follicular fluid. A review. Reprod Nutr Dev. 1998; 38(4): 345-62.

El-Fouly MA, Cook B, Nekola M, Nalbandov AV. Role of the ovum in follicular luteinization. Endocrinology 1979; 87-288.

Eppig JJ, Chesnel F, Hirao Y, O'Brien MJ, Pendola FL, Watanabe S, Wigglesworth K. Oocyte control of granulosa cell development: how and why. Hum Reprod. 1997; 12(11 Suppl): 127-32.

Eppig JJ. A comparison between oocyte growth in coculture with granulosa cells and oocytes with granulosa cell-oocyte junctional contact maintained in vitro. J Exp Zool. 1979; 209(2):345-53.

Eppig JJ. Coordination of nuclear and cytoplasmic oocyte maturation in eutherian mammals. Reprod Fertil Dev. 1996; 8(4): 485-9.

Eppig JJ. Intercommunication between mammalian oocytes and companion somatic cells. Bioessays. 1991; 13(11): 569-74.

Evans AC, Fortune JE. Selection of the dominant follicle in cattle occurs in the absence of differences in the expression of messenger ribonucleic acid for gonadotropin receptors. Endocrinology. 1997; 138(7): 2963-71.

Fagbohun CF, Downs SM. Metabolic coupling and ligand-stimulated meiotic maturation in the mouse oocyte-cumulus cell complex. Biol Reprod. 1991; 45(6): 851-9.

Fair T, Hyttel P, Greve T, Boland M. Nucleus structure and transcriptional activity in relation to oocyte diameter in cattle. Mol Reprod Dev. 1996 Apr; 43(4):503-12.Li R, Mather JP. Lindane, an inhibitor of gap junction formation, abolishes oocyte directed follicle organizing activity in vitro. Endocrinology. 1997; 138(10): 4477-80.

Fike KE, Wehrman ME, Bergfeld EG, Kojima FN, Kinder JE. Prolonged increased concentrations of 17beta-estradiol associated with development of persistent ovarian follicles do not influence conception rates in beef cattle. J Anim Sci. 1997; 75(5): 1363-7.

Fissore RA, He CL, Vande Woude GF. Potential role of mitogen-activated protein kinase during meiosis resumption in bovine oocytes. Biol Reprod. 1996; 55(6): 1261-70.

Fleming WN, Saacke RG. Fine structure of the bovine oocyte from the mature graafian follicle. Fertil. 1972; 29(2): 203-13.

Fogwell RL, Weems CW, Lewis GS, Butcher L, Inskeep EK. Secretion of steroids after induced luteal regression in beef heifers: effects of PGF2 alpha and removal of corpora lutea. J Anim Sci. 1978; 46(6): 1718-23.

Fortune, JE. Ovarian follicular growth and development in mammals. Biol Reprod. 1994; 50(2): 225-32

Frei RE, Schultz GA, Church RB. Qualitative and quantitative changes in protein synthesis occur at the 8-16-cell stage of embryogenesis in the cow. J Reprod Fertil. 1989; 86(2): 637-41.

Funkenstein B, Waterman MR, Simpson ER. Induction of synthesis of cholesterol side chain cleavage cytochrome P-450 and adrenodoxin by follicle-stimulating hormone, 8-bromo-cyclic AMP, and low-density lipoprotein in cultured bovine granulosa cells. J Biol Chem. 1984; 259(13): 8572-7.

Furger C, Pouchelet M, Zorn JR, Ferre F. Cell shape change reveals the cyclic AMP-mediated action of follicle stimulating hormone, human chorionic gonadotrophin and vasoactive intestinal peptide in primary cultured human granulosa-lutein cells. Mol Hum Reprod. 1996; 2(4):251-7.

Gabrielli BG, Roy LM, Maller JL. Requirement for Cdk2 in cytostatic factor-mediated metaphase II arrest. Science. 1993; 259(5102): 1766-9.

Galaktionov K, Beach D. Specific activation of cdc25 tyrosine phosphatases by B-type cyclins: evidence for multiple roles of mitotic cyclins. Cell. 1991 20; 67(6): 1181-94.

Gastal EL, Gastal MO, Ginther OJ. Experimental assumption of dominance by a smaller follicle and associated hormonal changes in mares. Biol Reprod. 1999; 61(3): 724-30.

Gerace L, Blobel G. The nuclear envelope lamina is reversibly depolymerized during mitosis. Cell. 1980; 19(1): 277-87.

Gilula NB, Epstein ML, Beers WH. Cell-to-cell communication and ovulation. A study of the cumulus-oocyte complex. J Cell Biol. 1978; 78(1): 58-75.

Ginther OJ, Wiltbank MC, Fricke PM, Gibbons JR, Kot K. Selection of the dominant follicle in cattle. Biol Reprod. 1996; 55(6): 1187-94.

Glencross RG. Effect of pulsatile infusion of gonadotropin-releasing hormone on plasma oestradiol-17 beta concentrations and follicular development during naturally and artificially maintained high levels of plasma progesterone in heifers. J Endocrinol. 1987; 112(1): 77-85.

Granot I, Dekel N. Cell-to-cell communication in the ovarian follicle: developmental and hormonal regulation of the expression of connexin43. Hum Reprod. 1998; 13 Suppl 4:85-97.

Granot I, Dekel N. Phosphorylation and expression of connexin-43 ovarian gap junction protein are regulated by luteinizing hormone. J Biol Chem. 1994; 269(48): 30502-9.

Greve T, Callesen H, Hyttel P, Hoier R, Assey R. The effects of exogenous gonadotropins on oocyte and embryo quality in cattle. Theriogenology; 1995; 43: 41-50.

Gudermann T, Birnbaumer M, Birnbaumer L. Evidence for dual coupling of the murine luteinizing hormone receptor to adenylyl cyclase and phosphoinositide breakdown and Ca2+ mobilization. Studies with the cloned murine luteinizing hormone receptor expressed in L cells. J Biol Chem. 1992; 267(7): 4479-88.

Gust CM, Deaver DR, Dailey RA, Inskeep EK. Relationships between LH and estradiol-17 beta after removal of luteal progesterone in the ewe. J Anim Sci. 1984; 58(2): 396-400.

Hagemann LJ, Beaumont SE, Berg M, Donnison MJ, Ledgard A, Peterson AJ, Schurmann A, Tervit HR. Development during single IVP of bovine oocytes from dissected follicles: Interactive effects of estrous cycle stage, follicle size and atresia. Mol Reprod Dev. 1999; 53(4): 451-458.

Hashimoto N, Kishimoto T. Regulation of meiotic metaphase by a cytoplasmic maturation-promoting factor during mouse oocyte maturation. Dev Biol. 1988; 126(2): 242-52.

Homa ST, Carroll J, Swann K. The role of calcium in mammalian oocyte maturation and egg activation. Hum Reprod. 1993 ; 8(8):1274-81.

Homa ST, Webster SD, Russell RK. Phospholipid turnover and ultrastructural correlates during spontaneous germinal vesicle breakdown of the bovine oocyte: effects of a cyclic AMP phosphodiesterase inhibitor. Dev Biol. 1991; 146(2): 461-72.

Homa ST. Calcium and meiotic maturation of the mammalian oocyte. Mol Reprod Dev. 1995; 40(1): 122-34.

Huchon D, Rime H, Jessus C, Ozon R. Control of metaphase I formation in Xenopus oocyte: effects of an indestructible cyclin B and of protein synthesis. Biol Cell. 1993; 77(2): 133-41.

Hunzicker-Dunn M, Scott JD, Carr DW. Regulation of expression of A-kinase anchoring proteins in rat granulosa cells. Biol Reprod. 1998; 58(6): 1496-502.

Hyttel P. Bovine cumulus-oocyte disconnection in vitro. Anat Embryol (Berl). 1987a; 176(1): 41-4.

Hyttel P, Xu KP, Smith S, Callesen H, Greve T. Ultrastructure of the final nuclear maturation of bovine oocytes in vitro. Anat Embryol (Berl). 1987b; 176(1): 35-40.

Ireland JJ, Roche JF. Effect of progesterone on basal LH and episodic LH and FSH secretion in heifers. J Reprod Fertil. 1982; 64(2): 295-302.

Ireland JJ, Roche JF. Development of nonovulatory antral follicles in heifers: changes in steroids in follicular fluid and receptors for gonadotropins. Endocrinology. 1983; 112(1): 150-6.

Ireland JJ, Roche JF. Hypotheses regarding development of dominant follicles during a bovine estrous cycle. In: Follicular growth and ovulation rate in farm animals, pp 1-18 (Eds JF Roche and D O' Callaghan) Martinus Nijoff Publishers, The Hague.

Johnson ML, Redmer DA, Reynolds LP, Grazul-Bilska A. Expression of gap junctional proteins connexin 43, 32, and 26 throughout follicular development and atresia in cows. Endocrine. 1999; 10(1): 43-51.

Johnson SK, Dailey R.A., Inskeep E.K., Lewis P.E. Effect of peripheral concentrations of progesterone on follicular growth and fertility in ewes. Domestic Anim Endo. 1996; 13:69

Jolly PD, Smith PR, Heath DA, Hudson NL, Lun S, Still LA, Watts CH, McNatty KP. Morphological evidence of apoptosis and the prevalence of apoptotic versus mitotic cells in the membrana granulosa of ovarian follicles during spontaneous and induced atresia in ewes. Biol Reprod. 1997a; 56(4): 837-46.

Jolly PD, Tisdall DJ, De'ath G, Heath DA, Lun S, Hudson NL, McNatty KP. Granulosa cell apoptosis, aromatase activity, cyclic adenosine 3',5'-monophosphate response to gonadotropins, and follicular fluid steroid levels during spontaneous and induced follicular atresia in ewes. Biol Reprod. 1997b; 56(4): 830-6.

Jolly PD, Tisdall DJ, Heath DA, Lun S, McNatty K. Apoptosis in bovine granulosa cells in relation to steroid synthesis, cyclic adenosine 3',5'-monophosphate response to follicle-stimulating hormone and luteinizing hormone, and follicular atresia. changes in diameter, insulin-like growth factor system, aromatase activity, steroid hormones, and apoptotic index. Biol Reprod. 1994a; 51(5): 934-44.

Jolly PD, Tisdall DJ, Heath DA, Lun S, McNatty KP. Apoptosis in bovine granulosa cells in relation to steroid synthesis, cyclic adenosine 3',5'-monophosphate response to follicle-stimulating hormone and luteinizing hormone, and follicular atresia. Biol Reprod. 1994b; 51(5): 934-44.

Kastrop PM, Bevers MM, Destree OH, Kruip TA. Protein synthesis and phosphorylation patterns of bovine oocytes maturing in vivo. Mol Reprod Dev. 1991 ;29(3):271-5.

Kaufman ML, Homa ST. Defining a role for calcium in the resumption and progression of meiosis in the pig oocyte. J Exp Zool. 1993; 265(1): 69-76.

Kinder JE, Kojima FN, Bergfeld EG, Wehrman ME, Fike KE. Progestin and estrogen regulation of pulsatile LH release and development of persistent ovarian follicles in cattle. J Anim Sci. 1996; 74(6): 1424-40.

Langan TA, Gautier J, Lohka M, Hollingsworth R, Moreno S, Nurse P, Maller J, Sclafani RA. Mammalian growth-associated H1 histone kinase: a homolog of cdc2+/CDC28 protein kinases controlling mitotic entry in yeast and frog cells. Mol Cell Biol. 1989; 9(9): 3860-8.

Lanuza GM, Fischman ML, Baranao JL. Growth promoting activity of oocytes on granulosa cells is reased upon meiotic maturation. Dev Biol. 1998 1;197(1):129-39.

LaVoie HA, Garmey JC, Veldhuis JD. Mechanisms of insulin-like growth factor I augmentation of follicle-stimulatinghormone-induced porcine steroidogenic acute regulatory protein gene promoter activity in granulosa cells. Endocrinology. 1999; 140(1): 146-53.

Lee TH, Solomon MJ, Mumby MC, Kirschner MW. INH, a negative regulator of MPF, is a form of protein phosphatase 2A. Cell. 1991 25;64(2):415-23.

Levesque JT, Sirard MA. Effects of different kinases and phosphatases on nuclear and cytoplasmic maturation of bovine oocytes. Mol Reprod Dev. 1995; 42(1): 114-21.

Levesque JT, Sirard MA. Resumption of meiosis is initiated by the accumulation of cyclin B in bovine oocytes. Biol Reprod. 1996; 55(6): 1427-36.

Li R, Mather JP. Lindane, an inhibitor of gap junction formation, abolishes oocyte directed follicle organizing activity in vitro. Endocrinology. 1997; 138(10): 4477-80.

Lonergan P, Carolan C, Mermillod P. Development of bovine embryos in vitro following oocyte maturation under defined conditions. Reprod Nutr Dev. 1994; 34(4): 329-39.

Lonergan P, Carolan C, Van Langendonckt A, Donnay I, Khatir H, Mermillod P. Role of epidermal growth factor in bovine oocyte maturation and preimplantation embryo development in vitro. Biol Reprod. 1996 Jun; 54(6): 1420-9.

Lorca T, Cruzalegui FH, Fesquet D, Cavadore JC, Mery J, Means A, Doree M. Calmodulin-dependent protein kinase II mediates inactivation of MPF and CSF upon fertilization of Xenopus eggs. Nature. 1993; 366(6452):270-3.

Lorca T, Fesquet D, Zindy F, Le Bouffant F, Cerruti M, Brechot C, Devauchelle G, Doree M. An okadaic acid-sensitive phosphatase negatively controls the cyclin degradation pathway in amphibian eggs. Mol Cell Biol. 1991; 11(2): 1171-5.

Lorenzo PL, Illera MJ, Illera JC, Illera M. Enhancement of cumulus expansion and nuclear maturation during bovine oocyte maturation in vitro by the addition of epidermal growth factor and insulin-like growth factor I. J Reprod Fertil. 1994; 101(3): 697-701.

Lorenzo PL, Illera MJ, Illera JC, Illera M. Influence of growth factors on the time-dependent meiotic progression of the bovine oocytes during their in vitro maturation. Rev Esp Fisiol. 1995; 51(2): 77-83.

Manikkam M, Rajamahendran R. Progesterone-induced atresia of the proestrous dominant follicle in the bovine ovary: changes in diameter, insulin-like growth factor system, aromatase activity, steroid hormones, and apoptotic index. Biol Reprod 1997; 57:580.

Marcote MJ, Knighton DR, Basi G, Sowadski JM, Brambilla P, Draetta G, Taylor SS. A three-dimensional model of the Cdc2 protein kinase: localization of cyclin- and Suc1-binding regions and phosphorylation sites. Mol Cell Biol. 1993; 13(8): 5122-31.

Masui Y, Markert CL. Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. J Exp Zool. 1971; 177(2): 129-45.

Mattheij JA, Swarts JJ, van der Heijden AJ, van Helvoort AL, Kusters IC. Advancement of meiotic resumption in graafian follicles reduces fertility in the rat. Gynecol Obstet Invest. 1993;36(3):129-35.

Matten W, Daar I, Vande Woude GF. Protein kinase A acts at multiple points to inhibit Xenopus oocyte maturation. Mol Cell Biol. 1994; 14(7): 4419-26.

Mattioli M, Gioia L, Barboni B. Calcium elevation in sheep cumulus-oocyte complexes after luteinising hormone stimulation. Mol Reprod Dev. 1998; 50(3): 361-9.

McConnell JM, Campbell L, Vincent C. Capacity of mouse oocytes to become activated depends on completion of cytoplasmic but not nuclear meiotic maturation. Zygote. 1995; 3(1): 45-55.

Mihm M, Baguisi A, Boland MP, Roche JF. Association between the duration of dominance of the ovulatory follicle and pregnancy rate in beef heifers. J Reprod Fertil. 1994; 102(1): 123-30.

Mihm M, Curran N, Hyttel P, Boland MP and Roche JF. Resumption of meiosis in cattle oocytes from preovulatory follicles with a short and long duration of dominance. 1994b J. Reprod. Fert.; 74:167-172.

Mihm M, Curran N, Hyttel P, Knight G, Boland MP, Roche RF. Effect of dominant follicle persistence on follicular fluid oestradiol and inhibin and on oocyte maturation in heifers. J Reprod Fert. 1999; 116:293-304.

Mihm M, Good TE, Ireland JL, Ireland JJ, Knight PG, Roche JF. Decline in serum follicle-stimulating hormone concentrations alters key intrafollicular growth factors involved in selection of the dominant follicle in heifers. Biol Reprod. 1997; 57(6): 1328-37.

Momont HW, Sequin SE. 10th International Congress on Animal Reproduction and Artificial Insemination, University of Illinois at Urbana-Champaign, Illinois, USDA, June 10-14, 1984. v. 3 p. 336.1-336.3.

Moor RM. Contact, signaling and cooperation between follicle cells and dictyate oocytes in mammals. Current problems in germ cell Differentiation. Symposium of British Society for developmental biology. 1983 pp 307-326.

Moor RM, Smith MW, Dawson RM. Measurement of intercellular coupling between oocytes and cumulus cells using intracellular markers. Exp Cell Res. 1980; 126(1): 15-29

Moor RM, Trounson AO. Hormonal and follicular factors affecting maturation of sheep oocytes in vitro and their subsequent developmental capacity. J Reprod Fertil. 1977; 49(1): 101-9.

Morris JK, Richards JS. Hormone induction of luteinization and prostaglandin endoperoxide synthase-2 involves multiple cellular signaling pathways. Endocrinology. 1993; 133(2): 770-9.

Motlik J, Sutovsky P, Kalous J, Kubelka M, Moos J, Schultz RM. Co-culture with pig membrana granulosa cells modulates the activity of cdc2 and MAP kinase in maturing cattle oocytes. Zygote. 1996; 4(3): 247-56.

Nagai T, Ding J, Moor RM. Effect of follicle cells and steroidogenesis on maturation and fertilization in vitro of pig oocytes. J Exp Zool. 1993 Jun 1;266(2):146-51. Nature. 1993; 366(6452): 270-3.

Oak B, Tyson JJ. Numerical analysis of a comprehensive model of M-phase control in Xenopus oocyte extracts and intact embryos. J Cell Sci. 1993; 106 (Pt 4):1153-68.

Odde KG. A review of synchronization of estrus in postpartum cattle. J Anim Sci. 1990; 68(3): 817-30.

O'Keefe SJ, Wolfes H, Kiessling AA, Cooper GM. Microinjection of antisense c-mos oligonucleotides prevents meiosis II in the maturing mouse egg. Proc Natl Acad Sci U S A. 1989; 86(18):7038-42.

Ookata K, Hisanaga S, Okumura E, Kishimoto T. Association of p34cdc2/cyclin B complex with microtubules in starfish oocytes. J Cell Sci. 1993; 105 (Pt 4):873-81.

Osborn JC, Moor RM, Crosby IM. Effect of alterations in follicular steroidogenesis on the nuclear and cytoplasmic maturation of ovine oocytes. J Embryol Exp Morphol. 1986; 98:187-208.

Osborn JC, Moor RM. The role of steroid signals in the maturation of mammalian oocytes. J Steroid Biochem. 1983; 19(1A): 133-7.

Parrish, J.J. Current concepts of cell-cycle regulation and its relationship to oocyte maturation, fertilization and embryo development. Theriogenology. 1992; 38:277-296.

Pierson RA, Ginther OJ. Follicular populations during the estous cycle in heifers I. Influence of day. 1987; Animal Reprod Sci 14:165.

Pincus G, Enzmann EV. The comparative behavior of mammalian eggs in vitro and in vivo. J Exp Med 1935; 62:655-675.

Preston SL, Parmer TG, Behrman HR. Adenosine reverses calcium-dependent inhibition of follicle-stimulating hormone action and induction of maturation in cumulus-enclosed rat oocytes. Endocrinology. 1987; 120(4): 1346-53.

Rae MT, Menzies GS, McNeilly AS, Woad K, Webb R, Bramley TA. Specific non-genomic, membrane-localized binding sites for progesterone in the bovine corpus luteum. Biol Reprod. 1998; 58(6): 1394-406.

Ratoosh SL, Richards JS. Regulation of the content and phosphorylation of RII by adenosine 3',5'-monophosphate, follicle-stimulating hormone, and estradiol in cultured granulosa cells. Endocrinology. 1985; 117(3): 917-27.

Resnick CE, Fielder PJ, Rosenfeld RG, Adashi EY. Characterization and hormonal regulation of a rat ovarian insulin-like growth factor binding protein-5

endopeptidase: an FSH-inducible granulosa cell-derived metalloprotease. Endocrinology. 1998; 139(3): 1249-57.

Revah I, Butler WR. Prolonged dominance of follicles and reduced viability of bovine oocytes. J Reprod Fertil. 1996; 106(1): 39-47.

Revah I, Presicce GA, Senatore E, Ignotz G, and Butler WR. MPF phosporylating activity in oocytes and development and function of corpora lutea derived from persistent follicles in dairy cows. J Reprod Fert 1997; Suppl 19: 172 (abstract).

Richard FJ, Sirard MA. Effects of follicular cells on oocyte maturation. II: Theca cell inhibition of bovine oocyte maturation in vitro. Biol Reprod. 1996; 54(1) 22-8.

Richard FJ, Sirard MA. Role of the cyclic adenosine monophosphate-dependent protein kinase in the control of meiotic resumption in bovine oocytes cultured with thecal cell monolayers. Biol Reprod. 1997; 56(6): 1363-9.

Richards JS, Russell DL, Robker RL, Dajee M, Alliston TN. Molecular mechanisms of ovulation and luteinization. Mol Cell Endocrinol. 1998; 25; 145(1-2): 47-54.

Rime H, Jessus C, Ozon R. Tyrosine phosphorylation of p34cdc2 is regulated by protein phosphatase 2A in growing immature Xenopus oocytes. Exp Cell Res. 1995; 219(1): 29-38.

Ripoll P, Carmena M, Molina I. Genetic analysis of cell division in Drosophila. Curr Top Dev Biol. 1992; 27:275-307.

Roche JF, Ireland JJ. The differential effect of progesterone on concentrations of luteinizing hormone and follicle-stimulating hormone in heifers. Endocrinology. 1981; 108(2): 568-72.

Rozell TG, Keisler DH. Effects of oestadiol on LH, FSH and prolactin in ovariectomized ewes. J Reprod Fertil. 1990; 90:127-135.

Sadhu K, Reed SI, Richardson H, Russell P. Human homolog of fission yeast cdc25 mitotic inducer is predominantly expressed in G2. Proc Natl Acad Sci U S A. 1990; 87(13):5139-43.

Sadighian JJ, Kearns WG, Waddell BJ, Dimino MJ. Effects of diacylglycerol and inositol trisphosphate on steroidogenesis by ovarian granulosa from pigs. Biol Reprod. 1989; 40(2):294-9.

Sagata N, Watanabe N, Vande Woude GF, Ikawa Y. The c-mos proto-oncogene product is a cytostatic factor responsible for meiotic arrest in vertebrate eggs. Nature. 1989 30;342(6249):512-8.

SAS. Sas user's guide: statistics (version 6 ed) SAS Inst. Inc., Cary, NC.

Scott JD, Faux MC. Use of synthetic peptides in the dissection of protein-targeting interactions. Methods Mol Biol. 1998; 88:161-85.

Scott JD. Dissection of protein kinase and phosphatase targeting interactions. Soc Gen Physiol Ser. 1997; 52:227-39.

Senger PL, Saacke RG. Unusual mitochondria of the bovine oocyte. J Cell Biol. 1970; 46(2):405-8.

Sheffel CE, Pratt BR, Ferrell WL, Inskeep EK. Induced corpora lutea in the postpartum beef cow. II. Effects of treatment with progestogen and gonadotropins. J Anim Sci. 1982 Apr; 54(4): 830-6.

Sherizly I, Galiani D, Dekel N. Regulation of oocyte maturation: communication in the rat cumulus-oocyte complex. Hum Reprod. 1988; 3(6):761-6.

Sirard MA, First NL. In vitro inhibition of oocyte nuclear maturation in the bovine. Biol Reprod. 1988; 39(2): 229-34.

Sirard MA, Florman HM, Leibfried-Rutledge ML, Barnes FL, Sims ML, First NL. Timing of nuclear progression and protein synthesis necessary for meiotic maturation of bovine oocytes. Biol Reprod. 1989; 40(6): 1257-63.

Sirois J, Fortune JE. Lengthening the bovine estrous cycle with low levels of exogenous progesterone: a model for studying ovarian follicular dominance. Endocrinology. 1990; 127(2):916-25.

Sirois J, Fortune JE. Ovarian follicular dynamics during the estrous cycle in heifers monitored by real-time ultrasonography. Biol Reprod. 1988; 39(2):308-17.

Sirotkin AV, Taradajnik TE, Makarevich AV, Bulla J. Effect of follicular cells, IGF-I and tyrosine kinase blockers on oocyte maturation. Anim Reprod Sci. 1998; 29; 51(4):333-44.

Sirotkin AV. Involvement of steroid hormones in bovine oocytes maturation in vitro. J Steroid Biochem Mol Biol. 1992; 41(3-8): 855-8.

Smith CL. Cross-talk between peptide growth factor and estrogen receptor signaling pathways. Biol Reprod. 1998; 58(3): 627-32.

Solomon MJ, Glotzer M, Lee TH, Philippe M, Kirschner MW. Cyclin activation of p34cdc2. Cell. 1990; 30; 63(5): 1013-24.

Stephens RJ, Bils RF. An atypical mitochondrial form in normal rat liver. J Cell Biol 1965; 24:500.

Stock AE, Fortune JE. Ovarian follicular dominance in cattle: relationship between prolonged growth of the ovulatory follicle and endocrine parameters. Endocrinology. 1993; 132(3): 1108-14.

Sugawara T, Kiriakidou M, McAllister JM, Kallen CB, Strauss JF 3rd. Multiple steroidogenic factor 1 binding elements in the human steroidogenic acuteregulatory protein gene 5'-flanking region are required for maximal promoter activity and cyclic AMP responsiveness. Biochemistry. 1997; 36(23):7249-55.

Sutovsky P, Flechon JE, Flechon B, Motlik J, Peynot N, Chesne P, Heyman Y. Dynamic changes of gap junctions and cytoskeleton during in vitro culture of cattle oocyte cumulus complexes. Biol Reprod. 1993; 49(6): 1277-87.

Taft R, Ahmad N, Inskeep EK. Exogenous pulses of luteinizing hormone cause persistence of the largest bovine ovarian follicle. J Anim Sci 1996; 74(12): 2985-91.

Taieb R, Thibier C, Jessus C. On cyclins, oocytes, and eggs. Mol Reprod Dev. 1997; 48(3): 397-411.

Tandler B, Krahenbuhl S, Brass EP. Unusual mitochondria in the hepatocytes of rats treated with a vitamin B12 analogue. Anat Rec. 1991; 231(1): 1-6.

Tesarik J, Mendoza C. Nongenomic effects of 17 beta-estradiol on maturing human oocytes: relationship to oocyte developmental potential. J Clin Endocrinol Metab. 1995; 80(4): 1438-43.

Tian XC, Berndtson AK, Fortune JE. Differentiation of bovine preovulatory follicles during the follicular phase is associated with increases in messenger ribonucleic acid for cytochrome P450 side-chain cleavage, 3 beta-hydroxysteroid dehydrogenase, and P450 17 alpha-hydroxylase, but not P450 aromatase. Endocrinology. 1995; 136(11):5102-10.

Tortonese DJ, Lewis PE, Papkoff H, Inskeep EK. Roles of the dominant follicle and the pattern of oestradiol in induction of preovulatory surges of LH and FSH in prepubertal heifers by pulsatile low doses of LH. J Reprod Fertil. 1990 ; 90(1):127-35.

Turzillo AM, Fortune JE. Effects of suppressing plasma FSH on ovarian follicular dominance in cattle. J Reprod Fertil. 1993; 98(1):113-9.

Ulberg LC, Christian RE, Casida LE. Ovarian response in heifers to progesteroe injections. J Anim Sci; 10: 752-759.

van Tol HT, van Eijk MJ, Mummery CL, van den Hurk R, Bevers MM. Influence of FSH and hCG on the resumption of meiosis of bovine oocytes surrounded by cumulus cells connected to membrana granulosa. Mol Reprod Dev. 1996; 45(2): 218-24.

Vanderhyden BC, Macdonald EA. Mouse oocytes regulate granulosa cell steroidogenesis throughout follicular development. Biol Reprod. 1998; 59(6): 1296-301.

Verde F, Labbe JC, Doree M, Karsenti E. Regulation of microtubule dynamics by cdc2 protein kinase in cell-free extracts of Xenopus eggs. Nature. 1990; 343(6255): 233-8.

Ward GE, Kirschner MW. Identification of cell cycle-regulated phosphorylation sites on nuclear lamin C. Cell. 1990 18; 61(4):561-77.

Watts T, Fuquay JW. Response and fertility of dairy heifers following injection with prostaglandin F2alpha during early, middle or late diestrus. Theriogenology. 1985. v. 23 (4).

Wehrman ME, Roberson MS, Cupp AS, Kojima FN, Stumpf TT, Werth LA, Wolfe MW, Kittok RJ, Kinder JE. Increasing exogenous progesterone during synchronization of estrus decreases endogenous 17 beta-estradiol and increases conception in cows. Biol Reprod. 1993; 49(2): 214-20.

Wehrman, M.E., Fike, K.E. Melvin E.J., Kojima, F.N., Kinder, J.E. Development of a persistent ovarian follicle and associated elevated concentrations of 17 beta-estradiol preceding ovulation does not alter the pregnancy rate after embryo transfer in cattle. Theriogenology. 1997. v. 47 (7).

Wert SE, Larsen WJ. Meiotic resumption and gap junction modulation in the cultured rat cumulus-oocyte complex. Gamete Res. 1989; 22(2): 143-62.

Wickramasinghe D, Albertini DF. Cell cycle control during mammalian oogenesis. Curr Top Dev Biol. 1993; 28:125-53.

Wiersman A, Hirsch B, Tsafriri A, Hanssen RG, Van de Kant M, Kloosterboer HJ, Conti M, Hsueh AJ. Phosphodiesterase 3 inhibitors suppress oocyte maturation and consequent pregnancy without affecting ovulation and cyclicity in rodents. J Clin Invest. 1998; 102(3): 532-7.

Wise T, Suss U, Stranzinger G, Wuthrich K, Maurer. RRCumulus and oocyte maturation and in vitro and in vivo fertilization of oocytes in relation to follicular steroids, prolactin, and glycosaminoglycans throughout the estrous period in

superovulated heifers with a normal LH surge, no detectable LH surge, and progestin inhibition of LH surge. Domest Anim Endocrinol. 1994; 11(1): 59-86.

Wrenzycki C, Herrmann D, Carnwath JW, Niemann H. Expression of the gap junction gene connexin43 (Cx43) in preimplantation bovine embryos derived in vitro or in vivo. J Reprod Fertil. 1996; 108(1):17-24.

Wu B, Ignotz G, Currie WB, Yang X. Dynamics of maturation-promoting factor and its constituent proteins during in vitro maturation of bovine oocytes. Biol Reprod. 1997; 56(1):253-9.

Wu TC, Wang L, Wan YJ. Detection of estrogen receptor messenger ribonucleic acid in human oocytes and cumulus-oocyte complexes using reverse transcriptase-polymerase chain reaction. Fertil Steril. 1993; 59(1):54-9.

Wu TC, Wang L, Wan YJ. Expression of estrogen receptor gene in mouse oocyte and during embryogenesis. Mol Reprod Dev. 1992; 33(4):407-12.

Whytock S, Moir RD, Stewart M. Selective digestion of nuclear envelopes from Xenopus oocyte germinal vesicles: possible structural role for the nuclear lamina. J Cell Sci. 1990; 97 (Pt 3):571-80.

Xu Z, Garverick HA, Smith GW, Smith MF, Hamilton SA, Youngquist RS. Expression of follicle-stimulating hormone and luteinizing hormone receptor messenger ribonucleic acids in bovine follicles during the first follicular wave. Biol Reprod. 1995; 53(4):951-7.

Yew N, Mellini ML, Vande Woude GF. Meiotic initiation by the mos protein in Xenopus. Nature. 1992 13; 355(6361):649-52.

Yoshimura Y, Hosoi Y, Atlas SJ, Dharmarajan AM, Adachi T, Wallach EE. Effect of the exposure of intrafollicular oocytes to clomiphene citrate on pregnancy outcome in the rabbit. Fertil Steril. 1988; 50(1):153-8.

Zamboni L, Thompson RS, Smith DM. Fine morphology of human oocyte maturation in vitro. Biol Reprod. 1972; 7(3):425-57.

Zelinski-Wooten MB, Hess DL, Wolf DP, Stouffer RL. Steroid reduction during ovarian stimulation impairs oocyte fertilization, but not folliculogenesis, in rhesus monkeys. Fertil Steril. 1994; 61(6): 1147-55.

Zuelke KA, Brackett BG. Luteinizing hormone-enhanced in vitro maturation of bovine oocytes with and without protein supplementation. Biol Reprod. 1990; 43(5): 784-7.