THE CHARACTERISTICS OF CORPORA LUTEA INDUCED BY

hCG IN CATTLE.

by

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ABSTRACT

The function and morphometry of human chorionic gonadotropin (hCG)-induced corpora lutea (CL) were examined. In experiment 1, ultrasonography and progesterone (P_4) were used to characterize the development and function of induced corpora lutea (CL). Between d 5 and 14, spontaneous CL were larger ($P < 0.01$) than induced CL. Among cows with induced CL only, P_4 concentration increased to reach peak concentrations on d 18 but was lower ($P < 0.01$) throughout the study period than for the spontaneous CL. Intrauterine infusion with Indomethacin, a prostaglandin synthase inhibitor, were examined in experiment 2. There were no differences ($P > 0.05$) in 13,14-dihydro-15-keto-prostaglandin $F_{2\alpha}$ (PGFM) concentrations. However, pulsatile PGFM secretion was abolished in one indomethacin treated cow sampled at 6 h intervals. Plasma P_4 and CL diameter were not different between indomethacin treated and control cows. In experiment 3, the morphometry and functional characteristics were compared. Weight of induced and spontaneous CL were not different ($P > 0.05$). Basal P_4 production was higher ($P < 0.01$) among induced than spontaneous CL. Human CG increased ($P < 0.01$) P_4 production at low but not at high concentrations. A higher ($P < 0.02$) number of large luteal cells (LLC) and a concomitant reduction in the number of small luteal cells (SLC) was observed among induced CL.

The effects of hCG on the morphometry and P_4 production ability of spontaneous CL exposed to hCG were investigated in experiment 4. Basal P_4 production was lower ($P < 0.01$) in hCG-exposed CL removed on d 12, than in the control CL. However, hCG-exposed CL removed on d 15 secreted more ($P < 0.01$) P_4 than d 12 hCG-exposed and control CL. Treatment with hCG was also associated with an increase ($P < 0.01$) in the number of LLC and a concomitant reduction in the number of SLC in hCG-exposed CL. Administration of hCG on day 7 of the

cycle was associated with alterations in follicular dynamics but did not affect cycle length (experiment 5). In conclusion, *in vivo* studies show that the induced CL are inherently subnormal in function. *In vitro* studies showed that P_4 production is significantly higher among hCG-induced and d 15 hCG-exposed spontaneous CL. Such increases in P_4 are associated with an increased differentiation of small into large luteal cells.

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GLOSSARY AND ABBREVIATIONS

3βHSD	3β-hydroxysteroid dehydrogenase	HBSS	Hank's balanced salt solutions
		hCG	Human chorionic gonadotropin
		IFN	Interferon
AI	Artificial Insemination	IP_3	Inositol 1,4,5-triphosphate
AMP	Adenosine monophosphate	IU	International units
ATP	Adenosine triphosphate	L/HDL	Low/High density lipoprotein
BSA	Bovine serum albumin	LH	Luteinizing hormone
CP	Crude protein	LHRH	Luteinizing hormone releasing hormone
CL	Corpus luteum/corpora lutea		
d	Day(s)	MHz	MegaHertz
DAG	Diacylglycerol	mRNA	Messenger ribonucleic acid
DNAase	Deoxyribonunclease	mM	Millimolar
dbcAMP	Dibutyryl 3':5'-cyclic adenosine monophosphate	$P\text{-}450_{scc}$	Cholesterol side chain cleavage (enzyme complex)
DMEM	Dulbecco's modified Eagle's medium	ng	Nanogram
		P_4	Progesterone
		PGE_2	Prostaglandin E_2
FSH	Follicle stimulating hormone	PGFM	13,14-dihydro-15-keto-$PGF_{2\alpha}$
E_2	Estradiol 17-β	$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
FBS	Fetal bovine serum	PIP_2	Phosphatidylinositol 4,5-biphosphate
GDP	Guanine diphosphate		
GTP	Guanine triphosphate		
GnRH	Gonadotropin releasing hormone	PKA(C)	Protein kinase A(C)
Gi/s	Inhibitory/stimulatory G-proteins		
h	Hour(s)		

USER DEFINED ABBREVIATIONS

CHOLE   Cholesterol
CONT   Control group of cows.
FORS   Forskolin

hCG-12/15   Cows treated with hCG on d 7 followed by enucleation of the spontaneous CL on d 12 or 15.

hCG-CONT Group of cows treated with hCG on d 7 of the cycle and allowed to retain the spontaneous CL for the duration of the cycle.

hCG-LUT   Group of cows treated with hCG on d 7 of the estrous cycle followed by luteectomy of the spontaneous CL on d 12.

INDOMETH Cows receiving *intrauterine* infusion with indomethacin, an inhibitor of prostaglandin synthase

PREGN   Pregnenolone

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DEDICATION

This thesis
is dedicated to my mother
for making the greatest sacrifice,
thus enabling me
to pursue my education
and to my father,
who set the standard and
for always believing it was possible.
Papa,
as I dot the last "i" and cross the last "t"
in this thesis,
I sadly learn you've silently
slipped into the next world.
May your dear soul
rest in eternal peace.
You'll forever
be missed.

CHAPTER 1

1.1. INTRODUCTION

The corpus luteum (CL) occupies a unique position among all known endocrine glands. It is a transient gland formed following the rupture and luteinization of the graafian follicle at the end of the follicular phase. Since its possible first identification and description by Vesalius in 1555 (Short, 1977), much attention has been paid to its role as an endocrine gland, particularly its necessity in the establishment and maintenance of pregnancy. The indispensability of the CL in the ontogeny of pregnancy has been known since early in the 20th century (Short, 1977).

The length of the obligatory requirement for the CL in the progression of pregnancy seems to vary from species to species. Among domestic ruminants, it has been shown in sheep (Casida and Warwick, 1945) that the CL must be present for at least the first 50 d of gestation after which time the placenta takes over the function of the CL. In the cow, on the other hand, the CL is required for the first 200 d of gestation (Estergreen et al., 1967). Some farm animals (e.g.. pig), however, require the presence and function of the CL for the entire duration of gestation (du Mensil du Buison and Dauzier, 1957). The CL is required because of progesterone (P_4), a steroid hormone, which it secretes. Infusion of P_4 into the ewe, cow and sow following luteectomy or surgical removal of the CL, is sufficient to prevent the loss of the pregnancy in these animals (Tanabe et al., 1968; Bindon, 1971; Ellicott and Dzuik, 1973).

It is now widely accepted that inadequate CL function is one of the major causes of infertility in domestic animals (Bulman and Lamming, 1978). Available data indicate that the losses in pregnancy, most of which occur early in gestation (Sreenan and Diskin, 1985), range from 20 to 85 % (Boyd et al., 1969; Roche et al., 1981). Such losses have been estimated to cost

farmers in the UK's dairy and beef industry nearly £ 300 million (Flint *et al.*, 1990) per annum.

Based on these observations, it is apparent that measures designed to correct aberrant CL function need to be formulated, perfected or both. The techniques that have been utilized thus far include P_4 supplementation (Loy *et al.*, 1960; Northey *et al.*, 1985), or use of gonadotropin-releasing hormone (GnRH) (Nakao *et al.*, 1983; Lewis *et al.*, 1990) and the luteotropins luteinizing hormone (LH) (Donaldson and Hansel, 1965) and human chorionic gonadotropin (hCG) (Wiltbank *et al.*, 1961; Wagner *et al.*, 1973; Holness *et al.*, 1982; Walton *et al.*, 1990). These have been administered at various times relative to breeding but have largely been characterized by considerable variability with regard to their effect on peripheral P_4 and pregnancy rate. Our recent investigations (Sianangama and Rajamahendran, 1992) suggest that hCG administration, especially when given on d 7 after breeding, increases pregnancy rates consistently and at higher rates than previously reported. In concurrence with published reports (Wiltbank *et al.*, 1961; de Los Santos-Valadez *et al.*, 1982), we observed that use of hCG is also associated with extensions in estrous cycle length among cows returning to estrus (Sianangama and Rajamahendran, 1992).

Clearly, there is need to examine the mechanisms underlying the hCG-induced increases in pregnancy rates as well as to investigate the factors predisposing the extensions in estrous cycle length among hCG-treated animals that return to estrus.

1.2. LITERATURE REVIEW

1.2.1. Corpus Luteum (CL) Formation

The CL is a transient endocrine gland that is formed from the cells of a ruptured follicle at ovulation. The theca and granulosa cells of the graafian follicle undergo a series of biochemical and morphological transformations induced by the LH surge at estrus. The process whereby cells of the follicle wall are transformed into luteal cells of the CL, is designated luteinization (Anderson and Little, 1985). It is now known that the CL consists of more than two types of cells (Hild-Petito et al., 1987). On the basis of their capability to secrete steroids, dissociated luteal cells can be separated into two sub-populations. The two sub-populations include cells that secrete steroids when cultured in-vitro (steroidogenic or steroidogenically competent cells) and those that do not (non-steroidogenic luteal cells).

The non-steroidogenic sub-population of luteal cells consists mainly of endothelial cells, pericytes, smooth muscle cells, fibrocytes, macrophages, and formed blood cells. Cytochemical techniques have also been used to demonstrate that steroidogenic luteal cells stain positive for the presence of the enzyme(s) 3ß-hydroxy Δ^5-steroid dehydrogenase (3ßHSD) while non-steroidogenic luteal cells do not. The 3ßHSD is part of an enzyme system (3ßHSD / $\Delta^{5\rightarrow4}$-isomerase) that catalyses the dehydrogenation of 3ß-hydroxy-5-ene-steroid and the subsequent isomerization of 3-oxo-5-ene-steroid (Sasano et al., 1990). The product of these physiological events is the synthesis of P_4 from its precursor pregnenolone. For this reason, localization and demonstration of this enzyme has been a routine method for distinguishing and quantifying steroidogenic luteal cells amidst other cells of the CL.

Based on size, steroidogenic cells comprise two cell populations variously referred to as

small (< 20-22 μm) and large (> 20-22 μm) luteal (lutein) cells or sometimes as thecal and granulosa luteal cells, respectively (Mossman and Duke, 1973). Generally, the origin of the large and small luteal cells is believed to be the granulosa and thecal cells, respectively. However, studies using monoclonal antibodies have suggested a cellular differentiation of small into large luteal cells (Gamboni *et al.*, 1984; Farin *et al.*, 1988) under the stimulation of circulating LH/hCG (Farin *et al.*, 1988). The culturing of dissociated luteal cells *in vitro* has confirmed earlier reports suggesting that the steroidogenic luteal cells are indeed the source of P_4 in the CL.

The steroidogenic luteal cells also differ in their ultrastructural characteristics. Large luteal cells contain numerous mitochondria and have numerous smooth endoplasmic reticula (Christensen and Gillim, 1969). The latter are mostly found at the periphery of the luteal cell. In addition, large luteal cells contain an extensive Golgi complex and electron-dense secretory granules bound to the membrane. Small luteal cells, on the other hand, contain an irregularly shaped nucleus, a small number of mitochondria, and large amounts of smooth endoplasmic reticulum. Small luteal cells are noted for not having any secretory granules and the Golgi complex is not as pronounced as observed in large luteal cells. Furthermore, small luteal cells also contain lipid droplets which have not been described in the large luteal cells.

1.2.2. Luteotropic Regulation Of CL Function

Steroidogenesis in luteal tissue of ruminant animals is directly regulated at least at three levels, namely the anterior pituitary (through its secretion of LH), the uterus (prostaglandins of uterine origin) and, during pregnancy, the conceptus elaborates luteotropic or antiluteolytic factors that influence CL function. Kaltenbach *et al.* (1968a), and Denmur *et al.* (1973), demonstrated that hypophysectomy on d 1 after a spontaneous or induced ovulation in sheep was associated

with failure of the CL to develop. Additionally, these workers reported the regression of the CL if hypophysectomy was performed 5 d after ovulation. These studies indicated the necessity for the anterior pituitary not only for normal CL function but also during CL formation as well. Thus, there is general agreement that an essential gonadotropin is LH but other products of the anterior pituitary may be necessary for proper function and lifespan of the CL. Several lines of evidence suggest a need for prolactin to ensure structural integrity of the CL (Denmur *et al.*, 1973). Other workers, however, have argued that prolactin exerts its effect only in the presence of an intact uterus (Kaltenbach *et al.*, 1968b). The elucidation of the role for prolactin in the function of the CL warrants further research.

Infusions of LH have been shown to enhance both the lifespan and function of the CL in ewes (Karsch *et al.*, 1971) and cows (Wiltbank *et al.*, 1961; Donaldson and Hansel, 1965). This luteotropic role of LH was further confirmed by *in situ* experiments showing enhanced P_4 secretion following infusions of LH (Karsch *et al.*, 1971). Evidence from *in vitro* data provide additional verification for the luteotropic influence of LH on CL function (Kaltenbach *et al.*, 1967). These workers demonstrated that LH stimulated P_4 secretion *in vitro*. Furthermore, Hansel *et al.* (1973) have shown that in the absence of LH, accomplished by administering daily injections of antiserum to LH, the CL regresses in cattle.

The molecular basis for steroidogenesis in luteal cells is a complex phenomenon, involving a number of possible pathways (Appendix 1 and 2). The synthesis and secretion of P_4 falls under two categories. The first is a basal P_4 production by which the steroid hormone is synthesized and secreted from luteal cells without external gonadotropic stimulation. Additionally, however, P_4 secretion is influenced by a number of secretogogues. Under basal (unstimulated) conditions, low density lipoproteins (LDL) serve as the primary substrate for P_4 synthesis in luteal cells. The steroidogenic cascade begins when lipoproteins bind to their specific cell surface

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receptors. The LDL-LDL receptor complex is internalized by the process of endocytosis. Cholesterol is liberated from the LDL following combining of the LDL-LDL receptor endocytotic product with lysosomes. This reversible step of cholesterol acquisition is mediated by the enzyme cholesterol esterase. Excess cholesterol is converted back to the esterified form by a process catalyzed by cholesterol ester synthase. The free cholesterol is then either stored as lipid droplets, esterified or used immediately for synthesis of other steroids.

An alternative, albeit not common, method of procuring cholesterol involves its *de novo* synthesis from acetate. Whichever method is used to acquire cholesterol, the free steroid is transported to the mitochondria wherein it is converted to pregnenolone by a process catalyzed by the enzyme cholesterol side chain cleavage (cytochrome $P-450_{scc}$). After secretion, pregnenolone is transported to the smooth endoplasmic reticulum where it is converted to P_4 through a process catalyzed by the enzyme complex 3β-hydroxysteroid dehydrogenase (3βHSD)/$\Delta^5 \rightarrow \Delta^4$ -isomerase. Progesterone is then secreted by either exocytosis or by diffusion from the cell.

Luteinizing hormone, a glycoprotein, regulates steroidogenesis by interacting with its specific receptor (Lee and Ryan, 1973) located in the cell plasma membrane of luteal cells. In almost all species examined to date, the general agreement is that small luteal cells have the most LH receptors. Large luteal cells have been reported to have a relatively fewer number of LH receptors. It is presently not clear what role, if any, these receptors play in steroidogenesis in large luteal cells. The binding of LH to its receptor activates the intramembrane enzyme adenylate cyclase (Marsh, 1975) through a process mediated by regulatory protein complexes (G-proteins) which are either stimulatory (G_s) or inhibitory (G_i). The G-proteins are heterotrimeric (α, β, and γ subunits) in structure but function as heterodimers due to the close association between the β and γ subunits. The activity of the G-proteins is guanine nucleotide binding-

dependent which, in turn, is a magnesium (Mg)-dependent step. Once activated, by binding of ligand (gonadotropin) to its receptor, GDP is released from the G-proteins. The release of GDP from the G-proteins allows GTP to bind to the sites previously occupied by GDP. After GTP binds to the G-protein, the α-subunit of the G_s is released into the surrounding plasma membrane where it activates the catalytic component of adenylate cyclase. An activated adenylate cyclase catalytic subunit stimulates the conversion of adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP).

After formation, cAMP binds to the regulatory subunit of the cAMP-dependent protein kinase (PKA) (Flockhart and Corbin, 1982) and dissociates (or activates) the catalytic subunit which, in turn, stimulates endogenous protein synthesis as well as activation of the synthesis of cholesterol esterase and P-450$_{scc}$ enzyme complex. Additionally, the activated catalytic subunit of the PKA in the cytoplasm promotes the transport of;

a) cholesterol into mitochondria,

b) pregnenolone out of the mitochondria into the smooth endoplasmic reticulum and

c) LDL into the cytosol which would serve to increase the availability of cholesterol as a substrate for pregnenolone synthesis.

The GTP bound to free α-subunit of G_s is converted to GDP by a process catalyzed by an intrinsic α-subunit-associated GTPase whose function appears to be a physiologically relevant means of timing the duration of the activation. The α-subunit-GDP complex then reassociates with the β and γ subunits to form the inactive α-β-γ trimer. Other signal transduction mechanisms mediating the actions of gonadotropins have been described and reviewed by Leung and Steele, (1992) and are shown in Appendix 2.

Human CG is a LH-like glycoprotein produced by the developing syncytiotrophoblast and is first detected in the maternal circulation approximately 9 to 12 d after conception. There are

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suggestions, however, that it my be secreted at an earlier time (d 7 post conception) than previously believed (Jones, 1976). The structure of hCG closely resembles that of LH but the former contains an extra 32 amino acids which are associated with the carbohydrate moieties on the hCG β-subunit. The α-subunits are essentially identical for the two hormones. Because of the extensive degree of glycosylation in its molecule, hCG has a much longer half-life than LH. Furthermore, it has been reported that once bound to the LH/hCG receptor, hCG is internalized at approximately 50 times slower compared to LH (Niswender et al., 1985). Thus, hCG exerts a more prolonged and intense stimulation of the cells.

The CL is under additional regulatory mechanisms during gestation. It is an established fact that the CL must function beyond its normal lifespan if the pregnancy is to be maintained. In animal species such as carnivores and marsupials gestation is as long as one estrous cycle. In livestock animals, however, gestation periods are considerably longer than an estrous cycle (Niswender and Nett, 1988). This necessitates that domestic animals develop mechanisms for protecting the CL for it to last the duration of gestation or until the luteo-placental shift. Alternatively, the luteal phase in these animals may be programmed for the duration of gestation and, thereby, animals must device mechanisms for shortening the duration of the luteal phase should pregnancy not ensue (hence the prevalence of luteolytic agents). Whichever the case may be, domestic animals produce a number of substances that are either anti-luteolytic or luteo-protective (or luteotropic). Various methods by which a conceptus prevents luteal regression are as follows:

a) synthesis of prostanglandin E_2 (PGE_2) which is known to extend luteal lifespan and function and suppresses the luteolytic effect of exogenous prostanglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) or estradiol 17-β (E_2) in cows and ewes

b) synthesis of conjugated estrogens by the porcine conceptus (Robertson and King, 1974).

Conjugated estrogens have been demonstrated to be associated with the sequestration of $PGF_{2\alpha}$ within the uterine lumen during pregnancy starting from approximately d 12 and peaking around d 25.

c) the synthesis of CG by the human syncytiotrophoblasts among women and non-human primates as early as 6 d after fertilization (Saxena *et al.*, 1974). Human CG is known for its luteotropic role.

1.2.3. Luteolytic Regulation Of CL Function

It is now generally accepted that the main luteolytic agent in most livestock animals is $PGF_{2\alpha}$ of uterine origin. Evidence to this end was provided by experiments showing that hysterectomy extends the lifespan of the CL beyond its normal duration or at least the duration of one gestation period (Loeb, 1923). Furthermore, active or passive immunization against $PGF_{2\alpha}$ in some livestock species, such as the cow (Fairclough *et al.*, 1981) and ewe (Scaramuzzi and Baird, 1976), prevents the spontaneous regression of the CL. This is in contrast with data from rhesus monkeys (Neill *et al.*, 1969) and humans (Doyle *et al.*, 1971), species among which the uterus is not required for luteal regression to occur. The mechanisms underlying the luteolytic action of $PGF_{2\alpha}$ include:

a) a rapid decrease in the blood supply to the ovary, consequently to the CL as well, (Niswender *et al.*, 1976; Nett *et al.*, 1976)

b) a decrease in the number of LH receptors and the subsequent cAMP-dependent signal transduction mechanism (Fletcher and Niswender, 1982)

c) the uncoupling of the LH receptor from the adenylate cyclase system (Fletcher and Niswender, 1982)

d) decreased utilization of lipoprotein for P_4 synthesis and suppression of luteal cholesterol synthesis (Pate and Condon, 1989)

e) reduced activity of enzymes involved in steroidogenesis (Caffrey *et al.*, 1979) and

f) changes in the membrane lipid composition and an increased membrane fluidity (Riley and Carlson, 1985).

g) direct cytotoxic effects (Silvia *et al.*, 1984)

Because utero-ovarian levels of $PGF_{2\alpha}$ increase before oxytocin pulses which occur starting on d 15 (Moore *et al.*, 1986), it has been suggested that uterine $PGF_{2\alpha}$ initiates oxytocin release from the CL. The $PGF_{2\alpha}$-induced oxytocin release is preceded by the E_2-stimulated induction of oxytocin receptor synthesis in the endometrium (McCracken *et al.*, 1984). Endogenous oxytocin then interacts with its endometrial receptor thereby stimulating $PGF_{2\alpha}$ secretion and initiating a positive feedback loop involving the ovarian oxytocin and uterine $PGF_{2\alpha}$. Luteolysis is initiated because of the counter-current transfer of $PGF_{2\alpha}$ between the ovarian artery and uterine vein, which ensures that $PGF_{2\alpha}$ reaches the ovary. McCracken and Schramm (1983) demonstrated that five, but not four, pulses of $PGF_{2\alpha}$ occurring over a 24 h period induces irreversible structural as well as functional luteal regression.

At the cellular level, the $PGF_{2\alpha}$-induced luteal regression has been shown to involve phosphoinositide metabolism (Leung *et al.*, 1986; West *et al.*, 1986) and is initiated by $PGF_{2\alpha}$ binding to its specific receptor on the large (but not small) luteal cells (Powell *et al.*, 1976). The receptor mediated binding of $PGF_{2\alpha}$ stimulates the hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP_2) resulting in the formation of inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). The release of the second messengers IP_3 and DAG causes the mobilization of calcium [Ca^{+2}] from endoplasmic reticulum as well as activation of calcium-dependent protein kinase C (PKC), respectively. The resultant effect of increased intracellular Ca^{+2} and DAG concentration

is an inhibition of the gonadotropin-sensitive adenylate cyclase response mechanism and dephosphorylation of steroidogenic enzymes and, consequently, suppression of steroidogenesis in luteal cells.

1.2.4. Inadequate CL Function

In cattle, as in many other mammals, the establishment and maintenance of early pregnancy depends on the continued secretion of P_4 by the CL. This process must continue beyond the time when luteal regression normally occurs in non-fertile reproductive cycles (Wiesak, 1989). There is ample evidence, however, suggesting that a significant portion of embryo losses in most mammalian species, is attributable to inadequacies in maternal luteal function (Lukaszewska and Hansel, 1980; Lamming et al., 1989). Inadequate CL function is characterized by estrous (reproductive) cycles of normal duration but exhibit P_4 concentrations that remain lower than threshold levels of approximately 1.0 to 1.5 ng/mL. A second type of inadequate CL function is typified by normal secretory patterns occurring during a shorter than normal estrous cycle (diZerega and Hodgen, 1981). In either case, it has been consistently shown that animals, typified by these examples, invariably lose their pregnancies prior to term (Hanly, 1961; Boyd, 1965; Short, 1979). It is estimated that losses in embryos cost the dairy and beef industry of the UK approximately £300 million per annum (Flint et al., 1990).

1.2.5. Measures For Correcting Inadequate CL Function

In order to reduce the incidence of early embryonic mortality, several corrective measures have been suggested. These include the use of P_4 supplementation from an exogenous source

(Frankel, 1903; Flint *et al.*, 1990), uterine infusion of embryonic vesicles or products thereof (Betteridge *et al.*, 1980; Beal *et al.*, 1981), or injection of luteotropic agents such as hCG (Wiltbank *et al.*, 1961; McDermott *et al.*, 1986; Price and Webb, 1989; Sianangama and Rajamahendran, 1992) or hypothalamic hormones eg. GnRH (Nakao *et al.*, 1983).

The rationale for the use of these regimens is as follows;

a) P_4 is provided from an exogenous source to compensate for the deficient endogenous P_4 production,

b) Preparations based on embryonic vesicles are infused into the uterus because embryos have been shown to secrete anti-luteolytic agents,

c) GnRH is given when hypothalamic support of the pituitary gland is believed to be inadequate

d) hCG is administered to provide a luteotropic agent in cases where luteal dysfunction is attributable to lack of adequate pituitary support, and finally

A close evaluation of the measures proposed as solutions to the problem of inadequate CL function reveals that they are characterized by inconsistent results with regard to their effect on endogenous P_4 levels as well as on pregnancy rates. When exogenous P_4 supplementation was used to correct the endogenous deficiency, some workers reported significant increases in peripheral P_4 concentrations (Northey *et al.*, 1985; Robinson *et al.*, 1989), while others failed to show this effect (Zimbelman *et al.*, 1959; Loy *et al.*, 1960; Walton *et al.*, 1990). Although uterine infusions of embryonic vesicles, or products thereof, have been shown to extend the duration of the luteal phase (Northey and French, 1980; Betteridge *et al.*, 1980), the adaptation of such a technique for practical farm use has its limitations and awaits further research. Similarly, the use of GnRH or its analogues have produced variable results (Nakao *et al.*, 1983; Lewis *et al.*, 1990).

A more viable method for augmenting the function of an inadequate CL has been the use

of the luteotropin hCG. In the manner it has been utilized in the past, even this method has been characterized by very inconsistent results. Significant increases in peripheral P_4 concentrations were reported in certain cases (McDermott *et al.*, 1986; Bennett *et al.*, 1989) but not in others (de Los Santos-Valadez *et al.*, 1982).

1.2.6. Model Technique For Improving Pregnancy Rates

In our previous experiments (Rajamahendran and Sianangama, 1992; Sianangama and Rajamahendran, 1992; Sianangama and Rajamahendran, 1992), we have described a model for supplementing endogenous P_4 production. These experiments considered administering hCG either at the time of breeding by artificial insemination (AI) at estrus (d 0), or on d 7 or 14 after breeding. The timing of giving the treatments was ascertained from earlier studies (Taylor and Rajamahendran, 1990) that revealed dominant (ovulatory size) follicles to be present at these specific times. The main objective was to attempt to ovulate (d 7 and 14) or ensure the ovulation of the follicle (d 0).

The highest number of induced ovulations and resultant pregnancy rates was recorded among animals receiving hCG on d 7 after breeding (Rajamahendran and Sianangama, 1992). This study provided further understanding of the mechanism of action following administration of hCG. This mechanism was found to be the induction of ovulation of the dominant follicle(s) present at the time of administering hCG (Rajamahendran and Sianangama, 1992).

Undoubtedly due to the preciseness in the timing of the hCG injections with respect to the developmental and physiological competence of the follicles, the resultant pregnancy rates were much higher than any reported previously. The d 7 follicle is reported to possess more LH receptors than other follicles that grow during the luteal phase (Ireland and Roche, 1983) and

attain maximum diameter between d 7 to 8 (Taylor and Rajamahendran, 1990). Therefore, it is more appropriate to inject hCG at a time when the follicle is at the confluence of physiological and morphological development. Being an *in vivo* experiment, we could not determine the functional status of hCG-induced CL. The ultrasonographic evidence (Rajamahendran and Sianangama, 1992) on the emergence and development of the induced structures was the first report on CL induced by treatment with hCG. That study revealed that the hCG-induced CL grew to a size indistinguishable from spontaneously derived CL and were still detectable as late as d 90 of gestation. Other studies (de Los Santos-Valadez *et al.*, 1982; McDermott *et al.*, 1986) allude only to peripheral concentration of P_4, which obviously does not permit one to separate the function of the induced structures from that of spontaneous CL which were present at the time of administering the treatments.

However, there is tendency for a delay in return to estrus among cows that either do not conceive or that fail to maintain pregnancy following treatment with hCG. Evidence indicates that as much as 60% (vs 24% in control cows) of cows treated with hCG exhibit extended estrous cycles (de Los Santos-Valadez *et al.*, 1982). It is estimated that the extensions in estrous cycle length range from 6 to 15 d (Wiltbank *et al.*, 1961; Morris *et al.*, 1976). The main causes of such extensions have never been investigated. Possible mechanisms by which estrous cycles are extended include a direct effect of hCG on the spontaneous CL present at the time of hCG administration (Wiltbank *et al.*, 1961; Donaldson and Hansel, 1965), or alterations in follicular dynamics (Sianangama and Rajamahendran, 1991). Additionally, estrous cycles can be extended by factors of embryonic origin (Northey and French, 1980). Clearly, extensions in estrous cycles are undesirable when associated with a corrective measure intended to reduce production costs incurred by farmers.

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1.2.7. *Rationale For The Proposed Studies*

Despite the observed increase in pregnancy rates following use of hCG at d 7 after breeding, the precise mechanism by which this is achieved remains largely inconclusive. Possible mechanisms include an increase in P_4 concentration due to 1) the stimulation of the spontaneous CL, 2) formation of accessory CL by ovulating dominant follicles present at the time of hCG administration, or 3) a combination of both mechanisms. Therefore, the objectives of the studies reported herein are as follows:

a) use an *in vivo* experiment to evaluate the development and function of CL induced by hCG given on d 7 of the estrous cycle in cows (Chapter 2).

b) to investigate what influence $PGF_{2\alpha}$ has on the development and function of hCG-induced CL (Chapter 3).

c) to use an *in vitro* system to characterize the function of both hCG-exposed spontaneous CL and CL induced by hCG given on d 7 of the cycle (Chapter 4 & 5)

d) to investigate the cytological effects of hCG on spontaneous CL as well as on CL induced by hCG given on d 7 of the cycle (Chapter 4 & 5).

Also, since the main objective of corrective measures is to increase the net income of farmers by reducing losses incurred through reproductive inefficiency, it is important that the causes of the extensions in estrous cycle length observed following use of hCG be determined. Therefore an additional objective of this investigation was

e) to examine the effect of hCG given on d 7 on follicular dynamics in noninseminated dairy cows (Chapter 6).

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CHAPTER 2

The Development And Function Of Corpora Lutea Induced By Human Chorionic Gonadotropin Given On Day 7 Of The Cycle In Dairy Cattle.

2.1. ABSTRACT

Ultrasonography and plasma progesterone (P_4) were used to characterize the development and function of corpora lutea (CL) induced by human chorionic gonadotropin (hCG) given on d 7 of the bovine estrous cycle (estrus = d 0). Eighteen lactating and regularly cycling Holstein cows were randomly assigned to serve as control (CONT, n = 6) or to receive hCG on d 7 with (hCG-LUT, n = 6) or without (hCG-CONT, n = 6) surgical removal of the spontaneous CL on d 12. Accessory CL were induced in all treated cows except one in the hCG-CONT group. Spontaneous CL diameter in all treatment groups was not different ($P > 0.05$) prior to d 12. Similarly, spontaneous CL diameter in CONT and hCG-CONT was similar ($P > 0.05$) during the remainder of the cycle. The diameter of induced and spontaneous CL of similar age did not differ between d 1 to 4, inclusive ($P > 0.05$). At d 5, CONT CL (29.3 ± 1.4 mm) were larger ($P < 0.04$) than hCG-LUT (24.5 ± 1.5 mm) or hCG-CONT (24.6 ± 1.7 mm). Similarly, CL diameter for hCG-LUT and hCG-CONT were smaller ($P < 0.01$) than CONT CL between d 10 to 14, inclusive. On d 14 of the cycle, P_4 concentration among hCG-LUT cows decreased ($P < 0.01$) to 1.1 ± 0.9 ng/mL but had increased to 3.1 ± 0.9 ng/mL by d 18. Corresponding values for hCG-CONT and CONT were 5.8 ± 0.8 and 4.2 ± 0.8 ng/mL and 4.5 ± 0.8 and 5.5 ± 0.8 ng/mL, respectively. The onset of both functional and structural regression of CL as well as estrous cycle length were similar ($P > 0.05$) for all treatment groups. These results demonstrate that hCG-induced CL are functional but appear to be smaller and secrete less progesterone compared to spontaneous CL of similar age.

2.2. INTRODUCTION .

A significant proportion of infertility in cattle has been attributed to an inadequate function of the CL (Wiebold, 1988; Lamming et al., 1989). Typically, inadequate CL function, also called luteal phase insufficiency or luteal dysfunction, is characterized by an estrous cycle of normal duration and low concentrations of peripheral progesterone (P_4) (diZerega and Hodgen, 1981; Lamming et al., 1989) or a shorter estrous cycle, with only a transient increase in P_4 concentration (Lamming et al., 1981). Henricks et al. (1971) demonstrated that cows that were bred by AI, but did not maintain their pregnancies, had lower peripheral P_4 concentrations between d 10 and 16, inclusive, than pregnant cows during the same time period. Similarly, Lamming et al. (1989) found lower milk P_4 concentrations during the same period among cows that did not establish pregnancies than for cows that established and maintained pregnancy.

Several measures have been developed to correct inadequate CL function, including the use of P_4 supplements from various exogenous sources (Robinson et al., 1989) or GnRH (Nakao et al., 1983) administered at various times following breeding. Results of those studies (Nakao et al., 1983; Robinson et al., 1989) remain inconsistent with regard to effect on pregnancy rates. Another method designed to augment the function of an inadequate CL function in cattle has been the use of gonadotropic hormones such as LH (Donaldson and Hansel, 1965) or hCG (Wiltbank et al., 1961; Holness et al., 1982; Rajamahendran and Sianangama, 1992) at various times following breeding. The use of hCG in cattle improved pregnancy rates in certain studies (Wiltbank et al., 1961; Holness et al., 1982; Sianangama and Rajamahendran, 1992), but not in others (Wagner et al., 1973; de Los Santos-Valadez et al., 1982; McDermott et al., 1986).

Despite the observed improvement in pregnancy rates following use of hCG, the precise mechanism by which such increases in pregnancy rates are realized remains largely inconclusive. Possible mechanisms of increases in pregnancy rates include the increased P_4 concentrations

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resulting from the stimulation of the spontaneous CL (Donaldson and Hansel, 1965; Wiltbank *et al.*, 1961), formation of accessory CL by ovulating the dominant follicle present at the time of administering hCG (de Los Santos-Valadez *et al.*, 1982; Price and Webb, 1989; Rajamahendran and Sianangama, 1992), or both.

The induction of ovulation and the subsequent formation of accessory CL has been confirmed by several researchers using different techniques, namely rectal palpation (de Los Santos-Valadez *et al.*, 1982), rectal palpation and examination of caesarian section material at term (Greve and Lehn-Jensen, 1982), laparoscopy (Price and Webb, 1989), and ultrasonographic imaging (Rajamahendran and Sianangama, 1992). The main rationale for use of hCG in cattle is to stimulate the spontaneous CL (Donaldson and Hansel, 1965; Wiltbank *et al.*, 1961), to induce formation of accessory CL (Price and Webb, 1989; Rajamahendran and Sianangama, 1992), or both. The overall effect of stimulating either a spontaneous CL or the development of accessory CL is increased P_4 concentrations. Reports on the effect of hCG on systemic P_4 concentrations vary considerably. Although several laboratories have reported significant increases in P_4 concentrations (McDermott *et al.*, 1986; Bennett *et al.*, 1989; Walton *et al.*, 1990), several other researchers could not demonstrate a similar effect of hCG on P_4 concentrations (de Los Santos-Valadez *et al.*, 1982; Sianangama and Rajamahendran, 1992). de Los Santos-Valadez *et al.* (1982) found that cows that developed accessory CL did not have higher progesterone concentrations compared with hCG-treated cows without accessory CL. Collectively, these data suggest that the hCG-induced accessory CL are either nonfunctional or subnormal in function. The objectives of this study, therefore, were to use an *in vivo* model to: 1) characterize the growth of accessory corpora lutea induced by hCG given on d 7 of the estrous cycle and 2) establish their functional characteristics.

2.3. MATERIALS AND METHODS

2.3.1. Cows

Eighteen lactating and regularly cycling cows (2½ to 5 yr old), approximately 60 to 90 d *postpartum*, were randomly selected from the dairy herd at the South Campus Teaching and Research Facility of the University of British Columbia (Vancouver, BC, Canada). The experiment was conducted between September 1992 and January 1993. During this period, ambient temperature ranged from - 5 to 15 °C. All cows received a standard dairy mixed ration consisting of lucerne cubes and barley and a Canola-based concentrate mixed in equal proportions to yield a diet of approximately 16 % CP. Rations were adjusted to meet requirements of individual cows according to production. The cows were milked twice daily between 0230 to 0530 and 1430 to 1730 h. Estrus observation was conducted at both milkings and for about 30 min midmorning. All observations were by experienced farm technicians. All animals used in this experiment and those used in subsequent experiments, were cared for according to quidelines outlined by the Canadian Council of Animal Care.

2.3.2. Treatments

At standing estrus (d 0), cows were assigned at random to serve as controls or to receive the hCG treatment. Control cows (CONT.; n = 6) received no treatment; hCG (1000 IU, APL®; Ayerst Laboratories, Montreal, PQ, Canada) was administered, intramuscularly, to the remainder of the cows on d 7. These animals were allowed to carry the spontaneous CL for the duration of the cycle (hCG-CONT; n = 6) or the spontaneous CL was removed surgically (luteectomized) on d 12 (hCG-LUT; n = 6).

2.3.3. Luteectomy

All cows in the hCG-LUT group were fasted for 12 to 16 h (starting on d 11 at 2130 h) before surgery. On the day of surgery, cows were anaesthetized using Lidocaine® HCl 2% (Langford Inc., Guelph, ON, Canada) to effect a *paralumbar* block. A flank incision was made, and the CL was located. The spontaneous CL was enucleated using gentle pressure applied to the area surrounding the gland. Care was taken to ensure that bleeding from the site of the enucleated CL had stopped before the flank incision was sutured. A single i.m. injection of antibiotic (30 ml; Ethacilin®; Rogar/STB Inc., London, ON, Canada) was administered immediately after surgery. Cows were then moved and housed in individual recovery free stalls, where they remained under continuous observation during the first 12 h, and were kept in these individual free stall pens for ≥ 3 d. Thereafter, the cows were returned to the general herd in the closed free-stall barns.

2.3.4. Ultrasonographic Examination

A real-time linear array ultrasound machine (Ultrasound scanner; model LS 300; Tokyo Keiki Company Limited, Tokyo, Japan), equipped with a 5-MHz transrectal transducer, was used to examine the ovaries as previously described (Rajamahendran and Taylor, 1990). Ultrasound imaging was conducted daily, starting at observed estrus until the subsequent observed standing estrus and ovulation signifying the end of the treatment cycle. Ovulations induced by hCG were verified as described previously (Rajamahendran and Sianangama, 1992) and defined as an acute disappearance of large follicles that were present at the time of hCG administration and the subsequent emergence of a luteal structure on a site previously occupied by the disappeared

follicles. Optimal scan images were frozen, and the diameter of the follicles, and spontaneous and accessory (induced) CL, was determined by measurement of the structures at their widest poles using a calibrated system of built-in electronic callipers. This system allowed for the measurement of structures to the nearest 1.mm. Printed copies of frozen images of the ovary were produced using a Mitsubishi Video copy processor (model P60U; Mitsubishi Electric Sales America Inc., Cypress, CA., USA) connected to the ultrasound machine.

2.3.5. Blood Samples And Radioimmunoassay

When ultrasound scanning was conducted, blood samples were collected via a *coccygeal* artery or vein using heparinized Vacutainer tubes (Becton Dickinson, Vacutainer Systems, Rutherford, NJ, USA). Blood samples were centrifuged at 4000 x g and plasma was separated within 30 min of collection. Plasma samples were stored at -20 °C until analyzed for P_4. Quantification of P_4 was achieved using a commercially available radioimmunoassay kit (Coat-A-Count®; Progesterone kit; Diagnostic Products Corporation, Los Angeles, CA, USA) previously validated to measure P_4 in bovine plasma samples (Rajamahendran and Taylor, 1990). The kit had a sensitivity of 0.05 ng/mL while the intra- and inter-assay coefficients of variation were 7.89 and 10.3 %, respectively.

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2.3.6. Statistical Analyses

For statistical analyses, data for P_4 concentrations and induced CL diameter among hCG-CONT and hCG-LUT groups were normalized such that the d of induced ovulation (disappearance of large follicle) was defined as d 1. By this transformation, the development and function of spontaneous and hCG-induced CL of similar age could be compared for all treatments. Thus, d 1 of the induced CL (d 9 of the estrous cycle) among hCG-CONT and hCG-LUT groups was compared with d 1 of the spontaneous CL among CONT cows. Spontaneous CL were compared only on nonnormalized data.

Data for CL diameter and progesterone concentration were analyzed as a split-plot experimental design with repeated measures. The model used to analyze these data included the following effects: treatment, days after estrus, cows nested within treatment, and interaction of treatment by d after estrus. The effect of cows nested within treatment was used as the error term to test the main effect treatment. When main effects were significantly different using the General Linear Models (GLM) of the Statistical Analysis System (SAS), the respective treatment means were compared using least squares ANOVA. The data for inter-estrus interval were compared using contingency tables in chi-square (χ^2). Unless stated otherwise, all data are presented as least square means ($\pm$ SEM). The same statistical models and software were used to analyze data for P_4 and CL for the nonnormalized (normal estrous cycle) data. Also, unless stated otherwise, all statistical analyses in the text refers to nonnormalized data.

2.4. RESULTS

2.4.1. Effect Of hCG On Dominant Follicles

Administration of hCG on d 7 resulted in the ovulation of large follicles (diameter = 20.0 ± 0.7 mm) in all cows in the hCG-LUT group. Ultrasound scanning conducted on d 9 after estrus (2 d after the hCG injection) revealed that all cows in the hCG-LUT group had two CL each, one having been induced by hCG. Similarly, in all but one cow in the hCG-CONT group, the large follicle (diameter = 21.0 ± 0.8 mm) that was present at the time of injection of hCG ovulated. One cow in the hCG-CONT group had four CL, two being induced by hCG. Among cows in the CONT group, none had spontaneous twin ovulations.

2.4.2. Effect Of hCG On Spontaneous CL

Treatment with hCG did not increase ($P > 0.05$) the diameter of spontaneous CL which were present at the time of treatment (Figure 2.1). The diameter of CL showed a significant ($P < 0.01$) increase over time and no interaction of treatment by time ($P > 0.05$) was detected. The diameter of spontaneous CL among CONT, hCG-CONT and hCG-LUT groups during the first 12 d of the estrous cycle (i.e. prior to luteectomy) showed no significant ($P > 0.05$) differences among treatment groups. Similarly, the diameter of spontaneous CL among CONT and hCG-CONT groups of cows did not differ ($P > 0.05$) during the experimental period.

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2.4.3. Development Of Induced (Accessory) CL

The emergence and development of spontaneous CL during the treatment cycle among CONT and of induced CL among hCG-CONT and hCG-LUT groups are shown in Figure 2.2a as well as in Plate 2. Also presented in Figure 2.2b are the same profiles, but normalized with respect to the d of spontaneous or induced ovulation. Accessory CL induced by hCG given on d 7 were detectable starting on d 9 (d 1 following induced ovulation) and were distinguishable from spontaneous CL by their relatively smaller diameter and reduced echogenicity at this stage of development. Spontaneous CL were characterized by a biphasic pattern of development during the first 15 d following formation and grew at an increasing rate of growth from d 1, when they measured 17.4 ± 1.5 mm, to reach a point of reduced rate of growth starting on d 6 (31.9 ± 1.8 mm). Minimal growth in diameter occurred between d 7 and 14 inclusive (32.6 ± 1.4 to 34.9 ± 1.6 mm, respectively). The emergence and growth of hCG-induced CL were similar to that of spontaneous CL during the first 6 d following induction of ovulation (Figure 2.2b). Induced CL among hCG-CONT and hCG-LUT groups grew from 19.4 ± 3.4 and 16.8 ± 1.2 mm on d 1 to 24.0 ± 1.1 and 27.8 ± 1.4 mm on d 6, respectively. Moderate induced CL growth was exhibited from d 7 to 9 among cows in hCG-CONT and hCG-LUT groups. Corresponding values during this period were 25.0 ± 1.5 and 26.3 ± 1.0 mm to 24.8 ± 1.0 and 28.2 ± 1.4 mm, among cows in hCG-CONT and hCG-LUT groups, respectively. After this time, all hCG-induced CL regressed.

The surgical removal of the spontaneous CL (29.7 ± 0.4 mm) appeared ($P > 0.05$) to facilitate the continued growth, albeit brief, of hCG-induced CL among cows in the hCG-LUT group (22.5 ± 0.4 mm). The corresponding average diameter of the CL among cows in the hCG-CONT group was 21.6 ± 0.5 mm. A significant ($P < 0.01$) interaction of time by treatment was detected.

Spontaneous CL among CONT cows were significantly larger ($P < 0.01$) than induced CL among cows in hCG-CONT and hCG-LUT groups of cows and this difference was mainly between d 9 to 14 following ovulation. During this period, induced CL among cows in hCG-CONT and hCG-LUT groups were similar (> 0.05).

2.4.4. Progesterone Concentrations In Plasma

Concentrations of P_4 increased steadily from d 0 to 7 in all treatment groups (Figure 2.3a). Analysis of the nonnormalized P_4 data revealed a significant ($P < 0.01$) interaction of time by treatment. However, profiles of P_4 concentrations were similar in all treatment groups until d 12. Following *luteectomy*, P_4 concentrations dropped (P < 0.01) from 8.1 ± 1.3 ng/mL to 2.6 ± 1.5 ng/mL on d 13 among cows of the hCG-LUT group. Corresponding values for cows in the CONT and hCG-CONT groups were 5.7 ± 1.1 and 5.8 ± 0.5 to 6.7 ± 1.4 and 5.1 ± 0.5 ng/mL on d 12 and 13, respectively. Concentrations of P_4 in plasma remained high until d 18 among cows in the CONT group (> 5.5 ± 2.0 ng/mL) or d 17 among cows in the hCG-CONT group (> 4.5 ± 1.8 ng/mL) and typically declined thereafter in both treatment groups. However, among cows in the hCG-LUT group, P_4 concentrations increased from 1.1 ± 0.9 ng/mL on d 14 and attained peak values (3.1 ± 0.9 ng/mL) on d 18, and P_4 concentrations in plasma declined thereafter. Corresponding values for cows in the hCG-CONT and CONT groups during the same period were 5.8 ± 0.8 and 4.2 ± 0.8 ng/mL, and 4.5 ± 0.8 and 5.5 ± 0.8 ng/mL, respectively.

When data for P_4 were normalized (Figure 2.3b) such that d 1 of the natural cycle among CONT cows was compared with d 9 among cows in the hCG-CONT and hCG-LUT groups, concentrations of P_4 were much higher ($P < 0.01$) among cows in the hCG-CONT and hCG-LUT groups between d 1 and 4. Starting at d 5, however, P_4 concentrations dropped significantly (P

< 0.01) among cows in the hCG-LUT group. Although the interaction between time and treatment was significant ($P < 0.01$), P_4 concentrations clearly were much lower ($P < 0.01$) among cows in the hCG-LUT group than those in the CONT group between d 7 and 11, inclusive, following ovulation (spontaneous or induced). Concentrations of P_4 declined after this period among cows in the hCG-CONT and hCG-LUT groups

2.4.5. The Functional And Structural Regression Of The CL

Regression of the CL in all treatment groups was defined as having started (onset) when the decrease in diameter, on a given d, was followed by two consecutive d showing a similar trend. Similarly, this process was considered to be complete when the decline in diameter (structural regression) ceased and was more often associated with concentrations of $P_4 < 0.05$ ng/mL. In cows in the CONT group, the onset of structural regression of the CL was detected on d 18.9 ± 0.6 after estrus. The hCG-induced CL among hCG-LUT group were regressing by d 17.7 ± 0.6 after estrus. Among cows in the hCG-CONT group, for which the spontaneous CL were left *in situ* until the end of the cycle, induced and spontaneous CL were regressing by d 18.2 ± 1.0 and 18.2 ± 0.6 d after estrus, respectively. Luteal regression was completed by d 22.1 ± .07, 21.6 ± 0.9 and 21.5 ± 0.6 in cows in the CONT, hCG-CONT and hCG-LUT groups, respectively. The spontaneous CL among cows in the hCG-CONT group were completely regressed by d 21.3 ± 1.0 d after estrus. Time to the onset and completion of luteal (structural) regression was not significantly different ($P > 0.05$) among treatment groups.

The onset of functional regression among cows in the CONT group was evident by d 18.0 ± 0.4 after estrus. Corresponding values for the onset of functional regression among cows in the hCG-CONT and hCG-LUT groups were 17.3 ± 0.9 and 17.8 ± 0.6 d after estrus, respectively.

Although cows in the CONT group exhibited behavioral signs of estrus by d 21.4 ± 0.3, those in the hCG-CONT and hCG-LUT groups were observed in estrus on d 21.7 ± 0.8 and 21.0 ± 1.0, respectively, after the initial estrus at the beginning of the experiment. Analysis of the data on the onset and completion of functional regression among the treatment groups revealed no significant ($P > 0.05$) differences.

2.5. DISCUSSION

Clearly, CL induced by hCG given on d 7 of the estrous cycle are functional but appear not only to be smaller, but also seem to secrete less P_4 than spontaneously developed CL of similar age. Such a finding is in contrast with results reported by Rusbridge et $al.$ (1992), who found no difference in the P_4 content of CL induced by GnRH administered on d 6 of the estrous cycle. The small size and subnormal function of hCG-induced CL could be attributed to an inadequate development of preovulatory follicles (diZerega and Hodgen, 1981; Ireland and Roche, 1983). Final maturation and development of follicles occurs during the follicular phase, a period that is characterized by low P_4 and increasing concentrations of estradiol (Ireland and Roche, 1983). This period is also characterized by a rise in concentration of FSH (d 17 to 19) and LH (after d 19) occurring several d before the LH surge. The increase in systemic concentrations of the gonadotropins is a phenomenon that may play an important role in the growth and differentiation of a preovulatory follicle (Ireland and Roche, 1983). Because none of these events has been described in cows treated with hCG or GnRH, the lack of a transient increase in gonadotropins immediately preceding ovulation could explain the smaller diameter among hCG-induced CL compared with spontaneous CL of similar age. Available data seem to suggest a need for the removal of high concentrations of P_4 in the circulatory system and an increase in

gonadotropin following luteolysis for normal follicular development to ensue. This hypothesis is supported by evidence showing that ovulation of first wave dominant follicles in heifers or cows after administration of $PGF_{2\alpha}$ early in the luteal phase (d 5 to 8) results in the formation of a CL of normal lifespan and function (Savio et al., 1990).

Because a CL is only a continuation of follicle development, the subnormal function of hCG-induced CL may be attributed to the influence of hCG on the newly developing luteal tissue. Evidence indicates that hCG influences the relative proportions of steroidogenic luteal cells constituting the CL (Farin et al., 1988; Wiesak, 1989). In studies conducted in sheep (Farin et al., 1988) and in pigs (Wiesak, 1989), hCG increased the number of large luteal cells and concurrently reduced the number of small luteal cells. Because small luteal cells possess the majority of LH/hCG receptors and are more responsive to luteotropic stimuli (Rodgers and O'Shea, 1982), reductions in the number of small luteal cells could explain the subnormal function of hCG-induced CL in the present study. Although unverified, reductions in small luteal cells lend the CL to diminished responsiveness to luteotropic stimulation in hCG-induced CL. The subnormal function in hCG-induced CL in the present experiment might also be due to the heterologous desensitization of the LH/hCG-stimulated adenylate cyclase activity (Jena and Abramowitz, 1989).

In the present study, hCG-induced CL also exhibited a compromised (shorter) lifespan, lasting about 11 to 13 d (regression occurred approximately on d 20 to 23). The hCG- induced CL were thus similar to the short-lived CL described in the first ovulatory estrus in *prepubertal* heifers (Berardinelli et al., 1979), *postpartum cows* (Corah et al., 1974), to the CL forming after early weaning in cattle (Odde et al., 1980), or to the CL induced during early *postpartum* using GnRH (Lishman et al., 1979) or hCG (Pratt et al., 1982). An explanation for the phenomenon observed in the current study might be the increase in large luteal cells (Farin et al., 1988;

Wiesak, 1989) which possess the high affinity receptors to the luteolysin $PGF_{2\alpha}$ (Fitz *et al.*, 1982). Other researchers have presented evidence *in vivo* (Howard and Britt, 1990; Rajamahendran and Calder, 1993) and *in vitro* (Rusbridge *et al.*, 1992) suggesting that hCG- or GnRH-induced CL have a luteolytic mechanism capable of responding to a luteolysin when spontaneously derived CL do not respond.

Furthermore, in previous experiments (Rajamahendran and Sianangama, 1992), we demonstrated that accessory (induced) CL, developed similarly, persisted at least until d 80 of pregnancy, when the release of the uterine luteolytic mechanism was suppressed by the establishment of pregnancy in fertile insemination. Collectively, the present and previous findings suggest a role for a uterine luteolytic mechanism as a contributing factor in the subnormal function and early demise of hCG-induced CL (see review by Hunter, (1991)).

2.6. CONCLUSIONS

Luteal structures induced by hCG given on d 7 of the cycle are smaller and are associated with lower systemic P_4 concentrations than spontaneously derived CL of similar age. Such subnormal function may be attributed to preovulatory and postovulatory causes. Questions regarding the contribution of postovulatory causes of the described subnormal luteal function should be addressed. These include the contribution of the luetolysin $PGF_{2\alpha}$.

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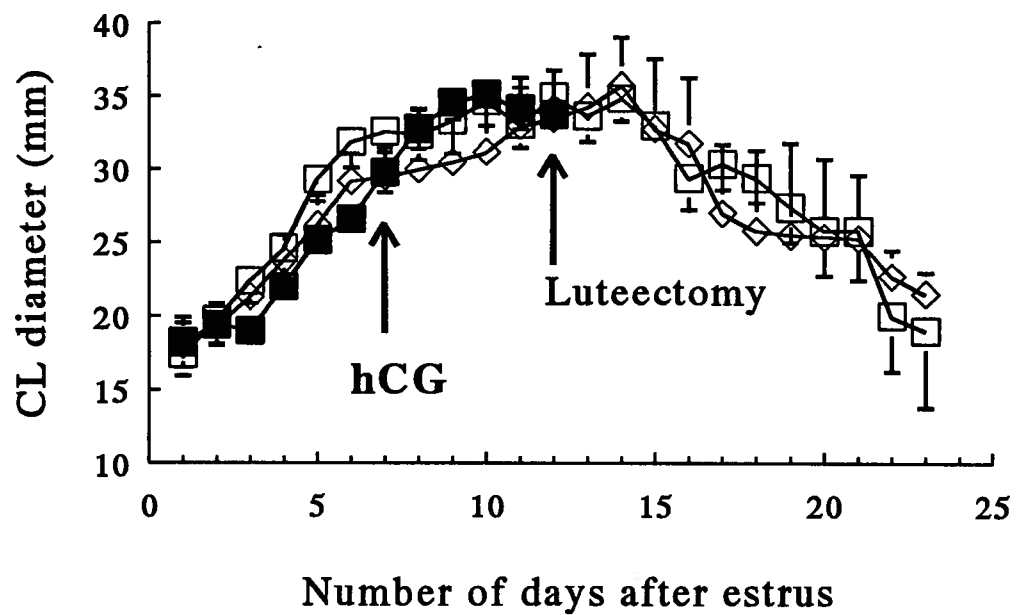
Figure 2.1. Least square means (± SEM) of spontaneous corpus luteum (CL) diameter among control (□) and cows treated with human chorionic gonadotropin (hCG) on d 7 and i) allowed to carry the spontaneous CL for the duration of the estrous cycle (◊), or ii) had the spontaneous CL removed on d 12 of the estrous cycle (■).
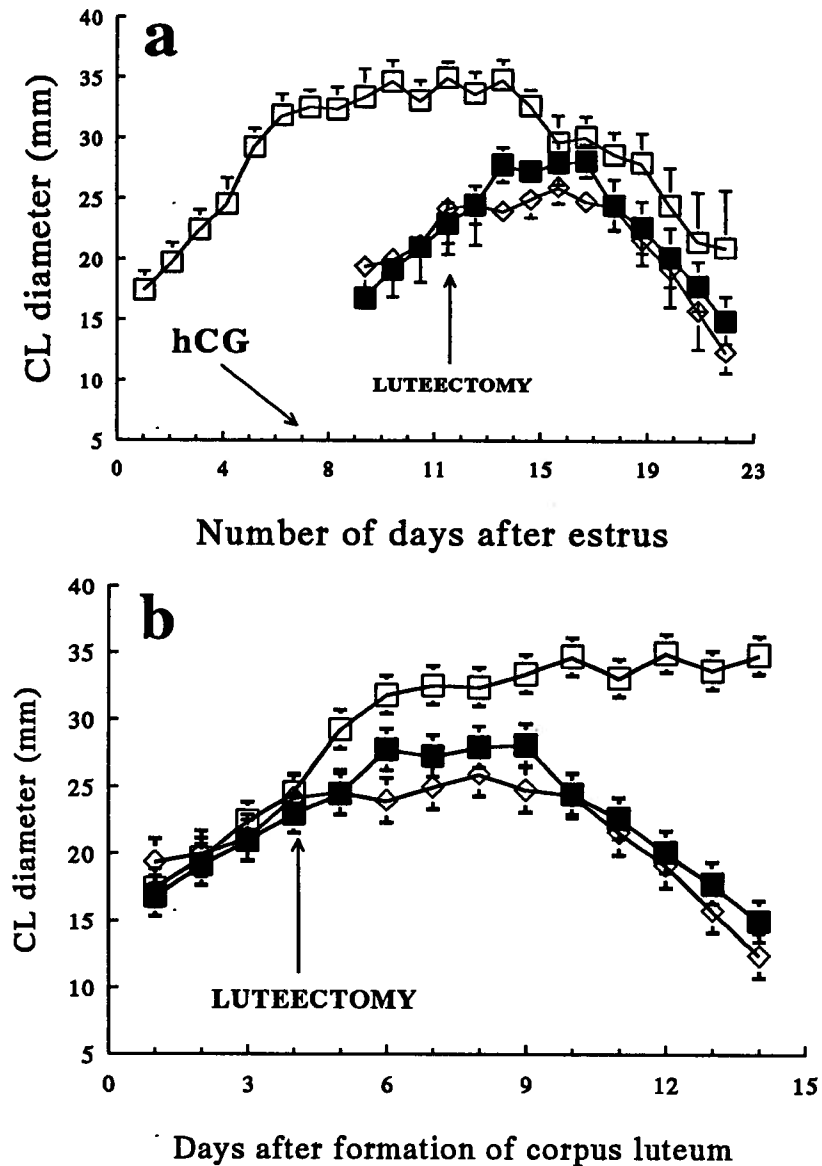
Figure 2.2. a) Least square means (± SEM) of spontaneous CL diameter among control (□) and induced CL diameter in cows treated with human chorionic gonadotropin (hCG) on d 7 followed by removal of the spontaneous CL on d 12 of the estrous cycle (■) or allowed to carry the spontaneous CL for the duration of the estrous cycle (◊). b) Least square means (± SEM) of CL diameter when normalized to the d of ovulation (spontaneous or induced) such that, among hCG-treated cows, d 9 of the estrous cycle ≡ d 1 of the normal (spontaneous) cycle among cows in the CONT group.
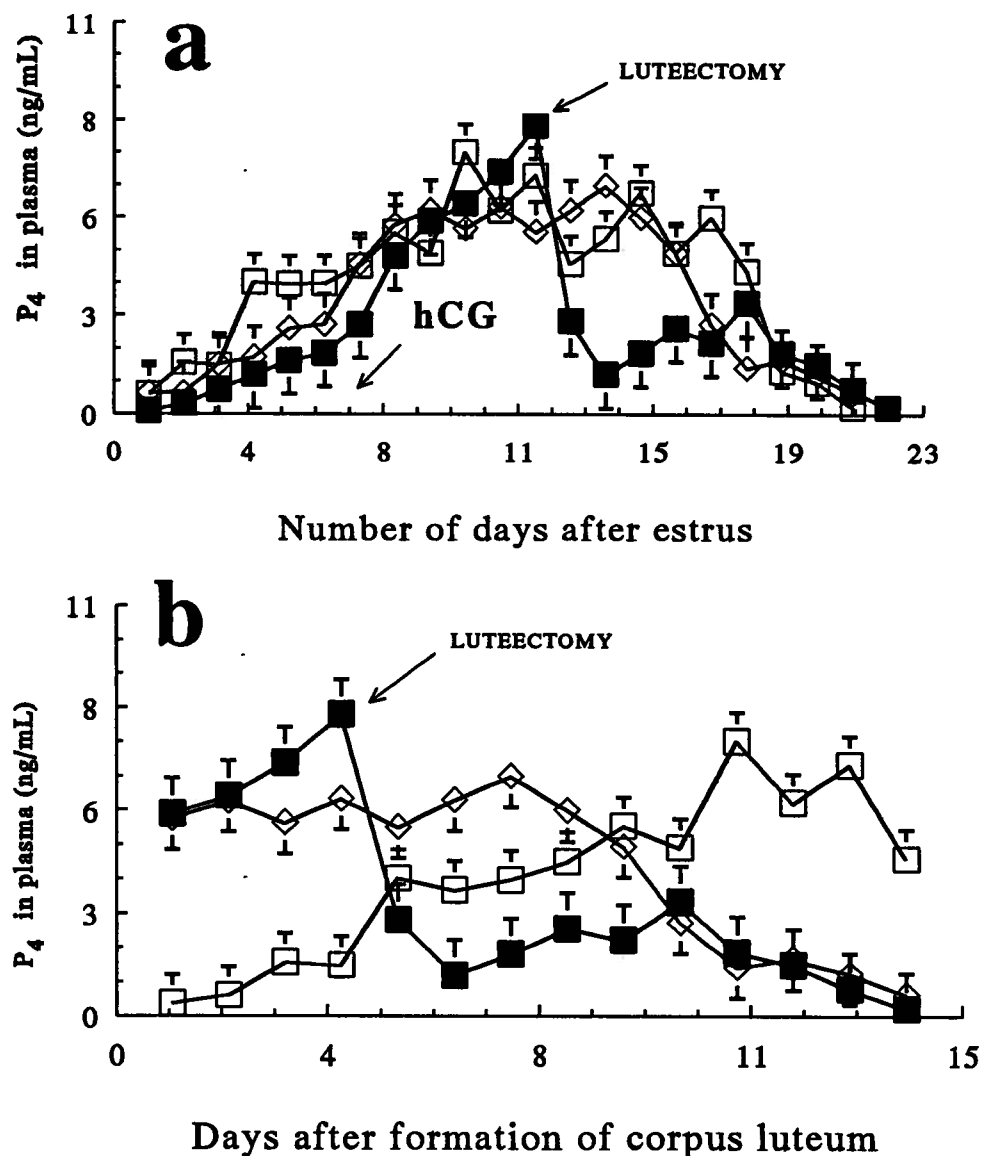
Figure 2.3. a) Least square means (± SEM) of progesterone (P₄) concentrations in control cows (□) and in cows treated with human chorionic gonadotropin (hCG) on d 7 and either allowed to carry the spontaneous corpus luteum (CL) for the duration of the cycle (◊) or removal of the spontaneous CL on d 12 of the cycle (■). b) Least square means (± SEM) of plasma progesterone when normalized to the d of ovulation (spontaneous or induced) such that, among hCG-treated cows, d 9 of the estrous cycle ≡ d 1 of the normal (spontaneous) cycle among cows in the CONT group.
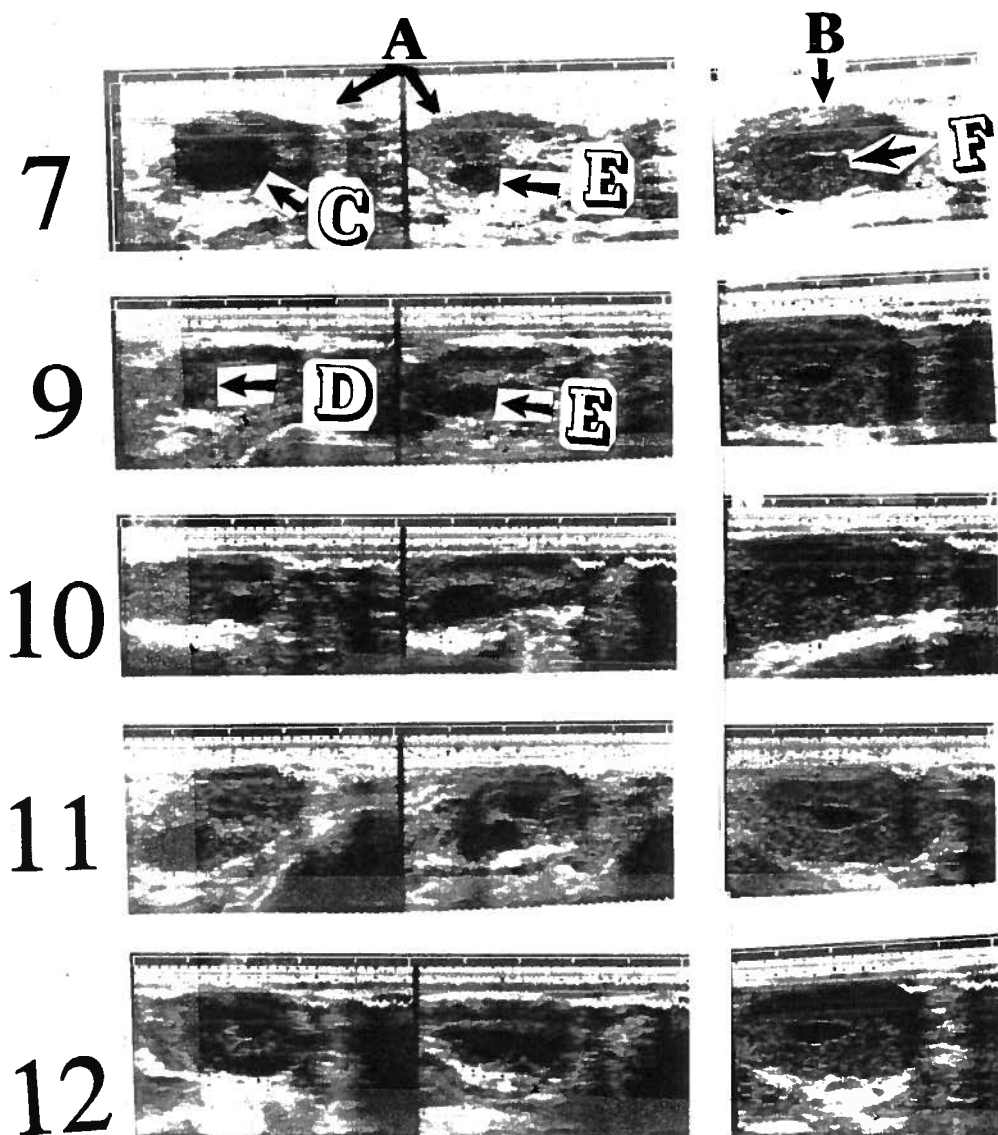
Plate 2.1. Ultrasound images of the left A) and right B) ovaries in a cow treated with human chorionic gonadotropin (hCG) on d 7 after estrus. The dominant follicle (C) present on d 7 ovulated by d 9 on which d the corpus haemorragicum was visible (D). Subordinate follicles (E), which were present when hCG was given, resumed growth following ovulation of the dominant follicle. The spontaneous CL is visible on the right ovary until d 12 (F) when luteectomy was conducted.
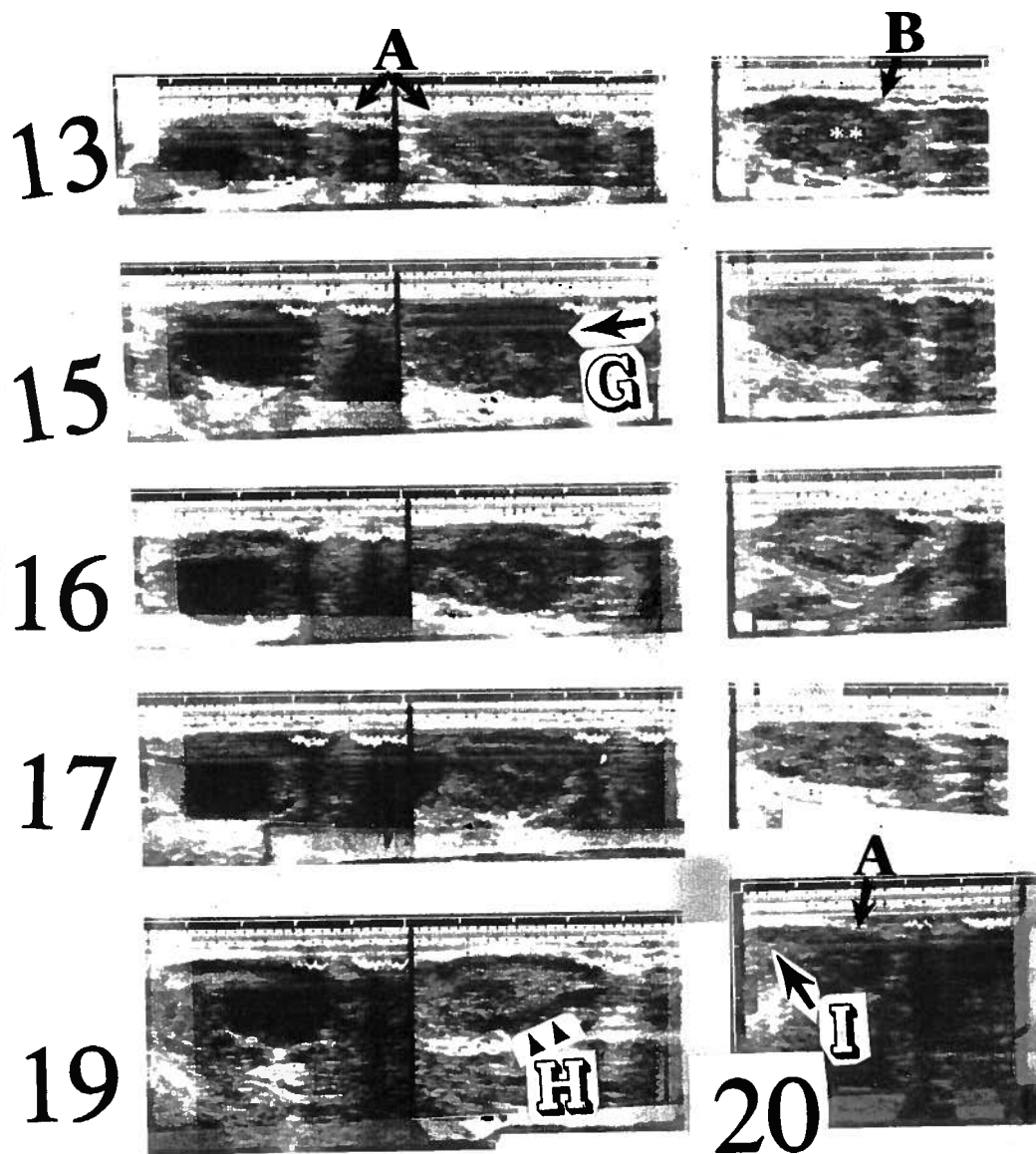
Plate 2.2. Ultrasound images of the left A) and right B) ovaries in a cow treated with human chorionic gonadotropin (hCG) on d 7 after estrus. Note the site of the enucleated spontaneous CL is identifiable on d 13 (**). The hCG-induced CL (G) was completely regressed by d 19 when a fresh pool of follicles (H) could be identified on the left ovary. Notice the continued growth of the first-wave subordinate follicle until its ovulation on d 20 (I).

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CHAPTER 3

The Influence of Prostaglandins On The Function Of Corpora Lutea Induced By Human Chorionic Gonadotropin Given On Day 7 Of The Cycle In Dairy Cattle.

3.1.   ABSTRACT

The effects of intrauterine infusions of indomethacin, an inhibitor of prostaglandin synthase, on the function and lifespan of corpora lutea (CL) induced by human chorionic gonadotropin (hCG) given on d 7 (estrus = d 0) of the cycle, were examined in 10 cyclic Holstein cows. Animals were examined by ultrasonography once daily to characterize CL and follicular dynamics. Blood samples for hormone assays were collected daily between d 9 and 23. In addition, a second sample was collected from each animal between d 13 and 18. Spontaneous CL were enucleated following flank laparotomy on d 12. Starting 24 h after surgical removal of the spontaneous CL, cows received either indomethacin (40 mg uterine/horn twice a d; INDOMETH, n = 5) or vehicle (CONTROL, n = 5). There were no differences ($P > 0.05$) in 13,14 dihydro-15 keto Prostaglandin $F_{2\alpha}$ (PGFM) concentrations between INDOMETH and CONTROL cows. However, a pulsatile PGFM secretory pattern was abolished in one INDOMETH cow sampled at 6 h intervals. Although CL development during the study period was different ($P < 0.01$), no effect of treatment ($P = 0.47$) or the interaction between time and treatment ($P = 0.96$) was detected. Plasma P_4 was similar ($P = 0.2$) between CONTROL and INDOMETH cows. However, a significant ($P = 0.01$) treatment by time interaction was detected. Both physiological and structural regression of CL were similar ($P > 0.05$) between INDOMETH and CONTROL cows. In summary, these results provide no evidence to support the hypothesis that $PGF_{2\alpha}$ causes the subnormal function and compromised lifespan of induced corpora lutea.

3.2. INTRODUCTION

It has been frequently suggested that inadequate corpus luteum (CL) function may predispose the demise of many embryos early in gestation (Lamming *et al.*, 1989). Consequently, such aberrant function has been challenged, *inter alia*, by gonadotropins given during the luteal phase to stimulate the function of spontaneous CL. Donaldson and Hansel, (1965) demonstrated that the lifespan of the CL could be extended and progesterone (P_4) concentrations increased with a single intramuscular injection of luteinizing hormone (LH) given during the luteal phase. Other workers have shown, also, that human chorionic gonadotropin (hCG), a hormone that not only shares a common receptor and signal transduction pathway but also mimics the effects of LH (Lee and Ryan, 1973), could be used to the same end. Wiltbank and co-workers, (1961) extended CL lifespan with a series of hCG injections given daily between d 14 to 34, inclusive, of the estrous cycle.

Evidence from studies based on ultrasonography (Rajamahendran and Sianangama, 1992), laparoscopy (Price and Webb, 1989) and rectal palpation (de Los Santos-Valadez *et al.*, 1982) or examination of the ovaries in animals delivering by caesarian section at term (Greve and Lehn-Jensen, 1982), clearly testifies to the induction of ovulation of follicles present at the time of administering hCG to animals. While investigating the development and functional characteristics of CL induced by hCG given on d 7 of the estrous cycle in cows, we (Sianangama *et al.*, 1994a) reported that these structures were smaller, appeared to secrete less P_4 and were short-lived compared to spontaneously derived CL of similar age. Howard and Britt, (1990) as well as Rajamahendran and Calder, (1993) have presented evidence showing that CL induced by hCG are capable of responding to a luteolytic dose of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) at a time when spontaneously derived CL remain unresponsive. Further, Rusbridge and co-workers, (1992) reported that CL induced by GnRH given on d 6 contained as much intracellular oxytocin as

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spontaneously derived CL, suggesting that components of the luteolytic mechanism are already established in these GnRH or hCG induced CL. However, the precise underlying mechanisms responsible for the subnormal function and shorter lifespan of CL induced by hCG have never been investigated. Collectively, our data (Sianangama et al., 1994a) and that of others (Howard and Britt, 1990; Rusbridge et al., 1992; Rajamahendran and Calder, 1993) enable us to propose that the diminished function and compromised lifespan of CL induced by hCG could be primarily due to the prostanoid $PGF_{2\alpha}$.

Indomethacin [1-(p-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid] is an inhibitor of prostaglandin synthesis (by inhibiting prostaglandin synthase) and has been shown to prevent estrogen-induced luteal regression in cows (Lewis and Warren, Jr, 1974). Furthermore, several workers have used indomethacin to either enhance CL function (Troxel et al., 1984) or prevent normal luteal regression (Lewis and Warren, Jr, 1977) in animals expected to have short luteal phases. Therefore, the objectives of this experiment were to:

a) use indomethacin to suppress $PGF_{2\alpha}$ production and to examine the consequences of this on (b) the development, function and lifespan of CL induced by hCG given on d 7 of the estrous cycle.

3.3. MATERIALS AND METHODS

3.3.1. Cows

Ten cyclic and nonlactating Holstein cows, ranging in age from 2½ to 5 yrs and which were approximately 60 to 90 d postpartum, were randomly selected from the dairy herd at the South Campus Teaching and Research Facility of the University of British Columbia (Vancouver,

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BC, Canada). The experiments were performed between September 1992 and December 1993 when ambient temperature ranged from -5 to 15°C. All cows received a standard dairy mixed ration consisting of lucerne cubes and barley as well as a Canola-based concentrate mixed in equal proportions to yield a diet of approximately 16 % CP. The cows were observed for estrus daily between 0230 and 0530 as well as between 1430 and 1700 h. Additionally, estrus observation was conducted for approximately 30 min during midmorning by farm technicians trained in estrus detection.

3.3.2. Treatments

The cows used in this study were selected from the general herd as they came into estrus (d 0). All cows received the hCG treatment (1000 IU APL®; Ayerst Laboratories, Montreal, PQ, Canada) as a single intramuscular injection given on d 7 after estrus.

3.3.4. Luteectomy

All cows in the INDOMETH group were exposed to a 12 to 16 h fasting period (starting on d 11 at 2130 h) prior to surgery. On the d of surgery, the cows were administered with Lidocaine® HCl 2 % (Langford Inc., Guelph, ON, Canada) to effect a *paralumbar* block. A flank incision was made and the spontaneous CL was located. The spontaneous CL was enucleated using gentle pressure applied to the area immediately surrounding the gland. Care was taken to ensure bleeding from the site of the enucleated CL had stopped before the flank incision was sutured. A single intramuscular injection of antibiotic (Ethacilin®; Rogar/STB Inc., London, ON,

Canada) was administered immediately following surgery. Cows were then moved and housed in individual recovery free stalls, where they remained under continuous observation for the first 12 h. They were kept in these individual free stalls for ≤ 4 d. Thereafter, the cows were returned to the general herd in the closed free-stall barns.

3.3.5. Ultrasonograhic Examination

A real-time linear array ultrasound scanning machine (Ultrasound scanner; model LS 300; Tokyo Keiki Company Limited, Tokyo, Japan), equipped with a 5-MHz transrectal transducer, was used to examine the ovaries as previously described (Rajamahendran and Taylor, 1990). Ultrasound imaging was conducted daily, starting at observed estrus until the subsequent observed standing estrus and ovulation.which signified the end of the treatment cycle. Ovulations induced by hCG were verified as described previously (Rajamahendran and Sianangama, 1992) and defined as an acute disappearance of large follicles that were present at the time of administering hCG and the subsequent emergence of luteal tissue on a site which was previously occupied by the disappeared follicles. Optimal scan images were frozen, and the diameters of the follicles, spontaneous and induced (accessory) CL, were determined by measurement of the structures at their widest poles using a calibrated system of built-in electronic callipers. This system of callipers allowed for the measurement of structures to the nearest 1 mm. Printed copies of frozen images were made using a Mitsubitshi Video copy processor (model P60U; Mitsubishi Electric Sales America Inc., Cypress, CA, USA) connected to the ultrasound machine.

3.3.6. Blood Sample Collection

Each cow was bled via the *coccygeal* vein or artery using Vacutainer tubes (Vacutainer Systems, Becton and Dickinson, Rutherford, NJ, USA) as described under section 2.3.5 once daily between d 9 and 23, inclusive, or until the next observed estrus and ovulation. In addition, a second blood sample was collected daily from each cow between d 13 and 18, inclusive. The two blood samples were collected approximately 12 h apart. The sampling frequency from one animal in each treatment group was increased to four samples collected 6 h apart between d 13 and 18, inclusive. Plasma samples were stored frozen at -20°C until required. Progesterone was determined only in the morning sample from each animal. Concentrations of 13,14 dihydro-15 keto prostaglandin $F_{2\alpha}$ (PGFM), a stable metabolite of $PGF_{2\alpha}$, were determined in all samples collected between d 10 and 19, inclusive.

3.3.7. Intrauterine Infusion With Indomethacin

Forty (40) mg of indomethacin (Sigma Chemical Co., St. Louis, MO) was dissolved in 400 µL of acetone and then diluted with 4.6 mL of buffer (0.2M phosphate buffer at pH 7.2) to bring the total volume to 5 mL, as previously described (Lewis and Warren, Jr, 1977). Indomethacin (40 mg) was infused twice daily (12 h apart) into each uterine horn (INDOMETH, n = 5) using an 18G Rusch catheter (Minitube of America, Inc., Madison, WI, USA). The infusions were conducted for 6 d starting on d 13 (24 h after luteectomy). Control cows received intrauterine infusion of the indomethacin solvent (acetone) and buffer (CONTROL, n = 5) as described for the indomethacin-infused cows. Each infusion, in both groups of cows, was followed by an additional 5 mL of buffer infused into each uterine horn to flush the initial treatment (indomethacin or acetone + buffer) completely through the Rusch catheter.

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3.3.8. Hormone Analyses

A commercially available radioimmunoassay kit (Coat-A-Count$^®$, Progesterone kit, Diagnostic Products Corporation [DPC] Los Angeles, CA, USA) was used to quantify P_4 as described under section 2.3.5: The intra- and interassay coefficients of variation (CV) were 7.89 and 10.3 %, respectively. The kit had a sensitivity of 0.05 ng/mL. Analysis for PGFM concentrations in individual plasma samples was performed as described in a previous protocol (Eley *et al.*, 1981) at Dr W. W. Thatcher's Laboratory at the University of Florida. Pooled PGFM plasma samples were used to calculate inter- and intraassay coefficients of variation (CV). The intraassay CV for the assays were 7.2 and 7.3 % for low (95.7 pg/mL) and high (260.5 pg/mL) pool samples, respectively. The interassay CV, for the low and high pools of samples were 1.6 and 8.7 %, respectively. The sensitivity of the assay was 25 pg PGFM/mL plasma.

3.3.9. Statistical Analyses

Duplicate estimates of plasma PGFM concentration were averaged before subjecting the data to statistical analysis. To reduce heterogeneity of variance, all data were exposed to logarithmic transformation prior to analysis. However, the data presented herein are not transformed. Due to the varying frequency of sample collection for PGFM data, the two sampling periods (morning [AM] and afternoon [PM]) were analyzed separately. Data for CL diameter, plasma P_4 (one sample per d) and PGFM concentrations were evaluated using ANOVA as a split-plot (in time) design for repeated measurements. The following effects were included in the model, viz: treatment (indomethacin vs acetone + buffer), cows nested within treatment, day of the cycle (i.e. repeated effect) and the interaction between treatment and day. In all instances,

cow nested within treatment was used as the error term to test the main effect treatment. All data analyses were done using the General Linear Models (GLM) option in the Statistical Analysis System (SAS). Differences between means, where applicable, were ascertained using least squares analysis in the GLM option of SAS. A statistical probability (P) of equal to or less than 0.05 was considered significant. Differences between means for all categorical data (Table 3.1 and 3.2) were ascertained using contingency tables in Chi-square (χ^2) analysis (GLM., SAS). A pulse was defined as a single value that was at least two standard deviations greater than the overall mean of PGFM concentration and above the preceding nadir for each sampling period (AM or PM). The peak was defined as the maximum value of each pulse and was accompanied by at least two other consecutive decreasing PGFM concentrations. The pulse frequency was the sum of all pulses occurring during a 24 h period.

3.4.   RESULTS

3.4.1.   Effect Of Indomethacin On Plasma PGFM

The effect of intrauterine infusions of indomethacin on systemic PGFM concentrations (mean ± SEM) in samples collected in the morning are presented in Figure 3.1a. There were no significant differences ($P = 0.31$) between indomethacin treated and control animals in PGFM concentrations in samples collected in the morning. Similarly, time ($P = 0.22$) and the interaction ($P = 0.93$) between d and treatment were also not significantly different.

Analysis of samples collected in the afternoon detected a slight but nonsignificant difference ($P = 0.15$; Figure 3.1b) between indomethacin infused animals compared to the control contemporaries. No differences were detected in PGFM concentrations during the period of

experimentation ($P = 0.25$) and the interaction between d and treatment was also not different ($P = 0.41$). In the two animals from which samples were collected every 6 h, the control cow (Figure 3.2a) showed several pulses of PGFM, the last one being at luteolytic concentrations. These pulses were abolished in the indomethacin-infused cow which exhibited only two pulses at luteolysis (Figure 3.2a). Interestingly, in another cow, the first PGFM peak occurred prior to the initiation of indomethacin infusions. This PGFM peak was associated with regression of CL and decrease in P_4 concentrations before d 17 of the cycle (Figure 3.2b).

An examination of the following characteristics (Table 3.1) found no significant ($P > 0.05$) difference between the two treatment groups, viz: the number of PGFM pulses occurring between d 11 to 18, inclusive, the PGFM pulse amplitude during d 11 to 12 (pre-infusion phase), d 13 to 18 (infusion phase), as well as the lowest concentrations of PGFM throughout the period of experimentation.

3.4.2. Effect Of Indomethacin On CL Growth

Profiles of CL diameter among control cows and cows infused with indomethacin are presented in Figure 3.3a. The intrauterine infusion with indomethacin did not ($P = 0.47$) enhance the emergence, growth or continued development of CL induced by hCG given on d 7. There was, however, a significant effect of d ($P < 0.01$). This effect appeared to be due to the delay in the initiation of luteal structural regression among cows infused with indomethacin. The interaction between treatment (indomethacin vs control) and d was not significant ($P = 0.96$), presumably due to the consistency in responsiveness to the effect of $PGF_{2\alpha}$ on CL development among cows which were infused with indomethacin.

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3.4.3. Effect Of Indomethacin On Plasma Progesterone

The profiles of systemic P_4 concentrations among control cows and cows that received intrauterine infusion with indomethacin are presented in Figure 3.3b. Profiles of P_4 concentrations were similar ($P = 0.2$) between controls and cows infused with indomethacin. The increases in P_4 concentrations during the experimental period were significant ($P < 0.01$). However, a significant ($P = 0.01$) treatment by d interaction was also detected. This could have been due to the slight extension in the duration of P_4 secretion as well as higher P_4 amplitude attained among cows infused with indomethacin compared to control cows (Figure 3.3b). The average peak concentrations of P_4 (Table 3.1) attained during the period of experimentation were not different ($P > 0.05$) between the two treatment groups.

3.4.4. Effect Of Indomethacin On Cycle Length

The initiation of physiological regression as well as the structural regression of the CL were delayed among cows infused with indomethacin, but not significantly different ($P > 0.05$) from that observed in control cows (Table 3.2). A similar trend ($P > 0.05$) was observed for the completion of both structural and physiological regression in the two treatment groups.

3.5. DISCUSSION

The infusion with indomethacin was expected to extend the lifespan and, consequently, the function of hCG-induced CL. The finding that the hCG-induced CL regressed despite the infusion of indomethacin was both surprising and unexpected. Although there was considerable variability in PGFM concentrations among cows, the intrauterine infusions of indomethacin failed

to suppress basal PGFM secretion. Concentrations of PGFM in the current experiment were lower among animals infused with indomethacin but not significantly different from those observed in control animals. Such a finding is in conformity with results reported by Troxel *et al.* (1984). In the second experiment of their study, both control and indomethacin-infused beef cows exhibited concomitant increases in systemic PGFM concentrations.

Although such a finding might be suggestive of lack of any inhibitory effect of indomethacin on $PGF_{2\alpha}$ secretion, evidence from two cows in our study which were bled at 6-h intervals shows that the treatment did abolish the pulsatile secretory pattern of PGFM occurring during the period of indomethacin infusion. Based on this observation, it is probable, therefore, that the lack of detecting significant differences in PGFM concentrations could have been due to several factors. Among these could be an inadequate sampling frequency employed in our study, as suggested by the profiles of PGFM in the more frequently sampled cow compared to the rest of the cows. Results previously reported by others (Kindahl et al., 1981; Thatcher *et al.*, 1984) tend to support this inference. It is noteworthy, however, that in these studies, it was imperative to detect acute responsiveness of the uterine endometrium, via its secretion of $PGF_{2\alpha}$, to exogenous challenge with estradiol-17β (E_2). Interestingly, evidence from previous studies wherein samples were collected at 3-d (Troxel *et al.*, 1984) or daily intervals (Lewis and Warren, Jr, 1977) tends to argue against an absolute requirement for a high sampling frequency in order to detect significant differences in PGFM profiles.

A second possibility might be that the dose of indomethacin infused, the timing of the infusion, or both, were inadequate to suppress uterine $PGF_{2\alpha}$ secretion as has been previously suggested (Troxel *et al.*, 1984). It is plausible that this inadequacy might be due to the suppression of only endometrial but not myometrial $PGF_{2\alpha}$ secretion. Differentially regulated $PGF_{2\alpha}$ secretory profiles have been described in cattle. While both endometrium and myometrium

contribute towards $PGF_{2\alpha}$ concentrations, only the former is reported to be under oxytocin regulation (Fuchs *et al.*, 1990). Furthermore, the contribution of the CL towards total $PGF_{2\alpha}$ concentration cannot be ruled out. Milvae and Hansel, (1983) suggested a role for locally produced $PGF_{2\alpha}$ in the regression of the CL. The precise mechanism by which indomethacin is modulating $PGF_{2\alpha}$ secretion remains unclear. Based on profiles from the cows sampled at 6-h intervals, it is, nonetheless, safe to assume that the change in the secretory pattern of PGFM was adequate in effecting our intended objective, namely suppressing (or altering) $PGF_{2\alpha}$ secretion.

The indomethacin-induced alteration in the $PGF_{2\alpha}$ secretion delayed the onset as well as completion of luteal regression. This effect of indomethacin on $PGF_{2\alpha}$ secretion was supported by the extended P_4 secretory profiles observed in cows infused with indomethacin. Although modest average peak P_4 concentrations were observed, some cows had profiles characteristic of d 7 (growing; ~7 ng/mL) CL suggesting that induced CL may be capable of secreting more P_4 than is observed in the average cow with hCG-induced CL. It is presently not clear why the induced CL appear to secrete less P_4 compared to their spontaneously derived contemporaries. Such a finding is consistent with our results reported recently (Sianangama *et al.*, 1994a). In this finding, our results differ from the *in vitro* data reported by Rusbridge *et al.* (1992) who found that CL derived in a similar manner but using GnRH, contained nearly equal amounts of luteal P_4 as did spontaneously derived CL.

The apparent inadequacy in secreting P_4 among hCG-induced CL could be due to a number of reasons. We have utilized hCG to induce the ovulation of large follicles present during the luteal phase (Rajamahendran and Sianangama, 1992). It is likely that the long half-life, and therefore long occupancy of the LH/hCG receptor, and the slow dissociation of ligand from the hormone-receptor complex (Lee and Ryan, 1973) causes refractoriness to further gonadotropic stimulation. Furthermore, the single ovulatory dose of hCG used to induce ovulations might

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potentially cause the inactivation of the gonadotropin-activated adenylate cyclase. The inactivation of adenylate cyclase has been reported in rats (Kirchick *et al.*, 1983) and rabbits (Kirchick and Birnbaumer, 1981) following use of gonadotropins. This inference is supported by the finding in this study that the size and lifespan were maintained and not different, respectively, from those of spontaneously derived CL (Sianangama *et al.*, 1994a) as well as results from the current experiment wherein use of indomethacin failed to improve the function of the hCG-induced CL.

Both the subnormal function and our finding that hCG-induced CL are also short lived might be indicative of inadequacies in the development of the ovulated follicle during the peri-ovulatory period (Ireland and Roche, 1983). Additionally, it is plausible that hCG-induced CL exhibit a shorter than normal lifespan ($\sim$ 11-13 d) due to alterations in luteal cell subpopulations. Such alterations have been reported to include a reduction in the number of small luteal cells and a concurrent increase in the number of large luteal cells (Farin *et al.*, 1988; Wiesak, 1989). Increases in the number of large luteal cells could predispose an increased sensitivity to the luteolysin $PGF_{2\alpha}$ (Niswender. *et al.*, 1985).

3.6.   CONCLUSIONS

In summary, there is no evidence in this study to support the hypothesis that the luteolysin $PGF_{2\alpha}$ causes the subnormal function and compromised lifespan exhibited by hCG-induced CL. These inadequacies may be due to factors intrinsic to the hCG-induced CL, thus *in vitro* investigations need to be conducted in order to elucidate the steroidogenic characteristics of induced CL.
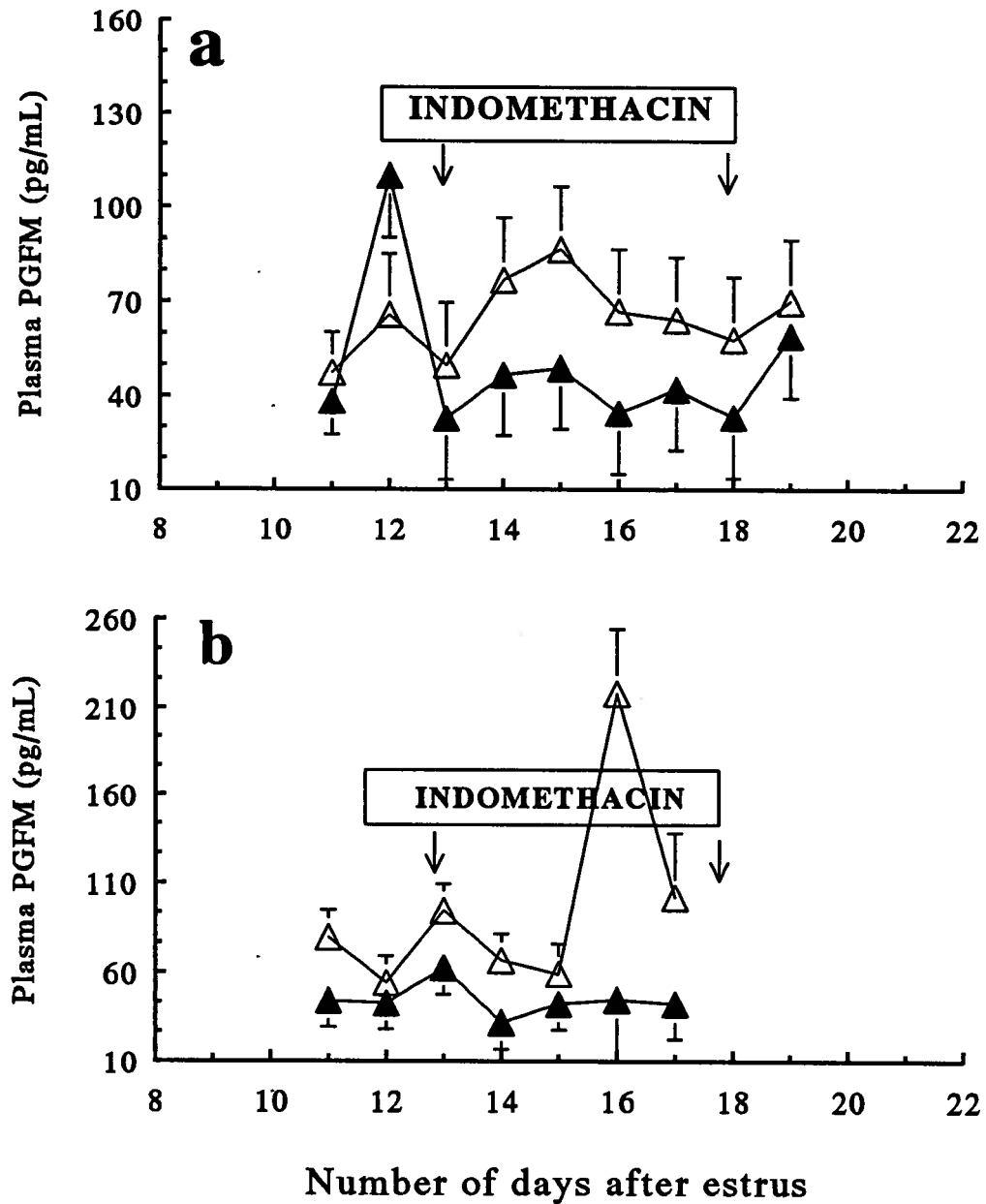
Figure 3.1. Least square means of 13, 14-dihyro-15-keto-prostaglandin $F_{2\alpha}$ (PGFM) concentrations in samples collected either in the a) morning or b) afternoon from cows infused with either indomethacin (▲) or vehicle (Δ) between d 13 and 18, inclusive. The infusions were conducted twice daily, 12 h apart. See Materials and Methods for sampling schedule.
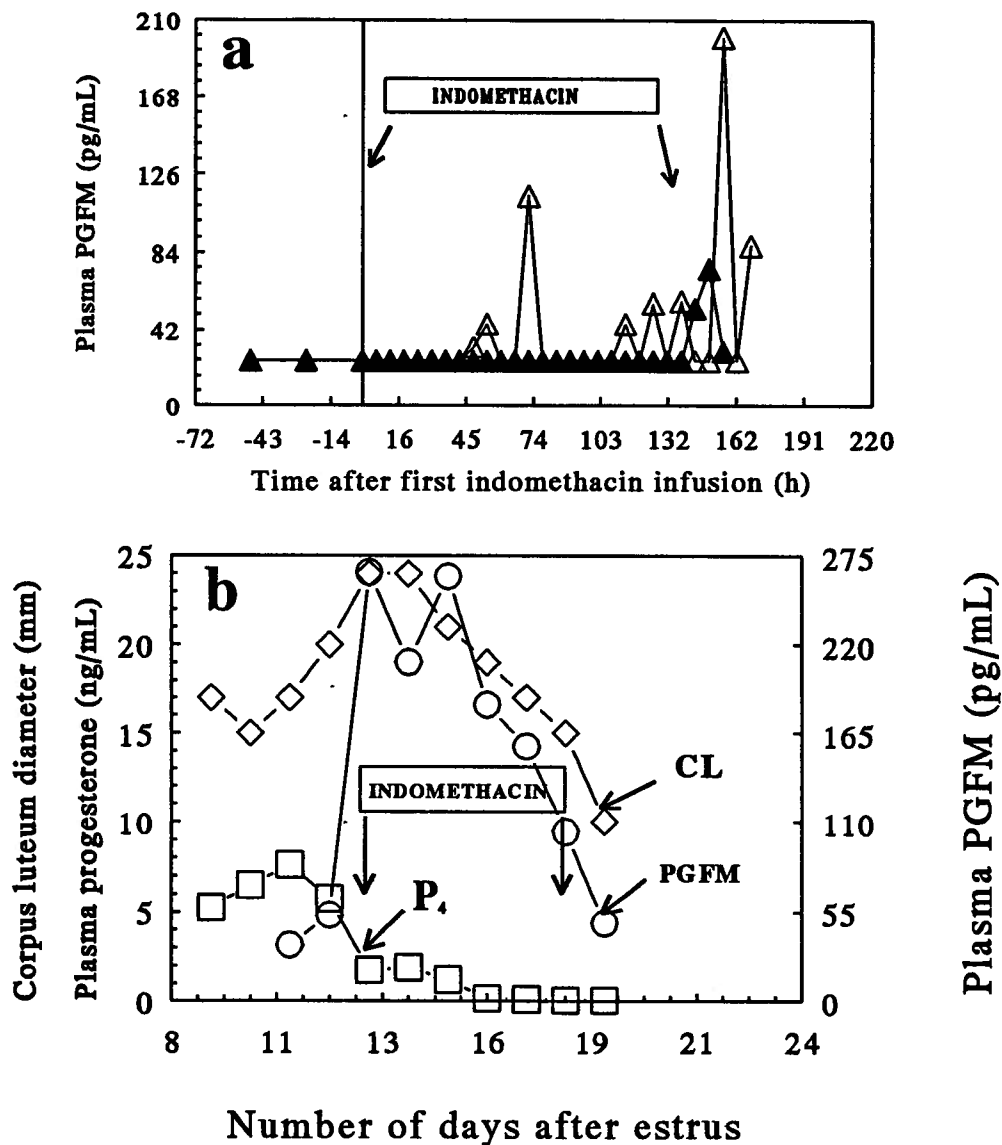
Figure 3.2. Individual profiles of plasma PGFM concentrations a) of two cows infused either with indomethacin (▲) or vehicle (Δ) at 12 h interval between d 13 to 18, inclusive. Note the absence of pulsatility in the pattern of PGFM secretion in the indomethacin infused cow (▲) compared to the cow infused with vehicle only (Δ). b) In one cow infused with indomethacin, PGFM peaks were observed prior to or coincident with the first indomethacin infusion and these were associated with regression of the CL and concomitant drop in progesterone concentrations.

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Table 3.1. Effects of intrauterine infusion with indomethacin on the number of pulses, peak pulse amplitude prior to (d 11-12), and during (d 13-18) infusion, and the lowest (basal) PGFM concentrations as well as the highest progesterone concentrations attained during the period of infusion.

Parameter	Treatment[z]	
	INDOMETHACIN	CONTROL
Number of cows	5	5
Number of pulses (d 13-18)	3.60 ± 0.51	4.75 ± 0.63
PGFM pulse amplitude (pg/mL)		
d 11-12	110.02 ± 47.76	54.65 ± 15.57
d 13-18	87.80 ± 18.65	146.85 ± 51.69
basal level	32.38 ± 4.13	46.43 ± 12.59
Peak progesterone (ng/mL)	3.91 ± 1.08	3.48 ± 0.58

[z]For all parameters examined, indomethacin vs control $P > 0.05$
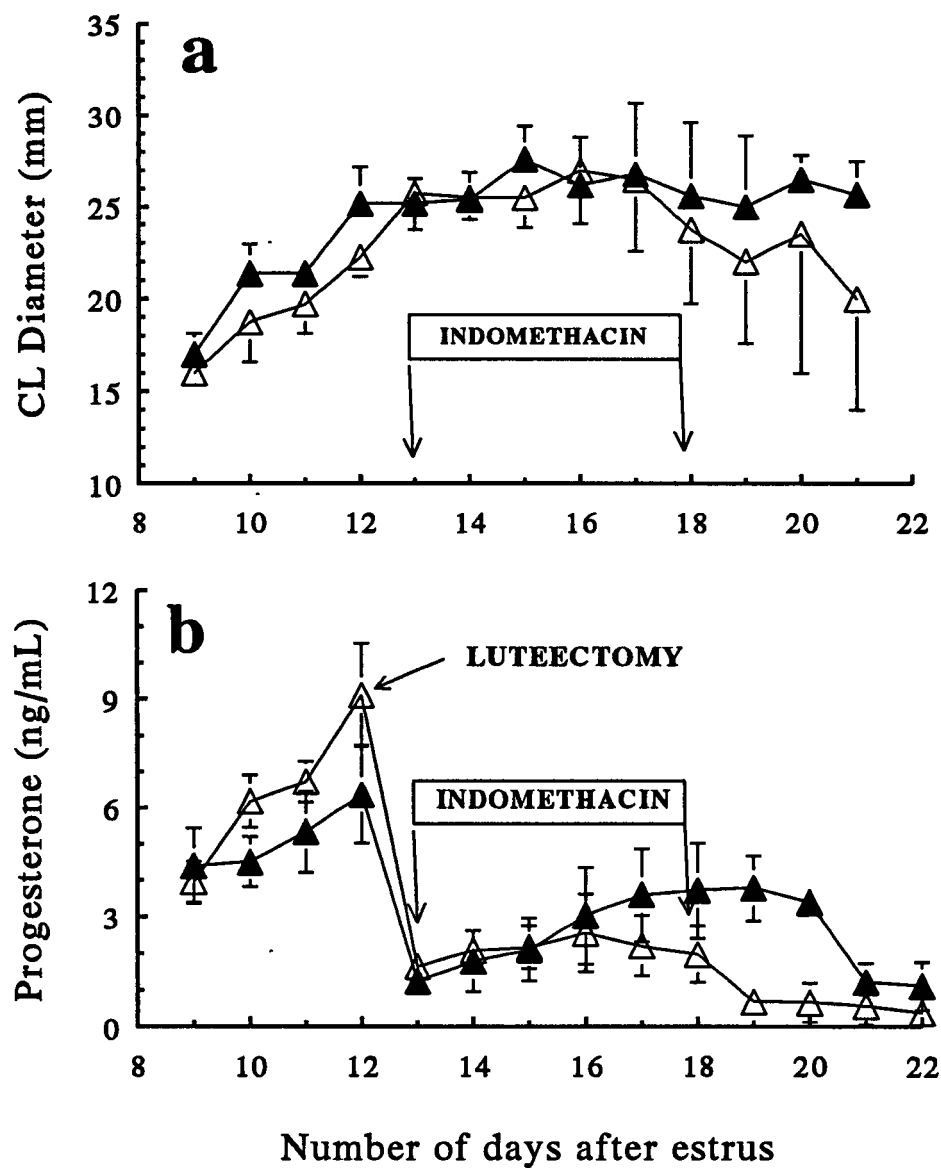
Figure 3.3. a) Least square means (± SEM) of CL diameter and b) plasma progesterone among cows infused either with indomethacin (▲) or vehicle (△) at 12 h intervals between d 13 to 18, inclusive.

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Table 3.2. Effects of intrauterine infusion with indomethacin on the structural and physiological regression of the hCG-induced corpora lutea (CL) in dairy cows.

Parameter	Treatment	
	INDOMETH	CONTROL
Number of cows	5	5
Physiological Regression*		
Initiation[z] (d)	17.0 ± 0.9	15.8 ± 0.9
completion[y] (d)	21.8 ± 1.5	18.8 ± 1.5
Structural regression*		
Initiation[x] (d) ·	17.3 ± 0.8	15.5 ± 0.8
completion[w] (d)	22.0 ± 1.4	19.0 ± 1.4

[z]P = 0.38 for indomethacin vs control cows
[y]P = 0.2 for indomethacin vs control cows
[x]P = 0.15 for indomethacin vs control cows
[w]P = 0.17 for indomethacin vs control cows
*Regression with respect to the previous estrus at the beginning of the experiment.

CHAPTER 4

The Morphometry And Function Of Corpora Lutea Induced By Human Chorionic Gonadotropin Given On Day 7 Of The Cycle In Dairy Cattle.

4.1. ABSTRACT

The cause of the subnormal function and shortened lifespan of human chorionic gonadotropin (hCG)-induced corpora lutea (CL) were examined in this study. Cows were injected with hCG on d 7 after estrus.(INDUCED, n = 6) or served as control (SPONT, n = 6). On d 7 after induced or spontaneous ovulation, CL were surgically removed and processed for morphometric evaluation or cell culture. Mixed luteal cells were cultured in the presence of hCG (0 - 5 IU/mL) for 12 or 24 h. Additionally, the maximum stimulatory dose of hCG (1 IU/mL) was used in cultures with forskolin (0 - 1 mM), dbcAMP (0 - 2 mM), cholesterol (0 - 1.25 mM) or pregnenolone (0 - 2.5 mM) for 12 h. The weight of INDUCED (4.21 ± 0.61 g) and SPONT CL (4.27 ± 0.86 g) were similar. Baseline progesterone (P_4) production was higher (P < 0.01) among INDUCED (280.3 ± 26 ng/mL) compared to SPONT CL (90.6 ± 25.8 ng/mL). However, significant CL-type by dose (P < 0.01) and CL-type by period of incubation (P < 0.01) interactions were detected. Progesterone production was dose dependently increased (P < 0.13) by forskolin among INDUCED but reduced among SPONT CL. Similarly, significant (P < 0.01) CL-type by dose interactions were detected when dbcAMP was used. Cholesterol and pregnenolone influenced (P ≤ 0.01) P_4 production among INDUCED and SPONT CL in a dose-dependent manner. A significantly higher (P < 0.01) number of large luteal cells (LLC) and a concomitant reduction in the number of small luteal cells (SLC) were observed among INDUCED

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CL. These results demonstrate that hCG-induced CL secrete more P_4 *in vitro* than do spontaneously derived CL of similar age and are associated with an increased frequency of LLC. Additionally, the data suggest an adenylate cyclase-receptor complex mediated locale for the *in vivo* subnormal function reported previously.

4.2. INTRODUCTION

Previous studies have provided evidence showing that follicular growth during each estrous cycle in cattle occurs in a wave-like pattern (Rajakoski, 1960). Use of ultrasonography confirmed the wave-like pattern and characterized either two or three successive waves of follicular growth (Rajamahendran and Walton, 1988; Savio *et al.*, 1988; Sirois and Fortune, 1988). Typically, a wave of follicular growth is characterized by the emergence and growth of a pool of small follicles. One or two of these follicles continue growing while the rest undergo the process of atresia. The largest (dominant) follicle reaches its maximum size (diameter) between d 6-8 and maintains this size until the onset of atresia during midcycle (Ginther *et al.*, 1989). That these follicles are capable of ovulating in response to exogenous human chorionic gonadotropin (hCG) has been a subject of a number of investigations (Price and Webb, 1989; Walton *et al.*, 1989; Rajamahendran and Sianangama, 1992). Collectively, these results demonstrate the potential use of hCG to ovulate dominant follicles present during the luteal phase. Our own data indicate that maximal ovulations occur when hCG is given on d 7 after breeding (Rajamahendran and Sianangama, 1992). Such a technique could be a viable tool for supplementing progesterone (P_4) concentrations and, consequently, increasing pregnancy rates in cattle (Sianangama and Rajamahendran, 1992).

Indeed, hCG has been used to augment the function of deficient corpora lutea (CL) and

results in cattle indicate improvements in pregnancy rates in some studies (Wiltbank *et al.*, 1961; Sianangama and Rajamahendran, 1992,) but not in others (Wagner *et al.*, 1973; de Los Santos-Valadez *et al.*, 1982). It has been postulated that the possible mechanisms by which such increases in pregnancy rates are achieved include the stimulation of the spontaneous CL (Wiltbank *et al.*, 1961; Donaldson and Hansel, 1965), ovulation of dominant follicles to produce accessory CL (Price and Webb, 1989; Walton *et al.*, 1990; Rajamahendran and Sianangama, 1992) or both.

The overall effect of either stimulating a spontaneous CL or the development of accessory CL would be an increase in P_4 concentrations. However, examination of published reports on the effect of hCG on P_4 concentrations reveals considerable variability. While several laboratories have reported significant increases in P_4 concentrations (McDermott *et al.*, 1986; Bennett *et al.*, 1989; Walton *et al.*, 1990), several other laboratories could not demonstrate a similar effect of hCG on P_4 concentrations (de Los Santos-Valadez *et al.*, 1982; Sianangama and Rajamahendran 1992). Furthermore, de Los Santos-Valadez and co-workers, (1982) found no difference in P_4 concentrations between animals developing induced CL compared to those failing to do so, suggesting that either induced CL were non-functional or merely subnormal in function. Recently, we (Sianangama *et al.*, 1994a) demonstrated that induced CL are functional. This study also revealed that induced CL are smaller in size and appeared to secrete less P_4 than spontaneous CL of similar age.

It is plausible that the observed subnormal function exhibited by hCG-induced CL could be due to the influence of luteolysin prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). In a more recent study (Sianangama *et al.*, 1994b), we found that reducing the concentration of $PGF_{2\alpha}$ or altering the pattern of $PGF_{2\alpha}$ secretion from the uterus by infusing indomethacin, a prostaglandin synthase inhibitor, could neither extend the lifespan nor effect any substantial improvement in the

development and function of hCG-induced CL. Since $PGF_{2\alpha}$ secretion was not completely eliminated in that study, the reported anomalous function in hCG-induced CL could be due to either an increased sensitivity to luteolysins or some inherent deficiency. The objective of this study, therefore, was to use an *in-vitro* model to establish the morphometry and steroidogenic characteristics of CL induced by hCG given on d 7 of the cycle.

4.3. MATERIALS AND METHODS

4.3.1. Materials

For cell culture experiments, Dulbecco's modified Eagle's medium (DMEM), Ca^{2+}-Mg^{2+}-free Hanks balanced salt solution (HBSS), fetal bovine serum (FBS), collagenase type IV, deoxyribonuclease (DNAase), Hepes, L-glutamine, $NaHCO_3$, antibiotic solution (Pen-Strep®) comprising Penicillin (100 IU/mL) and Streptomycin (100 μg/mL), bovine serum albumin (BSA, fraction V), N^6,2'-dibutryl 3':5'-cyclic adenosinemonophosphate (dbcAMP), forskolin, 25-hydroxycholestrol and 5-pregnen-3β-ol-20-one (pregenenolone), were purchased from Sigma Chemical Company (St. Louis, MO). Human chorionic gonadotropin (hCG, APL) was purchased from Ayerst (Montreal, PQ, Canada). Culture plates (3 mL, 24-well) were purchased from Falcon Plastics (Los Angeles, CA). Ethacilin® was bought from Rogar/STB Inc., (London, Ont, Canada). Lidocaine® HCl 2% was purchased from Langford Inc., (Guelph, Ont, Canada). All solutions were filter sterilized immediately before use for in cell culture experiments.

4.3.2. Cows

Twelve lactating and regularly cycling cows (2½ to 5 yrs old), approximately 60 to 90 d *postpartum*, were randomly selected from the dairy herd at the South Campus Teaching and Research Facility of the University of British Columbia. All animals received a standard dairy mixed ration consisting of lucerne cubes and barley, and a Canola based concentrate mixed in equal proportions to yield a diet of approximately 16 % crude protein. Rations were adjusted to meet requirements for individual animals according to production level. The cows were milked twice daily, between 0230 and 0530 and between 1430 and 1730 h. Estrus observations were conducted at milking and for about 30 min during mid-morning by experienced farm technicians.

4.3.3. Treatments And Surgery

At standing estrus (d 0), animals were assigned at random to serve either as untreated controls (SPONT, n = 6) or to receive an intramuscular (i.m.) injection of hCG (1000 IU, APL®, INDUCED, n = 6) on d 7 after estrus. Ovulations were confirmed as previously described (Rajamahendran and Sianangama, 1992). Invariably, ovulations had occurred between 36 - 48 h after administration of hCG. All animals were exposed to a 12 to 16 h fasting period (starting on d 6 (CONT) or 14 (INDUCED) at 2130 h) before surgery. On d 7 (CONT) or 15 (INDUCED) of the estrous cycle, animals were anaesthetized using Lidocaine® (2 % HCl) to effect a *paralumbar* block. A flank incision was made and the CL located. The CL was enucleated using gentle pressure applied to the area surrounding the gland. Care was taken to ensure that bleeding from the site of the enucleated CL had stopped before suturing the flank incision. A single i.m. injection of antibiotic (30 ml; Ethacilin®) was administered immediately after surgery. Animals

were then moved to individual recovery stalls where they remained under observation during the first 12 h. They were housed in these stalls for at least 3 d. Thereafter, the cows were returned to the general herd in a closed free-stall barn.

4.3.4. Dissociation Of Luteal Cells

Enucleated CL were transported to the laboratory within 5 min of collection in HBSS supplemented with antibiotics (HBSS-AB; 100 IU/mL penicillin and 100 μg/mL streptomycin) and Hepes (20 mM), $NaHCO_3$ (0.35g/L), and BSA (1 %). In the lab, CL were washed twice in fresh HBSS-AB, trimmed of the connective tissue capsule, weighed and then divided into 3 g portions. Each 3 g portion of luteal tissue was minced into small (< 2 mm) pieces using a pair of scissors, and then rinsed in HBSS-AB at least twice to remove erythrocytes and remnant connective tissue. Each minced portion was transferred to a 25 ml Erlenmeyer flask, gassed with a 5 % CO_2-balanced air, tightly capped and then placed in a humid incubator (also at 5 % CO_2). A luteal cell suspension was prepared by three sequential dissociations of the minced tissue. Each dissociation procedure was conducted for approximately 45 min at 37°C in 10 ml dissociation mixture containing collagenase (0.25 %) and DNAase (0.02 %) dissolved in HBSS supplemented with BSA (1 %).

Liberated cells were filtered through a 100 μm wire sieve and sterile gauze fitted to a cell dissociation grinder cup (Sigma Chemical Company, St Louis, MO), washed thrice in HBSS-AB and stored in 1 ml of DMEM culture media on ice until required for further use. After the third dissociation, the cells were pooled and filtered through a sterile 80-μm nylon mesh and 100 μm wire sieve fitted to the tissue grinder cup. The cell suspension was centrifuged at 2-300 x g for 5 min and the pellet resuspended in culture media. The culture medium (DMEM) was

supplemented with antibiotics (50 IU/mL penicillin and 50 μg/mL streptomycin), L-glutamine (0.584 g/L), NaHCO$_3$ (0.35 g/L) and fetal calf serum (10 %).

The viability of resuspended cells was estimated using the trypan blue (0.04 %) exclusion test. The pre- and post-incubation viability estimates ranged from 83 - 90 % and 75 - 84 %, respectively. The number of luteal cells was estimated using a haemocytometer.

4.3.5. Cell Culture And Treatments

Mixed steroidogenic luteal cells (approx. 8×10^5 to 1×10^6 per cow /well) were plated in triplicate in 24-well culture plates in 1 mL of incubation medium at 37°C in a humidified atmosphere with 5 % CO$_2$ and 95 % air. Mixed luteal cells were cultured for 12 or 24 h (hCG experiments). All treatments to the cell cultures were applied at the start of the incubation period. To investigate the responsiveness of the LH/hCG receptor to further gonadotropic stimulation *in vitro*, luteal cells were treated with hCG (hCG; 0 - 5 IU/mL). Forskolin (FORS; 0 - 1 mM) was used to investigate the physiological status of the adenylate cyclase generating system. Additional cell culture experiments were conducted using dbcAMP (0 - 2 mM) to determine whether or not the reported subnormal function was due to inadequate second messenger (cAMP) production. Also, 25-hydroxycholesterol (CHOLE; 0 - 1.25 mM) and 5-pregnen-3β-ol-20-one (PREGN; 0 - 2.5 mM) were used in separate cell culture experiments to determine whether the anomaly in P$_4$ production among INDUCED CL was mediated by inadequacies in enzymes involved in the *de novo* acquisition of cholesterol (P-450$_{scc}$) or conversion of pregnenolone to P$_4$ (3βHSD/$\Delta^{4\to5}$ isomerase), respectively. All FORS, cAMP, CHOLE, and PREGN experiments were conducted in the presence of the maximal stimulatory dose of hCG (1 IU/mL), as ascertained from previous

experiments, and were allowed to run for 12 h. Concentrations of treatments quoted above were the final concentration per well containing 1 mL of culture media and cell suspension. After incubation the media was removed, centrifuged at 1000 x g and the supernatant stored at -20°C until assayed for P_4 using a solid phase radioimmunoassay kit (Coat-a-Count®, Diagnostics Products Corporation, Los Angeles, CA).

4.3.6. Morphometric Analyses

A representative section from each CL (approx. 2-3 mm slice) was placed in 10 % phosphate buffered formalin. The tissues were embedded in paraffin wax, sectioned into thin slices (~5-6 μm) which were stained with haematoxylin and counter-stained with eosin. A video-active microcomputer planar morphometry system (Kontron, SEM-Image Processing System [IPS], Kontron Electronics Group, Munich, Germany) was used to determine the relative size (maximum and minimum diameter) as well as the area of each luteal cell type. This involved tracing the outline of each cell and the video-active system automatically calculated the cell area and diameter. Small (10.00-20.00 μm) and large (20.01- 65.00 μm) luteal cells were differentiated from each other based on their morphometric characteristics. Approximately 200 cells were counted in a total of 10 randomly selected sites on each CL at x 100 objective and oil. Only cells larger than 10 μm in diameter were included in the data analysis.

4.3.7. Statistical Analyses

Due to the heterogeneity of variance, P_4 data were exposed to log transformation prior to analysis using the General Linear Models (GLM) procedures in the Statistical Analysis System (SAS, Cary, NC.). The data for P_4 concentration in the culture medium were analyzed by ANOVA as a split-plot experimental design and included the following effect, viz; type of CL (INDUCED vs SPONT), cow nested within treatment, the dose used, and replicate as well as their higher level interaction terms. For all *in vitro* experiments with hCG, the model included the effect of period of incubation (12 vs 24 h). All morphometric data (i.e. cell area, minimum and maximum diameter of luteal cells) were analyzed by ANOVA after log transformation, to see if there were any differences between induced and spontaneously formed CL. Where main effects were indicated to be significantly ($P < 0.05$) different, appropriate means were compared using least squares analysis in the GLM procedure. Additionally, categorical data (frequency distribution of luteal cells) were analyzed using contingency tables in Chi-square (χ^2) under the PROC Frequency procedure in SAS.

4.4. RESULTS

There was no difference ($P > 0.05$) with regard to the weight of INDUCED (4.21 ± 0.61 g) compared to SPONT CL (4.27 ± 0.86 g). Baseline P_4 accumulation in culture media was significantly ($P < 0.01$, Figure 4.1a) higher among mixed luteal cells from INDUCED compared to SPONT CL. Incubation of luteal cells for 24 h was associated with a significantly ($P < 0.01$; Figure 4.1a) lower P_4 concentration among SPONT luteal cells compared to either SPONT luteal cells cultured for 12 h or INDUCED luteal cells cultured for either 12 or 24 h. Treatment with hCG maximally stimulated P_4 production by luteal cells from INDUCED CL at a dose of 1.5

IU/mL for the 12 h incubation period (Figure 4.1b; dose by period $P = 0.02$), INDUCED luteal cells cultured for 24 h were maximally stimulated at 0.5 IU hCG/mL of incubation medium (Figure 4.1c). A dose dependent response ($P < 0.01$) was observed in both types of CL. However, a significant ($P < 0.01$) dose of hCG by type of CL interaction was detected. Progesterone production was stimulated at lower concentrations of hCG (0.5 - 2 IU/mL) among INDUCED but not in SPONT CL. Addition of hCG (> 2 IU/mL) *in vitro*, dose dependently suppressed P_4 production among SPONT CL. Similarly among INDUCED CL, a dose dependent suppression of P_4 production was observed at concentrations of hCG higher than 2 IU/mL.

Treatment of mixed populations of luteal cells with FORS revealed no difference ($P > 0.05$) in the responsiveness of either INDUCED and SPONT luteal cells. Increasing the concentration of FORS did not affect ($P > 0.05$) the accumulation of P_4 in the culture medium from either luteal cell type. However, a significant type of CL by dose of FORS interaction was detected ($P < 0.01$ Figure 4.2a). Progesterone production was linearly increased by increasing doses of FORS among INDUCED CL. Among SPONT CL, a dose dependent suppression of P_4 production was observed.

When cells were challenged with dbcAMP, there was no difference ($P > 0.05$) between INDUCED and SPONT luteal cells either in their capacity to respond to low concentrations of the second messenger or to increments in the concentration of dbcAMP. A significant type of CL by dose of dbcAMP was observed ($P < 0.01$, Figure 4.2b). Progesterone concentration was initially suppressed at concentrations of dbcAMP lower than 0.02 mM but was increased at 0.2 mM.

There was no difference ($P = 0.09$, Figure 4.3a) in the accumulation of P_4 in media from INDUCED compared to SPONT CL following use of CHOLE as a substrate for P_4 synthesis. A significant dose dependent responsiveness ($P = 0.01$) was detected. In both types of CL, P_4

production was stimulated at lower (0.0125 mM) but not at higher concentration (0.125 mM) of CHOLE. Increasing the dose of CHOLE (1.25 mM) stimulated P_4 production to almost double the baseline level. Furthermore, treatment of the mixed luteal cells with PREGN caused no difference ($P > 0.05$, Figure 4.3b) between INDUCED compared to SPONT luteal cells. However, a significant ($P < 0.01$) effect of the dose of PREGN used was detected. In both types of CL, P_4 production was significantly increased in comparison to the baseline concentration for all concentrations of PREGN studied.

When the frequency distribution of luteal cell subpopulations was examined, INDUCED CL had significantly ($P < 0.01$, Figure 4.4a, Plate 4.1) more large luteal cells (LLC) than SPONT CL (52.43 vs 44.89 %). This effect was also associated with a concomitant reduction in the proportion of small luteal cells (SLC) among INDUCED (47.57 %) compared to SPONT CL (55.11 %). There were fewer SLC measuring 10 - 15 μm in diameter and more LLC in the 20 - 25 μm among INDUCED CL compared to SPONT CL. The average diameter of SLC was 16.56 $\pm$ 0.24 and 16.12 $\pm$ 0.23 μm among INDUCED and SPONT, respectively. Large LC, on the other hand, measured 26.69 $\pm$ 0.23 and 27.13 $\pm$ 0.25 μm among INDUCED and SPONT CL, respectively. The average minimum diameter of LLC (16.83 $\pm$ 0.18 vs 17.21 $\pm$ 0.20 μm) and SLC (11.78 $\pm$ 0.19 vs 11.29 $\pm$ 0.19 μm) was similar between INDUCED and SPONT CL, respectively. There was no difference ($P > 0.05$; Figure 4.4b) with regard to the area of SLC (186.85 $\pm$ 8.63 vs 177.13 $\pm$ 8.48 μm^2) and LLC (438.25 $\pm$ 8.22 vs 450.39 $\pm$ 9.18 μm^2) between INDUCED and SPONT CL, respectively.

4.5. DISCUSSION

The use of hCG to augment the function of aberrant CL has been shown to consistently result in the induction of accessory CL (de Los Santos-Valadez *et al.*, 1982; Rajamahendran and Sianangama, 1992) and to increase the frequency of luteal cells > 20 µm in diameter in spontaneously derived CL present when hCG was administered (Wiesak, 1989). More often, however, hCG treatment is also associated with inconsistencies with regard to peripheral concentrations of P_4 (de Los Santos-Valadez *et al.*, 1982; Rajamahendran and Sianangama, 1992; Sianangama and Rajamahendran, 1992). More recent studies suggest that hCG-induced CL might be inherently subnormal in function (Sianangama *et al.*, 1994a & b).

Morphometric evaluation of cells from spontaneous and hCG-induced CL in this study provide evidence supporting the postulate that LH induces the differentiation of small into large luteal cells in spontaneous CL (Donaldson and Hansel, 1965). The results show a bimodal distribution based on the size of luteal cells. Such a finding is consistent with previous reports in sheep (O'Shea *et al.*, 1979) but differ from studies recently conducted in cattle by Parkinson *et al.* (1994). The distribution into small and large luteal cells, in our study, was evident but more so only when the size grouping approached very narrow demarcations (categories) especially close to 20 µm. Interestingly, the data of Parkinson *et al.* (1994) did show a bimodal distribution but only for cows approximately 19 d into gestation.

The administration of hCG in the current study was associated with a significant increase in the number of large (> 20 µm) luteal cells as well as a concomitant reduction in the number of small (< 20 µm) luteal cells. This finding is consistent with available evidence both in pigs (Wiesak, 1989) and sheep (Farin *et al.*, 1988) following exposure of spontaneous CL to hCG during the luteal phase. However, the results reported herein are the first to document the increase in frequency of luteal cells > 20 µm in diameter among CL induced by hCG given during the

luteal phase, specifically d 7 after estrus in cattle.

The P_4 values obtained in the current study are consistent with those reported previously for dispersed luteal cells or 100 mg explants of luteal tissue (Kineman *et al.*, 1987; Fricke *et al.*, 1993). These results provide evidence that mean basal P_4 accumulation from mixed luteal cells was significantly higher among hCG-induced compared to spontaneously derived CL. Such a finding is in accordance with results reported by Rusbridge *et al.* (1994) for gonadotropin releasing hormone (GnRH)-induced CL of similar age. The data obtained in our current study was surprising because our recent *in vivo* data appeared to suggest synthesis and(or) secretory profiles being typically subnormal among hCG-induced CL (Sianangama *et al.*, 1994a). Hypothesizing that PGs are, in part, involved in suppressing the function of hCG-induced CL, our attempt at eliminating the confounding influence of ecosanoids could not improve both the lifespan and function of hCG-induced CL (Sianangama *et al.*, 1994b) suggesting an intrinsic source for the subnormal function. It seems reasonable, however, to suggest that ecosanoids, specifically the luteolysin $PGF_{2\alpha}$, may have contributed to the observed subnormal function among hCG-induced CL. The use of indomethacin, a PG synthase inhibitor, suppressed but did not eliminate $PGF_{2\alpha}$ secretion from the uterus (Sianangama *et al.*, 1994b). The finding that the function of GnRH-induced CL in hysterectomized ewes (Keisler and Keisler, 1989) was subnormal is in keeping with this interpretation of our *in vivo* data on hCG-induced CL (Sianangama *et al.*, 1994a & b). Additionally, bovine CL have been reported to secrete $PGF_{2\alpha}$ (Milvae and Hansel, 1983) which is known to de-activate the LH-sensitive adenylate cyclase (Fletcher and Niswender, 1982), thereby reducing the synthesis and secretion of P_4. Such sources of PGs might explain the premature demise and function of hCG-induced CL in our previous studies and the apparent difference between our *in vivo* data and the results being reported in the current *in vitro* study. The results from our P_4 studies being reported herein are also in contrast to those reported by

Fricke *et al.* (1993) who used 100 mg pieces of luteal explants from 6 d-old hCG-induced CL from cows and those reported by Rusbridge *et al.* (1992) whose GnRH-induced CL showed a tissue content of P_4 similar to spontaneously-derived CL in heifers. The cause of this dichotomy is presently not clear.

One plausible explanation for the increased basal concentrations of P_4 observed in our study could be the increase in number of large luteal cells accompanied by a concomitant decrease in the number of small luteal cells observed among hCG-induced CL. Recent evidence indicates that steroidogenesis in large luteal cells is regulated by the continuous transcription of mRNA for $P-450_{scc}$ enzyme which becomes constitutive (Oonk *et al.*, 1989) thereby supporting steroidogenesis without a requirement for further gonadotropic stimulation. Such an interpretation is consistent with reports showing that on a per cell basis, large luteal cells secrete as much as 20 times more unstimulated P_4 (Rodgers *et al.*, 1983) than do small luteal cells.

In an attempt to investigate the responsiveness of the LH/hCG receptor among hCG-induced and spontaneously derived CL, we treated cells with increasing doses of hCG. Our observation that hCG applied *in vitro* revealed a capacity to respond only among hCG-induced CL, is probably an indication that spontaneously derived CL are maximally stimulated as they approach midcycle. Such secretory characteristics further indicate that the subnormal function of hCG-induced CL exhibited *in vivo*, is most probably not due to the uncoupling of the LH receptor from the post-receptor signalling mechanism which is reported to occur in superovulated rats treated with an ovulatory dose of hCG (Kirchick *et al.*, 1983). Furthermore, Garverick and co-workers, (1988) demonstrated that subnormal CL were not due to inadequacy in gonadotropic support. In keeping with both interpretations, apparently subnormal CL in sheep and cattle do not differ with respect to the concentration of unoccupied and occupied LH receptors (McNeilly *et al.*, 1981; Smith *et al.*, 1986).

These results taken together support our hypothesis that the subnormal function *in vivo* is most probably mediated by $PGF_{2\alpha}$. The increasing $PGF_{2\alpha}$ concentrations observed after d 12 of the cycle in cattle (Flint and Sheldrick, 1983) may have prevented induced CL from reaching their maximal peak P_4 secretory potential. The possibility exists that this effect of $PGF_{2\alpha}$ was mediated by specific receptors which are abundant on large luteal cells (Wiltbank *et al.*, 1989) resulting in the subsequent activation of post-receptor mediators (phosphoinositol metabolism, Ca^{2+} mobilization and protein kinase C) and a concomitant uncoupling of the LH receptor from adenylate cyclase (Fletcher and Niswender, 1982; see review by Leung and Steele, 1992). It is noteworthy that large luteal cells have not been shown to possess an inherent mechanism for regulating an influx of Ca^{2+} and once initiated, such influx leads to functional luteolysis (Wiltbank *et al.*, 1989). The apparent decrease in P_4 secreted *in vitro* in the presence of increasing doses of hCG is probably an indication of the down regulation of the LH/hCG receptor (Schwall and Erickson, 1984) induced *in vitro* and mediated by increases in intracellular cAMP levels.

Anticipating an anomaly in the *in vivo* subnormal function of hCG-induced CL to reside at a site distal to the LH/hCG receptor, the influence of forskolin, dbcAMP, cholesterol and pregnenolone on the steroidogenic characteristics of hCG-induced and spontaneous CL was investigated. Forskolin revealed a dose dependent stimulation of P_4 secretion which increased linearly among hCG-induced CL. Increasing the dose of forskolin was associated with decreased P_4 secretion among spontaneously derived CL. Such a finding suggests the presence of a stimulable adenylate cyclase system among hCG-induced but not among the already maximally stimulated spontaneous CL. Forskolin is known to activate the gonadotropin-sensitive adenylate cyclase (Seamon and Daly, 1981) and increases gene expression and(or) stabilization of the message (mRNA) for the enzyme $P-450_{scc}$ (Lauber *et al.*, 1991). We propose, therefore, that the rate limiting step among the subnormally functioning hCG-induced CL *in vivo*, resides at sites

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proximal to the adenylate cyclase stage of P_4 biosynthesis. This suggestion is further supported by our previous finding (Sianangama *et al.*, 1994b) that infusion of indomethacin could not improve the function and lifespan of hCG-induced CL. It is not possible, however, from this study to determine whether this anomaly involves the stimulatory/inhibitory guanine nucleotide binding (G-) protein complex. Jena and Abramowitz, (1989) reported that the hCG-induced heterologous desensitization of the LH/hCG receptor function included alterations in G-protein function. We do not exclude the possibility that other mechanisms may be involved in the apparent subnormal function of hCG-induced CL as determined from *in vivo* experiments in our studies (Sianangama *et al.*, 1994a & b) as well as in those of other workers (Keisler and Keisler, 1989).

Addition of increasing doses of dbcAMP, to bypass the requirement for both the receptor and gonadotropin-dependent adenylate cyclase, failed to increase P_4 secretion in both types of CL. In fact, increasing the dose of dbcAMP appeared to suppress P_4 synthesis. Our findings on the effect of dbcAMP on P_4 secretory capacity of d 7 luteal cells differs from those reported by Rusbridge *et al.* (1994) who found significant stimulation of P_4 secretion in response to 0.5 mg/mL dbcAMP in the culture medium. They found a significant response among spontaneous but not GnRH-induced luteal cells. The lack of significant increases in P_4 secretion following use of dbcAMP may be a confirmation of previous reports suggesting that the effect of cAMP on luteal cells depends on the cell type being investigated. Large, but not small, luteal cells are reported to require an initial trigger of cAMP to induce the expression of mitochondrial steroidogenic enzymes following which the expression of the enzyme (P-450$_{scc}$) becomes constitutive (Oonk *et al.*, 1989). Small luteal cells require a continuous generation and, therefore, the presence of cAMP to induce enzyme expression. This hypothesis is consistent with our present observations that the number of large luteal cells were increased among hCG-induced CL.

Provision of luteal cells with cholesterol as a metabolizable substrate for P_4 biosynthesis *in vitro* was also associated with a mild increase in P_4 synthesis at lower concentrations. Increasing the concentration of cholesterol in the medium was accompanied by decreases in P_4 synthesis, although a further increase in cholesterol in the culture medium appeared to reverse this trend. The reason for the increase in P_4 synthesis observed at the higher concentration of dbcAMP and cholesterol is presently uncertain. The finding that, fundamentally, there was no difference in the response to both dbcAMP and cholesterol suggests that the second messenger and substrate, respectively, are normally not rate limiting in the function of the two types of CL. Relatively, much higher concentrations of P_4 were recorded among cells treated with cholesterol, presumably due to the ease with which this substrate is able to diffuse across the cellular and mitochondrial membranes (Mason *et al.*, 1978). Interestingly, however, the difference between the two types of CL (higher basal P_4 secretion among hCG-induced compared to spontaneous CL) was overcome with the provision of pregnenolone. Comparison with published reports with regard to the responsiveness of hCG- or GnRH-induced CL to the secretogogues forskolin, cholesterol and pregnenolone is presently not possible as none seem to have been conducted and reported as yet.

4.6. CONCLUSIONS

Alterations in the frequency distribution of luteal cell types were detected among hCG-induced CL but not in spontaneously derived CL. There was an increase in the proportion of large and a concomitant reduction in the fraction of small luteal cells. Corpora lutea induced by hCG secreted significantly more basal P_4 concentrations. Treatment with hCG in vitro was associated with increased P_4 secretion only among hCG-induced CL. Forskolin dose-dependently

increased among hCG-induced but decreased P_4 secretion among spontaneous CL. Cyclic AMP

and cholesterol did not increase P_4 secretion in both types of CL. While basal P_4 concentrations

were lower among spontaneous CL, provision of pregnenolone increased P_4 secretion among

spontaneous CL to concentrations equal to those observed among hCG-induced CL.
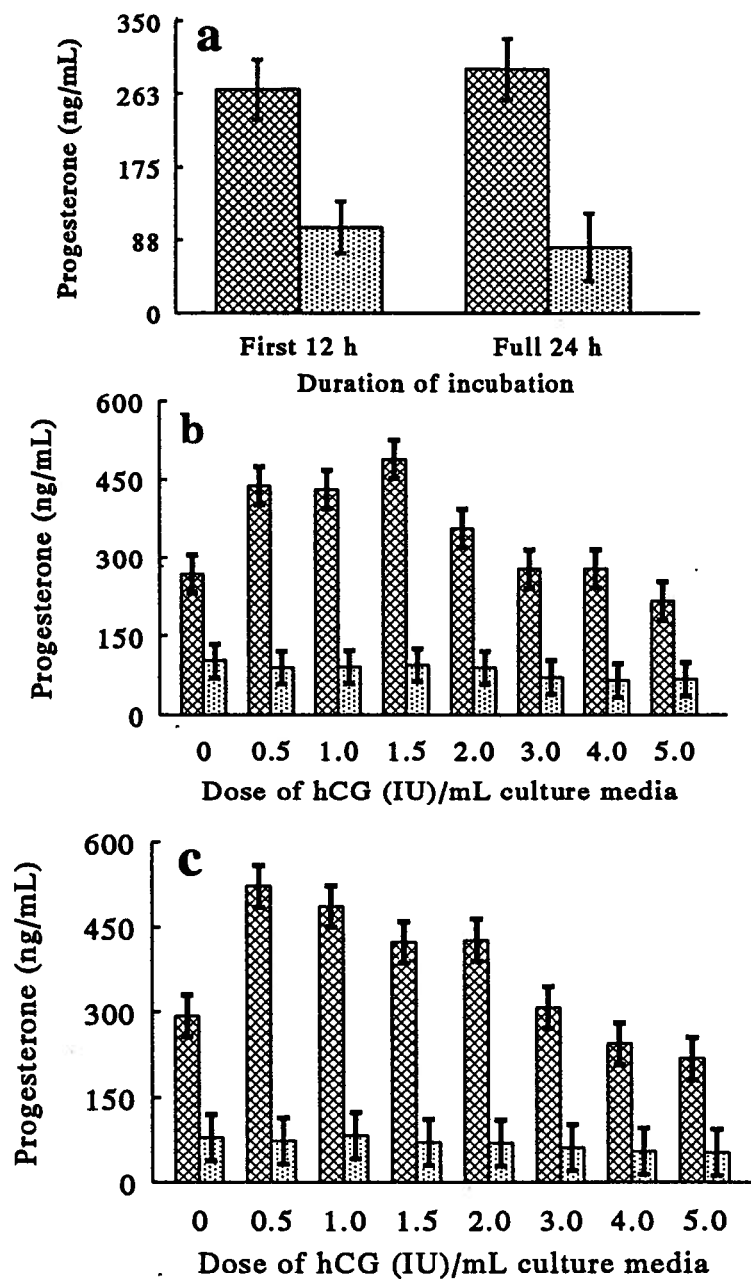
Figure 4.1. Baseline progesterone concentration after the first 12 or full 24 h of incubation a) and the effect of human chorionic gonadotropin (hCG) on *in vitro* progesterone production during 12 b) or 24 h c) of incubation among mixed luteal cells from INDUCED (▨) and SPONT CL (▨). Values are least square means (± SEM) of triplicate samples per treatment (n = 6 cows per treatment).
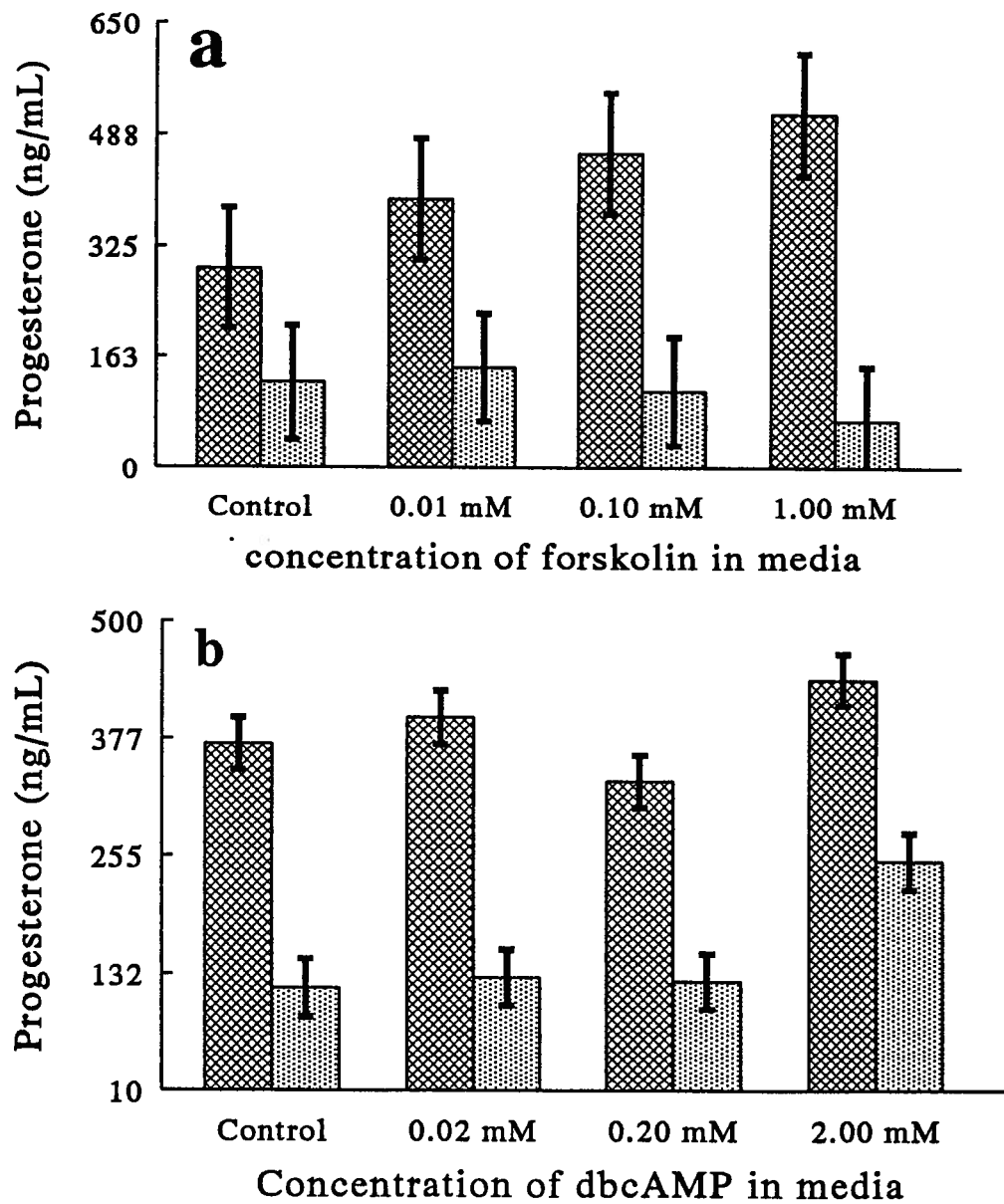
Figure 4.2. The effect of forskolin (a) and dbcAMP (b) on *in vitro* progesterone production by luteal cells from INDUCED (▩) and SPONT CL (▨) during a 12 h incubation period. Values are least square means (± SEM) of triplicate samples per treatment (n = 6 cows per treatment).
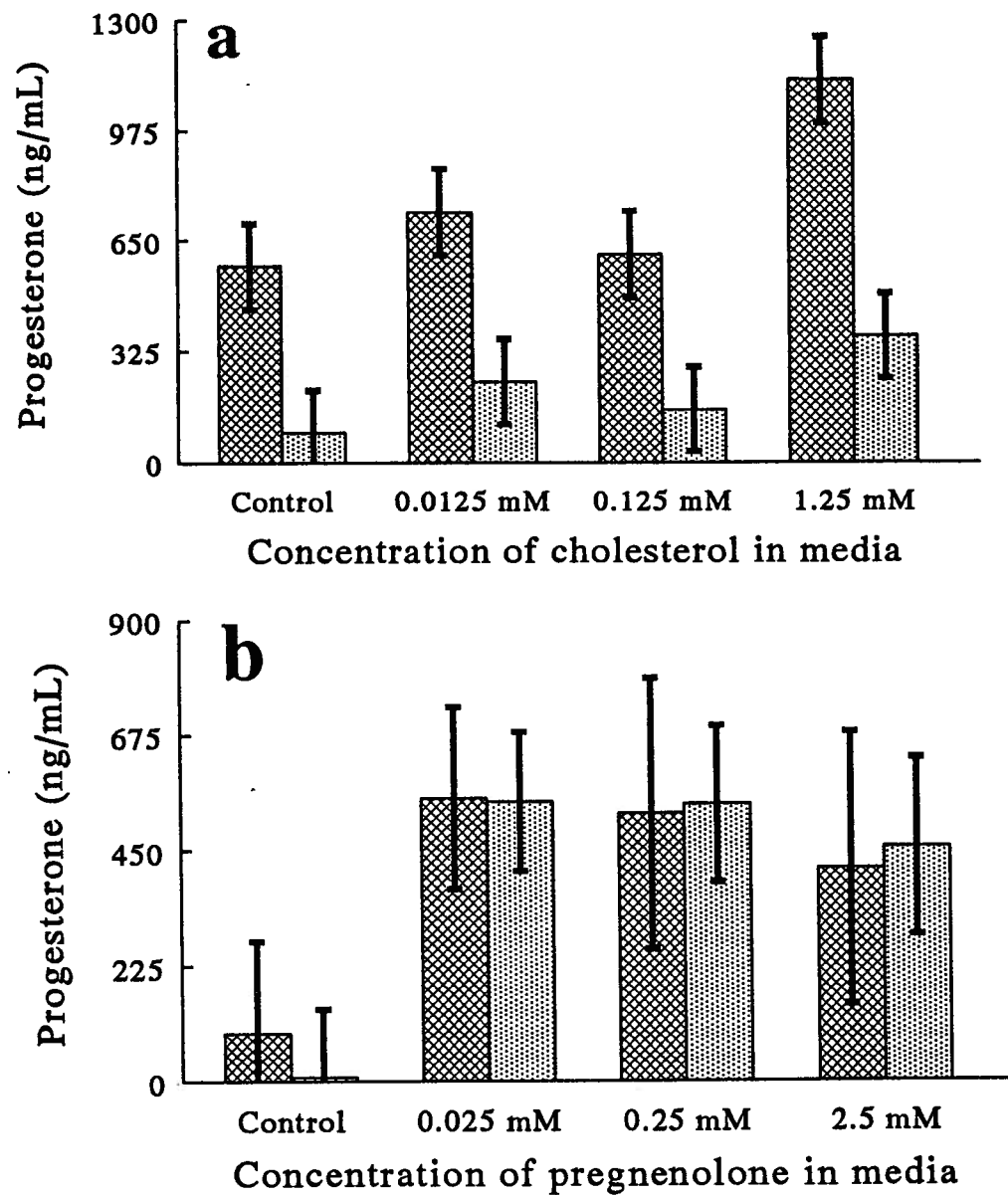
Figure 4.3. The effect of cholesterol (a) and pregnenolone (b) substrate on *in vitro* progesterone production by luteal cells from INDUCED (▨) and SPONT CL (▨) during a 12 h incubation period. Values are least square means (± SEM) of triplicate samples per treatment (n = 6 cows per treatment).
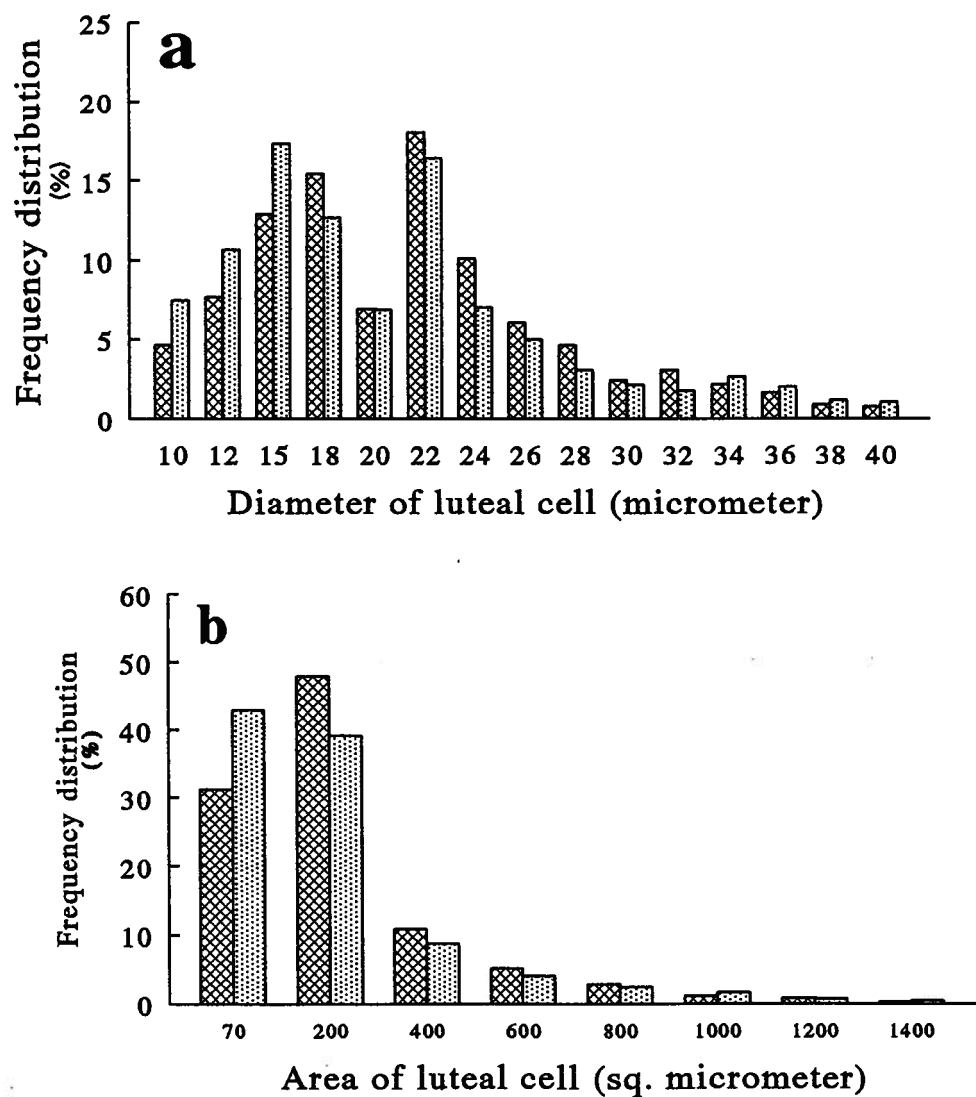
Figure 4.4. The frequency distribution (%) of the maximum diameter (μm; a) and area (μm²; b) of luteal cells from INDUCED (▨) and SPONT CL (▥). Each bar represents a mean of 200 observations per cow.
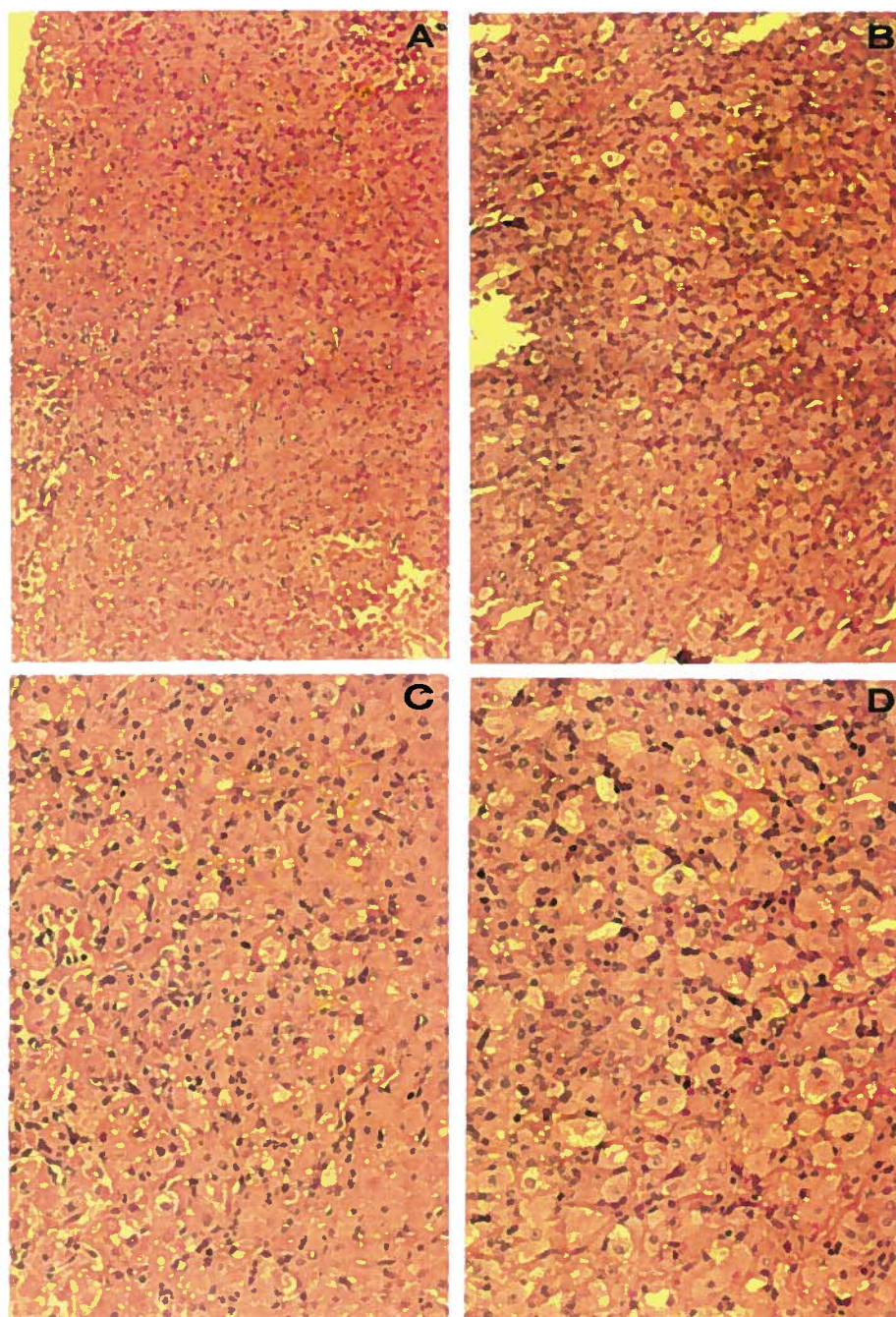
Plate 4.1. The effect of human chorionic gonadotropin (hCG) on luteal cell distribution among hCG-induced corpora lutea (CL). Note the prevalence of large luteal cells among hCG-induced CL (B, D) compared to spontaneous CL of similar age (A, C). The pictures were taken at a magnification of x 100 (A, B) or x 200 (C, D) from a representative area of the same slide.

The Morphometry And Function Of Corpora Lutea Previously Exposed To hCG Given On Day 7 Of The Cycle In Cows.

5.1. ABSTRACT

The objective of this experiment was to evaluate the morphometry and steroidogenic characteristics of luteal cells from spontaneous corpora lutea (CL) previously exposed to hCG *in vivo*. Cows were treated with 1000 IU of human chorionic gonadotropin (hCG) on d 7 (estrus = d 0) and the spontaneous CL were excised surgically on d 12 (hCG-12, n = 5) or 15 (hCG-15; n = 5). Six additional cows which did not receive the hCG treatment served as controls (CONT). Enucleated CL were weighed and processed for morphometric evaluation or cell culture. Dispersed, mixed luteal cells were cultured in the presence of hCG (0-10 ng/mL) for either 12 or 24 h. After incubation, luteal cells were separated by centrifugation and the supernatant was frozen at - 20°C until assayed for progesterone (P_4). There were no differences ($P > 0.05$) in the weight of CL among hCG-12 (6.03 ± 0.65 g), hCG-15 (7.76 ± 0.77 g) compared to CONT cows (5.96 ± 0.86 g). Baseline P_4 levels were lower ($P < 0.01$) among hCG-12 (23.55 ± 2.86 ng/mL) compared to CONT (32.12 ± 1.73 ng/mL) after 12 h of incubation. However, P_4 accumulation was significantly higher ($P < 0.01$) among hCG-15 (674.40 ± 48.91 ng/mL) compared to both hCG-12 and CONT cows. Although there was no effect of *in vitro* hCG ($P > 0.12$), control luteal cells tended to be more responsive to hCG stimulation when compared to hCG-12. The refractoriness in response to hCG was not observed among hCG-15. Treatment with hCG was associated with an increase in the number of large and a concomitant reduction in the number of small luteal cells ($P < 0.01$). However, there was no difference with regard to luteal cell area

and diameter of each luteal cell type. These results suggest that treatment with hCG during the luteal phase induces diminution in the responsiveness of luteal cells previously exposed to hCG. However, the refractoriness does not seem to persist until d 7 after the hCG treatment. The results also confirm that hCG is associated with an increased differentiation of small into large luteal cells.

5.2. INTRODUCTION

Infertility in cattle represents a major source of reproductive inefficiency and contributes substantially towards economic losses experienced in the animal production industry. It has been known for a long time that subnormal luteal function is a cause of infertility in cattle (Odde *et al.*, 1980; Pratt *et al.*, 1982). Typically, subnormal corpus luteum (CL) function, which is alternately called luteal phase insufficiency or luteal dysfunction, is characterized either by a normal estrous cycle length with low peripheral progesterone (P_4) concentrations (diZerega and Hodgen, 1981; Lamming *et al.*, 1989) or a shorter than normal lifespan of the CL showing only a transient increase in P_4 secretion (Lamming *et al.*, 1981). Consequently, several measures aimed at correcting inadequate CL function have been developed, including the use of P_4 supplements from various types of exogenous sources (Robinson *et al.*, 1989), gonadotrophin releasing hormone (GnRH) or analogues of GnRH (Nakao *et al.*, 1983) administered at various times in relation to the time of breeding. Results obtained from these studies remain inconsistent with regard to their effect on pregnancy rates.

An alternate method designed to augment the function of an inadequate CL function in cattle has been the use of gonadotropins such as luteinizing hormone (LH) (Donaldson and Hansel, 1965) or human chorionic gonadotrophin (hCG) (Wiltbank *et al.*, 1961; Sianangama and

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Rajamahendran, 1992) administered at various times relative to breeding. Although a number of researchers could not find any improvement following use of hCG (Wagner *et al.*,1973; de Los Santos-Valadez *et al.*, 1982; McDermott *et al.*, 1986), it has been shown that hCG improves pregnancy rates in cattle (Wiltbank *et al.*, 1961; Wagner *et al.*, 1973; Diskin and Sreenan, 1985; Sianangama and Rajamahendran, 1992). The precise mechanism by which pregnancy rates are improved in cattle remains unknown. It has, however, been postulated that the mechanisms by which increases in pregnancy rates are achieved include the stimulation of the spontaneous CL (Wiltbank *et al.*, 1961; Donaldson and Hansel, 1965). Sufficient evidence is now available clearly showing that hCG induces the ovulation of dominant follicles present at the time of its administration (de Los Santos-Valadez *et al.*, 1982; Price and Webb, 1989; Walton *et al.*, 1990; Rajamahendran and Sianangama, 1992).

The overall effect of either stimulating a spontaneous CL or developing induced CL would be an increase in P_4 concentrations. Examination of published data on the effect of hCG on P_4 concentrations reveals considerable variability. While several researchers have reported significant increases in P_4 concentrations (McDermott *et al.*, 1986; Bennett *et al.*, 1989; Walton *et al.*, 1990), a number of other researchers could not demonstrate a similar effect of hCG on P_4 concentrations (de Los Santos-Valadez *et al.*, 1982; Sianangama and Rajamahendran, 1992). de Los Santos-Valadez *et al.*, (1982) compared P_4 profiles of hCG-treated cows that developed induced CL to those that did not. In that study, there was no difference in P_4 concentrations between cows carrying induced CL compared to those that did not develop induced CL. Recently, we (Sianangama *et al.*, 1994a) reported that CL induced by hCG given on d 7 of the estrous cycle were smaller and secreted less P_4 compared to spontaneous CL of similar age. This difference between induced and spontaneous CL was even more profound 7 d after either induced or spontaneous ovulation. At this time, induced CL were invariably regressing. Reasoning that the

observed subnormal function and the shorter than normal lifespan of induced CL were caused by endogenous prostanoids, specifically prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), we used indomethacin (Sianangama *et al.*, 1994b), a prostaglandin synthase inhibitor, but could not improve the development and function of hCG-induced CL. This suggested that the increase in P_4 concentrations observed immediately following the administration of hCG could be a result of stimulation of the spontaneous CL. The objective of this study, therefore, was to use an *in-vitro* model to establish the steroidogenic characteristics of spontaneous CL previously exposed to hCG.

5.3.   MATERIALS AND METHODS

5.3.1. *Materials And Management Of Cows*

All materials used in this study were procured from the sources noted under section 4.3.1 of chapter 4. Sixteen lactating and regularly cycling cows (2½ to 5 yrs old), approximately 60 to 90 d *postpartum*, were randomly selected from the dairy herd at the South Campus Teaching and Research Facility of the University of British Columbia. The cows were managed as described under section 4.3.2. The surgical procedure was also conducted as outlined previously with the following changes; surgery was conducted either on d 12 or 15 after estrus.

5.3.2. Treatments And Surgery

At standing estrus (d 0), animals were assigned at random to receive an intramuscular (i.m.) injection with hCG (1000 IU, APL®) on d 7 after estrus and enucleation of the hCG exposed CL conducted either on d 12 (hCG-12, n = 5) or 15 (hCG-15, n = 5). An additional six cows which did not receive the hCG treatment served as control (CONT) and had the CL enucleated on d 12 of the cycle. All animals were exposed to a 12 to 16 h fasting period (starting on d 11 or 14 at 2130 h) before surgery. All other procedures were conducted as described under section 4.3.3.

5.3.3. Processing Of Luteal Cells And In Vitro Culture

Enucleated luteal tissue was handled as described under sections 4.3.4 and 4.3.5 with the modifications noted below. Mixed steroidogenic luteal cells (approx. 8×10^5 to 1×10^6 per cow /well) were placed in 24-well culture plates in 1 mL incubation medium at 37°C in a humidified atmosphere with 5 % CO_2 and 95 % air. All cultures from each of the 16 cows in the experiment were done in triplicate and allowed to run for either 12 or 24 h. All treatments to the cells were applied at the start of each incubation period. To investigate the responsiveness of the LH/hCG receptor to further gonadotropic stimulation *in vitro*, luteal cells were treated with hCG (hCG; 0 - 10 IU/mL). After incubation the media was removed, centrifuged at 1000 x g and the supernatant stored at -20°C until required for P_4 assays using a solid phase radioimmunoassay kit. Sections of the enucleated CL were processed for morphometric evaluation as previously described under section 4.3.6.

5.3.4. Statistical Analyses

Due to the heterogeneity of variance, P_4 data were log-transformation prior to analysis using the General Linear Models (GLM) procedures in the Statistical Analysis System (SAS, Cary, NC). The data were analyzed by ANOVA as a split-plot experimental design which included the following effects, viz; type of CL (hCG-12, hCG-15 vs CONT), cow nested within treatment, the dose used, period of incubation (12 vs 24 h), and replicate as well as their higher level interactions. Morphometric data (i.e. cell area and diameter) were also analyzed by ANOVA, after log transformation. Where main effects were indicated to be significantly different ($P < 0.05$) by ANOVA, the respective treatment means were compared using least squares analysis in the GLM. Categorical data were analyzed using contingency tables in χ^2 analysis (Proc Frequency, SAS) and were considered different if $P < 0.05$. Data are presented as least square mean ± standard error.

5.4. RESULTS

5.4.1. Effect Of hCG On In Vitro Progesterone Production

There was no difference ($P > 0.05$) with regard to the weight of the CL in all treatment groups. There was a tendency, however, for hCG-exposed CL to be heavier. The weights for CONT, hCG-12 and hCG-15 were 5.95 ± 0.86 g, 6.03 ± 0.65 g and 7.76 ± 0.77 g, respectively. Baseline P_4 production was significantly lower ($P = 0001$; Figure 5.1a) among hCG-12 luteal cells (23.55 ± 2.86 ng/mL) compared to CONT (32.12 ± 1.73 ng/mL). Baseline P_4 concentrations were much higher ($P < 0.01$) among hCG-15 (674.40 ± 48.91 ng/mL) compared to both hCG-12 and CONT luteal cells. However, significant ($P = 0.03$) interactions between type of CL by

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period of incubation by dose of hCG applied were detected. While no effect of hCG dose was detected for hCG-12 CL, hCG significantly increased P_4 production among both CONT and hCG-15 CL. Increasing the dose of hCG applied was associated with a dose dependent suppression of P_4 production. This effect of hCG was observed for both the 12 h (B) and 24 h (C) incubation periods.

5.4.2. *Effect Of hCG On Luteal Cell Size*

Treatment of cows with hCG was associated with a significant ($P < 0.01$; Figure 5.2a) increase in the number of large luteal cells (LLC) among hCG-12 (59.35 %) and hCG-15 (65.68 %) compared to CONT cows (31.32 %). This increase in the number of LLC occurred concomitantly with a reduction in the number of small luteal cells (SLC). The number of SLC among CONT, hCG-12 and hCG-15 were 68.68, 40.65, and 34.32 %, respectively.

There was no difference with regard to the absolute size of each luteal cell type. The maximum diameter for LLC among CONT, hCG-12 and hCG-15 was 27.36 ± 0.37 μm, 28.49 ± 0.27 μm and 28.48 ± 0.21 μm, respectively. The SLC, on the other hand, measured 16.32 ± 0.25 μm, 17.10 ± 0.33 μm, 17.42 ± 0.28 μm for CONT, hCG-12 and hCG-15, respectively. A significant (P < 0.01; Figure 5.2a) treatment by type of CL interaction was detected. The area of LLC among CONT (500.76 ± 13.75 μm^2), hCG-12 (480.35 ± 9.76 μm^2) and hCG-15 (470.02 ± 7.58 μm^2) was also not different. Similarly, no difference was detected in the area of SLC among CONT (177.92 ± 9.32 μm^2), hCG-12 (186.81 ± 12.00 μm^2) and hCG-15 (186.70 ± 10.40 μm^2). Significant ($P < 0.01$; Figure 5.2b) type of CL by treatment interactions were detected.

5.5. DISCUSSION

Recently, we (Sianangama *et al.*, 1994a) reported that CL induced by hCG given on d 7 of the estrous cycle are functional but appear not only to be smaller but also secrete less P_4 compared to spontaneously developed CL of similar age. This finding suggests that the spontaneous CL are the main source of the increased P_4 concentrations observed among hCG-treated cows. However, results obtained in this study only partially support this hypothesis. Compared to control, both basal and hCG-stimulated P_4 production was less among 12 d-old luteal cells previously exposed to hCG. Such a finding is in agreement with a recent study (Fricke *et al.*, 1993) in which 100 mg slices of spontaneous luteal tissue from 13 d-old CL were used. In that study, cows were treated with hCG on d 6. Therefore, the data being reported herein does support their data but only for the 12 d old CL. More importantly, however, our data clearly show that the refractoriness to further gonadotropic stimulation *in vitro* does not last until d 7 after the hCG treatment.

The subnormal function exhibited by 12 d-old hCG-exposed luteal cells in the present experiment might be due to the heterologous desensitization of the gonadotropin (LH/hCG) responsive adenylate cyclase activity, or down regulation of the LH/hCG receptor induced by the hCG administered *in vivo*. This interpretation of our data is in keeping with previously reported results from experiments conducted in the rat (Kirchick *et al.*, 1983) and rabbit (Kirchick and Birnbaumer, 1981). It is noteworthy, that the observed refractoriness was negated by 7 d after the hCG treatment was administered. Human CG is known to bind to the LH/hCG receptor from which it dissociates very slowly (Lee and Ryan, 1973). It is conceivable, therefore, that the initial (d 12) refractoriness may indeed represent a desensitization of the adenylate cyclase generating system and/or down regulation of the LH/hCG receptor. Additionally, the subsequent resensitization and up regulation of the adenylate cyclase system and receptor, respectively, are

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supported by the rebound in the function observed among the 15 d-old CL previously exposed to hCG. It is not clear from this experiment whether actual numbers of either total or the unoccupied and occupied LH/hCG receptors were down regulated and if so, to what extent. Previous work designed to elucidate the cause of subnormal CL function found no evidence to support the hypothesis that gonadotropin levels or the receptor dynamics predispose the subnormal function. Garverick and co-workers, (1988) found no difference in gonadotropin concentrations in animals with CL destined to be short lived compared to normal CL. Furthermore, there is evidence suggesting that even the number of unoccupied and occupied LH/hCG receptors are similar in animals exhibiting normal compared to those presenting subnormal CL function (McNeilly *et al.*, 1981; Smith *et al.*, 1986). Collectively, our data as well as those of others (McNeilly *et al.*, 1981; Smith *et al.*, 1986) suggest that the subnormal function may be mediated by events occurring at a site distal to the LH/hCG receptor. Our finding in this study, that increasing the dose of hCG applied *in vitro* actually decreased P_4 production, is suggestive of an *in vitro* induced down regulation of the LH/hCG receptor. This is a physiologically relevant mechanism by which luteal cells that are already maximally stimulated (d 10 - 15) are able to regulate their own function. This explanation, however, does not exclude the possibility that the lack of a stimulatory effect of hCG *in vitro* on P_4 production was related to the observed increase in the number of large luteal cells which are reportedly unresponsive to low doses of LH or hCG (Niswender *et al.*, 1985).

Several lines of evidence indicate that hCG influences the relative proportions of steroidogenic luteal cells constituting the CL (Farin *et al.*, 1988; Wiesak, 1989). In these studies conducted in sheep (Farin *et al.*, 1988) and pigs (Wiesak, 1989), hCG was reported to increase the number of large luteal cells while concurrently reducing the number of small luteal cells. Since small luteal cells possess the majority of LH/hCG receptors and are more responsive to

luteotropic stimuli (Rodgers and O'Shea, 1982), reductions in the number of small luteal cells might, in part, explain the subnormal function of 12 d-old hCG exposed-CL reported in the present study. This study also found a bimodal frequency distribution of luteal cells. This is consistent with previous studies in the bovine (Webley *et al.*, 1990) showing that the steroidogenic luteal cells consist of two populations. It is noteworthy, however, that Hild-Petito *et al.* (1987) and more recently Parkinson *et al.* (1994) failed to confirm the bimodal distribution as described both in our present study and in studies reported by others (Webley *et al.*, 1990). This difference may be due to the classification used to sort luteal cells into size groups prior to analysis. A narrower classification scheme as used in our study as well as that employed by Webley *et al.* (1990) appears to be a prerequisite in demonstrating the bimodal distribution.

It is paradoxical, however, that 15 d-old CL exposed to hCG *in vivo* and shown to also have been cytologically affected by hCG to the same magnitude, actually produced more P_4 *in vitro*. This discrepancy can be explained by our finding in this study that the 15 d-old CL previously exposed to hCG had significantly more large luteal cells. The large, unlike the small, luteal cell has been demonstrated to secrete steroids independent of gonadotropic support. Oonk *et al.* (1989) ascribed this phenomenon to the fact that the expression of steroidogenic enzymes in large luteal cells becomes a constitutive phenomenon after the first trigger. Specifically, they demonstrated that steroidogenesis in large luteal cells is driven by the continuous transcription of mRNA for cytochrome $P-450_{scc}$ enzyme which becomes constitutive, thereby supporting steroidogenesis without requirement for further gonadotropic stimulation (Oonk *et al.*, 1989). Furthermore, the large luteal cell is known to secrete as much as 20 times more P_4 under basal conditions (Rodgers *et al.*, 1983). Therefore, barring the effects of hCG on the receptor and the intracellular responsive and/or signalling mechanism as discussed above, it is reasonable to expect higher P_4 production from spontaneous CL previously exposed to hCG *in vivo*.

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From a production as well as physiological stand point, it is significant that the refractoriness to gonadotropic support is negated by d 15 of the cycle (7 d after hCG treatment) and that P_4 production is significantly higher among these hCG-exposed CL. In cattle, it has been shown by us (Sianangama and Rajamahendran, 1992) as well as others (Wiltbank *et al.*, 1961; Holness *et al.*, 1982) that hCG increases pregnancy rates in cattle. The establishment and maintenance of these pregnancies relies, in a large number of cases, on the adequate function of CL (Lamming *et al.*, 1989). Any increase in P_4 occurring at or around d 15 after estrus would ensure that an otherwise developmentally lagging embryo is not lost for failing to signal its presence at d 16 at which time maternal recognition of pregnancy is known to occur in cattle. The increased P_4 would "buy the embryo time" until it is able to signal its presence and, therefore, become an established pregnancy.

5.6.   CONCLUSIONS

Corpora lutea exposed to hCG given on d 7 of the cycle secrete less P_4 *in vitro* and appear to lose responsiveness to further gonadotropic stimulation. This refractoriness appears to last until d 15 of the cycle after which time hCG-exposed CL secrete even more P_4 than do control CL. Such subnormal function may be attributed to both receptor desensitization and cytological alterations in CL exposed to hCG *in vivo*. The increased P_4 production of 15 d old CL previously exposed to hCG may be due to the increase in the number of large luteal cells whose function is driven by constitutive mechanisms that are independent of tropic stimulation. Treatment with hCG is associated with increased differentiation of small into large luteal cells. These cytological alterations have profound implications on the subsequent function and lifespan of CL.
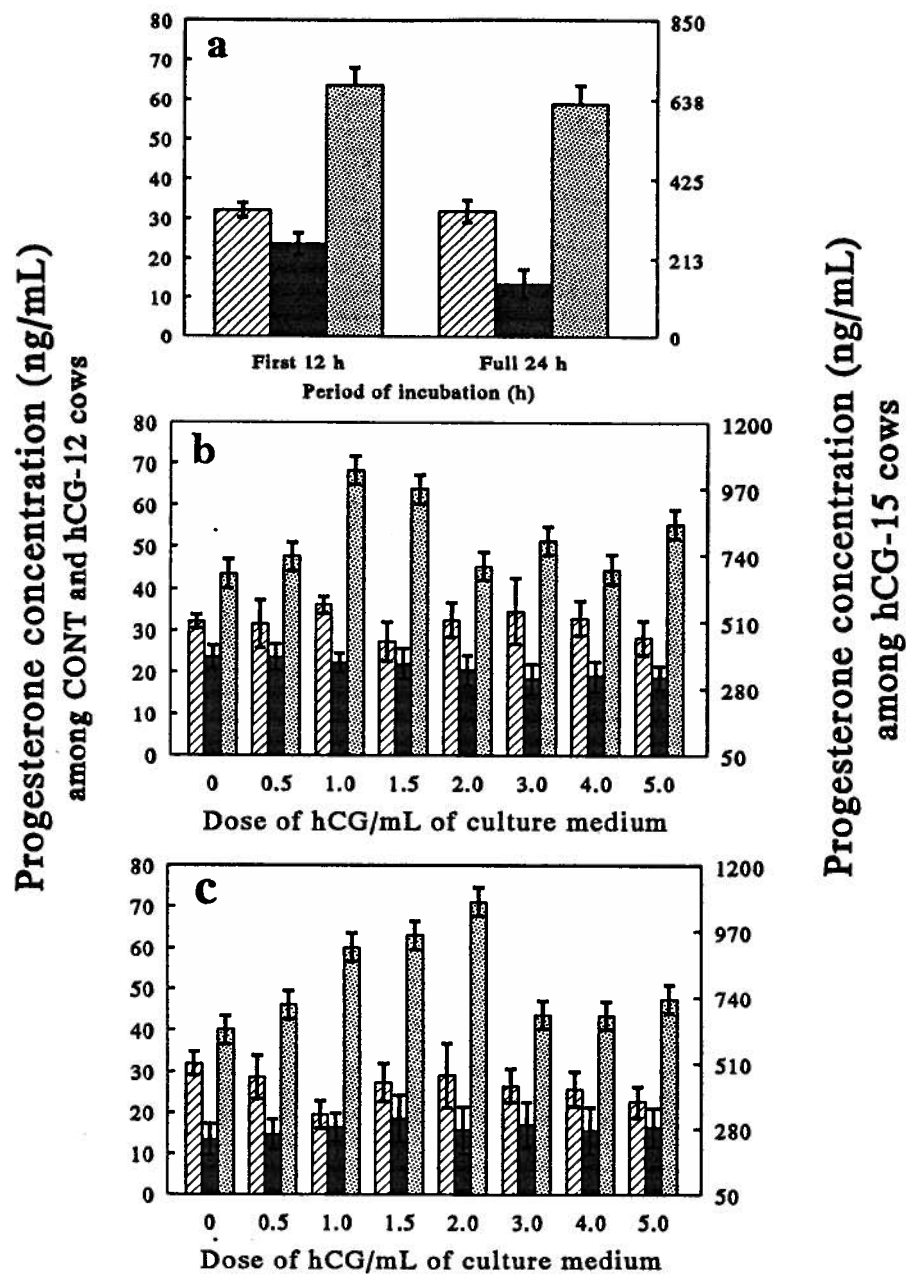
Figure 5.1. Baseline secretion of P_4 production among mixed luteal cells perviously exposed to hCG and enucleated either on d 12 (■) or 15 (▓) as well as among 12 d-old control luteal cells (▨) both during the first 12 h and 24 h (a) period of incubation. Progesterone production was influenced by the type of CL, dose of hCG applied in vitro as well as the period of incubation. Data are presented as least square means of P_4 production during the first 12 h (b) and total P_4 production during a 24-h (c) period of incubation.

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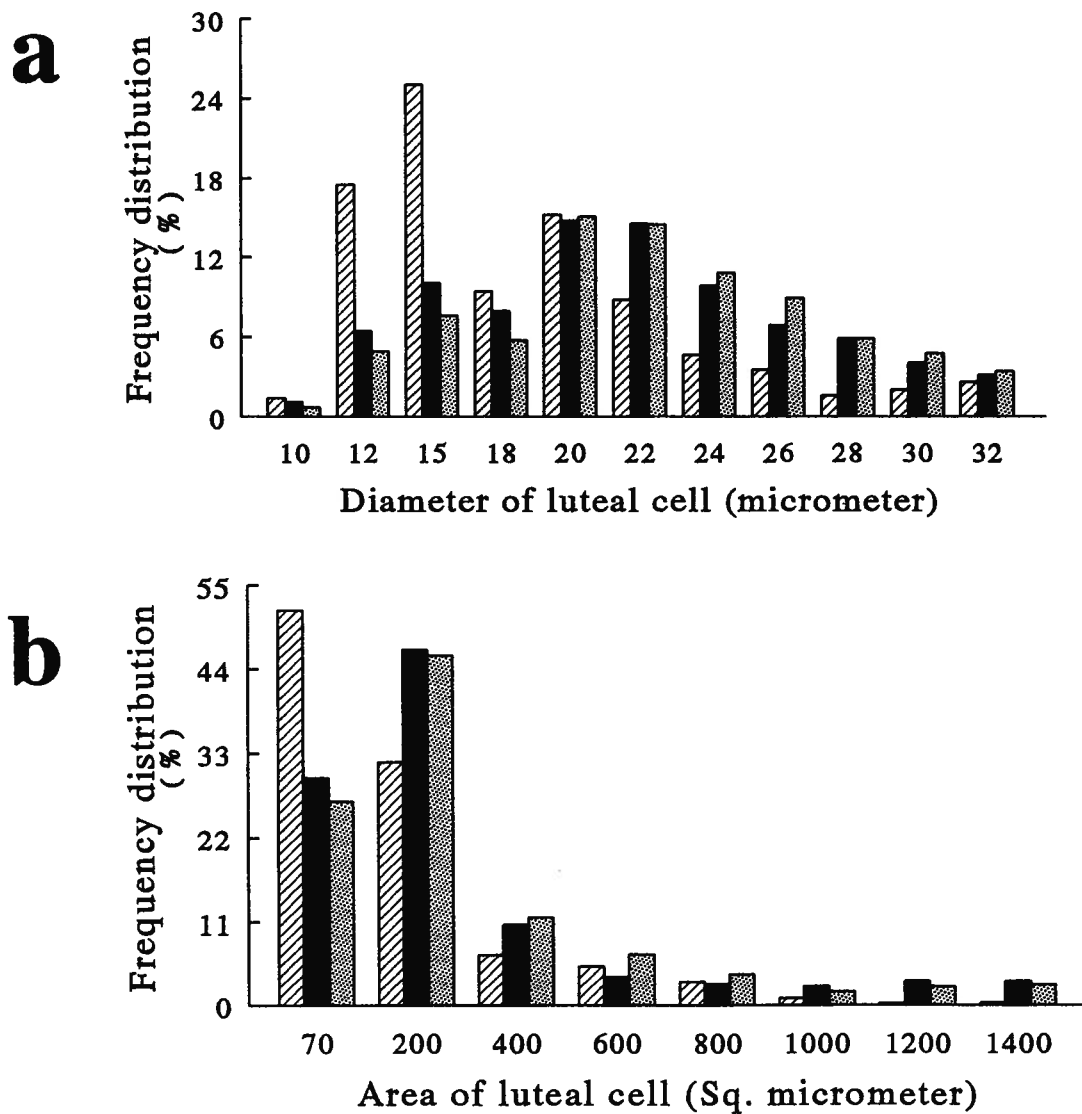
Figure 5.2. The frequency distribution of the maximum diameter (a) and area (b) of steroidogenic luteal cells from control cows (▨) as well as cows treated with hCG on d 7 and spontaneous CL enucleated on d 12 (■) or 15 after estrus (▩). Each bar represents a mean of 200 observations per cow.

CHAPTER 6

Effects Of Human Chorionic Gonadotropin Given On Day 7 Of The Cycle On Follicular Dynamics In Noninseminated Postpartum Dairy Cows.

6.1. ABSTRACT

The objective of this experiment was to determine whether the increase in estrous cycle length observed in hCG-treated cows that fail to maintain their pregnancies is due to changes in follicular dynamics. Twelve nonbred cows were randomly assigned to receive hCG on d 7 (n = 6) or serve as control (n = 6). Ultrasound scanning was conducted daily from d 0 until the onset of the next ovulation, to monitor follicular and CL dynamics. Ovulation of the d 7 follicle occurred in six hCG-treated cows. There was no difference ($P > 0.05$) with regard to the d of emergence of the second wave of follicular growth between hCG treated (d 10.8 ± 0.3) and control cows (d 12.7 ± 1.4). More follicles were observed on the ipsilateral than on the contralateral side to the spontaneous or induced CL . The dominant follicle from wave two was maintained longer (13.0 ± 1.8 vs 9.7 ± 2.1 d), had a slower growth rate (0.83 vs 1.31 mm/d), but was larger (19.6 ± 3.4 vs 15.7 ± 4.8 mm) among hCG-treated cows. The second wave dominant follicle was the ovulatory follicle in five control cows, but only in three hCG treated cows. Corpus luteum lifespan was not affected by hCG treatment (19.3 ± 2.7 vs 18.8 ± 1.2 d). The dominant follicle from the third wave ovulated in one control and three hCG treated cows. These results suggest altered follicular dynamics and delayed ovulation are probable causes for extensions in cycle length.

6.1. INTRODUCTION

In cattle, fertilization rates range from 85 to almost 100 % (Roche *et al.*, 1981). In contrast, pregnancy diagnosis conducted 60 d post breeding indicate that only 60 % of these embryos are maintained. One factor that contributes to the loss of embryos is subnormal corpus luteum (CL) function (Bulman and Lamming, 1978). Consequently, gonadotropic substances such as human chorionic gonadotropin (hCG), have been used to augment the function of inadequate CL in cattle (Wiltbank *et al.*, 1961; Morris *et al.*, 1976; Eduvie and Seguin, 1982). Although it has been demonstrated that hCG increases pregnancy rates, results have been very variable. Some reports suggest increases in pregnancy rate (Wagner *et al.*, 1973; de Los Santos-Valadez *et al.*, 1982). It is noteworthy that the percent change in pregnancy rates in these studies ranged from a net loss of 11 % to a net gain of 13 % (Diskin and Sreenan, 1985). In a recent study, we demonstrated that the greatest increase in pregnancy rates occurred if hCG was administered on d 7 after breeding (Sianangama and Rajamahendran, 1992). In that study, we showed that treatment with hCG on d 0, 7 or 14 was associated with 8, 49 and 35 percent point increase in pregnancy over the untreated control cows at the first of two farms. Similarly, a repeat of the same experiment at a second farm resulted in an even higher response to hCG given on 0, 7 or 14 after breeding. The corresponding percent increases at the second farm were 24, 57 and 40 for cows treated on d 0, 7 or 14, respectively.

Notably, however, the use of hCG has been associated with extensions in estrous cycle length among cows that either do not conceive or fail to maintain their pregnancies (Wiltbank *et al.*, 1961; Morris *et al.*, 1976; Sianangama and Rajamahendran, 1992). Recently, we (Sianangama and Rajamahendran, 1992) investigated the effect of hCG on milk P_4 and pregnancy rates. Among animals that did not conceive, we observed extensions in the cycle length. Interestingly, the extensions were influenced by the timing of hCG treatment in relation to the

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luteal phase. The frequency of extended cycles was higher and the duration of the extension longer among cows treated with hCG on d 14 compared to either d 0 or 7 after breeding. de Los Santos-Valadez and co-workers, (1982) reported that up to 60 % of the nonpregnant cows had extended cycles lasting more than 24.5 d compared to 24 % in cows not receiving the hCG treatment. The extensions in cycle length, which range from 6 to 15 d, are consistent with previous reports (Wiltbank *et al.*, 1961; Morris *et al.*, 1976; de Los Santos-Valadez *et al*, 1982).

It has been suggested that CL exposed to hCG function beyond their normal lifespan (Wiltbank *et al.*, 1961) and this has been the premise for the use of hCG to augment the function of subnormal CL in cattle. We noted from our studies (Sianangama and Rajamahendran, 1992) that there was no difference with regard to the number of d to the physiological and structural regression of CL between animals treated with hCG compared with untreated control contemporaries. Furthermore, among cows with hCG-induced CL, both the induced and spontaneous CL regressed simultaneously. This suggested that among animals that either do not conceive or fail to maintain their pregnancies, the extensions were not mediated by CL since both induced and spontaneous CL regressed simultaneously and on schedule (d 21 - 24). Notable, too, was the observation that estrus expression among hCG treated animals was markedly suppressed (Sianangama, 1990, unpublished observations).

A retrospective evaluation of a recent study (Sianangama and Rajamahendran, 1991) suggested that alterations in follicular turnover among hCG-treated cows, which were inseminated approximately 12 h after standing estrus, might predispose the observed extensions in cycle length. However, cycles in animals that were inseminated at estrus and treated with hCG at specific times relative to estrus, can be extended by a number of factors. These include the effect of antiluteolytic factors elaborated by the conceptus during early pregnancy. The removal of the bovine conceptuses from the uterus on d 17 extends the cycle to d 25 ± 1.2 compared to a control

cycle of 21 d (Northey and French, 1980). Proof that the conceptus predisposes the observed extension is provided by evidence showing that the intrauterine infusion of embryonic homogenates between d 15 - 17 extends both the interestrus interval and the lifespan of the CL (Northey and French, 1980). Taken together, both our results (Sianangama and Rajamahendran, 1991) as well as that presented by others (Northey and French, 1980) suggest that the observed extensions in cycle length could be attributable to either alterations in follicular dynamics or factors of embryonic origin.

The objective of this study was to investigate the effect of hCG administered 7 d post estrus on follicular dynamics in lactating dairy cows which are not inseminated.

6.3.   MATERIALS AND METHODS

6.3.1.   Animals And Materials

Regularly cycling postpartum cows ranging from 3 to 6 yrs of age, were randomly selected from the dairy herd at the University of British Columbia. They were housed in a closed free stall barn and received a standard dairy ration of alfalfa cubes and a barley/Canola based concentrate adjusted to meet requirements for milk production. Human chorionic gonadotropin (hCG, APL®) was purchased from Ayerst Laboratories (Montreal, PQ., Canada). Heparinized Vacutainer tubes were purchased from Becton Dickinson (Vacutainer Systems, Rutherford, NJ, USA). Progesterone (P_4) kit (Coat-A-Count®) was purchased from Diagnostic Products Corporation (Los Angeles., CA, USA).

6.3.2. Treatments And Blood Sample Collection

At standing estrus (d 0), 12 lactating and cycling cows were randomly assigned to either serve as untreated control (n = 6) or receive a single intramuscular hCG (1000 IU) injection on d 7 post estrus (n = 6). Blood samples were collected into 8 ml heparinized vacutainer tubes and plasma was harvested within 30 min of collection. Plasma samples were stored at -20°C until assayed for P_4 concentration as described under section 2.3.5. The intraassay and interassay coefficient of variation ranged from 2 to 6 and 9.86 to 11.0 %, respectively.

6.3.3. Ultrasonography

A real time linear-array ultrasound scanning device (model LS 300, Tokyo Keiki Company Limited, Tokyo, Japan) equipped with a 5-MHz transducer was used to characterize follicular and CL dynamics. Ultrasonography was conducted as previously described (section 2.3.4) modified for this experiment as noted below. Ultrasound scanning was conducted daily, starting at observed estrus until the subsequent ovulation was detected. The ovaries were located and uterine tone determined by rectal palpation prior to insertion of the transducer. The ovaries were scanned in several planes and follicles equal to or greater than 4 mm in diameter were individually identified daily at each ultrasound scanning. Optimum images were frozen and size of CL and follicle (antral diameter) was measured at the widest poles of each structure using a system of built-in calibrated callipers. Permanent copies of frozen images were made using the Mitsubishi video copy processor.

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6.3.4. Statistical Analyses

Data for follicle and corpus luteum diameter and P_4 concentration were analyzed as a split plot experimental design. The least squares analysis of variance in the General Linear Model Procedure (SAS, version 6.09) was used to test for differences between control and hCG-treated cows. The model used included the following effects; treatment, cows nested within treatment, d after estrus and the interaction between treatment and d after estrus. The cow (treatment) mean square was used to test treatment mean square, the rest were tested using the residual error mean square. Categorical data were analyzed using contingency tables in Chi-square (χ^2) analysis.

6.4. RESULTS

The size of the first wave dominant follicle was similar between hCG and CONT cows ($P > 0.05$). Treatment with hCG was associated with a significant increase ($\chi^2 = 12$, df = 2, $P < 0.01$) in the number of CL among hCG (n = 2.33 ± 0.23; range 1 - 4) when compared to CONT cows in which all cows ovulated only the single dominant follicle at estrus. Among hCG cows, five of six cows ovulated a single follicle in response to the hCG treatment. The sixth cow ovulated both the dominant and second largest follicle of the first wave present when hCG was administered. No difference ($P > 0.05$) was detected between control and hCG-treated cows with respect to the CL (Figure 6.1a) as well as P_4 concentrations (Figure 6.1b).

Profiles of the dominant follicle among control cows and cows treated with hCG are presented in Figures 6.2 and 6.3. Although the emergence of the second wave was advanced among hCG cows, the number of follicles observed on the ovary was significantly higher ($\chi^2 = 8.53$, df = 2, $P = 0.01$) on the ipsilateral (n = 1.94 ± 0.06) compared to the contralateral side (n = 1.50 ± 0.08) relative to the CL in both treatment groups. While 60 % of follicles emerged on

the ipsilateral side among hCG cows, this number corresponded to 57.14 % among CONT cows. Single follicles (> 10 mm) present on the ovary were more often detected on the contralateral side. As the number of follicles detected increased, there was a tendency for these to be located ipsilateral to the CL. The frequency of follicles located ipsilateral to the CL, when occurring as single, 2 or 3 follicles were 41.18, 63.77 and 100 %, respectively. Among hCG-treated cows, these percentages corresponded with 13.33, 75.56 and 11.11, respectively. In control cows, there were 44.44 and 55.56 percent of follicles occurring as one or two present on the ovary. No control cows had more than two follicles on the CL-bearing ovary. After the hCG treatment, there were more follicles (χ^2 = 16.71, df = 2, P < 0.01) greater than 10 mm which remained visible during the study period among hCG compared to CONT cows.

There was no difference (P > 0.05, Table 1) with regard to the d of emergence of the second wave between hCG (d 10.8 ± 0.3) and CONT cows (d 12.7 ± 1.4). This follicle was maintained for a longer period (13.0 ± 1.8 vs 9.7 ± 2.1 d) and was larger (19.6 ± 3.4 vs 15.7 ± 4.8 mm) in hCG treated cows. The second wave dominant follicle had a slower growth rate (0.83 vs 1.31 mm/d) among cows treated with hCG compared to control. However, the same follicle was the ovulatory follicle in 5 control cows, but only in 3 cows treated with hCG. The ovulatory follicle among hCG-treated cows took longer to ovulate (36 h) compared to CONT cows (10 h) after P_4 concentrations reached a nadir of 0.05 ng/mL. The dominant follicle from the third wave ovulated in one control and three hCG treated cows.

There was no effect (χ^2 = 4.46, df = 6, P = 0.62) of hCG with regard to the number of d to regression of the CL between hCG (d 18.8 ± 1.2) and to CONT cows (d 19.3 ± 2.7). Similarly, there was no difference (χ^2 = 3.09, df = 6, P = 0.8) with regard to the number of d to ovulation between hCG and CONT cows (Table 6.1).

6.5. DISCUSSION

The classical explanation for extensions in estrous cycle length following use of hCG, administered either at estrus or during the luteal phase, has been that the functional status of the CL is maintained beyond its normal duration. In a recent study, we (Sianangama and Rajamahendran, 1992), demonstrated that both spontaneous and induced CL regress simultaneously and at the normal time. That study showed also that the physiological (drop of P_4 concentration to < 1.0 ng/mL) and structural (decline in size of the CL) regression occurred coincident with control cows that did not receive the hCG treatment. Additionally, we (Sianangama and Rajamahendran, 1991) showed that such extensions in cycle length are probably due to persistence or maintenance of follicles. Such follicles were broadly classified under two categories, namely follicles that were exposed to hCG and failed to ovulate or follicles that emerge after the induced ovulation and are exposed to the high P_4 milieu. Both classes of follicles failed to ovulate following luteolysis. Such an effect was more pronounced when hCG was administered late (d 14) but not early (d 7) in the luteal phase. That study, however, could not exclude the effect of pregnancy as a predisposing factor causing extensions in estrous cycle length.

The present experiment evaluated the effect of hCG in non-bred cows and found no difference in the persistence of spontaneous versus induced CL. This finding is consistent with our previous results (Rajamahendran and Sianangama, 1992). Similarly, the decline of P_4 concentration to less than 1.0 ng/mL was also not different between the two treatment groups. However, a trend was observed for a more rapid and earlier decline in P_4 concentrations among hCG-treated compared to control cows. This is in contrast to P_4 concentration observed among cows which were inseminated and treated with hCG either at or after insemination (Sianangama and Rajamahendran, 1992). The earlier onset of physiological regression might be due to several

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factors. It has been suggested previously that hCG treatment is associated with increase in the number of large luteal cells and a concomitant reduction in the number of small luteal cells in spontaneous CL (Wiesak, 1989; Farin et al., 1988) previously exposed to hCG treatment in vivo. Furthermore, large (but not small) luteal cells are reported to possess receptors to the luteolysin $PGF_{2\alpha}$ (Niswender et al., 1985). Therefore, increasing the frequency of large luteal cells further increases the potential of the CL to be more responsive to $PGF_{2\alpha}$ and might suggest higher sensitivity of hCG-exposed CL to the effect of luteolysins in a nonbred estrous cycle. This increase in the number of large luteal cells and, therefore, potential increase in the sensitivity to $PGF_{2\alpha}$, would explain the premature regression of hCG-induced CL as well as the earlier regression of spontaneous CL in hCG-treated cows. Furthermore, it has previously been demonstrated that if spontaneously formed CL are exposed to hCG during the period of formation (d 0 - 3), estrous cycles and the lifespan of the CL were shorter than normal (Battista et al., 1984). Thus, our observation in this study that treatment of cows with hCG was associated with reduced cycle length is to be expected.

Although not statistically different, the time of emergence of the second wave of follicular growth was advanced in cows developing induced CL. Such a finding is in agreement with the hypothesis that (physiologically) dominant follicles suppress the emergence of new waves of follicular growth (Adams et al., 1993). These authors demonstrated that the removal of the dominant follicle is followed by the resurgence of the subordinate follicle(s) which would have otherwise been lost to the process of atresia. Removal of the suppressive effect of the dominant follicle, by way of induced ovulations, created a permissive environment, thus allowing a new wave of follicles to emerge. The dominant follicle from the second wave, among hCG-treated cows, took longer to ovulate following regression of the CL and decline of peripheral P_4 concentrations to < 1.0 ng/mL. The maintenance of the wave two dominant follicle, which

emerged after the hCG-induced ovulation of the first wave dominant follicle, could be ascribed to the carry-over effects of hCG on the second wave dominant follicle and possible luteinization of the same follicle in three of the cows.

During the normal cycle, LH secretory profiles at the end of the cycle (follicular or proestrus phase) have been described as being typically of high LH pulse frequency, low LH pulse amplitude (Schallenberger et al., 1985). It is not clear from this study how LH profiles during the follicular phase were influenced by the hCG treatment given on d 7 and by the resultant P_4 concentrations during the luteal phase. The fact that the dominant follicle emerging after the induced ovulation took longer to ovulate following the regression of the CL suggests an altered biochemical environment in these follicles. The plausible explanations for this delay in attaining ovulation might relate to an altered P_4:estradiol 17-β (E_2) ratio. These steroids have been shown to act in concert to regulate gonadotropin secretion in the cow (Beck et al., 1976). In fact, recent evidence indicates that P_4 acts by inhibiting or reducing LHRH-induced LH release. However, the ability to reduce the tonic release of LH from the pituitary is an E_2 dependent phenomenon (Baratta et al., 1994). Although half of the cows ovulated the second wave dominant follicle, our observation that this follicle regressed in the other three cows suggests that the maintained follicles may have become atretic. A prerequisite for this is a decline in LH levels brought about by low pulse amplitude profiles characteristic of the diestrus phase (Schallenberger et al., 1985). Such a decline in LH concentrations might be due to the effect of high P_4 during the luteal phase. After luteolysis, however, the LH pulse frequency might have increased to reach the follicular phase secretory characteristics. This inference is consistent with the hypothesis that high LH pulse frequency causes the maintenance of the dominant follicle (Roberson et al., 1989).

Due to the onset of atresia in the second wave dominant follicles, a third wave was

required to provide an ovulatory follicle, hence the observed trend toward more cows exhibiting three waves of follicular growth among hCG treated cows. The 24 h extension in time to ovulation observed in this study is consistent with previous reports suggesting that approximately 4 - 6 d are necessary for a new wave of follicles to occur (Sirois and Fortune, 1988) and attain physiological maturity. This need for a third wave may explain the extension in the estrous cycle length and is in agreement with previous reports (Ginther *et al.*, 1989) that have demonstrated that three-wave cycles are associated with longer luteal phases. Nonetheless, such a delay in the expression of estrus is less than what has been reported when hCG was given later in the luteal phase of the cycle (Wiltbank *et al.*, 1961; Morris *et al.*, 1976; Sianangama and Rajamahendran, 1992). Fewer antral follicles larger than 10 mm in diameter underwent atresia following treatment with hCG.

Follicles observed in this study were more often located on the CL-bearing ovary and the frequency of this phenomenon was higher among cows treated with hCG. One plausible explanation for the preferential orientation of follicles, with respect to the location of the CL, is the increase in the blood supply to the ovary bearing the CL (Ford and Chenault, 1981). This observation is further supported by our finding that there were more follicles developing among hCG-treated cows. The precise mechanism by which this preference of follicular development for the ipsilateral ovary still remains to be elucidated. It could, however, be due to the readily available precursors for steroidogenesis in the blood supply to the CL-bearing ovary.

6.6. CONCLUSIONS

The current study found alterations in follicular dynamics among cows treated with hCG. The emergence of the second wave was advanced among hCG-treated cows compared to control cows. The treatment was associated with increased follicular development, more so on the ovary bearing the CL. There was no difference in cycle length, although there was a trend towards earlier luteal regression among hCG-treated cows. The interval to ovulation following luteolysis was delayed among cows treated with hCG. These changes might be predisposed by alterations in the LH secretory pattern caused by increases in P_4 concentrations following treatment with hCG. Further studies are needed to examine the physiological status of the second wave dominant follicle as well as the temporal association between P_4 profiles and LH following treatment with hCG.

Table 6.1. The effect of human chorionic gonadotropin (hCG) on the incidence of accessory corpus luteum formation, characteristics of follicular waves and cycle length among lactating Holstein cows.

Characteristics	Treatment	
	hCG (n = 6)	CONT (n = 6)
Cows with accessory CL (n)	6	0**
Number of CL (range)	1 - 4	1
Number of waves (n)	2.50 ± 0.23	2.14 ± 0.14
Emergence of second wave (d)	10.80 ± 0.26	12.70 ± 1.44
Time to ovulation from emergence of wave-2 (d)	13.01 ± 1.80	9.71 ± 2.11
Time to low P_4 conc. (< 0.1 ng/ml)	21.83 ± 0.54	23.29 ± 1.04
Time to ovulation (d)	23.33 ± 1.45	23.71 ± 1.21***
Cows ovulating wave-2 follicle (n)	3	5

** $\chi^2 = 12$; df = 2; P < 0.01
*** $\chi^2 = 3.09$; df = 6; P = 0.8

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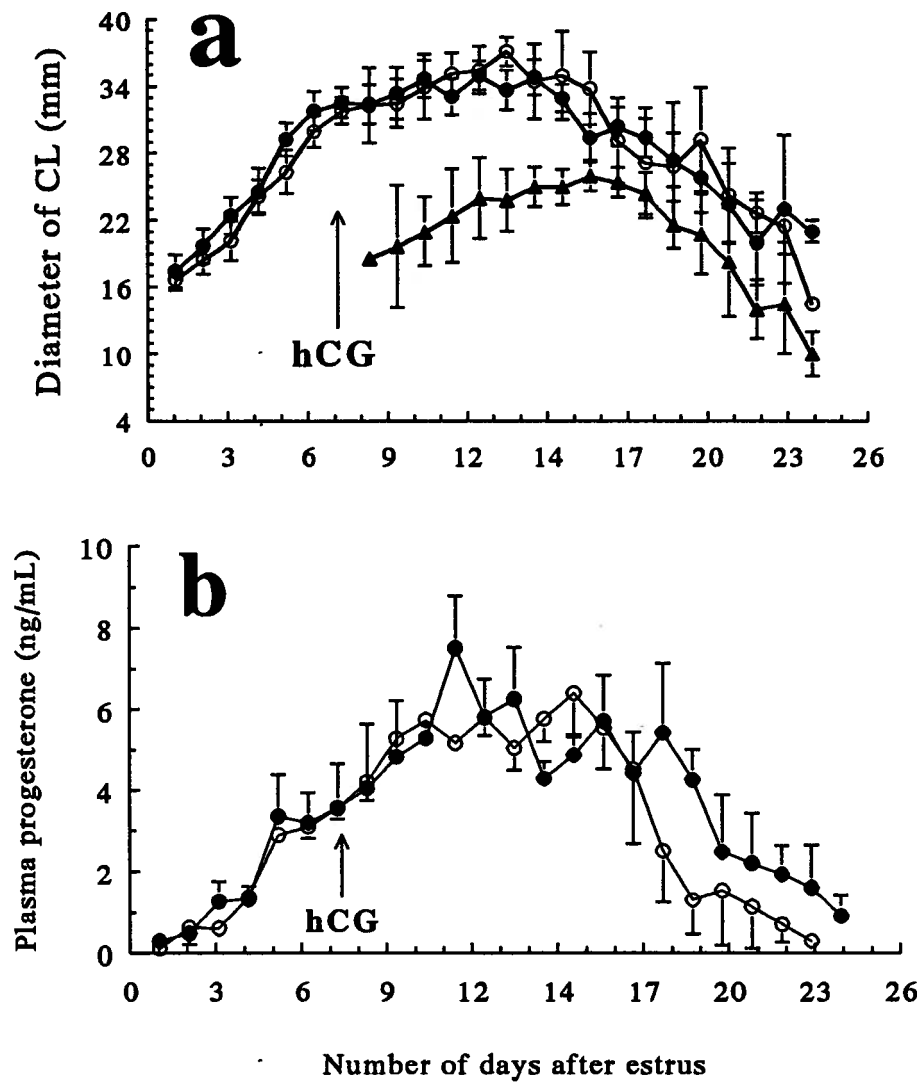
Figure 6.1. Least square means (± SEM) of spontaneous corpus luteum diameter (a) among control cows (●) and cows treated with hCG on d 7 (○) as well as well as the diameter of induced CL among hCG-treated cows (▲). Profiles of least square means for progesterone concentration among control (●) and hCG-treated cows (○) are also presented in panel b.
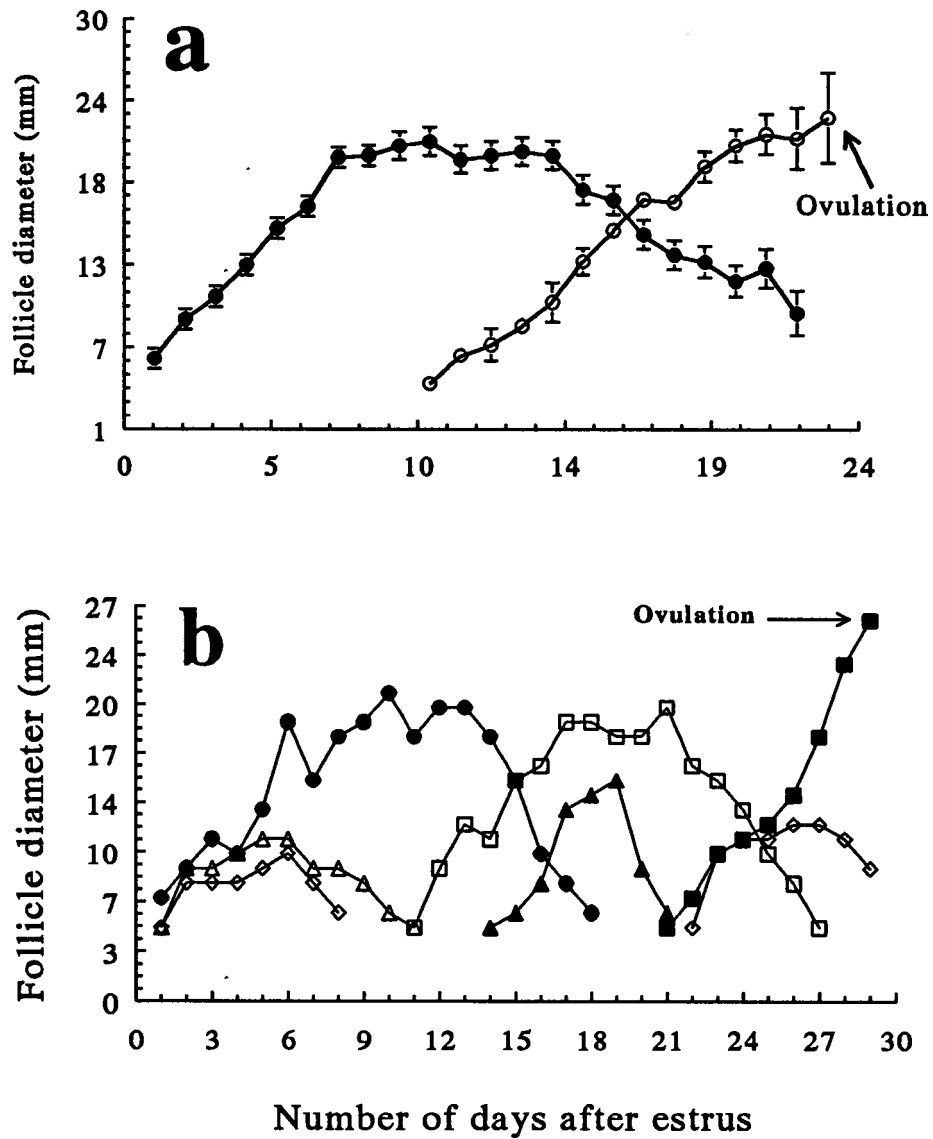
Figure 6.2. Least square means (± SEM) of the diameter of the dominant follicle of the first (●) and second (○) wave among the five control cows that presented a two-wave pattern of follicular growth (a). One control cow had three waves of follicular growth (b).

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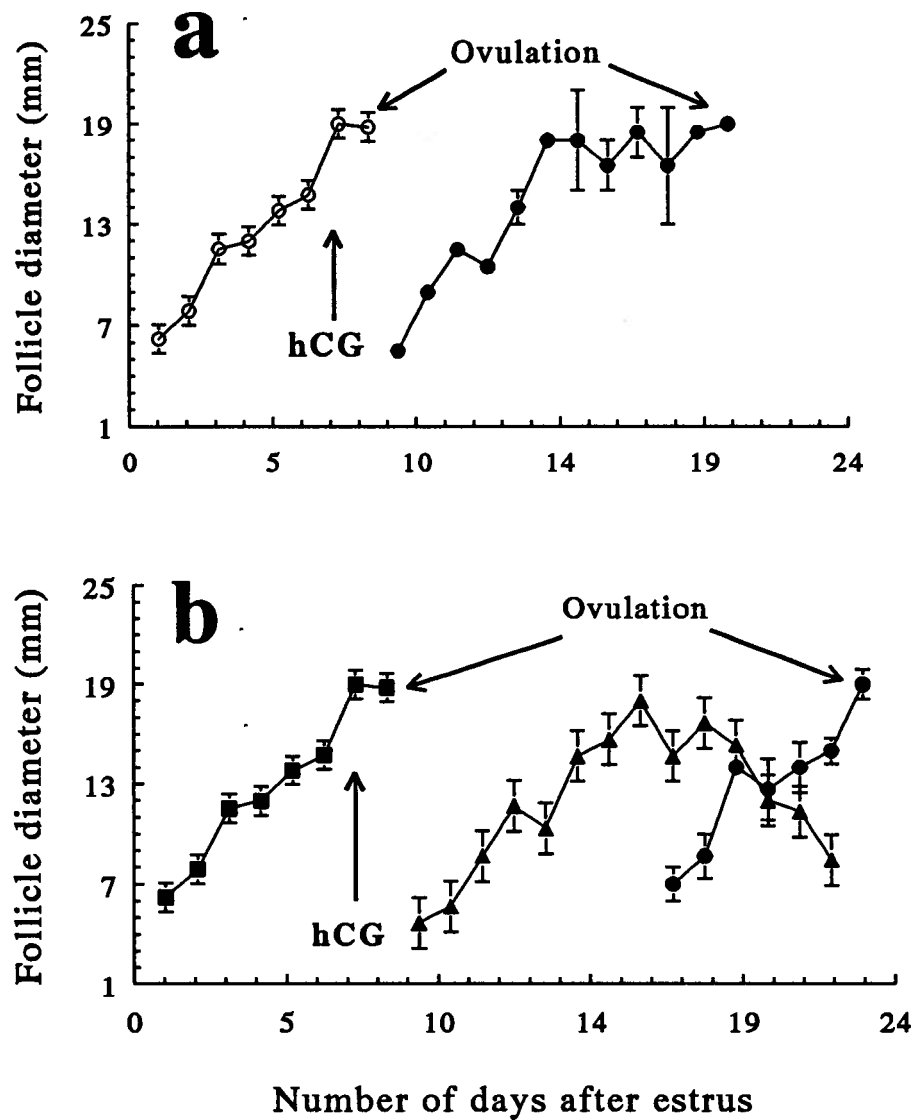
Figure 6.3. Treatment of cows with hCG given on d 7 after estrus resulted in the ovulation of the first-wave dominant follicle. A new wave emerged following ovulation of the dominant follicle. The dominant follicle from the new (second) wave was ovulatory follicle in three cows (a). A third wave emerged to provide the ovulatory follicle in the remainder of the cows (b).

CHAPTER 7

7.1. GENERAL DISCUSSION

The mechanism by which hCG increases pregnancy rates is not clearly understood. Previous studies have clearly demonstrated that administration of hCG induces the development of accessory CL by ovulating the ovulatory-size follicle(s) present at the time of giving the hCG treatment (de Los Santos-Valadez *et al.*, 1982; Price and Webb, 1989). More recent data suggest that the optimum time to administer hCG is 7 d after breeding (Rajamahendran and Sianangama, 1992). These studies revealed that induced ovulations and resultant pregnancy rates are maximal when hCG is given at this time. Available data are, however, inconclusive on the functional status of hCG-induced CL. de Los Santos-Valadez and co-workers, (1982) found no difference in the P_4 concentrations between hCG-treated cows which ovulated the dominant follicle compared to those that did not. Other researchers reported similar results (Sianangama and Rajamahendran, 1992; Rajamahendran and Sianangama, 1992). These results when taken together, raises questions as to whether or not hCG-induced CL are functional and if so at what level in comparison to spontaneous CL of similar age. There is no question, however, that the CL induced by hCG given during the luteal phase remains *in situ* for the duration of pregnancy (Greve and Lehn-Jensen, 1982). Our own studies (Rajamahendran and Sianangama, 1992) demonstrated that these structures are visible as late as 80 d after breeding. Also, the increased pregnancy rates following hCG would have increased P_4 production from spontaneous CL.

The series of experiments outlined in this thesis attempted to answer these questions along with investigating whether extensions in estrous cycle length observed in hCG-treated cows are due to alterations in follicular dynamics. Clearly hCG-induced CL secreted less P_4 under *in vivo* conditions. Additionally the lifespan of hCG-induced CL (13-15 d) was considerably shorter than

the normal lifespan of spontaneous CL (22-24 d; Sianangama *et al.*, 1994a). This finding is probably to be expected because the secretory profiles of the luteolysin $PGF_{2\alpha}$ are typically luteolytic (Moore *et al.*, 1986). This inference is supported by the finding that in at least one cow, the hCG-induced CL regressed within 2 d of surgical removal of the spontaneous CL. Such a finding is not surprising because the regression of the CL in this particular cow occurred coincident with two peaks of PGFM concentrations, both at luteolytic levels. Thus, it is reasonable to suggest that the subnormal function observed in the *in vivo* experiments was predisposed by $PGF_{2\alpha}$ production which has been reported (Moore *et al.*, 1986) to be increasing at the time the hCG-induced CL were evaluated in the studies outlined in this thesis.

However, during pregnancy luteal sensitivity to $PGF_{2\alpha}$ is significantly lower than observed in nonpregnant cows. The decreased sensitivity to the luteolysin is believed to be due to a mechanism that includes a reduction in the number of high affinity luteal receptors to $PGF_{2\alpha}$ as well as a reduction in the peripheral concentration of $PGF_{2\alpha}$ (Moeljono *et al.*, 1977; Silvia and Niswender, 1986; Gadsby *et al.*, 1993). Such a decreased sensitivity to $PGF_{2\alpha}$ serves to protect the CL from regressing and thus ensures a continued secretion and availability of P_4 which is essential for the maintenance of pregnancy. The failure to show any improvement in the function of hCG-induced CL following intrauterine infusion with indomethacin may simply reflect the fact that basal concentrations of PGFM were not significantly reduced. Interestingly, some studies suggest that basal $PGF_{2\alpha}$ is actually increased during early pregnancy (Williams *et al.*, 1983; Thatcher *et al.*, 1984), despite the absence of the high-amplitude surges of $PGF_{2\alpha}$ commonly observed at luteolysis in nonpregnant animals (Kindahl *et al.*, 1976). Clearly, the mechanism by which the CL is protected during pregnancy still remains poorly defined. However, the observation in this thesis that the hCG-induced CL regressed prematurely in response to the endogenous $PGF_{2\alpha}$ when taken together with reports suggesting increased $PGF_{2\alpha}$ concentrations

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during pregnancy, further confirms the hypothesis that the CL of pregnancy is rescued by factors, some of which are of embryonic origin. Evidence clearly shows that developing conceptuses rescue the CL by either reducing $PGF_{2\alpha}$ synthesis as well as by changing the mode of secretion from being characteristically endocrine to being exocrine in nature (Bazer and Thatcher, 1977). Furthermore, data are now accumulating in support of a direct role for the conceptus in the rescue of the CL, specifically developing blastocysts secrete interferons (INFs) of the $-\alpha_{II}$ or $-\omega$ subfamily (Flint et al., 1991). The infusion of embryonic homogenates (Northey and French, 1980), or secretory proteins obtained from the in vitro culture of embryos (Martal et al., 1979) improved luteal function and extended luteal lifespan. Thus, the CL is destined to regress if challenged with the luteolysin $PGF_{2\alpha}$ at an appropriate time during the luteal phase. This effect of $PGF_{2\alpha}$ is negated if a conceptus is present in utero. Because the cows used in these experiments were not inseminated, it seems reasonable to suggest that the subnormal function exhibited by hCG-induced CL was due to the luteolytic effect of $PGF_{2\alpha}$.

That the subnormal function observed in vivo can be ascribed to the luteolysin is further supported by the finding that the same hCG-induced CL secreted more P_4 in vitro, once removed from the unconducive endogenous environment. Additionally, the finding that hCG dose-dependently increased P_4 production is not in agreement with studies suggesting a down-regulation of the hCG receptor (Kirchick et al., 1983) but is consistent with others who found no difference with regard to the number of LH receptors in animals with subnormal CL function (McNeilly et al., 1981; Smith et al., 1986). That forskolin, and not dbcAMP or cholesterol, dose-dependently increased P_4 production only among hCG-induced CL is an indication that the endogenous subnormal function could have been mediated at a site proximal to the LH/hCG-sensitive adenylate cyclase and cAMP-dependent signal transduction (Fletcher and Niswender, 1982). Other workers have reported that $PGF_{2\alpha}$ induces functional regression by either decreasing

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the utilization of lipoprotein for P_4 as well as luteal cholesterol synthesis (Pate and Condon, 1989). Furthermore, Caffrey *et al.* (1979) reported that $PGF_{2\alpha}$ may suppress P_4 production by reducing the activity of enzymes involved in steroidogenesis. In addition, studies reported in this thesis found alterations in the frequency distribution of luteal cells. This is the first report citing a differentiation of SLC into LLC among hCG-induced CL in the bovine. Such alterations could explain the higher basal P_4 production observed in this study.

Although the main focus of this study was to evaluate the morphometry and function of hCG-induced CL, the thesis includes data on similar parameters among spontaneous CL exposed to hCG *in vivo*. When these studies were initiated, it was not clear whether the increase in systemic P_4 concentrations following hCG was due to the stimulation of the spontaneous CL or synthesis and secretion by the hCG-induced CL. The finding in the *in vivo* studies that most increases in systemic P_4 concentrations were observed only at specific times (d 15 - 18) is consistent with the observation that systemic P_4 concentrations from spontaneous CL is low (< 0.1 ng/mL) during the first 4 d after ovulation. The times for increased P_4 concentrations reported herein coincide with ds when P_4 begins to increase and reach concentrations > 0.1 ng/mL among newly formed CL. It is reasonable, therefore, to assume that any increase occurring prior to d 15 of the cycle is primarily due to stimulation of the spontaneous CL. Such a view is consistent with the observation that baseline P_4 production was approximately double among d 15 CL compared to d 7 CL induced by hCG. Furthermore, this increased P_4 production is also in keeping with the observation that d 15 CL had more LLC compared to d 7 CL induced by hCG. Large LC are known to secrete as much as 20 times more P_4, on a per cell basis, compared to SLC (Rodgers *et al.*, 1983).

When we (Sianangama and Rajamahendran, 1992) reported that hCG increases pregnancy rates when administered as a single intramuscular injection given at specific times during the

luteal phase, there was a concern that a number of animals failing to conceive did not return to estrus on schedule. This proportion of animals was more prevalent when hCG was given on d 14 than on either d 0 or 7 after breeding. Any animals not returning to estrus on schedule not only deny the farmer an opportunity to re-inseminate the animal, but also represents increased costs through the increased interestrual interval. This is clearly an undesirable trait in any remedial technique intended to assist farmers in reducing losses incurred due to reproductive inefficiency. Because the administration of hCG on d 7 leads to nearly 90 % increase in pregnancy rates and because we have encouraged the use of hCG to be restricted to d 7 after breeding, this thesis attempted to investigate whether the extension in estrous cycle length was caused by alterations in follicular dynamics. This was undertaken in order to exclude the effect of conceptus derived products which are also known to extend the cycle if present *in utero* up to and including d 16 in cattle.

Following the ovulation of the dominant follicle(s) with hCG given on d 7, a fresh pool of follicles emerged earlier than in the untreated control animals. Since the new wave wasn't necessarily a fresh pool of follicles recruited to continue growing, this finding is in keeping with data presented by Adams *et al.* (1993). These workers found that the removal of the suppressive factors from the dominant follicle leads to the resurgence in the growth of the subordinate follicle(s). Such a view is also consistent with the long held hypothesis that the large follicle exerts dominance over its subordinates thereby preventing their continued growth (Ireland and Roche, 1987). Other investigations also revealed that removal of the dominant follicle, by cauterization, delays the regression of the subordinate follicle(s) (Ko *et al.*, 1991). It is perhaps more important to note that the use of hCG to induce ovulation leads to alterations in follicle turnover (earlier emergence of wave-two and an increase in the number of follicles growing on the CL-bearing ovary), although these changes did not increase the cycle length appreciably. In

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fact, the delay to the next ovulation was just 24 h. This is a positive finding if hCG administration at d 7 after estrus is to be encouraged for adoption as a tool for increasing pregnancy rates in cattle.

7.2. GENERAL CONCLUSIONS

Some answers to the initial questions emerged from these studies on the function and morphometry of both hCG-induced and spontaneous CL which were exposed to hCG *in vivo*, as well as to the effect of hCG on follicular dynamics in non-inseminated cows. The conclusions from these studies are that;

1) the *in vivo* data suggest that hCG-induced CL are inherently subnormal in function.

2) the *in vitro* studies showed that P_4 production is significantly higher among hCG-induced as well as hCG-exposed CL.

3) hCG administration is associated with increased differentiation of SLC into LLC both among hCG-induced and spontaneous CL exposed to hCG on d 7 and removed on d 15 after estrus.

4) the apparent hCG-induced diminution in responsiveness of hCG-exposed spontaneous CL to further gonadotropic stimulation is evident up to d 5 but not 7 d after administering hCG.

5) hCG given on d 7 is associated with alterations in follicular dynamics but does not extend cycle length.

7.3. FUTURE CONSIDERATIONS

Some of these observations have not been previously reported and therefore will require confirmation by other laboratories. So far only basal as well as cAMP stimulated P_4 production has been reported by others. Specifically arising from these studies include;

1) where in the steroidogenic pathway is $PGF_{2\alpha}$ influencing the function of hCG-induced CL *in vivo*?

2) can hCG-induced CL alone maintain pregnancy to term among pregnant cows?

3) what are the LH/hCG and $PGF_{2\alpha}$ receptor dynamics among hCG-induced CL as well as spontaneous CL exposed to hCG.

4) does the switch from a predominantly two- to a three-wave cycle represent a direct effect of hCG on follicular turnover? Too few animals were used in this study to warrant a general conclusion on this observation.

5) Does increased P_4 concentration following hCG administration lead to amplification of the embryonic signal at maternal recognition of pregnancy?

CHAPTER 8   LITERATURE CITED

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CHOLESTEROL ESTER(S) CHOLESTEROL

PROGESTERONE PREGNENOLONE
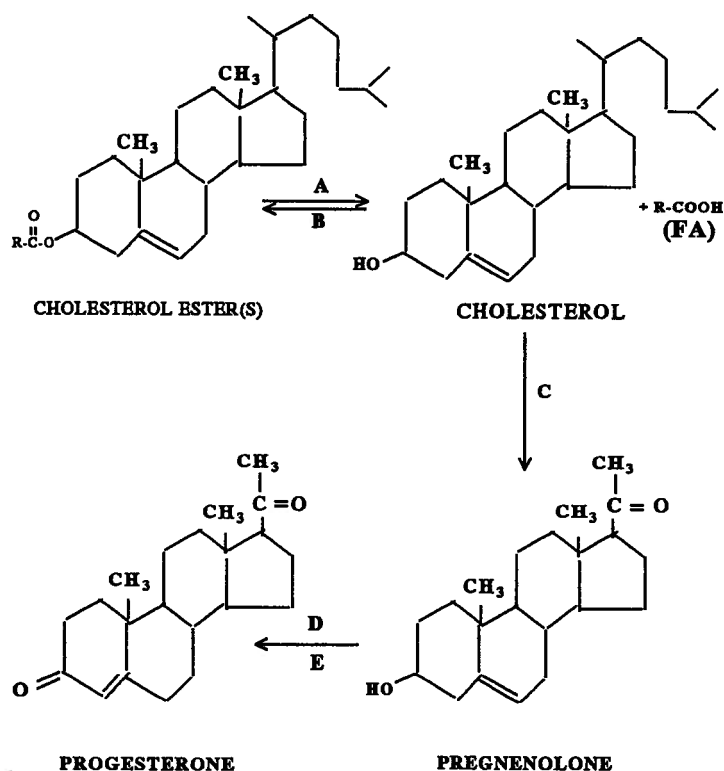
Appendix 1. The conversion of cholesterol esters to cholesterol and fatty acid is catalyzed by cholesterol esterase (A). The process can be reversed by the enzyme cholesterol ester synthase (B). Once formed, cholesterol is transported into mitochondria where it is converted to pregnenolone by the enzyme complex cholesterol side chain cleavage (cytochrome P-450$_{scc}$; C). Pregenenolone is subsequently converted to progesterone by the enzyme complex 3β-hydroxysteroid dehydrogenase (3βHSD)/^{5}Δ→^{4}Δ isomerase (D/E). Progesterone is then released to the systemic circulation by either diffusion or exocytosis.
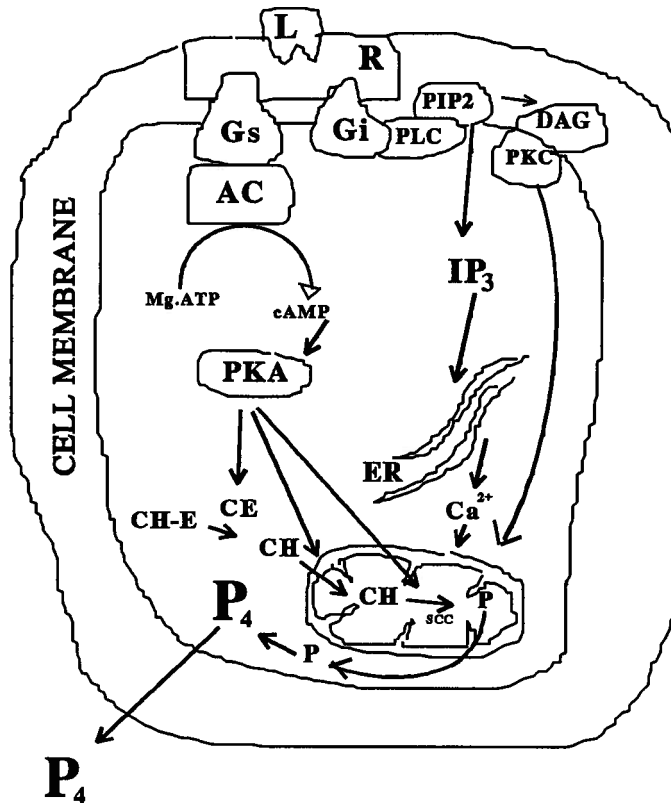
Appendix 2. Steroidogenesis in a generic luteal cell. The binding of ligand (LH, hCG) activates the adenylate cyclase (AC), a step mediated by stimulatory G-proteins (G_s). Once activated, AC converts ATP to cAMP which activates protein kinase A (PKA). An activated PKA is a potent stimulater of the synthesis of several enzymes involved in steroidogenesis, namely cholesterol esterase (CE) which converts the esterified form of cholesterol (CH-E) into free cholesterol (CH). PKA also facilitates the transport of CH into the mitochondria from the cytosol. Another site of PKA action is the activation of the enzyme complex cholesterol side chain cleavage (cytochrome $P-450_{scc}$; SCC) which generates pregnenolone (P) from CH. Pregnenolone is converted to progesterone (P_4) by a step catalyzed by 3β-hydroxysteroid dehydrogenase (3βHSD)/$^5\Delta \rightarrow ^4\Delta$ isomerase. Alternatively, $PGF_{2\alpha}$ binds to a specific receptor and activates phospholipase C (PLC) formation by a step by inhibitory G-proteins (G_i). Phospholipase C hydrolyses phosphatidyl inositol 4,5-biphosphate (PIP_2). This results in the formation of inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). Inositol-P_3 mobilizes calcium (Ca^{2+}) from the endoplasmic reticulum (ER) by binding to its receptor. Calcium is also recruited from extracellular sources by opening of calcium channels. DAG, on the other hand, activates protein kinase C (PKC) which suppresses P_4 synthesis. Collectively, an increased intracellular Ca^{2+} and the generation of PKC lead to the suppression of P_4 synthesis.