STUDIES ON OVARIAN GnRH-R AND GnRH mRNA, AND THE DIRECT EFFECTS OF GnRH ON OVARIAN FUNCTION IN THE BOVINE

by

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ABSTRACT

In the bovine species, available information from a limited number of studies has resulted in contradicting opinions with respect to the intra-ovarian presence of GnRH-GnRH receptor system or direct effects of GnRH on ovarian function. Therefore, experiments were carried out to examine: if GnRH-R and GnRH mRNA are expressed in bovine ovary; the direct effects of a GnRH agonist (buserelin) on steroid hormone secretion from granulosa cells, luteal cells and luteal tissues; the effects of buserelin on mRNA expression for steroidogenic enzymes (StAR protein, P450scc, 3β-HSD) and the apoptotic genes (Bcl2, Bax), and; the effects of post-breeding GnRH administration on corpus luteum (CL) function and pregnancy outcome in Holstein cows.

Results from present study revealed GnRH-R mRNA expression in granulosa cells of small, medium, and large follicles as well as in the CL. The sequence analysis of RT-PCR-amplified products from granulosa cells and CL tissues revealed a complete homology to that of bovine pituitary GnRH receptor cDNA sequence. RT-PCR studies also revealed the possible evidence for presence of GnRH mRNA expression in granulosa cells from different size follicles. Buserelin elicited a dose-dependent biphasic response on E2 production from granulosa cells. A similar trend in P4 secretion from luteal cells and luteal tissues was observed following buserelin treatment. GnRH antagonist alone ($P = 0.07$) or in combination with buserelin resulted in a significant ($P = 0.004$) stimulatory responses on P4 output from CL tissues. In terms of luteal steroidogenic machinery, GnRH-a treatment of luteal tissues showed a mild (nonsignificant) stimulatory response on mRNA levels for StAR protein and

P450scc mRNA although; tendency for significance (P = 0.12) could be seen only in the case of 3β-HSD. Buserelin treatment did not affect mRNA levels of Bax and Bcl2 in CL tissues. In response to post-breeding GnRH administration, despite the slight (P > 0.05) elevation in P4 levels, no improvement in pregnancy rates was observed.

In conclusion, the present findings reveal evidence for the presence of GnRH-receptor mRNA expression in bovine ovarian follicles and CL. Buserelin (GnRH-a) caused a dose dependent biphasic response on steroid output from bovine granulosa cells, luteal cells and luteal tissue. However, the mRNA levels of StAR protein, P450scc, and 3β-HSD observed following buserelin treatment do not provide the definitive evidence for the direct interaction of GnRH with its receptor in the above cell types and tissue. Post-breeding GnRH administration did not result in improved CL function or pregnancy outcome in diary cattle.

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LIST OF ABBREVIATIONS

°C	degrees Celsius
µg	microgram
µl	microlitre
µm	micrometer
3β-HSD	3β-hydroxy steroid dehydrogenase
AI	Artificial insemination
BSA	bovine serum albumen
cAMP	cyclic adenosine monophosphate
CE	cholesterol esterase
CL	corpus luteum
DAG	1, 2-diacylglycerol
DF	dominant follicle
DMSO	dimethyle sulfoxide
DNA	deoxyribonucleic acid
E2	estradiol-17β
FSH	follicle stimulating hormone
g	gram
G	gravity
G3PDH	glyceloraldehyde 3-phosphate dehydrogenase
GAP	GnRH-associated peptide
GDP	guanosine diphosphate
GnRH	gonadotropin releasing hormone
GnRH-a	GnRH-agonist
GnRH-I, II, III	gonadotropin releasing hormone type I, II, III
GnRH-R	gonadotropin releasing hormone receptor
GTP	guanosine triphosphate
h	hour(s)
hCG	human chorionic gonadotropin
HDL	high-density lipoprotein
HDIbp	high-density lipoprotein binding protein
HPO	hypothalamo-pituitary-ovarian axis
IM	intramuscular
INF-t	interferon tau
IP_3	inositol-1,4,5-triphosphate
LD	ladder (molecular)
LDL	low-density lipoprotein
LDLr	low-density lipoprotein receptor
LF	large follicle
LH	luteinizing hormone
LHRH	luteinizing hormone-releasing hormone
MF	medium follicle
mg	milligram
Mg^{+2}	magnesium

MGA	melengestrol acetate
mL	millilitre
mm	millimeter
mRNA	messenger ribonucleic acid
n	sample size or animal numbers
ng	nanogram(s)
P4	progesterone
P450scc	cytochrome P450 side chain cleavage enzyme
PBS	phosphate buffered saline
PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGHS-2	prostaglandin G/H synthase
PIP_2	phosphatidyl inositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PR	pregnancy rate(s)
-R	sample without reverse transcriptase enzyme
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
s	second(s)
SEM	standard error of the mean
SER	smooth endoplasmic reticulum
SF	small follicle
SLC	small luteal cells
SSC	standard Sodium Citrate
StAR	steroidogenic acute regulatory protein
-T	sample without template
UTR	untranslated region.

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CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1. INTRODUCTION

Gonadotropin releasing hormone (GnRH), also known as luteinizing hormone releasing hormone (LHRH), is a vital requirement for the state of homeostasis of reproductive processes. GnRH is a neuronal secreting decapeptide, principally produced by neuronal cells in the medial basal hypothalamus (MBH), and released in a pulsatile manner into the hypophysial portal circulation. Upon binding to its receptors on pituitary gonadotropes, GnRH initiates a series of physiological events leading to the synthesis and release of gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). In a highly coordinated fashion, both the LH and FSH in turn control the processes of gametogenesis and steroidogenesis (Conn and Crowley, 1994; Stojilkovic and Catt, 1995). In addition, it is believed that GnRH may have a role as a modulator of the activity of diverse systems in the brain and many peripheral organs (Hsueh and Jones, 1981; Hsueh et al., 1981; Emons and Schally, 1994). Several reports suggest that there is also an extra-hypothalamic origin of GnRH, as well as the extra-pituitary presence of GnRH receptors (GnRH-R) across different types of tissues in the body (Fig. 1.1).

With respect to the reproductive system, several reports reveal evidence for the presence of GnRH and GnRH-R systems in different organs such as the ovary, oviduct, endometrium, placenta, and testes, across different species of animals, including humans

(Reviewed by Janssens et al., 2000). From both *in vivo* and *in vitro* model studies in rodents, primates and humans, it is becoming increasingly evident that GnRH or its synthetic analogues, could exert direct effect (s) through an autocrine or paracrine manner, eliciting a variety of responses depending on the type of target tissue and physiological conditions (Reviewed by Janssens et al., 2000; Steele and Leung, 2003). Thus, in recent years the extra-pituitary roles of GnRH have attracted special interests in the field of reproductive biology and clinical reproductive medicine. However, there is an apparent paucity of such information with regard to the bovine species. Despite the wealth of accumulated information on multifunctional roles of GnRH and its applications in bovine reproductive management, substantial variability exists in terms of reproductive responses following administration of GnRH or its super-agonist analogues. Hypothetically, the broader perception is that these variations could, in part, be due to the direct effects of GnRH on reproductive tissues (Hsueh and Jones, 1982; Jones and Hsueh, 1982; Gillian et al., 1981; D'Occhio and Aspden, 1999). Hence, there is apparent need for more research and thorough understanding of GnRH or its super-agonist-induced biological effects on the reproductive system in the bovine species. Further, it would be highly intriguing if mechanisms that are reported in other species, can be shown to exist in the bovine species which is more often subjected to GnRH (or its analogues) treatment in day-to-day reproductive management practices. Therefore, due to the apparent lack of information, there is a clear indication that more systematic and in-depth studies are required to explore the intra-ovarian presence of GnRH and GnRH-R system and its possible involvement in modulation of ovarian function and reproductive performance in the bovine species.

1.2. LITERATURE REVIEW

This section provides background information pertaining to this thesis, and is presented in two parts. Part I covers the review on GnRH, GnRH-R, and their different forms, and distribution in different body tissues with special emphasis on reproductive system. Part II covers an overview of the ovarian function in cattle with the main emphasis on applications of GnRH in bovine reproductive management. The rationale for the present study, general hypothesis tested, and the objectives of the study are presented at the end of this section.

PART I

1.2.1. GnRH and its types

The hypothalamic GnRH, which is also referred to as GnRH-I or type one mammalian GnRH (mGnRH), was first isolated and sequenced during the early seventies by Drs. A. Schally, R. Guillemin, R. Yalow and co-workers who became the Nobel Laureates in Medicine, in 1977 (Reviewed by Rivier, 2001). Presently, about 14 structurally variant forms of hypothalamic GnRH are known to regulate reproduction across a wide variety of organisms ranging from yeast to mammals. At least a dozen of these different GnRH forms have been fully sequenced and characterized (Sherwood et al., 1993; Sealfon et al., 1997). All these forms are of classical 10 amino acid peptides, (pGlu-His-Trp-Ser-Tyr-Gly-Len-Ser-Pro-Gly-NH2) with a pyro-glutamyl-modified amino terminus, an amidated corboxy terminus, and conserved amino acids in positions 1, 2, 4, 9, and 10 (Powell et al., 1994). The most widely recognized and common structural variation among the different forms of GnRH resides in amino acids between 5 and 8 in the sequence.

The second type, a midbrain GnRH or GnRH-II was first identified in chicken brain (Miyamoto et al., 1984). Thus, it is also referred to as chicken GnRH-II or cGnRH-II (Millar et al., 2001) and is structurally conserved in species ranging from teleost fish to humans (White et al., 1998). The third type, a telencephalic GnRH, also called type III GnRH or GnRH-III, predominantly exists in the terminal part of the olfactory neuronal cell in the brain (Millar et al., 2001). It is clear that each form has its unique locations within the brain, which suggests a difference in developmental origin and/or adult function (Sherwood et al., 1993; 1994; White et al., 1995; Lescheid et al., 1997; Dubois et al., 2002). Most importantly, the GnRH-II peptide is the one that appears to be predominantly expressed in other tissues such as the reproductive system; thereby, attracting the special interest of today's reproductive biologists. However, the exact function of these non-hypothalamic GnRH molecules is yet to be defined (Lescheid et al., 1997; Gore, 2002; Leung et al., 2003).

1.2.2. GnRH agonists

Besides the multifunctional properties of GnRH, knowledge on peptide sequence and its shorter half-life in general circulation has led to the discovery of synthetic GnRH, super-agonist analogues with greater stability against enzymatic degradation, increased receptor affinity and biological potency (review by Karten and Rivier, 1986). Initially, the original concept of potent GnRH-agonists (GnRH-a) was to treat hypogonadism resulting from insufficient endogenous secretion of GnRH. However, the multidimensional properties of GnRH analogues, and their potential application in reproductive physiology and medicine were quickly realized. Typical structural features that distinguish GnRH-a from the natural sequence of GnRH are substitution of glycine at position 6 of the peptide with a D-amino

acid (e.g. D-tryptophan), and the removal of glycine from the amino terminus. Substitution with a D-amino acid at position 6 increases the half-life of GnRH-a in circulation, and removal of the amino terminal glycine increases affinity for the GnRH-receptor (Karten and River, 1986). Some of the GnRH analogues or GnRH-a that are currently in use for clinical or experimental purposes include: gonadorelin (native-like GnRH; gonadorelin diacetate tetrahydrate or gonadorelin hydrochloride), buserelin (D-serine at position 6 and ethylamide at position 10), fertirelin acetate (ethylamide at position 10) and deslorelin (D-tryptophan at position 6 and ethylamide at position 10).

1.2.3. GnRH receptor and its types

Cloning of the GnRH-R from several mammalian species including the mouse (Tsutsumi et al., 1992), rat (Eidne et al., 1992; Kaiser et al., 1992; Reinhart et al., 1992), cow (Kakar et al., 1993), human (Kakar et al., 1992; Chi et al., 1993), sheep (Brooks et al., 1993; Illing et al., 1993) and pig (Weesner and Matteri, 1994) and a non-mammalian species (catfish: Tensen et al., 1997) has revealed that the GnRH-R is a member of the large super family of seven transmembrane domain receptors that bind to G-proteins. The GnRH-R has seven transmembranal helical domains, characteristic of the guanine protein coupled receptor (GPCR) super family. However, a unique feature of the GnRH-R is the absence of a corboxy-terminal cytoplasmic tail, which is thought to be engaged in desensitization and internalization in other GPCRs. In conjunction with the existence of the other two forms of GnRH, GnRH-II and GnRH-III, it is also suggested that separate and cognate receptor types would also be present in vertebrates (Troskie et al., 1998). The recently discovered Type II GnRH-R from the marmoset and human (Millar et al., 2001; Neill et al., 2001) is shown to be

highly selective for GnRH-II, and is widely expressed in reproductive tissues and in the central nervous system (Millar, 2002). In addition, GnRH-II receptor is expressed in the majority of gonadotropes suggesting it has a role in the regulation of gonadotropin secretion. Contrast to the Type I receptor, the Type II receptor get rapidly internalized and has a distinctly different signaling pathway and preferentially stimulates FSH secretion. This has led to the suggested hypothesis that GnRH-II and the Type II receptor have a specific role in the regulation of the gonadotrope function (Millar, 2002). The localization of the Type II receptor to the brain region is known to affect sexual arousal, and suggests a role in reproductive behaviors. Similarly, the presence of the Type II receptor in reproductive tissues (e.g. gonads, prostate, endometrium/placenta, and mammary glands) suggests an existence of distinct role(s) for type II GnRH molecules in these tissues.

1.2.4. Mode of GnRH and GnRH-R interactions in the pituitary

Upon binding to its receptors on gonadotropes, GnRH activates the Gq/G11 subfamily of G-proteins. This causes an increase in phospholipase C (PLC) activity that results in phosphoinositide breakdown and generation of inositol 1, 4, 5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 releases Ca2+ from intracellular stores whereas DAG activates protein kinase C (PKC). These events lead to the synthesis and release of gonadotropins, LH and FSH (Stojilkovic and Catt 1995). The frequency and amplitude of GnRH release from hypothalamic neuronal cells is a critical and rate-limiting step for the control and maintenance of gonadotropin secretion from pituitary gonadotropes. The GnRH pulse and amplitude changes, in turn, depend on feedback exerted by sex steroids and gonadal peptides produced throughout the reproductive cycle (Shupnik, 1996). Both GnRH-

R synthesis and LHβ synthesis is favored at high GnRH pulse frequencies (one pulse every 30 min), whereas FSHβ synthesis is favored at low GnRH pulse frequencies (one pulse every 120 min) as observed by Kaiser et al. (1997). Further, it is suggested that the modulation of the GnRH pulse frequency enables GnRH to regulate multiple physiological effects that could result in the differential activation of signal transduction pathways, thereby eliciting different cellular processes. This phenomenon was demonstrated by Kaiser et al. (1995) where GnRH-stimulated LHβ promoter activity was at the optimal point of stimulation in coincidence with that of relatively high GnRH-R numbers, while FSHβ promoter activity was optimally stimulated at relatively low GnRH-R numbers. The recent studies by Haisenleder et al. (1997) suggest that in addition to GnRH-R numbers, the modulation of the frequency of intracellular calcium pulses may also play a role in the differential regulation of LHβ and FSHβ mRNA synthesis.

1.2.5. Extra-hypothalamic GnRH and GnRH-R system in reproductive tissues

The first and earliest reports revealing the evidence for the presence of ovarian GnRH-R, or high affinity binding sites for GnRH in rodent species, dates back to the late seventies. Through radioligand binding assays, ligand specific binding sites were demonstrated on granulosa and luteal cells (Clayton et al., 1979, 1980; Harwood et al., 1980; Reeves et al., 1980; Jones et al., 1980; Pieper et al., 1981), oocyte (Dekel et al., 1988; Ny et al., 1987), testicular interstitial tissue (Bourne et al., 1980, 1982), and Leydig cells (Lefebvre et al., 1980; Sharpe and Fraser, 1983; Clayton et al., 1990). These findings were further confirmed in subsequent studies by several other researchers (Latouche et al., 1989; Whitelaw et al., 1995). Expression of GnRH mRNA has been demonstrated in human

reproductive tissues and cell lines (Dong et al., 1993; Dong and Roberts et al., 1996), rat gonads (Oikawa et al., 1990; Bahk et al., 1995), and endometrial cells of human (Raga et al., 1999). In-situ hybridization studies revealed the localization of GnRH mRNA in granulosa cells of primary, secondary, and tertiary follicles in the ovary (Clayton et al., 1992; Whitelaw et al., 1995). Presence of mRNA for GnRH-receptors has also been identified in human granulosa luteal cells (hGLCs) using reverse transcription polymerase chain reaction (RT-PCR) techniques (Minaretzis et al., 1995; Olofsson et al., 1995; Kang et al., 2000; Ramakrishnappa et al., 2003).

In testicular tissue, from both fetal (Botte et al., 1998) and mature rats, and in adult humans, seminiferous tubular cells were found positive for GnRH mRNA, whereas mRNA for GnRH-R was found in interstitial cells, including the Leydig cells (Bahk et al., 1995; Clayton et al., 1990). Recently, using Northern hybridization procedure, three distinctive, different sized GnRH-R mRNA transcripts have been demonstrated in rat and mouse testicular germ cells (Bull et al, 2000). In addition, type II GnRH-R exon 1-containing transcripts were detected by in situ hybridization in human mature sperm, and in postmeiotic germ cells, and were considered to be closely related with spermatogenesis, sperm maturation, and fertilization (van Biljon et al, 2002). Through nucleotide sequence analysis, it was confirmed that both rat and human ovarian and testicular GnRH-R have sequences identical to those found in the pituitary (Kakar et al., 1992; Peng et al., 1994; Moumni et al., 1994; Olofsson et al., 1995; Kottler et al., 1999; Ramakrishnappa et. al., 2003). In parallel, the knowledge of hypothalamic GnRH structure and its physiological concentrations (Nett et al., 1974) or its short half-life in the general circulation (Eskay et al., 1977, Hsueh and Jones,

1981), has led to the idea of exploring the presence of GnRH or GnRH like molecules in gonads. In this regard, several researchers were successful in demonstrating the existence of GnRH-like molecules in Sertoli cells by competitive binding studies and immuno-histochemistry (Sharpe and Fraser 1983; Paull et al., 1981; Bhasin and Swerdloff, 1984). GnRH or GnRH-like molecules have been detected in human follicular fluid (Ying et al., 1981), bovine (Aten et al., 1987a; Ireland et al., 1988) and human ovary (Aten et al., 1987b), in human seminal plasma (Izumi et al., 1985), in testicular interstitial fluid of hCG-treated rats (Sharpe and Fraser, 1980a,b), and in rat germ cells (Paull et al, 1981). It has also been suggested that these molecules are probably being synthesized in the gonads (Oikawa et al., 1990; Sharpe and Cooper, 1982a, b) and in the prostate (Azad, et al., 1993). With growing evidence of the endogenous presence of GnRH-GnRH-R systems in reproductive tissues, intensive studies have been focused on elucidating autocrine or paracrine mechanisms that may exist within reproductive tissues.

1.2.6. Mode of GnRH and GnRH-R interaction in reproductive tissues

In the ovary, GnRH has been shown to elicit a mixed effect of both inhibitory and stimulatory responses affecting ovarian function (review by; Sharpe, 1982; Janssens et al., 2000). GnRH is believed to exert its direct effects either on its own, or in conjunction with other factors such as PGF2α, angiotension II or luteinizing hormone (Fig. 2). Stimulation of one or more signaling pathways such as phospholipase C (PLC), phospholipase A2 (PLA2) and phospholipase D (PLD), or activation of protein kinase C (PKC) causes either inhibitory or stimulatory effects on ovarian cellular steroid output. These dual effects have been clearly demonstrated with *in vivo* experiments in adult male and female hypophysectomized rats

where exogenous GnRH or GnRH-a could either stimulate or inhibit gonadal functions in terms of steroidogenesis (Hsueh and Jones, 1981). In adult male rats, a lower dose GnRH-a administration for a short-term duration was shown to stimulate testosterone secretion (Sharpe et al., 1982), whereas the effect was opposite when the agonist was administered at a higher dose or for long-term durations (Arimura et al., 1979, Hsueh and Erickson, 1979). Similarly, other reports have demonstrated that GnRH modulates, both basal and gonadotropin, stimulated steroidogenesis (Olofsson et al., 1995) in the ovary. The inhibitory action of GnRH, or its agonists, on gonadal steroidogenesis involves suppression of gonadotropin receptors or intermediary enzymes involved in steroidogenic pathway. Reports suggesting GnRH-a induced suppression of FSH and LH receptors (Tilly et al., 1992; Piquette et al., 1991; Guerrero et al., 1993), gonadotropin-induced cAMP levels (Richards, 1994; Knecht et al., 1985), steroidogenic enzyme activity such as peripheral-type benzodiazepine receptor, steroidogenic acute regulatory protein, P450scc enzyme, and 3β-HSD (Sridaran et al., 1999a; Sridaran et al., 1999b) or no effect (Casper et al., 1984) of GnRH on progesterone (P4) production in human granulosa-lutein cells (hGLCs) have been documented.

In the bovine species, similar observations revealed inhibitory effects of GnRH-a, buserelin on P4 secretion from *in vitro* cultured luteal cells (Milvae et al., 1984). *In vivo* studies by D'Occhio et al., (2000) suggest that the suppressed ovarian function in heifers treated long-term with GnRH-a may have been due, in part, to a direct action of deslorelin (GnRH implant) on the ovaries. On the other hand, there are several reports, both from *in vitro* or *in vivo* studies, in rodent, primate and *in vitro* human granulosa cell culture models,

to provide contradicting data on the above mentioned inhibitory effects of GnRH-a at the gonadal level. Liu et al. (1991) reported dose-related stimulatory effects of GnRH-a on aromatase activity and P4 production in monkey granulosa cell cultures. They also demonstrated GnRH antagonist suppression of GnRH-a stimulatory effect on granulosa cell steroidogenesis in culture. Similar reports demonstrating GnRH-a induced steroidogenesis in cultured human granulosa cells have been reported (Ranta et al., 1982; Parinaud et al., 1992; Bussenot et al., 1993). Parinaud et al. (1988) suggest that GnRH-a could modulate steroidogenesis by a direct ovarian action. The agonist, buserelin, increased basal and decreased LH-induced P4 secretion *in vitro*. Guerrero et al. (1993) found an increase in P4 and decrease in estradiol-17β (E2) production, which seemed to be related to a decrease of LH receptor numbers and aromatase activity in GnRH agonist-treated granulosa cells.

In the male gonads GnRH has been shown to cause a direct stimulatory effect on basal steroidogenesis, and an inhibitory effect on gonadotropin–stimulated androgen biosynthesis (Hsueh and Jones, 1982; Hsueh et al., 1984). Short-term *in vitro* treatments of adult rat Leydig cells with GnRH resulted in increased testosterone production (Sharpe and Copper, 1982b; Molcho et al., 1984), while long-term incubation decreased the response to hCG (Browning et al., 1983). GnRH-a or GnRH-like material also revealed positive effects on the testosterone production and spermatogonial multiplication in frogs (Minucci et al., 1986; Zerani et al., 1991). In contrast, it has been shown that high dose or long-acting GnRH-a have a direct inhibitory effect on the testosterone secretion in hypophysectomized rat. In immature and hypophysectomized rats, *in vivo* administration of GnRH and GnRH-a had inhibitory effects on reproductive function (Bambino et al., 1980; Kerr et al., 1986), and

GnRH-a inhibited the basal and LH-stimulated steroidogenesis in rat fetus, and cultured testicular cells (Dufau and Knox, 1985; Habert, 1992). This suggests that GnRH-a has a direct effect on testis without the action of gonadotropin (Arimura et al, 1979). Therefore, physiological functions induced by GnRH or its agonists may be derived from the direct effects of GnRH and/or indirect effects via the variations of gonadotropin and testosterone levels (Botte et al., 1999).

Hormonal regulation of GnRH and GnRH-R mRNA appears to be of crucial importance for elucidating GnRH-GnRH-R system in testis. In adult rats, long-acting GnRH-a (triptorelin) could reduce the levels of both GnRH, and GnRH-R mRNA 24 hrs after injection. However, GnRH-antagonist (antarelix) significantly increased the levels of GnRH-R mRNA 8 hrs after injection, but had no effect on GnRH mRNA expression in rat testis (Botte et al, 1999). The mechanism by which the GnRH-antagonist elevated the levels of GnRH-R mRNA remains to be elucidated. On the other hand, treatment with FSH had no effect on the GnRH and GnRH-R mRNA expression in rat testis, and LH can down-regulate GnRH-R, but not GnRH, mRNA levels(Botte et al, 1999). Administration of testosterone could increase GnRH-R, but not GnRH mRNA levels, and this stimulation was mediated by LH secretion (Botte et al, 1999). Thus, the regulation of GnRH mRNA appeared to be independent of gonadotropins in rat testis, and LH is thought to be a key regulator of GnRH-R mRNA expression. In addition, in hypophysectomized rat, administration of LH diminished the effect of post-hypophysectomy-induced increase of GnRH-R (Bourne and Marshall, 1984). These findings suggest that endogenous regulation in the testis is different from that in pituitary gland where GnRH and steroid regulate GnRH-R expression.

Furthermore, it is suggested that GnRH and its analogs may modulate spermatozoa-zona pellucida binding in humans (Morales and Llanos, 1996). Short time (5 min) exposure of spermatozoa to a 20 nM GnRH, or its agonist buserelin, caused a significant increase in the number of zona bound sperm count (by 300 to 350 fold). This effect was completely preventable by prior exposure of spermatozoa to GnRH antagonist (Morales and Llanos, 1996). These findings suggest that the spermatozoa may interact with GnRH, or GnRH-like molecules, that they may come in contact with during their journey through the male and female reproductive tracts (Morales, 1998; Bull et al., 2000). The interaction may occur: (a) during spermatogenesis by local, intra-testicular production (Hedger et al; 1985; Hsueh and Schaeffer, 1985; Verhoeven and Cailleau, 1985); (b) during sperm maturation in the epididymis; (c) during ejaculation, upon mixing with seminal plasma during transport to the site of fertilization in the oviduct. In the oviduct, the spermatozoa may interact with GnRH secreted locally, or transported by the products of ovulation (follicular fluid, granulose cells) from the ovary (Ying et al., 1981; Aten et al., 1987a, b; Ireland et al., 1988; Oikawa et al., 1990). In addition, it is also suggested that GnRH is involved in the process of fertilization. GnRH-a has been shown to increase the cleavage rate of bovine oocytes *in vitro* (Funston et al., 1995), whereas Casan et al. (2000) demonstrated the presence of both GnRH mRNA and protein expression in the human fallopian tube during the luteal, but not follicular, phase of the menstrual cycle. GnRH-a in supra-physiological or long-acting doses appear to exert an inhibitory effect on each step of spermatogenesis, and it is thought to be mediated by suppressing FSH, LH and intratesticular testosterone levels. Interestingly, GnRH-a or GnRH antagonist, treatment enhanced the regeneration of spermatogenesis from damaged testes in irradiated rats (Meistrich and Kangasniemi, 1997; Shuttlesworth et al, 2000), cryptorchid rats

(Koichi et al., 2002), cytotoxic therapy rat (Meistrich, et al., 1999), and juvenile spermatogonial depletion (jsd) mutant mice (Matsumiya et al, 1999). It is believed that GnRH-a treatment would stimulate spermatogonial proliferation resulting in then regeneration of spermatogenesis. The exact mechanism for this is still uncertain, however, reduced intra-testicular testosterone levels by GnRH-a may play a role since intra-testicular testosterone level was elevated after irradiation or chemical insult (Meistrich and Kangasniemi, 1997). Testosterone is necessary for spermatogenesis, but suppressive to spermatogonial proliferation (Koichi et al., 2002).

Mixed opinion exists with regard to the presence of the GnRH-GnRH-R system and its autocrine or paracrine effect in the mammary system. GnRH mRNA has been detected in the mammary gland of pregnant and lactating rats, but not in that of virgin rats (Palmon et al., 1994; Ikeda et al., 1995). Due to its conspicuous presence strictly during the period of pregnancy or lactation, it was suggested that GnRH expression might be regulated by prolactin (Palmon et al., 1994). It has been speculated that the presence of biologically active peptides in milk suggests that the target of mammary GnRH may be the offspring (Gore, 2002).

1.2.7. Direct effects of GnRH and programmed cell death in the ovary

As in the majority of other cell types in the body, occurrence of programmed cell death or apoptosis in ovarian follicle and luteal tissues has been considered a physiological process that selectively eliminates unwanted cells, whilst maintaining normal physiological processes (Shikone et al., 1996; Yuan and Giudice., 1997). In all the species studied so far,

the initiation of apoptosis in granulosa cells is one of the earliest signs of follicular demise (Tilly and Hsueh, 1993; Juengal et al. 1993; Tilly, 1996). The occurrence of apoptosis in granulosa cells of atretic follicles has been documented based on morphological (Palumbo and Yeh, 1994; Yang and Rajamahendran, 2000a; Saito et al., 2000) and biochemical criteria (Hughes and Gorospe, 1991; Nahum et al., 1996; Manikkam and Rajamahendran 1997, and Yang and Rajamahendran 2000a, b). Evidence suggests a role for GnRH in inducing follicular atresia (Piquette et al., 1991; Billig et al., 1994; Wong and Richards, 1992). During follicular phase, atretic follicles in rats showed a high mRNA expression for GnRH-receptors (Whitelaw et al., 1995). During *in vitro* cultures, GnRH either inhibited DNA synthesis (Saragueta et al., 1997) or induced apoptosis in rat granulosa cells (Billig et al., 1994). Studies have provided the evidence for GnRH-induced remodeling of the extra-cellular matrix by inducing structural luteolysis in superovulated rats. This was accomplished through stimulation of matrix metalloproteinase (MMP-2) and membrane type 1-MMP expression in matured CL, which degraded collagens type IV, type I and III, respectively (Goto et al., 1999). During early pregnancy in the rat, GnRH-a was shown to suppress serum P4 levels, which was associated with an increased degree of DNA fragmentation in the CL (Sridaran et al., 1998; Dharmarajan et al., 1994; Rueda et al., 1995a,b; Rueda et al., 1997). Similar effects of GnRH-a-induced increased number of apoptotic bodies in human granulosa cells (obtained during oocyte retrieval for *in vitro* fertilization) was demonstrated by Zhao et al (2000).

1.2.8. GnRH and reproductive tissue tumors

It has been hypothesized that GnRH may play an autocrine or paracrine regulatory role in the growth of reproductive tissue tumors (ovarian carcinoma and endometrial

carcinomas). Recent studies have shown that GnRH was more abundantly present in ovarian and endometrial carcinomas than in normal ovaries and endometrium (Furui et al., 2002). GnRH peptide expression has been identified in certain tumors including ovarian (Arencibia and Schally, 2000; Kang et al., 2000; Irmer et al., 1995; Ohno et al., 1993), endometrial (Irmer et al., 1994), prostatic (Limonta et al., 1993; Bahk et al., 1998; Lau et al., 2001) and breast cancers (Harris et al., 1991; Kottler et al., 1997). Further, an autocrine growth-regulatory system that attenuates cancer development through direct effect in the ovary and the endometrium, both *in vivo* and *in vitro*, has been hypothesized based on GnRH production and its receptor expression in endometrial and ovarian cancers (Imai et al., 2000; Arencibia and Schally 2000; Emons and Schulz, 2000; Grundker et al., 2002b).

The most recent studies by Grundker et al. (2002b) demonstrated that a second GnRH system exists in primates. Further, the anti-proliferative effects of GnRH-II or GnRH-I agonist on cell cycle and apoptosis were analyzed in endometrial cancer cell lines (HEC-1A and Ishikawa) and ovarian cancer cell lines (EFO-21 and NIH: OVCAR-3), and was shown to be positive for expression of GnRH-II receptor mRNA. The proliferation of those GnRH-II receptor positive cell lines was dose- and time-dependently reduced by authentic GnRH-II, and these effects were found significantly higher than the anti-proliferative effects of equimolar doses of GnRH-I agonist, Triptorelin. Although there is still much research to be done, available evidence suggests that direct anti-proliferative effects of GnRH analogs hold promise as novel therapeutic remedies for the treatment of reproductive cancers (Grundker et al., 2002a; Volker et al., 2002). In summary, there are at least two distinctive types of GnRH that have been fully characterized in mammals, and these seem to be structurally conserved

for over 500 million years. The wide tissue distribution, particularly of GnRH-II, suggests that it may have a variety of reproductive and nonreproductive functions that are yet to be identified (Millar, 2002; Leung et al., 2003). The existence of distinctive GnRH forms suggests the presence of distinctive cognate receptor types in vertebrates; therefore, it will be a productive area of research in coming years. Like both GnRH-I and GnRH-II, GnRH-III may also play a role in the management of autocrine and paracrine effects within reproductive tissues (Grundker et al., 2002a). The existence of additional GnRH systems in brain, pituitary and reproductive organs, as well as different mechanisms of action in these tissues, may contribute to the development of "new generation" GnRH analogues (agonists and antagonists) with highly selective and controlled actions on these different types of receptors. Nonetheless, it is now much easier to delineate different mechanisms of action in these tissues; therefore, target-specific GnRH analogs could be developed in the near future that allow better control over manipulation of various reproductive processes. For example, target-specific GnRH analogs that only act either at the pituitary level, or at the gonadal level, could be developed. Although much remains to be elucidated with regard to the significance of different GnRH-GnRH-R systems in reproductive tissues, it is certain that the future looks promising for the research and development of innovative and novel therapeutic measures utilizing the GnRH system. Further, it is possible that additional roles for GnRH in governing one or more physiological events in the body could be unveiled in the future.

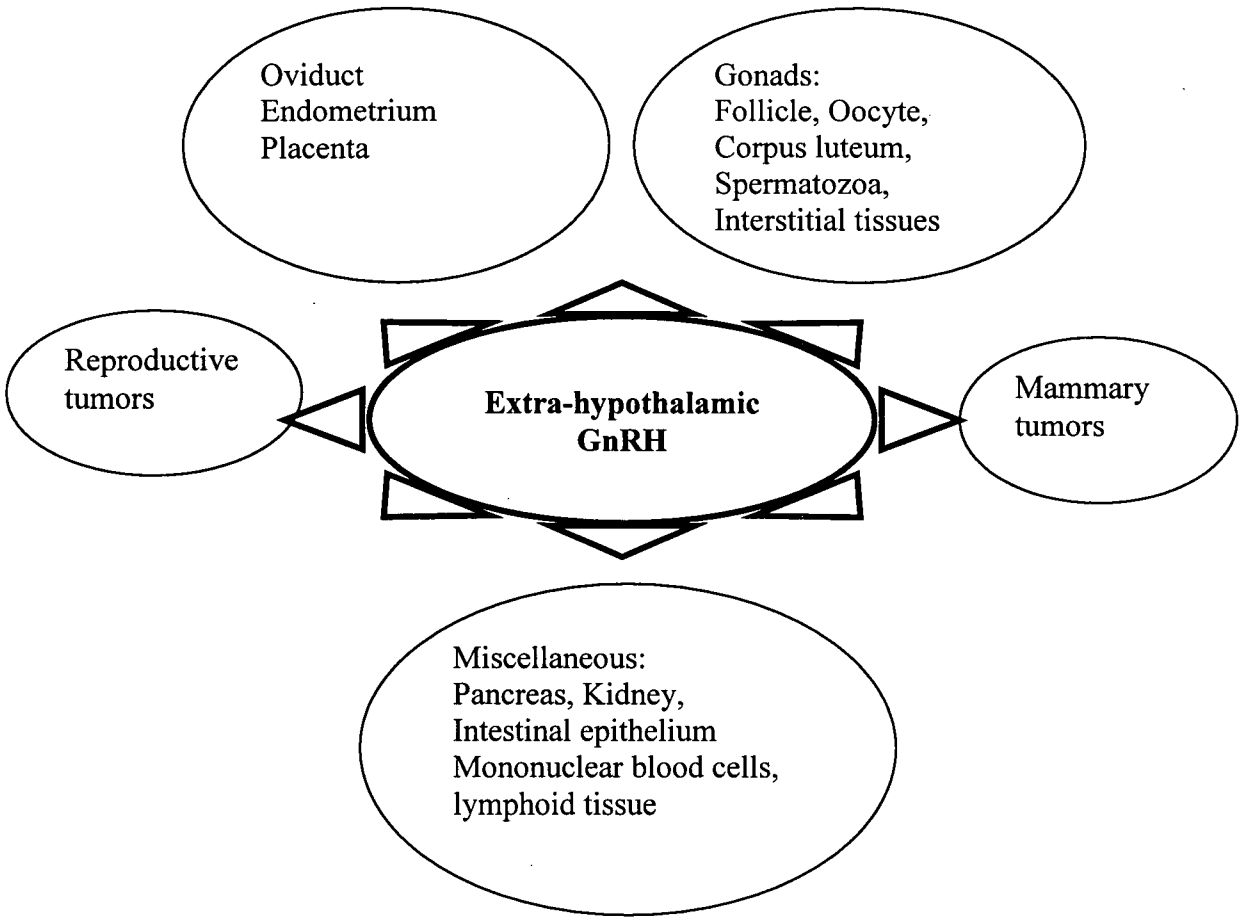

FIGURE 1.1. Extra-hypothalamic GnRH and its possible target sites in different tissues.

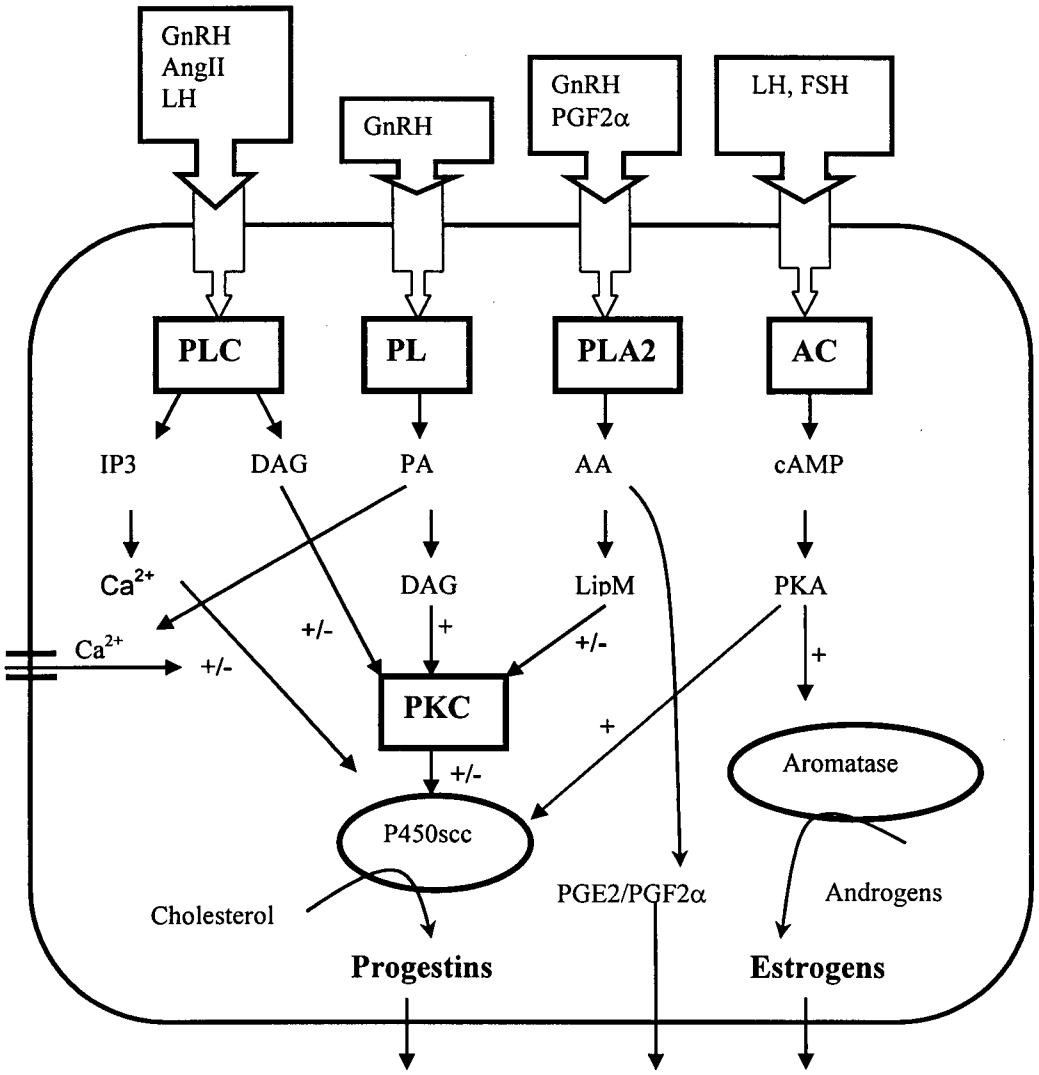

FIGURE 1.2. Hypothetical model of GnRH involvement in stimulation or suppression of multiple signaling pathways in association with various endocrine factors in the ovary. Potential interactions of several of these pathways culminating in the modulation of estrogen, progestins, and prostaglandin (PGE2/PGF2α) production are depicted. AC, adenylate cyclase; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; PLC, phospholipase C; PLA2, phospholipase A2; PLD, phospholipase D; IP3, inositol trisphosphate; DAG, diacylglycerol; AA, arachidonic acid; PA, phosphatidic acid; PKA, protein kinase A; Ca^{2+}, free calcium; PKC, protein kinase C; P450scc, cytochrome P450 side-chain cleavage enzyme. [Adapted from: Steele and Leung, 1993; with modifications].

PART II

Since this thesis mainly focuses on ovarian expression of GnRH and GnRH-R, and the direct influence of GnRH-a on bovine ovarian function, this section provides the overview on ovarian function in cattle. Emphasis has been placed on the current knowledge of applications of GnRH in bovine reproductive management.

1.2.9. Ovarian follicular and CL dynamics in the bovine species

Mammalian folliculo genesis is a highly complex process and only a very small portion of the follicles (~0.1%) survive apoptosis during their primordial follicle stages. In cattle, less than 1% of the 100,000 follicles present at puberty will develop to maturity and ovulate. The main processes involved in the growth and maturation of an ovulatory follicle includes recruitment, selection and dominance of an ovulatory follicle. The advanced technologies, such as a non-invasive ultrasound monitoring of ovarian structures, are an invaluable tool that have enabled researchers to map the follicular and luteal dynamics precisely during the bovine estrous cycle. In a typical estrous cycle of a cow, ovarian follicular growth occurs in two or three waves (Rajamahendran et al., 1994). The first follicular wave begins with the emergence of a cohort of follicles (4-mm diameter) from which a single follicle acquires dominance and continues to grow while the others undergo atresia (Lucy et al., 1992; Savio et al., 1993; Rajamahendran et al., 1994; Ginther et al., 1996). The first wave dominant follicle (DF) remains active until approximately Day 10-11 of the estrous cycle. At this time, dominance of the first wave follicle is arrested, since a recruitment of second wave follicles begins at Day 16 of estrous cycle. The second DF ovulates in a two-wave cycle; alternatively, it may undergo atresia in a three-wave cycle

(Ginther et al., 1996). Under normal circumstances, the ovulatory follicle arises from the final wave; therefore estrus gets delayed since the second DF must regress, and the third DF requires additional time to complete development before ovulation. Cows exhibiting three-wave pattern follicular growth tend to have longer inter-estrous intervals compared with those of a two-wave pattern (Ginther et al., 1989a, b; Taylor and Rajamahendran 1991). The primary functions of ovarian follicles are: 1) to support and nurture a developing oocyte that is capable of being fertilized after ovulation, 2) to secrete steroid hormones that regulate the morphology and function of the reproductive organs, as well as reproductive behavior during estrus, and 3) to provide the precursor cells that will luteinize and form the CL after ovulation (Irianni and Hodgen, 1992).

The CL is a transitory endocrine gland, or a yellowish body developed from a Graafian follicle after ovulation (Reviewed by Niswender et al. (2000). As described by Regnier de Graaf (1641–1673), Corpora (bodies) lutea (yellow) were named by Marcello Malpighi (1628–1694). The yellow color is attributed to the high levels of an antioxidant, β-carotene (Graves-Hoagland et al., 1989). In the bovine species, luteal formation begins with an LH surge resulting in successful ovulation and luteinization of follicular granulosa and thecal cells, which shifts steroid biosynthesis from estrogens to progestins. The follicular cells undergo morphological and biochemical changes that are related to luteinization (Niswender and Nett, 1988). Among the changes are: 1) the breakdown of the basement membrane which separates the theca from the granulosa cells, and 2) invasion of blood vessels into the ruptured wall of the antral follicular space, resulting in the development of an extensive vascular network (Niswender et al., 1994). Blood flow increases with the increase

in the CL weight (Damber et al., 1987). Thus, the complete development of a CL takes about three days (Day 2 to 5 of a cycle). The bovine CL increases in size until mid-luteal stage, at which time it plateaus before decreasing in diameter during the late diestrus stage (Mares et al., 1962). In a non-pregnant cycle (at around Day 16 to 18 after ovulation), or in failure of pregnancy, the CL undergoes luteolysis in response to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) secreted by uterine endometrium (Garverick et al., 1992).

1.2.10. Ovarian steroidogenesis

Steroids are a group of compounds that share the cyclopentanoperhydrophenanthrene ring system of sterols, and are interconverted by specific enzymes in different cells (Review by Hanukoglu, 1992). The two-cell, two-gonadotropin model (Fig. 3) has been proposed to explain ovarian steroidogenic function (Armstrong and Dorrington, 1979). The predominant pathways of steroidogenesis are governed by the specific enzymes produced by the thecal and granulosa cells (Fig. 4). The key enzymes governing follicular steroidogenesis include cytochrome P450 enzymes and hydroxysteroid dehydrogenases. The thecal and granulosa cells differ in the synthesis of cytochrome P450 enzymes, depending on the stage of follicular development. The cytochrome P45017α is expressed by thecal cells at all stages of follicular development; however, granulosa cells do not express cytochrome P45017α (Sasano et al., 1989). The cytochrome P450arom is expressed by granulosa cells (and not by thecal cells), but this expression is low in small antral follicles and increases markedly with follicular growth (Sasano et al., 1989). The cytochrome P450scc is expressed by thecal cells at all stages of follicular development, but cytochrome P450scc is only expressed by granulosa cells following luteinization (Sasano et al., 1989). The steroid environment of

small antral follicles is androgenic; however, as the follicle develops, the steroid environment will become progressively estrogenic, and then following luteinization, P4 synthesis increases markedly.

Progesterone is the primary steroid product of the CL, and it is an essential requirement for the process of embryo implantation and maintenance of pregnancy. Concentration of P4 begins to increase progressively during Day 3 to 12 of the estrous cycle, and then stabilizes until Day 16. Concentrations rapidly decrease following regression of the CL (Hansel and Echternkamp, 1972; Rajamahendran et al., 1976; Niswender et al., 2000). The functional lifespan of the CL is mainly dependent on the balance between luteotropic and luteolytic hormone, LH and PGF2α, respectively. It is a well-established phenomenon that both LH and PGF2α act in two different mechanisms to exert their luteotropic and luteolytic effects on existing CL (Reviewed by Niswender et al., 2000). Upon binding its receptors on the luteal cell membrane, LH causes the synthesis and release of P4 into the general circulation (Fig. 5). The LH-receptor complex activates a secondary messenger system, cAMP, which in turn initiates a cascade of events through a specific signaling pathway called protein kinase-A pathway (PKA pathway). PKA system is known to activate esterase enzymes in the cytoplasm, subsequently processing the conjugated cholesterol into a free form cholesterol. Cholesterol is mainly derived from low-density lipoproteins (LDL) or high-density lipoproteins (HDL) found in the blood (Anderson and Dietschy, 1978; Azhar and Meanon, 1981), acetate or lipid droplets in luteal cells (Hinshelwood et al., 1993; Hansel et al., 1987). It may also exist in unesterified forms in small and large luteal cells (Hinshelwood et al., 1993). Lipoproteins (either LDL or HDL) bind to their luteal plasma

membrane-specific receptors to stimulate the release of cholesterol. The free form cholesterol is further transported into the inner mitochondrial membrane by an acute regulatory protein called steroid acute regulatory protein (StAR). This protein can induce acute changes in the rate of steroidogenesis by mobilizing cholesterol, and subjecting it to the enzymes that are already present. These acute change can happen within matter of minutes. In the mitochondria, a special type of enzyme called cytochrome side chain cleavage enzyme (P450scc), that is present within the inner mitochondrial membrane, converts the free form cholesterol into pregnenolone through cleavage of the cholesterol side chain (Milvae et al., 1996). Further, pregnenolone is released into cytoplasm where another key enzyme, 3β-hydroxysteroid dehydrogenase (3β-HSD), exists in the smooth endoplasmic reticulum, and converts pregnenolone into P4. The resultant P4 is then processed in the Golgi apparatus and released into general circulation (Reviewed by Niswender et al., 2000).

Luteolytic hormone, or its analogues, exerts the cytotoxic effects that eventually kill luteal cells resulting in both functional and structural regression of CL tissue. It is univocal that PGF2α is a potent luteolytic agent that exerts its actions through a specific signaling pathway called protein kinase C (PKC pathway). Upon its activation, PKC is believed to exert its action in one or two ways. Firstly, PKC activation is believed to suppress the production and activity of a key acute regulatory protein, StaR, which is responsible for the transport of free cholesterol from outer mitochondrial membrane into inner mitochondrial membrane. Secondly, a cytotoxic effect alters the cell membrane potential and causes increased intracellular calcium, something that is detrimental to cell survivability.

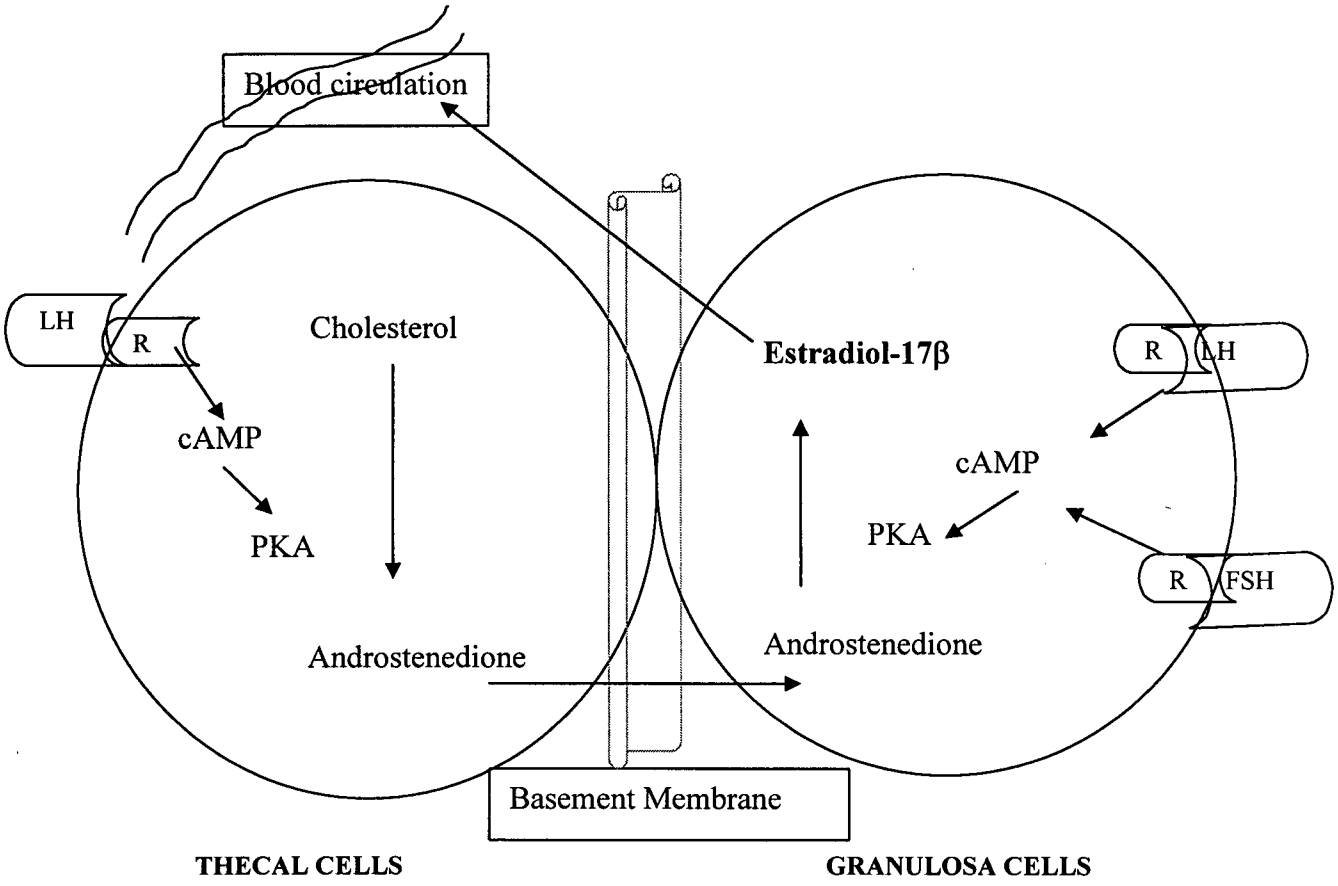

FIGURE 1.3. Diagrammatic illustration of the two-cell–two-hormone theory. Luteinizing hormone stimulates thecal cells to produce androgens, and follicle-stimulating hormone stimulates granulosa cells to produce estrogens from androgens. LH, Luteinizing hormone; FSH, Follicle stimulating hormone, cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; R, receptor.

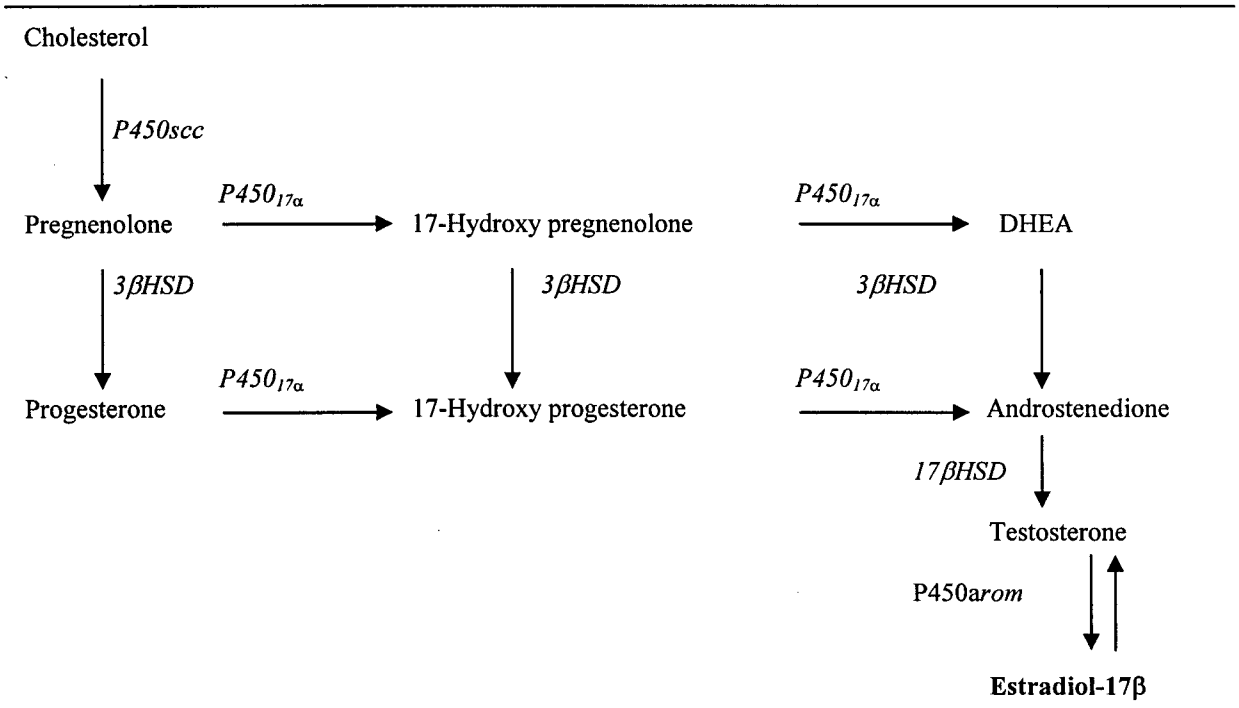

FIGURE 1.4. Follicular biosynthesis of estradiol-17β. The key steps involved in follicular steroidogenic pathways and their respective enzymes are illustrated. P450scc, cytochrome P450 side chain cleavage enzyme; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; P450$_{17\alpha}$, 17α-hydroxylase cytochrome P450; P450arom, cytochrome 450aromatase; DHEA, dihydroepiandrostenedione.

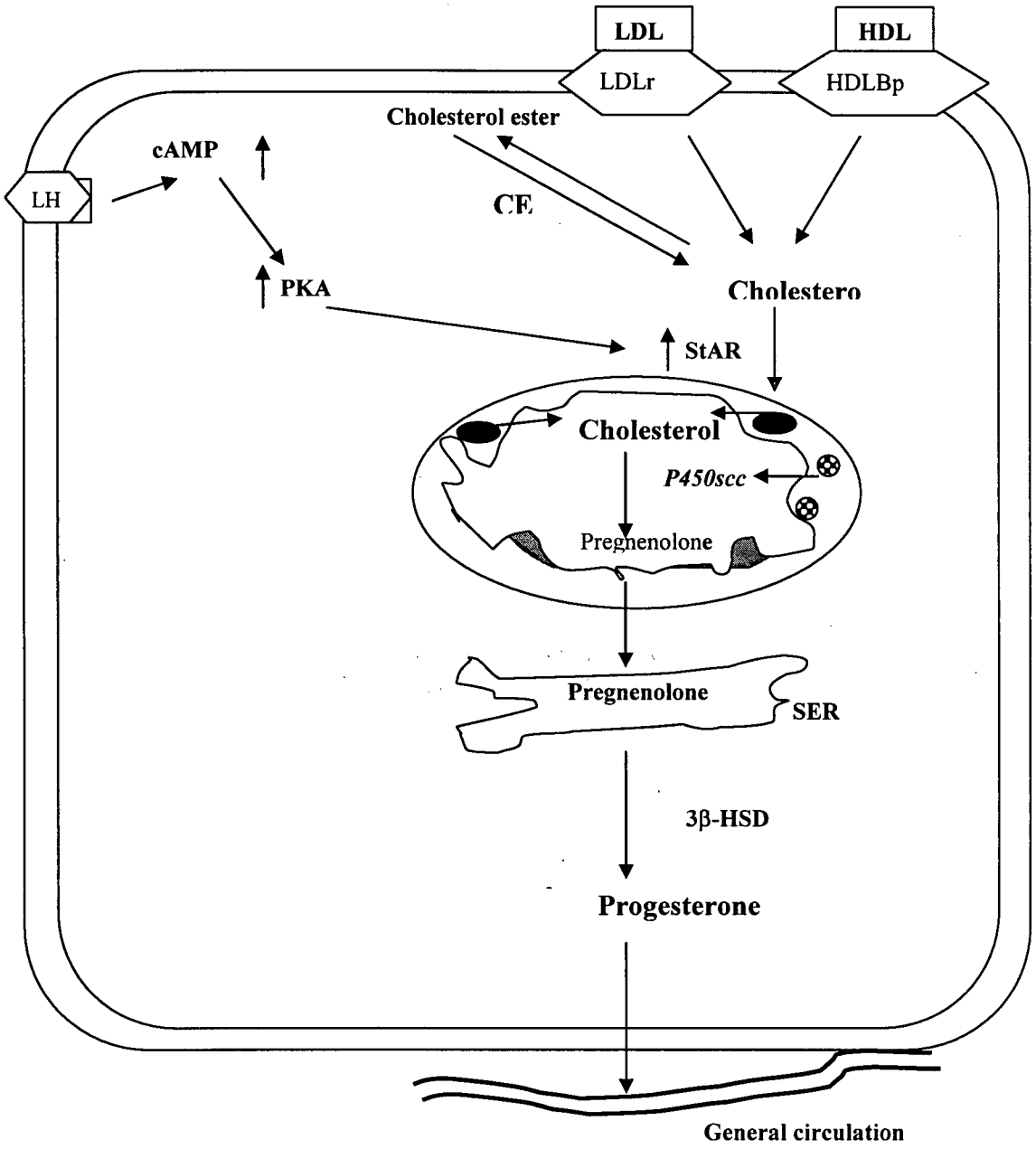

FIGURE 1.5. Schematic representation of intracellular events occurs during progesterone (P4) biosynthesis in CL. Three source of cholesterol can be utilized for substrate: 1) low-Density lipoprotein (LDL), 2) high-density lipoprotein (HDL), or 3) hydrolysis of cholesterol esters by cholesterol esterase. Free cholesterol is transported to inner mitochondrial membrane by the help of steroid acute regulatory protein (StAR). Cholesterol is converted to pregnenolone by cytochrome P450 side chain cleavage enzyme (P450scc). Then pregnenolone transported out of mitochondria, and converted to P4 by 3β-hydroxy steroid dehydrogenase (3β-HSD) in the smooth endoplasmic reticulum (SER). P4 is believed be diffuse out from the cell into general circulation. LH, luteinizing hormone; PKA, protein kinase A; cAMP, cyclic adenosine monophosphate; LDLr, low-density lipoprotein receptor; HDLBp, high-density lipoprotein binding protein; CE, cholesterol esterase. Modified from Niswender et al., 2000.

1.2.11. Applications of GnRH and GnRH analogues in bovine reproduction

Combining the multifunctional roles of GnRH, and the availability of potent, long acting synthetic GnRH analogues broaden the scope for GnRH applications in the field of reproductive biology and medicine. In veterinary practice, the versatile functional properties of GnRH have been exploited to manage a variety of reproductive disorders with varying degree of success (Reviewed by Thatcher et al., 1993; D'Occhio and Aspden, 1999; Rajamahendran et al., 2001). For instance, the acute effect of GnRH causing endogenous release of LH and FSH is an important feature that is being employed in manipulating the ovarian activity. GnRH-induced LH release is associated with numerous physiological effects such as an altered CL function, and ovulation or luteinization of ovarian follicles, induction and development of new follicles. Further, GnRH or its analogues are routinely employed in various treatment regimens including synchronization of estrus and ovulation, induction of postpartum ovulation, and reversible suppression of ovarian activity for an extended periods (D'Occhio and Aspden, 1999). The accomplishment of the later phenomena has become much easier since the availability of slow releasing subcutaneous implants of GnRH analogues. Furthermore, this feature of a chronic effect appears to be a promising tool for the development of safer methods of contraception through temporary suppression of ovarian function. The following paragraphs provide an overview of the roles for GnRH or its analogues in the field of farm animal reproduction, with special emphasis on the bovine species.

1.2.12. Neuroendocrine response to GnRH analogues in cattle

The acute response to GnRH-a, irrespective of dosage, was characterized by increased gonadotropin secretion (Rodger and Stormshak, 1986; Chenault et al., 1990). Chronic treatment with relatively high doses of GnRH-a resulted in the reversible suppression of gonadotropin secretion (Lahlou et al., 1987). The latter was due to the down-regulation of GnRH-receptors on gonadotrophes (Hazum and Conn, 1988; Grospe and Conn, 1988). Knowledge of direct effects of GnRH-a on hypothalamic GnRH secretary pattern in bovine species is limited. However, in rams, treatment with GnRH-a did not influence GnRH secretary patterns (Caraty et al., 1990). It would appear, therefore, that GnRH-a treatment does not influence the activity of hypothalamic GnRH secreting neurons, at least in rams.

The major direct effects of GnRH-a within the reproductive axis appear to be action on pituitary gonadotropes (Hazum and Conn, 1988; Huckle and Conn 1988; Vizcarra et al., 1997). Bulls treated with GnRH-a (nafarelin) for 15 days had reduced pituitary GnRH-receptors (Melson et al., 1986). This was consistent with the classical down-regulation of GnRH-receptors induced by GnRH-a (Hazum and Conn, 1988). However, there are several studies in the bovine species to contradict these findings, where bulls and heifers treated with GnRH-a maintained basal secretion of LH. These results differ from the significant reduction in circulating LH seen in most species during agonist treatment (D'Occhio and Aspden, 1996). Reduced levels of pituitary LH and LH mRNA was demonstrated in intact bulls (Melson et al, 1986) and cows (Vizcarra et al., 1997) treated with GnRH-a. This was associated with a lack of pulsatile secretion of LH but maintenance of basal LH, secretion (D'Occhio and Aspden, 1996; Aspden et al., 1997a, b; Rajamahendran et al., 1998; Davis et

al., 2003). Studies on LH secretary patterns in bulls treated with nafarelin revealed that basal LH secretions in bulls were slightly, but significantly, increased during treatment (Jimenez-Severiano et al., 1998, 2003). Similar increases in basal plasma LH was observed in heifers treated with a long-acting GnRH-a, leuprolide (Evans and Rawlings, 1994), as well as in cows treated with buserelin (Gong et al., 1995, 1996; Schmitt et al., 1996a; Rajamahendran et al., 1998).

GnRH-a-responsive pituitary LH secretion in cattle was further demonstrated using castrated bulls, which naturally have an increased secretion of LH. Although plasma LH was reduced in castrated bulls treated with agonist, basal secretion was maintained, similar to the findings in intact bulls (Aspden et. al., 1996). The understanding that has emerged from GnRH-a studies in cattle, therefore, is that pulsatile secretion of LH is blocked, which is consistent with down-regulation of GnRH-receptors (Vizcarra et al., 1997). However, basal LH secretion is tonically increased (Evans and Rawlings, 1994, Schmitt et al., 1996b; Rajamahendran et al., 1998; Jimeneze-Severiano et al., 1998 and 2003). The exact mechanism(s) that promotes increased basal secretion of LH in cattle treated with GnRH-a is not known. However, it has been hypothesized that the basal secretion could be constitutive and does not require typical second messenger pathways (Huckle and Conn 1988). Alternatively, GnRH-a may stimulate second messenger pathways in cattle to maintain increased basal LH secretion; nevertheless this assumption might be considered inconsistent with the down-regulation of GnRH-receptors in cattle during agonist treatment. Endogenous GnRH is not required for continued secretion of LH in bulls treated with GnRH-a, as bulls

treated with agonist, and simultaneously actively immunized against GnRH, maintained basal secretion of LH (Aspden et al., 1997a, b).

The response of the pituitary to the natural sequence GnRH secretion was reinstated over several weeks after treatment with GnRH-a in bulls (Bergfeld et al., 1996a) and heifers (Bergfeld et al., 1996b). It was not known whether the gradual recovery of pituitary responsiveness to GnRH is related to a gradual replenishment of GnRH-receptors on gonadotroph cells, or to a gradual re-establishment of second messenger pathways within gonadotroph cells (Grospe and Conn, 1988). Consistent with a gradual return to normal pituitary function after GnRH-a treatment, post-pubertal heifers treated with a deslorelin (GnRH-a) bio-implant for 10, 28, or 56 days, ovulated approximately 20 days after the end of treatment (D'Occhio and Kinder, 1995; D'Occhio et al, 1996). Heifers infused with buserelin for 48 days displayed estrus and preovulatory LH surge 8-11 and 22 days after cessation of treatment, respectively (Gong et al., 1996). In young bulls, treatment with leuprolide from 6 to 20 weeks of age delayed the occurrence of a pre-pubertal rise in plasma LH and testosterone by 4 weeks, from 20 weeks to 24 weeks (Chandolia et al., 1997).

1.2.13. Gonadal response to GnRH analogues in cattle

The acute increase in plasma LH that occurs at initiation of GnRH-a treatment (Chenault et al., 1990) can induce ovulation of a growing preovulatory follicle, and can also induce a new follicular wave (Macmillan and Thatcher, 1991). Luteinization, without ovulation, also can be induced by treatment with GnRH-a (Macmillan and Thatcher, 1991; Rettmer et al., 1992). In Brahman heifers, ovulation was induced with GnRH-a administered

on Day 4 and Day 6 of the estrous cycle, but not on Day 2 or Day 8 as reviewed by D'Occhio and Aspden (1999). Similar finding were reported by Schmitt et al. (1996a) and Rajamahendran et al. (1998), where ovulation was consistently induced by treatment with GnRH-a at about Day 5 to Day 6 of the estrous cycle. Long-term and continuous administration of GnRH-a starting from Day 5 of the estrous cycle in cattle has resulted in suppressed plasma FSH levels (Gong et al., 1996), and follicle growth was restricted to early stages of development (<4mm) (Gong et al., 1996; Rajamahendran et al., 1998; D'Occhio et al., 2000).

In prepubertal heifers, treatment with deslorelin for 28 days was associated with increased plasma concentrations of E2, similar to that of increased testosterone secretion in bulls treated with GnRH-a (Bergfeld et al., 1996a). Similar trends were observed with respect to P4 secretion by the CL in heifers and cows treated with GnRH-a early in the estrous cycle (Thatcher et al., 1993; D'Occhio et. al 1996; Schmitt et al., 1996a; Rajamahendran et al., 1998). Increased gonadal steroidogenesis in cattle receiving GnRH-a was thought to be due to maintenance of tonically increased basal LH secretion (Evans and Rawlings, 1994; Schmitt et al., 1996a; Jimenez-Severiano et al., 1998: Rajamahendran et al., 1998; Taponen et al., 1999). Contrary to this assumption, several other authors have shown that GnRH-a administered at different known time points during the estrous cycle elicited different effects on steroidogenic capabilities of the follicle or ensuing CL. It was suggested that the acute increase in LH that occurs when GnRH-a treatment is initiated at the mid luteal phase of the estrous cycle (Day 12-13) causes luteinization of follicles, accounting for the reduced the secretion of E2 (Thatcher et al., 1993; Rettmer et al., 1992). For instance, heifers treated

chronically with GnRH-a failed to initiate an endogenous preovulatory LH, and ovulation did not occur (D'Occhio et al., 2000 and 2002).

1.2.14. Effects of GnRH-a on preovulatory follicle development and CL function

Following luteolysis, increased LH pulse frequency (moderate rise in FSH levels), with concomitant transitory rise in E2 levels, favors the final transformation of granulosa and thecal cells into luteal cells, acquiring enhanced steroidogenic potentials to secrete enough P4 from the ensuing CL. The transient increase in FSH and E2 levels prior to ovulation is believed to play a pivotal role in follicular cell proliferation, and acquisition of de nova LH-receptors before differentiating into fully luteinized cell mass.

Consequent to the advent of the concept of estrus synchronization in farm animals, vigorous efforts are under way to find out an efficient hormonal treatment regimen to induce synchronized follicular development, ovulation and an effective CL formation in cattle. Recent reports from several laboratories have given new hope to effective manipulation of synchronized ovulation leading to an enhanced CL function through usage of GnRH or its synthetic analogues. One such recently developed protocol, referred to as "ovsynch" (Pursley et al., 1995), involving GnRH on Day 0, $PGF_{2\alpha}$ on Day 5-7 and second GnRH on Day 7-9, has been shown to be a promising method of estrus synchronization and ovulation. This method is being widely practiced in timed insemination protocols. However, due to limited knowledge of the multifunctional roles of GnRH or its analogues in the reproductive tissues, it is not yet possible to come up with an appropriate treatment regimen for GnRH or GnRH-a in the field of both human and domestic animal reproductive medicine. Experimental studies

have revealed that while low pulse frequency of GnRH supports FSH synthesis and release. On the other hand, it is not as effective in increasing LH levels. Whereas high GnRH pulse frequency inhibits FSH synthesis and release (Vizcarra et al., 1999). Size of the largest ovarian follicle was greater in heifers treated with GnRH-a (Pursley et al., 1997, and Taponen., 1999 and 2000), which was associated with increased plasma concentrations of E2 (Bergfeld et al., 1996b; Maclellan et al., 1997: Dufour et al., 1999). Conversely, when a second dose of GnRH-a was administered 24 h after the prostaglandin injection, it seemed to cause an LH surge, which stopped the E2 secretion in preovulatory follicle. This seems to occur even though the follicle had not reached the final maturity (Taponen et al, 1999, 2002, 2003).

Administration of GnRH or GnRH-a in the early follicular phase has resulted in an inadequate or defective CL (Lucy and Stevenson, 1992; Murdoch and Van Kirk, 1998; Taponen et al., 1999, 2003). There are more than one theory to explain that consequent to GnRH-a administration, LH concentrations reach beyond their threshold levels causing down-regulation of LH-receptors in preovulatory follicle cells; thus, ensuing luteal defect. Similar postulations indicate lack of FSH priming granulosa cells would fail to acquire aromatizing capabilities; hence decreased E2 levels could lead to decreased granulosa cell proliferation, and thus, the luteal insufficiency (Dieleman and Blankenstein 1984). In heifers, chronic administration of GnRH-a commencing early in the estrous cycle (Day 3) resulted in a larger CL, and secreted more P4 than in untreated heifers (D'Occhio et al., 1997). Similar observations were made when GnRH-a was administered during mid-follicular phase (Murdoch and Van Kirk, 1998, Momcilovic et al., 1998; Taponen, 1999), or early estrous

cycle, resulting in an enhanced CL function (Twagiramungu et al., 1995). Treatment with deslorelin is associated with an increased plasma P4 levels in cattle (Schmitt et al., 1996a and Rajamahendran et al., 1998), which is believed to be attributed in part to the increased size and function of the CL. The CL in heifers treated with deslorelin had a greater content of StAR protein, and the steroidogenic enzyme P450scc, (reviewed in D'Occhio and Aspden, 1999). Treatment of heifers with buserelin early in the estrous cycle caused an increase in the relative numbers of large luteal cells in the CL (Twagiramungu et al., 1995, Schmitt et al., 1996a). On the other hand, heifers treated with GnRH-a from about Day 4 to Day 6 of the estrous cycle can ovulate and develop an accessory CL which may be a contributory factor towards increased plasma P4 (Schmitt et al., 1996a; Rajamahendran and Sianangama, 1992; Rajamahendran et al., 1998). Following insemination, administration of GnRH-a early in the estrous cycle to increase plasma P4 would enhance the likelihood of conception, pregnancy recognition and embryo survival (Ullah et al., 1996).

1.2.15. Effects of GnRH analogues on pregnancy outcome in cattle

A considerable number of studies have examined whether GnRH treatments given at the time of AI are able to improve pregnancy rates in cattle. In most of the experiments GnRH treatment (gonadorelin-native GnRH or buserelin-agonistic analogue) was given at the time of first or second AI. The dosage is generally around 100 - 125 μg for native GnRH and 8-10 μg for buserelin. Generally, these treatment are employed around the time of AI, or during the mid-luteal phase to attempt to establish enhanced CL function. The majority of experimental results show a numerically increased P4 production, and in some instances, pregnancy rate, after GnRH treatment. However, the difference is often statistically non-

significant in comparison with untreated animals. Overall, the studies demonstrate an increase in pregnancy rates in cows treated with GnRH at the time of first AI (Mee et al., 1990; Ullah et al., 1996). However, according to Thatcher et al. (1993), changes in pregnancy rates, in response to GnRH administration at the time of first service of dairy cows, varies from -7 to +17%; therefore, it is difficult to accept that GnRH is eliciting a predictable increase in fertility that can be reliably applied from herd to herd.

Repeat breeding has considerable impact on the economy of dairy farmers. The definition of repeat breeding includes pregnancy failures occurring after three or more inseminations performed at estrus with normal inter-estrous intervals in the absence of detectable abnormalities (Zemjanis, 1980). However, in most studies dealing with the effect of GnRH given at the time of AI in repeat breeders, they were classified as cows that return to estrus for a third or further service. The native GnRH or GnRH agonists are often the choice of treatment to tackle this problem, and this treatment is usually employed at the time of AI, or 12-16 hours before AI (Stevenson et al., 1990). In conclusion, it is suggested that the administration of GnRH and its agonistic analogues at the time of AI would increase pregnancy rates, despite the fact that type of animal, management and physiological status can contribute to the differential fertility responses to GnRH treatments (Thatcher et al., 1993; Ullah et al., 1996; Cam et al., 2002).

In a nutshell, both acute and chronic phases of the LH response in cattle to GnRH-a administration broaden the scope for fine-tuning the practical applications for GnRH analogues in bovine reproduction. The acute increase in plasma LH that occurs at the

beginning of agonist treatment has been an invaluable tool in the treatment of cystic follicles, development of estrous synchronization protocols, and new super-ovulation programs (review by Thatcher et al., 1993; D'Occhio and Aspden, 1999). Further, the exercise of administering GnRH analogues at various time points at AI and embryo transfer to enhance conception rates, is an ongoing effort in anticipation of more precise answers. Based on the facts, the absence of a pre-ovulatory LH surge in animals treated chronically with GnRH-a is a promising feature. It could be applied to the development of long-acting contraceptive methods using GnRH-a bio-implants, which could be used in farm animal practice or for veterinary use (D'Occhio and Aspden, 1999; D'Occhio et al., 2002; Trigg et al., 2001; Munson et al., 2001).

A significant proportion (30 - 40%) of pregnancy failures or infertility problems in cattle, have been attributed to inadequate functioning of the CL (Pursely et al., 1997). Studies from several researchers reveal that animals destined to carry a conceptus following insemination, produce more P4 during the second half of the diestrum period. The opposite is true for those animals destined for conception failure or early pregnancy loss. Most of the conception failures or early pregnancy losses are associated with defective CL function or luteal function, which is due to a lack of adequate luteotropic support following ovulation. Several corrective measures have been developed to combat inadequate CL function in cattle. For example, induction of increased LH plasma concentrations either through direct LH administration or administration of hCG, could exert LH like activity. Efforts have also been made to compensate for luteal insufficiency through exogenous P4 administration. Consequently, they have yielded poor results due to unknown reasons besides practical

difficulties. In spite of continued efforts from several researchers, little improvement has been achieved towards successful treatment of luteal insufficiency in bovine species. Results from several studies has revealed that the administration of GnRH or its agonist analogues, which posses higher affinity for GnRH receptors compared to the endogenous GnRH, yielded a mixed response towards luteal enhancement following ovulation. Due to the versatile nature of GnRH function depending on physiological status of an individual, it is not yet possible to utilize its promising beneficial effects towards luteal enhancement protocols. Supportive results from existing GnRH protocols include administration of GnRH at various time points before and after ovulations, and induction of accessory CL, resulting in increased plasma P4 concentration. GnRH agonists administered either in early diestrum, or in late diestrum period, resulted in successful induction of accessory CL and increased plasma P4 concentration. However, results are often inconsistent, and therefore, the search for suitable time-point(s) for GnRH-a administration during estrous cycle continues.

1.3. RATIONALE FOR THE STUDY

GnRH has an undisputable key role in hypothalamus-pituitary-gonadal axis. As well, accumulated information clearly suggests an importance for the use of GnRH in both management and treatment of variety of reproductive disorders. The availability of potent and long-acting synthetic GnRH analogues have led to an unveiling of diverse roles for GnRH in mammalian reproduction. In addition, there are evidences to suggest autocrine or paracrine roles of GnRH in reproductive tissues. Direct effects of GnRH, or its analogues, have been documented in different reproductive cell types in several species of animals (primates, pigs, rodents), including humans. However, several of those reports, in one or

more of these species, have yielded mixed results on the direct effects of GnRH on ovarian function; therefore, it is a continued topic of research interest. In lieu of these intriguing findings from other species, it would be highly interesting to explore if any such mechanisms exist in the bovine species, where these animals are often subjected to GnRH or GnRH-a treatment during their reproductive life span.

The expected outcome from this study will be a strengthening of our knowledge regarding the direct effects of GnRH-a on ovarian cellular function in the bovine species. In addition, any such knowledge gained through these studies would aid in the rationalization of the usage of GnRH, or it potent synthetic analogues, in both domestic animal and human reproductive management. More importantly, the information gained through these studies will further broaden the basis for additional studies with respect to pro-and anti-fertility effects, as well as therapeutic roles, for GnRH or its analogues in the field of reproductive medicine.

1.4. HYPOTHESIS

As pointed out in the preceding section, literature review, there is an apparent paucity of information with respect to the presence of GnRH and GnRH-R molecules in bovine reproductive tissues, and their possible involvement in modulation of reproductive function(s). Therefore, this thesis proposes the hypothesis in a broader perspective that the bovine ovarian structures (follicle and CL tissue), would express mRNA transcripts for GnRH-R and GnRH, and that the direct effects of GnRH are exhibited through altered

ovarian function in the bovine species. As well, post-breeding GnRH administration would enhance CL function and pregnancy outcome in this species.

1.5. OBJECTIVES

In order to test the above hypothesis, a series of experiments were performed with the following specific objectives:

(a) To explore the possibility of mRNA expression for GnRH-R and GnRH in bovine ovarian structures (follicles and CL) [CHAPTER 2].

(b) To examine the direct effects of GnRH-a on steroid hormone production in granulosa cells, dispersed luteal cells, and CL tissues *in vitro* [CHAPTER 3].

(c) To assess the direct influence of GnRH-a on molecular mechanisms of luteal steroidogenic machinery in the bovine CL [CHAPTER 4].

(d) To examine the direct influence of GnRH-a on apoptotic process in bovine CL [CHAPTER 4] and

(e) To determine the influence of post-breeding GnRH administration on *in vivo* CL function and pregnancy outcome in dairy cattle [CHAPTER 5].

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CHAPTER 2

GnRH-R AND GnRH mRNA EXPRESSION IN THE BOVINE OVARY

2.1. ABSTRACT

This study was undertaken to investigate the mRNA expression for GnRH receptor and its ligand, GnRH, in the bovine ovary. Granulosa cells from small (<4 mM), medium (5-8 mM) and large follicles (>8 mM) and tissues from different staged CL [Stage I (Day 1-4), Stage II (Day 5-10), Stage III (Day 11-17), and Stage IV (Day 18-21, days after ovulation)], were harvested from bovine ovaries collected at a local abattoir. The mRNA isolated from representative samples was subjected to RT-PCR using gene sequence specific primers. The expected sized PCR amplicons were detected through southern hybridization procedure, and the sequence identity (GnRH-R) was confirmed through nucleotide sequence analysis. The results were shown to be positive for the presence of GnRH-R mRNA expression in both follicles and CL. Moderately higher levels of GnRH-R transcripts were evident in granulosa cells from small follicles, whereas slightly lower, but uniform, expression levels were evident in both medium and large follicles. Among different staged CL, GnRH-R transcripts were clearly detectable in stage III luteal tissues, where as the expression levels were slightly lower in stage II, and barely or not detectable in stage I and IV luteal tissues. With respect to GnRH mRNA expression in bovine follicles, preliminary findings from RT-PCR studies revealed the evidence for the possible presence of GnRH mRNA expression in granulosa cells from different staged follicles.

2.2. INTRODUCTION

GnRH is a neuronal secretary decapeptide that plays a central role in reproductive processes through signaling, synthesis and release of gonadotropins, LH and FSH, from the anterior pituitary. Although it is an indisputable fact that hypothalamus and pituitary are respectively the principal source of GnRH and its target sites, recent reports confirm the extra-hypothalamic origin of more than one type of GnRH peptide, as well as the presence of its cognitive receptor types in extra-pituitary tissues in the body. Reproductive tissues such as ovaries, placenta, endometrium, oviducts, and testes have been shown to be positive for the GnRH and GnRH-R mRNA, or functional forms of respective protein molecules, across different laboratory species (rats, pigs, and monkeys) and in humans. Numerous studies have also demonstrated the direct effects of GnRH at the ovarian cellular level (section 1.2.5 and 1.2.6; CHAPTER 1). The earliest report of evidence for the presence of ovarian GnRH receptors was the demonstration of high affinity binding sites for GnRH in rodent species (Clayton et al., 1979; Jones et al., 1980; Pieper et al., 1981; Dekel et al., 1988). Despite initial conflicting reports, subsequent studies in humans have successfully demonstrated GnRH receptor expression in the ovary. Presence of high affinity GnRH receptors on granulosa cells (Latouche et al., 1989), ands low affinity binding sites in corpus luteum (CL) (Latouche et al., 1985), has been confirmed through binding or auto-radiographic studies. Further, the availability of powerful tools in the field of molecular biology has enabled researchers to gain deeper insight with respect to GnRH or GnRH receptor gene expression, and its regulation. The nucleotide sequence of human GnRH receptors cDNA was deduced using the RT–PCR technique (Kakar et al., 1992). Later, similar studies confirmed the nucleotide sequence of GnRH and its receptor in human granulosa cells (Peng et al., 1994;

Minaretzis et al., 1995). Studies on the regulation of the ovarian GnRH receptor mRNA showed that GnRH up-regulates, and hCG down-regulates the GnRH receptor gene expression (Peng et al., 1994). Further, several observations from both *in vivo,* and *in vitro* model studies in rodents, primates and humans, provided evidence that GnRH can affect the fate and function of ovarian cells through its direct action on them (reviewed by Janssens et al., 2000; Leung et al., 2003).

Interestingly, there is an apparent paucity of information with regards to the possible existence of GnRH-R and GnRH system, and its direct involvement in modulation of ovarian function locally in bovine species. Based on only a limited number of studies, the conclusion was drawn that the bovine species lacks the ovarian expression of GnRH receptor mRNA, or its functional protein (Brown and Reeves, 1983; Nett et al., 1987; Kakar et al., 1993). However, there is at least one study that has revealed the presence of GnRH receptor mRNA in bovine cumulus-oocyte complex cells by using RT-PCR technique (Funston and Seidel, 1995). Therefore, there is a clear indication that more studies are needed in order to explore the presence of GnRH-R and GnRH system in bovine ovarian structures, follicle and CL. Hence, the current study was undertaken with the objective of exploring for the presence of mRNA expression for GnRH receptor and its ligand, GnRH, in bovine ovarian structures, follicle and CL.

2.3. MATERIALS AND METHODS

2.3.1. Collection of bovine ovaries and classification of follicles and CL

Bovine ovaries were collected at a local abattoir within 15 to 20 min after exsanguinations. Paired ovaries from each animal were separately packed in whirl pack plastic bags containing ice-cold DMEM/F12 culture medium. Ovaries were transported to the laboratory on ice within 3 to 4-h after collection. Immediately upon arrival at the laboratory, each pair of ovaries was examined carefully, and the existing follicles were classified into different categories according to previously established criteria (Kruip and Dieleman, 1982; Yang and Rajamahendran, 2000). Based on follicle diameter and morphological appearance, follicles were classified as small (<4 mm), medium (5-8 mm) and large follicles (>8 mm diameter). The stage of existing CL was determined and categorized into one of four stages based on their gross appearance, size of the crown, and shape (Ireland et al., 1980). The four stages were, Stage I (Day, 1-4); Stage II (Day, 5-10); Stage III (Day, 11-17) and Stage IV (Day, 18-21), after ovulation. Batches of 6 to 8 pairs of ovaries were obtained during each visit to the abattoir, and at least 5 different pools of granulosa cells from each stage of follicles, and at least 6 different tissue samples from each stage of CL were examined in this study.

2.3.2. Aspiration of follicles and granulosa cell processing

Follicular fluid containing granulosa cells was aspirated from different sized follicles using a 10-mL plastic syringe with 18 gauge needle. Follicular fluid from each category of follicles was pooled and kept separately in heparin-dusted 15 mL Falcon tubes. For small follicles, about 1 mL of sterile phosphate buffered saline (PBS) solution was used in order to

facilitate retrieval of granulosa cells into the syringe. Medium and large follicles were aspirated without using PBS. During aspiration of each follicle, follicular fluid was repeatedly aspirated and expelled into the follicle several times to maximize the recovery of granulosa cells. Tubes containing follicular fluid were held on ice during the period of aspiration. Follicular aspirates were centrifuged at 400 x G for 10 min at 4°C and the supernatants were discarded. Depending on the size of the remaining granulosa cell pellet, 1 to 2 mL of Tri Reagent solution (Sigma-Aldrich Canada Ltd. Oakville, ON) was added directly to the Falcon tubes. Contents were thoroughly mixed by repeated pipetting until the complete dissolution of cell pellet into to a uniform homogenate. At this stage, individual samples were either processed immediately, or stored at –75°C until the next day, for continued with total RNA isolation.

2.3.3. Processing of CL tissues

Upon identifying the stage of each CL, about 200 mg of luteal tissue was harvested from the central portions of each CL mass, and immediately transferred into a 50 mL Falcon tubes containing 2 to 3 mL of Tri Reagent solution. Care was taken to avoid using CL with larger cavities, or from pregnant animals during the collection process. The luteal tissues were then homogenized using a low speed motor-operated tissue homogenization metallic probe. The resultant homogenate mixtures were either used immediately for total RNA isolation, or stored at -75°C for subsequent use.

2.3.4. Collection and processing of bovine pituitary and hypothalamus tissues

A bovine (cow) head with an intact skull was collected at the local abattoir and transported to the laboratory on ice. The skull bone was cut using a hand-held electric saw, and the intact brain, along with pituitary gland, was carefully lifted from the skull cavity, and thoroughly washed with ice-cold physiological saline solution. The pituitary gland was carefully separated from its capsule and surrounding vascular structures, and immediately frozen on dry ice. Cerebral hemispheres were cut apart through the mid-sagital section using a scalpel and brain tissue explants from the hypothalamus area were harvested and immediately frozen on dry ice. Both the pituitary and hypothalamus samples were either stored at –75°C, or immediately processed for total RNA and mRNA isolation, in a similar fashion to those of CL tissues.

2.3.5. Total RNA and mRNA isolation from granulosa cells, CL, pituitary, and hypothalamus tissues

Total RNA was isolated following a single-step RNA isolation method (Chomczynski and Sacchi, 1987) using a total RNA isolation solution, Tri Reagent. Briefly, 1 mL of homogenate mixture prepared from granulosa cells, or respective tissue samples (CL, pituitary and hypothalamus) were transferred to sterile 1.5 mL micro-centrifuge tubes, and samples were allowed to sit for 5 min at room temperature to facilitate complete dissolution of nuclear proteins and cell wall components. Two hundred µl of chloroform per each 1 mL of initial volume of Tri Reagent solution was added to each tube, and samples were agitated vigorously for 30 s. Again, the samples were allowed to stand at room temperature for 10 to 15 min, and then centrifuged at 12000 x G for 15 min at 4°C. The top layer of a clear

solution containing total RNA molecules was carefully transferred into a new set of sterile micro-centrifuge tubes. For each tube, 0.5 mL of isopropanol (per each 1 mL initial volume of Tri Reagent solution) was added and samples were allowed to stand for 15 min at room temperature. Samples were again centrifuged at 12000 x G for 10 min at 4°C, and the supernatant solution was carefully discarded . The resultant RNA pellet was washed two times with 75 % ethanol and finally the RNA pellet was dissolved in sterile, DEPC treated water. The quantity and quality of the total RNA was assessed by both spectrophotometry, and by observing clear bands for 28S and 18S ribosomal RNA species on ethidium bromide stained, 0.8% agarose gels. Total RNA was either used immediately for mRNA isolation or stored at –75°C for subsequent use.

Using total RNA from the representative samples of follicles, CL, and pituitary tissues, Poly (A^+) RNA was isolated using a commercially available mRNA Isolation Kit (Roche Molecular Biochemicals, Laval, QC). Briefly, a known amount of total RNA from respective samples was hybridized with a biotin-labeled oligo (dT)20 probe. This mixture was added to separate micro-centrifuge tubes containing streptoavidin magnetic particles that were prepared earlier. Tubes were incubated for 5 min at 37°C. The resultant Poly (A^+) RNA-Streptoavidin magnetic particle hybrid mixture was separated from the rest of the RNA species by subjecting to a Magnetic Particle Separator (Cat. No. 1641794, Roche Molecular Biochemicals, Laval, QC). Further, the magnetic particle-Poly (A^+) RNA mixture was washed twice with washing buffer. Finally the bound Poly (A^+) RNA was eluted into the RNase-free water, by incubating 2 min at 65°C, followed by separation of magnetic particles from the fluid portion, which contains Poly (A^+) RNA molecules. The quality and quantity of

Poly (A^+) RNA was determined spectrophotometrically. Poly (A^+) RNA samples were either used immediately for RT-PCR or stored in aliquots at –75°C. All buffers used in this protocol were supplied as part of the kit.

2.3.6. Detection of GnRH-R mRNA expression in granulosa cells and CL tissue

2.3.6.1. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Unless otherwise specified, the RT-PCR protocols used in this study were accomplished by utilizing a commercially available RT-PCR-20 Kit (Sigma-Aldrich Canada Ltd. Oakville, ON). Initially, the amount of template mRNA, magnesium concentration, compatibility of primer pairs, and PCR cycle number were tested to determine optimal conditions for RT-PCR (data not shown). About 200 to 500 ng (depending on the source, e.g. 200 ng from pituitary and 500 ng from granulosa cells and CL tissues) of Poly (A^+) RNA from each sample was reverse transcribed (RT) for 1 hour at 42°C, as per kit guidelines. The gene sequence specific 3' end antisense primers for both GnRH-R and a housekeeping gene, G3PDH, were utilized for the RT step. The internal control, G3PDH, was co-amplified with the GnRH receptor sequence in order to ascertain the quality of the template RNA, and to insure the success of RT- PCR reactions. Components included in RT reaction were: 50 mM Tris-HCl (pH 8.3), 40 mM KCl, 8 mM $MgCl^2$, 1 mM DTT, 0.5 mM each dNTP, 20 U Enhanced AMV reverse transcriptase, 20 U RNase inhibitor, and mRNA from follicular granulosa cells or luteal tissues. A 5 µl of the RT sample representing about 50 ng (pituitary) or 125 ng (granulosa cells or luteal tissue) of Poly (A^+) RNA, was used to co-amplify both GnRH-R and G3PDH sequences in the 50 µL reaction volume. PCR mix was comprised of 1 x PCR buffer (10 mM Tris-HCl/ pH 8.3, 50 mM KCl, 1.5 mM $MgCl^2$, 0.001 % gelatin, 2 %

DMSO v/v), 200 μM each dNTP, 400 nM of each gene specific primers (forward and reverse), 2.5 U of AccuTaq LA DNA polymerase, and the required amount of water to make up the final volume of 50 μL reaction mix. The primer pairs derived from respective cDNA sequences, bovine pituitary GnRH-R (Access#: U00934) and bovine G3PDH (Access #: AJ000039) were used to amplify an approximate size of a 920 bp and a 320 bp PCR fragments for GnRH-R and G3PDH, respectively (Table 1.1). In order to maintain the exponential PCR conditions for both GnRH-R and G3PDH, different sets of PCR cycles were performed for follicles (33 & 20), CL (35 & 21), and pituitary (26 & 20) samples (data not shown). Primers for G3PDH were dropped in to PCR reactions at a specified cycle number during PCR amplification (Wong et al., 1994). Control reactions included Poly (A^+) RNA from bovine pituitary, without mRNA template, and without reverse transcriptase enzyme, to ensure the successful amplification of the expected PCR amplicon, lack of cross contamination, and the absence of genomic DNA amplification, respectively. After an initial denaturation step of 3 min at 95°C, the PCR was carried out for the appropriate number of cycles of: a denaturing step of 1 min at 94°C, an annealing step of 1 min at 58°C, and an extension step of 1 min at 72°C, followed by a final extension step of 10 min at 72°C.

2.3.6.2. Southern transfer of PCR products

Aliquots (pituitary 10 μL, granulosa cells 15 μl and luteal tissues 20 μl) of PCR products were electrophoresed on a 1.5 % agarose gel and transferred onto the nylon membranes. Briefly, the agarose gel-resolved PCR amplicons were subjected to the alkaline denaturation for 20 min in 0.4 M sodium hydroxide solution, and the denatured DNA strands were transferred onto positively charged nylon membranes (Hybond-N^+, Amersham Biotech

UK Ltd. Buckinghamsire, England) by following the capillary transfer method in 0.5 M sodium hydroxide solution. Following overnight transfer, the transfer set up was dismantled, the membranes were identified, and the individual lanes were marked. In order to remove agarose residues, the membranes were washed in 2 x SSC solution for 2 min at room temperature, air-dried, and the membranes were either used immediately for hybridization or stored at -20°C.

2.3.6.3. Preparation of chemiluminescent probe and hybridization

RT-PCR amplified gene specific sequences of both GnRH-R (750 bp) and G3PDH (320 bp) served as the source of material for preparation of chemiluminescent DNA probes (ECLTM, Direct Nucleic Acid Labeling Detection System, Amersham Biotech UK Ltd. Buckinghamsire, England). Briefly, DNA fragments of an approximate size, 750 bp GnRH-R and a 320 bp G3PDH, were generated through RT-PCR by using mRNA isolated from bovine pituitary and luteal tissues, respectively. The DNA fragments were resolved on 1 % agarose gel, and extracted using gel extraction kit (QIAquick Gel Extraction Kit, Quiagen Inc. Mississauga, ON). The sequence identity of the respective PCR amplicons was confirmed through nucleotide sequence analysis. The required amount of gel purified DNA (10 ng/ mL) was denatured for five min in a boiling water bath and immediately placed on ice for 5 min. Tube contents were centrifuged briefly and an equal amount of labeling reagent was added and thoroughly mixed by repeated pipetting. Subsequently, an equal amount of glutaraldehyde solution was added, mixed and once again centrifuged briefly to concentrate tube contents at the bottom. Labeling was continued by incubating the tubes at 37°C for 20

min, and then the probe was used immediately, or stored in 50 % glycerol at –20°C for subsequent use.

Hybridization of resultant DNA blots and signal detection was carried out by using an ECL Direct Nucleic Acid Labeling kit (ECLTM, Direct Nucleic Acid Labeling Detection System, Amersham Biotech UK Ltd. Buckinghamsire, England) as per manufacture's instructions. Briefly, the hybridization buffer was prepared freshly by mixing blocking agent (supplied with the kit) and analytical grade NaCl to the concentration of 5 % and 0.5 M, respectively. Buffer contents were mixed thoroughly by stirring for 1 hour at room temperature, followed by mixing at 42°C for 30 min. Membranes were pre-hybridized for 1 hour at 42°C, then a labeled probe was added at the concentration of 10 ng/mL of hybridization buffer. A further hybridization process was carried out over night at 42°C with gentle shaking. Blots were washed (2 x 20 min cycle) in primary wash buffer (0.5 x SSC, pH 7.0, 0.4 % SDS, 6 M urea) at 42°C, followed by a secondary wash two times with 2 x SSC at room temperature. Each secondary wash lasted for 5 min with gentle agitation. Following washing, the detection solution was added to the membranes for one min, and the excess detection reagent was quickly blotted dry from the membranes. Signals were recorded through autoradiography by using ECL films (Amersham Biotech UK Ltd. Buckinghamsire, England). The probe-target specificity and the absence of cross reaction between G3PDH and GnRH-R probes were examined by hybridizing the uncut membranes either with only G3PDH probe, or GnRH-R probe alone in separate hybridization steps (data not shown).

2.3.6.4. Sequence analysis of PCR amplicons (GnRH-R)

The PCR amplified GnRH-R fragments (~920 bp) generated during the first round RT-PCR (from bovine granulosa cells, CL and pituitary tissues) step were subjected to a nested PCR amplification. The identity of the nested PCR amplified, DNA products were confirmed through nucleotide sequence analysis (Fig. 2.3).

2.3.6.5. Nested PCR amplification of GnRH-R sequence and nucleotide analysis

The internal primers, flanking a 750 bp sized PCR fragment within the sequence length of a 920 bp first round RT-PCR, GnRH-R DNA fragment used are illustrated in Fig. 2.2A and B. The internal primer pair (Table. 1.1) used in nested PCR was previously tested and published (Funston and Seidel, 1995). About 2 µl of first round RT-PCR DNA sample, representing each small follicle and CL tissue, was used to amplify the second round GnRH-R DNA sequences (750 bp) in 50 µL reaction volumes. PCR mix was comprised of 1 x PCR buffer (10 mM Tris-HCl/ pH 8.3, 50 mM KCl, 1.5 mM $MgCl^2$, 0.001 % gelatin, 2 % DMSO v/v), 200 µM each dNTP, 400 nM of each gene specific primers (forward and reverse), 2.5 U of AccuTaq LA DNA polymerase and the required amount of water to make up the final volume of 50 µL reaction mix. Control reactions including first round RT-PCR DNA representing bovine pituitary, without reverse transcriptase enzyme in RT samples from small follicle granulosa cells and CL tissue, and without DNA template were included to ensure successful amplification of the expected PCR amplicons, the absence of genomic DNA amplification, and lack of cross contamination, respectively. The PCR conditions were: initial denaturation for 3 min at 95°C, and 45 sec at 94°C, 1 min at 58°C, and 1 min at 72°C

followed by final polymerization for 10 min at 72°C. A total of 30 cycles were allowed during the nested PCR step.

The nested PCR amplicons were resolved on 1% agarose gel, and respective bands (Fig. 2.2) were carefully excised from the gel and purified using a commercially available gel extraction kit (QIAquick Gel Extraction Kit, Quiagen Inc. Mississauga, ON). Further, the purified, nested PCR DNA samples representing small follicles, CL and pituitary, were subjected to nucleotide sequence analysis (Nucleic Acid Protein Services, Biotechnology Laboratory, The University of British Columbia). The identity of the products was confirmed through nucleotide sequence query using NCBI standard nucleotide-nucleotide BLAST [blastn]. At least 3 samples each of nested PCR products representing different pools of granulosa cells, and different CL tissue, were analyzed.

2.3.7. Detection of GnRH mRNA in granulosa cells

Due to the lack of information on bovine GnRH coding sequence, three different sets of primers degenerate primers, primers designed based on ovine GnRH sequence (partial), and a primer set derived from human hypothalamus GnRH-I were utilized in this part of the experiment. RT-PCR was performed using mRNA isolated from bovine granulosa cells of different size ovarian follicles.

2.3.7.1. Selection of degenerate GnRH primers

Degenerate primers are oligonuleotides primers that have a number of options at several positions in the sequence, so as to allow annealing to, and amplification of, a variety

of related sequences. Degenerate primers are used in PCR amplification to isolate distantly related sequences encoding the conserved amino acid sequence (Rose et al., 2003). During this study, a set of degenerate primers (Table 1.1) was derived based on the most conserved regions or motifs of amino acids in hypothalamus GnRH sequence among bovine, sheep and human. The online version of the degenerate primer designing software (CODEHOP Version 12/17/99.1: Fred Hutchinson Cancer Research Center, Seattle, WA, USA) was utilized to generate GnRH degenerate primers for this study. Degenerate primers generated by this software consist of a all possible nucleotide sequences encoding 3 to 4 highly conserved amino acids within a 3' degenerate core, and a longer 5' non-degenerate clamp region that contains the most probable nucleotide predicted for each flanking codon (Rose et al., 2003).

A second and third pair of primer sets were derived based on ovine and human hypothalamus GnRH (GnRH-I) cDNA sequences and were utilized during the RT-PCR procedure. RT-PCR, Southern transfer-hybridization, and signal detection methods were similar to those explained under the RT-PCR procedure for GnRH-R mRNA identification in bovine granulosa cells and CL tissues, except for the following modifications. About two μg of total RNA representing the hypothalamus and granulosa cells from different size follicles was reverse transcribed (RT) using 3' end specific antisense primer in 20 μl reaction (RT-PCR-20, Sigma-Aldrich Canada Ltd. Oakville, ON). The RT-PCR reaction components were the same as outlined for the amplification of the GnRH receptor sequence, except for the template RNA and different primer pairs used during different RT-PCRs. Both the sense and antisense oligonucletide primers (degenerate, or ovine and human GnRH gene sequence specific primers (Table 1.1) were used to amplify an expected sized (sheep-152 bp and

human-380 bp) PCR fragment. PCR conditions were, initial denaturation for 3 min at 95° C followed with 45 s at 94° C, 1 min at 55° C, [degenerate primes, 2 min at 65° C; ovine primers, 1 min at 65° C] and 1 min at 72° C for 40 cycles, and final polymerization for 10 min at 72° C. Wherever applicable, parallel reactions comprising a positive control (human granulosa lutein cell cDNA), without RNA template, and without reverse transcriptase enzyme, were included to ensure successful amplification of the expected PCR amplicon, lack of cross contamination, and the absence of genomic DNA amplification, respectively. Human granulosa lutein cell cDNA and the primers used in this part of the RT-PCR experiment, were obtained as a gift from Dr. P.C.K. Leung's Lab, Faculty of Medicine, The University of British Columbia.

2.3.7.2. Sequence analysis of PCR amplicons (GnRH)

RT-PCR fragments of GnRH, representing both bovine granulosa cells, hypothalamus tissue and human granulosa lutein cells, was resolved on ethidium bromide stained 2% agarose gel and respective bands (Fig. 2.4) were carefully excised from the gel, and purified by using a commercially available gel extraction kit (QIAquick Gel Extraction Kit, Quiagen Inc. Mississauga, ON). Further, the purified PCR DNA samples were subjected to nucleotide sequence analysis (Nucleic Acid Protein Services, Biotechnology Laboratory, The University of British Columbia). The 3' end (reverse) primer from human hypothalamus GnRH-I, which was used in RT-PCR step, was used as sequencing primer.

2.4. RESULTS

2.4.1. GnRH-R mRNA Expression in bovine granulosa cells and luteal tissue

Results from the present study revealed GnRH-R mRNA expression in granulosa cells from small, medium, and large follicles, as well as in the CL. A representative autoradiogram showing GnRH-R mRNA expressions in different size follicles (Fig. 2.1B), and from different stage CL (Fig. 2.1C), are presented. Among different stage CL, the GnRH-R transcripts were clearly detectable in stage III (Day 11 – 17), whereas low expression in stage II (Day 5 – 10), and very weak expression or no signal was evident from stages I (Day 1 – 4) and IV (Day 18 – 21) luteal tissue (Fig. 2.1C). As shown in the Fig. 2.1B and C, the expected sized (~920 bp) PCR fragments of GnRH-R were successfully amplified using gene specific primers designed, based on the bovine pituitary GnRH-R cDNA sequence. Further, nucleotide sequence analysis of nested PCR, DNA fragments (Fig. 2.2) generated from both granulosa cells and CL tissue mRNA revealed a complete homology to that of bovine pituitary GnRH-R sequence (Fig. 2.3).

Due to a weak fluorescence signal on ethidium bromide-stained agarose gels, Southern blot hybridization step was undertaken in order to enhance the signal intensity and detection limits. This step was an added advantage for identification of PCR amplified products using a gene sequence specific probe. The expected sized PCR fragments were detectable as clear, discrete and single bands on resultant autoradiograms. Non-specific binding of the chemilumeniscent probe, or cross-reaction between GnRH-R and G3βDH probes, was not detectable during southern blot procedure (Fig. 2.1B and C). The possibility of genomic DNA amplification or cross-contamination was ruled out as no PCR products

were detectable from negative control samples without RT enzyme and without template during RT and PCR amplification steps, respectively.

2.4.2. GnRH mRNA Expression in bovine granulosa cells

RT-PCR technique in an attempt to identify GnRH mRNA expression in bovine granulosa cells, using degenerate primers or a primer set derived based on the ovine GnRH cDNA sequence (partial), was not successful. However, the preliminary evidence from RT-PCR using the primers derived from human hypothalamus GnRH-I revealed possible evidence for GnRH mRNA expression in bovine granulosa cells obtained from small, medium, and large follicles (Fig. 2.4). The expected sized PCR amplicon was evident on the ethidium bromide stained agarose gels. However, further confirmatory steps through Southern blot procedure or sequence analysis were not successful due to lack of signal during either step.

2.5. DISUCSSION

Intra-ovarian expression of GnRH receptor-ligand system and its direct involvement in alteration of ovarian cellular functions, is a well-documented fact in several species of animals and humans but not in the bovine species. To our knowledge, current results are the first to successfully demonstrate the presence of GnRH receptor mRNA expression in both follicles and CL tissues in the bovine species. However, present studies cannot rule out granulosa cell contamination with cumulus oocyte complexes where the latter cell types have been shown positive for the presence of GnRH-R mRNA expression (Funston and Seidel, 1995). In addition, although our present findings are a step forward in the right direction,

demonstration of the functional form of GnRH-R protein molecules is an imperative step before attributing any possible direct effects of GnRH or its agonists at ovarian cellular level in bovine. Failure of previous attempts to demonstrate GnRH receptor protein (Brown and Reeves, 1983; Nett et al., 1987), or GnRH receptor mRNA, in the bovine ovary (Kakar et al., 1993) may be attributable to the investigative methods used. They may not be sensitive enough, or because of the type of experimental materials used were inadequate. In the former study (Brown and Reeves, 1983), the whole ovarian homogenates used for single point saturation analysis in association with low abundance receptor concentrations, could have undermined the detection limits. In the study of Kakar et al. (1993), the procedure of using whole ovarian tissue as a starting material might not have been adequate for detecting the GnRH-R message if it is present only in a few cell types of the ovary.

With respect to bovine ovarian expression of GnRH mRNA, although the current findings are very preliminary, RT-PCR results show possible evidence for presence of GnRH mRNA in bovine ovarian granulosa cells. PCR products resolved on agarose gel (ethidium bromide stained) revealed clear bands of expected sized PCR amplicons that run at a similar level with the positive control samples (human granulosa lutein cells). Despite the limitations of the materials, and the experimental approach used in this part of the study, failure of southern hybridization procedure and nucleotide sequencing steps may be attributable to one or more factors. These include the amount of target DNA available on the blot, the percentage of probe target complimentary sequence, the stability of the probe sequence to withstand post-hybridization washing steps, and finally, the nature of the signal itself. In the present experiment, the low amount of target DNA, and the partial complimentary probe

sequence of relatively shorter length in the whole plasmid used, and the chemiluminescent signaling method could have undermined the detection ability. In the case of the sequencing step, the direct PCR product used as template DAN, and the same 3' end primer which was used both during RT-PCR and during sequencing step, would have been the major contributing factor for the absence of the signal output during sequencing step. Further steps, such as cloning of RT-PCR fragments into a suitable vector, propagation in a plasmid, and sequence analysis using universal sequencing primers such as M13 could have been a more productive approach in identifying GnRH sequence. The preliminary evidence from present study may be supported by the related information from earlier findings where the presence of GnRH-like protein molecules in bovine ovaries has been suggested (Stojilkovic and Catt, 1995; Ireland et al., 1988), and demonstrated in human and rat ovaries (Aten et al., 1987). Therefore, we believe that the RT-PCR technique, using human primers has revealed evidence for GnRH mRNA expression in bovine granulosa cells of different sized follicles.

Several studies from other species not only reveal the presence of GnRH-R-GnRH system in intra-ovarian structures (follicles and CL tissue), but there are evidences suggesting intra-ovarian functional roles for GnRH. GnRH-a has been shown to induce a dose-dependent stimulatory effect on aromatase activity and P4 production in monkey granulosa cells, *in vitro* (reviewed by Janssens et al., 2000). On the other hand, inhibitory actions of GnRH or its agonists on gonadal steroidogenesis involving suppression of gonadotropin receptors, or intermediary enzymes in steroidogenic pathway such as StAR, P450scc enzyme, and 3-βHSD, were also reported in rodent species (Sridaran et al., 1999). In the bovine species, GnRH-a has been shown to cause an inhibitory effect on P4 secretion from *in vitro*

cultured luteal cells (Milvae et al., 1984). It is also noteworthy to that bovine oocytes exposed to the GnRH agonist, buserelin, had increased cleavage potential after *in vitro* fertilization (Funston and Seidel, 1995). The reason for these varying effects of GnRH on reproductive tissues across different species is not known. Therefore, in order to gain a deeper perspective subsequent studies (CHAPTER 3) have focused on investigating the direct effects of GnRH on the bovine ovary.

2.5. CONCLUSION

In conclusion, the present findings reveal definitive evidence for the presence of GnRH-R mRNA expression in bovine ovarian structures (the follicle, and the CL). It is worth noting that our preliminary studies also provided evidence for GnRH mRNA expression in bovine granulosa cells.

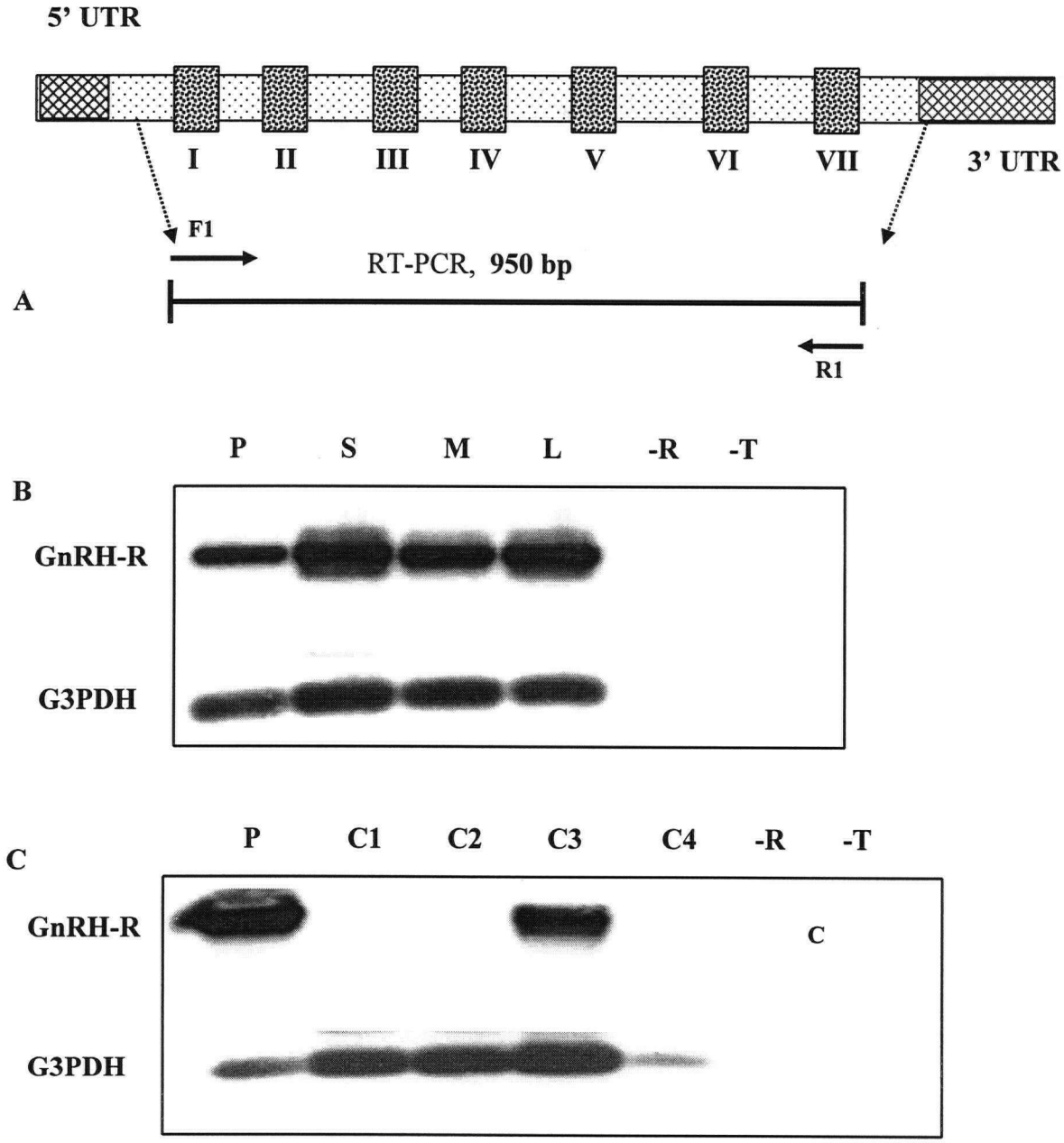

FIGURE 2.1. RT-PCR detection of GnRH-R mRNA in bovine granulosa cells and CL tissue. A. The locations of the primers selected from bovine GnRH-R cDNA sequence are indicated (not to scale). B. Representative auto radiogram showing GnRH-R (upper panel) mRNA transcripts in different staged follicular granulosa cells and pituitary tissue; (P) bovine pituitary, (S) small follicles, (M) medium follicles, (L) large follicles, (-R) sample without reverse transcriptase enzyme, (-T) sample without mRNA template. Lower panel represents the internal control, G3PDH. C. Representative auto radiogram showing GnRH-R (upper panel) mRNA transcript in different staged CL and in pituitary tissue; (P) bovine pituitary, (C1) stage I CL, (C2) stage II corpus luteum, (C3) stage III CL, and (C4) stage IV CL, (-R) sample without reverse transcriptase enzyme, (-T) sample without mRNA template. Lower panel represents the internal control, G3PDH.

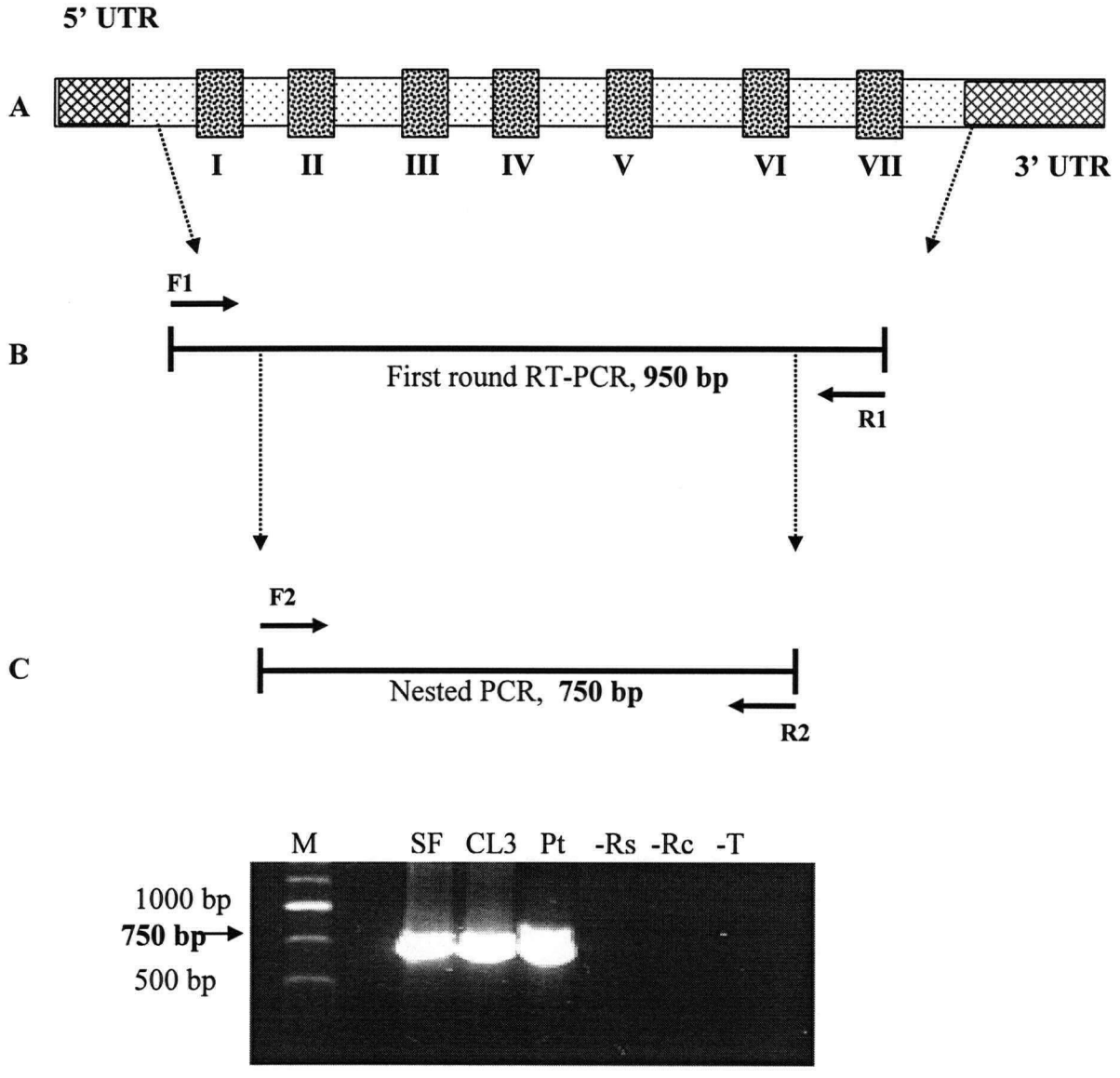

FIGURE 2.2. Ethidium-bromide stained agarose gel showing the expected sized nested PCR fragments (GnRH-R). A. Locations of first round PCR (F1, R1). B. Locations of the internal primers (F2, R2). (M) 250 bp ladder; (SF), small follicle; (S) small follicle; (CL3) stage III CL; (Pt) pituitary; (-Rs) without reverse transcriptase from small follicle; (-Rc) sample from small follicles without reverse; (-Rc) sample from CL3 without reverse transcriptase; (-T) negative control without template.

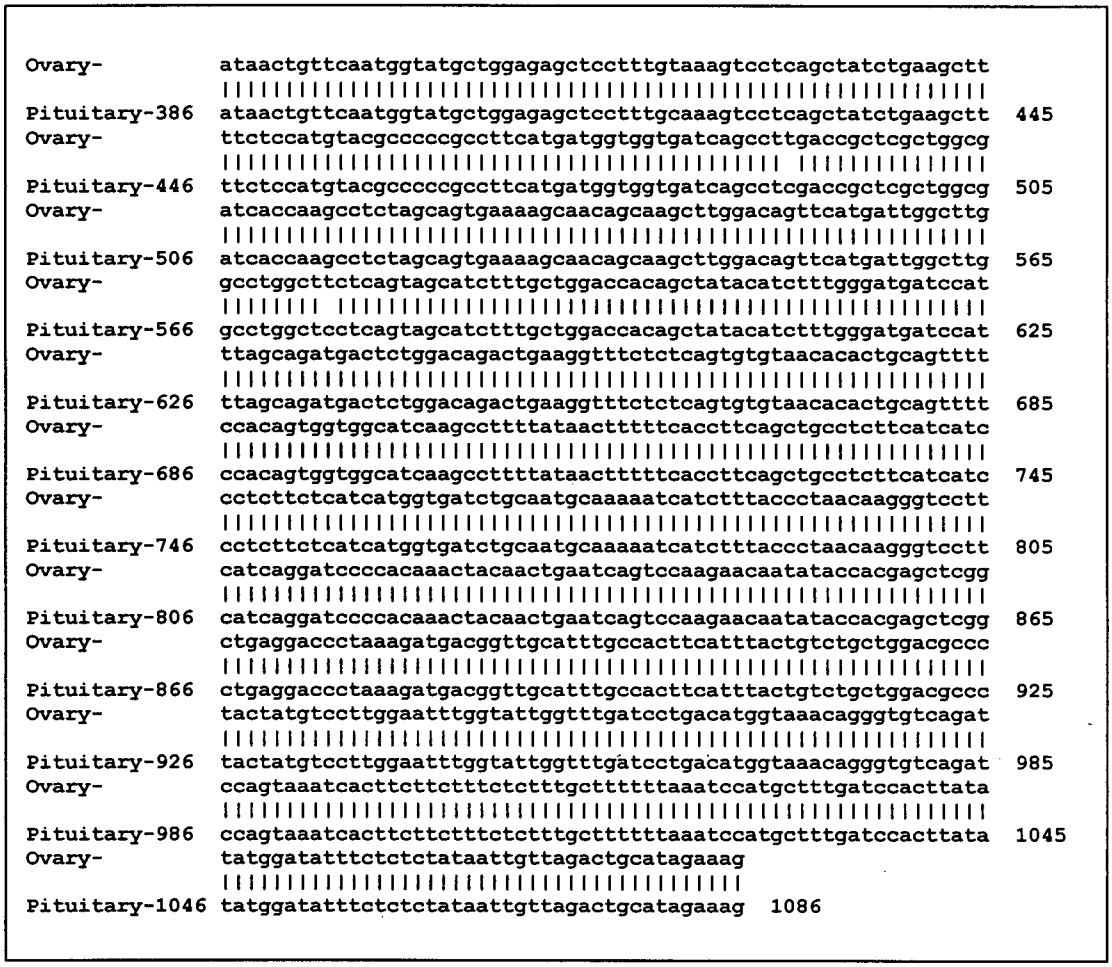

FIGURE 2.3. Bovine granulosa cell and luteal tissue GnRH-R cDNA (partial) sequence and its homology to bovine pituitary GnRH-R cDNA sequence. About 750 bp sized nested PCR products from granulosa and luteal tissues were gel extracted and subjected for nucleotide sequence analysis. Sequence results were subjected to sequence query using NCBI standard nucleotide-nucleotide BLAST [blastn].

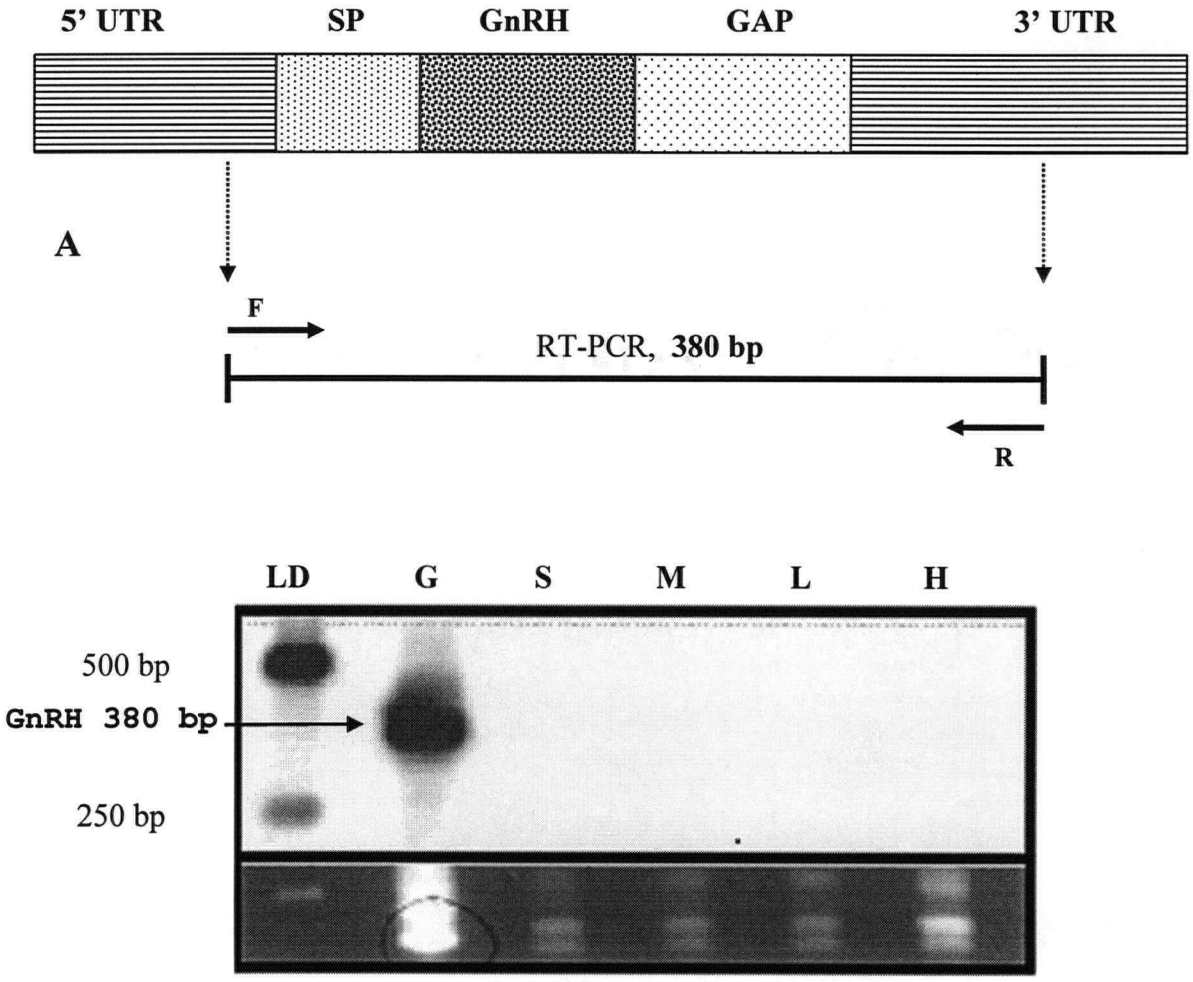

FIGURE 2.4. Representative autoradiogram (upper panel) and corresponding photograph of ethidium bromide stained agarose gel (lower panel) showing GnRH mRNA transcripts in different staged follicular granulosa cells. (LD) 250 bp ladder; (G) human granulosa lutein cells; (S) small follicle; (M) medium follicle; (L) large follicles; (H) bovine hypothalamus. A. Location of the forward and reverse primers on human GnRH-I cDNA are indicated (not to scale). SP, signal peptide sequence; GAP, GnRH-associated peptide; 3'/5' UTR, 3' and 5' untranslated region.

Table 2.1. Oligonucletide primer pairs and their respective PCR amplicons for GnRH-R, GnRH and G3PDH mRNA transcripts form bovine ovary.

Name	Primer Sequence Forward 5'-3' Reverse 5'-3'	Fragment Length (bp)
G3PDH [318 bp]	5'-TGTTCCAGTATAGATTCCACC-3' 5'-AGGAGGCATTGCTGACAATC-3'	318
GnRH-R [918 bp]	5'- GAGTAGCAGTTACTTTCTTCC-3' 5'- AGGAAGAAGCGTAACATTACC-3'	918
GnRH-R [748 bp]	5'-ACTCTGATTGTTATGCCACTG-3' 5'-CCTTTCTTTGACTTTCTATGC-3'	748
GnRH [degenerate]	5'- CGGCTTGCGGCCNGGNGGNAA-3' 5'-TCGCGGGCTGGTCNACYTCYTTNRC-3'	??
GnRH [ovine]	5'-GAATTCGCCCTGGAGGAAAGAGAAAT-3' 5'-GAATTCACTTTCCAGAGCTGCCTTCA-3'	152
GnRH-I [human]	5'-ATTCTACTGACTTGGTGCGTG-3' 5 –GGAATATGTGCAACTTGGTGT-3'	380

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CHAPTER 3

DIRECT EFFECTS OF GnRH-a ON *In Vitro* STEROID HORMONE PRODUCTION IN BOVINE GRANULOSA CELLS AND CORPUS LUTEUM

3.1. ABSTRACT

The present study investigated the direct effects of the GnRH-a (buserelin) on *in vitro* steroid hormone secretion from granulosa cells, dispersed luteal cells and CL tissue from the bovine species. Granulosa cells harvested from medium and large follicles were cultured in a serum-supplemented growth medium for 48 h. Thereafter, cell cultures were switched to serum-free culture conditions, and subjected to increased dose levels of buserelin (0, 10, 50, 200, 500 or 1000 ng/mL) for the next 24 h. Dispersed luteal cells were prepared from stage III CL (Day 11 - 17) and incubated in serum-supplemented growth medium for 24 h. Thereafter, luteal cell cultures were switched to serum free-culture conditions, and treated with increased doses of buserelin (0, 10, 50, 200, 500 or 1000 ng/mL) for the next 24 h. Minced luteal tissue samples (100 mg), prepared from stage III CL, were subjected to buserelin treatment. Treatments (ng/mL) were comprised of medium alone (CON), LH 100 ng, buserelin (BUS) 200 ng , BUS 1000 ng, LH 100 ng + BUS 200 ng, LH 100 ng + BUS 1000 ng, antide (ANT) 500 ng, ANT 500 ng + BUS 200 ng, $PGF_{2\alpha}$ 500 ng, or $PGF_{2\alpha}$ 500 ng + BUS 1000 ng. Granulosa and luteal cell cultures were incubated at 37°C and minced tissues were incubated at 38°C in a humidified atmosphere of 95 % air, and 5 % CO_2. Spent culture medium was collected at the end of each treatment period and assessed for steroid hormone concentrations by following a radioimmunoassay procedure. A dose-dependent, biphasic response of buserelin was evident on E2 accumulation during *in vitro* treatment of

granulose cells. Buserelin, at a dose of 200 - 500 ng/mL, exerted a stimulatory response ($P < 0.05$) on E2 secretion from granulosa cells. A further higher dose of buserelin, 1000 ng/mL, caused a mild inhibiltory effect on E2 output. Combined treatment of antide and buserelin resulted in the reversal of buserelin caused stimulatory response on E2 output in granulosa cells. P4 concentrations revealed a similar trend, except that the buserelin response on P4 output was not significantly different from that of P4 levels in untreated control samples.

Dispersed luteal cells treated with buserelin exhibited a dose-dependent, biphasic response in terms of P4 production. Maximal stimulatory response was evident at the dose range of 50 - 500 ng/mL of buserelin ($P = 0.1$). A higher dose of buserelin, 1000 ng/mL, showed a mild inhibitory effect on P4 levels; although the difference was not significantly different from that of P4 levels in untreated control samples. Buserelin treatment of bovine luteal tissue (minced CL tissue) *in vitro* exhibited a similar response in terms of P4 output. The dose-dependent stimulatory response of buserelin 200 ng/mL ($P = 0.19$) and 1000 ng/mL ($P = 0.14$) tended to be different from that of P4 levels in untreated control samples. Treatment with antide alone ($P = 0.07$), or a combination of buserelin ($P = 0.004$), showed a maximal stimulatory response in terms of P4 output. LH treatement, at the dose of 100 – 500 ng/mL caused a stimulatory response on P4 output in luteal tissue ($P = 0.05$). , Buserelin in combination with LH or $PGF_{2\alpha}$ treatment had no influence on P4 output in luteal tissue. In summary, the GnRH-a (buserelin) exerted a dose dependent-biphasic treatment effect on steroid hormone output from granulosa cells, dispersed luteal cells, and luteal tissue during *in vitro* culture.

3.2. INTRODUCTION

Gonadotropin releasing hormone (GnRH) is a hypothalamic neuronal secretory decapeptide that plays a pivotal role in reproduction. On the pituitqry gonadotrope cells, GnRH binds to a single class of G protein coupled receptors (GPCR), and initiates a cascade of biochemical and molecular events that leads to synthesis and the release of gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH), into general circulation. Both the LH and FSH, in turn control the processes of gamate production and ovarian steroidogenesis (Conn and Crowley, 1994; Stojilkovic and Catt, 1995). In addition, it has been suggested that GnRH may have a role as a modulator of the activity of the diverse systems in the brain, and many peripheral organs (Jones et al., 1980; Hsueh and Jones, 1981; Emons and Schally, 1994). Although it is an indisputable fact that the hypothalamus and pituitary are the principal source and target site for GnRH, respectively, several reports have suggested an extra-hypothalamic source of GnRH, as well as an extra-pituitary presence of GnRH receptors (GnRH-R) across different types of tissues in the body. With respect to the reproductive system, it is becoming increasingly evident that there is a functional GnRH-GnRH-R system that exist in different laboratory species (rats, pigs, and monkeys) and in humans. (CHAPTER 1; 1.2.5 and 1.2.6). Further, there are several reports suggesting that the direct effects of GnRH, in an autocrine or paracrine manner, elicit a variety of responses depending on the type of target tissue and physiological conditions. Therefore, the experiments present in this chapter were undertaken to further verify our previous findings of the presence of GnRH receptor mRNA expression in bovine granulosa cells and in CL tissue (CHAPTER 2). We tested the hypothesis that the direct effects of GnRH-a at ovarian cellular levels are exhibited through the altered *in vitro* steroid hormone production in follicular and

luteal cells, or CL tissue during *in vitro* culture. Experiments were performed in different steps with the following specific objectives; (i) to examine the direct effect of buserelin on *in vitro* production of estradiol-17β (E2) and progesterone (P4) in bovine granulosa cells, and (ii) to examine the direct effect of buserelin on *in vitro* P4 production in bovine luteal cells, and minced CL tissue, in the presence or absence of luteinizing hormone (LH) and luteolytic hormone [prostaglandin F2 alpha ($PGF_{2\alpha}$)] and the GnRH antagonist, antide (ANT).

3.3. MATERIALS AND METHODS

3.3.1. Cell culture reagents and related materials

Dulbecco's Modified Eagles medium (D-MEM), and Ham's F-12 (1:1) containing 15 mM HEPES buffer (15 mM), L-glutamine, and pyridoxine HCl were purchased from GIBCO (Burlington, Ontario, Canada). Falcon Primaria™ multi-well (6 and 24-well) tissue culture plates were purchased from Becton Dickinson and Company, CA. Percoll™, density-gradient medium was purchased from Pharmacia Biotech, Morgan, Canada. Bovine LH was obtained as a gift from NHPP (USDA-LH-B5; Beltsville, MD, USA). $PGF_{2\alpha}$ (Lutalyse®; Pharmacia & Upjohn, Orangeville, ON, Canada). RIA kits were purchased from Diagnostic Products Corporation (Coat-A-Count, Diagnostic Products Corp., Los Angeles, CA, USA). Unless specified, all other cell culture reagents and materials utilized during these studies were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada).

3.3.2. Direct effect of GnRH-a on *in vitro* E2 and P4 synthesis of bovine granulosa cells

This part of the study was performed to determine the direct effect of GnRH-a (buserelin) on *in vitro* cultured bovine granulosa cells, in terms of their steroidogenic potentials.

3.3.2.1. Collection of bovine ovaries and laboratory processing

Bovine ovaries were obtained from a local slaughterhouse within 20 to 30 min after exsanguinations. Ovaries were held in the thermos flask containing warm (30°C to 35°C) normal saline supplemented with streptomycin (100,000 μg/L) and penicillin (100,000 IU/L). Saline was replaced 2 to 3 times during waiting period at the slaughterhouse, and ovaries were transported to the laboratory within 3 to 4 hrs after collection. In the laboratory, ovaries were washed in saline, and held in warm saline solution, while aspirating follicles from individual ovaries for granulosa cell collection.

3.3.2.2. Isolation of granulosa cells and in vitro culture conditions

Follicles were classified into three different categories based on their diameter and morphological appearance (Kruip and Dieleman, 1982; Yang and Rajamahendran, 1998); medium (5-8 mm) and large follicles (>8 mm diameter). Care was taken to avoid aspiration of atretic and hemorrhagic follicles. Follicular fluid was aspirated using a 10 CC plastic syringe with an 18 guage needle. Follicular fluid from each category of follicles was pooled separately in heparin-dusted 15 mL Falcon tubes. While aspirating follicles, follicular fluid was gently aspirated and expelled into follicle several times in order to maximize granulosa cell recovery. During the period of aspiration (20–30 min), tubes containing follicular fluid

were held at 37°C in a water bath. Follicular aspirates were centrifuged at 300 x G for 10 min, and the supernatant was discarded. In order to eliminate the red blood cells (RBC), the cellular pellets were re-suspended in calcium and magnesium-free Hank's Balanced Salt Solution (HBSS). They were then gently over-layered on a continuous percoll gradient (40%) medium, and centrifuged at 600 x G for 20 min at room temperature. The interphase, cellular layer was carefully aspirated using a 1 mL pipette, and transferred to sterile 15-mL Falcon tubes. Cells were mixed in 5 to 6 mL of HBSS, and centrifuged in order to remove the contaminated Percoll solution. Cell pellets were re-suspended in a primary growth medium, plated in 35 mm 6 well cell culture plates, and incubated for 48 h. The primary growth medium consisted of 1:1 (v/v) DMEM/F-12 with L-glutamine, pyridoxine HCL, sodium bicarbonate, and HEPES. Growth medium was supplemented with 10% heat-inactivated fetal bovine serum (FBS), human transferrin (5 μg/mL), sodium selenite (4 ng/mL) and an antibacterial and antimycotic mixture (penicillin 100 IU/mL; streptomycin 100 μg/mL and amphoterocin B 2.5 ng/mL).

3.3.2.3. Examination of granulosa cell viability and cell counting

After 48 h of incubation in the primary growth medium, cells were washed 2 to 3 times to free the dead or unattached cells, and cell debris, using warm HBSS. The attached cell layer was over layered with 0.7 mL of non-enzymatic cell dissociation solution. Cultures were incubated for 10 to 15 min, or until the cells were completely detached from the plate surface. Some cultures required more than 15 min incubation, depending the cell density and degree of attachment. Detached cells were diluted with 4 to 5 mL of HBSS, pooled in 15 mL Falcon tubes, and centrifuged at 300 x G for 8 min. Based on the size of the cell pellet, cells

were re-suspended in 0.5 to 2 ml of culture medium, and cell viability was examined by the trypan blue (0.2%) exclusion method (Simmons et al., 1976). Aliquot of cells with required dilution was mixed with trypan blue, and incubated 2 to 3 min at room temperature. Total cell count and percentage cell viability were determined by following Hemocytometer cell counting method. The cell viability of >85% was observed during different trials of experiments.

3.3.2.4. GnRH-a treatment of granulosa cells during in vitro culture

Serum-free, experimental cell cultures were prepared in 24 well tissue culture plates (Falcon Primaria^{TM}), and granulosa cells were seeded at the density of 5 x 10^5 cells/mL/well. Each treatment well contained a final volume of 1 mL of culture medium, including experimental treatments. The cell culture medium included supplements (v/v): androstenedione (10^{-7} M), human transferrin (5 μg/mL), sodium selenite (4 ng/mL) and gentamicin (50 μg/mL). The buserelin treatment comprising of different doses (0, 10, 50, 200, 500 or 1000 ng/mL) was applied to duplicate or triplicate wells, depending on total cell yield. Additional cell cultures were also treated with buserelin in the presence of an antide, a GnRH antagonist (500 ng/mL), when sufficient cells were available. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2, and 95% air, for 24 h. After 24 h incubation, 700 μl of spent medium was harvested from each treatment well, and immediately stored at – 20°C. Care was taken not to disturb the cellular layer while collecting the spent medium from culture wells. Each experiment comprising of duplicate or triplicate wells for each level of treatments, was performed using a different pool of granulosa cells prepared from the groups of ovaries collected at different days. Experiments were repeated at least 5 times.

3.3.3. Direct effect of GnRH-a on *in vitro* P4 synthesis of bovine CL

In this part of the study, the direct effects of buserelin on *in vitro* P4 production in dispersed luteal cells, and minced luteal tissue was examined. Experiments were conducted in two different steps. In the first step, a dispersed luteal cell culture system was utilized to determine the dose effect of buserelin on *in vitro* P4 output. In the second step, minced luteal tissues were utilized to examine the possible direct effects of buserelin on P4 secretion in the presence or absence of LH, and PGF2α.

3.3.3.1. Isolation of luteal cells and in vitro culture conditions

Bovine CL (Stage III; Ireland et al., 1980) were obtained at a local abattoir and transported in ice-cold DMEM-F12 medium supplemented with antibacterial (penicillin 100,000 IU/L, streptomycin 100,000 μg/L) and antimycotic (amphoterocin B 2.5 ng/mL) agents. In the laboratory, CL were washed in cold saline, subjected to a quick dip in 70% ethanol, and immediately washed with cold saline. Dispersed luteal cells from individual CL were prepared by following the procedures outlined reports in the literature (Simmons et al., 1976; Okuda et al., 1992; Petroff et al., 2001; Kamada and Ikumo, 1997), with necessary modifications. Briefly, each CL was separated from its capsule, and about 1.5-2 g of tissue was aseptically dissected into a 50 mL Falcon centrifuge tube containing 15-20 mL of ice cold DMEM-F12 culture medium. Tissues were minced into smaller pieces using a sterile scalpel, and was washed 2 times with ice cold DMEM-F12 culture medium. Further, the tissue samples were incubated (37°C for 30 min) in DMEM-F12, with gentle agitation in order to free the red blood cells and cellular debris. The medium was carefully decanted and

incubation was continued with DMEM-F12, containing 0.5% BSA, and collagenase (400 IU/ mL). After 45- 60 min of incubation, dissociated cells were removed, fresh media containing collagenase was added, and incubation was continued for further 45 – 60 min. Tissue clumps were dispersed by repeated up and down pipetting using a glass Pasteur pipette. The resultant cell suspension was filtered through a multi-layered cheesecloth to remove the remaining tissue clumps. Further, the cells were washed in 10 mL of fresh medium by centrifugation at 300 G, for 5 min in 3 cycles. The supernatant was discarded, and the cells were resuspended in 2-3 mL of fresh medium, and subjected to Percoll discontinuous gradient centrifugation to remove remaining cell debris, RBC, and excessive lipid content. The discontinuous Percoll gradients composed of 3 mL each of 50, 25, and 12.5% Percoll column were layered in the same tubes (Kamada and Ikumo, 1997). About 3 mL of enriched cell suspension was carefully layered on the top Percoll column, and centrifuged at 400 G for 20 min at 4°C. After centrifugation, the purified luteal cells in the 25% Percoll layer were carefully aspirated, and washed 2 times with fresh culture medium. Immediately, the cells were sparsely plated in a 6-well culture plates and incubated in primary growth medium containing heat inactivated 10% FBS, for the first 24 h.

3.3.3.2. Examination of luteal cell viability and cell counting

After 24 h of culture in the primary growth medium, cells were washed with warm HBSS, 2 to 3 times, to free the dead or unattached cells and cell debris. The attached cell layer was over layered with 0.7 mL of a non-enzymatic cell-dissociation solution, and incubated for 10 to 15 min (or until the cells were completely detached from the plate surface). Some cultures required more than 15 min incubation, depending on the cell density

and degree of attachment. Cultures were diluted with 4 to 5 mL of HBSS, pooled into sterile 15 mL Falcon tubes, and centrifuged at 300 x G for 8 min. Depending the size of cell pellet, cells were re-suspended in 0.5 to 2 mL of culture medium, and cell viability, were examined by the trypan blue (0.2%) exclusion method. Briefly, an aliquot of cells, with required dilutions, were mixed with trypan blue, and incubated 2 to 3 min at room temperature. Total cell count, and percentage cell viability, were determined by following the Hemocytometer cell count method. Cell viability was observed in the range of 80 to 90% during different days of luteal cell preparation for the experimental treatments.

3.3.3.3. GnRH-a treatment of dispersed luteal cells

Dispersed luteal cell cultures prepared as per the procedure outlined in the above paragraphs. Luteal cells were plated at the density of 5 x 10^5 cells/mL/well, in 24 well tissue culture plates. Each treatment well contained a final volume of 1 mL of culture medium, including experimental treatments, and culture medium supplements (v/v): 25-hydroxycholestrol (20 μg/mL), human transferrin (5 ng/mL), selenium (4 ng/mL) and gentamicin (50 μg/mL). Different doses of buserelin (0, 10, 50, 200, 500 or 1000 ng/mL), was applied in duplicate or triplicate culture wells. Treatment cultures were incubated at 37°C in a humidified atmosphere, saturated with 95 % air and 5 % CO2 for the next 24 h. After 24 h, the incubation was terminated, and a sample (0.5 mL) of spent culture medium was harvested from each treatment well, and stored at –20°C for P4 measurement. More than a dozen CL were utilized in this study, and on each day of experiment, 1 to 3 CL were processed, depending on the number of CL obtained during each visit to the slaughterhouse.

3.3.3.4. Preparation of minced luteal tissue and *in vitro* culture conditions

Bovine ovaries were obtained at a local abattoir, and transported in ice-cold DMEM-F12, supplemented with antibacterial (penicillin 100,000 IU/L, streptomycin 100,000 μg/L), and antimycotic (amphoterocin B 2.5 ng/mL) agents. In the laboratory, CL were washed in cold saline, subjected to a quick dip in 70% ethanol, and immediately washed with a sterile cold saline. Tissue samples were harvested from 2-3 CL, pooled together and minced into smaller pieces in a large petri dish containing ice cold HBSS. Minced CL tissue was washed 2-3 times with 10 mL of ice cold HBSS. During washing steps, the petri dish contents were subjected to gentle swirling, and HBSS solution, including floating tissue debris, was aspirated using Pasture pipette. The excess amount of liquid from minced tissue was removed by briefly placing the tissue on multi-layered sterile Kim wipes. Immediately, about 100 mg of tissue was weighed, and randomly distributed into individual wells (24-well tissue culture plates, Falcon PrimariaTM) containing 1 mL of cold HBSS solution. The tissue samples in individual culture wells were given an additional wash by gentle agitation, and removal of HBSS. Care was taken to avoid the removal of tissue while aspirating HBSS during washing steps. Immediately, the tissue samples in each well were supplied with the warm DMEM-F12 medium, which was supplemented with 25-hydroxycholestrol (20 μg/mL), human transferrin (5 ng/mL), selenium (4 ng/mL) and gentamicin (50 μg/mL). The final volume of culture medium, including respective treatments in each treatment well was adjusted to 1 mL.

3.3.3.5. Effect of LH on in vitro P4 synthesis in luteal tissue

The minced luteal tissue cultures were prepared as outlined in the previous paragraph. The different doses of LH (0, 50, 100, or 500 ng/ mL) were applied in triplicate culture wells,

and the treatment cultures were incubated at 38°C in a humidified atmosphere, saturated with 95 % air, and 5 % CO2 for the next 6 h. At the end of 6 h, treatment cultures were terminated, and a sample (0.5 mL) of spent culture medium was collected from each well and stored at –20°C for P4 measurement. At least 4 individual CL collected on different days were utilized in this part of the experiment.

3.3.3.6. Effect of GnRH-a on in vitro P4 synthesis in luteal tissue

The different treatments were comprised of medium alone (CON), LH-100 ng, buserelin (BUS) 200 ng, BUS 1000 ng, LH 100 ng + BUS 200 ng, LH 100 ng + BUS 1000 ng, Antide (ANT) 500 ng, and ANT 500 ng + BUS 200 ng. Each treatment culture well contained a final volume of 1 mL of culture medium, including experimental treatments, and culture medium supplements (v/v): 25-hydroxycholestrol (20 μg/mL), human transferrin (5 ng/mL), selenium (4 ng/mL) and gentamicin (50 μg/mL). Treatment cultures were incubated for 6 h at 38°C, with humidified atmosphere saturated with 95 % air and 5 % CO2. Cultures were terminated at the end of the sixth hour of incubation, and spent medium was harvested and stored at –20°C for P4 measurement. At least five trials that were conducted on separate occasions were included in this study.

3.3.3.7. Effect of GnRH-a on in vitro P4 synthesis in luteal tissue, in presence of LH, or $PGF_{2\alpha}$

This part of the study was a replicate of the above experiment (3.3.3.6), with different treatment combinations using different batches of CL. This study was undertaken to examine the direct effect GnRH-a on *in vitro* P4 production in bovine luteal tissue, in the presence of LH, or d PGF2α. The different treatments (ng/mL) were comprised of control (medium

only), LH 100 ng, buserelin (BUS) 200 ng, BUS 1000 ng, LH 100 ng + BUS 200 ng, LH 100 ng + BUS 1000 ng, PGF2α 500 ng, and PGF2α 500 ng + BUS 1000 ng. Treatment cultures were incubated for 6 h at 38°C, in a humidified atmosphere, saturated with 95 % air and 5 % CO2. Cultures were terminated at the end of sixth hour incubation and spent medium was harvested and stored at –20°C for P4 measurement. In this part of the study, at least 4 trials were conducted using CL that were harvested on different days.

3.3.4. Steroid hormone assay

Steroid hormones (E2 and P4 concentrations in spent culture medium) were measured by using a commercially available, solid phase radioimmunoassay (RIA) kits. These kits were previously validated for the measurement of both E2, and P4, concentrations in spent cell culture medium in our laboratory (Manikkam and Rajamahendran, 1997). Briefly, during initial steps, RIA were performed on serial diluted spent culture medium samples (data not shown). During subsequent steps, the spent culture medium samples were diluted in a phosphate buffered saline (1:100), and the respective concentrations of E2 and P4 were measured during separate RIA steps. Sampling of the spent culture medium (diluted), the kit standards (E2 and P4 calibrators) and incubation of reaction components were similar to that of kit guidelines. Upon addition of buffered I^{125}-labeled P4 or E2 (1.0 ml), the tube contents were mixed by gentle agitation, and then incubated at room temperature for 3 h. At the end of 3 h incubation, the tubes were decanted, and the remaining radio-activity was counted for 1 min using a gamma counter (Packard Auto gamma 500, Packard Instruments, Downers Grove, IL, USA). The assay sensitivity, intra- and inter- assay coefficient of variation for E2 were 5 pg /mL, 7.6% and 12.3% (n = 3), respectively. Whereas for P4, the assay sensitivity,

intra- and inter-assay coefficient of variation were 0.01 ng/mL, 6.3% and 10.2% (n = 6), respectively.

3.3.5. Data analysis

RIA data for steroid hormones (E2 and P4 measurements), were converted to a percentage of control, and presented as the mean ± SEM. This step was necessary due to the variation observed in the steroid output among different day's cultures, and different pools of granulosa cells, luteal cells, and different batches of minced CL tissue cultures. Each culture well, in duplicate or triplicate treatment, was considered as an independent experimental unit. The data were analyzed using one-way analysis of variance (ANOVA), followed by pair-wise comparison using a Student-Newman-Keuls test. Results were considered significant at $P < 0.05$ and with an approaching significance at $P > 0.05$ to $P < 0.15$. The respective P values are indicated wherever applicable. All data were analyzed using NCSS Statistical Software - trail version (Kaysville, Utah).

3.4. RESULTS

3.4.1. Effect of GnRH-a on *in vitro* E2 and P4 synthesis in granulosa cells

Buserelin elicited a dose-dependent, biphasic response on *in vitro* E2 secretion, in granulosa cells harvested from both medium, and large, follicles (Fig. 3.1 and 3.2). The maximum, significant stimulatory response of buserelin on E2 release was observed at the doses of 200 ng/mL ($P = 0.002$), and 500 ng/mL ($P = 0.07$), in granulose cells from medium follicles and 200 ng/mL in large follicular granulosa cells ($P < 0.05$). In terms of P4 output, there was an apparent tendency towards mild stimulatory response, although the results were

not significantly different from that of untreated control samples. Higher dose levels of buserelin, 1000 ng/mL appeared to be slightly inhibitory on both E2 and P4 output, irrespective of the follicle size (Fig. 3.1 and 3.2). In the cultures co-treated with both buserelin and its antagonist (antide, ANT), the antagonistic effect of the latter was tended to be clearly evident in terms of the reversal of buserelin effect on granulosa cell steroid output (Fig. 3.3).

3.4.2. Effect of GnRH-a on *in vitro* P4 synthesis in luteal cells and tissue

3.4.2.1. Effect GnRH-a on in vitro P4 synthesis in luteal cells

GnRH-a exhibited a dose-dependent, biphasic response on P4 production from bovine luteal cells treated during *in vitro* culture (Fig. 3.4). The maximum stimulatory response was found at the dose range of 50 ng - 200 ng/mL of buserelin ($P = 0.1$), whereas higher dose of buserelin (1000 ng/mL) showed a mild inhibitory effect on P4 levels.

3.4.2.2. LH response on in vitro P4 synthesis in luteal tissue

Minced luteal tissues were cultured *in vitro*, and treated with different doses of bovine LH. The LH (100 - 500 ng/mL) response on *in vitro* P4 release in luteal tissue was significantly higher ($P < 0.05$), compared to that of P4 levels from untreated control samples (Fig. 3.5).

3.4.2.3. Effect of GnRH-a on in vitro P4 synthesis in luteal tissue

In luteal tissue, buserelin treatment caused a dose-dependent, stimulatory response on P4 output (Fig. 3.6) similar to that of from dispersed luteal cells (Fig. 3.4). Buserelin caused

stimulatory response on P4 output at 200 ng/mL (P = 0.19), and 1000 ng/mL (P = 0.14), tended to be different from that of untreated control samples. Treatment of bovine luteal tissue with antide alone (P = 0.07), or in combination with buserelin (P = 0.004), showed the maximal stimulatory response in terms of P4 output (Fig. 3. 6).

3.4.2.4. Effect of GnRH-a on in vitro P4 synthesis in luteal tissue, in presence of LH, or $PGF_{2\alpha}$

Treatment of minced luteal tissue with buserelin (200 or 1000 ng/mL) resulted in a non-singnificant rise in P4 levels. Whereas, buserelin treatment combinations either with LH, or PGF2∝ alone did not affect the P4 output in luteal tissue (Fig. 3. 7). LH (100 ng/mL) response on P4 ouput was significantly different (P = 0.05) from that of P4 levels in untreated samples.

3.5. DISCUSSION

The series of experiments presented in this chapter investigated the direct influence of GnRH-a on steroid hormone secretion in bovine granulosa cells, dispersed luteal cells and in minced luteal tissue during *in vitro* culture. It was evident that GnRH-a exerts a dose-dependent, biphasic effect on E2 output from *in vitro* cultured granulosa cells. At lower dose levels (10 to 200 ng/mL), GnRH-a caused an increased (E2) output, whereas at a higher dose level (1000 ng/mL), the accumulated E2 levels were slightly lower in comparison with that of E2 levels from untreated control cultures. Although the effect of buserelin of P4 output was similar to that of E2 output, none of the buserelin doses (10 to 1000 ng/mL) caused a singnificant rise in P4 output in granulosa cells. This is confirmatory evidence that under the present culture conditions granulosa cells were fully estrogenic and did not undergo

luteinization process. The present observations are in agreement with similar reports that have demonstrated that GnRH-a would induce steroidogenesis in human granulosa cells *in vitro* (Ranta et al., 1982; Parinaud et al., 1992; Olsson et al., 1990; Bussenot et al., 1993). Parinaud et al. (1988) suggested that GnRH-a could modulate steroidogenesis by a direct ovarian action. Guerrero et al. (1993) found an increase in P4, and decrease in E2 production, which seemed to be related to a decrease of LH receptor numbers and aromatase activity in GnRH agonist-treated cells. The present study does not provide definitive evidence for the presence of functional GnRH receptors that could mediate a GnRH-a response affecting steroidogenic potentials of bovine granulosa cells treated *in vitro*. Nonetheless, based on the response elicited by GnRH antagonist, reversal of GnRH-a effect on E2 output does suggest the possible existence of GnRH ligand specific target sites on granulosa cells.

In efforts to examine the direct effects of GnRH-a on *in vitro* P4 production in bovine CL, both dispersed luteal cell cultures, and luteal tissue (organ) culture systems, were utilized. Both dispersed cell culture systems and organ/tissue culture systems possess their own merits and demerits. Dispersed cell culture conditions allow examination of the specific type, or a specific of cell population in question, whereas an organ or tissue culture system allows examination of the organ, which represents, more or less, the intact system in the body. From the literature, it is evident that the minced luteal tissues, or organ culture system, would preserve the cellular interaction, and retain histological and biochemical differentiation of the CL tissue (Harrison et al., 1987; Del Vecchio et al., 1995a,b). Therefore, we utilized both dispersed luteal cell culture, and tissue/organ culture systems, to examine the possible direct influence of GnRH-a on CL function in bovine species. During

the initial steps, the functional viability of minced luteal tissue and the culture conditions were ascertained by examining the LH responsive P4 secretion (Fig. 3.5); There was a dose-dependent LH stimulatory response on P4 secretion. Therefore the present culture conditions were considered appropriate for the purpose of examining GnRH-a effect on CL P4 output during *in vitro* culture.

GnRH-a exhibited a dose-dependent, stimulatory response on P4 output from both dispersed luteal cells and luteal tissue. However, the higher dose levels of buserelin (1000 ng/mL) showed a mild inhibitory effect on P4 levels in luteal cells. It is not clear whether the suppressed P4 level at higher dosage levels is in fact due to inhibitory roles of GnRH-a, or autoregulatory mechanisms such as receptor desensitization, or down-regulation of its own receptors, as reported in other studies (Olofsson et al., 1995; Volker et al., 2002). Treatment of luteal tissues with antide alone, or in combination of buserelin, elicited a maximal stimulatory response in terms of P4 output. Unlike other reports, as well as in the present study (antide alone or the combined effect of buserelin and antide on granulosa cell steroid output; 3.3.6), a maximal stimulatory response was elicitd in terms of P4 output in CL tissue, when treated as antide alone, or in combination with buserelin. The reason for this atypical response is not known. However, evidence suggests that the GnRH antagonist (antide) could elicit a agonist-like activity in certain type of ovarian cancer cells (Grundker and Emons, 2003). In addition, there may be other unknown factors within CL tissue, in conjunction with antide that might have been responsible for this atypical response. It is also interesting to note that granulosa cells from women that were treated with GnRH antagonist (cetrorelix) responded earlier to the *in vitro* hormone stimulation, in terms of P4 accumulation, than

women treated with the GnRH agonist (buserelin) as reported by Lin et al. (1999). Their results indicate that luteal function is less impaired in GnRH antagonist treatment, than in GnRH agonist, treatment. Further, evidence from porcine granulosa cell cultures suggests that GnRH antagonist, combined with follicular fluid, could enhance LH-stimulated P4 secretion (Ledwitz-Rigby, 1989). In addition, the biphasic effects of GnRH antagonist on rat granulosa cell *in vitro* steroidogenesis that varies with exposure time, showed the initial response being stimulatory, and the latter inhibitory. Their studies show that 20 alpha-OHP secretion in the same cultures was potentiated by the combined presence of FSH, and GnRH-a (Sheela Rani et al., 1983). These authors suggest that these types of responses could have been due, to some extent, the maturational stage of the granulosa cells. From this evidence, it is tempting to suggest that GnRH antagonist may mediate their effects independent of GnRH target sites and they may also interact with some unknown factors in ovarian cell types. This brings attention to several of previous reports, which suggest the presence of GnRH-like molecules in gonadal structures (Ying et al., 1981; Aten et al., 1987a, b; Ireland et al., 1988; Izumi et al., 1985). However, there is no concrete evidence that suggests any functional role for such molecules in the ovary or any other reproductive tissues.

Results from the present study differ from those of Milvae et al. (1984), who reported a dose-dependent suppression of P4 secretion from *in vitro* cultured, bovine luteal cells. In that study, the authors suggest that it is very unlikely that GnRH mediated its effects through the mechanism of ligand-specific receptor interaction on bovine luteal cells. The reason for entirely different types of responses observed between these studies, could have been due to the variations in experimental conditions. As it seems to be there are only two such studies

that were carried out in bovine species, with two different observations, it is difficult to draw a definitive conclusion with respect to the direct effects of GnRH or its analogues on bovine ovarian function. However, it is noteworthy that in the bovine species, *in vivo* administration of GnRH-a during late-mid luteal phase has resulted in increased P4 output (Thatcher et al., 1993; D'Occhio and Aspden, 1999; D'Occhio et al., 2000; Rajamahendran et al., 1998, 2001).

In the ovary, GnRH has been shown to elicit a mixedtype of response that may affect ovarian function (review by; Sharpe, 1982; Janssens et al., 2000; Leung et al., 2003). Several reports, from different experimental models, have suggested the functional modulator roles for GnRH, or its analogues, at the gonadal level. Guerrero et al. (1993) obsereved an increase in P4 and decrease in E2 production, which seemed to be related to a decrease in LH receptor numbers and aromatase activity in GnRH agonist-treated cells. It is also reported that the GnRH agonist, buserelin, causes a biphasic effect, such as increased basal P4 secretion, or decreased LH-induced P4 secretion, *in vitro*. GnRH is believed to exert its direct effects on its own, or in conjunction with other factors such as PGF2α, angiotension II or luteinizing hormone (Reviewed by Steele and Leung, 1993). However, in this study buserelin treatment in combinations of LH or PGF2α, had no influence on P4 output from luteal tissue. Stimulation of one or more signaling pathway, such as phospholipase C (PLC), phospholipase A2 (PLA2) and phospholipase D (PLD), and/or activation of protein kinase C (PKC) have been hypothesized to cause either inhibitory, or stimulatory effects on ovarian cellular steroid output. These dual effects have been demonstrated during *in vivo* experiments in adult male and female hypophysectomized rats, where exogenous GnRH or GnRH-a could

both stimulate or inhibit gonadal functions in terms of steroidogenesis (Hsueh and Jones, 1981, 1982). In adult male rats, a lower dose of GnRH-a administration for a short duration, was shown to stimulate testosterone secretion (Sharpe et al., 1982). However, the effect was opposite when the agonist was administered at a higher dose, or for a longer durations (Arimura et al., 1979, Hsueh and Erickson, 1979). Other reports have demonstrated that GnRH modulates both basal and gonadotropin-stimulated steroidogenesis (Olofsson et al., 1995) in the ovary. The inhibitory action of GnRH, or its agonists, on gonadal steroidogenesis involves suppression of the gonadotropin receptors, or intermediary enzymes, involved in the steroidogenic pathway. Reports suggesting GnRH-a-induced suppression of FSH and LH receptors (Tilly et al., 1992; Piquette et al., 1991; Guerrero et al., 1993), gonadotropin-induced cAMP levels (Richards, 1994; Knecht et al., 1985) or steroidogenic enzyme activity such as StAR protein, P450scc enzyme, and 3β-HSD (Sridaran et al., 1999a; Sridaran et al., 1999b), or no effect (Casper et al., 1984) of GnRH on P4 production in human granulosa-lutein cells (hGLCs), have been documented. Researchers have also reported that GnRH and its potent agonists could stimulate meiosis *in vitro*, in follicle-enclosed oocytes, in a dose-dependent manner (Hillensjo and LeMaire, 1980). Similarly, in the bovine species, oocytes that were pre-exposed to the GnRH-a (buserelin) had increased cleavage potentials during *in vitro* fertilization (Funston and Seidel, 1995). The reasons for these complex, and varied responses of reproductive tissues across different species (to the GnRH, or its analogues) are not understood. Therefore, in attempts to verify our present observations, further studies were focused on examining the GnRH-a influence on molecular mechanisms of steroidogenic machinery in the bovine CL (CHAPTER 4).

3.6. CONCLUSIONS

Based on the results presented in this chapter, GnRH-a caused a dose-dependent stimulatory response on steroid hormone output from bovine granulosa cells, dispersed luteal cells, and from luteal tissue treated *in vitro*. However, these findings do not provide the definitive evidence for a direct interaction of GnRH-a with its receptor on the above cell types. Nevertheless, the evidence gained through these studies, not only form the basis for further studies, but also refines the research strategies in gaining further knowledge with regard to the direct effects of GnRH in mammalian reproductive tissues.

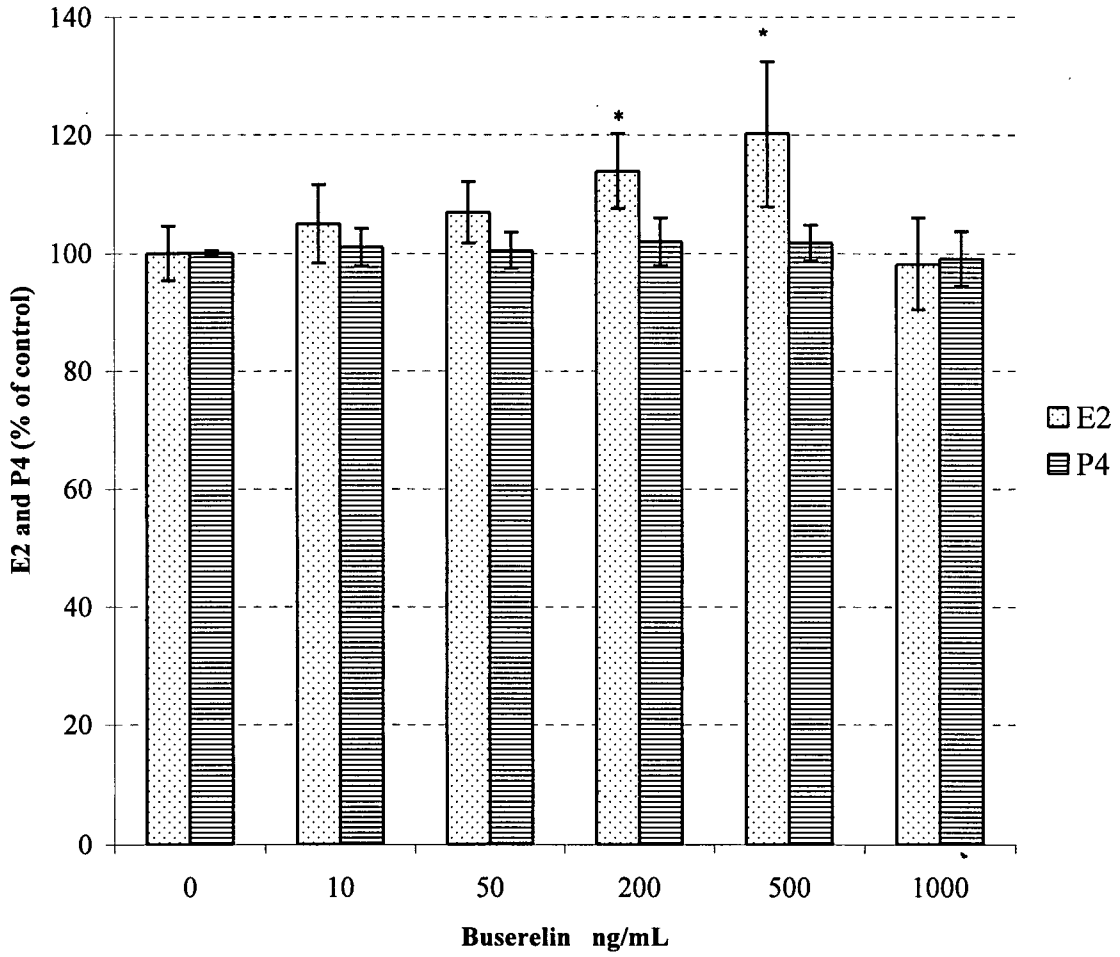

FIGURE 3.1. Effect of GnRH-a on *in vitro* synthesis of estradiol-17β (E2) and progesterone (P4) in bovine granulosa cells from medium follicles. Cells were cultured in primary growth medium for 48 h and then treated with different doses of GnRH-a (0 ng to 1000 ng/mL) for the next 24 h in serum-free cultures supplemented with 10^{-7} M androstenedione as the precursor for E2 synthesis. E2 and P4 values were expressed as a percentage of control. Each data point represents the mean ± SEM from at least 5 individual trials. * different ($P < 0.05$) from untreated control samples.

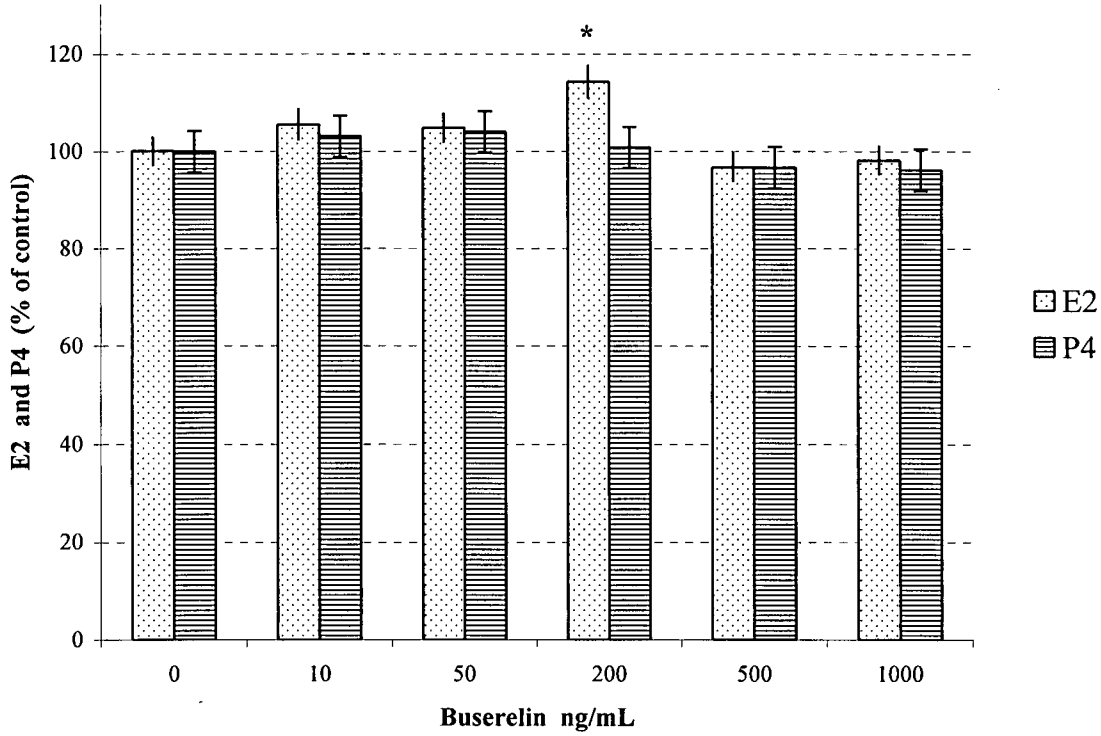

FIGURE 3.2. Effect of GnRH-a on *in vitro* synthesis of estradiol-17β (E2) and progesterone (P4) in bovine granulosa cells from large follicles. Cells were cultured in primary growth medium for 48 h and then treated with different doses of GnRH-a (0 ng to 1000 ng/mL) for the next 24 h in serum- free cultures supplemented with 10^{-7} M androstenedione as the precursor for E2 synthesis. E2 and P4 values were expressed as a percentage of control. Each data point represents the mean ± SEM from at least 5 individual trials. * different ($P < 0.05$) from untreated control samples.

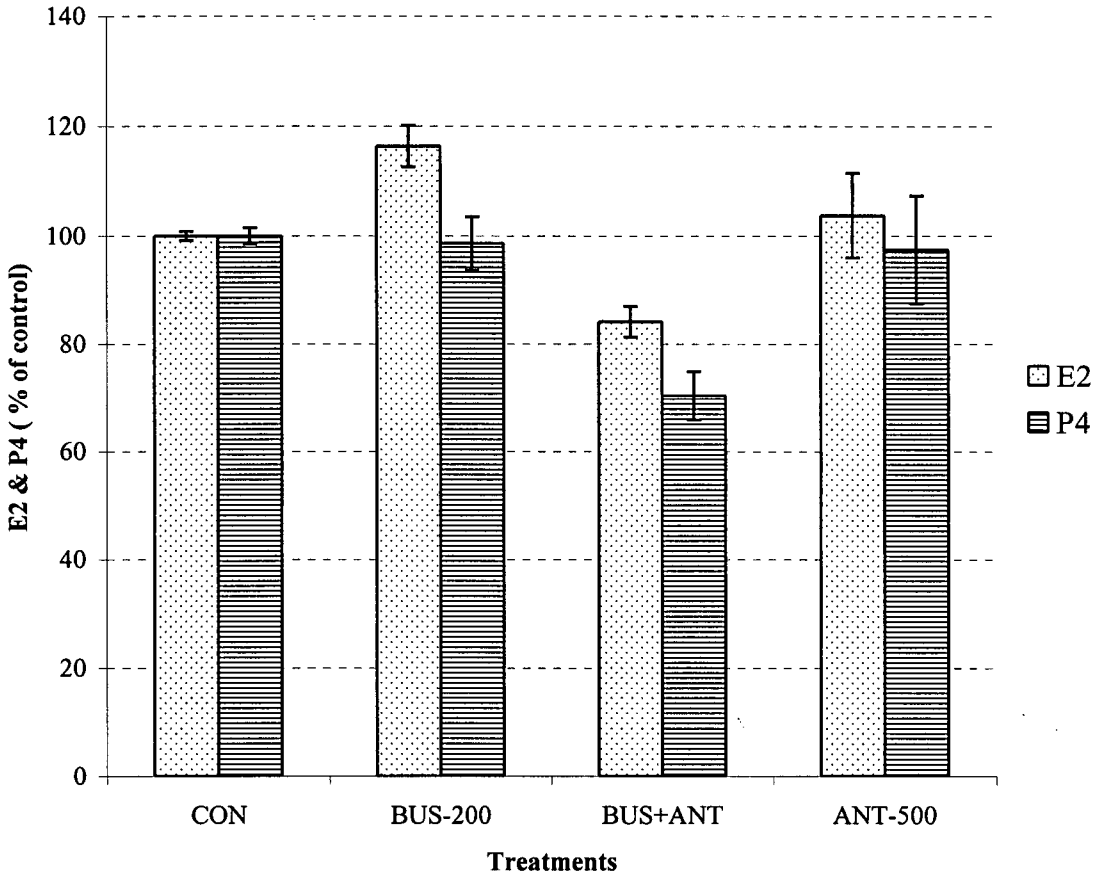

FIGURE 3.3. Effect of GnRH-a in presence or absence GnRH antagonist on *in vitro* estradiol-17β (E2) and progesterone (P4) synthesis in bovine granulosa cells from large follicles. Cells were cultured in primary growth medium for 48 h and then treated with of GnRH-a in presence of absence GnRH antagonist, antide (ANT) for the next 24 h. Serum-free cultures supplemented with 10^{-7} M androstenedione as a precursor for E2 synthesis. Treatments were consisted of medium alone (CON), Buserelin (BUS) 200 ng, BUS 200 ng + ANT 500 ng and ANT 500 ng/mL. Each data point represents the mean ± SEM from two different trials. No statistical analysis was performed due to less er number of trials.

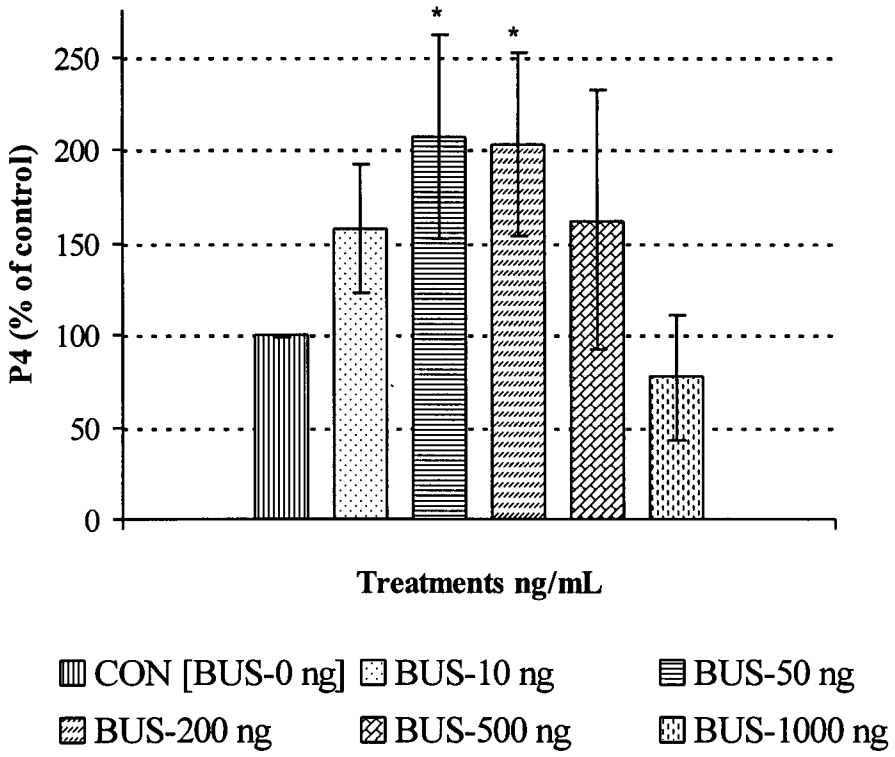

FIGURE 3.4. Effect of GnRH-a on *in vitro* P4 synthesis from bovine luteal cells. Dispersed luteal cells were cultured in primary growth medium for 24 h and then treated with different doses of buserelin (BUS) for the next 24 h in serum free culture conditions. Treatments were comprised of medium alone (CON), BUS (0, 10, 50, 200, 500, 1000 ng/mL). Data represents the mean ± SEM from more than a dozen of individual CL that were processed (1-3 CL/ trial) on different occasions. * different (P = 0.1) from control samples.

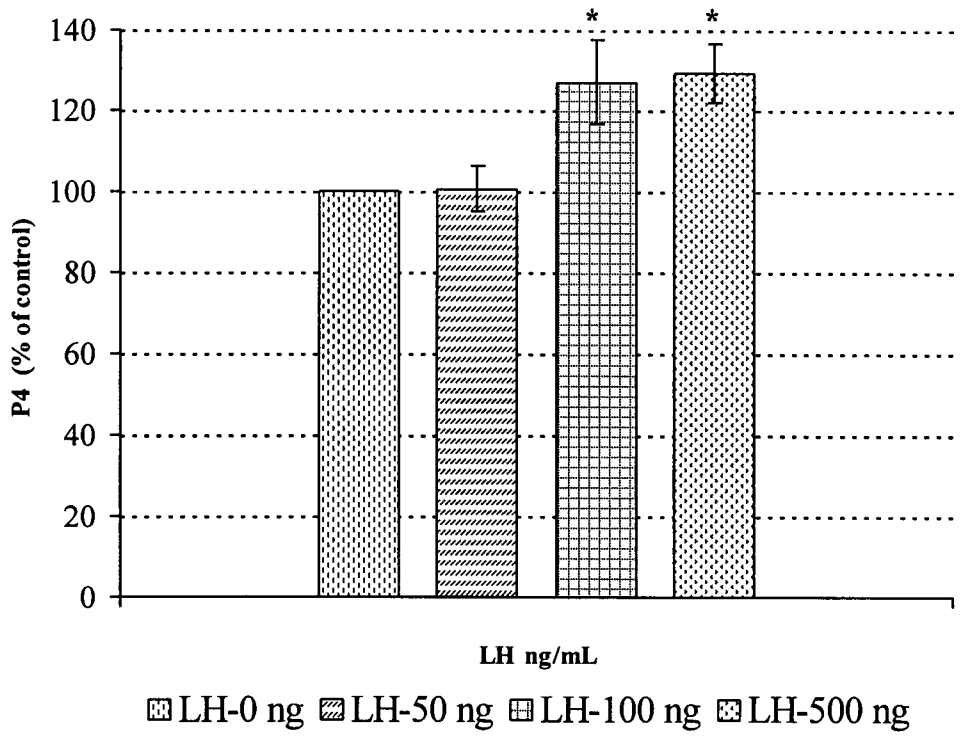

FIGURE 3.5. LH dose-response on *in vitro* progesterone (P4) synthesis in luteal tissue. Minced luteal tissue samples were cultured *in vitro* and treated with different doses of LH (0, 50, 100, and 500 ng/ mL) for 6 h. Data represents the mean ± SEM of 4 individual CL (stage III). * different ($P < 0.05$) from untreated control samples (LH-0 ng/mL).

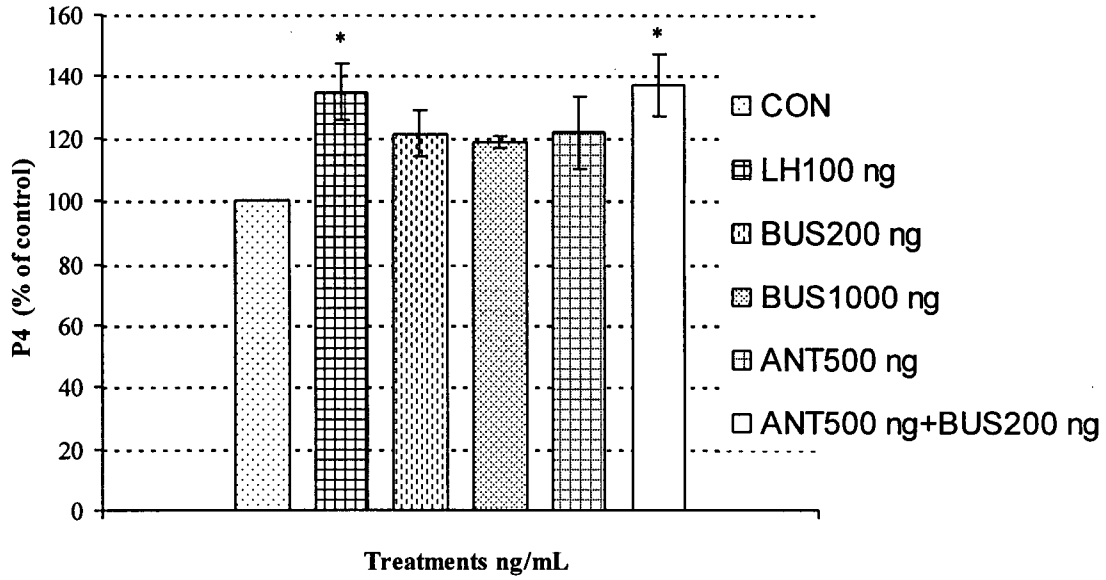

FIGURE 3.6. Effect of GnRH-a on progesterone (P4) synthesis in bovine luteal tissue. Minced luteal tissue samples were cultured *in vitro* and treated with different doses of buserelin (BUS) for 6 h. Treatments consisted of only medium (CON), LH100 ng, BUS 200 ng, BUS 1000 ng, Antide (ANT) 500 ng, ANT + BUS 200 ng, Data represents the mean ± SEM of 5 trials. * different ($P < 0.05$) from control samples.

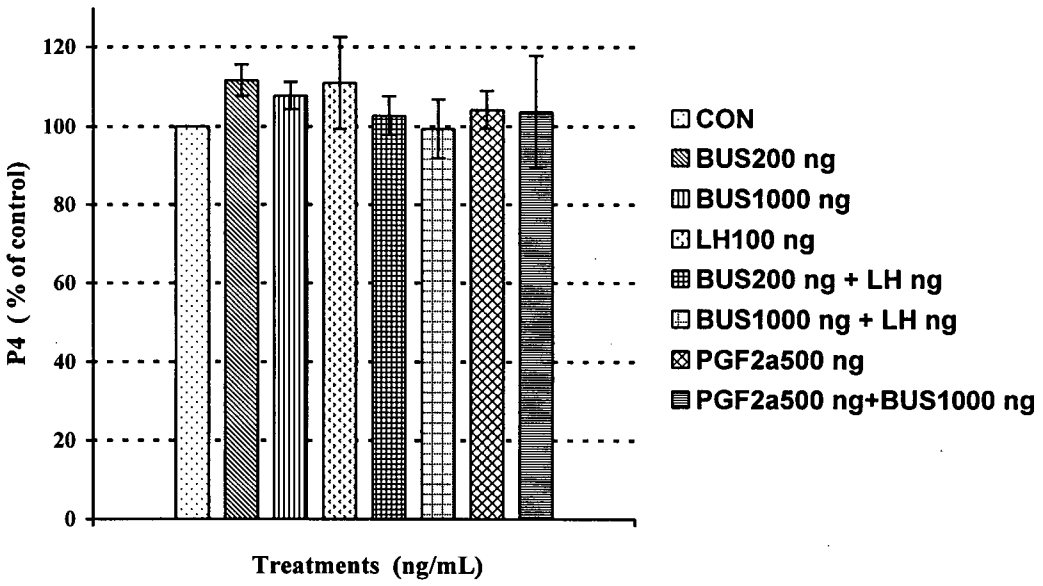

FIGURE 3.7. Effect of GnRH-a in combinations of LH and $PGF_{2\alpha}$ on progesterone (P4) synthesis in bovine luteal tissue. Minced luteal tissue was cultured *in vitro* and treated with buserelin (BUS) in combinations of LH or $PGF_{2\alpha}$ for 6 h. Treatments consisted of medium alone (CON), LH100 ng, BUS200 ng, BUS1000 ng, BUS200 ng + LH100, BUS1000 ng + LH100 ng, $PGF_{2\alpha}$500 ng, BUS1000 ng + $PGF_{2\alpha}$500 ng. Data represents the mean ± SEM of 4 trials.

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CHAPTER 4

EFFECTS OF GnRH-a ON StAR PROTEIN, P450scc, 3β-HSD, Bcl2 AND Bax mRNA EXPRESSION IN BOVINE CORPUS LUTEUM

4.1. ABSTRACT

This study investigated the direct effects of the GnRH-a (buserelin) on mRNA expression levels for the steroidogenic molecular machinery; StAR protein, P450scc, 3β-HSD, and assessed the influence of buserelin on mRNA levels for the pro- and anti-apoptotic molecules Bax and Bcl2 in bovine corpus luteum (CL). Bovine CL (stage III or matured CL) were obtained from a local abattoir and transported to the laboratory in ice-cold DMEM-F12 culture medium. Minced luteal tissue samples (100 mg) were prepared (pooled tissue from 2 or 3 CL) and subjected to buserelin treatment *in vitro*. Treatments were comprised of untreated control (medium only), LH-100 ng, buserelin (BUS) 200 ng, BUS 1000 ng, GnRH antagonist (antide; ANT) 500 ng, and ANT 500 ng + BUS 200 ng. Treatment cultures were incubated for 6 h at 38°C, in a humidified atmosphere of with 95 % air, and 5 % CO2. At the end of the treatment period, incubation was terminated and the culture medium was decanted from the treatment wells. The remaining luteal tissue in culture wells was subjected to snap freezing in liquid nitrogen, and then stored at –75°C for total RNA isolation at a later time. The mRNA levels were assessed by the semi-quantitative RT-PCR method. Results revealed that despite the tendency for a stimulatory response, the effect of buserelin on mRNA levels for StAR protein and P450scc were not different from that of control samples. However, GnRH-a caused a stimulatory response on 3β-HSD mRNA levels that tended to be different ($P = 0.12$) from untreated control samples. Buserelin treatment had no influence on the

mRNA levels for pro-and anti-apoptotic molecules (Bax and Bcl2) in luteal tissue. In conclusion, GnRH-a treatment exhibited an apparent tendency towards a stimulatory response on steroidogenic machinery molecules in bovine CL. The present study revealed no evidence of any adverse effects of GnRH-a treatment in terms of inducing apoptotic or lutelytic process in bovine luteal tissue.

4.2. INTRODUCTION

The hypothalamic gonadotropin releasing hormone (GnRH) is released into the portal circulation, and binds to its receptors on pituitary gonadotropes and causes synthesis and release of FSH and LH into systemic circulation. Both the FSH and LH, in turn, act on the gonads governing the process of gametogenesis and steroidogenesis (Conn and Crowley, 1994; Stojilkovic and Catt, 1995). In addition, it has been suggested that GnRH may have a role as a modulator of the activity in diverse systems in the brain, and many peripheral organs (Jones et al., 1980; Hsueh and Jones, 1981; Emons and Schally, 1994). Several reports have suggested an extra-hypothalamic origin of GnRH, as well as the extra-pituitary presence of GnRH receptors (GnRH-R) in different types of tissues in the body. With respect to the reproductive system, it is becoming increasingly evident that there is a functional GnRH-GnRH-R system that exists in different laboratory species (rats, pigs, and monkeys) and in humans (CHAPTER 1; 1.2.5 and 1.2.6). Further, there are numerous reports that suggest there are direct effects of GnRH that may act in an autocrine or paracrine manner, eliciting a variety of responses in reproductive tissues. Several studies have demonstrated that GnRH modulates, both basal and gonadotropin, stimulated steroidogenesis in the ovary (Olofsson et al., 1995). It has been shown that GnRH-a administration causes suppression of

FSH and LH receptors (Tilly et al., 1992; Piquette et al., 1991; Guerrero et al., 1993) or gonadotropin-induced cAMP levels in rats (Richards, 1994; Knecht et al., 1985). Further,*in vivo* administration of GnRH-a (buserelin) in rats has resulted in suppression of steroidogenic enzyme activity, such as steroidogenic acute regulatory protein (StAR), cytochrome P450 side chain cleavage enzyme (P450scc), and 3β-hydroxy steroid dehydrogenase (3β-HSD), in the CL of rats (Sridaran et al., 1999a; Sridaran et al., 1999b). Contrarily, no effect of GnRH was found on P4 production in human granulosa-lutein cells (Casper et al., 1984). Collectively, it is evident that GnRH, or its analogues, elicit widely varying responses, depending on the type of tissue or physiological status of the body.

It is a widely accepted fact that apoptosis, or programmed cell death, is of central importance for development and morphological homeostasis in the body (Steller, 1995). Hence, apoptosis has been considered as one of the key mechanisms that occur during the process of luteal demise in different species, including the bovine species (Juengal et al., 1993; Chun et al., 1994; Quirk et al., 1995; Rueda et al., 1995 and 1997). In the ovary, there are several specific regulators of apoptosis, including hormones, growth factors and cytokines (Chun and Hsueh, 1998). Lack of exposure, or overexposure, to some of the hormones may induce apoptosis by causing changes in the intracellular environment. For example, gonadotropins are known as follicle survival factors. GnRH, on the other hand, is suggested to play a physiological role associated with follicular atresia and CL demise in the vertebrate ovary, possibly via stimulation of apoptosis (Billig et al., 1994; Kogo et al., 1995; Sridaran et al., 1998; Zhao et al., 2000). Further, reports have indicated that GnRH-I and GnRH-II can directly induce apoptosis in the ovarian cell types in both mammalian, and non-

mammalian, vertebrates (Imai et al., Yano et al., 1997; Andreu-Vieyra and Habibi, 2000). However, no such information has been reported in the bovine species. In the wake of this interesting evidence from other species, it is of great interest to examine if similar mechanisms take part in the bovine ovary cellular level, where GnRH, or its agonists, are employed on a routine basis in reproductive management practices.

Therefore, the experiments presented in this chapter were carried out to further examine the results from our previous study where the tendency for a GnRH-a stimulatory response on *in vitro* steroid output was observed in bovine CL. In addition, we examined the possible direct influence of GnRH-a on the apoptotic process in bovine CL. The specific objectives of these studies were as follows: (i) To determine the GnRH-a induced alterations in mRNA expression levels for key regulatory molecules in steroidogenic machinery: StAR protein, 3β-HSD, and P450scc enzyme, (ii) To examine the direct influence of GnRH-a on mRNA expression levels for pro (Bax) and anti (Bcl2) apoptotic molecules in the bovine CL.

4.3. MATERIALS AND METHODS

4.3.1. Preparation of minced CL tissue and *in vitro* culture conditions

Bovine ovaries were obtained at a local abattoir, and transported in ice-cold DMEM-F12 supplemented with antibacterial (penicillin 100,000 IU/L, streptomycin 100,000 μg/L) and antimycotic (amphoterocin B 2.5 ng/mL) agents. In the laboratory, ovaries were washed with cold saline, subjected to a quick dip in 70% ethanol, and then immediately washed once again with cold saline in order to remove the ethanol residue. The individual CL were inspected carefully, and Stage III CL (mature CL) were selected as per the procedure

outlined by Ireland et al. (1980). Tissue samples were harvested from 2 or 3 CL, pooled together, and minced into smaller pieces in a large petri dish containing ice cold HBSS. Minced tissue was washed 2 to 3 times in cold HBSS. During each washing cycle, the excessive HBSS and floating tissue fragments were aspirated using a Pasture pipette. Further, any remaining liquid content from minced tissue was removed by briefly placing the tissue on multi layered sterile Kim wipes. Immediately, about 100 mg of tissue was weighed and randomly distributed into individual wells (24-well tissue culture plates, Falcon PrimariaTM) containing 1 mL of cold HBSS solution. The tissue samples in individual culture wells were given an additional wash in cold HBSS with gentle agitation, and excess HBSS was removed. Care was taken to avoid the removal of tissue while aspirating HBSS during each washing step. Immediately, the tissue sample in each well was supplied with warm DMEM-F12 culture medium. DMEM-F12 medium was supplemented with 25-hydroxycholestral (20 μg/mL), human transferrin (5 ng/mL), selenium (4 ng/mL) and gentamicin (50 μg/mL). Each treatment well contained a final volume of 1 mL DMEM-F12 including the treatments (v/v). During the initial steps, the viability of the minced luteal tissue culture conditions was ascertained by examining the LH dose response on P4 accumulations in spent culture medium samples (CHAPTER 3; 3.3.3.5. and 3.4.2.2).

4.3.2. GnRH-a treatment of luteal tissue

The different treatments were comprised of untreated control (medium only, CON), LH-100 ng, buserelin (BUS) 200 ng, BUS 1000 ng, Antide (ANT) 500 ng, and ANT 500 ng + BUS 200 ng. Treatment cultures were incubated for 6 h at 38°C, in a humidified atmosphere of 95 % air, and 5 % CO2. At the end of treatment (6 h), incubations were

terminated, and the spent culture medium was decanted from culture wells. The remaining tissue samples in culture wells were immediately subjected to "snap" freezing in liquid nitrogen, and stored at –75°C for total RNA isolation at a later time. At least five trials that were conducted on separate occasions were included in this study. Each treatment levels were comprised of triplicate samples.

4.3.3. Total RNA isolation from GnRH-a treated luteal tissue

From post-treatment luteal tissues, total RNA was isolated by following a single step RNA isolation method (Chomczynski and Sacchi, 1987), using a commercially available total RNA isolation solution, Tri Reagent. Briefly, about 100-150 mg of luteal tissue was pooled from replicate treatment wells. Using mortar and pestle, the tissue sample was pulverized in liquid nitrogen, and immediately transferred into sterile 1.5 mL micro-centrifuge tubes containing 1 mL of Tri Reagent solution. The tube contents were mixed thoroughly, and allowed to stand for 5 min at room temperature in order to facilitate complete dissolution of nuclear proteins, and cytoskeletal components. For each mL (initial volume) of Tri Reagent solution, 200 µl of chloroform was added to each tube, and samples were agitated vigorously for 30 s. Samples were allowed to stand at room temperature for 10 to 15 min, and then centrifuged at 12000 x G for 15 min at 4°C. The top layer with clear and transparent solution containing total RNA molecules was carefully transferred into a new set of sterile micro-centrifuge tubes. Each tube was supplied with a 0.5 mL of isopropanol, mixed, and samples were allowed to stand for 15 min at room temperature. The samples were centrifuged at 12000 x G for 10 min at 4°C, and supernatant was discarded carefully. The resultant pellet containg total RNA at the bottom of the tube was washed twice in ice-cold

75% ethanol by centrifuging at 12000 x G for 5 min. The resultant total RNA pellets were air-dried for 10-15 min, and finally dissolved in sterile DEPC-treated water. The quantity and quality of total RNA were assessed by both spectrophotometry, and by observing clear bands for 28S, and 18S, ribosomal RNA species on ethidium bromide stained agarose gel (0.8%). Total RNA was either used immediately for cDNA preparation, or stored at –75°C for subsequent use.

4.3.4. Semi-quantitative Reverse Transcription-Polymerase Chain Reaction

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) used in this study was accomplished by utilizing the commercially available first strand cDNA Synthesis kits (The Cells-to-cDNA II Kit, Ambion, Inc. The RNA Company, Austin, Texas, USA), and PCR kits (JumpStart RED Taq Ready mix kit, Sigma-Aldrich Canada Ltd. Oakville, ON, Canada). RT-PCR reactions were carried out as per the kit manufacturer's protocol, with necessary modifications. During initial attempts, the amount of template RNA, magnesium concentration, and compatibility of primer pairs was tested to determine optimal conditions for RT-PCR (data not shown). Using Kit supplied random decamer primers, 2 μg of the total RNA sample was reverse-transcribed in 20 μl reaction volumes. The following components were included in the RT reaction: 10X RT buffer pH 7.4 (2 μl), dNTP (0.5 mM each) 1 μl, M-MLV reverse transcriptase 1μl (10 U), RNase inhibitor 1 μl (10 U), Random decamers 5 μM, and total RNA 2 μg. The final volume was adjusted to 20 μl using nuclease-free water. The RT step was performed at 42°C for 60 min, and then incubated at 95°C for 10 min to inactivate the reverse transcriptase enzyme. RT samples were stored at -20°C for future use in PCR amplification.

During PCR amplification, 2 μl of RT sample (first strand cDNA) were used to co-amplify the housekeeping gene, G3PDH (as an internal standard), in the following combinations of different gene transcripts: G3PDH, StAR and P45oscc; G3PDH and 3β-HSD; G3PDH, Bcl2 and Bax. The PCR mix (JumpStart RED Taq ReadyMix) was comprised of 20 mM Tris-HCl, pH 8.3, 100 mM KCl, 4 nM $MgCl^2$, 0.002% gelatin, 0.4 mM each dNTP (dATP, dCTP, dGTP, dTTP), inert dye, stabilizers, 0.06 U/μl Taq DNA Polymerase, JumpStart Taq antibody, 400 nM of each primer (forward and reverse), and the required amount of nuclease-free water to make up the final volume of 25 μL reaction mix. The primer sequence and their respective expected sized PCR amplicons are presented in Table 4.1. The PCR conditions were as follows: initial denaturation for 3 min at 94°C, (G3PDH, StAR, P450scc, 3β-HSD, Bcl2, and Bax), followed by subsequent cycles with denaturation at 93°C for 40 sec, annealing at 58°C for 50 sec (G3PDH, StAR, P450scc, 3β-HSD), at 62°C for 50 sec (Bcl2, Bax, G3PDH) and extension for 1 min at 72°C, and an additional extension for 10 min at 72°C. The annealing temperature for G3PDH was found to be in the range of 55°C to 65°C (data not shown). For each gene of interest, PCR amplification was calibrated to determine the optimal number of cycles that would allow detection of the appropriate mRNA transcripts, while still keeping the amplification of these genes in the log phase. The different gene transcript, PCR combinations including StAR, P450scc, G3PDH; 3β-HSD, G3PDH; and Bcl2, Bax, G3PDH were allowed a total of 23, 22, and 27 cycles, respectively.

An aliquot (equal volume) from each reaction mixture (PCR) was electrophoresed on a 2% agarose gel containing ethidium bromide, and photographed under ultraviolet

illumination. The optical density of individual bands (inverse image) was analyzed by using Scion Image computerized densitometry software (Scion Image, http://www.scioncorp.com).

4.3.5. Data analysis

Each experiment was repeated at least five times. The data represent the mean ± SEM, and is presented as relative fold change to the control values. Densitometry values were normalized against corresponding, internal control G3PDH values, analyzed by one-way ANOVA, and followed with a pair-wise comparison using a Tukey-Karmer test. Any difference in treatment effects are indicated with respective P values. All data were analyzed using NCSS Statistical Software-trail version (Kaysville, Utah).

4.4. RESULTS

4.4.1. Validation of semi-quantitative RT-PCR

Semi-quantitative RT-PCR method was used to determine the mRNA levels for StAR protein, P450scc, 3β-HSD, Bcl2 and Bax in luteal tissue. A linear relationship (log phase), PCR amplification for different gene transcripts and the internal control, G3PDH was found between 19 to 32 cycles (Fig. 4.1, 4.2, 4.3 for StAR and P450scc, 3β-HSD, Bcl2 and Bax, respectively). The expected sized PCR amplicons for different gene transcripts StAR, P450scc, 3β-HSD, Bcl2, Bax, and G3PDH were 590 bp, 362 bp, 360 bp, 154 bp, 362 bp, and 850 bp, respectively. This method of semi-quantitative mRNA measurement using G3PDH as an internal control has been previously described (Mamluk et al., 1998; 1999). The possibility of PCR cross-contamination, or genomic DNA amplification, was ruled out

because no PCR products were observed in negative controls (without template and without reverse transcriptase enzyme during RT step).

4.4.2. Effects of GnRH-a on StAR protein, P450scc, 3β-HSD, Bcl2 and Bax mRNA levels in the bovine CL

The semi-quantitative RT-PCR results of luteal tissue mRNA levels for StAR protein, P450scc, 3β-HSD, and Bax and Bcl2 are shown in figures 4.4, 4.5, 4.6, and 4.7 respectively. Despite the tendency for a mild stimulatory response, GnRH-a (buserelin) treatment did not affect mRNA levels for StAR protein and P450scc, whereas buserelin effect on mRNA levels for 3β-HSD tended to be different ($P = 0.12$) from that of untreated control samples (Fig. 4.6). Treatment of luteal tissue with GnRH-a (Buserelin, 200 ng, 1000 ng), in combination of antide, or antide alone (500 ng/mL), did not affect the mRNA levels for pro- and anti-apoptotic gene transcripts, Bax and Bcl2 (Fig. 4.7), respectively

4.5. DISCUSSION

The present studies investigated the direct effects of GnRH-a on molecular steroidogenic machinery, as well as GnRH-a influence on mRNA expression for pro- and anti-apoptotic molecules in bovine CL. During *in vitro* culture, GnRH-a treatment of CL tissues caused a mild stimulatory response on mRNA levels of StAR, P450scc and 3β-HSD although an approaching significance levels could be seen only in case of 3β-HSD. On the other hand, GnRH-a treatment of bovine luteal tissues during *in vitro* culture did not affect the mRNA levels for either anti- or pro-apoptotic molecules, Bcl2 and Bax, respectively. Overall, GnRH-a caused an apparent mild stimulatory response on mRNA transcripts for the

above-specified key regulatory molecules of CL steroidogenic machinery. This was a typical of the response observed in terms of P4 output from luteal cells and CL tissues, following GnRH-a treatment *in vitro* (CHAPTER 3).

The present findings are in agreement with earlier reports where the CL in heifers administered deslorelin (GnRH-a) had a greater content of StAR protein, and the steroidogenic enzyme, P450scc (Pitcher et al., 1997 cited by D'Occhio and Aspden, 1999). The same group of researchers also reported a significant increase in testosterone levels and mRNA expression level for StAR, P450scc, 3β-HSD, and P45017∝, in testicular tissues of bulls that were administered deslorelin (Aspden et al., 1998). From recent studies, it is evident that the GnRH-a (leuprolide acetate) in combination of eCG administration in mice caused a significant increase in not only StAR mRNA, but also StAR protein and P4 level in ovarian follicles (Irusta et al., 2003). Overall, the present findings are in agreement with similar observations where increased plasma P4 levels were a characteristic feature in many of the studies with GnRH administration during the luteal phase in cattle (Thatcher et al 1993, D'Occhio and Aspden, 1999; D'Occhio et al., 2000; Rajamahendran et al., 1998, 2001). Further, these studies confirm the important fact that GnRH-a administration during luteal phase does not adversely affect the CL function in bovine species, as it has been reported as an opposite phenomenon in the majority of the studies in other species of animals including humans (Reviewed by Janssens et al., 2000). The present findings differ from the similar studies where the GnRH-a, buserelin (Sridaran et al., 1999a; Sridaran et al., 1999b) or leuprolide acetate (Andreu et al, 1998), treatment caused a significant suppressive effect of StAR, 3β-HSD and P450scc in rat ovaries.

The steroidogenic machinery molecules, StAR protein, cytochrome P450scc, and 3β-HSD, are key and rate limiting factors that govern P4 synthesis in the CL. In luteal cells, StAR protein facilitates the transfer of free-form cholesterol into the inner mitochondrial membrane. This protein can induce acute changes in the rate of steroidogenesis by mobilizing cholesterol, and subjecting it to the enzymes that are already present. These acute changes can happen within a matter of minutes. In the mitochondria, the special type of enzyme (P450scc) that is present within the inner mitochondrial membrane converts the free-form cholesterol into pregnenolone through cleavage of cholesterol side chain (Milvae et al., 1996). Further, pregnenolone is released into the cytoplasm where another key enzyme, 3β-HSD, that exists in smooth endoplasmic reticulum, converts pregnenolone into P4. The resultant P4 is then processed in the Golgi apparatus, and released into general circulation (Reviewed by Niswender et al., 2000).

In the present studies, the exact mechanisms that affected a mild stimulatory response on mRNA expression levels StAR, P450scc and 3β-HSD are not known. In addition, these findings do not confirm whether the GnRH-a effect was, indeed, due to its direct interaction with the GnRH specific receptor on luteal cell types in bovine CL. More studies are needed to further explore such a possibility, and confirmation of any functional forms of GnRH receptor molecules and their mode of interaction with its ligand is needed. However, it has been suggested that GnRH could mediate its direct effects in conjunction with other hormonal agents such as PGF2α, or LH, that are well known to regulate ovarian function (Reviewed by Steele and Leung, 1993). Stimulation of one or more signaling pathways such as phospholipase C (PLC), phospholipase A2 (PLA2) and phospholipase D (PLD), and

activation of protein kinase C (PKC) has been hypothesized as causing either inhibitory, or stimulatory effects on ovarian cellular steroid output. These dual effects have been clearly demonstrated during *in vivo* experiments in adult male and female hypophysectomized rats, where exogenous GnRH or GnRH-a could either stimulate or inhibit gonadal functions in terms of steroidogenesis (Hsueh and Jones, 1981, 1982). GnRH has been shown to elicit mixed responses affecting ovarian function (reviewed by Sharpe, 1982; Janssens et al., 2000; Leung et al., 2003). The inhibitory action of GnRH, or its agonists, on gonadal steroidogenesis involves suppression of the gonadotropin receptors, or intermediary enzymes, involved in the steroidogenic pathway. GnRH-a induced suppression of FSH and LH receptors (Tilly et al., 1992; Piquette et al., 1991; Guerrero et al., 1993), and GnRH-a caused suppression of gonadotropin-induced cAMP levels, has been reported (Richards, 1994; Knecht et al., 1985).

From the present studies, *in vitro* treatment of luteal tissue with GnRH-a did not affect the mRNA levels for pro- and anti-apoptotic molecules, Bax and Bcl2. These findings differ from several other studies of different species where GnRH-a was shown to induce apoptotic process in ovarian cell types. During *in vitro* culture, GnRH inhibited DNA synthesis (Saragueta et al., 1997), or induced apoptosis in rat granulosa cells (Billig et al., 1994). Studies have shown evidence for GnRH-induced remodeling of the extra-cellular matrix. Where GnRH induced structural luteolysis in superovulated rats through stimulation of matrix metalloproteinase (MMP-2), and membrane type 1-MMP expression (in a developed CL), which degraded collagens type IV, type III, and type I, respectively (Goto et al., 1999). During early pregnancy in the rat, GnRH-a has been shown to suppress serum P4

levels, which is associated with an increased degree of DNA fragmentation in the CL (Sridaran et al., 1998). Similar effects of GnRH-a-inducing increased number of apoptotic bodies in human granulosa cells (obtained during oocyte retrieval for *in vitro* fertilization) was demonstrated by Zhao et al. (2000).

In the ovary, there are several specific regulators of apoptosis, including hormones, growth factors and cytokines (Chun et al., 1994; Chun and Hsueh, 1998; Yang and Rajamahendran, 2000). Transforming growth factor β (TGFβ) (Martimbeau and Tilly 1997), as well as some locally produced cytokines, are believed play a role in regulating apoptosis in the ovary (Richards, 1994). For example, in cultured rat follicles, IL-1β suppressed follicle apoptosis in a dose dependent manner (Chun et al., 1995). In contrast, IL-6 was shown to induce apoptosis in cultured rat granulose cells (Grospe and Spangelo, 1993). Although the exact mechanism of apoptotic cell death is not clear, some of the processes involved appear to be highly conserved. Different signaling pathways, that are cell specific, ultimately converge to activate a common or similar apoptotic death program (Hsu and Hsueh, 1998).

Two important families of regulators of the apoptotic process are the molecules of Bcl-2 families and caspases (Hengartner, 2000). The Bcl-2 family of genes includes both apoptosis promoting (e.g. Bax, Bok, and Bad), and apoptosis inhibiting (e.g. Bcl-2 and Bcl-XL, Mc1-1) members (Antonsson and Martinou, 2000). The Bcl-2 family members are located in the outer membranes of the mitochondria, and can bind to each other in different pair-wise conditions. In most cases, the ratio of pro-apoptotic to anti-apoptotic Bcl-2 homologues within a cell determines whether the cell undergoes apoptosis or not (Gajewski

and Thompson, 1996; Antonsson et al., 1997). The main function of the Bcl-2 family seems to be regulating the release of pro-apoptotic factors, particularly cytochrome C, from the mitochondria into the cytosol (Antonsson and Martinou, 2000). Many members of the Bcl-2 family have been isolated in the ovary, including Bad, Mcl-1, Bcl-XL and Bok (Hsu and Hsueh, 2000). Caspases are cytosolic proteases, and are synthesized as inactive precursors that are cleaved by other caspases, or autocatalytically in order to become activated. An important function of caspases is to activate caspase-activated DNase (CAD), the endonuclease responsible for inter-nucleosomal DNA fragmentation, one of the most frequently used hallmarks of apoptosis (Yuang, 1997; Nagata, 1997). CAD and its inhibitory subunit, inhibitor of caspase-activated DNase (ICAD), are constantly expressed in the cells. Caspase-mediated cleavage of the inhibitory subunit results in release and activation of the endonuclease (Yuang, 1997; Nagata, 1997).

The present study reveals no evidence of any adverse effects of GnRH-a in terms of inducing programmed cell death in bovine luteal tissue, as reported in other species. Besides species difference, the reason for this differential response of GnRH-a on luteal tissue or cell types is not known. However, findings from these studies do not reveal any conclusive evidence with respect to the lack of a GnRH-a effect causing programmed cell death, contrary to the findings from other species. More studies involving, individual cell types, rather than whole organ/tissue culture systems and different dose levels of GnRH-a, may provide more concrete and reliable evidence. It is also important to undertake comprehensive investigations utilizing different approaches such as assessing the hall mark of apoptosis,

inter-nucleosomal DNA fragmentation, or examining the early responder of apoptotic process, FAS and FAS ligand system.

4.6. CONCLUSION

From the present studies, results revealed a mild stimulatory response of GnRH-a, buserelin on mRNA expression levels for StAR, P450scc, and 3β-HSD, which, are key regulatory molecules of steroidogenic machinery mechanism in bovine CL. However, statistical significance could not be achieved. Secondly, unlike results from other species, GnRH-a treatment did not cause any adverse effect on CL function by inducing or augmenting the apoptotic process and luteolysis.

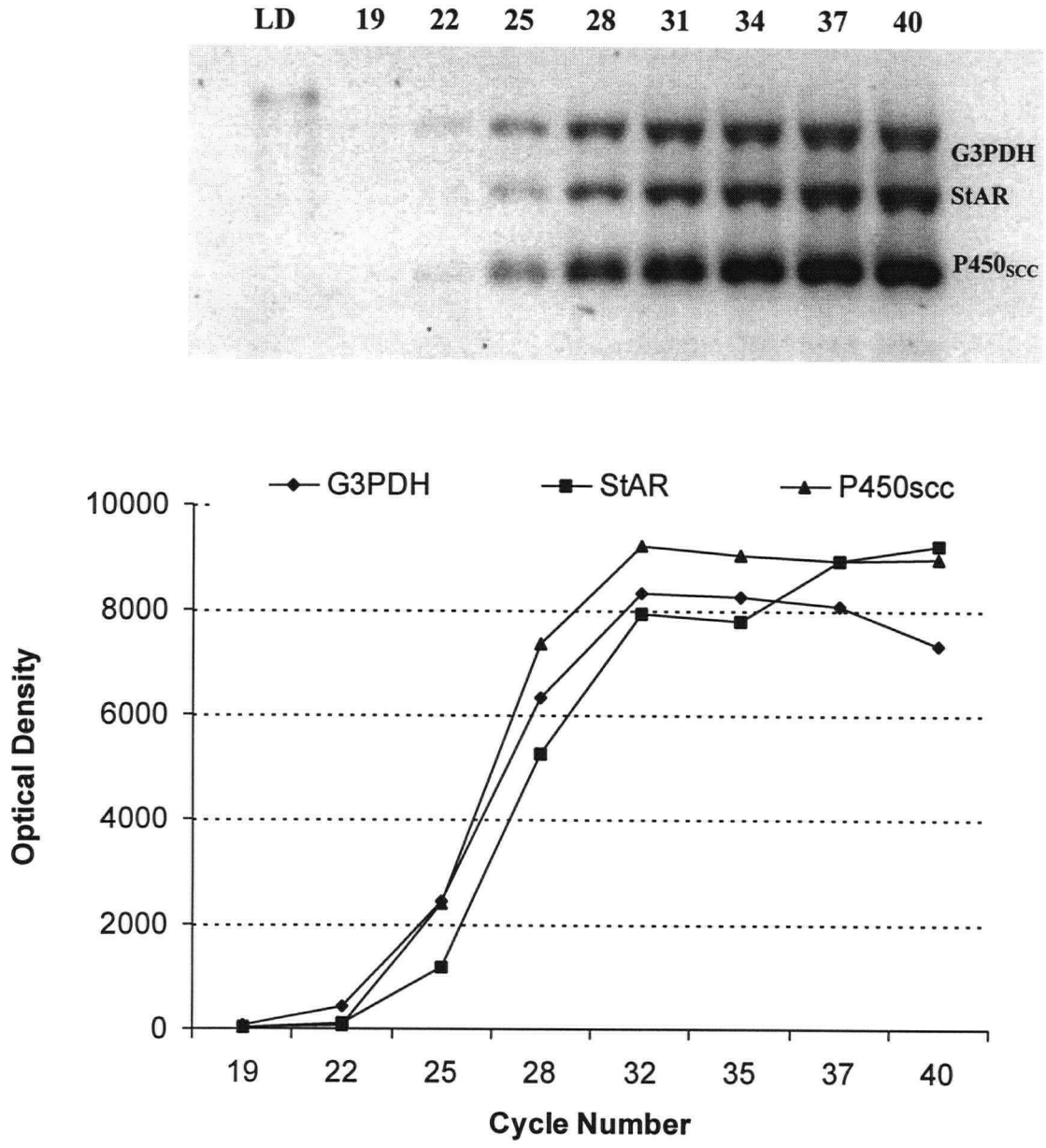

FIGURE 4.1. Characterization of semiquantitative RT-PCR for G3PDH, StAR protein, and P450scc mRNA transcripts from bovine luteal tissue. Total RNA was extracted from experimental samples, reverse transcribed, and amplified using a thermal cycler as described under Materials and Methods. PCR products were electrophorosed on 2% agarose gel, stained with ethidium bromide and photographed. Inverse images (upper panel) were analyzed by densitometry and the linear relationship between PCR products and amplification cycles is shown in the lower panel. LD, molecular weight base pair (ladder).

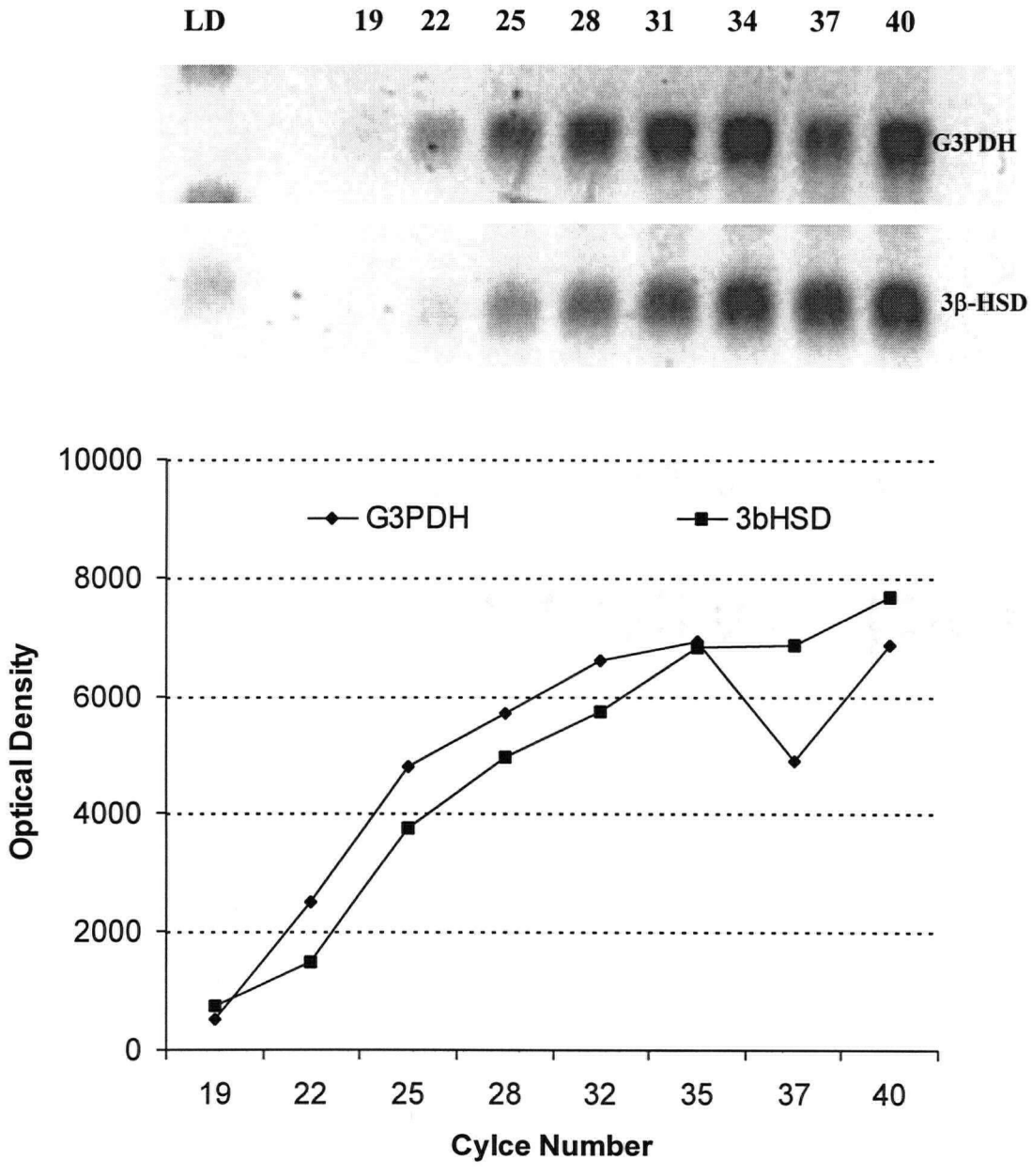

FIGURE 4.2. Characterization of semiquantitative RT-PCR for G3PDH and 3β-HSD mRNA transcripts from bovine luteal tissue. Total RNA was extracted from experimental samples, reverse transcribed, and amplified using a thermal cycler as described in Materials and Methods. PCR products were electrophorosed on 2% agarose gel, stained with ethidium bromide and photographed. Inverse images (upper panel) were analyzed by densitometry and the linear relationship between PCR products and amplification cycles is shown in the lower panel. LD, molecular weight base pair (ladder).

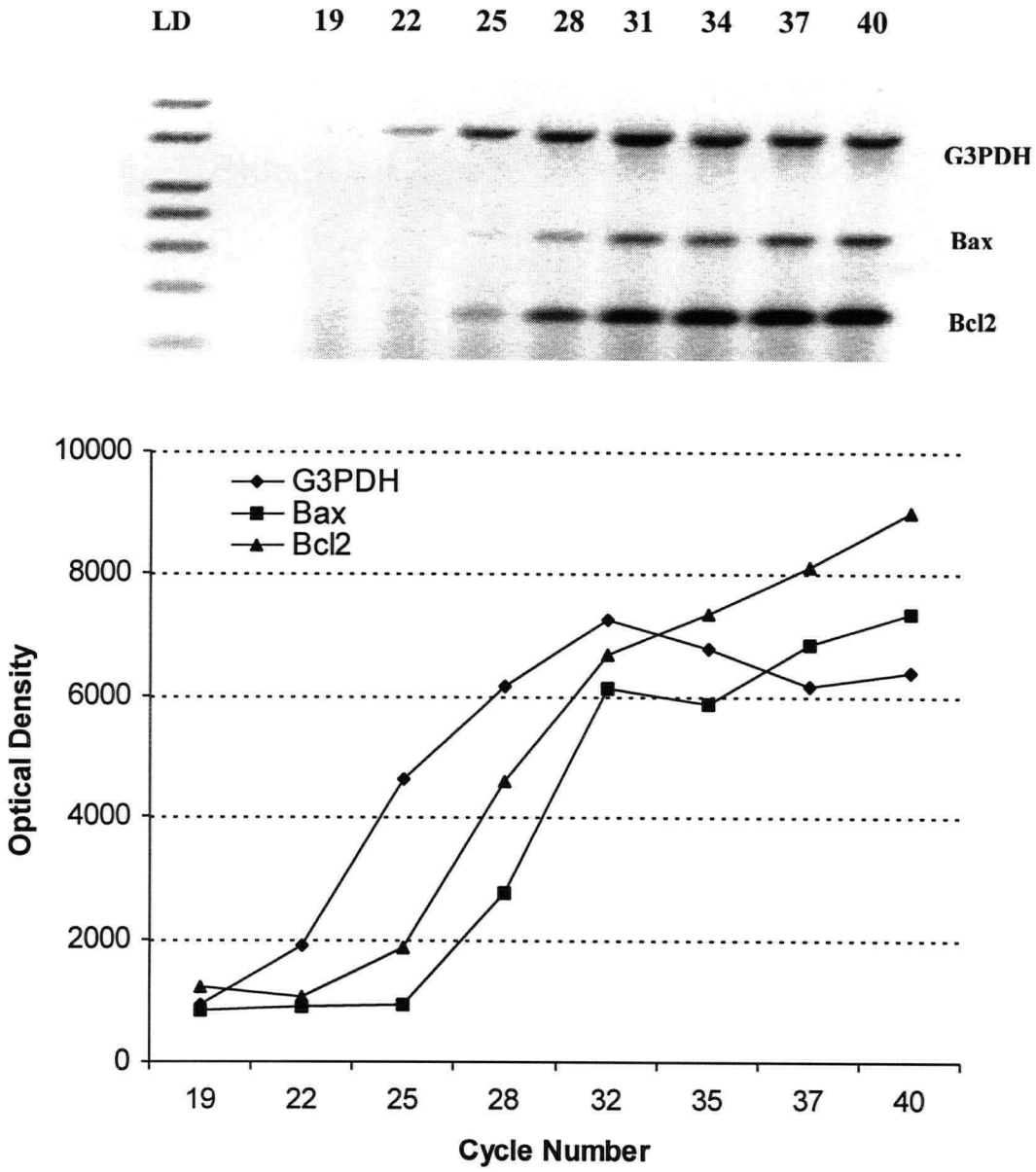

FIGURE 4.3. Characterization of semiquantitative RT-PCR for G3PDH, Bcl2, and Bax mRNA transcripts from bovine luteal tissue. Total RNA was extracted from experimental luteal tissue, reverse transcribed, and amplified using a thermal cycler as described in Materials and Methods. PCR products were electrophorosed on 2% agarose gel, stained with ethidium bromide and photographed. Inverse images (upper panel) were analyzed by densitometry and the linear relationship between PCR products and amplification cycles is shown in the lower panel. LD, molecular weight base pair (ladder).

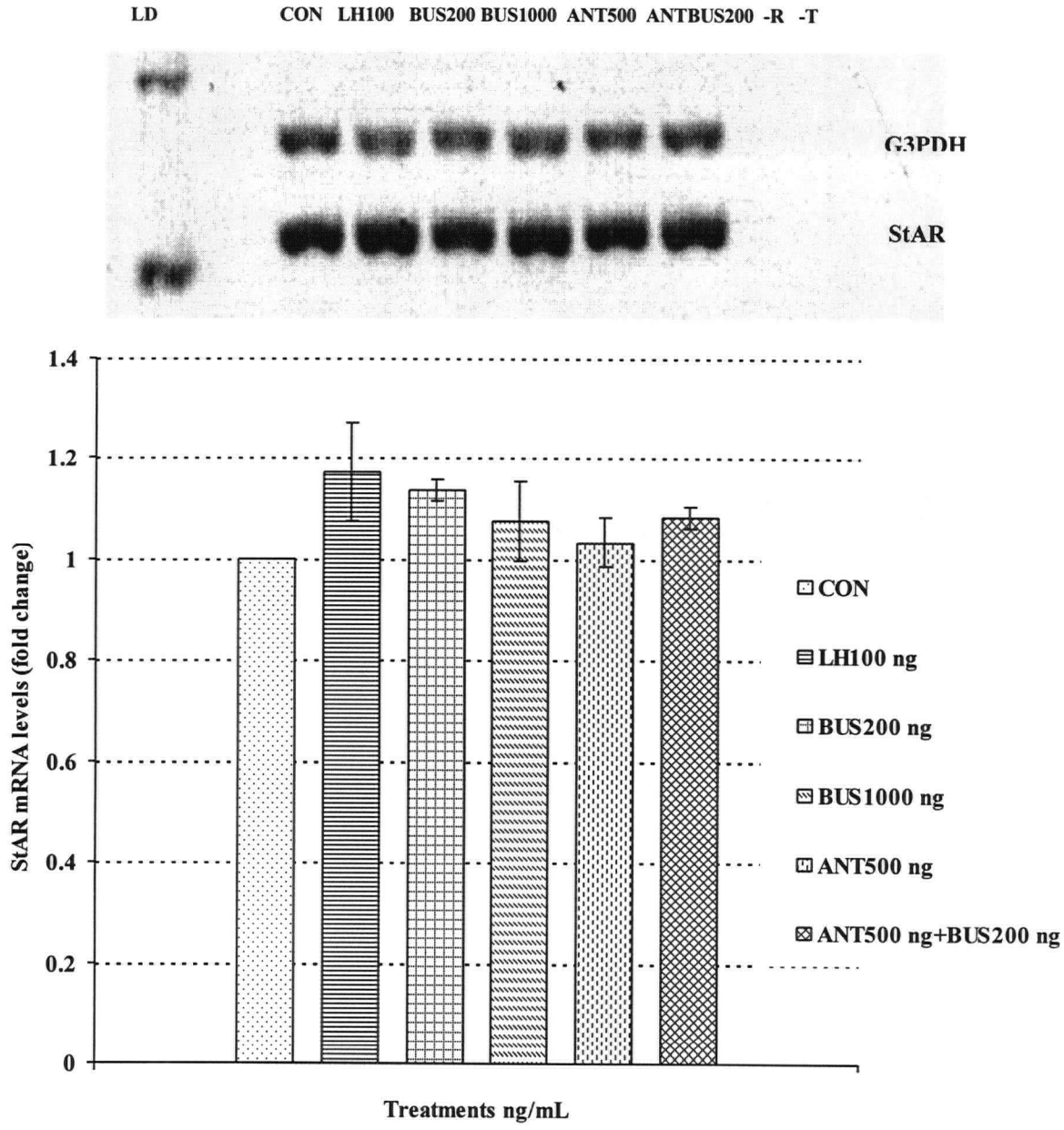

FIGURE 4.4. Effect of GnRH-a on StAR protein mRNA levels in bovine luteal tissues. Minced luteal tissue samples were cultured *in vitro* and treated with different doses of buserelin medium only (CON), LH 100 ng, buserelin (BUS) 200 ng, and BUS 1000 ng, Antide (ANT) 500 ng, ANT 500 ng + BUS 200 ng. BUS dose response on StAR protein mRNA levels (fold change from CON) is shown in the histogram. LD, molecular weight base pair (ladder); -R, minus RTase; -T, minus template.

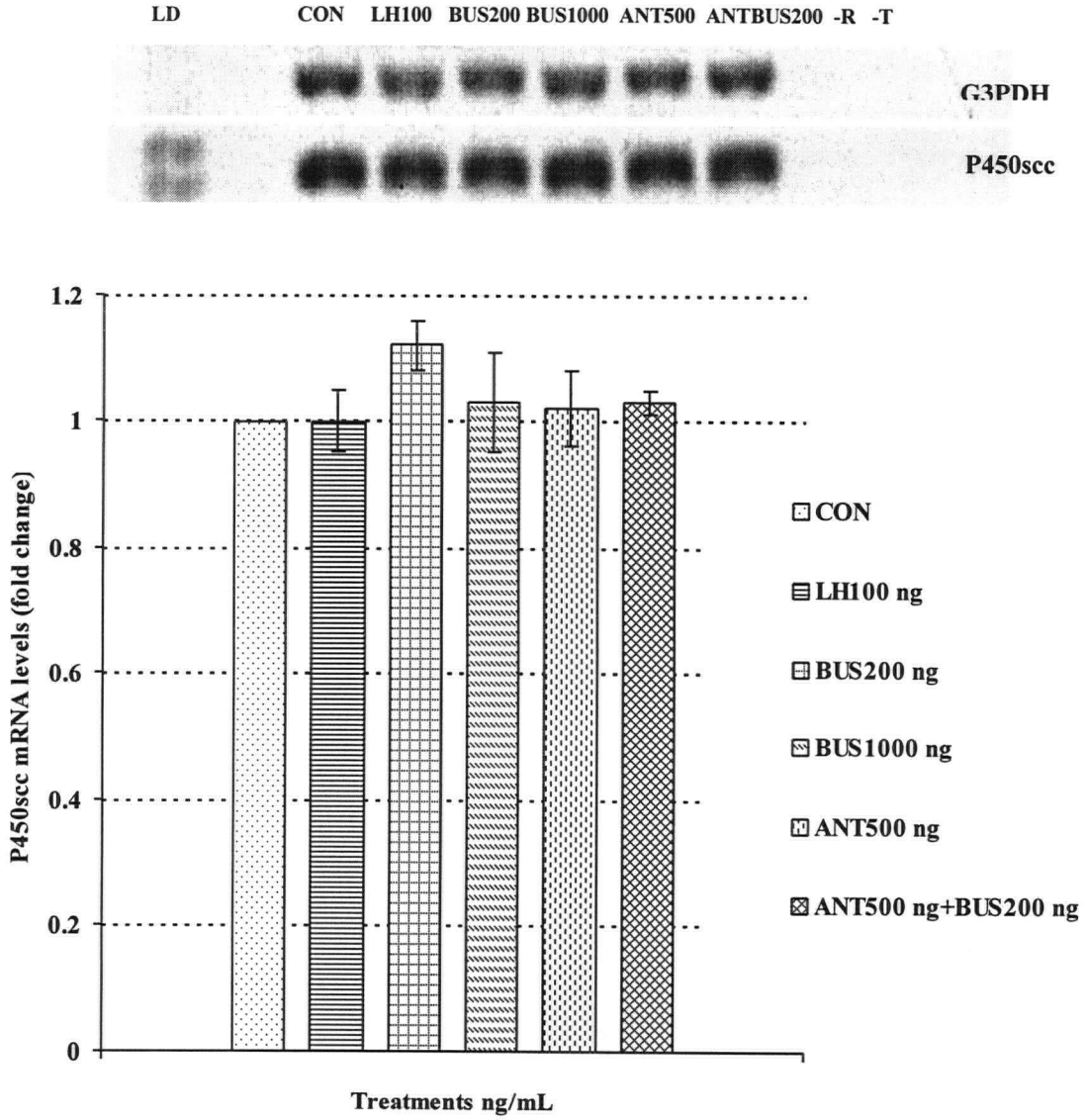

FIGURE 4.5. Effect of GnRH-a on P450scc mRNA levels in luteal tissues. Minced luteal tissue samples were cultured *in vitro* and treated with different doses of buserelin. Untreated control (CON), LH 100 ng, buserelin (BUS) 200 ng, BUS 1000 ng, Antide (ANT) 500 ng, ANT 500 ng + BUS 200 ng. BUS dose response on P450scc mRNA levels (fold change from CON) is shown in the histogram. LD, molecular weight base pair (ladder); -R, minus RTase; -T, minus template.

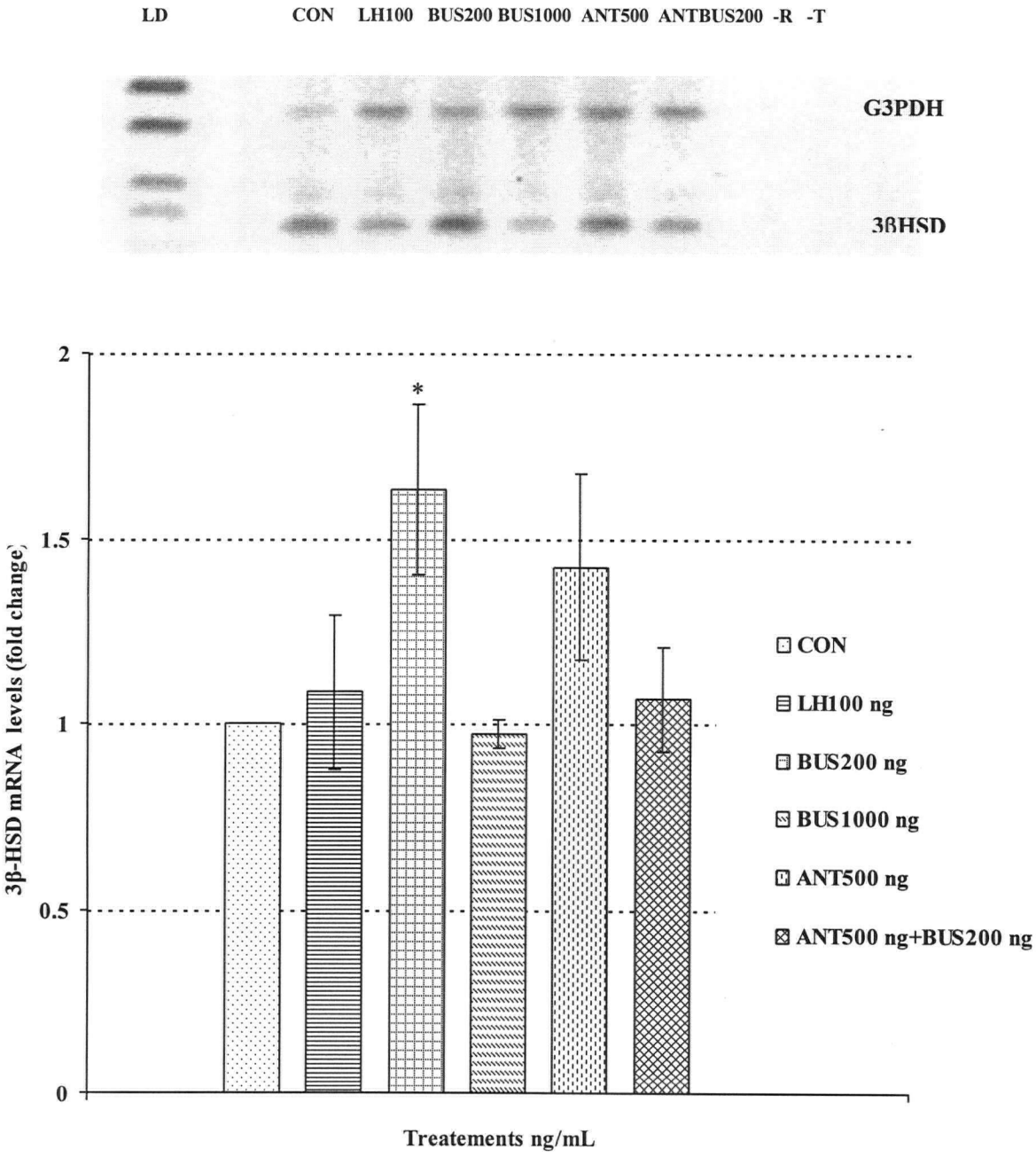

FIGURE 4.6. Effect of GnRH-a on 3β-HSD mRNA levels in luteal tissue. Minced luteal tissue samples were cultured i*n vitro* and treated with different doses of buserelin. Untreated control (CON), LH100 ng, buserelin (BUS) 200 ng, BUDS 1000 ng, Antide (ANT) 500 ng, ANT 500 ng + BUS 200 ng. BUS dose response on 3β-HSD mRNA levels (fold change from CON) is shown in the histogram. * different ($P = 0.12$) from that of control samples.LD, molecular weight base pair (ladder); -R, minus RTase; -T, minus template.

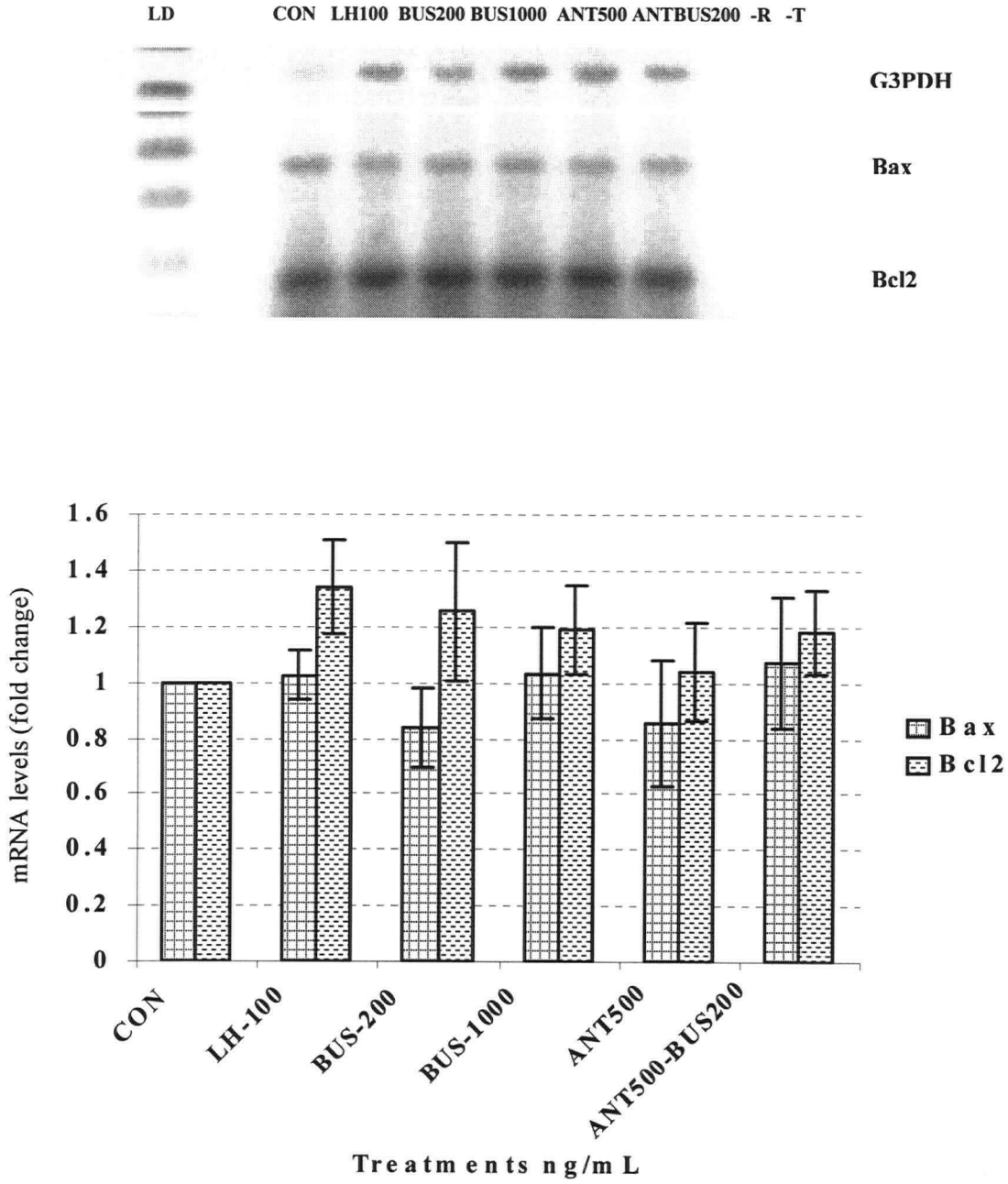

FIGURE 4.7. Effect of GnRH-a on Bcl2 and Bax mRNA levels in bovine luteal tissue. Minced luteal tissue samples were cultured *in vitro* and treated with different doses of buserelin (BUS). Medium only (CON), LH 100 ng, BUS 200 ng, BUS 1000 ng, Antide (ANT) 500 ng, ANT 500 ng + BUS 200 ng. BUS dose response on Bcl2 and Bax mRNA levels (fold change from CON) is shown in the histogram. LD, molecular weight base pair (ladder); -R, minus RTase; -T, minus template.

Table 4.1. Oligonucletide primer pair and their respective PCR amplicons for StAR protein, P450scc, 3β-HSD, Bax, Bcl2 and G3PDH mRNA transcripts from bovine luteal tissue.

Gene sequence	Primer sequence Forward 5'-3' Reverse 5'-3'	Fragment length (bp)
G3PDH	5'-TGTTCCAGTATAGATTCCACC-3' 5-TCCACCACCCTGTTGCTGTA-3'	850
StAR	5'-CATGGTGCTCCGCCCCTTGGCT-3' 5'-CATTGCCCACAGACCTCTTGA-3'	590
P450scc	5'-AACGTCCCTCCAGAACTGTACC-3' 5'-CTTGCTTATTGCTCCCTCTGCC-3'	362
3β-HSD	5'-TGTTGGTGGAGGAGAAGG-3' 5'-GGCCGTCTTGGATGATCT-3'	360
Bax	5'-CCTTTTGCTTCAGGGTTTCATCCAG-3' 5'-CGAAGGAAGTCCAATGTCCAGC-3'	362
Bcl2	5'-TTCGCCGAGATGTCCAGTCAGC-3' 5'-TTGACGCTCTCCACACACATGAC-3'	154

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CHAPTER 5

INFLUENCE OF POST-BREEDING GnRH ADMINISTRATION ON CORPUS LUTEUM FUNCTION AND PREGNANCY IN DAIRY CATTLE

5.5. ABSTRACT

The objectives of the present study were to examine the influence of post-breeding GnRH administration on *in vivo* CL function in terms of progesterone (P4) secretion, and pregnancy outcome, in dairy cattle. One hundred and four Holstein cattle (cows = 74; heifers = 30) were included in this study. Lactating cows (completed at least 90 days postpartum period), and heifers, that were observed in standing natural estrus were inseminated and subjected to one of the three treatments randomly. Treatments were comprised of control (untreated, cows n=25; heifers n= 10), GnRH 100 μg (cows n=25; heifers n= 10), or hCG 2500 IU (cows n=24; heifers n= 10). All treatments were administered(intramuscular) on day 7 post-breeding. Starting from the day of breeding (Day 0), milk (cows) and blood (heifers) samples were collected at weekly intervals until the 5th week post-breeding for P4 measurements. Pregnancy diagnosis was determined by ultrasound scanning on Day 35 after breeding. P4 concentration from milk and plasma samples was determined by following a RIA procedure. Based on P4 concentrations and pregnancy outcome, no significant differences were observed between sham treated control and GnRH, or GnRH and hCG, treatment groups. However, hCG treatment group had significantly ($P < 0.05$) higher levels of P4 (from Day 14 until 35 post-breeding), but pregnancy rates were not different from

control or GnRH groups. In summary, post-breeding GnRH administration, did not result in improved pregnancy rates in dairy cattle.

5.2. INTRODUCTION

Gonadotropin releasing hormone (GnRH) is a vital requirement for the maintenance of reproductive homeostasis. The versatile functional properties of GnRH have been extensively studied, and are a continued topic of interest in reproductive physiology and medicine. For instance, the acute effects of GnRH causing endogenous release of follicle stimulating hormone (FSH), and luteinizing hormone (LH) is an invaluable feature that aids in manipulating ovarian activity in a predictable manner (Conn and Crowley, 1994; Stojilkovic and Catt, 1995). GnRH-induced gonadotropin release exert numerous physiological effects such as wave emergence and development of new follicles, ovulation or luteinization of ovarian follicles, and an altered corpus luteum (CL) function. Numerous studies have examined whether GnRH treatments given at the time of artificial insemination (AI) are able to improve pregnancy rates in cattle (Reviewed by Thatcher et al., 1993; D'Occhio and Aspden, 1999; Rajamahendran et al., 2002). In most experiments, GnRH treatments were administered around the time of AI (Schmitt et al., 1996a) or during the mid-luteal phase (reviewed by Thatcher et al., 1993; D'Occhio and Aspden et al., 1999; Rajamahendran et al., 2002) in attempts to establish enhanced CL function. The majority of experimental results show a numerically increased P4 concentration, and in some instances, increased pregnancy rate after GnRH treatment. However, the difference is often statistically non-significant in comparison with untreated control animals. Administration of GnRH agonist analogues (GnRH-a) during mid-follicular phase (Murdoch and Van Kirk, 1998,

Momcilovic et al., 1998), or early estrus (Macmillan and Thatcher et al., 1991) period, has been reported to result in an enhanced CL function following ovulation in some studies. On the other hand, no change has been noticed in other studies and studies have reported that GnRH, or GnRH-a, administered in the early follicular phase has resulted in an inadequate or defective CL following ovulation (Lucy and Stevenson, 1992; Murdoch and Van Kirk, 1998; Taponen et al., 1999, 2000). Theoretically, it is believed that consequent to GnRH-a administration, LH concentration reaches beyond its threshold level causing a down-regulation of LH-receptors in preovulatory follicle cells; and thus, ensuing luteal defect. Similar postulations suggest the lack of FSH priming granulosa cells would fail to acquire aromatizing capabilities; thereby, the decreased estradiol-17β (E2) levels could lead to decreased granulosa cell proliferation, and consequently the luteal insufficiency. In contrary to these experiments, in rodent species, experiments have proved that ovarian super-stimulation during follicular phase has resulted in suppression of E2 augmented pituitary LH response, preventing ovulation. Further, it has been shown that the LH surge inhibits E2 secretion by the preovulatory follicle (Dieleman and Blankenstein 1984).

A significant proportion of pregnancy failures (30 – 40%) and infertility problems in cattle have been attributed to inadequate functioning of the CL (Reviewed by Rajamahendran et al., 2002). Most of the conception failures, or early pregnancy losses, are associated with the defective CL, due to lack of adequate luteotropic support following ovulation. Several corrective measures have been developed to combat inadequate CL function in cattle. Either direct LH administration, or administration of human chorionic gonadotropin (hCG) that exerts an LH-like luteotropic effect leading to enhanced CL function (Reviewed by

Niswender et al., 2000). However, repeated use of hCG in cattle can induce antibody formation; thus, it cannot be used as the best alternative treatment regimen on a routine basis, in contrast to GnRH-based protocols (Reviewed by Rajamahendran et al., 2002). In addition, efforts have been made to compensate for luteal insufficiency through exogenous P4 administration, but it has often yielded poor results (Rhodes et al., 1999). In spite of continued efforts from several researchers, little improvement has been achieved towards the successful treatment of luteal insufficiency in bovine species. Therefore, based on the supportive evidence from present GnRH protocols, administration of GnRH at various time points before and after ovulation and induction of an accessory CL leading to increased plasma P4 concentrations is still being considered as a promising method to achieve enhanced pregnancy outcome in cattle. In this direction, including the present laboratory, there have been numerous studies that have reported a varying degree of success in pregnancy rates (PR) following different time-point GnRH administration during the estrous cycle in cattle. Therefore, it is a clear indication that more studies are needed to gain further knowledge with respect to the GnRH response on *in vivo* CL function, and, in, turn, the eventual PR in cattle. Further, most of the studies cited above were conducted in combination with estrous synchronization protocols. Only limited information is available on post-breeding GnRH administration in animals that were bred at the time of natural estrus with spontaneous ovulations. Therefore, the present study was undertaken to assess CL function, and pregnancy outcome following post-breeding GnRH administration in dairy cattle that were inseminated at natural estrus. In order to establish the effects of post-breeding GnRH treatment on *in vivo* CL function, hCG treatment was included as positive a control group during the present experiment. Besides the numerous reports from the literature, previous

studies from this laboratory have suggested that hCG treatment at 7 days after insemination could be used to induce an accessory CL, raise plasma P4 and reduce the incidence of early embryonic mortality in cattle (reviewed by Rajamahendran et al., 2002).

5.3. MATERIALS AND METHODS

5.3.1. Animals and treatments

This study was conducted at the University of British Columbia Dairy Education and Research Center, Agassiz, British Columbia, Canada, during the months of January to May 2003. Handling and management of animals were in accordance with the guidelines of the Canadian Council on Animal Care (1993).

Animals were housed in a free-stall system that allowed a considerable amount of interaction among herd mates. A total of one hundred and four Holstein dairy cattle (lactating cows, n = 74; heifers, n = 30) were included in this study (Table 5.1). Lactating cows were in the stage of first to sixth lactation, and completed at least a 90-days post-partum period. Animals with a history of injury or reproductive problems, were excluded from the experiment. Both cows and heifers were artificially inseminated at the time of natural estrus, and then randomly allocated to one of the three treatment groups: control (no treatment; cows, n = 25; heifer, n = 10), GnRH 100 μg (cows, n = 25; heifers, n = 10), and hCG (cows, n = 24; heifers, n = 10). GnRH (Factrel®) and hCG (Chorulon) were purchased from the Fort Dodge Laboratories, Fort Dodge, IA and Intervet, Canada, respectively. All treatments were administered (intra-muscular route, IM) on the 7th Day post-breeding (Fig. 5.1).

5.3.2. Milk and blood sample collection for P4 measurement

Whole milk samples (cows) and blood samples (heifers) were collected on Day 0 (day of breeding), and Days 7, 14, 21, 28, and 35, post-breeding, for P4 determination. Blood samples (10 mL) were collected from each animal (heifers) via the coccygeal vessels in heparinized vacutainer tubes (Becton Dickinson, Ruthfield New Jersey, USA). Samples were centrifuged (1000 x G, 30 min) within 1 h of collection, and the plasma separated. Both milk and plasma samples were stored at –20°C until assayed for P4.

5.3.3. Radioimmunoassy for P4 measurement

Concentration of P4 in milk and plasma samples was measured using a commercially available, solid phase, radioimmunoassay (RIA) kits (Coat-A-Count, Diagnostic Products Corp., Los Angeles, CA). These kits were previously validated for the measurement P4 concentration in milk and plasma samples (Taylor and Rajamahendran, 1991; Sianangama and Rajamahendran, 1992) in this laboratory. Briefly, at the time of assay preparation, an aliquot of 100 µl of the P4 standards was transferred into duplicate set of tubes (antibody-coated) that were labeled as AA, BB, CC, DD, ED, FF, and GG, corresponding to the P4 concentrations of 0.0, 0.1, 0.5, 2.0, 10.0, 20.0, and 40.0 ng/mL. Similarly, the required number of additional tubes (antibody-coated) was supplied with an aliquot of 100 µl of experimental samples (milk and plasma), and each tube was assigned an unique identification number to match their respective samples (milk or plasma). Then, all tubes were arranged in serial order (Tube A to Tube N in numbers), and each tube was supplied with P4-buffered I^{125}-labeled P4 (1.0 ml). The tube contents were mixed by gentle agitation, and incubated at room temperature for 3 h to achieve the equilibrium of antibody-antigen reaction. At the end

of the 3 h incubation, the liquid portion of the assay mixture was decanted from the tubes and any remaining liquid residue was removed by holding the tubes in an inverted position on a blotting surface (paper) for an additional 10 min. The solid portion of assay mixture (in tubes) that contained radioactivity was counted for 1 min using a gamma counter (Packard Auto gamma 500, Packard Instruments, Downers Grove, IL, USA). The assay sensitivity, that is the intra- and inter- assay coefficienst of variations for P4, were 8.6% and 10.8%, respectively.

5.3.4. Reproductive status of the animals based on P4 concentration

Number of animals included in P4 analysis, number of animals that were in estrus based P4 <1 ng/mL on the day of AI and ovulation rates based on P4 >1 ng/mL on Day 7 post-breeding, in each treatment group are presented in Table 5.2. Individual animals were considered as pregnant providing P4 levels were <1 ng/mL on Day 0 and maintained >1 ng/mL on Day 7 through 21 post-AI. The presumptive PR was calculated based on the percentage of animals that became pregnant in relation to the total number that were in estrus on Day 0 (day of breeding), and had a successful ovulation (based on P4 levels < 1 ng/mL on Day 0, and >1 ng/mL on Day 7 post-breeding).

5.3.5. Pregnancy diagnosis

On Day 35 after breeding, pregnancy diagnosis was carried out using a per-rectal ultrasound-scanning device, as described by Rajamahendran and Taylor (1990). A real-time ultrasound-scanning device (Aloka 500 V, Aloka Co. Ltd., Tokyo, Japan), equipped with a 7.5 MHz trans-rectal transducer was used. During scanning, the uterine horns and body of the

uterus were scanned in several planes in order to examine uterine contents, and to identify embryonic vesicles and the embryo proper, if pregnant. The overall PR in each treatment group was calculated based on the total number of animals that were allocated per treatment group (control n = 35; GnRH n = 35; hCG n = 34), on the day of breeding (Fig 5.4).

5.3.6. Data analysis

PR was defined as the percentage of animals that became pregnant in relation to the total number treated. The post-breeding GnRH effect on the *in vivo* CL function was determined based on the comparative P4 levels from that of the control, and hCG treatment groups. The GnRH treatment effect on presumptive PR was assessed based on P4 levels at different time points during the post-GnRH treatment period (Day 21, 28, and 35). Finally, the GnRH treatment effect on overall PR was calculated based on ultrasound scanning data from Day 35, post-AI. The percentage of estimated embryonic mortality was calculated based on animals with P4 levels < 1 ng/mL on Day 0, and > 1 ng/mL at Days 7, 14, 21 and any drop in P4 levels <1ng/mL on 28, and 35, post-AI. RIA data for P4 concentrations was analyzed using one-way analysis of variance (ANOVA), followed by pair-wise comparison using a Fisher's LSD method. *In vivo* CL function (presumptive PR based on P4 concentrations), and overall PR data from ultrasound scanning results, were analyzed using Chi-Square method. The level of significance was set at $\alpha = 0.05$.

5.4. RESULSTS

5.4.1. CL function and presumptive pregnancy rates based on P4 concentration

Ovulation occurred in 69 out of the 74 (93.24%) animals that were in estrus on the day of breeding (based on P4 < 1 ng/mL; Table 5.2). The mean ± SEM of combined (cows and heifers) P4 profiles during post-breeding period (Day 7 through 35) indicated no difference ($P > 0.05$) in CL function between sham control, and GnRH treatment groups (data not shown). However, when P4 data was analyzed for only those animals that were in estrus (P4 <1 ng/mL) on day of AI and had a successful ovulation (based on P4 levels >1 ng/mL on Day 7 post-breeding), the tendency for a non-significant ($P > 0.05$) increase in P4 levels was evident in the GnRH treatment group (Fig. 5.2A). The hCG treatment group maintained a significantly higher levels P4 concentrations when analyzed either as combined data both cows and heifers (Fig. 5.2A), or based on the type or parity of the animals, heifers (Fig. 5.2B) and cows (Fig. 5.2C).

For the purpose presumptive PR calculation, only those animals that were in estrus on the day of breeding (P4 <1 ng/mL), and had a successful ovulation (P4 >1 ng/mL on Day 7 post-breeding), were included (Table 5.3). Presumptive PR was calculated based on P4 levels >1 ng/mL on Day 21, 28, and 35 post-breeding. The combined (Fig. 5.3A), percentage of presumptive PR in different treatment groups was as follows: Day 21 (control 68%, GnRH 80%, hCG 91.67%), Day 28 (control 52%, GnRH 65%, hCG 66.67%), and Day 35 (control 25%, GnRH 60%, hCG 62.5%). The percentage of presumptive PR in heifers and cows are presented in Fig. 5.3B and Fig. 5.3C, respectively. When P4 levels were considered,

irrespective of treatment groups, the cumulative presumptive PR (overall PR) were 79.71%, 60.87%, and 57.59% on Day 21, 28, and 35 post-breeding, respectively (Fig. 5.3A)

5.4.2. Overall pregnancy rates on Day 35 post-AI

Based on ultrasound scanning data (Day 35 post-breeding), the combined (cows and heifers) PR was calculated based on the number of animals pregnant in relation to the total numbers of animals that were initially allocated to each treatment group on the day of breeding (control, n = 35; GnRH, n = 35; hCG, n = 24). The combined PRs in the different treatment groups were 42.86%, 42.86% and 40.00% for control, GnRH, and hCG groups, respectively (Fig. 5.4) In terms of pregnancy outcome, no difference was observed among control, GnRH, and hCG ($P > 0.05$) treatment groups. When PR was analyzed based on parity or type of the animals (cows and heifers), PR in heifer groups was significantly higher ($P < 0.05$) from that of PR in cows (Fig. 5.4).

5.4.3. Embryonic death or pregnancy loss

The percentage of estimated embryonic mortality, on Day 28 and 35 after breeding, was calculated based on the animals with P4 levels < 1 ng/ml on Day 0, and > 1 ng/ml at Days 7, 14, 21, and any drop in P4 levels to <1 ng/mL on 28 or 35 post-AI. In other words animals that were in estrus on Day 0, had successful ovulation, maintained P4 levels > 1 ng/mL until at least Day 21 post-breeding (presumptively pregnant), and had P4 levels droped to <1 ng/mL on Day 28 or 35 post-breeding was considered as pregnancy loss. The breakdown of presumptive PR and pregnancy losses, based on animal type (cows and heifers) are presented in Table 5.3. The estimated pregnancy loss or early embryonic

mortality that occurred between Day 21 and 35 post-breeding in different treatment groups were 16%, 20%, and 29.17% for control, GnRH and hCG, respectively. Among the three, the hCG treatment group had more number of animals that lost pregnancy between Day 21 and 35 post-breeding. When overall data was considered (irrespective of the treatment groups), the presumptive pregnancy loss that occurred between Days 21 and 35 post-breeding was 21.74%.

5.5. DISCUSSION

The present findings are in agreement with several related studies reported in the literature. GnRH, administered at various time-points during the post-breeding period in cattle, had a mixed response in terms of P4 output, and PR. For example, no effect on P4 concentrations was observed when cycling cows were injected with GnRH-a on Day 7 after estrus (Twagiramungu et. al., 1995). Similarly, GnRH (100 μg, natural GnRH) on Day 2 or 8 of the estrous cycle did not affect any increase in plasma P4 levels (Martin et al., 1990). GnRH, or GnRH-a, administered in the early follicular phase, has resulted in an inadequate or defective CL following ovulation (Lucy and Stevenson, 1992; Taponen et al., 1999, 2000). Treatment with GnRH on Day 2 or 10 caused a reduction in serum P4 levels on Days 12, 14 and 16 of the cycle in cattle (Rodger and Stormshak, 1986). Whereas mid-follicular (Murdoch and Van Kirk, 1998: Momcilovic et al., 1998; Taponen et al., 2003), or early estrus (Twagiramungu et al., 1994) GnRH-a administration has been shown to result in an enhanced CL function following ovulation. It was evident that buserelin administered (heifers) early in the estrous cycle, caused an increase in the relative numbers of large luteal cells in the CL (Twagiramungu et al., 1995; Schmitt et al., 1996b). GnRH-a administration at

about Day 4 to Day 6, following breeding, could ovulate first wave follicle and develop an accessory corpus luteum as a contributory factor towards increased serum P4 levels (D'Occhio et al., 1999; Ambrose et al., 1999, 2000; Rajamahendran et al., 2001).

Pregnancy rates following post-breeding GnRH administration have been reported with variable results. The present study does not indicate any increase of PR due to GnRH administration on Day 7 after breeding. Whereas from other studies (Macmillan et al., 1986), it is evident that there was an increase in PR following mid-cycle GnRH administration in heifers. Ultrasound examinations, during the luteal phase of the estrous cycle (Harvey et al., 1994), revealed that treatment with GnRH induced an accessory CL in approximately half of the treated animals that subsequently had increased conception rates. However, the same authors also suggested that a sudden increase in the serum concentrations of LH followed by a concomitant increase in P4 concentration, after mid-cycle GnRH administration. Further, this effect caused an extended estrous cycle length among cows returning to estrus and that did not become pregnant. In the present study, GnRH treatment did result in significant differences in P4 concentrations, or PR rates, compared to that of the control or hCG treatment groups.

Although the hCG-treated animals maintained significantly higher P4 levels ($P < 0.05$) in this study, there was no significant difference observed in terms of PR from that of either the control, or GnRH group. It is suggested that the higher P4 levels in the hCG-treatment animals could have been attributed to the accessory CL formation as well as the direct effect of hCG on spontaneous CL. Similar observations have been reported from

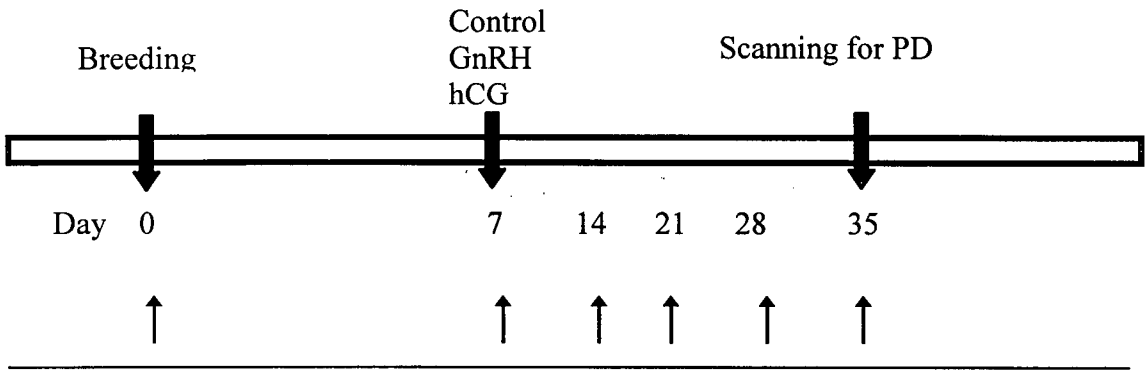
previous studies where hCG was administered during mid-luteal stage in anticipation of improved pregnancy rate, but it was often associated with inconsistent increase in peripheral concentrations of P4 (Rajamahendran and Sianangama, 1992; Rajamahendran and Sianagama, 1992; Schmitt et al., 1996b). Some authors (Edmonson et al., 1989) attributed these variable results to the condition (parity) of animals utilized for such experiments. It is reported that first parity cows usually have higher conception rates than older cows (Lean et al., 1989; Folman et al., 1990; Eicker et al., 1996). The present findings are in agreement with the above stated observations. The overall PR in the heifer group was significantly higher than the cows ($P < 0.05$). This may be attributable to the adverse effects of high milk yields; and thus stress on early pregnancy as observed in multiparous animals (Lean et al., 1989). However, contrary to this, Lucy et al. (1992) suggested that multiparous cows may have advanced ovulation and enhanced PR, due to one or more reasons under the free stall heard management system. Older cows (multiparous) consume more dry matter during postpartum period than younger cows (heifers and primiparous) (Lucy et al., 1992; Grant and Albright, 1995), which may be attributable to larger sizes and social dominance, which multiparous cows usually possess over primiparous cows when housed in a free-stall type of facility (Grant and Albright, 1995).

In this study, the estimated pregnancy loss or early embryonic mortality that occurred between Days 21 and 35 post-breeding, in different treatment groups was, 16%, 20%, and 29.17% for control, GnRH and hCG groups, respectively. Among the three treatment groups, the hCG group had higher number of animals that apparently lost their pregnancy between Days 21 and 35 post-breeding. The reason for this higher pregnancy loss in the hCG group is

not clear. However, this may be also attributable to an extended estrous cycle length due to hCG treatment (Sianangama and Rajamahendran, 1992). This might have contributed to more animals returning to estrus between Days 28 and 35, when compared to control or GnRH-treated groups. However, the percentage range of pregnancy loss observed in this study is in agreement with the findings of Vasconcelos (1999), who reported a 24% embryonic loss between 25 Days post-AI and calving time in GnRH-based synchronization protocols (Ovsynch). Similarly, Moreira et al. (2000) have reported an estimated 35% loss of embryos between Day 20 and 27, and an 8% loss between Day 27 and 45, post-AI. Loss of CL function around the time of implantation (Day 14 to 16) could prevent implantation and could lead to a loss of pregnancy. Further, it has been reported that 40% of the total embryonic mortality occurred between Days 8 and 17 of pregnancy, which may be attributed to the inability of certain conceptuses to secrete interferon-tau (IFN-t), which inhibits the uterine PGF2α secretion during early pregnancy (Thatcher et al., 2001). Including this study, the reported variability in pregnancy rates in response to GnRH administration at different time-points after breeding in dairy animals, may be, in part, due to the type of the herd, geographical location (including weather patterns), and overall management practice. However, it is an univocal assumption that administration of GnRH and its agonistic analogues at the time of AI or during post-breeding period would increase pregnancy rates in cattle (Thatcher et al., 1993; Ullah et al., 1996; Rajamahendran et al., 1998; D'Occhio and Aspden, 1999; D'Occhio et al., 2000; Cam et al., 2002). Therefore, in order to fully utilize the benefits of GnRH in bovine reproductive practice, further studies are needed to unravel any inherent, as well as external, factors that my be contributing to the present variability in terms of pregnancy outcome in cattle.

5.6. CONCLUSION

In conclusion, administration of GnH or hCG on Day 7 post-breeding in the present study did not increase the overall pregnancy rates in dairy cattle. For more than one decade, the present laboratory in association with other research collaborators has carried out extensive work on post-breeding hormonal treatments to improve embryo survival with varying degrees of success. However, based on first hand research experience, the previous findings from present laboratory suggest that it is possible to induce the ovulation of a first-wave dominant follicle using GnRH preparations, or hCG at various time-points post-breeding, thus improving the possibility of pregnancy outcome in dairy cattle.



Time points of milk and blood sample collection for P4 determination.

FIGURE 5.1. Experimental protocol for post-breeding GnRH administration. One hundred and four Holstein dairy cattle (lactating cows, n = 74; heifers, n = 30) were included in this study. Lactating cows were in the stage of 1- 6th lactation, and completed at least a 90-day post partum period. Both the cows and heifers were bred at the time of natural estrus (Day 0) and randomly assigned to one of the three treatment groups, untreated (Control), GnRH (100 μg, IM,), and hCG (2500 IU, IM) group. All treatments were administered on Day 7, post-breeding. Milk (cows) and blood (heifers) samples were collected on Day 0, and on Day 7, 14, 21, 28 and 35 post-breeding, for P4 determination. Ultrasound scanning for pregnancy diagnosis (PD) was carried out on Day 35 post-breeding.

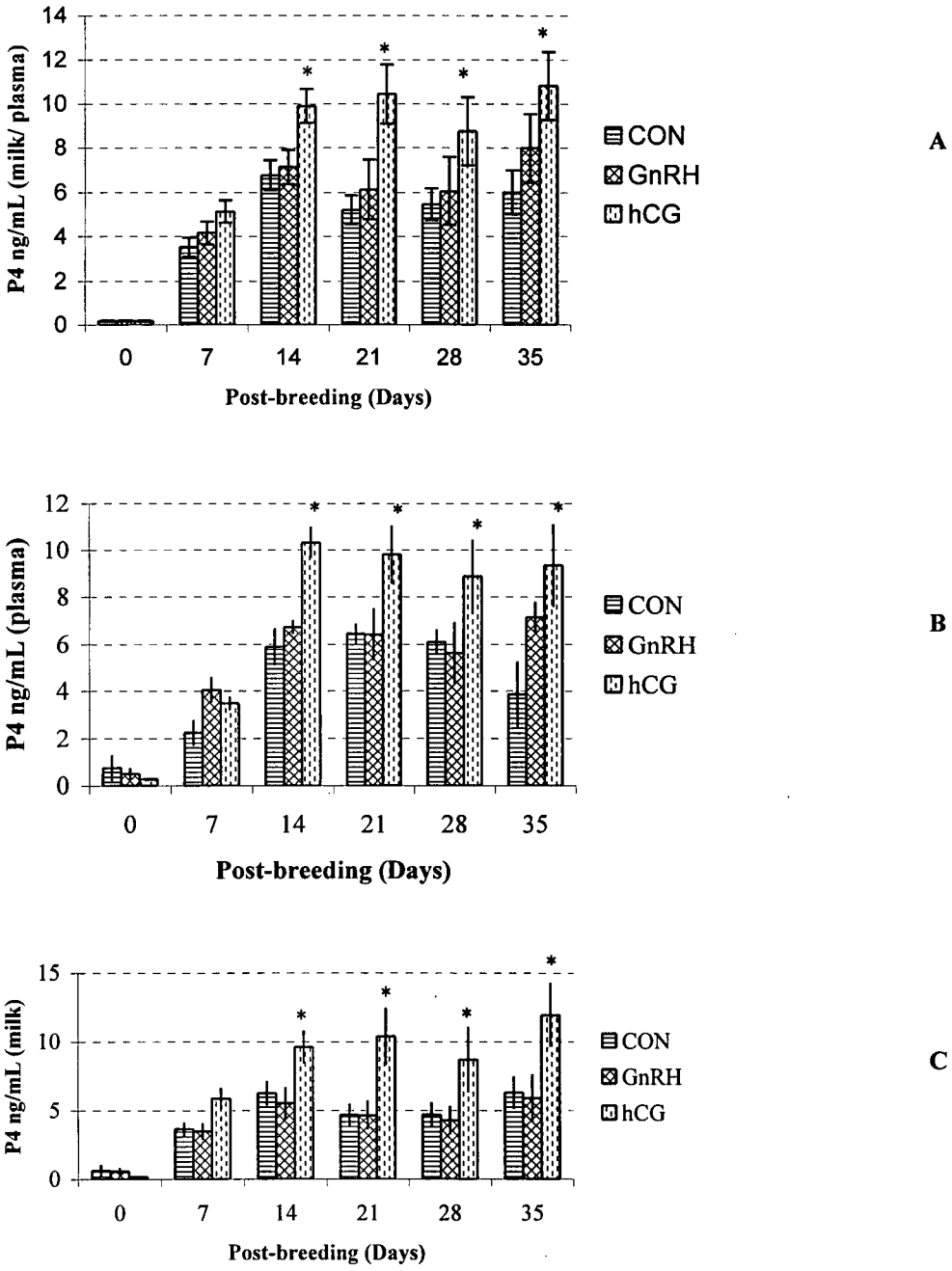


FIGURE 5.2. Post-breeding mean progesterone (P4) levels in different treatments groups. **A.** Combined (heifers and cows N=81: CON=29, GnRH=27, hCG=25). **B.** Heifers (CON=9, GnRH=10, hCG=10). **C.** Cows (CON=20, GnRH=17, hCG=15). Animals were bred on the day of natural estrus (Day 0) and randomly assigned to one of the three treatment groups, untreated or control (CON), GnRH (100 μg, IM,), and hCG (2500 IU, IM) group. All treatments were administered on Day 7 (Day 7), post-breeding. Whole milk and blood samples were collected on Day 0, and on Day 7, 14, 21, 28, and 35 post-breeding, for P4 determination. * different from other treatments ($P < 0.05$), within the same time point.

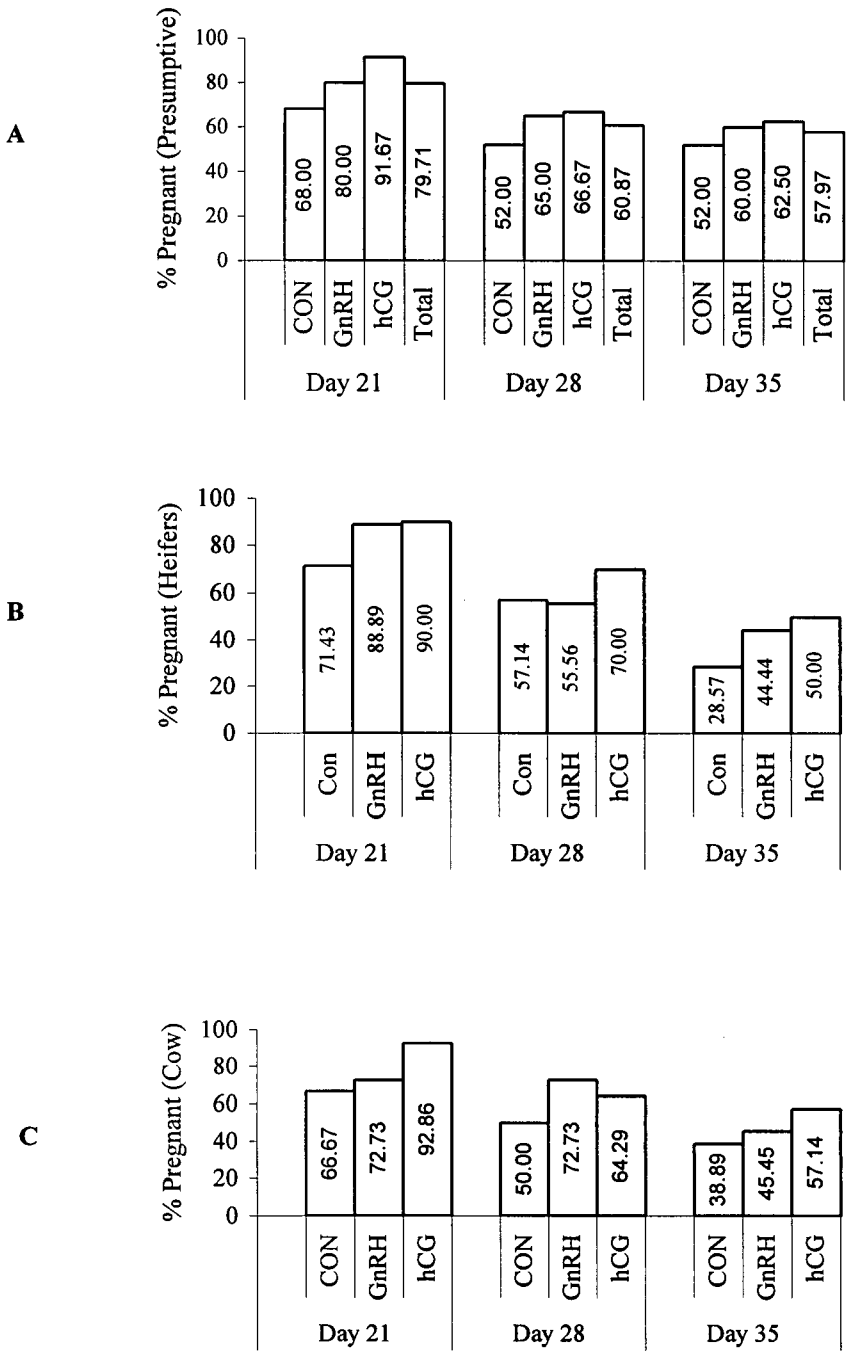


FIGURE 5.3. Presumptive pregnancy rates in lactating dairy cows and heifers subjected to different treatments: untreated control (CON), GnRH (100 μg, IM), and hCG (2500 IU, IM) on Day 7 post-AI. Milk (cows) and serum (heifers) P4 profiles measured at different time points during post-AI period, was used as an indicator of presumptive pregnancy rates and expressed in percentage. **(A)** Combined (heifers and cows), **(B)** Heifers, and **(C)** Cows. Animals considered pregnant based on $P_4 < 1$ ng/mL on Day 0, and $P_4 > 1$ ng/mL on Days 7, 14, 21, 28 and 35 post-AI.

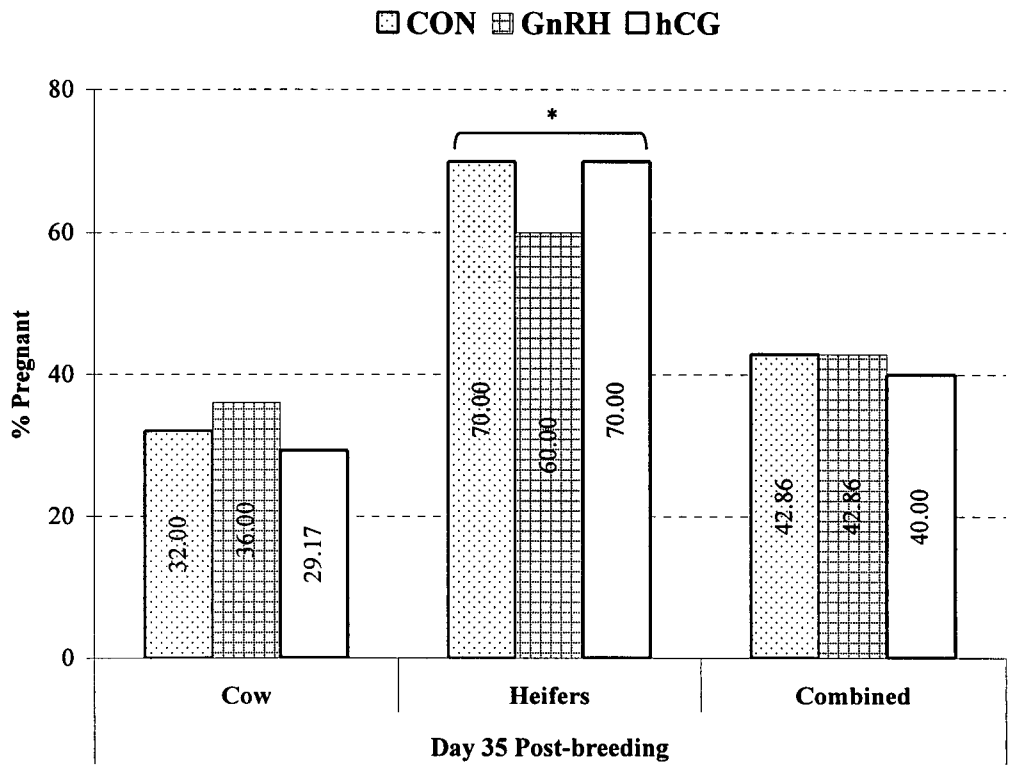


FIGURE 5.4. Overall pregnancy rates in lactating dairy cows and dairy heifers based on the scanning results on Day 35 post-AI. All animals were inseminated at the time of natural estrus and randomly allocated to one of the three treatments: control (CON = 35; GnRH 100 μg, n = 35; hCG 2500 IU, n = 34). Treatments were administered on Day 7 post-AI. On Day 35 post-AI, all animals were scanned for pregnancy diagnosis. * different ($P < 0.05$) from PR in cows or combined (cows and heifers).

Table 5.1. Post-breeding experimental treatments and number of animals per treatment group. All animals that were in natural estrus were inseminated (Day 0) and randomly allocated into one of the three treatment groups(control, GnRH or hCG).

Treatments	No. of Cows	No. of Heifers
Control	25	10
GnRH	25	10
hCG	24	10

Table 5.2. Number of animals included in P4 analysis, number of animals that were in estrus based on P4 <1 ng/mL on the Day of AI, and ovulation rates based on P4 >1 ng/mL on Day 7 post-AI, in each treatment group are listed.

Animal Type	**Treatments**	**Animals included in P4 analysis**	**Animals in Estrus**	**Animals Ovulated**
Cows N=52	Control	20	19	18 (94.74%)
	GnRH	17	14	11 (78.57%)
	hCG	15	14	14 (100%)
Heifers N=29	Control	9	8	7 (87.5%)
	GnRH	10	9	9 (100%)
	hCG	10	10	10 (100%)

Table 5.3. Presumptive pregnancy rates (PR) and embryonic losses in cows and heifers subjected to different treatments: untreated control, GnRH (100 μg, IM), and hCG (2500 IU, IM) on Day 7 post-AI. Milk (cows) and plasma (heifers) P4 profiles measured at different time points during post-AI, was used as an indicator of percentage presumptive pregnancy rates (based on $P_4 < 1$ ng/ml on Day 0, and $P_4 > 1$ ng/ml on Day 7, 14, 21, 28 and 35 post-AI), and percentage estimated embryonic loss (the difference of % PR on Day 21 and Day 35 post-AI).

Pregnancy status	Treatments	Animal type	
		Heifers (%)	Cows (%)
Estimated PR on Day 21 post-AI	Control	71.43	66.67
	GnRH	88.89	72.73
	hCG	90.00	92.86
Estimated PR on Day 35 post-AI	Control	28.57	38.89
	GnRH	44.44	45.45
	hCG	50.00	57.14
Estimated embryonic loss (%) on Day 35 post-AI	Control	42.86	27.78
	GnRH	44.45	27.27
	hCG	40.00	35.71

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CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

6.1. GENERAL DISCUSSION

In recent years, numerous reports have gathered evidence on an extra-hypothalamic origin of GnRH, as well as the presence of an extra-pituitary GnRH-R in different types of tissues in the body. With respect to the reproductive system, several reports reveal evidence for the presence of GnRH and GnRH-R systems in the ovary, oviduct, endometrium, placenta, and testes, in different species of animals, including humans (Reviewed by Janssens et al., 2000; Ramakrishnappa et al., 2004). From both *in vivo* and *in vitro* model studies in rodents, primates, and in humans, it is becoming increasingly evident that GnRH, or its synthetic analogues, could exert direct effects through an autocrine or paracrine manner, in target tissue (Reviewed by Janssens et al., 2000; Ramakrishnappa et al., 2004). Surprisingly, in the bovine species, there were no such information available, nor was there any evidence of systematic efforts that have been made to examine such possibilities. In lieu of these intriguing findings from other species, we were prompted to explore if any such mechanisms exist in bovine species. Therefore, the series of experiments outlined in this thesis were designed to examine if GnRH-R and GnRH mRNA are expressed in the bovine ovary, as well as to examine the direct effect of GnRH-a on ovarian steroidogenic machinery, apoptotic process in CL, and post-breeding GnRH administration on *in vivo* CL function and pregnancy outcome in dairy cattle.

In Chapter 1, using RT-PCR methods, we examined the presence of GnRH-R mRNA expression in granulosa cells from different stage follicles and CL, as well as GnRH mRNA expression in granulosa cells. To our knowledge, the present results are the first to successfully demonstrate the presence of GnRH-R mRNA expression in follicles (small, medium and large), and CL tissues (stage III) in bovine species. Although these findings are, a step forward demonstration of the functional form GnRH-R protein molecules is an imperative step needed before attributing any possible direct effects of GnRH, or its agonists, at ovarian cellular level in the bovine species. Preliminary evidence from RT-PCR results suggest for the presence of GnRH mRNA expression in bovine granulosa cells of different size follicles. Due to the limitations of the materials, and the experimental approach used in this part of the study, our further confirmatory attempts such as southern blot hybridization, and nucleotide sequencing steps, were not successful. This may be attributed to one or more factors such as the low amount of target DNA on blots, the partial complimentary probe sequence that was of relatively shorter length in the whole plasmid used, and the chemiluminescent signaling method could have undermined the detection ability. In the case of the sequencing step, the direct PCR product used as template DNA, and the same 3' end primer that was used both during RT-PCR, and during the sequencing step, could have been the possible contributing factors for the absence of the signal output during the sequencing step. Further steps, such as cloning of RT-PCR fragments, and sequence analysis using universal sequencing primers, may aid in identifying the GnRH sequence. The preliminary evidence from the present study supported by previous reports that suggested the presence of GnRH-like protein molecules in bovine ovaries (Stojilkovic and Catt, 1995; Ireland et al., 1988). In addition, similar evidence has been demonstrated in human and rat ovaries (Aten et

al., 1987a). Therefore, we do believe that the RT-PCR technique, using human primers, reveals evidence for GnRH mRNA expression in bovine granulosa cells.

In Chapter 3, a series of trials were performed to investigate the direct effects of GnRH-a (buserelin) on steroid hormone secretion from bovine granulosa cells, dispersed luteal cells, and from minced luteal tissue during *in vitro* culture. Based on the results, it was evident that GnRH-a exerted a dose-dependent, biphasic effect on E2 output from *in vitro* cultured granulosa cells. At lower dose levels (200 – 500 ng/mL), GnRH-a caused an increased steroid output, whereas at higher dose levels (1000 ng/mL), the accumulated steroid levels were slightly lower or not different from E2 levels in untreated controls. Although P4 concentrations revealed a similar trend, the buserelin response on P4 output was not different from that of in untreated control samples. The present observations are in agreement with reports that have demonstrated GnRH-a-induced steroidogenesis from *in vitro* cultured human granulosa cells (Ranta et al., 1982; Parinaud et al., 1992; Olsson et al., 1990; Bussenot et al., 1993). Previously, it was suggested that GnRH-a could modulate steroidogenesis bya direct ovarian action (Parinaud et al., 1988). Guerrero et al. (1993) found an increase in P4 and a decrease in E2 production, which seemed to be related to a decrease of LH receptor numbers and aromatase activity in GnRH-a-treated cells. The present study, do not provide definitive evidence for the presence of functional GnRH receptors that could mediate a GnRH-a response, affecting steroidogenic potentials of bovine granulosa cells *in vitro*. Nonetheless, based on the response elicited by a GnRH antagonist, reversal of the GnRH-a effect on E2 output does suggest the possible existence of GnRH ligand specific target sites on granulosa cells. Demonstration of the functional form of GnRH-R protein

molecules is an imperative step before attributing any possible direct effects of GnRH, or its agonists, at the ovarian cellular level in the bovine species.

In efforts to examine the direct effects of GnRH-a on *in vitro* P4 production in bovine CL, both dispersed luteal cell cultures, and luteal tissue or organ culture systems, were utilized. GnRH-a exhibited a dose-dependent, biphasic response in terms of P4 synthesis from *in vitro*-treated dispersed luteal cells, and from luteal tissues. However, in luteal cells, the higher dose levels of buserelin (1000 ng/mL) showed a slightly inhibitory effect on P4 levels. It is not clear whether the suppressed P4 levels at higher dosage levels is in fact due to inhibitory roles of GnRH-a, or auto-regulatory mechanisms such as receptor desensitization, or down regulation of its own receptors, as reported in other studies (Olofsson et al., 1995; Volker et al., 2002). The present findings differ from that of Milvae et al. (1984), who reported a dose-dependent suppression of P4 secretion from *in vitro* cultured bovine luteal cells. In that report, authors suggest that it is very unlikely that GnRH mediates its effects through the mechanism of ligand specific-receptor interaction on bovine luteal cells. However, the reason for the different types of response observed between these two studies could have been due to the variations in experimental conditions. As there seems to be only two such studies that were carried out in the bovine species, it is difficult to draw a definitive conclusion as to whether GnRH, or its analogues, could exert direct effects at ovarian cellular levels in the bovine species. However, it is note-worthy that in the bovine species, *in vivo* administration of GnRH-a during late-mid luteal phase has resulted enhanced P4 output (Thatcher et al., 1993; D'Occhio and Aspden, 1999; D'Occhio et al., 2000; Rajamahendran et al., 1998, 2001).

In the present study GnRH-a treatment in combination with LH or $PGF_{2\alpha}$, had no influence on P4 output from luteal tissues. However, unlike with other reports [as well as in the present study (antide and GnRH-a effect on granulosa cell steroid output)], antide treatment elicited a maximal stimulatory response on P4 output in CL tissue, when treated as antide alone, or in combination with GnRH-a. However, this effect was not reflected in terms of a concomitant increase in mRNA levels of key enzymes (StAR protein, P450scc, and 3β-HSD) that are involved in the steroidogenic pathway. The reason for this atypical response is not known. Perhaps, this could be due to some other unknown factors within CL tissue, that in conjunction with antide that may have been responsible for this atypical response. It is also reported that granulosa cells from women that were administered with GnRH antagonist (cetrorelix) responded earlier to the *in vitro* hormone stimulation, in terms of P4 accumulation, than women treated with the buserelin (Lin et al. (1999). Their results indicate that luteal function is less impaired in GnRH antagonist treatment, than in GnRH agonist treatment. Further, evidence from porcine granulosa cell cultures suggests that a GnRH antagonist, combined with follicular fluid, could enhance LH-stimulated P4 secretion (Ledwitz-Rigby, 1989). In addition, the biphasic effects of GnRH antagonist on *in vitro* steroidogenesis (rat granulosa cells) that varied with exposure time showed the initial response being stimulatory, and the later inhibitory (Sheela Rani et al., 1983). They also showed that 20 alpha-OHP secretions in the same cultures were potentiated by the combined presence of FSH, and GnRH-a. These authors suggested that these types of responses could have been due to the maturational stage of the granulosa cells. From this evidence, it is tempting to suggest that the GnRH antagonist may not only mediate their effects independent

of GnRH target sites, but they may also interact with some other unknown factors in ovarian cell types. This brings attention to several previous reports that suggest the presence of GnRH-like molecules in gonadal structures (Ying et al., 1981; Aten et al., 1987a, b; Ireland et al., 1988; Izumi et al., 1985), which may have a regulatory role on ovarian cellular function, locally. However, to date there is no concrete evidence for a functional role for such molecules in the ovary or any other reproductive tissue.

In Chapter 4, we examined the direct effect of GnRH-a on molecular steroidogenic machinery, as well as GnRH-a influence on mRNA expression for pro- and anti-apoptotic molecules in bovine CL. During *in vitro* culture, GnRH-a treatment of CL tissue caused a mild stimulatory response on mRNA levels of StAR, P450scc and 3β-HSD; although an approaching significance ($P = 0.12$) could be achieved only in the case of 3β-HSD. This was typical of the response observed in terms of P4 output from luteal cells and tissues, following GnRH-a treatment *in vitro* (CHAPTER 3). The present findings are in agreement with earlier reports where the CL in heifers that were administered with long-acting GnRH-a (deslorelin), had a greater content of StAR protein, and the steroidogenic enzyme, P450scc (Pitcher et al., 1997 cited by D'Occhio and Aspden, 1999). The same group of researchers also reported a significant increase in testosterone levels, and mRNA expression level for StAR, P450scc, 3β-HSD, and $P450_{17\alpha}$, in testicular tissues of bulls that were administered deslorelin (Aspden et al., 1998). GnRH-a (leuprolide acetate), in combination with eCG administration, has been reported to cause a significant increase in StAR mRNA and also StAR protein and P4 levels, in ovarian follicles of mice (Irusta et al., 2003). Overall, the present findings are in consistent with observations where increased plasma P4 levels were a characteristic feature

in many of the studies with GnRH administration during the luteal the phase in cattle (Thatcher et al., 1993; D'Occhio and Aspden, 1999; D'Occhio et al., 2000; Rajamahendran et al., 1998, 2001). Further, these studies indicate that GnRH-a administration during the luteal phase does not adversely affect the CL function in the bovine species, contrary to reports in the majority of the studies in other species (Reviewed by Janssens et al., 2000). The present findings differ from similar studies where buserelin (Sridaran et al., 1999a,b), or leuprolide acetate (Andreu et al, 1998), treatment caused a significant suppressive effect on StAR, 3β-HSD and P450scc in rat ovaries. In the present study, the exact mechanisms for a mild stimulatory response in mRNA expression levels for StAR, P450scc and 3β-HSD are not known. In addition, these observations do not confirm whether the effect of GnRH-a treatment was due to its direct interaction with the GnRH specific receptor on luteal cell types in bovine CL. More studies are needed to explore such a possibility, and confirmation of any functional forms of GnRH receptor molecules, and their mode of interaction with its ligand system. It has been suggested that GnRH could mediate its direct effects in conjunction with other hormonal agents such as $PGF_{2\alpha}$, or LH, that are well known to regulate ovarian function (Reviewed by Steele and Leung, 1993). Stimulation of one or more signaling pathways, such as phospholipase C (PLC), phospholipase A2 (PLA2), and phospholipase D (PLD), and activation of protein kinase C (PKC) has been hypothesized to cause either inhibitory, or stimulatory, effects on ovarian cellular steroid output. These dual effects have been clearly demonstrated with *in vivo* experiments in adult male, and female, hypophysectomized rats, where exogenous GnRH or GnRH-a could either stimulate or inhibit gonadal functions in terms of steroidogenesis (Hsueh and Jones, 1981a, b; Hsueh and Jones,1982). GnRH has been shown to elicit mixed responses pertaining to ovarian function

(reviewed by Sharpe, 1982; Janssens et al., 2000; Leung et al., 2003). The inhibitory action of GnRH, or its agonists, on gonadal steroidogenesis involves suppression of the gonadotropin receptors, or intermediary enzymes, involved in the steroidogenic pathway. GnRH-a induced suppression of FSH and LH receptors (Tilly et al., 1992; Piquette et al., 1991; Guerrero et al., 1993), and GnRH-a caused suppression of gonadotropin-induced cAMP levels, has been reported (Richards, 1994; Knecht et al., 1985).

In the present study, *in vitro* treatment of luteal tissues with GnRH-a did not affect the mRNA levels for pro-and anti-apoptotic molecules (Bax and Bcl2). This is consistent with the other findings from present study where GnRH-a exerted a positive influence on *in vitro* steroid output, a mild increase in mRNA levels for steroidogenic enzymes. However, these findings differ from several other studies in different species where GnRH-a was shown to induce apoptotic process in ovarian cell types. During *in vitro* cultures, GnRH inhibited DNA synthesis (Saragueta et al., 1997), and induced apoptosis in rat granulosa cells (Billig et al., 1994). GnRH has been shown to induce structural luteolysis in superovulated rats through stimulation of matrix metalloproteinase (MMP-2), and membrane type 1-MMP expression (in a developed CL), which degraded collagens type IV, type III, and I respectively (Goto et al., 1999). During early pregnancy in the rat, GnRH-a has been shown to suppress serum P4 levels, which was associated with an increased degree of DNA fragmentation in the CL (Sridaran et al., 1998). A similar effect of GnRH-a inducing an increased number of apoptotic bodies in human granulosa cells (obtained during oocyte retrieval for *in vitro* fertilization) has been reported by Zhao et al. (2000).

The present study reveals no evidence of any adverse effects of GnRH-a in terms of inducing programmed cell death (apoptosis) in bovine luteal tissues, as has been reported in other species. Besides species differences, the reason for this differential response of GnRH-a on luteal tissue or cell types is not known. On the other hand, findings from the present study do not suggest any conclusive evidence with respect to the lack of a GnRH-a effect causing programmed cell death, contrary to the findings from other species. More studies involving, individual cell types rather than whole organ/tissue culture systems, and different dose levels of GnRH-a, may provide more concrete and reliable evidence. It is also important to undertake comprehensive investigations through different approaches such as assessing inter-nucleosomal DNA fragmentation and examining the early responder of apoptotic process such as FAS and FAS ligand, or P^{53} system.

In chapter 5, we investigated the influence of post-breeding GnRH administration on *in vivo* CL function in terms of P4 secretion, and pregnancy outcome, in dairy cattle. Results from this study indicate no significant differences between sham-treated control and GnRH, or GnRH and hCG, treatment groups. However, in comparison with control animals, cows in the GnRH treatment group maintained slightly higher levels (non-significant) of P4 from Day 7 to 35 post-breeding, however, no difference was observed in pregnancy rates. While the hCG treatment group had significantly ($P < 0.05$) higher levels of P4 from Day 14 until 35 post-breeding, the pregnancy rate was not different from both the control and GnRH groups. The present findings are in agreement with several related studies reported in the literature. GnRH administered at various time points during the post-breeding period in cattle had a mixed response in terms of P4 output and PR. For example, no increase in P4 concentrations

was observed when cycling cows were injected with GnRH-a on Day 7 after estrus (Twagiramungu et. al., 1995). Similarly, GnRH (100 μg) on Day 2 and 8 of the estrous cycle did not affect plasma P4 levels (Martin et al., 1990). GnRH, or GnRH-a, administered in early the follicular phase has resulted in an inadequate or defective CL following ovulation (Lucy and Stevenson, 1992; Taponen et al., 1999, 2003). Treatment with GnRH on either Day 2, or Day 10, caused a reduction in serum P4 levels than on Days 12, 14 and 16 of the cycle in cattle (Rodger and Stormshak 1986). Whereas, mid-follicular (Murdoch and Van Kirk, 1998, Momcilovic et al., 1998; Taponen et al., 1999), or early estrus (Twagiramungu et al., 1994), GnRH-a administration has been shown to result in an enhanced CL function following ovulation. Heifers treated with buserelin early in the estrous cycle were shown to have an increase in the relative numbers of large luteal cells in their CL (Twagiramungu et al., 1995; Schmitt et al., 1996b). GnRH-a administration at about Day 4, to Day 6, following breeding could induce ovulation of first wave dominant follicle and development of an accessory CL as a contributory factor towards increased serum P4 levels (D'Occhio and Aspden et al., 1999; Ambrose et al., 1999, 2000; Rajamahendran et al., 2001).

Pregnancy rates following post-breeding GnRH administration have been reported with variable results. The present study does not indicate any increase of PR from GnRH administered on Day 7 after breeding. However, there was an increase in PR following mid-cycle GnRH administration in heifers in other studies (Macmillan et al., 1986; Thatcher et al., 1993). Ultrasound examinations during the luteal phase of the estrous cycle (Harvey et al., 1994) revealed that treatment with GnRH induced an accessory CL in approximately half of the treated animals that subsequently had an increase in PR. In addition, these authors

suggest that there is a sudden increase in the serum concentrations of LH followed by a concomitant increase in P4 concentration after mid-cycle GnRH administration. Further, this effect can cause an extended estrous cycle among nonpregnant cows. However, the present study did not show any difference in P4 concentrations or PR rates following GnRH compared to that of the control or hCG treatment groups.

In this study, although the hCG treated animals tended to maintain significantly higher P4 levels ($P < 0.05$) there was no significant difference observed in terms of PR from that of either the control or GnRH group. It is suggested that the higher P4 levels in the hCG treatment animals could have been attributed to the accessory CL, and the direct effects of hCG on existing CL at the time of administration. Similar observations have been reported from previous studies where hCG was administered during the mid-luteal stage in anticipation of improved pregnancy rate, but it was often associated with inconsistent peripheral concentrations of P4 (de Los Santos-Valadez et. al., 1982; Rajamahendran and Sianangama, 1992; Schmitt et al., 1996b). Some authors (Edmonson et al., 1989) attributed variable results to the type of animals utilized for such experiments. It has been reported that first parity cows usually have higher conception rates than older cows (Lean et al., 1989; Folman et al., 1990; Eicker et al., 1996). In the present study, overall PR in the heifer group was significantly higher than in cows ($P < 0.05$). This may be attributable to the adverse effects of high milk yields; and thus stress on early pregnancy, as reported by Lean et al. (1989).

In this study, the estimated pregnancy loss, or early embryonic mortality, that occurred between Day 21 and 35 post-breeding in the different treatment groups was 16%, 20%, and 29.17% for control, GnRH and hCG, respectively. Among the three treatment groups, the hCG group had more animals that apparently lost pregnancy between Days 21 and 35 post-breeding. The reason for this higher pregnancy loss in the hCG group is not clear. However, this may be attributable to an extended estrous cycle length due to an hCG caused delay in luteolyis (Sianangama and Rajamahendran, 1992). This might have contributed to the fact that more animals returned to heat between Days 28 and 35, compared to the control or GnRH treated groups. However, the percentage range of pregnancy loss observed in this study is in agreement with the findings of Vasconcelos (1999), who reported a 24% embryonic loss between 25 Days post-AI, and calving time, in GnRH based synchronization protocols (Ovsynch). Similarly, Moreira et al. (2000) recently reported an estimated 35% loss of embryos between Day 20 and 27, and an 8% loss between Day 27 and 45 post-AI. Loss of CL maintenance around the time of implantation (Day 14 to 16) could prevent implantation, and could lead to loss of the pregnancy. Further, Thatcher et al. (2001) reported that 40% of the total embryonic mortality occurred between Days 8 and 17 of pregnancy, which may be attributed to the inability of certain conceptuses to secrete interferon-tau (IFN-t) that inhibits the uterine $PGF_{2\alpha}$ during early pregnancy (Thatcher et al., 2001). In summary, including this study, changes in pregnancy rates, in response to GnRH administration at different time points after breeding in dairy animals may be in part due to the type of the herd, geographical location, including weather patterns, and overall management practice. However, it was an univocal assumption that administration of GnRH and its agonistic analogues at the time of AI, or during post-breeding period, would increase

pregnancy rates in cattle (Thatcher et al., 1993; Ullah et al., 1996; Rajamahendran et al., 1998; D' Occhio and Aspden, 1999; D'Occhio et al., 2000; Cam et al., 2002). Therefore, in order to utilize the beneficial effects of GnRH to its fullest extent, further studies are needed to unravel any inherent, as well as external, factors that may be contributing to the present variability, in terms of pregnancy outcome in cattle.

6.2. GENERAL CONCLUSIONS

To the best of our knowledge, some of the findings that emerged from these studies are the first to be reported in the literature. In particular, this study provided the clear evidence for the presence of GnRH-R mRNA expression in both follicles, and CL in the bovine species. In these studies GnRH-a (buserelin) shown to cause a dose-dependent, biphasic effect on steroid hormone output from *in vitro* cultured granulosa cells, luteal cells and in CL tissues. In luteal tissues, treatment with a GnRH antagonist (antide) alone or in combination with buserelin showed a highly significant stimulatory response on P4 output. The reason for this atypical response is unknown, and further research using different GnRH antagonistic preparations is warranted. In terms of luteal steroidogenic machinery, GnRH-a treatment of luteal tissues showed a mild (nonsignificant) stimulatory response on mRNA levels for StAR protein and P450scc mRNA although; tendency for significance ($P = 0.12$) could be seen only in the case of 3β-HSD. Consistent with above findings, the present study revealed no evidence for any adverse effects of GnRH-a treatment, in terms of inducing apoptotic process, and luteolysis, unlike that reported in other species. In response to post-breeding GnRH administration, although animals in the GnRH treatment group maintained

slightly (non-significant) higher P4 levels from Day 7 to 35 post-breeding, no difference was observed in pregnancy rates when compared to the control and hCG treatment groups.

Collectively, based on the present observations of GnRH-R mRNA expression, and GnRH-a effect on steroid hormone output, and GnRH-a effect on molecular steroidogenic machinery, we believe that GnRH-a could cause a dose-dependent biphasic response on steroid hormone output from bovine granulosa cells, dispersed luteal cells and CL tissue. However, these findings do not provide definitive evidences to whether these results are, in fact due to the direct interaction of GnRH-a with its receptors on the above cell types. GnRH antagonist caused atypical stimulatory response on P4 output from CL tissues; is an interesting feature in this study. Consistent with above findings, and unlike in other species, GnRH-a treatment did not cause any adverse effects on CL function by inducing or augmenting the apoptotic process, or luteolysis. In the present study, although GnRH administration on Day 7 post-breeding caused a slight elevation in peripheral P4 levels pregnancy, outcome was not different from untreated control animals. This simply implies that the beneficial effects of exogenous GnRH on the bovine reproductive system are widely variable; and therefore, it is important to continue further research in order to unravel the inherent beneficial effects of GnRH, and its super-agonistic analogues, on bovine reproductive function.

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