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The equine corpus luteum: *In vivo* and *in vitro* responsiveness to gonadotropin stimulation

Kelly, Christopher Mark, M.S.

The University of Arizona, 1987
THE EQUINE CORPUS LUTEUM:
IN VIVO AND IN VITRO RESPONSIVENESS TO
GONADOTROPIN STIMULATION

by

Christopher Mark Kelly

A Thesis Submitted to the Faculty of the
DEPARTMENT OF ANIMAL SCIENCES
In Partial Fulfillment of the Requirements
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1987
STATEMENT BY AUTHOR

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ABSTRACT

Gonadotropins were used to stimulate luteal function, as determined by progesterone secretion, in both in vitro and in vivo systems. LH and hCG were capable of significantly stimulating progesterone secretion in the in vivo systems. Stimulation of progesterone secretion by hCG was greater than that for LH. PMSG failed to increase progesterone production at any level of treatment. hCG was also used to stimulate progesterone production by the corpus luteum in mares during early gestation. hCG administration resulted in a significant (p<0.10) increase in peripheral progesterone levels in treatment mares through day 14 post-estrus. Peripheral progesterone concentrations were also higher in hCG treated mares for days 15 through 30 post-estrus in mares that conceived. hCG treatment had no influence on anterior pituitary release of LH.
CHAPTER 1

INTRODUCTION

In domestic animals, it is estimated that 20 to 60 percent of all potential young die early during gestation. Embryonic death often results in reabsorption of the conceptus with no external clue that pregnancy has occurred as ovarian cyclicity is maintained at a normal duration. Few attempts have been made to evaluate the physiological factors associated with the maintenance of pregnancy in the mare. In the horse industry obtaining optimal pregnancy rates is hindered by a number of factors. Obstacles such as the absence of selection for fertility potential, the determination to breed geriatric mares and the disparity between physiological and arbitrary breeding seasons, all make it difficult to achieve optimal conception rates. The economical potential for improving pregnancy rates in the mare are enormous considering the value of genetically superior horses.

Both maternal and embryonic factors may contribute to early embryonic death. One of the key maternal regulators of embryonic development is progesterone. In mammals, the anterior pituitary gland secretes Luteinizing Hormone (LH) which appears to be the primary regulator of
progesterone secretion by the corpus luteum. Progesterone is in turn responsible for providing the embryo with an optimum uterine environment, which is required for implantation and maintenance of pregnancy in all mammals. It is thought that by increasing progesterone levels during the critical time for maternal recognition of pregnancy, a uterine environment which is more conducive to embryonic survival will be created.
CHAPTER 2

REVIEW OF LITERATURE

Maintenance of pregnancy

It is well accepted that some source of progesterone is necessary for the maintenance of pregnancy in mammals. The maternal ovaries with a functional corpus luteum appear necessary almost throughout gestation in the rabbit, rat, sow, cow, goat and bitch but for a much shorter duration of gestation in the ewe, guinea pig, mare and primates. These latter species derive their progesterone from the placenta during gestation (Gomes and Erb, 1965). Although the equine placenta contains high levels of progesterone (Short, 1957), peripheral blood levels reflect ovarian sources of the hormone during early pregnancy when corpora lutea are functional (Squires, Wentworth and Ginther, 1974; Holtan, Nett and Estergreen, 1975a). The placenta presumably takes over progesterone production at some stage during the first half of gestation (Squires and Ginther, 1975a). The work of Holtan et al. (1979) indicates that the ovaries of the mare become dispensable between days 50 and 70 of gestation.

The critical time for the recognition of pregnancy in most domestic farm species appears to be from day 11 to day 16. Therefore any attempts to decrease embryonic
mortality rates need to be made before this time. In the classical studies conducted by Moor and Rowson (1966a, 1966b), embryo removal was utilized to study the relationship between the corpus luteum and the preimplantation blastocyst. In the pregnant ewe, blastocyst removal after day 12 post-estrus resulted in extended estrous cycles as compared to blastocyst removal before day 12 (Moor and Rowson, 1966a). Also, ovine embryos transferred to recipient ewes by day 12 post-estrus resulted in pregnancy, whereas embryos transferred to recipients after day 12 did not (Moor and Rowson, 1966b).

Surgical removal of pig embryos on days 13 and 16 after the first day of estrus resulted in extended interestrus intervals as compared with embryo removal on day 8 or in non-pregnant gilts which were surgically flushed (French and Strauss, 1976). This agrees with other studies demonstrating the critical time for recognition of pregnancy in pigs occurs between days 11 to 13 (Dindsa and Dziuk, 1968; Ford et al., 1982).

In the mare, surgical removal of the embryo on day 24 after ovulation resulted in pseudopregnancy, evidenced by a prolonged interval from removal to the next estrus. Prolonged progesterone production and extended maintenance of uterine and cervical tone were also prevalent (Kooistra and Ginther, 1976). Similar observations have been made by Hershman and Douglas (1979) using blastocyst removal, by
uterine flushing, on days 10, 12, 14, and 16 after ovulation. Blastocyst removal on day 16 resulted in pseudopregnancy, again characterized by a prolonged interestrous interval, prolonged interovulatory interval, maintained uterine tone and continued progesterone production. Whereas, blastocyst removal on days 10, 12 and 14 after ovulation did not result in pseudopregnancy, indicating that the critical time for the maternal recognition of pregnancy in the pony mare appears to be confined to the period between days 14 and 16 after ovulation.

Progesterone is referred to as the hormone of pregnancy because of its function to prevent the female from cycling when pregnant and to provide an optimum uterine enviroment for the developing embryo. It has also been shown (Day et al., 1959) that exogenous progesterone can enhance embryo survival in ovariectomized gilts and in the mare (McDowell et al., 1985).

Under natural conditions ovulation does not occur during physiological states in which plasma progesterone levels are high, i.e. the luteal phase, pregnancy and psuedopregnancy. Progesterone may suppress development of ovulatory follicles through negative feedback effects on gonadotropin secretion (Greenwald, 1978; Heap et al., 1978). Progesterone has been shown to inhibit the development of ovulatory follicles in rats when given prior
to or in conjunction with gonadotropins (Fukada et al., 1980; Buffler and Roser, 1974; Moore and Greenwald, 1974). Moor and Walters (1979) reported that co-culture with luteal tissue decreased estradiol production by sheep follicles. These reports taken together suggest that progesterone may be acting on follicular steroidogenesis both indirectly, through effects at the brain level, and directly, through local actions within the ovary.

The early experiments of Loy and Swan (1966) were the first to show that progesterone and progestagens are potent inhibitors of estrous behavior and ovulation in mares. Theses conclusions have been supported by the work of other investigators (Webel, 1975; Garcia and Ginther, 1976; Holtan et al., 1979; Munro et al., 1979; Loy et al., 1981).

Progesterone binding proteins have been found in cytosols from human, bovine, rat ovarian and rat granulosa cells (Jacobs et al., 1980; Milwidsky et al., 1980; Jacobs and Smith., 1980; Schreiber and Hseuh, 1979; Schreiber and Erickson, 1979) and in porcine follicular fluid (Flemming and McGaughey, 1982). Naess (1981) has demonstrated in rats translocation of rodent cytoplasmic progesterone receptors to the nucleus of the granulosa cells. Fortune and Vincent (1983) showed that progesterone dramatically supressed the effects of FSH on the aromatase activity of granulosa cells from hypophysectomized rats. This is in agreement with the work
of Schreiber et al. (1980, 1982) showing that progesterone and a synthetic progestin depress the action of FSH in inducing aromatase and LH receptors in granulosa cells from immature, hypophysectomized, DES-treated rats. Therefore, it appears that intraovarian or intrafollicular environments that are high in progesterone are detrimental to the development of preovulatory follicles. Therefore, it appears that progesterone may also be regulating one or more aspects of ovarian function, by possibly reducing the ability of the granulosa cell to produce estrogen.

Since estrogen acts to stimulate granulosa cell mitosis and to induce LH receptors, it exerts intrafollicular positive feedback on its own production and therefore on the differentiation of the follicle to preovulatory status. Because of these positive feedback effects, even a slight reduction in the capacity to secrete estradiol, as seen with increased progesterone levels, might be sufficient to predispose a follicle to atresia.

The results of Fortune and Vincent (1983) suggest that progesterone primarily acts distal to the formation of cyclic AMP, but prior to the induction aromatase activity in rat granulosa cells.

For the successful maintenance of pregnancy, progesterone must provide a uterine environment that will optimize the chances for embryo survival. Progesterone acts on the uterine endometrium causing the epithelium to become
columnar and stimulating the mucosal glands to become branched, coiled and actively secretory.

The results of Brinsfield et al. (1974) show that the ultrastructure of the endometrial stromal cells changed during the estrous cycles of ewes. Progesterone prevented the changes in stromal cell ultrastructure which otherwise occurred late in the estrous cycle. The effects presumably reflect maintenance of the corpus luteum, although there is a possibility of direct effects of the embryo on the stromal cell.

Knight and coworkers (1973a) demonstrated a positive relationship between quantity of luteal tissue and quantity of recoverable uterine protein on day 15 of the estrous cycle of gilts. They also detected a positive correlation between level of exogenous progesterone administered and quantity of uterine protein recovered (Knight, et al., 1973b). Therefore indicating that progesterone is stimulating protein secretion into the uterine lumen.

Uterine protein is a major constituent of histotrophe, or uterine milk, which is believed to be responsible for supplying the embryo with adequate nutrition in swine (Bazer et al., 1969). Since the mare and the sow have the same diffuse epitheliochorial type of placenta it can probably be assumed that they have the same type of nutrient supply system for the developing embryo. The work
of McDowell and coworkers (1985) demonstrated that administration of an exogenous progestin in mares, even after luteolysis and declining levels of progesterone resulted in maintenance of pregnancy. This is believed to be due to maintained levels of histotrophe for embryonic survival.

Knight et al. (1974) presented data which indicated that increased uterine secretory activity, which resulted from increased progesterone levels, enhances placental development in gilts. This may be accomplished by an increase in allantoic fluid volume which leads to an increase in the placental surface area which is in contact with the uterine endometrium, particularly the placenta areola surface area which may serve as the area for nutrient and water absorption (Chen et al., 1973).

The spontaneous appearance of gap junctions in the uterine myometrium of rats in vitro was shown to be prevented or delayed by progesterone administration (Garfield et al., 1978, 1980) which would help prevent uterine contractile activity which would be undesirable during pregnancy. Burghardt et al. (1984) also demonstrated the antagonistic effect of progesterone on the number of estrogen induced gap junctions present in the uterine myometrium of rats.
Regulation of progesterone secretion

It is believed that in mammals luteinizing hormone (LH) is the stimulus for luteal function and progesterone production. This pituitary gonadotropin induces luteinization and is capable of extending the functional life span of the corpus luteum in several species (Wiltbank, 1961; Donaldson and Hansel, 1965; Ross et al., 1970; Karsh et al., 1970; Vande Wiele et al., 1971).

LH has also been shown to increase the synthesis of progesterone by the bovine corpus luteum in vivo (Simmons and Hansel, 1964; Donaldson et al., 1965; Schomberg et al., 1967; Brunner et al., 1969; Carlson et al., 1971) or when incubated with bovine luteal slices or dissociated luteal cells (Mason and Savard, 1964; Armstrong and Black, 1966; Kaltenback, 1967; Williams and Marsh, 1978; Hixon and Hansel, 1979; Milvae and Hansel, 1980; Milvae et al., 1983).

Previous studies indicate that LH treatment stimulated progesterone secretion by porcine luteal tissue in vitro (Cook et al., 1967; Watson and Leask, 1975; Watson and Wrigglesworth, 1975; Lemon and Loir, 1977; Lemon and Mauleon, 1982) and in vivo (Cook et al., 1969).

The presence of luteal LH receptors has been reported for the mare (Stewart and Allen, 1979) and it is generally accepted that LH is the physiological luteotropin in cyclic mares. Further support for this concept was provided by the
work of Pineda, Ginther and McShaw (1972) demonstrating that antibodies to LH given during the luteal phase of the estrous cycles of mares induced a premature regression of the corpus luteum, indicating that LH is required for corpus luteum maintenance and function in the equine.

The mechanism by which LH acts upon lutel cells and stimulates progesterone production is thought to be initiated by the binding of LH to a specific plasma membrane receptor. The LH-receptor complex then interacts with adenylate cyclases located on the cytoplasmic side of the plasma membrane to catalyze the conversion of adenosine triphosphate (ATP) to adenosine 3'5' monophosphate (cyclic AMP). Increased intracellular levels of cyclic AMP bind and activate the cyclic AMP-dependent protein kinase which stimulates the conversion of cholesterol to pregnenolone, the rate limiting step in steroidogenesis, by protein phosphorylation (Catt and Dufau, 1976; Marsh, 1976).

To lend support to the theory that LH stimulates progesterone production by the functional corpus luteum through a cyclic AMP pathway, Watson and Wrigglesworth (1975) demonstrated that cyclic AMP treatment of porcine luteal slices mimicked LH stimulation of progesterone secretion. LH has also been shown to increase adenylate cyclase activity in porcine luteal tissue homogenates (Anderson et al., 1974).
LH stimulation of progesterone synthesis has been shown to be mediated by a stimulation of the adenylate cyclase cyclic AMP system in vitro for the cow (Marsh, 1976; Godkin et al., 1977), the rat (Carnegie and Tsang, 1984) and the monkey (Eyster and Stouffer, 1985).

It has been reported (Condon and Black, 1976; Jordan et al., 1978) that catecholamines stimulate progesterone production by the bovine and ovine corpus luteum in vitro. The catecholamines are thought to achieve this increase in steroidogenesis by a mechanism similar to that of LH, binding to a membrane receptor and stimulation of the adenylate cyclase-cyclic AMP system.

In order to obtain an increase in the production of progesterone by the corpus luteum, increased amounts of LH or a compound that would bind to the luteal LH receptors with a binding affinity similar to that of LH would be required. Another hormone which has been shown to bind to luteal LH receptors is human chorionic gonadotropin (hCG). LH and hCG are similar in both structure and function. Both are glycoprotein hormones composed of two subunits, an alpha and beta chain. The primary structure of the alpha subunits are identical within species and the amino terminal end of the beta subunits of LH and hCG show extensive homology (Pierce, 1971; Albertson and Vaitukaitis, 1977.) In many instances, hCG is more effective than LH in stimulating progesterone secretion despite the fact that the amino acid
sequences of the two peptides are similar. This difference appears to be due to the longer half-life of hCG (eight hours for hCG versus thirty to sixty minutes for LH). Differences in the carbohydrate substituents of the molecule are important determinants for half-life. HCG contains about 31% carbohydrate versus 16% for LH. Also, hCG contains 9% sialic acid, which is not present in the LH molecule (Daughaday, 1974).

In the ovary of the rat, Moyle (1980) demonstrated that LH and the human placental analog hCG, bind to specific luteal receptors in a similar fashion and induce progesterone production. It has also been shown (Ziecik et al., 1980) that hCG competes for binding sites on porcine luteal particulates, whereas other hormones such as follicle stimulating hormone (FSH) (Bramely, 1981; Ziecik et al., 1980) and thyroid stimulating hormone (TSH) (Ziecik et al., 1980) did not.

Receptors in primate corpora lutea have been shown to bind LH and hCG leading to a stimulation of the adenylate cyclase system (Cameron and Stouffer, 1981; Ottobre et al., 1984; Eyster and Stouffer, 1985). The studies of Wilkes and Noble (1983) and Ottobre and Stouffer (1983) showed that when hCG was given to nonpregnant monkeys, the function of the corpus luteum was similar to that of pregnancy: the lifespan of the corpus luteum was extended and peripheral progesterone levels were increased.
Roser and associates (1982) demonstrated in their study that only equine LH and hCG would bind to luteal membrane preparations from mares, whereas the other protein hormones equine FSH, ovine prolactin, pregnant mare serum gonadotropin (PMSG) and a gonadotropin releasing hormone (GnRH) analog did not.

The corpus luteum

Following ovulation, blood vessels and fibroblasts grow into the collapsed follicle. This results in the formation of the corpus luteum. The corpus luteum has a single purpose which is to produce progesterone, which quiets the reproductive system and maintains pregnancy.

The receptors for LH and hCG are localized in the plasma membrane of the luteal cell and in the rat it has been shown (Anderson et al., 1979) that the majority of binding sites are localized along regions of the cell surface facing capillaries and characterized by microvillus folds, whereas the basolateral surfaces characterized by junctional complexes contain very few binding sites.

Two luteal cell types have been demonstrated in the ewe (O'Shea et al., 1979), the sow (Lemon and Loir, 1977) and the cow (Chegini et al., 1984) which differ in size and can be distinguished on a morphological basis. They have been classified as either small (10-25um) or large (25-50)um based on their diameters. Both cell types stain
positively for 3 beta hydroxysteroid dehydrogenase (HSD) activity (O'Shea et al., 1979; Fitz et al., 1982) implying that both cell types are steroidogenic. Small luteal cells have irregular shaped nuclei, variable sized mitochondria, a golgi apparatus, some smooth endoplasmic reticulum and rough endoplasmic reticulum, a low cytoplasmic to nuclear ratio and some granules and lipid droplets. By comparison the large luteal cells contain more smooth endoplasmic reticulum, less rough endoplasmic reticulum, more granules and lipid droplets, more mitochondria which are of a regular size and a higher cytoplasmic to nuclear ratio than the small luteal cells. These differences in cellular structure suggest that there are possible differences in cell function in terms of steroid production and secretion. The cytoplasmic enzyme 3 beta HSD catalyzes the conversion of pregnenolone to progesterone. The enzymes that are involved in the synthesis of cholesterol and steroids are generally associated with the smooth endoplasmic reticulum and mitochondria. Therefore, an increase in the number and size of those two structures would suggest increases in steroid production.

Small luteal cells have been shown to be more responsive to LH stimulation of progesterone production than large luteal cells (Lemon and Loir, 1977; Ursely and Leymairie, 1979; Fitz et al., 1982; Rodgers et al., 1983; Hoyer et al., 1984). Also, the basal production of
progesterone has been shown to be greater for large luteal
cells than for small luteal cells (Lemon and Loir, 1977;
Ursely and Leymairie, 1979; Fitz et al., 1982; Rodgers et

It has also been demonstrated that the two luteal
cell types have different origins. Alila and Hansel (1984)
concluded that granulosa cells become large luteal cells and
thecal cells become small luteal cells after luteinization.
Once formed, the corpus luteum undergoes dynamic changes as
the large - granulosa derived luteal cells are lost and the
small theca - derived luteal cells become large luteal
cells.

In vivo studies have also suggested that hCG (Camboni
et al., 1984) is capable of stimulating the conversion of
small luteal cells to large luteal cells. If this conversion
did take place, progesterone secretion would be increased
since total production of progesterone is much greater by
large than small luteal cells.
CHAPTER 3

IN VITRO AND IN VIVO RESPONSIVENESS OF THE EQUINE CORPUS LUTEUM TO GONADOTROPIN STIMULATION

Summary

Gonadotropins were used to stimulate luteal function, as determined by progesterone secretion, in both in vitro and in vivo systems. Dispersed equine luteal cells were used in two types of cell culture systems to evaluate the ability of equine luteinizing hormone (LH), human chorionic gonadotropin (hCG) and pregnant mare serum gonadotropin (PMSG) to stimulate progesterone production in vitro. The luteal cells were incubated for two hours with hormone challenge as suspended cell cultures or allowed to attach to culture plates and then challenged with hormone for two hours. LH and hCG significantly (p<0.01) stimulated progesterone production at all levels of treatment in suspended cell incubations. Stimulation of progesterone secretion by HCG was greater (p<0.05) than LH over the range of doses utilized. For culture plate incubations, LH was capable of significantly (p<0.01) stimulating progesterone secretion above the control, or no hormone challenge, at all levels of treatment. hCG stimulated progesterone
production in the culture plate system \((p<0.01)\) at the two highest levels of treatment. PMSG failed to increase progesterone production at any level of treatment for either suspended cell incubations or cultured plates.

HCG was also used to stimulate progesterone production by the corpus luteum in mares during early gestation. Fourteen mature mares were used for this experiment. Mares were either postpartum or had not been in foal for the previous year. The animals were randomly assigned to one of two treatment groups; control or hCG treated (treatment). The control animals received no injection and the treatment mares received an intramuscular injection of 1000 I.U. of hCG on days 3, 4 and 5 post estrus. hCG administration resulted in a significant \((p<0.10)\) increase in peripheral progesterone levels in treatment mares over control mares beginning on day 4 post-estrus and continuing through day 14. Peripheral progesterone concentrations were also higher in hCG treated mares for days 15 through 30 post-estrus in mares that conceived. There was no effect of reproductive status (post partum vs. previously open) and no interaction between treatment and reproductive status on progesterone levels. hCG treatment had no influence on anterior pituitary release of LH for any of the parameters tested (mean LH, number of pulses, pulse amplitude, period or frequency).
Introduction

Embryonic mortality during early gestation is of major concern to horse breeders. Since progesterone is critical for pregnancy maintenance (Holtan et al., 1979) a deficiency of this hormone has often been implicated as the cause of early pregnancy loss. Thus, it has been common practice to administer progesterone to abortion prone mares. The results of several experiments (Gunzel and Merkt, 1979; Hawkins et al., 1979; Shidler et al., 1981; Parry and Holtan, 1985) indicate that the majority of progesterone regimens used are usually inadequate to sufficiently increase progesterone levels for pregnancy maintenance, and that adequate dosages and frequency of administration are often impractical to use.

LH has been demonstrated to stimulate progesterone production by luteal cells in vitro for several species (Lemon and Mauleon, 1982; Milvae et al., 1983; Carnegie and Tsang, 1984; Eyster and Stouffer, 1985). hCG has also been shown to stimulate progesterone secretion by luteal cells in vitro for a number of species (Moyle, 1980; Ziecik et al., 1980; Ottobre and Stouffer, 1983). While PMSG will bind to rat, cow and pig gonadotropin receptors with approximately the same efficiency, on a molar basis, as hCG and pituitary gonadotropins, PMSG was found to bind to equine LH receptors with about one-tenth the affinity of other species.
(Stewart and Allen, 1979). The study by Roser and coworkers (1982) demonstrated that only equine LH and hCG would bind to corpus luteum membrane preparations from mares, whereas PMSG does not.

Administration of hCG during various stages of the estrous cycle in sheep (Gamboni et al., 1984) has been shown to elevate serum progesterone levels. The influence of hCG on the development and function of the equine corpus luteum, however, has not been explored.

The objectives of the present studies were threefold; (1) to determine if more than one steroidogenic cell type exists in the equine corpus luteum, (2) to examine the effectiveness of LH, hCG and PMSG to stimulate progesterone secretion in vitro and (3) to determine if hCG treatment during the early luteal phase of the mare would potentiate progesterone secretion throughout the estrous cycle.

Materials and Methods

Experiment one

A total of seven corpora lutea were collected from mares during the mid luteal phase of the estrous cycle. After ovariectomy the ovaries were immediately placed in sterile Medium 199 (Gibco Laboratories, Grand Island, N.Y.), pH 7.5. The corpora lutea were dissected free of the ovary,
and interstitial tissue removed. The corpora lutea were then sliced and placed in dissociation media which consisted of Hank's medium (calcium and magnesium free), 0.4% collagenase, 0.005% deoxyribonuclease and 5% bovine serum albumin. The tissue was incubated at 37 C in an atmosphere of 95% O2 - 5% CO2 for two to ten hours with frequent agitation by aspiration to disperse cells.

Cell viability was determined by trypan blue exclusion and varied from 70 to 99% and was used in determining the number of cells for culture.

Cells were stained for 3 beta hydroxysteroid dehydrogenase (3 beta HSD) activity to determine steroidogenic capacity according to the procedure of Payne et al. (1980), and cell diameters were measured by an ocular micrometer. Cell diameters were measured on approximately 300 stained cells per CL.

Suspended cell cultures (25,000 cells/ml) were incubated in 12x75 mm glass tubes containing 2 ml m-199 at 37 C for two hours in an atmosphere of air with gentle shaking. The incubation tubes were sealed to prevent evaporation. The medium contained equine LH (3.0 x 10^-6 mmol/tube to 3.0 x 10^-10 mmol/tube) or hCG (9.0 x 10^-7 mmol/tube to 9.0 x 10^-11 mmol/tube) or PMSG (4.0 x 10^-6 mmol/tube to 4.0 x 10^-10 mmol/tube) or no hormone challenge. At the end of incubation, the cells were pelleted by centrifugation (400xg, 3 min.) and medium was collected and
stored at -20 C until ready for radioimmunoassay for progesterone.

Culture plates were set up by allowing 50,000 viable cells to attach to culture plates with two ml of M-199. Hormone challenges (2 hour) were at the same dosages as for suspended cell incubations. At the end of the challenge, medium was collected and store at -20 C until assayed for progesterone.

Experiment two

Fourteen sexually mature, post partum or cycling mares which were not pregnant for the year prior to the experiment were randomly assigned to one of two treatment groups; control mares or treatment mares. The control mares received no injection and the treatment mares received an intramuscular injection of 1000 I.U. hCG on days 3, 4 and 5 post estrus.

The mares were checked for estrus once daily by teasing with a stallion and when found to be in estrus were artificially inseminated every other day until the cessation of estrus.

Daily blood samples for progesterone analysis were taken via jugular venipuncture. Serial blood samples for LH analysis were collected at 12 minute intervals for an eight hour time period using an indwelling jugular catheter on days 4 or 5 and days 12 or 13 post estrus. Serum was stored
at -20 C until assayed. Progesterone (Niswender, 1973) and LH (Niswender et al., 1969) were determined by radioimmunassay. Characteristics of pulsatile LH release were determined by the cycle detection method described by Clifton and Steiner (1983).

Multiple linear regressions were used to determine differences between different hormone treatments in vitro, to detect differences between the different levels of the hormone treatment an analysis of variance was performed as described by Iman and Conover (1983). For the in vivo experiments an analysis of variance was used to compare progesterone values between treatment groups, on a day to day basis. An analysis of variance was also conducted to detect differences in LH parameters between treatment groups (Iman and Conover).

**Results**

Experiment one

There appears to be at least two populations of steroidogenic cells in the equine corpus luteum (fig. 1) these populations are separated on the basis of cell diameter only. Whether other differences exist needs to be determined by future studies.

In suspended cell incubations LH and hCG significantly (p<0.01) stimulated progesterone production at
Figure 1. Relative distribution of luteal cells based on cell diameters.
Figure 2. Progesterone secretion by luteal cells in suspended cell incubations. Dose responses to hCG, LH and PMSG.
Table 1. Progesterone production by suspended cells in response to LH challenge

<table>
<thead>
<tr>
<th>Dosage (mmol/tube)</th>
<th>Progesterone (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0 x 10⁻⁶</td>
<td>2.96a</td>
</tr>
<tr>
<td>3.0 x 10⁻⁷</td>
<td>2.40b</td>
</tr>
<tr>
<td>3.0 x 10⁻⁸</td>
<td>1.70c</td>
</tr>
<tr>
<td>3.0 x 10⁻⁹</td>
<td>1.40d</td>
</tr>
<tr>
<td>3.0 x 10⁻¹⁰</td>
<td>.60e</td>
</tr>
<tr>
<td>0.0</td>
<td>.20f</td>
</tr>
</tbody>
</table>

a, b, c, d, e, f values differ at p<0.05

Table 2. Progesterone production by suspended cells in response to hCG challenge

<table>
<thead>
<tr>
<th>Dosage (mmol/tube)</th>
<th>Progesterone (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.0 x 10⁻⁷</td>
<td>4.7a</td>
</tr>
<tr>
<td>9.0 x 10⁻⁸</td>
<td>3.8b</td>
</tr>
<tr>
<td>9.0 x 10⁻⁹</td>
<td>2.6c</td>
</tr>
<tr>
<td>9.0 x 10⁻¹⁰</td>
<td>2.0c,d</td>
</tr>
<tr>
<td>9.0 x 10⁻¹¹</td>
<td>1.6d</td>
</tr>
<tr>
<td>0.0</td>
<td>.2e</td>
</tr>
</tbody>
</table>

a, b, c, d, e values differ at p<0.05
Table 3. Progesterone production by suspended cells in response to PMSG challenge

<table>
<thead>
<tr>
<th>Dosage (mmol/tube)</th>
<th>Progesterone (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.0 x 10^-6</td>
<td>.18</td>
</tr>
<tr>
<td>4.0 x 10^-7</td>
<td>.20</td>
</tr>
<tr>
<td>4.0 x 10^-8</td>
<td>.17</td>
</tr>
<tr>
<td>4.0 x 10^-9</td>
<td>.15</td>
</tr>
<tr>
<td>4.0 x 10^-10</td>
<td>.14</td>
</tr>
<tr>
<td>0.0</td>
<td>.20</td>
</tr>
</tbody>
</table>

Table 4. Progesterone production of cultured cells in response to LH challenge

<table>
<thead>
<tr>
<th>Dosage (mmol/tube)</th>
<th>Progesterone (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0 x 10^-6</td>
<td>1.3a</td>
</tr>
<tr>
<td>3.0 x 10^-7</td>
<td>.8b</td>
</tr>
<tr>
<td>3.0 x 10^-8</td>
<td>.7c</td>
</tr>
<tr>
<td>3.0 x 10^-9</td>
<td>.7c</td>
</tr>
<tr>
<td>3.0 x 10^-10</td>
<td>.3d</td>
</tr>
<tr>
<td>0.0</td>
<td>.1e</td>
</tr>
</tbody>
</table>

a,b,c,d,e values differ p<.05
all levels of treatment (fig. 2). At all levels of LH there was a significant (p<0.05) increase over the lower dosage (table 1). hCG followed a similar pattern except that a dose of $9.0 \times 10^{-9}$ mmol. did not differ from a dose of $9.0 \times 10^{-10}$ mmol., and this dose did differ from a dose of $9.0 \times 10^{-11}$ mmol. Over the range of doses, hCG was more effective on a millimolar basis in stimulating progesterone release (fig. 2). There were no levels of PMSG which resulted in a significant increase in progesterone production over controls (table 3).

Two hour hormone challenges to attached cells in culture plates showed responses similar to those with suspended cell incubations (fig. 3, tables 4, 5 and 6) except responses were lower at all levels of treatment.

Experiment two

hCG significantly (p<0.10) increased progesterone secretion in all treated mares for days 4 through 14, progesterone levels remained elevated over days 15 to 30 in those mares that conceived (fig. 4).

The levels of progesterone for the control group (fig. 4) are similar to those reported by other investigators for early gestation (Ganjam et al., 1975; Holtan et al., 1975; Squires and Ginther, 1975b; Terqui and Palmer, 1979; Nett and Pickett, 1979; Kindhal et al., 1982).
Figure 3. Progesterone secretion by luteal cells attached to culture plates. Dose responses to hCG, LH and PMSG.
### Table 5. Progesterone production of cultured cells in response to hCG challenge

<table>
<thead>
<tr>
<th>Dosage (mmol/tube)</th>
<th>Progesterone (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.0 x 10^{-7}</td>
<td>1.8a</td>
</tr>
<tr>
<td>9.0 x 10^{-8}</td>
<td>.4b</td>
</tr>
<tr>
<td>9.0 x 10^{-9}</td>
<td>.3b,c</td>
</tr>
<tr>
<td>9.0 x 10^{-10}</td>
<td>.3b,c</td>
</tr>
<tr>
<td>9.0 x 10^{-11}</td>
<td>.3b,c</td>
</tr>
<tr>
<td>0.0</td>
<td>.2c</td>
</tr>
</tbody>
</table>

a, b, c values differ p<.05

### Table 6. Progesterone production by cultured cells in response to PMSG challenge

<table>
<thead>
<tr>
<th>Dosage (mmol/tube)</th>
<th>Progesterone (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.0 x 10^{-6}</td>
<td>.2</td>
</tr>
<tr>
<td>4.0 x 10^{-7}</td>
<td>.1</td>
</tr>
<tr>
<td>4.0 x 10^{-8}</td>
<td>.2</td>
</tr>
<tr>
<td>4.0 x 10^{-9}</td>
<td>.2</td>
</tr>
<tr>
<td>4.0 x 10^{-10}</td>
<td>.3</td>
</tr>
<tr>
<td>0.0</td>
<td>.2</td>
</tr>
</tbody>
</table>
Figure 4. Serum progesterone values for mares that conceived
Figure 5. Serum progesterone values for all mares for days one through fourteen.
Table 7. LH parameters for control mares during early and late luteal phase

<table>
<thead>
<tr>
<th>parameter</th>
<th>early</th>
<th>late</th>
</tr>
</thead>
<tbody>
<tr>
<td>mean (ng/ml)</td>
<td>28.6</td>
<td>22.7</td>
</tr>
<tr>
<td>amplitude (ng/ml)</td>
<td>54.5</td>
<td>53.3</td>
</tr>
<tr>
<td># of pulses/8 hr</td>
<td>5.0</td>
<td>2.6</td>
</tr>
<tr>
<td>frequency (mins)</td>
<td>1.1</td>
<td>2.9</td>
</tr>
<tr>
<td>period (mins)</td>
<td>116.0</td>
<td>211.2</td>
</tr>
</tbody>
</table>

Table 8. LH parameters for hCG treated mares during early and late luteal phase

<table>
<thead>
<tr>
<th>parameter</th>
<th>early</th>
<th>late</th>
</tr>
</thead>
<tbody>
<tr>
<td>mean (ng/ml)</td>
<td>59.4</td>
<td>33.0</td>
</tr>
<tr>
<td>amplitude (ng/ml)</td>
<td>81.6</td>
<td>53.8</td>
</tr>
<tr>
<td># of pulses/8 hr</td>
<td>4.5</td>
<td>4.8</td>
</tr>
<tr>
<td>frequency (mins)</td>
<td>1.6</td>
<td>3.1</td>
</tr>
<tr>
<td>period (mins)</td>
<td>156.0</td>
<td>190.0</td>
</tr>
</tbody>
</table>
The hCG treatment had no effect on any of the parameters of LH release tested (overall mean, number of pulses, pulse amplitude (average concentration at peak of pulse), period (average time between pulses) or frequency (average duration of pulse), table 7 and 8).

Although the hCG treatment did raise peripheral progesterone levels there was no difference in pregnancy rate. In the treatment group 2 out of 7 animals became pregnant versus 3 out of 7 in the control group.

There were no interactions detected between treatment and reproductive status (post partum vs. cycling) for either progesterone or LH values.

Discussion

On the basis of cell diameter there appear to be two distinct luteal cell populations in the mare (fig. 1). This is consistent with reports in other species such as the ewe (O'Shea et al., 1979) the sow (Lemon and Loir, 1977) and the cow (Chegnini et al., 1984). Whether, the responsiveness of these cell types to LH is different as it is in other species (Lemon and Loir, 1977; Ursely and Leymarie, 1979; Fitz et al., 1982; Rodgers et al., 1983; Hoyer et al., 1984) is unknown since mixed cell populations were incubated with LH in this study. In the present study hCG effectively stimulated progesterone production by the equine corpus luteum in vitro and in vivo. This may be due to the ability of the hCG molecule to bind to the LH
receptor on the corpus luteum which has been demonstrated in the rat (Moyle, 1980), pig (Ziecik et al., 1980), monkey (Eyester and Stouffer, 1981) and the mare (Roser et al., 1982). In vitro, hCG appears to be much more effective than LH in stimulating progesterone secretion, at least in suspended cell cultures. In cultured rat Leydig cells Segaloff et al., (1981) also found that hCG was more effective than was LH. This difference in effectiveness may be related to the rate of internalization of the two proteins. The results of Mock and Niswender (1983) demonstrated that hCG bound to the LH receptor in ovine luteal cells is internalized 50 times slower than is LH bound to the same receptor. Mock et al. (1983) also demonstrated that it is the beta subunit that is responsible for the different rates of internalization, by making different combinations of the subunits of the two molecules. The study by Niswender et al. (1985) demonstrated a difference in the rate of lateral mobility of the luteal LH receptor when bound by LH versus hCG. Presumably, this reduced lateral mobility of the LH receptor in the luteal cell membrane when bound to hCG is responsible for the decreased rate of internalization. These results taken together with the previously reported decreased rates of internalization of the hCG-LH receptor complex (Mock and Niswender 1983) indicate that a prolonged residence of the hCG-LH receptor complex in the membrane of the luteal cell may be resulting in extended steroidogenic response to hCG treatment.
It has been shown that continued occupancy of the LH receptor by hCG is required for continued activation of the adenylate cyclase system (Abramowitz and Birnbaumer, 1982) and that removal of the hormone from the receptor results in decreased adenylate cyclase activity (Amir-Zaltsman and Solomon, 1980). Bourdage et al. (1984) demonstrated that either a short pulse or constant treatment with hCG resulted in prolonged secretion of steroid, while LH only stimulated steroidogenesis briefly.

The inability of PMSG to stimulate progesterone production at any level of treatment is not surprising since equine luteal LH receptors apparently do not bind PMSG (Roser et al., 1982). The inability of PMSG to stimulate progesterone in the present study suggests that this hormone does not act through a different receptor system to stimulate progesterone secretion in the equine corpus luteum.

If in vivo hCG treatment early in the cycle did result in a conversion of small luteal cells to large luteal cells an overall increase in progesterone should occur. This would explain the elevated progesterone concentration observed in the mares given hCG since large cells have a greater basal level of progesterone secretion (Fitz et al., 1982).

Since there were no significant effects on the endogenous release of LH, the increased levels of progesterone due to the hCG treatment were not elevated enough to result in any feedback inhibition on endogenous gonadotropin release. There was no difference observed in the,
number of LH pulses in the treated mares from day 5 to day 12. It would be expected in animals which did not conceive to exhibit an increase in the number of pulses later in the cycle as they approached the preovulatory period. This has been demonstrated in the ewe and cow (Baird and Scaramuzzi, 1976; Rahe et al., 1980). Animals which did conceive would not be expected to demonstrate any changes in the number of pulses. In the control mares the number of pulses actually decreased. This could possibly be due to the sampling frequency (12 minute intervals) resulting in an underestimation of the number of pulses by missing some high frequency pulses. This has been demonstrated to occur in the ewe (Karsch et al., 1983). Another possible explanation would be that the long half life of equine LH could be masking low amplitude peaks.

Although progesterone production was enhanced there was no apparent increase in pregnancy rate with the administration of hCG, it is believed that this is not indicative of the whole population. There was only a small number of animals used in this experiment and some of these did have histories of reproductive problems.

Semen from the stallion was evaluated at every breeding and mares were inseminated with at least 3.5 million progressively motile spermatozoa. Therefore the low pregnancy rates can not be attributed to the stallion.
CHAPTER 4

GENERAL DISCUSSION

In the equine corpus luteum there are at least two distinct luteal cell populations, on the basis of cell diameter. It would be expected that these two populations probably differ from each other functionally as well, as is the case with the other domestic species. Several experiments will need to be carried out in order to confirm or dispel this hypothesis. The luteal cell populations will need to be separated from each other and comparisons made between them in terms of morphology and functionality. Ultrastructural determinations of the luteal cell types will reveal if they differ from each other in the manner as other species. Characterization of relative amounts of endoplasmic reticulum, granules and lipid droplets present, numbers of mitochondria and cytoplasmic to nuclear ratio needs to be completed. Experiments need to be conducted to measure basal progesterone production by the two luteal cell types as well as the ability of gonadotropic hormones to stimulate progesterone production by the two different luteal cell types. It would be interesting to determine whether that as with other species, large equine luteal cells produce significantly greater amounts of progesterone than
small cells and are not responsive to gonadotropic hormone stimulation whereas the small equine luteal cells are responsive to gonadotropic stimulation and basal progesterone secretion is minimal.

Also of interest would be experiments to determine if the origin of the luteal cell types is the same as in other species. This would be expected to be the case with the small equine luteal cells arising from the thecal cells and the large luteal cells originating from the granulosa cells of the preovulatory follicle.

Of much interest is the observation that human chorionic gonadotropin (hCG) is more effective in stimulating progesterone secretion by dispersed luteal cells than was luteinizing hormone (LH). It appears that, at least in the ovine species, the LH-receptor complex is internalized into the luteal cell fifty times as quickly as the hCG-receptor complex (Mock and Niswender, 1983). This delay in internalization of hCG is believed to due to an immobilization of the hormone receptor complex resulting from the binding of the hCG molecule. Unoccupied LH receptors and the LH-receptor complex appear to move at the same rate while the lateral mobility of the hCG-receptor complex is virtually undetectable. The steroidogenic response of the luteal cell is stimulated only during the time when the LH receptor is occupied by hormone. When the hormone receptor complex is internalized this ends the
stimulation for steroidogenesis. Bourdage et al. (1984) also suggested that the reason for greater responsiveness of luteal cells to hCG than LH is due to differences in hormone stimulated receptor internalization. Therefore, with the immobilization of the hormone receptor complex due to the binding of hCG, the signal for steroidogenesis is greatly prolonged and this would explain the increased biological activity of the hCG molecule. This would account for the differences seen in two hour challenges to equine luteal cells if the LH occupied receptor is internalized much more rapidly than the hCG occupied receptor in the equine species as well.

hCG, in vivo, was effective in stimulating progesterone production in mares but did not appear to have any effect on pregnancy rate. This is probably not indicative of the whole equine population, however. More experimentation needs to be conducted using many more animals before any conclusions regarding the effect of hCG on pregnancy rate can be drawn. It would be expected that hCG would lower the incidence of early embryonic death loss in abortion-prone mares. If hCG can stimulate the conversion of small luteal cells to large luteal cells as has been suggested to be the case in the ewe (Gamboni et al., 1984) this could provide the maternal corpus luteum a greater sensitivity to the luteotropic signal from the embryo for recognition of pregnancy. Also, with increased levels of progesterone, the uterine environment in which the embryo is
developing is more conducive to optimum growth. This condition results in the development of a healthier embryo and this embryo is capable of producing a stronger signal to trigger the maternal recognition of pregnancy. Therefore, with increased progesterone levels the survival rate of viable embryos is enhanced. However, under conditions of low circulating progesterone levels a uterine environment which is less than optimum is created and the resulting development of the embryo would be retarded. This underdeveloped embryo would not be capable of generating as strong a signal for maternal recognition as an embryo developing in a maternal animal with higher progesterone levels. This condition would result in decreased survival rates of viable embryos.

More experimentation is necessary before an hCG treatment regimen can be designed for use in lowering the incidence of early embryonic death loss in mares. This experiment used only one dose of hCG treatment. Experiments need to be conducted to determine the response over a wide range of dosages and days of treatment.

These experiments have demonstrated, however, that hCG can be an effective treatment for raising progesterone levels and should decrease the incidence of early embryonic death loss in some cases. This treatment also has the advantage of being less expensive than traditional treatments for mares in terms of cost and labor
involved since treatment is required only during the early stage of gestation and prolonged treatment is not necessary.
CHAPTER 5

LITERATURE CITED


