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Does Recombinant Bovine Growth Hormone-Releasing Factor or Recombinant Bovine Somatotropin Alter the Dominant Follicle Process in Dairy Cows?

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DOES RECOMBINANT BOVINE GROWTH HORMONE-RELEASING FACTOR OR RECOMBINANT BOVINE SOMATOTROPIN ALTER THE DOMINANT FOLLICLE PROCESS IN DAIRY COWS?

By

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ABSTRACT

DOES RECOMBINANT BOVINE GROWTH HORMONE-RELEASING FACTOR OR RECOMBINANT BOVINE SOMATOTROPIN ALTER THE DOMINANT FOLLICLE PROCESS IN DAIRY COWS?

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The dominant follicle process is characterized by two or three "waves" of development of ovarian follicles, ovulation of the dominant follicle from one of the waves, and formation of a corpus luteum during the bovine estrous cycle. The objective of this study was to evaluate the effects of recombinant bovine growth hormone (GH)-releasing factor (rGRF) or recombinant bovine somatotropin (rbST) on growth and function of the first-wave dominant follicle and corpus luteum. In Experiment I, 20 primiparous Holstein cows (117 days postpartum) were infused with 12 mg/d of rGRF or 29 mg/d of rbST for 63 days, and 10 non-infused cows were controls. At slaughter on Day 5 of the estrous cycle, blood and ovaries were collected and analyzed. Treatment with rGRF or rbST increased GH in serum and insulin-like growth factor-I (IGF-I) in serum and follicular fluid similarly compared with controls. In contrast, rbST-treated cows had higher intrafollicular concentrations of GH compared with rGRF-treated and control cows. Recombinant bST, but not rGRF, increased number and decreased size of estrogen-active (EA; estradiol > progesterone in FF) follicles, increased estradiol in FF from second and third largest follicles, increased IGF binding proteins-2, -3 and -4 in FF from EA follicles, and increased number but decreased size of each corpus luteum and decreased concentration of progesterone in serum compared with controls. Because rbST increased estradiol levels in FF, a follow up *in vitro* study evaluated the effects of rbST on estradiol-producing capacity of bovine granulosa cells from the first-wave

dominant and subordinate follicles. Addition of rbST to culture media did not alter basal estradiol production by granulosa cells from any follicle type. However, rbST blocked FSH-induced estradiol production by granulosa cells from the largest follicle. In conclusion, long-term infusion of rbST disrupts the dominant follicle process in cattle. While the mechanism is unknown, it is speculated that sustained high intrafollicular levels of GH disrupts dominance by preventing atresia of subordinate follicles during a follicular wave. Based on the present *in vivo* and *in vitro* studies, rbST may prevent atresia by stimulating thecal rather than granulosa cell function and(or) enhancing intrafollicular levels of IGFBPs or net IGF-I bioactivity, which in turn, stimulates follicular estradiol production.

To my parents,

to my wife, Gabriela for her understanding

and

for Fermín and Mariana de Jesús

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LIST OF ABBREVIATIONS

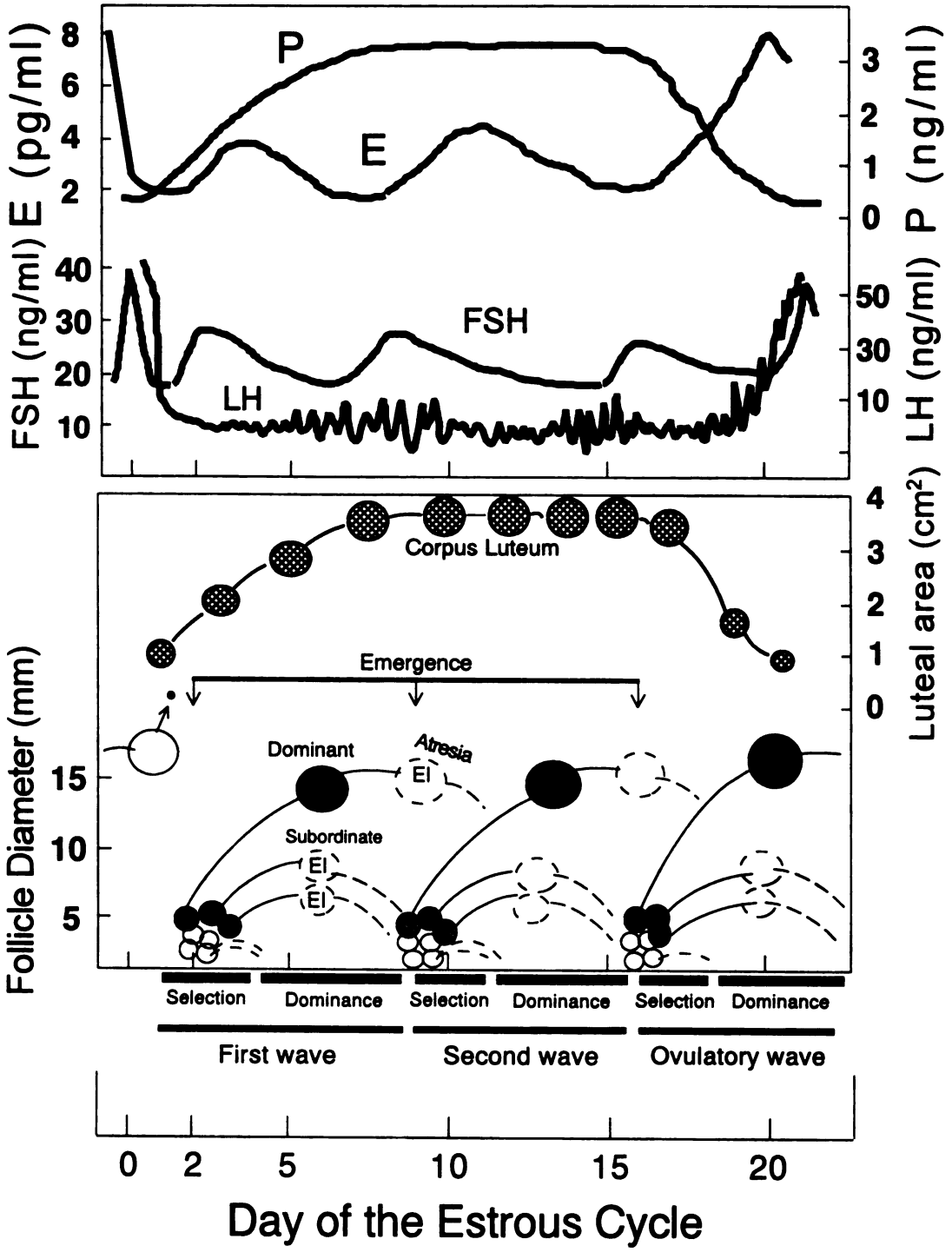
A	Androstenedione
CL	Corpus luteum or corpora lutea
CV	Coefficient of variation
DOM	Dominant follicle
E	Estradiol-17β
EA	Estrogen-active follicles
EI	Estrogen-inactive follicles
F1	Largest follicle on the ovary
F2	Second largest follicle on the ovary
F3	Third largest follicle on the ovary
F4	All remaining follicles on the ovary, but larger than 5 mm in diameter
FF	Follicular fluid
FSH	Follicle-Stimulating Hormone
GH	Growth Hormone
GRF	Growth Hormone-Releasing Factor
IGF-I	Insulin-like Growth Factor-I
IGFBP	Insulin-like Growth Factor Binding Protein(s)
LH	Luteinizing Hormone
n	number
P	Progesterone
PBS	Phosphate Buffered Saline
PG	Prostaglandin(s)
pp	postpartum
PRL	Prolactin
rbST	Recombinant Bovine Somatotropin
rGRF	Recombinant Bovine Growth Hormone-Releasing Factor
RIA	Radioimmunoassay
SEM	Standard error of the mean
SUB	Subordinate follicle

INTRODUCTION

The dominant follicle process is characterized by two or three "waves" of development of ovarian follicles, ovulation of the dominant follicle from one of the waves, and formation of a corpus luteum during the bovine estrous cycle (Figure 1; Rajakoski, 1960; Matton et al., 1981; Ireland and Roche, 1987; Fortune, 1993; Ginther et al., 1996; Roche, 1996). Based on ultrasonic imaging, follicular waves begin on Days 2 and 11 of the estrous cycle in heifers with two waves (Ginther et al., 1989), and on Days 2, 9 and 16 in heifers with three waves (Figure 1; Savio et al., 1988; Sirois and Fortune, 1988). Each wave consists of the simultaneous growth of a group of follicles to 4-6 mm in diameter (Sirois and Fortune, 1988; Fortune et al., 1991; Bodensteiner et al., 1996a), which is referred to as follicular emergence (Figure 1; Ginther et al., 1996). Within 1 to 2 days, one of the newly emerged follicles grows larger than the others in the cohort and becomes the dominant follicle. The other follicles in the cohort regress (Sirois and Fortune, 1988; Savio et al., 1988; Knopf et al., 1989) and are referred to as subordinate (Figure 1). In addition to use of ultrasonic analysis to characterize dominant follicle development, each wave of dominant follicle development includes three theoretical phases of growth: recruitment, selection, and dominance. During the recruitment phase, a group of primordial follicles begins to grow and gains ability to respond to gonadotropins (not shown in Figure 1). Selection is the physiological process by

Figure 1. The dominant follicle process in the cow. The dominant follicle process is characterized by two or three "waves" of development of ovarian follicles, ovulation of the dominant follicle from one of the waves, and formation of a corpus luteum during the bovine estrous cycle. Explanation provided in Introduction. (Figure based on reviews by Ireland and Roche, 1987; Kastelic et al., 1990a; Ginther et al., 1996; Roche, 1996).

Figure 1.


P
E
FSH
LH
FSH (ng/ml) E (pg/ml)
LH (ng/ml) P (ng/ml)
Luteal area (cm²)
Follicle Diameter (mm)
Corpus Luteum
Emergence
Dominant
Atresia
EI
Subordinate
Selection
Dominance
First wave
Second wave
Ovulatory wave
Day of the Estrous Cycle

which the "excess" recruited follicles are reduced to the ovulatory quota, whereas dominance is a process that enables the "selected" follicle(s) to suppress further growth of other follicles and to continue to grow until ovulation or atresia (Figure 1; Goodman and Hodgen, 1983). Intrafollicular concentrations of hormones are also used to characterize dominant follicles. Specifically, the newly emerged and the dominant follicle secrete greater amounts of estradiol compared with all other follicles, thus they are considered estrogen-active (EA) because of higher intrafollicular ratios of estradiol to progesterone concentrations (Figure 1). The subordinate follicles contain more progesterone than estradiol in follicular fluid, and are referred to as estrogen-inactive (EI) (Figure 1; Ireland and Roche, 1982; 1983a; 1983b; Sunderland et al., 1994). Understanding how the dominant follicle process (Figure 1) is regulated may provide information important to develop new methods to improve estrous cycle regulation and fertility in cattle. In addition, monitoring of the dominant follicle process can be used to evaluate whether treatments of cattle with various drugs may alter reproductive efficiency.

In heifers, a transient increase in basal concentration of follicle-stimulating hormone (FSH) in serum precedes each follicular wave (Figure 1; Adams et al., 1992; Sunderland et al., 1994), thus FSH is probably the physiological "trigger" that stimulates the cyclic follicle growth pattern. Selection of the dominant follicle is associated with the disappearance of this transient FSH increase (Ginther et al., 1996; Roche, 1996). In contrast, luteinizing hormone (LH) is important for dominant follicle growth when FSH levels are low, and during atresia or ovulation (Figure 1). Specifically, as the dominant follicle grows, it gains LH receptors (Ireland and Roche, 1982; 1983a; 1983b; Xu et al., 1995b; Bodensteiner et al.,

1996b), and becomes dependent on LH to ensure its survival in a hormonal milieu that inhibits growth of subordinate follicles (Ginther et al., 1996; Roche, 1996). However, within 3 to 7 days after emergence of a dominant follicle, a decrease in frequency of episodic LH secretion is associated with atresia of the dominant non-ovulatory follicle (Cupp et al., 1995), whereas an increase in episodic LH secretion causes the dominant ovulatory follicle to ovulate (Figure 1; Rahe et al., 1980; Cupp et al., 1995; Evans et al., 1997).

Although FSH and LH are the primary hormones that regulate ovarian follicle growth and function (Richards, 1980; Niswender and Nett, 1994), numerous intraovarian factors, such as insulin-like growth factor-I and its binding proteins, modulate gonadotropin actions on granulosa and theca cell proliferation and steroidogenesis (Adashi et al., 1985; Ireland, 1987; Spicer and Echternkamp, 1995). Furthermore, bovine somatotropin or growth hormone, which is used to increase milk production in dairy cows (Bauman et al., 1985), has direct effects on gonadal function and follicular growth (Gong et al., 1991; De la Sota et al., 1993) and modulates the insulin-like growth factor system in the cow (Dahl et al., 1993; VanderKooi et al., 1995). Therefore, the compelling overall hypothesis for my thesis research was that administration of recombinant bovine growth hormone-releasing factor (rGRF) or recombinant bovine somatotropin (rbST) to increase milk production (Dahl et al., 1993; Binelli et al., 1995) would also alter the process of dominant follicle development (Spicer and Enright, 1991; Webb et al., 1994), and, in turn, affect reproductive efficiency in cattle (Burton et al., 1990; Cole et al., 1991; Morbeck et al., 1991; Stanisiewski et al., 1994). The primary objective of my dissertation research was to determine whether administration of rGRF, which stimulates GH production, or rbST disrupted the dominant follicle process in lactating dairy

cows.

This dissertation is in two parts. The first part is the review of literature which will emphasize the role of growth hormone-releasing factor, growth hormone, insulin-like growth factor (IGF)-I and IGF-binding proteins on follicular development in cattle (Chapter 1). The second part will present the results of the two experiments that were the research for my dissertation:

a) Effects of administration of rGRF or rbST on follicle and corpus luteum growth and function during growth of the **first-wave dominant follicle** (Figure 1) in lactating dairy cows (Chapter 2); and

b) Evaluation of direct effects of rGRF or rbST on *in vitro* estradiol-producing capacity of bovine granulosa cells collected from follicles during the period of development of the first-wave dominant follicle (Chapter 3).

Part I

CHAPTER 1

REVIEW OF LITERATURE

Growth Hormone-Releasing Factor

Chemical Properties and Synthesis

Growth hormone-releasing factor (GRF) is a 44 amino acid hypothalamic polypeptide which was first isolated and characterized from extracts of human extra-hypothalamic tumors in 1982 (Guillemin et al., 1982; Rivier et al., 1982) and from rat (Spiess et al., 1983) and bovine (Esch et al., 1983) hypothalamic extracts in 1983. GRF-like immunoactivity is also in several extra-hypothalamic sites including testes (Berry and Pescovitz, 1988), ovaries (Moretti et al., 1990), corpus luteum (Moretti et al., 1989), and in media after culture of rat granulosa cells (Bagnato et al., 1992). Furthermore, the GRF gene is expressed in the rat ovary and its product is translated into a peptide with an amino acid sequence similar to hypothalamic GRF (Bagnato et al., 1992). Whether the bovine ovary produces GRF is unknown.

Receptors in Ovaries

GRF receptors are in rat granulosa cells (Moretti et al., 1990; Bagnato et al.,

1991), but not in human ovaries (Tang et al., 1995). Whether the bovine ovary has receptors for GRF is unknown.

Ovarian Actions

Follicle Growth and Function. GRF potentiates FSH-induced production of progesterone and estradiol and LH receptor formation by rat granulosa cells (Moretti et al., 1990; Hughes et al., 1996). The addition of GRF to culture medium accelerates maturation of follicle- and cumulus-enclosed oocytes obtained from immature rats (Apa et al., 1995). GRF may also have a role in ovulation, since addition of GRF to culture media increases rat granulosa cell plasminogen activator activity (Karakji and Tsang, 1995). In dairy cows, administration of GRF for 86 days increases size of follicles larger than 8 mm in diameter and concentration of progesterone in medium-size follicles (4 to 7.9 mm), but has no effect on concentration of IGF-I in follicular fluid of follicles larger than 1 mm in diameter (Spicer and Enright, 1991). In support of the stimulatory effects of exogenous GRF on follicular growth, Spicer et al. (1992) found that GRF increases *in vitro* proliferation of bovine granulosa cells collected from small follicles (1 to 5 mm in diameter). In contrast to rats (Moretti et al., 1990), GRF does not alter progesterone production by bovine granulosa cells *in vitro* (Spicer et al. 1992). In summary, GRF actions in ovaries may be species specific. In cattle, GRF acts directly on small follicles to increase proliferation of granulosal cells, whereas GRF injections selectively enhance progesterone production by medium-size follicles.

Corpus Luteum Growth and Function. A direct effect of GRF on growth or function of the bovine corpus luteum has not been reported.

Reproductive Efficiency

Administration of GRF to pregnant beef cows increases serum concentrations of growth hormone (GH) after parturition (Simpson et al., 1992). In addition, high serum GH concentrations are associated with a greater postpartum body weight loss and delayed return to estrus (Simpson et al., 1992). Immunoneutralization of GRF decreases GH concentration in serum (Simpson et al., 1991), delays onset of puberty (Simpson et al., 1991), reduces number of antral follicles (≥ 7 mm) in heifers (Cohick et al., 1996), and decreases fertility in rats (Gruaz et al., 1994). However, injection of GH to heifers immunized against GRF does not restore normal time for onset of puberty compared with controls (Stanko et al., 1994a). Taken together, these findings imply that the effects of exogenous GRF or immunoneutralization of GRF are probably mediated by alterations in GH secretion and synthesis, although direct effects of GRF on ovarian function cannot be ruled out.

Summary

Although results of only a few studies in cows have been reported, GRF produced by the ovary or treatments with GRF could directly or indirectly (via stimulation of GH) promote growth and function of the follicle and(or) corpus luteum.

Growth Hormone

Two theories regarding the mechanism of action of GH exist. The GH hypothesis (Thorner et al., 1992) states that GH actions occur by specific binding of GH to its receptor on target tissue which triggers a specific response. Alternatively, the somatomedin

hypothesis (Underwood and Van Wyk, 1992) implies that effects of GH are mediated by IGF-I and IGF-II and IGF binding proteins, which are secreted primarily by the liver.

Chemical Properties and Synthesis

Bovine GH is produced by the anterior pituitary and released in a pulsatile manner (Thorner et al., 1992). Four variants of GH are produced naturally by cattle. Each variant has 190 or 191 amino acids and either a valine or a leucine at position 127 (Santome et al., 1976; Charrier and Martal, 1988; Wood et al., 1989). Synthesis and release of GH are regulated by two hypothalamic peptides, GRF and somatostatin. Somatostatin inhibits whereas GRF stimulates secretion of GH (Frohman et al., 1992). GH exerts specific actions in several tissues, such as skeletal muscle, adipose tissue, liver, mammary gland and the ovary (Thorner et al., 1992). The physiological effects of GH are manifested in (i) anabolic effects (such as, nitrogen accretion in growing animals and milk synthesis in lactating animals), (ii) alterations in carbohydrate metabolism, and (iii) growth of cartilage and bone (Thorner et al., 1992).

Receptors in Ovaries

The majority of receptors for GH in the cow are in the liver (Hauser et al., 1990). Binding of GH to its receptors induces transcription of IGF-I mRNA that leads to synthesis and secretion of IGF-I. In addition to liver, GH receptors are in large luteal cells (Tanner and Hauser, 1989; Lucy et al., 1993a), small follicles (1 to 5 mm in diameter; Lucy et al., 1993a), and granulosa cells from preovulatory follicles of rbST-treated cows (Cameron

et al., 1990). Administration of GH down-regulates GH receptor mRNA in the corpus luteum (Kirby et al., 1996). GH receptor immunoreactivity is in the granulosa layer of human antral follicles (Carlsson et al., 1992; Tamura et al., 1994; Sharara and Nieman, 1994) and in rat granulosa cells (Lobie et al., 1990; Carlsson et al., 1993). In contrast, the GH receptor mRNA is not in preantral follicles, theca interna, theca externa, oocytes, or ovarian stroma of women (Sharara and Nieman, 1994). The presence of GH receptors in ovaries of cattle and other species implies that GH has a physiological role in regulation of development and function of corpora lutea and follicles. In support of a role for GH in ovarian function, reduced GH receptor function in miniature Brahman cattle (*Bos indicus*) is associated with decreased number of follicles and reduced size of dominant follicles and corpora lutea (Lucy et al., 1996).

Ovarian Actions

Follicle Growth and Function. Administration of rbST to heifers increases number of small antral follicles (2 to 5 mm; Gong et al., 1991, 1993a). In marked contrast to heifers, in lactating dairy cows rbST increases number of medium (6 to 10 mm) and large (10 to 15 mm) follicles (De la Sota et al., 1993), decreases size of the largest follicle (Lucy et al., 1994a, 1994b), and increases size of the second largest follicle (De la Sota et al., 1993; Lucy et al., 1993b, 1994a, 1994b). The reason for differences in effects of rbST treatments on follicle populations between heifers and lactating cows is unknown.

The presence of GH receptors in bovine follicles (Cameron et al., 1990) implies that rbST may have a direct effect on follicular growth and function. However, the

effects of rbST on ovarian function are controversial and inconsistent. For example, rbST stimulates estradiol production by rat, human and bovine granulosa cells in a dose-dependent fashion (Hutchinson et al., 1988; Barreca et al., 1993; Gong et al., 1994), and rbST increases proliferation of bovine granulosa cells from large follicles (>10 mm) *in vitro* (Gong et al., 1993c). In contrast, pharmacological doses of rbST (300 ng/ml) either inhibit FSH-induced estradiol production by bovine granulosa cells collected from small (1 to 5 mm) and large (≥ 8 mm) follicles (Spicer and Steward, 1996a) or have no effect on aromatase activity in rat granulosa cells (Jia et al., 1986). In addition, rbST increases (Langhout et al., 1991; Gong et al., 1994), inhibits (Sirotkin and Nitray, 1994), or has no effect (Spicer and Steward, 1996a) on progesterone secretion during culture of bovine granulosa cells. These conflicting effects of rbST on granulosa cell function *in vitro* may be due to differences in species, experimental conditions, or stage of differentiation of follicles.

In cultures of theca-interstitial cells obtained from immature rats, GH stimulates androsterone synthesis in a dose- and time-dependent manner, but does not increase cell number (Apa et al., 1996). Addition of antibodies against IGF-I to culture media does not modify the GH effect, thus suggesting that GH does not require IGF-I to stimulate androgen production (Apa et al., 1996). Similarly, in cultures of bovine theca cells that exhibit a >3-fold increase in LH-induced androstenedione production, addition of rbST further increases androstenedione production (Spicer and Steward, 1996a). Results from these studies suggest that rbST could affect regulation of follicular function by enhancing availability of substrate for estradiol production.

Corpus Luteum Growth and Function. The effects of bST on function of the

corpus luteum are both controversial and complex. For example, Lucy et al. (1994b) found that daily injection of heifers with rbST for 19 days increases serum concentrations of progesterone during the first 10 days of the estrous cycle. In contrast, progesterone is unaltered during the luteal phase in heifers injected with rbST for 21 (Lucy et al., 1994a) or 43 days (Gong et al., 1991; 1993a). In lactating cows, administration of rbST beginning soon after parturition (29 to 44 days post partum, dpp) for >130 days transiently increases progesterone concentration during the luteal phases of the first and second estrous cycles after treatments begin (Schemm et al., 1990; Gallo and Block, 1991). In contrast, administration of rbST after 55 days postpartum for 19 to 24 days does not affect progesterone concentration (De la Sota et al., 1993; Lucy et al., 1993b; Dalton and Marcinkowski, 1994; Kirby et al., 1997a). In support of a stimulatory role for rbST on luteal function, *in vitro* perfusion with rbST stimulates production of progesterone by bovine corpora lutea from cycling and pregnant cows, although a higher response is observed when corpora lutea are obtained during the early luteal phase (Liebermann and Schams, 1994). In summary, the stimulatory effects of rbST on progesterone production by the bovine corpus luteum are variable and may depend on duration of treatment, age of animal, stage post partum, stage of luteal development, and energy balance.

Reproductive Efficiency

Measures of Reproductive Efficiency. The effect of rbST on reproduction in cows is controversial. For example, administration of rbST during early lactation to dairy cows increases the intervals from parturition to first detected estrus (Stanisiewski et al.,

1992), to first breeding and to conception (Burton et al., 1990; Aguilar et al., 1991), and increases number of artificial inseminations required for pregnancy (McGuffey et al., 1991). Longer intervals to first estrus and breeding and a high number of inseminations result in longer calving intervals (Burton et al., 1990; Aguilar et al., 1991; McGuffey et al., 1991). In contrast to negative effects of rbST on reproductive efficiency, first service conception and pregnancy rates are higher in cows treated with lower doses of rbST (5 mg/day; Stanisiewski et al., 1992) compared with cows treated with higher doses or untreated controls (Stanisiewski et al., 1992). In further contrast to the negative or positive effects of rbST, other studies show that administration of rbST does not alter reproductive efficiency (Eppard et al., 1987; Grings et al., 1990; De la Sota et al., 1993).

In contrast to early lactation, most cows treated with rbST during mid lactation are pregnant. Thus, the effects of rbST on reproductive parameters are difficult to evaluate. However, pregnancy and conception rates in non-pregnant multiparous cows treated during mid lactation decrease (Esteban et al., 1994a; Stanisiewski et al., 1994) and interval from calving to conception increases (Esteban et al., 1994b) with increasing doses of rbST

Problems associated with detection of estrus or anestrus are reported to occur in dairy cows treated with rbST during early (Burton et al., 1990; Morbeck et al., 1991; Stanisiewski et al., 1992) and mid lactation (Stanisiewski et al., 1994; Kirby et al., 1997a). Success of detecting estrus decreases with increasing dose of rbST, resulting in longer intervals to first insemination or longer inter-estrus intervals (Morbeck et al., 1991; Stanisiewski et al., 1994). The lower rate of estrus detection in rbST-treated cows may be associated with reduced expression of estrus (Morbeck et al., 1991), since administration of

rbST depresses expression of estrogen-induced estrus in ovariectomized heifers (Lefebvre and Block, 1992).

Twinning. In dairy cows, the normal percentage of twinning is 3.5 to 5.8 % (Cady and Van Vleck, 1978; Roberts, 1986; Markusfeld, 1987), and the major factor limiting this phenomenon is ovulation rate (Anderson et al., 1982). Rhind and Schanbacher (1991) suggest that differences in ovulation rates in sheep breeds are a reflection of the number of large ovarian follicles that are that have estradiol levels > 10 ng/ml. Furthermore, in cattle selected for high twinning rate, two or more large (> 8 mm) estrogen-active follicles emerge within each follicular wave (Echternkamp et al., 1996). It is suggested that GH is a co-gonadotropin that amplifies FSH hormonal action on follicular growth and function in several species (Katz et al., 1993; Findlay, 1995). In cattle, pretreatment with rbST enhances superovulatory responsiveness to a subsequent injection of equine chorionic gonadotropin, thus leading to an increase in ovulation rate in heifers (Gong et al., 1993b) and cows (Herrler et al., 1994). Whether rbST alone increases ovulation or twinning rate in cattle is controversial. For example, administration of rbST does not increase number of corpora lutea in heifers (Gong et al., 1991; 1993) or cows (De la Sota et al., 1993; Lucy et al., 1993b; Kirby et al., 1997a), or twinning rate in lactating cows (Burton et al., 1990; Stanisiewski et al., 1994). In contrast, some studies indicate that administration of rbST increases twinning rate in lactating cows (Butterwick et al., 1988; Cole et al., 1991; Wilkinson and Tarrant, 1991; Oldenbroek et al., 1993; Esteban et al., 1994c).

Summary

Administration of rbST to increase milk production in dairy cattle can enhance ovarian function and(or) decrease reproductive efficiency. Although the reasons for conflicting results among different laboratories are unclear, differences in energy balance among dairy cows during rbST treatments could explain the inconsistent results. For example, approximately 80% of postpartum dairy cows experience negative energy balance during early lactation (Villa-Godoy et al., 1988), and the duration of negative energy balance may extend from calving until 16 weeks postpartum (Villa-Godoy et al., 1988; Butler and Smith, 1989). In dairy cows, negative energy balance delays resumption of ovulation (Butler and Smith, 1989) and accounts for an increase in number of days to first ovulation, days open and lactational anestrus (Villa-Godoy et al., 1988; Beam and Butler, 1997). Therefore, administration of rbST, which decreases energy balance in lactating cows (Bauman et al., 1985; Lucy et al., 1992b), may also negatively affect reproductive parameters in some dairy cows by decreasing energy balance below levels required for normal reproductive performance.

Intraovarian Insulin-like Growth Factor System

Several studies suggest the existence of an intraovarian insulin-like growth factor (IGF) system complete with ligands, receptors and binding proteins (Adashi et al., 1985; Giudice, 1992; Spicer and Echternkamp, 1995). Also, IGFs enhance proliferation and steroidogenesis of murine (Adashi et al., 1985), porcine (Hammond et al., 1991; Findlay, 1995), human (Giudice, 1992) and bovine (Webb et al., 1994; Spicer and Echternkamp, 1995)

ovarian cells. Although IGFs have direct effects, IGFs may also amplify the actions of gonadotropins (Giudice, 1992; Spicer and Echternkamp, 1995). It is hypothesized that GH is acting through its receptor to stimulate IGF release (Underwood and Van Wyk, 1992), which, in turn, results in augmentation of gonadotropin-supported growth and differentiation of granulosa and theca cells. In addition, administration of rbST increases serum concentration of IGF-binding proteins in heifers (Stanko et al., 1994a; 1994b) and cows (VanderKooi et al., 1995) which, in turn, regulates net IGF-I availability.

Insulin-like Growth Factors

Chemical Properties and Synthesis

Insulin-like growth factor-I and IGF-II are low molecular weight mitogenic peptides that are structurally related to proinsulin. In 1978, IGF-I and IGF-II were purified and sequenced by Riderkneckt and Humbel (1978). There is a high degree of amino acid homology between insulin, IGF-I and IGF-II; structural similarities are also high (greater than 93%) among bovine, ovine, porcine, human and rat peptides (Spicer and Echternkamp, 1995). The presence of IGFs in ovaries was first reported in pigs (Hammond et al., 1982), then in follicular fluid and ovaries from several species, including cows (Spicer et al., 1988).

Production of IGF-I in the liver and other tissues is GH-dependent (Giudice, 1992). Administration of rbST increases ovarian production of IGF-I in rabbits and pigs (Yoshimura et al., 1994; Samaras et al., 1996) and increases concentration of IGF-I in follicular fluid from bovine dominant and subordinate follicles (Spicer et al., 1993; Stanko et al., 1994b). In contrast, *in vitro* production of IGF-I by bovine granulosa cells from small

follicles is unaltered by rbST or FSH, or is inhibited by co-treatments with rbST and insulin (Spicer et al., 1993). These observations imply that GH does not stimulate production of ovarian IGFs.

Insulin-like growth factor-I and its mRNA are in granulosa cells of growing follicles in rats and cows (Hernandez et al., 1989; Oliver et al., 1989; Spicer et al., 1993), and IGF-I is in bovine follicular fluid of different size follicles (Badinga et al., 1992; Spicer et al., 1993; Steward et al., 1996). IGF-II is in theca-interstitial cells of rat follicles (Hernandez et al., 1990). Concentration of IGF-I in bovine follicular fluid is correlated positively with diameter of estrogen-active follicles (Spicer et al., 1988; Echternkamp et al., 1994a). Concentrations of IGF-I in follicular fluid from the dominant and two or three largest subordinate follicles collected during the first follicular wave are similar (De la Sota et al., 1996).

Immunoreactive IGF-I, but not IGF-II, is in bovine luteal cells (Amselgruber et al., 1994). In addition, bovine luteal tissue expresses IGF-I mRNA during the estrous cycle and gestation (Einspanier et al., 1990).

Receptors in Ovaries

IGF-I interacts with specific cell surface receptors in target tissues. The Type I IGF receptor primarily recognizes IGF-I, but also binds IGF-II and insulin, and has a peptide-binding subunit with a molecular weight of 130 kDa (Van Wyk, 1984). Specific IGF receptors and their mRNAs exist in various types of bovine ovarian cells including granulosa (Spicer et al., 1994; Steward et al., 1996), theca (Spicer and Steward, 1996b; Steward et al.,

1996) and large luteal cells (Sauerwein et al., 1992; Chakravorty et al., 1993). Binding of IGF-I to its receptor stimulates a cascade of tyrosine kinase-mediated events in the granulosa and luteal cells (Chakravorty et al., 1993).

Ovarian Actions

Follicle Growth and Function. IGFs have an autocrine/paracrine effect on granulosa and theca cell function. For example, infusion of a IGF-I analog, which has reduced affinity for IGFBPs, increases ovarian estradiol secretion in ewes (Campbell et al., 1995). During cell culture, IGF-I increases bovine granulosa (Spicer et al., 1993; Gong et al., 1993c; Gutierrez et al., 1997) and theca cell proliferation (Steward et al., 1995; Spicer and Steward, 1996b), and stimulates estradiol and progesterone production by rat (Adashi et al., 1985), bovine (Spicer et al., 1993) and porcine (Hsu and Hammond, 1987) granulosa cells. Moreover, IGF-I stimulates basal or FSH-induced progesterone (Spicer et al., 1993; Gong et al., 1994; Armstrong et al., 1996b) or estradiol (Gong et al., 1994; Gutierrez et al., 1997) production by granulosa cells collected from different sized follicles. In addition, in the presence of LH, IGF-I stimulates production of progesterone and(or) androstenedione by theca cells (Steward et al., 1995; Spicer and Steward, 1996b). In summary, IGFs stimulate bovine granulosa and theca cell mitosis and steroidogenesis. These effects are consistent with possible roles of IGF-I on follicle growth and function.

Corpus Luteum Growth and Function. Although *in vivo* effects of IGFs have not been tested in cattle, IGF-I increases progesterone production during culture of the bovine corpus luteum (McArdle and Holtorf, 1989; Sauerwein et al., 1992). In sheep, infusion

of IGF-I increases oxytocin release from the corpus luteum (Fleet et al., 1994).

Reproductive Efficiency

Administration of GRF or rbST increases the concentration of IGFs in serum (Dahl et al., 1993; VanderKooi et al., 1995; Yung et al., 1996) from cows in positive energy balance, but does not affect IGF-I concentration in heifers in negative energy balance (Yung et al., 1996). IGF-I is postulated to be a mediator of the effects of GH on reproduction (Giudice, 1992; Spicer and Echternkamp, 1995). However, administration of IGF-I to rats, which increases concentration of IGF-I in serum, does not increase progesterone concentration, ovulation rate or number of fetuses (Kerr and Kirkwood, 1992), suggesting that an increase in circulating IGF-I does not alter ovarian function. Whether IGF-I alters reproductive efficiency in cattle is unknown. Nevertheless, alterations in serum IGF concentrations in cattle are associated with various reproductive events. Specifically, cows selected for twinning have greater concentrations of IGF-I in serum and follicular fluid compared with control cows (Echternkamp et al., 1990). In addition, concentration of IGF-I is lower during anestrus induced by food restriction in beef and dairy cows (Richards et al., 1991; Lucy et al., 1992a), and delayed puberty in GRF-immunized heifers is associated with suppression of follicular growth and decreased concentrations of IGF-I in serum and follicular fluid (Armstrong et al., 1992).

Summary

IGF-I stimulates granulosa cell proliferation, aromatase activity, and

progesterone biosynthesis in several species, including cows. In addition, higher concentrations of IGF-I in serum are associated with twinning and enhanced ovarian function in cattle. Moreover, gonadotropins stimulate IGF-I production by bovine granulosa cells. Whether IGF-I production by bovine granulosa cells is stimulated by GH is unclear, even though administration of rbST increases IGF-I concentration in serum.

Insulin-like Growth Factor Binding Proteins

Insulin-like growth factor binding proteins prolong the half-life, act as systemic transport proteins, and regulate availability of IGFs (Giudice, 1992; Spicer and Echternkamp, 1995).

Chemical Properties and Synthesis

Six unique species of insulin-like growth factor binding proteins (IGFBP) have been identified (Giudice, 1992; Spicer and Echternkamp, 1995). The liver produces IGFBPs along with other tissues, including the ovary (Ui et al., 1989; Hammond et al., 1991; Ricciarelli et al., 1991; 1992; Spicer and Echternkamp, 1995). Immunoprecipitation analysis indicates that four IGFBPs (IGFBP-2, -3, -4, -5) exist in bovine follicular fluid (Cohick et al., 1996; De la Sota et al., 1996; Funston et al., 1996), and IGFBPs are secreted by bovine theca and granulosa cells (Sakal et al., 1992).

Insulin-like growth factor binding proteins are autocrine or paracrine regulators of granulosa cell function (Adashi, 1992; Giudice, 1992), and administration of GH may affect the ovarian production of IGFBPs. Specifically, GH increases ovarian IGFBP-3

mRNA in rats (Ricciarelli et al., 1992) and IGFBP-2 and IGFBP-3 in pigs (Samaras et al., 1990). Administration of rbST to lactating cows increases IGFBP-3, but decreases IGFBP-2 in serum (VanderKooi et al., 1995). Active immunization of heifers against GRF, which decreases concentration of GH in serum, also decreases IGFBP-3 and increases IGFBP-2 levels in follicular fluid (Cohick et al., 1996). Serine proteases and metalloproteases are involved in IGFBP degradation (Besnard et al., 1996; 1997). Proteolytic activity changes the levels of IGFBPs during growth and atresia of ovine, porcine and human follicles (Besnard et al., 1996; Iwashita et al., 1996; Besnard et al., 1997), and the amounts of these proteolytic enzymes change during folliculogenesis (Besnard et al., 1996). Also, FSH stimulates production of IGFBP proteases by rat granulosa cells (Fielder et al., 1993; Liu et al., 1993). Since IGF-I activity is regulated by the binding protein levels (Hammond et al., 1991; Giudice, 1992), different amounts of IGFBPs in follicular fluid may alter net IGF-I biological activity.

Receptors in Ovaries

Whether IGFBPs regulate cell function by binding to specific surface receptors is unknown. Several reports suggest that IGFBPs primarily affect ovarian function by binding to IGF-I and regulating its bioavailability (Hammond et al., 1991; Giudice, 1992; Spicer and Echternkamp, 1995). However, IGFBP-2 may interact with cell surface integrins via its Arg-Gly-Asp sequence (Giudice, 1992).

Ovarian Actions

Follicle Growth and Function. Amounts of insulin-like growth factor binding protein-2, -4, and -5 in bovine follicular fluid decrease as follicles develop and become estrogen-active or dominant. In contrast, IGFBP-2, -4, and -5 levels increase when follicles undergo atresia (De la Sota et al., 1996; Funston et al., 1996). Furthermore, there is a negative correlation between the amounts of low molecular weight IGFBPs (IGFBP-2, -4 and -5) and concentration of estradiol in follicular fluid in cattle (Echternkamp et al., 1994a). In contrast, IGFBP-3 levels in follicular fluid does not change with follicular size and(or) differentiation (Spicer and Echternkamp, 1995; De la Sota et al., 1996).

IGFBPs have anti-gonadotropic activity. For example, IGFBPs inhibit FSH-induced [^{3}H]-thymidine uptake (Bicsak et al., 1990; Ricciarelli et al., 1992) and FSH-induced estradiol synthesis (Ui et al., 1989; Mason et al., 1992) in rat and human granulosa cells in culture. These observations suggest that IGFBPs regulate the biological activity of the IGFs, possibly by sequestering extracellular IGFs thereby limiting peptide access to specific cell receptors (Giudice, 1992). Thus, IGFBPs may be important local factors that modulate follicular growth and function.

Corpus Luteum Growth and Function. A paucity of information exists on the role of IGFBPs in the corpus luteum. However, IGFBP-3 mRNA is localized in rat luteal cells (Nakatami et al., 1991). In addition, IGFBP-3 mRNA expression is increased during regression of the corpus luteum in rats (Erickson et al., 1993). Thus, IGFBP-3 may have a role in modulation of IGF-I-stimulated progesterone production by rat luteal cells (Parmer et al., 1991) and perhaps the bovine corpus luteum (McArdle and Holtorf, 1989; Sauerwein

et al., 1992).

Reproductive Efficiency

The effect of IGFBPs on reproductive efficiency in cattle is unknown. However, administration of IGFBP-3 inhibits ovulation in laboratory species (Bicsak et al., 1991; Yoshimura et al., 1996).

Summary

IGFBPs are produced by granulosa, theca and luteal cells in several species, including cows. Amounts of IGFBP-2, -4, and -5 in follicular fluid are associated negatively with follicular growth and positively with atresia in cattle. These findings imply that IGFBPs may alter IGF-I bioactivity and, in turn, modulate estradiol production.

Overall Summary

Strong evidence exists that the GRF-GH-IGF-IGFBP response system has an important role in regulation of gonadal function in numerous species, including cattle. However, the effects of rbST on ovarian function and reproductive efficiency in dairy cattle are controversial. Many studies indicate that rbST increases number of follicles (Gong et al., 1991), reduces expression of estrus and fertility (Esteban et al., 1994a; 1994c; Stanisiewski et al., 1994; Kirby et al., 1997b), and increases twinning in dairy cattle (Cole et al., 1991; Wilkinson and Tarrant, 1991; Esteban et al., 1994c). However, other studies report no effects of rbST on reproduction (Eppard et al., 1987; Burton et al., 1990; Grings et al., 1990). While

the reason for discrepancies among laboratories is unknown, it may be related to a complex interaction between energy balance and dose, duration and frequency of treatment with age and parity of animal, as already mentioned. Moreover, few studies have thoroughly examined the effects of GRF or rbST treatments on the dominant follicle process in dairy cows, which could explain its effects on reproductive efficiency. The dominant follicle process is well understood in cattle (Figure 1). Thus, monitoring the effects of GRF or rbST treatments on dominant follicles *in vivo* and *in vitro* may provide new information, and a physiologically relevant model to understand whether and how rbST and GRF treatments alter ovarian function in dairy cows.

Based on the review of literature, the overall hypothesis for my thesis research is that rGRF or rbST treatment alters the dominant follicle process. This hypothesis was tested as follows: The first objective was to examine the effects of rGRF or rbST on 1) occurrence of ovulation after prostaglandin treatment, 2) ovulation rate, 3) luteal function, and 4) follicular hierarchy and function during the first-wave dominant follicle in primiparous dairy cows. To this end, follicle population and number of corpora lutea, and concentrations of ovarian steroids, gonadotropins, growth hormone, IGF-I and IGFBPs were measured in serum and(or) follicular fluid (Chapter 2). The second objective was to determine the mechanism of action of rGRF or rbST by testing whether rGRF or rbST alters the capacity of bovine granulosa cells to release estradiol into culture media (Chapter 3). Taken together, the results of these studies will establish whether treatments with rGRF or rbST disrupt the dominant follicle process in cattle by increasing estradiol production by granulosa cells.

Part II

CHAPTER 2

A. Effect of Long-Term Infusion with Recombinant Growth Hormone-Releasing Factor and Recombinant Bovine Somatotropin on Development and Function of the Dominant Follicle and Corpus Luteum in Holstein Cows.

Introduction

Recombinant growth hormone-releasing factor (rGRF) and recombinant bovine somatotropin (rbST), which are used to increase milk production in dairy cows (Bauman et al., 1985; Dahl et al., 1991), also alter ovarian function (Webb et al., 1994; Findlay, 1995; Burton et al., 1996). For example, rGRF increases follicle size and intrafollicular concentrations of progesterone in dairy cows (Spicer and Enright, 1991), and stimulates proliferation of bovine granulosa cells (Spicer et al., 1992). Administration of rbST increases number (Gong et al., 1991) and size of follicles (Lucy et al., 1992b, 1994a), growth (Lucy et al., 1994b) and progesterone production by the corpus luteum (CL; Schemm et al., 1990; Gallo and Block, 1991; Lucy et al., 1994b), rate of twinning (Cole et al., 1991; Wilkinson and Tarrant, 1991; Oldenbroek et al., 1993; Esteban et al., 1994c), reduces expression of estrus (Lefebvre and Block, 1992; Esteban et al., 1994c) and increases the

interval from calving to conception and calving intervals (McGuffey et al., 1991; Bauman, 1992).

Since growth hormone receptors (GH-R) are in the CL (Lucy et al., 1993a), follicles (Lucy et al., 1993a) and in granulosa cells from preovulatory follicles of rbST-treated cows (Cameron et al., 1990), the effects of rbST on follicular growth and function may be direct. Moreover, abnormal GH-R function in miniature Brahman cattle results in small body size, fewer ovarian follicles, and smaller dominant follicles and CL sizes (Lucy et al., 1996). Together, these data demonstrate that rGRF or rbST can modify follicular growth and function, and the subsequent growth and function of the corpus luteum.

The dominant follicle process is characterized by two or three "waves" of development of ovarian follicles, ovulation of the dominant follicle from one of the waves, and formation of a corpus luteum during the bovine estrous cycle. The long-term effects of rGRF or rbST on development and function of the dominant follicle and the CL in dairy cows have not been examined. Therefore, the present study will test the hypothesis that rGRF or rbST treatment alters the dominant follicle process in dairy cows. Whether the dominant process is altered will be monitored by examining the effects of rGRF or rbST on 1) the occurrence of ovulation after $PGF_2\alpha$ treatment, 2) ovulation rate, 3) luteal function, and 4) follicular hierarchy and function during development of the first-wave dominant follicle.

Materials and Methods

Recombinant Hormones

The recombinant form of bovine GRF (rGRF) used in this study was $rGRF^{1-45}$

(Leu^{27}, Homoserine^{45}-bGRF$^{1-45}$ lactone, Pharmacia and Upjohn Inc., Kalamazoo, MI). This GRF analog differs from natural GRF at position 27 where a Leu is substituted for Met, and this GRF analog contains homoserine which makes it one amino acid longer than natural GRF (Kirschner et al., 1989).

Pituitary GH exists as four variants consisting of 190 or 191 amino acids with heterogeneity at the amino terminus (Phe or Ala-Phe) and at position 127 (Val or Leu) (Wallis, 1973; Santome et al., 1976; Charrier and Martal, 1988; Wood et al., 1989). In the present study, the rbST (Somavubove, Pharmacia and Upjohn Inc., Kalamazoo, MI) used to treat cows is identical to the pituitary variant that has an Ala at its amino-terminus and a Leu at position 127 (Wallis, 1973; Juskevich and Guyer, 1990; Stanisiewski et al., 1994).

Animals, and Blood and Tissue Collection

Thirty primiparous Holstein cows weighing 523 ± 7 kg were housed in tie stalls and fed a total mixed ration (Appendix A; NRC, 1989; VanderKooi, 1993). Eight days before treatments began, an infusion catheter (VETport, Thermedics, Woburn, MA) was implanted surgically into each cow's left jugular vein as described previously (Dahl et al., 1990).

Cows were infused beginning 117 days postpartum (day 1 of treatment) with rGRF (12 mg/day; n=10 cows) or rbST (29 mg/day; n=10 cows) for 63 days, and ten cows were untreated controls (Binelli et al., 1995). Prostaglandin $F_2\alpha$ (Lutalyse, Pharmacia and Upjohn Inc., Kalamazoo, MI) was injected into all cows on days 43 and 54 of treatment to synchronize estrus. Estrus was not observed because infusion pumps were attached to each

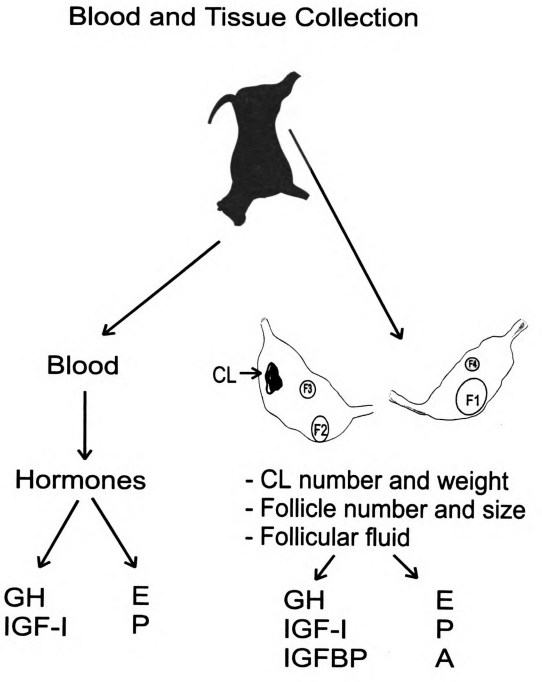
cow's back, and cows were tied in stalls except during milking. On day 57 of treatment, blood was sampled at 20-minute intervals for 6 h and LH and FSH were measured. All cows were slaughtered on day 63 of treatment (180 days postpartum), one 50-ml blood sample was collected from trunk blood, and the ovaries were collected and weighed (Figure 2). Stage of the estrous cycle was estimated based on appearance of the corpus luteum (CL; Ireland et al., 1980) to verify that at the time of ovary collection cows were between Days 5 to 7 of the estrous cycle (9 days after the last $PGF_2\alpha$ injection). Number and weight of corpora lutea, number and size of follicles > 5 mm in diameter (Figure 2; F1= largest, F2= second largest, F3= third largest and F4= remaining follicles > 5 mm), and size of five follicles ≤ 5 mm in diameter (not shown in Figure 2) were recorded. Follicular fluid (FF) was collected from all follicles measured. Serum and FF were stored at -20 °C in polypropylene tubes until assayed for concentration of hormones (Figure 2; GH= growth hormone, IGF-I= insulin-like growth factor-I, IGFBP= insulin-like growth factor binding proteins, E= estradiol, P=progesterone, A= androstenedione)

Radioimmunoassays

Progesterone (P) in unextracted serum and FF, and estradiol (E) in unextracted FF were determined using commercially available radioimmunoassay (RIA) kits (Diagnostic Products Corporation, Los Angeles, CA) as validated previously (Turzillo and Fortune, 1990; Ireland et al., 1994). Progesterone was quantified in three assays, whereas E was analyzed in five assays. Sensitivity of the P assay was 0.1 ng/ml, and intra- and inter-assay coefficients of variation (CV) were 5% and 9%, respectively. Cross-reactivity of P antiserum with various

Figure 2. Diagram of the procedure for blood and tissue collection and hormones assayed for rGRF-, rbST-treated and control cows.

Figure 2.


Blood and Tissue Collection
Blood
Hormones
GH
IGF-I
E
P
CL
F3
F2
F4
F1
- CL number and weight
- Follicle number and size
- Follicular fluid
GH
IGF-I
IGFBP
E
P
A

steroids follows: 11-deoxycortisol =2.4%, 20α-dihydroprogesterone =2.0%, 11-deoxycorticosterone =1.7%, 5β-pregnan-3,20-dione =1.3%, and < 1% with other steroids, such as androstenediol, corticosterone, cortisol, estradiol, 17α-hydroxy-progesterone, pregnenolone, and testosterone (Diagnostic Products Corp. Los Angeles, CA). Sensitivity of the E assay was 0.5 pg/ml, and intra- and inter-assay CV were 6 and 7% respectively. Cross-reactivity of E antiserum with various steroids follows: estrone = 12.5%, 17β-estradiol-3β-D-glucoronide = 6%, d-equilenin = 4.2%, 1,3,5(10)-estratrien-17α-methyl-3,17β-diol 3-methyl ether = 3.5%, estrone-β-D-glucoronide = 1.6%, 4-estren-17β-ol-3-one = 1.8%, and < 1% with other steroids, such as androstenedione, progesterone, testosterone, 19-hydroxy-androstenedione, estriol, cortisone, and corticosterone (Diagnostic Products Corp. Los Angeles, CA)

Estradiol in ether-extracted serum was quantified in a single assay using a different RIA kit (Serono Diagnostics, Allentown, PA) with modifications as validated previously (Prendiville et al., 1995). Sensitivity of the E assay was 0.19 pg/ml, and intra-assay CV was 4%. Cross-reactivity of E antiserum with various steroids follows: 17β-estradiol-3-benzoate = 2.5%, estrone = 1.8%, and < 0.5% with other steroids, such as estriol, testosterone, progesterone, androstenedione, dehydroepiandrosterone, estradiol-17β-glucoronide, 17β-estradiol-3 sulfate, and cortisol, (Serono Diagnostics, Allentown, PA).

Androstenedione in FF was quantified by a solid-phase RIA kit (Diagnostic Products Corporation, Los Angeles, CA) per manufacturer's instructions with the following modifications. Five microliters of FF were extracted once with 1 ml diethyl ether. Extraction efficiency was 86%, thus concentration of androstenedione was not adjusted for recovery.

After evaporation of solvent for 60 min at 37 °C, residues were dissolved in 500 µl of buffer supplied with the kit, incubated at 37 °C for 30 min, and then duplicate 200 µl aliquots were assayed. Increasing volumes (0.4 to 40 µl) of ether-extracted FF depressed binding parallel to the standard curve (Appendix B). All samples were analyzed in a single assay. Sensitivity of the assay was 20 pg/ml and intra-assay CV was 6%. Cross-reactivity of androstenedione antiserum with various steroids follows: androsterone = 6.3%, DHEA = 4.3%, and < 1% with other steroids such as corticosterone, cortisol, cortisone, estrone, progesterone, and testosterone (Diagnostic Products Corp. Los Angeles, CA).

Insulin-like growth factor-I (IGF-I) was quantified by RIA in formic acid-ethanol extracted serum and FF (Hammond et al., 1988; Bruce et al., 1991; Sharma et al., 1994). Extraction efficiency was 86%, and concentration of IGF-I was not adjusted for recovery. Recombinant human IGF-I (DGR012, Bachem, Inc. Torrance, CA) was radioiodinated using chloramine T (Etherton et al., 1987), and the international reference for human IGF-I (Bristow et al., 1990) was used as standard. Antiserum against human IGF-I (NIH-AB UBK487, kindly supplied by L. Underwood, University of North Carolina, Chapel Hill, NC) was diluted 1:10,000 in assay buffer (0.3 M phosphate buffer, 0.01 M EDTA, pH 7.5). Increasing volumes (50 to 200 µl) of extracted FF depressed binding parallel to the standard curve (Appendix C). All samples were analyzed in a single assay. Sensitivity of the assay was 12.5 pg/tube, and intra-assay CV for serum and FF were 6 and 5%, respectively. Cross-reactivity with IGF-II is < 0.5% (Bristow et al., 1990).

Growth hormone (GH) in serum and FF were quantified by a heterologous RIA (Gaynor et al., 1995) with the following modifications. Recombinant bST (U-72104;

Pharmacia and Upjohn Inc., Kalamazoo, MI) was used as standard or radioiodinated using chloramine T (Gaynor et al., 1995). Antiserum to ovine GH (NIADDK-anti-oGH-2) was diluted 1:80,000 in 1:400 normal rabbit serum (Gibco Laboratories, Madison, WI) in 0.01 M phosphate buffered saline (PBS, 0.05 M EDTA, pH 7.0). Assay buffer was 1% bovine serum albumin (BSA) in 0.01 M PBS, pH 7.0. Duplicate 100 µl rbST standard (0.001 to 1.0 ng), serum or follicular fluid (diluted 1:20 in assay buffer) were added to 12 x 75 mm glass tubes followed by 50 µl of antiserum. Each tube was vortexed, incubated for 24 h at 4 °C, and then ^{125}I-rbST (50 µl, 0.011 µCi) was added. Tubes were incubated for an additional 48 h at 4°C. One hundred microliters of *S. aureus* protein A (Boehringer Mannhein Biochemical, Indianapolis, IN, Cat. # 100 061) diluted 1:100 in 0.01 M PBS (with 2.5 mM EDTA, pH 7.4) was added to separate bound from free hormone. Increasing volumes (1 to 100 µl) of serum or follicular fluid samples depressed binding parallel to the standard curve (Appendix D). The recovery of 0.01 to 0.3 ng of rbST added to 5 µl of FF was 113 to 137%. Concentration of GH in serum and FF samples were determined in a single assay. Sensitivity of the assay was 30 pg/tube, and intra-assay CV for serum and FF were 14 and 15%, respectively. Cross reactivity of NIADDK-anti-oGH-2 antibody with bovine prolactin (PRL; USDA-bPRL-B1) was < 0.01%.

The concentration of FSH in serum was determined by a previously validated heterologous RIA (Glencross et al., 1992; Sunderland et al., 1994) using ^{125}I-ovine FSH (USDA-oFSH-19-SIAFP-I-2), bovine FSH (USDA-bFSH-I-2) as standard, and rabbit anti-ovine FSH-1 (AFP-C5288113) for antiserum. Samples were analyzed in a single assay. The sensitivity of the assay was 30 pg/ml, and intra-assay CV was 9%. Cross-reactivity with EHC-

bLH-1 is < 1% (Glencross et al., 1992).

The concentration of LH was determined by RIA (Matteri et al., 1987) using bovine LH (USDA-bLH-I-2, AFP-5500) for radioiodination and as standard, and a monoclonal anti-bovine LH antibody (B-518B7, kindly donated by Dr. J. F. Roser, University of California, Davis, CA). Samples were analyzed in a single assay. Sensitivity of the assay was 95 pg/ml, and intra-assay CV was 11%. Cross-reactivity with bovine FSH is < 0.04% (Matteri et al., 1987).

Ligand Blot Analysis

To evaluate whether rGRF or rbST altered amount of IGFBPs, follicular fluid from estrogen-active follicles was analyzed by ligand blot analysis. Estrogen-active follicles were examined because administration of rbST increased number of estrogen-active rather than estrogen-inactive follicles and intrafollicular concentrations of estradiol. Each FF sample from all estrogen-active follicles (n=25) was diluted 1:100 in double distilled water, and a spectrophotometer (Model DU-64, Beckman Instruments, Palo Alto, CA) was used to determine total protein concentrations (mg/ml; A_{280} = 1 for 0.1% yeast enolase solution; Warburg and Christian, 1942) in duplicate samples of FF from each follicle.

Recombinant human IGF-I (5 μg; H-5555, Bachem, Torrance, CA) was iodinated using 1 mCi $Na^{125}I$ (NEN, NEZ-033H) and 50 μg Iodogen (Pierce, Rockford, IL) for 10 minutes (De la Sota et al., 1996). Iodinated IGF-I was purified on a Sephadex G-25 column (PD-10, 5 x 1.6 cm prepacked column, Pharmacia, Piscataway, NJ) previously equilibrated with 25 ml of column buffer (0.01 M Na_2HPO_4, pH 7.2, 3% BSA), and aliquots

were stored at 4 °C until used. Specific activity was 97.7 μCi/μg protein.

Bovine FF samples (25 μg of protein/lane; n=25) were mixed (1:4, vol:vol) with non-reducing sample buffer (0.125 M Tris [pH 6.8], 0.2% glycerol, 10% sodium dodecyl sulfate (SDS), 0.0025% bromophenol blue), heated for 20 min at 80 °C, and subjected to 15% SDS-polyacrylamide gel electrophoresis (200 V, n=5 gels; Laemmli, 1970). Proteins on each gel were transferred to an Immobilon P membrane (0.2-μm pore size, polyvinylidene difluoride [PVDF], Millipore Co, Bedford, MA) for 30 min at 90 V using CAPS electroblotting buffer (10 mM 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS), 10% methanol; pH 11.0) and a Bio-Rad Mini-Protean II Transfer Apparatus (Bio-Rad, Richmond, CA). Membranes were air-dried, washed twice with TBS (10 mM Tris, 0.15 M NaCl, and 0.05% sodium azide, pH 7.4) for 15 min, and incubated with 1% nonfat dry milk in TBS on a rocking platform for 1 h at room temperature (Good et al., 1995). Each membrane was washed three times in TTBS (10 mM Tris, 0.15 M NaCl, 0.05% sodium azide, and 0.1% Tween 20, pH 7.4) for 10 min and then incubated with 25 ml ^{125}I-IGF-I (200,000 cpm/ml) in trace buffer (10 mM Tris, 0.15 M NaCl, 0.05% sodium azide, and 0.1% Tween 20, 1% BSA, pH 7.4) in Seal-A-Meal pouches for 24 h at 4 °C on a rocking platform (Hossenlopp et al., 1986; Davenport et al., 1990). Membranes were washed twice with TTBS and twice with TBS on a rocking platform for 15 min per wash. Molecular weight estimates were based on protein standards (Bio-Rad, Richmond, CA) after silver staining, and intensities of bands after ligand blotting were determined using the Molecular Analyst Software for Bio-Rad GS 250 Molecular Imager after exposure of blots for 82 h to a Bio-Rad GS 250 Imaging Screen-BI (Bio-Rad, Richmond, CA), as explained previously (Good et al., 1995). A representative

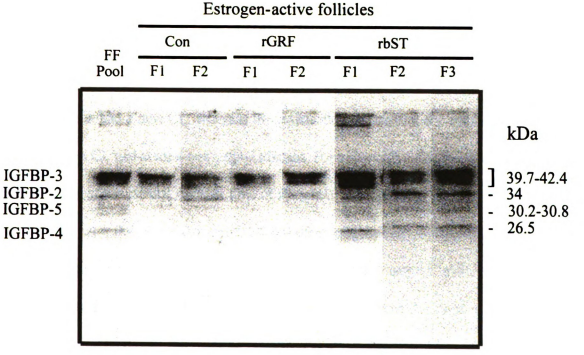
ligand blot of IGFBPs detected in follicular fluid from estrogen active follicles of rGRF-, rbST-treated and control cows is shown in Figure 3.

Statistical Analysis

One cow from each treatment group was removed from analysis because $PGF_2\alpha$ injection was given only once. In addition, one control cow, three cows infused with rbST, and four cows infused with rGRF did not have corpora lutea at slaughter, thus data were not analyzed. Data from the cows with corpora lutea (control, n = 8; rbST, n = 6; rGRF, n = 5) were analyzed using the General Linear Model procedure of SAS (SAS, 1991). The concentration of serum hormones, number and weight of corpus luteum, number of follicles, and number of estrogen-active (EA) follicles were analyzed as a random block design (Gill, 1978). The model included the effects of treatment, block and residual. Concentration of hormones in follicular fluid, diameter of follicles > 5 mm, and diameter of EA follicles were analyzed as a split block design with repeated measurements in space (Gill, 1986). The model included the effects of treatment, block, treatment by block, follicular class (ratio of estradiol to progesterone or size classification), treatment by follicular class, block by follicular class and residual. Significance of treatment and block effects were tested using treatment by block as the error term. Amount of IGFBPs in follicular fluid from estrogen-active follicles was analyzed by ANOVA (Gill, 1978). Mean comparisons were done by the Bonferroni-*t* test (SAS, 1991). Differences in proportion of cows that responded to $PGF_2\alpha$ treatment, cows with more than five follicles, and cows with EA follicles within each follicle class were analyzed by chi-square. Unless stated otherwise, the significance was $P < 0.05$. Values of

Figure 3. A representative ligand blot of IGFBPs detected in follicular fluid from estrogen-active follicles. Primiparous Holstein cows were infused with rGRF or rbST from 117 to 180 days postpartum. Ovaries were collected on Day 5 of an estrous cycle after 63 days of treatment. Follicular fluid (25 μg of protein) from each estrogen-active follicle was subjected to SDS-PAGE, transferred to Immobilon P membranes, incubated with ^{125}I-IGF-I, and intensity of each band determined as described in Material and Methods. Samples include a pool of follicular fluid from ovaries collected at a slaughterhouse (run as a positive control on all gels); and follicular fluid from estrogen-active follicles (E> P in FF) from controls (Con), and rGRF- and rbST-treated cows. Follicles were classified by size: F1 =largest, F2 = second largest, and F3=third largest follicles. Identity of IGFBPs (listed on the left side of figure) was determined by comparison with previous studies (De la Sota et al., 1996; Funston et al., 1996) and based on estimated mass (kDa) of each protein (right side of figure). Coefficient of variation for blot to blot was 20.4%.

Figure 3.


Estrogen-active follicles
Con
rGRF
rbST
FF Pool
F1
F2
F1
F2
F1
F2
F3
kDa
IGFBP-3
IGFBP-2
IGFBP-5
IGFBP-4
39.7-42.4
34
30.2-30.8
26.5

estradiol, progesterone, androstenedione, IGF-I and GH in follicular fluid were log transformed to satisfy assumptions of normally distributed errors before statistical analysis. Actual values are reported.

Results

Occurrence of ovulation after Prostaglandin $F_2\alpha$ treatment. Number of cows with a CL after PGF2α injection tended ($P < 0.10$) to be lower in the treated groups (rGRF + rbST) compared with controls (69% vs 100%). Cows that ovulated in response to synchronization had a new CL, whereas those that did not respond did not have a CL. Each cow without a CL had at least one corpus albicans. For cows with a CL, the average (± SEM) estimated day of the estrous cycle at time of slaughter for controls, rGRF- and rbST-treated groups was 5.4 ± 0.4, 4.3 ± 0.5 and 5.2 ± 0.4 days, respectively. Treatments did not affect ($P > 0.10$) estimated day of the estrous cycle, thus day of the estrous cycle will hereafter be referred to as Day 5 postestrus. Note, estimated day of the estrous cycle in this study was similar to expected day of the cycle based on previous studies that evaluated effects of two injections of $PGF_2\alpha$ spaced 11 days apart on synchronization of estrus in postpartum dairy cows (Rowson et al., 1972; Lauderdale et al., 1974; Niswender and Nett, 1994).

Concentration of Hormones in Serum. On Day 57 of treatment, LH or FSH measured at 20-minute intervals for 6 h did not differ ($P > 0.10$) among treatment groups (Figure 4).

After 63 days of treatment, concentrations of GH and IGF-I in serum were greater ($P < 0.05$) in rGRF- and rbST-treated cows compared with controls (Table 1). In

Figure 4. Effect of recombinant bovine growth hormone-releasing factor (rGRF) or recombinant bovine somatotropin (rbST) on the concentration of FSH and LH in Holstein cows. Primiparous cows were infused with rGRF (○) or rbST (■) from 117 to 180 days postpartum, and serum was collected at 20-minute intervals for 6 h on Day 57 of treatment. Each point represents the mean (± SEM) concentration of number of cows in each group. The number of cows for control (+), rGRF- and rbST-treated groups follows: n=8, 5 and 6 cows, respectively. There were no differences due to treatments ($P > 0.10$). Note: Two cows in the rbST-treated group had average FSH concentrations that were two-fold higher than controls, thus increasing overall variation and mean values.

Figure 4.

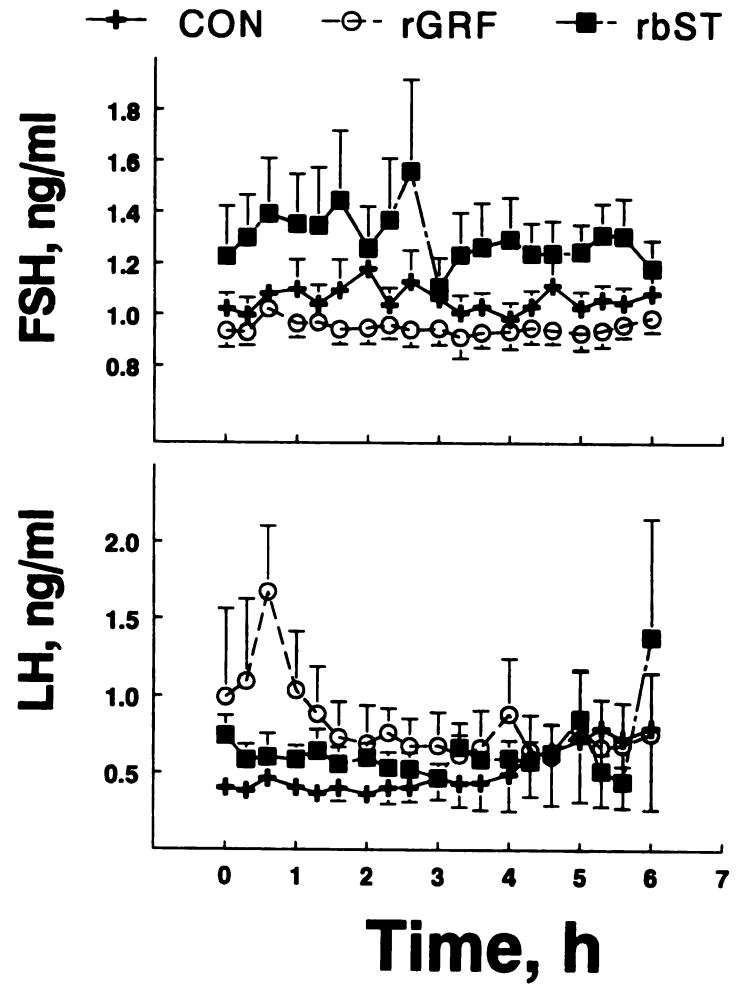

CON
rGRF
rbST
FSH, ng/ml
1.8
1.6
1.4
1.2
1.0
0.8
LH, ng/ml
2.0
1.5
1.0
0.5
0
1
2
3
4
5
6
7
Time, h

TABLE 1 **Effects of recombinant bovine growth hormone-releasing factor (rGRF) or recombinant bovine somatotropin (rbST) on serum hormone concentrations in Holstein cows [1].**

HORMONE [2]	CONTROL	rGRF	rbST
GH (ng/ml)	12.8 ± 2.3^{a} (8)[3]	18.7 ± 2^{b} (5)	26.4 ± 3.4^{b} (6)
IGF-I (ng/ml)	238 ± 8^{a}	520 ± 64^{b}	669 ± 71^{b}
E (pg/ml)	2.0 ± 0.7	1.2 ± 0.1	1.7 ± 0.5
P (ng/ml)	2.2 ± 0.2^{a}	1.4 ± 0.4^{ab}	0.9 ± 0.3^{b}
LH (ng/ml)	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
FSH (ng/ml)	1.1 ± 0.1	1.0 ± 0.1	1.2 ± 0.1

[1] Cows were infused with rGRF or rbST from 117 to 180 days postpartum. Concentration of hormones was determined for serum collected on Day 5 of an estrous cycle after 63 days of treatment.

[2] GH= growth hormone, IGF-I= insulin-like growth factor-I, E= estradiol, P= progesterone, LH= luteinizing hormone, FSH= follicle-stimulating hormone.

[3] Number of cows.

[a] Means with dissimilar superscripts in a row are different ($P < 0.05$).

addition, GH concentrations tended ($P < 0.10$) to be greater in rbST-treated cows compared with rGRF-treated cows. In contrast, concentration of progesterone was lower ($P < 0.05$) in rbST-treated cows compared with controls, even though all cows were on Day 5 of the estrous cycle. Concentrations of LH, FSH and estradiol did not differ ($P > 0.10$) among treatment groups.

Corpora Lutea. Number of CL was greater ($P < 0.05$), but weight per CL was lower in rbST-treated cows compared with controls ($P < 0.05$) or rGRF-treated ($P < 0.10$) cows (Table 2). However, total amount of luteal tissue per cow did not differ ($P > 0.10$) among treatments.

Follicles. Number of follicles per cow and diameter of follicles > 5 mm did not differ ($P > 0.10$) among treatments (Table 3). However, more cows in the rbST group tended ($P < 0.10$) to have five or more follicles > 5 mm in diameter compared with controls or rGRF-treated cows. Size of follicles and volume of follicular fluid from follicles ≤ 5 mm in diameter did not differ ($P > 0.10$) among treatments (Appendix E).

Concentration of GH in follicular fluid in rGRF- and rbST-treated cows was greater ($P < 0.05$) compared with controls (Table 4). In addition, GH was greater ($P < 0.10$) in rbST- compared with rGRF-treated cows. Androstenedione was higher ($P < 0.05$) in control cows compared with rGRF-treated cows. Concentrations of IGF-I, estradiol, and progesterone did not differ ($P > 0.10$) among treatments.

Ratios of concentration of estradiol to progesterone in follicular fluid were used to classify follicles into two different stages of differentiation: estrogen-active (EA; estradiol > progesterone) and estrogen-inactive (EI; progesterone > estradiol) (Figure 1;

TABLE 2 **Effects of recombinant bovine growth hormone-releasing factor (rGRF) or recombinant bovine somatotropin (rbST) on number and weight of corpora lutea (CL) in Holstein cows [1].**

	CONTROL	rGRF	rbST
Number of CL/cow	1.0 ± 0[a] (8)[2]	1.0 ± 0[a] (5)	1.7 ± 0.2[b] (6)
Weight/CL (g)	6.2 ± 0.5[a]	4.8 ± 1.1[a]	2.9 ± 0.4[b]
Total CL weight (g)	6.2 ± 0.5	4.8 ± 1.1	4.9 ± 0.9

[1] Cows were infused with rGRF or rbST from 117 to 180 days postpartum. Measurements were made on Day 5 of an estrous cycle after 63 days of treatment.
[2] Number of cows.
[a] Means with dissimilar superscripts in a row are different ($P < 0.05$).

TABLE 3 **Effects of recombinant bovine growth hormone-releasing factor (rGRF) or recombinant bovine somatotropin (rbST) on number and diameter of follicles > 5 mm in Holstein cows [1].**

	CONTROL	rGRF	rbST
Number of follicles/cow	4.0 ± 0.8 (8)[3]	4.2 ± 1.0 (5)	5.9 ± 0.9 (6)
Follicular diameter (mm)	10.7 ± 0.9	11.0 ± 1.1	10.5 ± 0.5
Cows with 5 or more follicles (%)	37	25	87
Estrogen-active [2]			
Number/cow	0.9 ± 0.2[a]	1.0 ± 0[ab]	2.2 ± 0.4[b]
Diameter (mm)	16.1 ± 1.2[a]	15.2 ± 1.7[ab]	12.5 ± 0.6[b]
Estrogen-inactive			
Number/cow	3.1 ± 0.7	3.2 ± 1.0	3.8 ± 0.9
Diameter (mm)	10.5 ± 1.3	9.7 ± 1.5	9.5 ± 1.2

[1] Cows were infused with rGRF or rbST from 117 to 180 days postpartum. Measurements were made on Day 5 of an estrous cycle after 63 days of treatment.

[2] Estrogen-active follicles have concentration of E > P in FF; estrogen-inactive follicles have concentration of P > E in FF (Ireland and Roche, 1983a; Sunderland et al., 1994).

[3] Number of cows.

[a] Values with dissimilar superscripts in a row are different ($P < 0.05$)

TABLE 4 **Effects of recombinant bovine growth hormone-releasing factor (rGRF) or recombinant bovine somatotropin (rbST) on follicular fluid hormone concentrations (ng/ml) in Holstein cows [1].**

HORMONE	CONTROL	rGRF	rbST
GH [2]	6.1 ± 0.9^{a} (30)[3]	14.9 ± 1.5^{b} (21)	22.6 ± 1.4^{b} (35)
IGF-I	137 ± 13	125 ± 9	173 ± 15
E	49 ± 12	38 ± 18	67 ± 21
P	53 ± 12	57 ± 28	85 ± 35
A	64 ± 19^{a}	19 ± 4^{b}	30 ± 5^{ab}

[1] Cows were infused with rGRF or rbST from 117 to 180 days postpartum. Concentration of hormones was determined in FF from follicles collected on Day 5 of an estrous cycle after 63 days of treatment.
[2] GH= growth hormone, IGF-I= insulin-like growth factor-I, E= estradiol, P= progesterone, A= androstenedione.
[3] Number of follicles.
[a] Means with dissimilar superscripts in a row are different ($P < 0.05$).

Ireland and Roche, 1983a; 1983b; Sunderland et al., 1994). In addition, follicles were also classified based on diameter as follows: largest (F1), second largest (F2), third largest (F3), and all remaining (F4) follicles (Savio et al., 1988; Badinga et al., 1992)

EA and EI Follicles. Number of EA follicles per cow was greater ($P < 0.05$), whereas size of EA follicles was reduced ($P < 0.05$) in rbST-treated cows compared with controls (Table 3). Number and size of EI follicles did not differ ($P > 0.10$) among treatment groups.

Treatment with rGRF or rbST increased ($P < 0.05$) GH in EA and EI follicles compared with controls, but insulin-like growth factor-I did not differ ($P > 0.10$) for EA and EI follicles (Table 5). Administration of rbST increased ($P < 0.05$) the amount of IGFBP-2, -3 and -4 in follicular fluid from EA follicles compared with controls and(or) rGRF-treated cows (Figure 5, also refer to Figure 3 for blot). Amounts of IGFBPs in EI follicles were not evaluated, as explained in Methods. Androstenedione was lower ($P < 0.05$) in EA and EI follicles in rGRF- and(or) rbST-treated cows compared with controls (Table 5).

Follicle Size Classes. Size of F1 follicles in rbST-treated cows was smaller ($P < 0.05$) compared with controls or rGRF-treated cows, whereas sizes of F2, F3 or F4 follicles were similar ($P > 0.10$) for all treatments (Figure 6, *top panel*). As expected, F1 follicles were larger ($P < 0.05$) than F2, F3 and F4 follicles in controls or rGRF-treated cows. However, in rbST-treated cows, size of the largest follicle (F1) was similar ($P > 0.10$) to F2 follicles, but larger ($P < 0.05$) than F3 and F4 follicles.

A greater percent of F2 and F3 follicles tended ($P < 0.10$) to be EA in rbST-treated cows compared to controls or rGRF-treated cows (Figure 6, *bottom panel*).

Table 5 **Effects of recombinant bovine growth hormone-releasing factor (rGRF) or recombinant bovine somatotropin (rbST) on follicular fluid hormone concentrations (ng/ml) from follicles classified as estrogen-active or estrogen-inactive in Holstein cows [1].**

	CONTROL	rGRF	rbST
Estrogen-active (EA) follicles [2]			
GH [3]	5.2 ± 1.2[a]	14.5 ± 3.3[b]	22.6 ± 2.3[b]
IGF-I	127 ± 23	142 ± 34	170 ± 24
E	209 ± 55	146 ± 56	177 ± 41
P	24 ± 6	20 ± 4	25 ± 4
A	122 ± 68[a]	21 ± 13[b]	43 ± 11[ab]
Estrogen-inactive (EI) follicles			
GH	6.3 ± 1.1[a]	15 ± 1.7[b]	22.5 ± 1.7[b]
IGF-I	140 ± 16	121 ± 7	176 ± 22
E	1.8 ± 0.9	3.9 ± 1.3	1.4 ± 0.5
P	62 ± 15	68 ± 36	120 ± 54
A	45 ± 12[a]	18 ± 4[b]	22 ± 4[b]

[1] Cows were infused with rGRF or rbST from 117 to 180 days postpartum. Measurements were made in FF from follicles collected on Day 5 of an estrous cycle after 63 days of treatment.

[2] EA follicles have concentration of E > P in FF; whereas EI follicles have concentration of P > E (Ireland and Roche, 1983a; Sunderland et al., 1994).

[3] GH= growth hormone, IGF-I= insulin-like growth factor-I, E= estradiol, P= progesterone, A= androstenedione.

[a] Means with dissimilar superscripts in a row are different ($P < 0.05$).

Figure 5. Effect of recombinant bovine growth hormone-releasing factor (rGRF) or recombinant bovine somatotropin (rbST) on insulin-like growth factor binding proteins (IGFBP) in follicular fluid from estrogen-active follicles in Holstein cows. Cows were infused with rGRF or rbST from 117 to 180 days postpartum. Ovaries were collected on Day 5 of an estrous cycle after 63 days of treatment. Follicular fluid (25 μg of protein) from each estrogen-active follicle was subjected to SDS-PAGE, transferred to Immobilon P membranes, incubated with ^{125}I-IGF-I, and intensity of each band determined as described in Methods. Bars depict mean (± SEM) intensity (arbitrary units) for IGFBP-2 (34 kDa), IGFBP-3 (39.7-42.4 kDa), IGFBP-4 (26.5 kDa), and IGFBP-5 (30.2-30.8 kDa). Number of estrogen-active follicles for control (CON), rGRF- and rbST treated cows were 7, 5 and 13. Bars with dissimilar letters among treatments are statistically different ($P < 0.05$).

Figure 5.

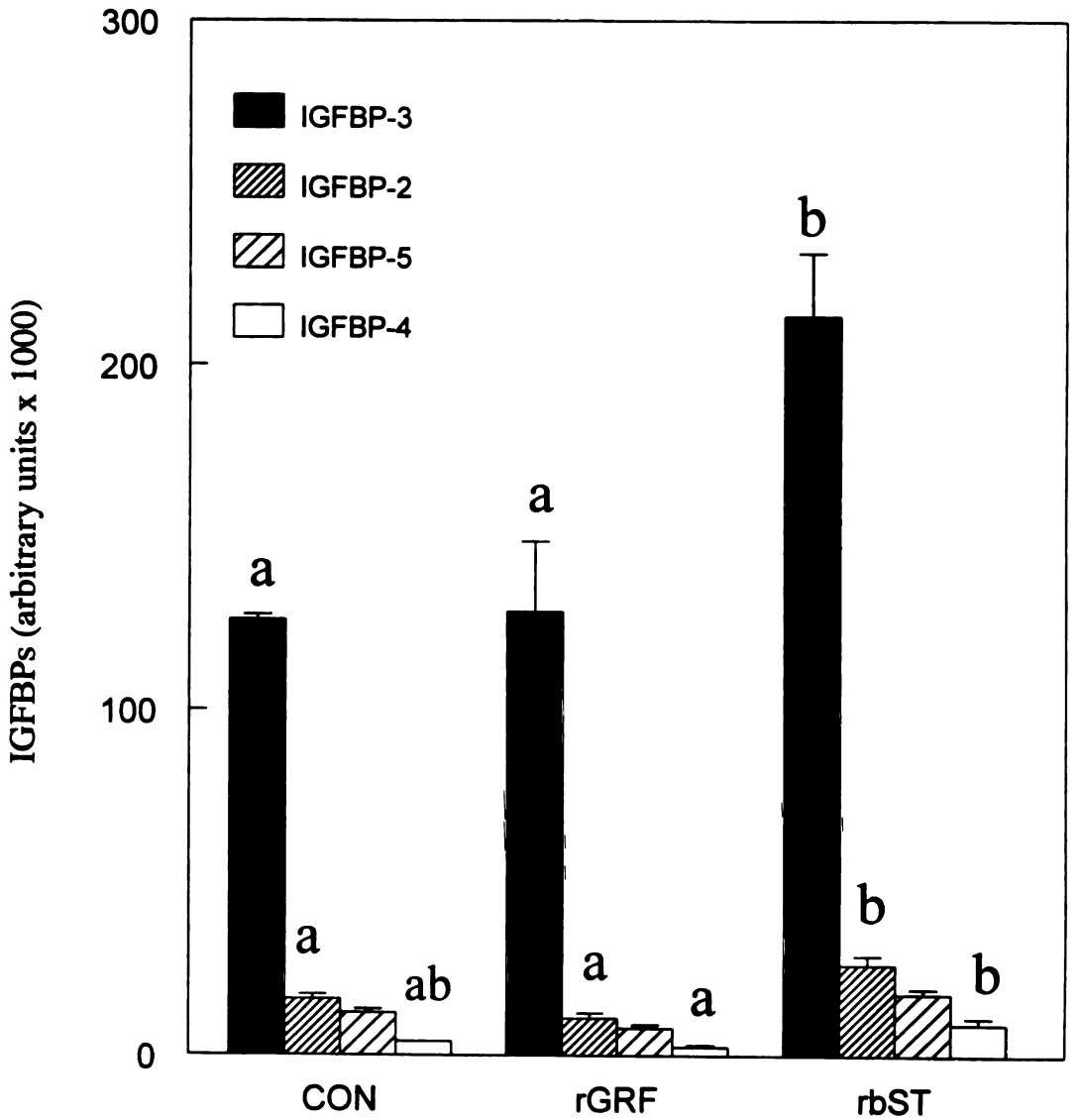
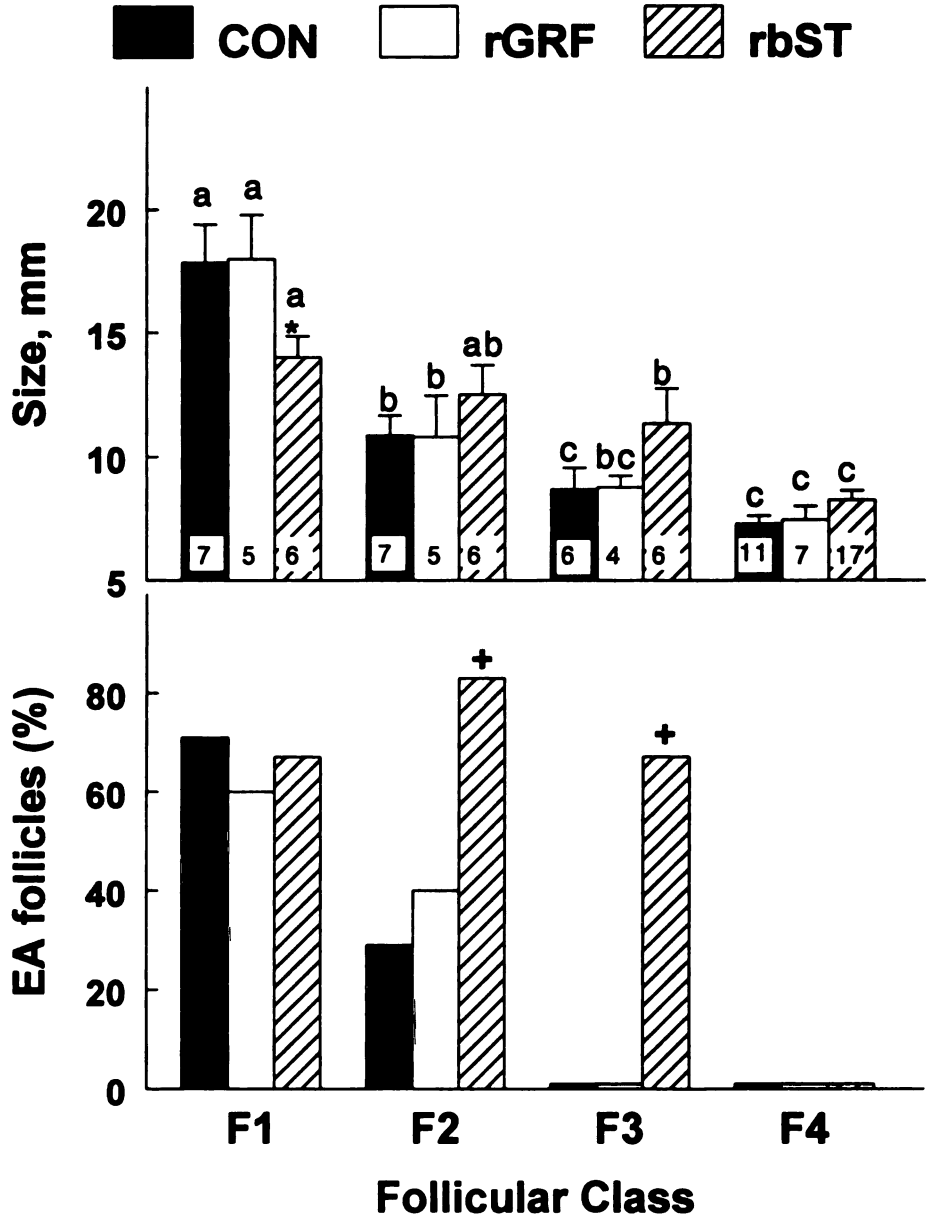

300
200
100
0
IGFBPs (arbitrary units x 1000)
IGFBP-3
IGFBP-2
IGFBP-5
IGFBP-4
a
a
ab
a
a
a
b
b
b
CON
rGRF
rbST

Figure 6. Effect of recombinant bovine growth hormone-releasing factor (rGRF) or recombinant bovine somatotropin (rbST) on follicular size and percent of estrogen active (EA) follicles within each size class of follicles in Holstein cows. Cows were infused with rGRF or rbST from 117 to 180 days postpartum. Ovaries were collected on Day 5 of an estrous cycle after 63 days of treatment. *Top panel*: Diameter of follicles > 5 mm was measured using calipers. Follicles were classified by size: F1=largest follicle, F2=second largest, F3=third largest, and F4= all remaining follicles. Bars depict mean (± SEM) diameter. Number of follicles is indicated by number inside bars. Asterisk indicates that size of F1 follicle was smaller ($P < 0.05$) in rbST-treated cows compared with controls (CON) or rGRF-treated cows. Bars with dissimilar letters among follicular classes, but within the same treatment, are different ($P < 0.05$). *Bottom panel*: Proportion of EA (concentration of E > P in follicular fluid) follicles within each follicle class was calculated. The plus sign above each bar indicates differences (χ^2; $P < 0.10$) in proportion of EA follicles within each follicle size class.

Figure 6.


CON
rGRF
rbST
Size, mm
20
15
10
5
a
a
a
*
b
b
ab
c
bc
b
c
c
c
7
5
6
7
5
6
6
4
6
11
7
17
EA follicles (%)
80
60
40
20
0
+
+
F1
F2
F3
F4
Follicular Class

Concentration of GH was higher ($P < 0.05$) in all follicle size classes in rbST-treated cows compared with controls, whereas IGF-I concentration did not differ for all follicle size classes (Table 6). In addition, GH was higher ($P < 0.05$) in F4 follicles in rGRF-treated cows compared with controls.

Treatment with rbST increased estradiol ($P < 0.05$) in F2 and F3 follicles compared with controls and rGRF-treated cows (Table 6). Also, estradiol was higher ($P < 0.05$) in F4 follicles in rGRF-treated cows compared with controls. Concentration of progesterone was lower ($P < 0.05$) in F4 follicles in rGRF-treated cows compared with control cows. Concentration of androstenedione was lower ($P < 0.05$) in F1 and F4 follicles in rGRF-treated cows, and in F1 follicles in rbST-treated cows compared with controls.

Discussion

The dominant follicle process is characterized by two or three "waves" of development of ovarian follicles, ovulation of the dominant follicle from one of the waves, and formation of a corpus luteum during the bovine estrous cycle (Figure 1). The dominant follicle process is regulated by a complex feedback between follicles, the CL and pituitary. The novel findings in the present study show that: 1) long-term infusion (63 days) of rbST disrupts the dominant follicle process, and 2) rGRF treatment did not alter the dominant follicle process, despite an increase in serum and intrafollicular growth hormone, and increased steroids levels in F4 follicles. In support of the disruptive effects of rbST on the dominant follicle process, others report that rbST increases number of small (2-5 mm; Gong et al., 1991; 1993a), medium (6-9 mm; De la Sota et al., 1993; Kirby et al., 1997a), and large

Table 6 **Effects of recombinant bovine growth hormone-releasing factor (rGRF) or recombinant bovine somatotropin (rbST) on follicular fluid hormones (ng/ml) from follicles classified according to size in Holstein cows [1].**

FOLLICLE SIZE CLASS [2]	HORMONE	CONTROL	rGRF	rbST
F1, Largest follicle	GH [3]	5.5 ± 1.2[a]	11.7 ± 1.8[ab]	22.9 ± 1.9[b]
	IGF-I	162 ± 34	159 ± 34	137 ± 4
	E	177 ± 66	125 ± 64	103 ± 64
	P	26 ± 6	131 ± 108	135 ± 82
	A	123 ± 68[a]	21 ± 12[b]	31 ± 21[b]
F2, Second Largest	GH	6.7 ± 2.2[a]	15.1 ± 3.6[ab]	24.8 ± 2.6[b]
	IGF-I	124 ± 12	115 ± 9	174 ± 18
	E	35 ± 22[a]	23 ± 1[a]	169 ± 76[b]
	P	29 ± 11	15 ± 0.6	26 ± 3
	A	49 ± 16	17 ± 8	44 ± 15
F3, Third largest	GH	7.3 ± 3.1[a]	12.1 ± 3.1[ab]	22.2 ± 4.5[b]
	IGF-I	118 ± 34	131 ± 15	189 ± 51
	E	3 ± 3[a]	0.8 ± 0.8[a]	112 ± 47[b]
	P	27 ± 2	84 ± 74	19 ± 5
	A	70 ± 37	23 ± 8	36 ± 9
F4, All remaining	GH	5.3 ± 1.1[a]	18.7 ± 2.7[b]	21.8 ± 2.1[b]
	IGF-I	139 ± 23	105 ± 4	180 ± 28
	E	0.6 ± 0.5[a]	7 ± 3[b]	1 ± 0.4[ab]
	P	100 ± 28[a]	18 ± 4[b]	111 ± 65[ab]
	A	25 ± 5[a]	15 ± 8[b]	22 ± 4[ab]

[1] Cows were infused with rGRF or rbST from 117 to 180 days postpartum. Measurements were made in FF from follicles collected on Day 5 of an estrous cycle after 63 days of treatment.

[2] Follicles were classified according to size (Badinga et al., 1992).

[3] GH= growth hormone, IGF-I= insulin-like growth hormone-I, E= estradiol, P= progesterone, A= androstenedione.

[a] Means with dissimilar superscripts in a row are different ($P < 0.05$).

(10-15 mm; De la Sota et al., 1993) follicles, decreases size of F1 follicles (De la Sota et al., 1993; Lucy et al., 1994a), increases size of F2 follicles (De la Sota et al., 1993; Lucy et al., 1993b; 1994a), increases (De la Sota et al., 1993) or decreases (Lucy et al., 1994a) estradiol concentration in serum during growth of preovulatory follicles, hastens emergence of the second-wave dominant follicle growth (Lucy et al., 1994b; Kirby et al., 1997a), and increases twinning (Cole et al., 1991; Wilkinson and Tarrant, 1991; Esteban et al., 1994c). Moreover, rbST increases (Lucy et al., 1994b) or decreases (Kirby et al., 1997a) CL size, and increases (Schemm et al., 1990; Gallo and Block, 1991; Lucy et al., 1994b) or decreases (Waterman et al., 1993; Dalton and Marcinkowski, 1994; Kirby et al., 1997a) progesterone concentration in serum. Of practical significance, disruption of the dominant follicle process may explain the decrease in reproductive efficiency after rbST treatment in dairy cattle (Burton et al., 1990; Cole et al., 1991; McGuffey et al., 1991; Morbeck et al., 1991; Wilkinson and Tarrant, 1991; Lefebvre and Block, 1992; Waterman et al., 1993; Esteban et al., 1994a; 1994b; 1994c; Kirby et al., 1997b).

In stark contrast to previous studies and results of the present experiment, some reports indicate that rbST treatment does not alter follicular or luteal development and function in dairy cows (Schemm et al., 1990; Gong et al., 1991; De la Sota et al., 1993; Gong et al., 1993a; Lucy et al., 1993b; 1994a; Stanisiewski et al., 1994; Yung et al., 1996). Although the reason for conflicting results among studies is unknown, confounding factors such as parity, stage of lactation, energy balance, intraovarian concentration of GH achieved during treatments, stage of the estrous cycle, and(or) length of treatments could explain the discrepancies. Several examples follow: 1) Negative energy balance (Lucy et al., 1992a;

1992b; Yung et al., 1996) and lactation (De la Sota et al., 1993) inhibit follicle and CL development and steroidogenesis. In addition, rbST treatment prolongs negative energy balance in lactating cows (Peel and Bauman, 1987; Lucy et al., 1992b), but does not alter energy balance in heifers or non-lactating cows (Eisemann et al., 1986; Lucy et al., 1992b). Consequently, the effect of rbST on ovarian function in lactating cows may be modified by energy balance status. Since the non-pregnant cows in the present study were in positive energy balance when ovaries were removed (Binelli et al., 1995; VanderKooi et al., 1995), rbST treatment may have altered follicular and CL growth and function without the confounding effect of negative energy balance. 2) A threshold level of GH may be required to disrupt the dominant follicle process. For example, serum and intrafollicular GH concentrations were lower in rGRF- compared with rbST-treated cows in the present study, perhaps explaining why the dominant follicle process was not disrupted in rGRF-treated cows. Although several reports indicate that administration of rGRF or rbST increases GH levels in serum (Dahl et al., 1993; Binelli et al., 1995; Gong et al., 1997), intrafollicular concentrations of GH were not reported. 3) The absence of (Stanisiewski et al., 1994), or an increase (Cole et al., 1991; Wilkinson and Tarrant, 1991; Esteban et al., 1994c) in twinning in rbST-treated cows may depend on timing of $PGF_2\alpha$ injection relative to stage of a follicular wave and number of estrogen-active follicles, which are not reported in previous studies. For example, the use of $PGF_2\alpha$ to induce CL regression during Day 7 of the estrous cycle causes ovulation of the first-wave dominant follicle (Kastelic et al., 1990b; Savio et al., 1990). In the present study, a high proportion of F1, F2 and F3 follicles were estrogen-active in rbST-treated cows on Day 5 of the estrous cycle, which if induced to ovulate, may form two CL.

In support of this speculation, the number of estrogen-active follicles is higher in cattle selected for twinning (Echternkamp et al., 1996), and most of the rbST-treated cows in the present study that had two CL also had two or more estrogen-active follicles. Taken together, this implies that number of estrogen-active follicles on Day 5 of the estrous cycle is a reliable marker for ovulation rate. 4) Duration of rbST treatment may differentially affect CL function as measured by progesterone concentration. For example, administration of rbST increases progesterone in serum during the first and second estrous cycles compared with controls (Schemm et al., 1990; Gallo and Block, 1991; Lucy et al., 1994a). However, during the third and subsequent estrous cycles, concentration of progesterone is similar, or lower in rbST-treated cows compared with control cows (Schemm et al., 1990; Gallo and Block, 1991; Kirby et al., 1997a) as shown in the present study. Of potential practical importance, if rbST-treated cows do not become pregnant during the first two estrous cycles after parturition, the possibility exists that cows will become anestrus (Waterman et al., 1993; Esteban et al., 1994c) because low concentrations of progesterone reduce expression of estrus and(or) ovulation (Lefebvre and Block, 1992; Kirby et al., 1997b). Also, reduced expression of estrus and ovulation could explain increased calving intervals after rbST treatment (McGuffey et al., 1991; Esteban et al., 1994b). In further support of a negative effect of long-term high serum levels of GH, number of cows that did not have a CL following a $PGF_2\alpha$ injection were greater in cows treated with rGRF and rbST compared with controls in the present study.

While evidence from the present study clearly shows that administration of rbST alters the dominant follicle process, the mechanism is unclear. A decline in serum FSH concentrations triggers selection of the dominant follicle in cows (Figure 1; Adams et al.,

1993; Mihm et al., 1997). Thus, if growth hormone reaches intrafollicular threshold levels after rbST treatment, it may act as a co-gonadotropin and synergize with FSH to increase number of estrogen active follicles. The presence of mRNA for GH receptors on granulosa cells from preovulatory follicles (Cameron et al., 1990) and CL (Lucy et al., 1993a) in dairy cows suggests that rbST can directly alter follicular and CL growth and function. For example, administration of rbST increased intrafollicular concentration of growth hormone in the present study. Thus, rbST may have directly increased estradiol concentration in F2 and F3 follicles during the first follicular wave. In support of this possibility, rbST treatment increases estradiol concentration in serum at a greater rate during the follicular phase compared with control cows (De la Sota et al., 1993), and rbST increases granulosa cell estradiol production *in vitro* in a dose-dependent fashion (Gong et al., 1994; Sirotkin, 1996). Direct positive effects of rbST on granulosa cell estradiol production may increase number of estrogen-active follicles, and therefore, explain the diminishment of the putative dominance effect of first-wave dominant follicle after rbST injections (Lucy et al., 1994b; Kirby et al., 1997a). Although androstenedione concentrations were lower in some follicles from rGRF- or rbST-treated cows compared with controls in the present study, estradiol concentrations were not reduced. This finding suggests that androgens were rapidly aromatized to estradiol, rather than implying a negative effect of rGRF or rbST on androgen production. In support of a positive effect, rbST treatment increases thecal androgen production *in vitro* (Apa et al., 1995; Spicer and Steward, 1996a).

Dominant follicles were smaller in rbST-treated cows compared with controls in the present study. While the mechanism explaining this result is unknown, others report that

rbST treatment decreases bovine granulosa cell proliferation *in vitro* (Gong et al., 1993c; Spicer and Steward, 1996a). In addition, rbST decreases LH pulse frequency (Schemm et al., 1990), which is associated with smaller dominant follicles (Savio et al., 1993). Also, administration of rbST increased CL number, but reduced their size and function in the present study. As explained earlier, it is likely that this effect of rbST is a consequence of its positive action on stimulating growth of multiple estrogen-active follicles, coupled with timing of PGF2α injection to induce ovulation of multiple EA follicles. Moreover, long-term treatment with rbST may have decreased CL function in cows in the present study, perhaps by reducing number of LH receptors (Yuan et al., 1996; Pinto Andrade et al., 1996) and(or) down-regulating GH receptor mRNA (Kirby et al., 1996) in luteal cells.

Administration of rbST can alter follicular and luteal function indirectly by modifying the intrafollicular IGF-IGFBP system. For example, although IGF-I concentrations in follicular fluid were similar in the present study, intrafollicular levels of IGFBP-2, -3 and -4 were higher in rbST-treated cows compared with controls and(or) rGRF-treated cows, as previously reported (Echternkamp et al., 1994b; Stanko et al., 1994a). This finding suggests that rbST treatment alters net intrafollicular IGF-I biological activity. Although increased amounts of IGFBP-3 in follicular fluid may be due to higher circulating IGFBPs in rbST-treated cows (Stanko et al., 1994a; VanderKooi et al., 1995), theca cells expresses mRNA encoding for IGFBP-2 and -4 (Armstrong et al., 1996a), perhaps implying that rbST increases ovarian production of low molecular weight IGFBPs. Moreover, IGFBPs can either inhibit (Ui et al., 1989; Bicsak et al., 1990; Hammond et al., 1991; Giudice, 1992; Monget and Monniaux, 1995) or potentiate (Blum et al., 1989; Jones et al., 1993; Stewart et al., 1993;

Jones and Clemmons, 1995) IGF action at the target cell. Consequently, the physiological role of higher concentrations of IGFBP-2, -3 and -4 in follicular fluid from rbST-treated cows in the present study is uncertain. Nevertheless, co-infusion of IGF-I antibodies and IGF-I potentiates IGF-I action *in vivo* (Stewart et al., 1993), which could explain why rbST-treated cows, which had higher intrafollicular IGFBPs compared with controls, also had higher estradiol levels.

The reason why the dominant follicle process was not altered in the dairy cows in this study following long-term infusion of rGRF, despite increased serum and intrafollicular levels of GH, and increased estradiol, but decreased progesterone in F4 follicles, is unknown. GRF effects on ovarian function may be mediated by growth hormone and(or) IGF-I and its binding proteins. Thus, the most likely explanation for why administration of rGRF in the present study did not alter follicular hierarchy is because intrafollicular threshold levels of GH were not achieved. Alternatively, exogenous rGRF stimulates release of four GH variants, whereas the rbST injected into cows mimics only one of the GH variants. Thus, differences in biological potencies of GH variants could also explain why rGRF- and rbST-treated cows responded different in the present study. Finally. the possibility exists that rGRF may inhibit rbST action on the ovary. In contrast to our findings, treatment with GRF analogs increases size of large follicles and progesterone concentration in medium-sized follicles in mature heifers (Spicer and Enright, 1991). However, GRF did not alter follicular hierarchy (Spicer and Enright, 1991), a finding that supports results of the present study. In rats, GRF increases estradiol and progesterone production by rat granulosa cells (Moretti et al., 1990; Hughes et al., 1996). Taken together, GRF effects may be species specific and(or) depend on stage of

follicular differentiation.

In summary, daily infusions with rGRF or rbST for 63 days beginning 117 days postpartum to primiparous Holstein cows results in the following: 1) both rGRF and rbST increased GH and IGF-I, but did not alter LH, FSH or estradiol concentrations in serum; 2) treatment with rbST increased number of CL, but decreased CL weight and serum concentration of progesterone compared with controls; 3) rbST increased: number of cows with more than five follicles (> 5 mm), number of EA follicles, intrafollicular concentrations of GH in follicles > 5 mm, amount of IGFBP-2, -3 and -4 in EA follicles, estradiol in F2 and F3 follicles, and percent of F2 and F3 follicles that were EA compared with controls and(or) rGRF-treated cows; 4) rbST decreased size of EA and F1 follicles and androstenedione in F1 and EI follicles; and 5) treatment with rGRF did not alter follicle size or CL weight and function compared with controls or rbST-treated cows, despite increased GH in all follicles, increased intrafollicular concentration of estradiol in F4 follicles, and decreased intrafollicular concentrations of progesterone in F4 follicles and androstenedione in EA, EI, F1 and F4 follicles compared with controls. These findings lead to the conclusion that long-term treatment with rbST disrupts the dominant follicle process in cattle. However, administration of rGRF did not disrupt the dominant follicle process probably because intrafollicular GH concentration were lower in the rGRF-treated compared with rbST-treated cows.

CHAPTER 3

B. Effect of Recombinant Bovine Somatotropin on the Estradiol-producing Capacity of Granulosa Cells Collected During Development of the First-wave Dominant Follicle of the Bovine Estrous Cycle.

Introduction

Several *in vivo* and *in vitro* studies (De la Sota et al., 1993; Gong et al., 1993a; Gong et al., 1993b; Gong et al., 1994; Sirotkin, 1996; Lucy et al., 1993b; Kirby et al., 1997b) show that recombinant bovine somatotropin (rbST) treatments increase either basal or gonadotropin-induced estradiol production. In Experiment I for this thesis, administration of rbST increased intrafollicular concentrations of estradiol from the second and third largest follicles compared with control and(or) recombinant bovine growth hormone-releasing factor (rGRF)-treated cows. Moreover, intrafollicular concentrations of growth hormone (GH) were higher in rbST-treated cows suggesting that GH may have a direct effect on estradiol production. In contrast, others report that rbST decreases (Lucy et al., 1994b; Spicer and Steward, 1996a) or does not alter (Schemm et al., 1990; Gong et al., 1991; Pinto Andrade et al., 1996; Kirby et al., 1997a) estradiol concentration in serum or in culture media. The reason for the differences in effects of rbST on granulosa cell estradiol production among

laboratories is unknown. However, previous studies have evaluated the regulatory roles of growth hormone on granulosa and theca cells isolated from follicles of different sizes collected at random stages of the estrous cycle (Gong et al., 1994; Spicer and Steward, 1996a). Consequently, the controversial effects of rbST on estradiol producing capacity of bovine granulosa cells may be because cells are isolated from follicles from different follicular waves or from follicles at different stages of differentiation within a wave (Figure 1; Ireland and Roche 1987; Ginther et al., 1996; Roche, 1996). Moreover, culture conditions, including presence of serum, growth factors, attachment factors, and time of culture, may also alter the response of granulosa cells to hormonal treatments. Because of the aforementioned concerns, the objectives of this study were to: 1) develop a serum-free cell culture system that mimics the differential *in vivo* capacity of bovine granulosa cells from follicles at different stages of development within a follicular wave to produce steroids, and 2) determine whether rbST alters the estradiol-producing capacity of bovine granulosa cells.

Materials and Methods

Reagents and Hormones

Ham's F-12 medium, sodium bicarbonate, penicillin, streptomycin and trypan blue were obtained from Gibco (Grand Island, NY) and 19-OH androstenedione from Sigma (St. Louis, MO). Ovine FSH (USDA-oFSH-18, 65x USDA-oFSH-S1) was obtained from the National Hormone and Pituitary Program (Baltimore, MD), and rbST (Somavubove) and recombinant bovine growth hormone-releasing factor (Leu^{27}, $Homoserine^{45}$-$bGRF^{1-45}$ lactone) were kindly provided by Pharmacia and Upjohn Inc. (Kalamazoo, MI). Culture media was

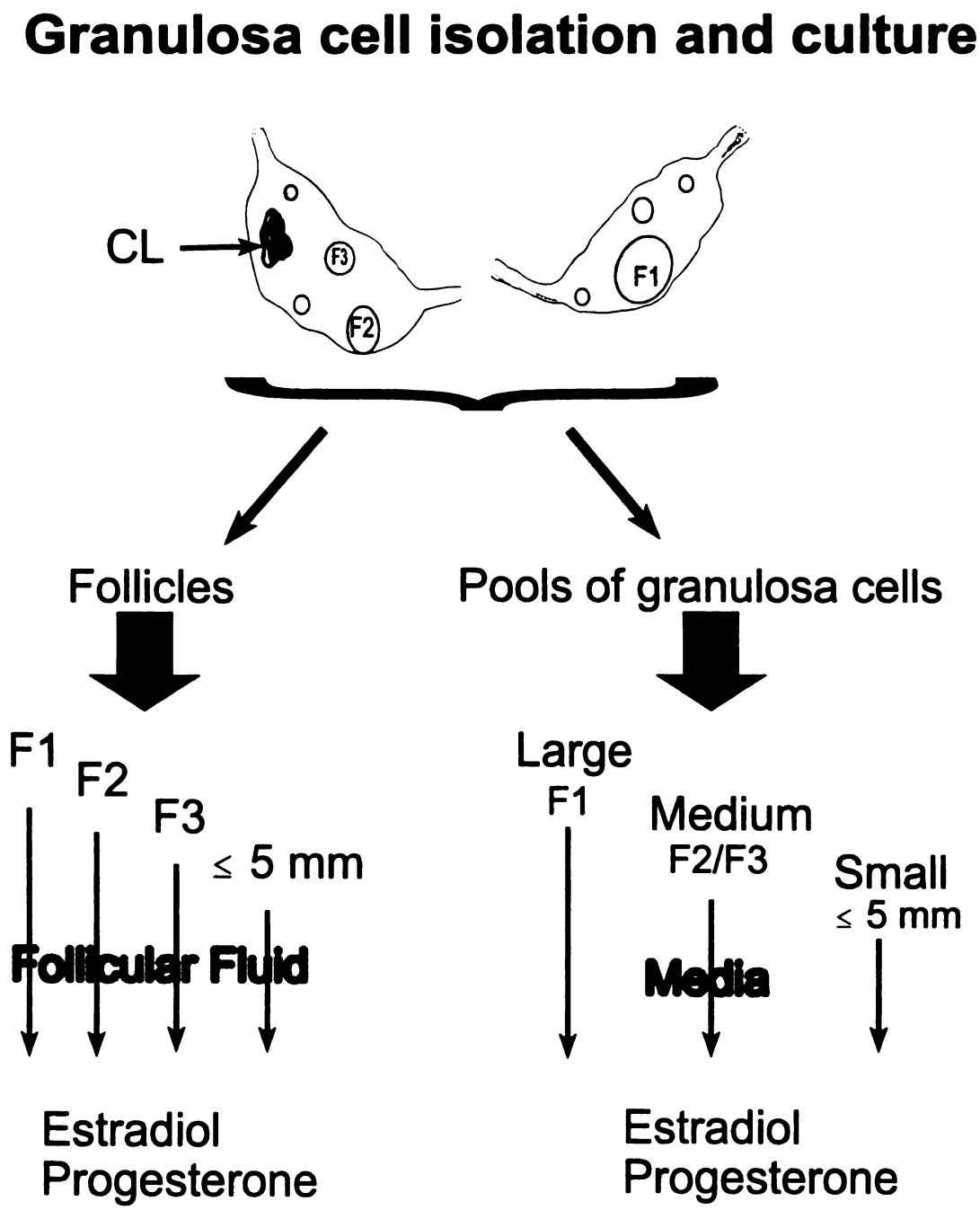
supplemented with sodium bicarbonate (0.01 M), penicillin/streptomycin solution (1.5 mg/ml penicillin and 2.5 mg/ml streptomycin) and 19-OH androstenedione (1 μM).

Validation of the Bovine Granulosal Cell Culture System

Ovaries from beef and dairy cows were collected from MURCO Inc., a slaughterhouse in Plainwell, Michigan. Stage of the estrous cycle was estimated based on the external and internal appearance of the corpus luteum (CL; Ireland et al., 1980). Ovaries from each cow were collected during Days 2 to 5 or 6 to 10 of the estrous cycle, when the first-wave dominant follicle develops (Figure 1), and placed separately into a bottle containing ice-cold phosphate buffered saline solution (0.01 M monobasic phosphate, 0.04 M dibasic phosphate, 0.1 M NaCl, 0.02 M EDTA; pH 7.4). Ovaries were then transported to the laboratory within 4 h of slaughter. In the laboratory, one to five small follicles (≤ 5 mm in diameter), one to four medium follicles (> 5 mm in diameter; F2, F3) and the largest follicle (F1) per cow were dissected (Figure 7). Ovarian stroma was removed from each follicle and individual follicle diameter was recorded. Each follicle was punctured with a 22-gauge needle attached to a 3-ml syringe then processed as follows: FF from small follicles (≤ 5 mm) was pooled, whereas FF from medium and the largest follicle were kept separate (Figure 7). Follicular fluid samples were stored at -20 °C for subsequent analyses of steroids. The follicle shell was bisected under sterile conditions and granulosa cells from the inner wall of each follicle were scraped gently into media supplemented with 19-OH androstenedione, thus leaving the basement membrane and theca cell layers intact. Preliminary experiments showed that scraping granulosa cells into media containing androgens increased their subsequent

Figure 7. Diagram of procedures for follicular fluid aspiration and granulosa cell isolation from different size follicles.

Figure 7.


Granulosa cell isolation and culture
CL
F3
F2
F1
Follicles
Pools of granulosa cells
F1
F2
F3
≤ 5 mm
Follicular Fluid
Large
F1
Medium
F2/F3
Small
≤ 5 mm
Media
Estradiol
Progesterone
Estradiol
Progesterone

estradiol-producing capacity (data not shown). Granulosa cells from each follicle size category were pooled, and then washed and centrifuged (400 x g for 5 min at 4 °C) twice with supplemented medium. Number of cells was estimated using a hemocytometer. Cell viability was assessed with trypan blue exclusion dye by mixing one part of a 1:10 dilution of the cell suspension with one part of a 1:5 dilution of 0.4 % of trypan blue, and then incubating the mixture for 2 to 3 min at room temperature. Stained cells were considered dead.

One million cells in 50 to 150 µl of medium were added to Falcon Primaria plates (24-well plates; Becton Dickinson and Co., Lincoln Park, NJ) containing 1 ml of supplemented media previously equilibrated at 37 °C. Granulosa cells were incubated at 37 °C in a humidified atmosphere (5% CO_2 and 95% air) for 48 h.

After 48 h in culture, medium was removed, centrifuged (400 x g for 5 min at RT) to remove cellular debris, transferred to 1-ml Eppendorf tubes and stored at -20°C until assayed. Percent of live cells at the end of the experiment was estimated using trypan blue exclusion dye as described above.

To determine whether *in vitro* estradiol-producing capacity mimicked *in vivo* estradiol production, concentrations of estradiol and progesterone in media (supplemented with 19-OH androstenedione) after 48 h of culture of granulosa cells from small, medium or the largest follicle on Days 2 to 5 or 6 to 10 of the estrous cycle were compared with the corresponding intrafollicular concentrations of estradiol and progesterone from each follicle size category.

Experiments

Granulosa cells were collected from small, medium and the largest follicles during Days 2 to 5 and 6 to 10 and subjected to serum-free cell culture for 48 h in media supplemented with 19-OH androstenedione as described above. Effects of various treatments administered at time of plating on accumulation of estradiol in media was examined as follows: In Experiment 1, cells were treated with different doses of FSH (0, 0.1, 1, 10 or 100 ng/well). The doses were selected based on previous *in vitro* studies (Berndtson et al., 1995), and intrafollicular concentrations of FSH (Fortune and Hansel, 1985; Niswender and Nett, 1994). In Experiment 2, granulosa cells were treated with different doses of rbST (0, 10, 25, 50, 250 ng/well). Doses were selected based on the concentrations of GH in follicular fluid from untreated or rbST-treated cows (Chapter 2). Experiment 3 tested whether rbST (0, 50 ng/well) altered FSH-induced estradiol accumulation. Experiment 4 examined whether recombinant growth hormone-releasing factor (0, 2, 10 ng/well) altered FSH- and rbST-induced estradiol production. The effect of rGRF was tested because administration of rGRF, despite an increase in endogenous GH, did not alter the number of EA follicles and intrafollicular concentration of estradiol, suggesting that rGRF may have an inhibitory role on rbST action. Doses of rGRF were based on previous studies (Spicer et al., 1992).

Hormone Determinations

Concentrations of estradiol and progesterone were determined in unextracted follicular fluid or media by radioimmunoassay using commercially available kits (Diagnostic Product Co., Los Angeles, CA) as previously validated (Turzillo and Fortune, 1990; Ireland

et al., 1994). Progesterone was analyzed in nine assays, whereas estradiol was analyzed in eleven assays. Sensitivity of the progesterone assay was 0.1 ng/ml, and intra- and inter-assay coefficients of variation (CV) were 5 and 9%, respectively. Cross-reactivity of progesterone antiserum with other steroids follows: 11-deoxycortisol =2.4%, 20α-dihydroprogesterone =2.0%, 11-deoxycorticosterone =1.7%, 5β-pregnan-3,20-dione =1.3%, and < 1% with other steroids, such as androstenediol, corticosterone, cortisol, estradiol, 17α-hydroxy-progesterone, pregnenolone, and testosterone (Diagnostic Products Corp. Los Angeles, CA) Sensitivity of the estradiol assay was 0.5 pg/ml, and intra- and inter-assay CV were 6 and 7%, respectively. Cross-reactivity of estradiol antiserum with various steroids follows: estrone = 12.5%, 17β-estradiol-3β-D-glucoronide = 6%, d-equilenin = 4.2%, 1,3,5(10)-estratrien-17α-methyl-3,17β-diol 3-methyl ether = 3.5%, estrone-β-D-glucoronide = 1.6%, 4-estren-17β-ol-3-one = 1.8%, and < 1% with other steroids, such as androstenedione, progesterone, testosterone, 19-hydroxy-androstenedione, estriol, cortisone, and corticosterone (Diagnostic Products Corp. Los Angeles, CA).

Statistical Analysis

Experimental data are expressed as arithmetic means (± SEM) of measurements of triplicate culture wells. Each experiment was replicated two or more times with different pools of granulosa cells collected from 95 cows. Main effects and interactions were examined using the general linear model procedure, and dose effects were tested using a linear regression model of Statistical Analysis System (SAS, 1991). The main effects were day of the cycle, follicle size, hormone treatment (FSH, rbST or rGRF dose) and their

interactions. When steroid accumulation for 48 h was expressed as pg or ng per million live cells, the number of live cells at the beginning of the experiment was used for this calculation. Mean comparisons were done by the Bonferroni-t test (SAS, 1991). Differences in the proportion of live cells were analyzed by Chi-square. Unless stated otherwise, significance was $P < 0.05$.

Results

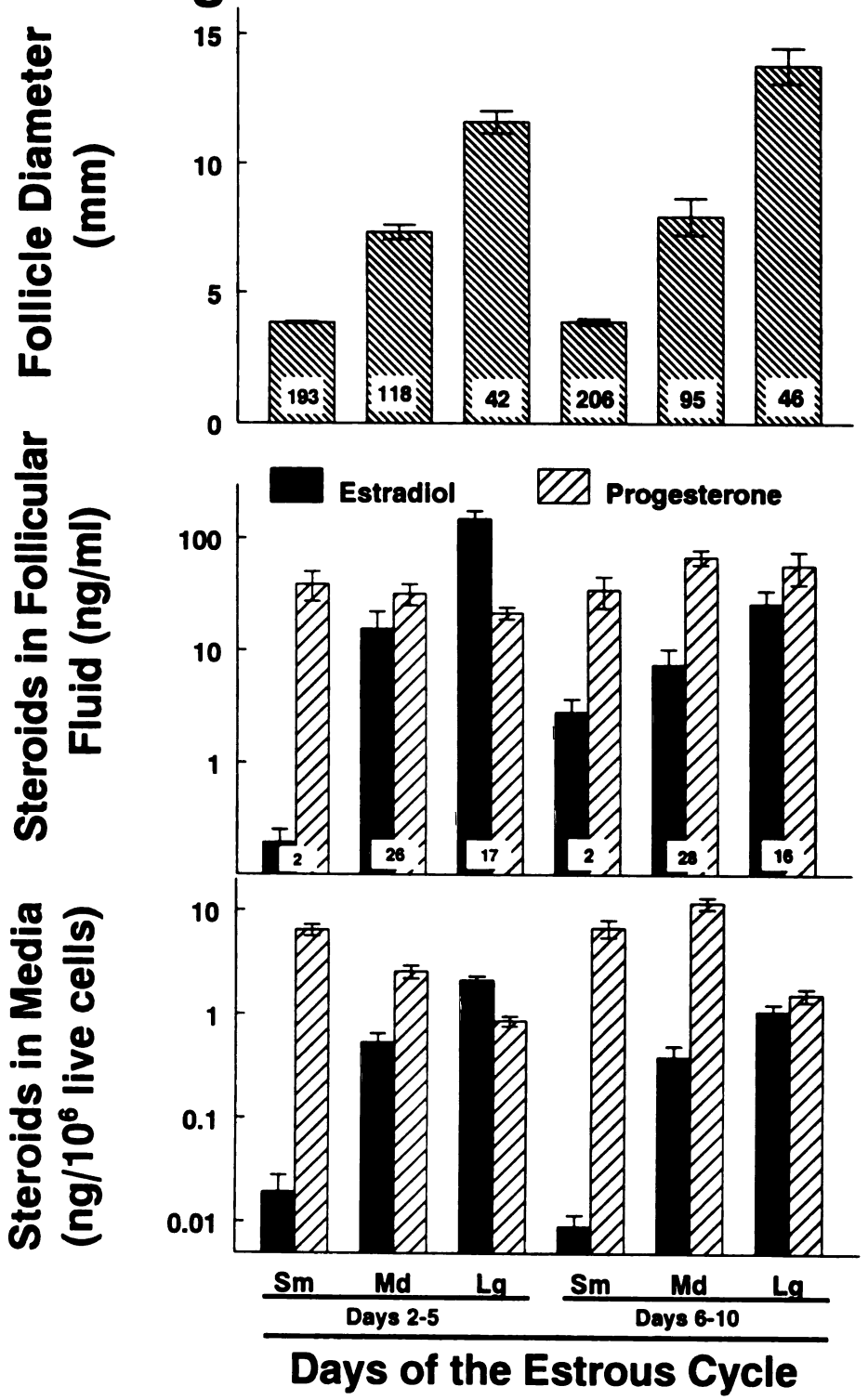
Validation

As expected, diameters of follicles collected during Days 2 to 5 or 6 to 10 of the estrous cycle were different ($P < 0.05$) among follicle sizes (Figure 8, top panel). Size of small or medium follicles from Days 2 to 5 were similar ($P > 0.10$) to size of small or medium follicles from Days 6 to 10 of the estrous cycle. However, the largest follicle from Days 2 to 5 was smaller ($P < 0.05$) compared with the largest follicle during Days 6 to 10 of the estrous cycle.

Concentration of estradiol in follicular fluid from the different follicle sizes increased ($P < 0.05$) with follicle size during both stages of the estrous cycle (Figure 8, middle panel). Concentration of progesterone in FF from follicles collected during Days 2 to 5 decreased as follicle size increased ($P < 0.05$), whereas progesterone increased ($P < 0.05$) in FF from follicles collected from Days 6 to 10 of the estrous cycle. Based on intrafollicular estradiol to progesterone concentration ratios, only the largest follicles collected during Days 2 to 5 of the estrous cycle were estrogen-active (estradiol > progesterone in FF; Figure 8, middle panel).

Figure 8. Association of follicle diameters with concentration of steroids in follicular fluid and capacity of bovine granulosa cells from each different size follicle to produce estradiol and progesterone during cell culture. Bovine ovaries from Days 2 to 5 and 6 to 10 of the estrous cycle were collected from a slaughterhouse. Follicles were isolated from each pair of ovaries and classified according to size: small (Sm, ≤ 5 mm in diameter; n = 1 to 4 follicles per cow), medium (Md, > 5 mm; n=1 to 5 follicles per cow), and the largest (Lg) follicle per cow (*x*-axis). Follicular fluid (FF) collected from small follicles was pooled, whereas FF from individual medium and the largest follicle were kept separate. Granulosa cells were scraped from each follicle size, pooled within follicle size and cultured in serum-free Ham's F-12 medium supplemented with 1 μM of 19-OH androstenedione for 48 h. Concentrations of estradiol and progesterone were measured in FF and media. Each bar depicts mean (± SEM) values for five to eight separate experiments. The number in the bars indicates total number of dissected follicles (top panel), number of FF pools (Sm) or number of individual FF (Md or Lg) samples assayed (middle panel). In the bottom panel, each bar depicts mean values for 18 replicates.

Figure 8.


Follicle Diameter (mm)
15
10
5
0
193
118
42
206
95
46
Steroids in Follicular Fluid (ng/ml)
Estradiol
Progesterone
100
10
1
2
26
17
2
28
16
Steroids in Media (ng/10⁶ live cells)
10
1
0.1
0.01
Sm
Md
Lg
Sm
Md
Lg
Days 2-5
Days 6-10
Days of the Estrous Cycle

During granulosa cell culture, concentration of estradiol in media increased ($P < 0.05$), whereas progesterone decreased ($P < 0.05$) as the follicle size increased in both stages of the estrous cycle (Figure 8, lower panel). Also, only culture media from granulosa cells isolated from the largest estrogen-active follicles during Days 2 to 5 of the estrous cycle had more estradiol than progesterone.

Concentration of estradiol in media was correlated positively with concentration of estradiol in FF (r = 0.94, $P < 0.01$) and follicular diameter (r = 0.81, $P < 0.01$). Concentration of progesterone in media was correlated positively with concentration of progesterone in FF (r = 0.61, $P < 0.05$), but correlated negatively with concentration of estradiol in media (r = -0.66, $P < 0.05$) or FF (r = -0.58, $P < 0.05$), and with follicular diameter (r = -0.56, $P < 0.05$).

Granulosa cell viability before plating was similar ($P > 0.10$; Figure 9) among different follicle sizes or between the two stages of the cycle (37% vs. 36% for Days 2 to 5 and 6 to 10, respectively). Compared with initial viability, proportion of live cells diminished between 45 and 75% after 48 h in culture (Figure 9). Since treatments did not affect viability, all data were pooled for the 48-h means. Final viability was higher (17.2% vs. 12.7%, $P < 0.05$) when granulosa cells were scraped from follicles collected during Days 2 to 5 compared with follicles collected during Days 6 to 10 of the estrous cycle.

Experiments

Experiment 1: FSH. Addition of FSH (0.1 - 10 ng/well) increased ($P < 0.05$) estradiol concentration in media during culture of granulosa cells from the largest follicle

collected during Days 2-5 of the estrous cycle (Figure 10).

Experiment 2: rbST. Addition of rbST (10 - 250 ng/well) did not alter ($P >$ 0.10) estradiol concentration in media during culture of granulosa cells from any size follicles (Figure 11).

Experiment 3: FSH and rbST. Addition of 50 ng/well of rbST did not alter ($P > 0.10$) FSH-induced estradiol concentration in media from small or medium size follicles (Figure 12). However, the 50 ng of rbST blocked the FSH-induced increase in estradiol concentration in media of granulosa cells from the largest follicle obtained during Days 2 to 5 of the estrous cycle [Figure 12; compare (Lg)(Days 2-5)(0 ng rbST) *vs* (Lg)(Days 2-5)(50 ng rbST)].

Experiment 4: rGRF. Addition of rGRF (2 and 10 ng/well) did not alter (P > 0.10) estradiol concentration in media from FSH- and rbST-treated granulosa cells (Figure 13).

Figure 9. Effect of time in culture on viability of bovine granulosa cells. Granulosa cells were isolated from small, medium and the largest follicles per cow as explained in Figure 8, and then cultured for 48 h in serum-free Ham's F-12 media supplemented with 1 μM of 19-OH androstenedione. Percent of live cells were estimated using trypan blue exclusion dye before plating (0 h), and at the end of the experiment (48 h). Since addition of trypsin, FSH, rbST and(or) rGRF did not affect viability, all data were pooled for the 48-h means. Values are overall means (± SEM) of five separate experiments. Each bar depicts mean values for 5 or 69 replicates for measurements done at 0 or 48 h, respectively.

Figure 9.

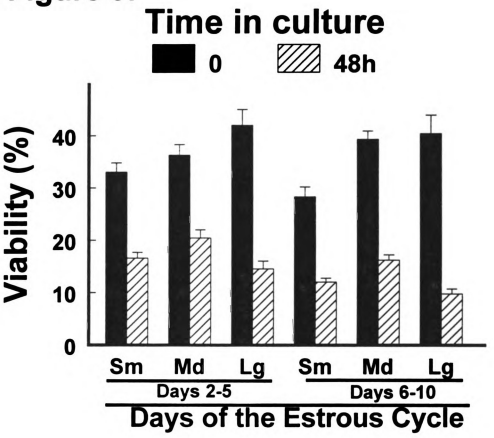

Time in culture
0
48h
Viability (%)
40
30
20
10
0
Sm
Md
Lg
Sm
Md
Lg
Days 2-5
Days 6-10
Days of the Estrous Cycle

Figure 10. Effect of FSH on accumulation of estradiol in media during 48 h of culture of bovine granulosa cells. In three separate studies, granulosa cells were isolated from small, medium and the largest follicles as explained in Figure 8, then cultured for 48 h in serum-free Ham's F-12 media supplemented with 1 μM of 19-OH androstenedione and different doses of FSH. When 19-OH androstenedione was not added, estradiol levels were undetectable (data not shown). Results from a representative study are presented. Bars depict mean (± SEM) values for three replicates. Asterisk indicates a significant ($P < 0.05$) effect of treatment.

Figure 10.

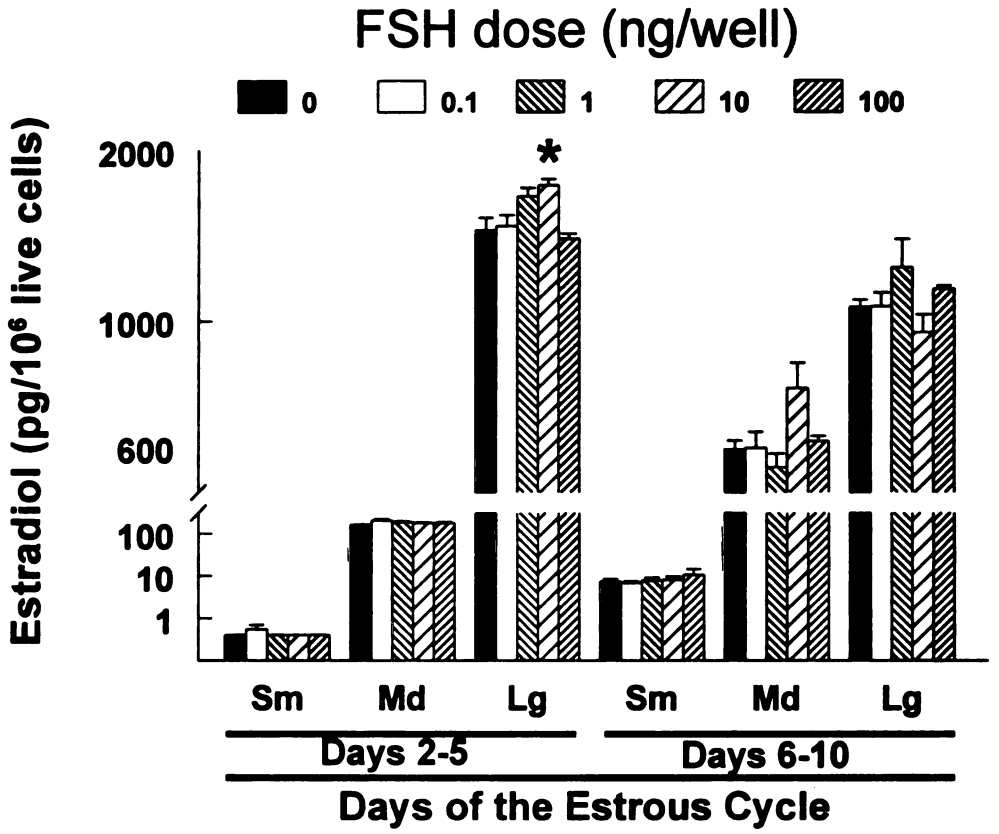

FSH dose (ng/well)
0
0.1
1
10
100
Estradiol (pg/10⁶ live cells)
2000
1000
600
100
10
1
*
Sm
Md
Lg
Sm
Md
Lg
Days 2-5
Days 6-10
Days of the Estrous Cycle

Figure 11. Effect of recombinant bovine somatotropin (rbST) on accumulation of estradiol in media during 48 h of culture of bovine granulosa cells. In two separate studies, granulosa cells were isolated from small, medium and the largest follicles as explained in Figure 8, then cultured for 48 h in serum-free Ham's F-12 media supplemented with 1 μM of 19-OH androstenedione and different doses of rbST. Results from a representative study are presented. Bars are mean (± SEM) values of three replicates.

Figure 11.

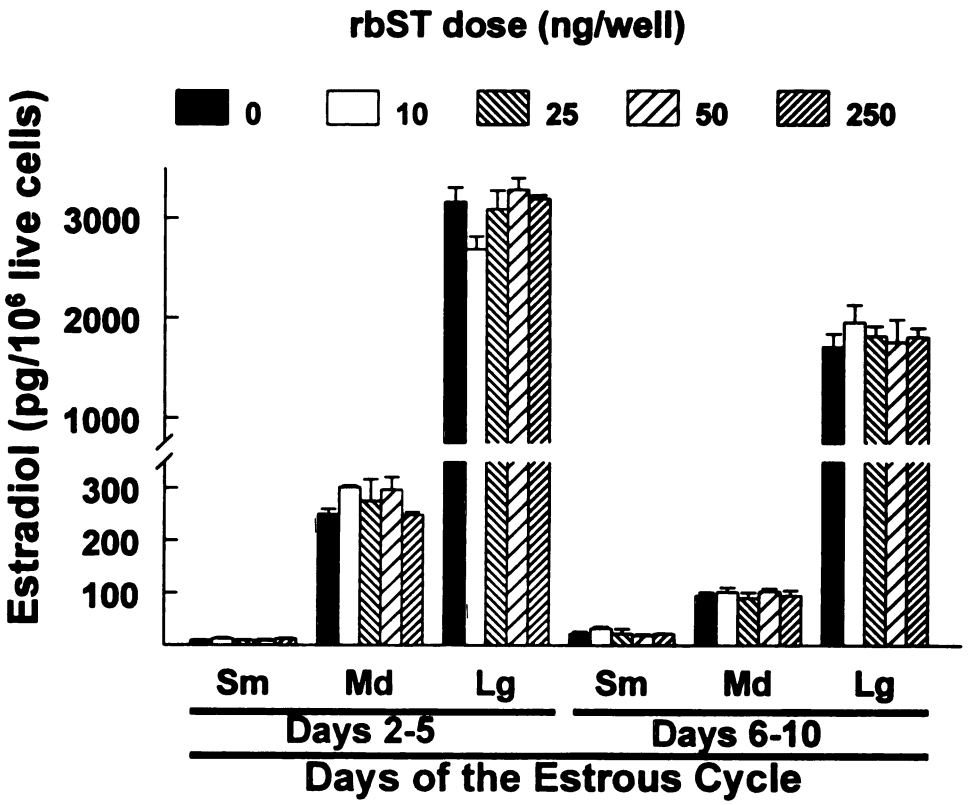

rbST dose (ng/well)
0
10
25
50
250
Estradiol (pg/10⁶ live cells)
3000
2000
1000
300
200
100
Sm
Md
Lg
Sm
Md
Lg
Days 2-5
Days 6-10
Days of the Estrous Cycle

Figure 12. Effect of recombinant bovine somatotropin (rbST) on FSH-induced accumulation of estradiol in media during 48 h of culture of bovine granulosa cells. In two separate studies, granulosa cells were isolated from small, medium and the largest follicles as explained in Figure 8, and then cultured for 48 h in serum-free Ham's F-12 media supplemented with 1 μM of 19-OH androstenedione with or without various doses of FSH and(or) rbST. Results from a representative study are presented. Bars are mean (± SEM) values of three replicates. Asterisk indicates significant ($P < 0.05$) difference of mean compared with control (0 FSH) within each follicle size class.

Figure 12.

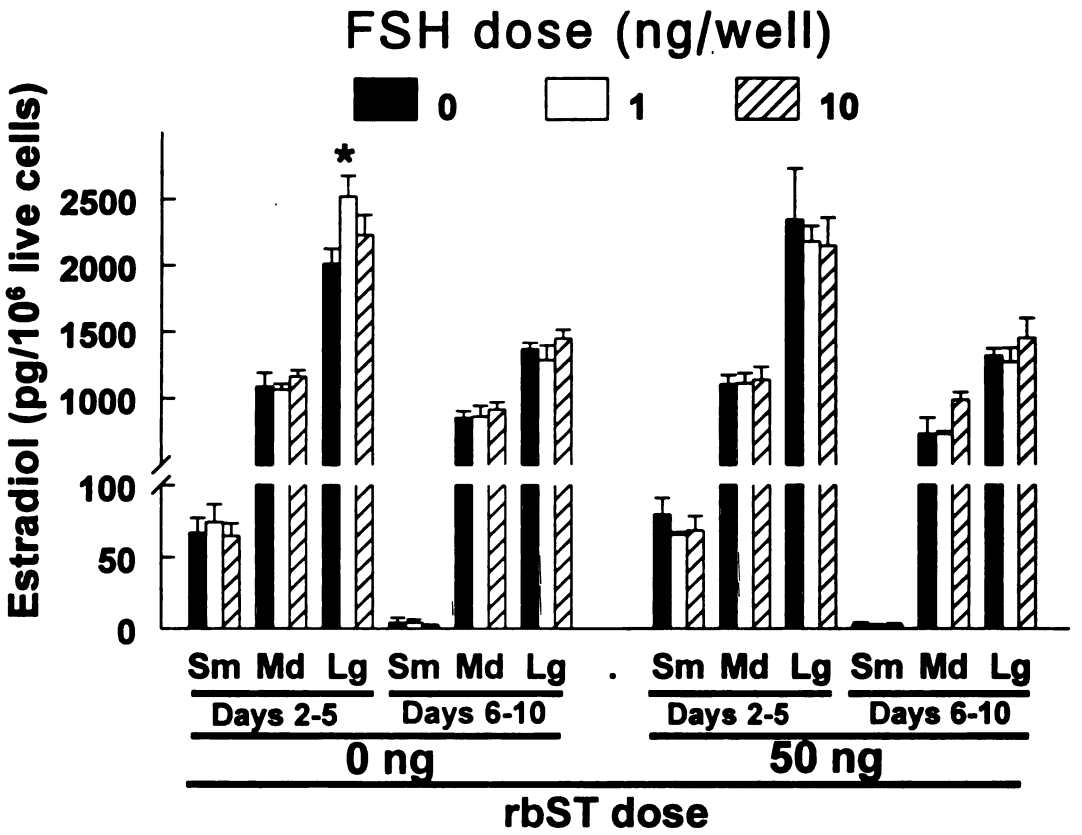
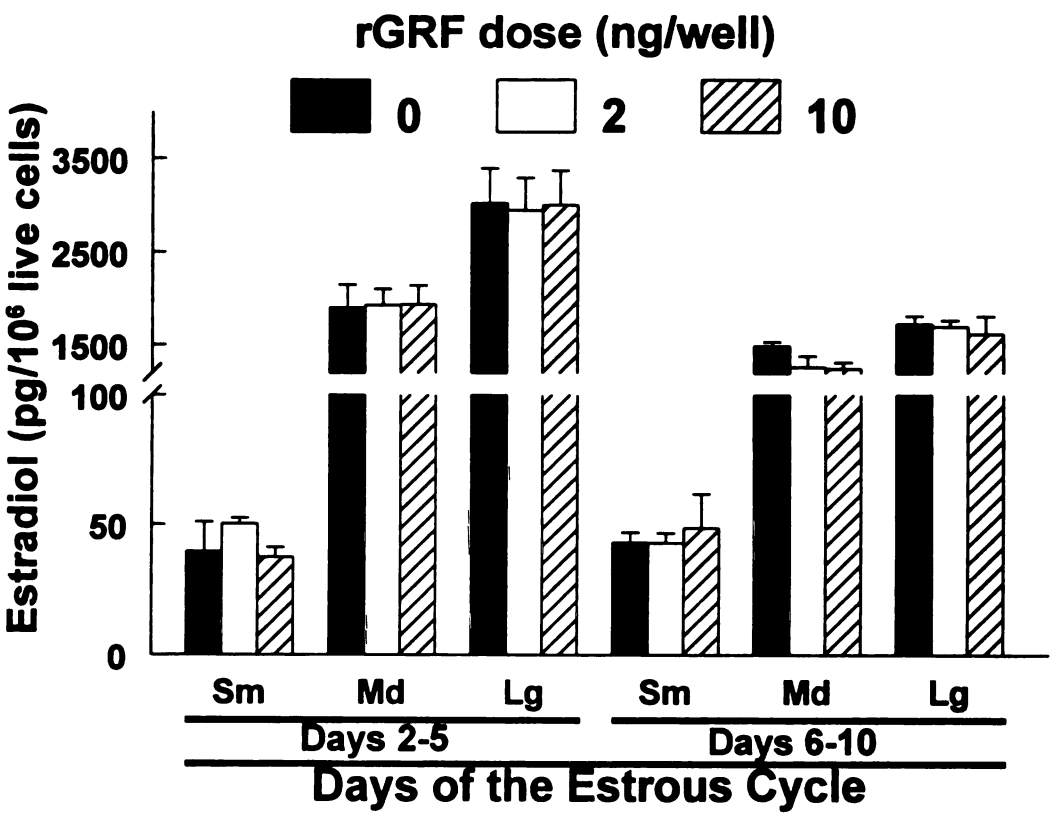

FSH dose (ng/well)
0
1
10
Estradiol (pg/10⁶ live cells)
2500
2000
1500
1000
100
50
0
*
Sm Md Lg Sm Md Lg . Sm Md Lg Sm Md Lg
Days 2-5
Days 6-10
Days 2-5
Days 6-10
0 ng
50 ng
rbST dose

Figure 13. Effect of recombinant bovine growth hormone-releasing factor (rGRF) on accumulation of estradiol in media during 48 h of culture of FSH- and recombinant bovine somatotropin (rbST)-treated bovine granulosa cells. In two separate studies, granulosa cells were isolated from small, medium and the largest follicles as explained in Figure 8, and then cultured for 48 h in serum-free Ham's F-12 media supplemented with 1 μM of 19-OH androstenedione, 10 ng of FSH and 50 ng of rbST with or without various doses of rGRF. Results from a representative study are presented. Bars are mean (± SEM) values of three replicates.

Figure 13.


rGRF dose (ng/well)
0
2
10
Estradiol (pg/10⁶ live cells)
3500
2500
1500
100
50
0
Sm
Md
Lg
Sm
Md
Lg
Days 2-5
Days 6-10
Days of the Estrous Cycle

Discussion

The major findings of this study were: 1) the primary serum-free cell culture system described in the present study is the first demonstration that *in vitro* steroid production by granulosal cells isolated from different size follicles reflected the original *in vivo* estradiol- and progesterone-producing status of the first-wave dominant and subordinate follicles; 2) there were remarkable differences in capacity of granulosa cells isolated from follicles at different stages of development during the first wave to produce estradiol, thus pooling of cells for *in vitro* studies could confound interpretation of results; 3) FSH enhanced estradiol producing-capacity of granulosal cells from the first-wave dominant follicle obtained on Days 2 to 5, but not 6 to 10 of the estrous cycle; 4) rbST blocked the FSH-induced increase in estradiol production by granulosa cells collected from the first-wave dominant follicle on Days 2 to 5; and 5) rGRF did not affect estradiol production by granulosa cells treated with FSH and rbST.

There was a high correlation of *in vitro* with *in vivo* steroid producing capacity by granulosal cells in the present serum-free culture system. But, viability of granulosal cells decreased during cell culture, and viability differed for granulosal cells from different days of the estrous cycle. While the reason for the decreased viability over time is unknown, others also report that granulosa cell viability (Skinner and Osteen, 1988; Langhout et al., 1991; Bao et al., 1995) and(or) estradiol production (Skinner and Osteen, 1988; Luck et al., 1990; Berndtson et al., 1995; Gutierrez et al., 1997) diminish with time in culture, especially in the absence of serum (Skinner and Osteen, 1988; Luck et al., 1990; Berndtson et al., 1995) and(or) growth factors (Langhout et al., 1991; Saumande, 1991; Gutierrez et al., 1997). In

addition, granulosa cells can de-differentiate during long-term culture (> 48 h) in serum-free media. For example, capacity of granulosal cells to produce estradiol or inhibin decreases after 48 hours of culture, whereas oxytocin and progesterone begin to increase (Skinner and Osteen, 1988; Luck et al., 1990; Chandrasekher and Fortune, 1990; Wrathall and Knight, 1993; Berndtson et al., 1995; Rouillier et al., 1996; Gutierrez et al., 1997). In contrast, others report that capacity of granulosal cells from small and medium size follicles (< 10 mm in diameter) to produce basal, or FSH-, insulin-, and IGF-induced estradiol increases after 48 hours in culture (Wrathall and Knight, 1993; Rouillier et al., 1996; Gutierrez et al., 1997). These findings, coupled with results of the present study, show that stage of differentiation of the follicle markedly alters its capacity to produce estradiol and respond to hormonal treatments. Since granulosal cells de-differentiate during long-term culture in serum-free conditions (Skinner and Osteen, 1988; Luck et al., 1990; Chandrasekher and Fortune, 1990; Wrathall and Knight, 1993; Berndtson et al., 1995; Rouillier et al., 1996; Gutierrez et al., 1997), short-term culture periods (≤ 48 hours) may be more likely to reflect the original state of differentiation of follicles. The short-term serum-free culture system used in the present study mimicked the estradiol and progesterone producing capacity of different follicle types from the first follicular wave. Therefore, this cell culture system was used to evaluate the effects of rGRF and rbST on estradiol producing capacity of bovine granulosa cells.

In the present study, only granulosal cells from the estrogen-active, first-wave dominant follicle on Days 2 to 5 of the estrous cycle responded positively to 1 or 10 ng of FSH. Since previous studies show that number of FSH receptors are low or absent in estrogen-inactive follicles (Ireland and Roche, 1983b), this may explain why only granulosal

cells from estrogen-active follicles responded positively to FSH in the present study. Others using markedly different serum-free culture systems report that treatment with low doses (1 to 2 ng/ml) of FSH (bovine, porcine, ovine) in the presence of insulin or IGF-I for 3 to 6 days, coupled with media changes every 24 to 48 hours, stimulates granulosal cells to produce estradiol, regardless of whether cells are isolated from preovulatory follicles (Berndtson et al., 1995), follicles from PMSG-primed prepubertal heifers (Saumande, 1991; Rouillier et al., 1996), or from non-atretic small (<4 mm), medium (4 to 8 mm) or large follicles (>8 mm) from random stages of the bovine estrous cycle (Gutierrez et al., 1997). The ability of low doses of FSH to stimulate estradiol production by granulosal cells is enhanced markedly in a linear fashion by a wide range of concentrations of insulin (10 to 1000 ng/ml; Saumande, 1991; Gutierrez et al., 1997) or IGF-I (1 to 1000 ng/ml; Gutierrez et al., 1997). However, relatively high doses (>10 ng/ml) of FSH (Wrathall and Knight, 1993; Berndtson et al., 1995; Rouillier et al., 1996; Gutierrez et al., 1997), as shown in the present study, or insulin (> 1 μg; Saumande, 1991; Spicer et al., 1993; Wrathall and Knight, 1993; Berndtson et al., 1995; Gutierrez et al., 1997) can diminish FSH-induced estradiol production. This finding may explain why others do not report positive effects of FSH on estradiol production by bovine granulosal cells (Chandrasekher and Fortune, 1990; Wrathall and Knight, 1993; Tian et al., 1995). Whether culture conditions similar to those described above (Saumande, 1991; Berndtson et al., 1995; Gutierrez et al., 1997) would stimulate granulosal cells from first-wave subordinate follicles to respond to FSH and produce estradiol has not been examined.

Other factors that may enhance capacity of FSH to stimulate granulosal cells to produce estradiol include follicle size (Gutierrez et al., 1997), location of granulosa cells

within the follicle (antral>mural, Rouillier et al., 1996), increased culture period (> 48 h; Skinner and Osteen, 1988; Wrathall and Knight, 1993; Berndtson et al., 1995; Berndtson et al., 1996; Rouillier et al., 1996; Gutierrez et al., 1997), and addition of serum to culture media before (Spicer et al., 1993; Baratta et al., 1996) or during (Berndtson et al., 1996; Hynes et al., 1996) treatments. Also, coating culture wells with serum is practiced in several laboratories that show positive effects of FSH on estradiol production (Bao et al., 1995; Gutierrez et al., 1997), although it is controversial whether this procedure enhances FSH action (Wrathall and Knight, 1993). In summary, numerous factors can influence the capacity of granulosal cells to produce estradiol in response to FSH during cell culture. However, the short-term serum-free granulosal cell culture system developed in the present study mimics the original *in vivo* estradiol-producing capacity of different types of follicles from the first wave. Thus, the present culture system was considered a reliable *in vitro* model system to investigate whether rbST or rGRF altered basal or FSH-induced estradiol production by granulosal cells isolated from first-wave dominant and subordinate follicles.

In the present study, rbST had no effect on basal estradiol production, but did inhibit FSH-induced estradiol production by granulosal cells from estrogen-active, first-wave dominant follicle on Days 2 to 5 of the estrous cycle. Similar to FSH, the effects of growth hormone on capacity of granulosal cells to produce estradiol are controversial among laboratories. For example, several laboratories report that growth hormone does not alter granulosa cell estradiol-producing capacity by bovine (Pinto Andrade et al., 1996; Spicer and Steward, 1996a), porcine (Rajkumar et al., 1993; Xu et al., 1995a; Xu et al., 1997) or rat (Jia et al., 1986; Hutchinson et al., 1988) granulosa cells. In contrast, others report that growth

hormone increases basal or gonadotropin-induced estradiol production by bovine (Gong et al., 1994; Sirotkin, 1996), rabbit (Yoshimura et al., 1993), human (Mason et al., 1990; Barreca et al., 1993) or rat (Jia et al., 1986; Hutchinson et al., 1988) granulosa cells. In addition, other laboratories also showed that growth hormone inhibits FSH-induced estradiol production by bovine (Spicer and Steward, 1996a) and porcine (Rajkumar et al., 1993) granulosa cells. The reasons for the inconsistent effects of growth hormone on estradiol production among laboratories are unknown, but may be related to differences in species, stage of follicle differentiation, culture conditions, growth hormone dose or isoform and(or) media additives, similar to explanations already mentioned for the conflicting reports among laboratories for FSH action. Specifically, the effects of rbST have been evaluated only in bovine granulosa cells from different size follicles collected at unknown stages of the estrous cycle (Gong et al., 1994; Pinto Andrade et al., 1996; Sirotkin, 1996; Spicer and Steward, 1996a), and then cultured in presence of fetal calf serum, insulin and(or) IGF-I (Gong et al., 1994; Sirotkin, 1996; Spicer and Steward, 1996a). Since administration of rbST alters serum and intrafollicular concentrations of insulin and IGF-I, the effects of rbST on estradiol production by granulosa cell may be confounded by addition of insulin and(or) IGF-I to culture media. Also, physiological doses of rbST do not alter basal production of estradiol by bovine granulosa cells isolated from small, medium or large follicles (Gong et al., 1994; Spicer and Steward, 1996a), whereas pharmacological doses of rbST either increase basal (Gong et al., 1994; Sirotkin, 1996) or decrease FSH-induced estradiol (Spicer and Steward, 1996a) production by granulosa cells collected from large follicles.

After treatment of dairy cattle with rbST, high intrafollicular concentrations

of estradiol in F2 and F3 first-wave follicles were associated with high intrafollicular concentrations of GH (Chapter 2). However, the findings that rbST did not alter basal and blocked FSH-induced estradiol production in the present study do not support the hypothesis that rbST alone or in combination with FSH directly increases estradiol-producing capacity of bovine granulosa cells isolated from first-wave dominant and subordinate follicles. Nevertheless, administration of rbST could indirectly stimulate *in vivo* estradiol production by acting on the thecal cells to increase aromatizable estrogen substrates. For example, during cell culture, rbST increases androgen production by bovine (Spicer and Steward, 1996a) and rat (Apa et al., 1996) theca cells. In addition, growth hormone increases porcine granulosa cell estradiol production when cells are concomitantly treated with IGF-I (Xu et al., 1997), suggesting that IGF-I may enhance and(or) mediate the effects of growth hormone on granulosa cell estradiol-producing capacity.

Addition of rGRF to bovine granulosa cells *in vitro* did not alter their capacity to produce estradiol in the present study. These results contrast with the stimulatory effect of rat GRF on estradiol production by rat granulosa cells (Bagnato et al., 1991), but agree with previous studies using bovine granulosa cells (Spicer et al., 1992). Taken together, these results imply that direct effects of GRF on gonadal function are species-specific. Although administration of rGRF to dairy cows increases progesterone production by medium size follicles (Spicer and Enright, 1991; Chapter 2), these stimulatory effects may be due to alterations in concentrations of GH, IGF-I and(or) IGFBPs in serum or follicular fluid.

In summary, a granulosa cell serum-free culture system that mimics *in vivo* estradiol-producing capacity of bovine subordinate and dominant follicles from the first-wave

was developed. By using this culture system, it was demonstrated that neither rGRF or rbST enhanced basal or FSH-induced estradiol-producing capacity of bovine granulosa cells from subordinate or dominant follicles. Based on these results, it was concluded that rbST does not directly stimulate aromatase activity during serum-free culture of bovine granulosa cells.

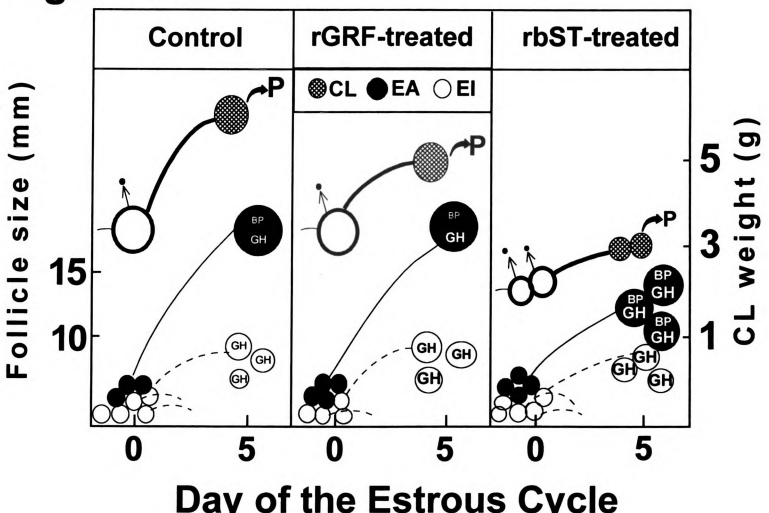
OVERALL SUMMARY AND SPECULATIONS

The results of daily infusions of rGRF or rbST for 63 days in primiparous Holstein cows beginning 117 days post-partum (Figure 14), or treatment of bovine granulosa cells with rGRF or rbST follow:

1) Compared with controls fewer cows treated with rGRF and rbST had CL after synchronization of estrus with prostaglandin $F_2\alpha$ (data not shown);
2) both rGRF and rbST increased GH and IGF-I, but did not alter LH, FSH or estradiol concentrations in serum;
3) treatment with rbST increased number of CL, but decreased CL weight and serum concentration of progesterone compared with controls;
4) rbST increased number of cows with more than five follicles, number of EA follicles, intrafollicular concentrations of GH in all follicles, amount of IGFBP-2, -3 and -4 in EA follicles, and estradiol concentration and percent of EA in F2 and F3 follicle size classes compared to controls and(or) rGRF-treated cows;
5) rbST decreased size of EA and F1 follicles and androstenedione in F1 and EI follicles;

Figure 14. Summary of effects of treating primiparous postpartum Holstein cows with rGRF or rbST for 63 days.

Figure 14.


Control
rGRF-treated
rbST-treated
CL EA EI
P
BP
GH
Follicle size (mm)
15
10
CL weight (g)
5
3
1
0
5
Day of the Estrous Cycle

6) treatment with rGRF increased endogenous GH, but did not alter follicle size or CL weight and function compared with controls or rbST-treated cows.

7) rGRF treatment increased concentration of estradiol, but decreased progesterone in F4 follicles, and decreased androstenedione in EA, EI, F1 and F4 follicles compared with controls;

8) rGRF or rbST treatment did not alter basal or FSH-induced estradiol production by granulosa cells collected from subordinate follicles of the first-wave, or by granulosa cells from the dominant follicle of the first-wave on Days 6 to 10 of the estrous cycle; and

9) rbST blocked the FSH-induced increase in estradiol during culture of granulosa cells from the first-wave dominant follicle on Days 2 to 5.

These findings lead to the conclusion that long-term treatment with rbST disrupts the dominant follicle process in cattle. Administration of rGRF did not affect the dominant follicle process probably because intrafollicular GH concentration did not achieve the high concentrations observed in rbST-treated cows. Independent of the mechanism, the major effect of rbST on the dominant follicle process appears to be to increase survival of subordinate follicles and to increase ability of subordinate follicles to produce estradiol.

There are several possible explanations for the effects of rbST on the dominant follicle process. Firstly, administration of rbST may alter selection of the dominant follicle rather than inhibit dominant follicle function. Selection of the dominant follicle occurs at the time of decline in circulating concentrations of FSH (See Figure 1; Ginther et al., 1996; Roche, 1996). Injections of FSH when endogenous FSH is decreasing (Days 2 to 3 of the

estrous cycle) delays the selection process (Adams et al., 1993; Mihm et al., 1997), as indicated by an increase in number of estrogen-active follicles (Mihm et al., 1997). I hypothesize that high intrafollicular concentrations of GH act as a co-gonadotropin and synergize with FSH to increase number of estrogen-active follicles as shown in the present study, thus delaying the selection process for the first-wave dominant follicle. Administration of rbST increases estradiol concentration in serum (De la Sota et al., 1993; Lucy et al., 1993b) and in follicular fluid (Chapter 2; Pinto Andrade et al., 1996) from dairy and beef cows. Whether rbST directly stimulates estradiol production is unclear, since physiological doses of rbST do not enhance basal or FSH-induced estradiol production by bovine granulosa cells *in vitro* (Gong et al., 1994; Spicer and Steward, 1996a; Sirotkin, 1996; Chapter 3), although pharmacological doses of rbST either enhance (Gong et al., 1994; Sirotkin, 1996) or inhibit (Spicer and Steward, 1996a; Chapter 3) estradiol production. In addition, administration of rbST increases insulin and IGF-I in serum (Gong et al., 1993a; VanderKooi et al., 1995; Gong et al., 1997) and follicular fluid (Spicer and Echternkamp, 1995). Since both insulin and IGF-I increase basal and FSH-induced estradiol by granulosa cells (Spicer and Echternkamp, 1995), rbST-induced increases in insulin or IGF-I may enhance the positive effects of GH on follicular estradiol production (Xu et al., 1997).

Alternatively, the increased number of estrogen-active follicles could be due to inhibition of follicular atresia. For example, administration of ovine and bovine growth hormone suppresses apoptosis in preovulatory rat follicles (Eisenhauer et al., 1995), and transgenic mice expressing the bovine growth hormone have reduced numbers of atretic follicles (Mayerhofer et al., 1990). Moreover, high concentrations of estradiol in follicular

fluid may decrease follicular atresia since administration of estrogens inhibits ovarian granulosa cell apoptosis in rats (Billig et al., 1993). Consequently, administration of rbST could increase number of estrogen-active follicles by increasing estradiol production and(or) decreasing follicular atresia.

Second, administration of rbST could indirectly stimulate estradiol production by increasing thecal cell steroidogenesis to provide more aromatizable estrogen substrate. For example, during cell culture, rbST increases androgen production by bovine (Spicer and Steward, 1996a) and rat (Apa et al., 1996) theca cells. Addition of anti-IGF-I antibodies does not inhibit the stimulatory effect of GH on androgen production in the rat thecal cell system (Apa et al., 1996), implying a direct positive effect of GH on thecal steroidogenesis. Moreover, estradiol production during the follicular phase is due, in part, to an increase in the availability of aromatizable substrate (Tian et al., 1995), and estradiol increases thecal cell androstenedione production (Roberts and Skinner, 1990). These findings indicate that a local feedback loop exists in ovarian follicles. However, in the present study (Chapter 2), despite lower levels of androgens in the largest follicle from rbST-treated cows compared with controls, capacity of granulosa cells in rbST-treated cows to produce estradiol was unaltered. Furthermore, insulin (Spicer et al., 1993) or IGF-I (Gong et al., 1994; Gutierrez et al., 1997) increased progesterone production by granulosa cells, which, in turn, may be used by thecal cells to increase androgen production (Lischinski and Armstrong, 1983; Liu and Hsueh, 1986). Consequently, administration of rbST may have indirectly increased estradiol production by enhancing androgen production by thecal cells, rather than directly enhancing granulosal cell function.

Although cyclic ovarian follicular development is regulated by FSH and LH, the GRF-GH-IGF-IGFBPs system plays an important role on ovarian follicle development and steroidogenesis (Hammond et al., 1991; Giudice, 1992; Jones and Clemmons, 1995; Monget and Monniaux et al., 1995; Spicer and Echternkamp, 1995). *In vitro* studies indicate that IGF-I at physiologic levels stimulates granulosa and theca cell mitogenesis and steroidogenesis (Spicer et al., 1993; Spicer and Echternkamp, 1995; Gutierrez et al., 1997), and that their effects are regulated by the presence of at least six specific forms of IGFBPs within the ovary (Giudice, 1992; Spicer and Echternkamp, 1995). Thus, a decrease in intrafollicular IGFBPs activity together with an increase in IGF-I as follicles growth may enhance net IGF-I bioactivity. The increased IGF-I activity should, in turn, stimulate follicular growth and granulosa and thecal cell differentiation. Administration of rbST may indirectly alter follicle growth and function by regulating both IGF-I and IGFBPs levels in serum (Gong et al., 1993a; Spicer and Echternkamp, 1995; VanderKooi et al., 1995) and follicular fluid (Echternkamp et al., 1994b; Stanko et al., 1994a; Chapter 2). However, the mechanism that regulates IGFBPs in FF, and the exact role of IGFBPs on ovarian function, are unclear. Intrafollicular IGFBPs may both regulate availability of IGF-I (Giudice, 1992; Jones et al., 1993; Monget and Monniaux, 1995; Spicer and Echternkamp, 1995) and directly affect cell growth and differentiation by a mechanism that does not involve sequestering circulating IGF-I (Rechler, 1997). For example, the anti-gonadotropic effects of IGFBP-2, -3 and -4 are achieved by sequestering IGF-I, thereby limiting the availability of IGF to synergize with gonadotropins acting on the ovary. Alternatively, cell membrane-bound IGFBP-2 and(or) -3 may enhance rather than inhibit IGF-I action on granulosa cells by acting as a reservoir for

IGFs, thus facilitating its delivery to type I receptor and potentiating IGF-I action (Jones et al., 1993; Monget and Monniaux, 1995). Consequently, administration of rbST may have indirectly stimulated granulosa cell estradiol production by enhancing IGFBPs activity, which in turn, increased net IGF-I activity.

In summary, while the mechanism is unknown, I speculate that sustained high intrafollicular levels of GH disrupts follicular dominance by preventing atresia of subordinate follicles during a follicular wave. Based on the present *in vivo* and *in vitro* studies for my thesis research, it is further speculated that rbST may prevent atresia by stimulating thecal rather than granulosa cell function, and(or) by enhancing intrafollicular levels of IGFBPs or net IGF-I bioactivity, which in turn, stimulates follicular estradiol production.

APPENDICES

APPENDIX A

Table 7. Ingredient composition of diets fed during the treatment period.

Ingredient	Percent DM
Alfalfa haylage	21.5
Corn silage	20.5
High moisture shell corn	19.0
Ground shell corn	10.5
Soybean meal	11.7
Whole cotton seed	10
Mega-Lac	0.3
Energy Balancer	1.5
Bypass protein	2.0
Mineral/Vitamin mix	2.0

Table 8. Chemical composition of diets fed during the treatment period. [1]

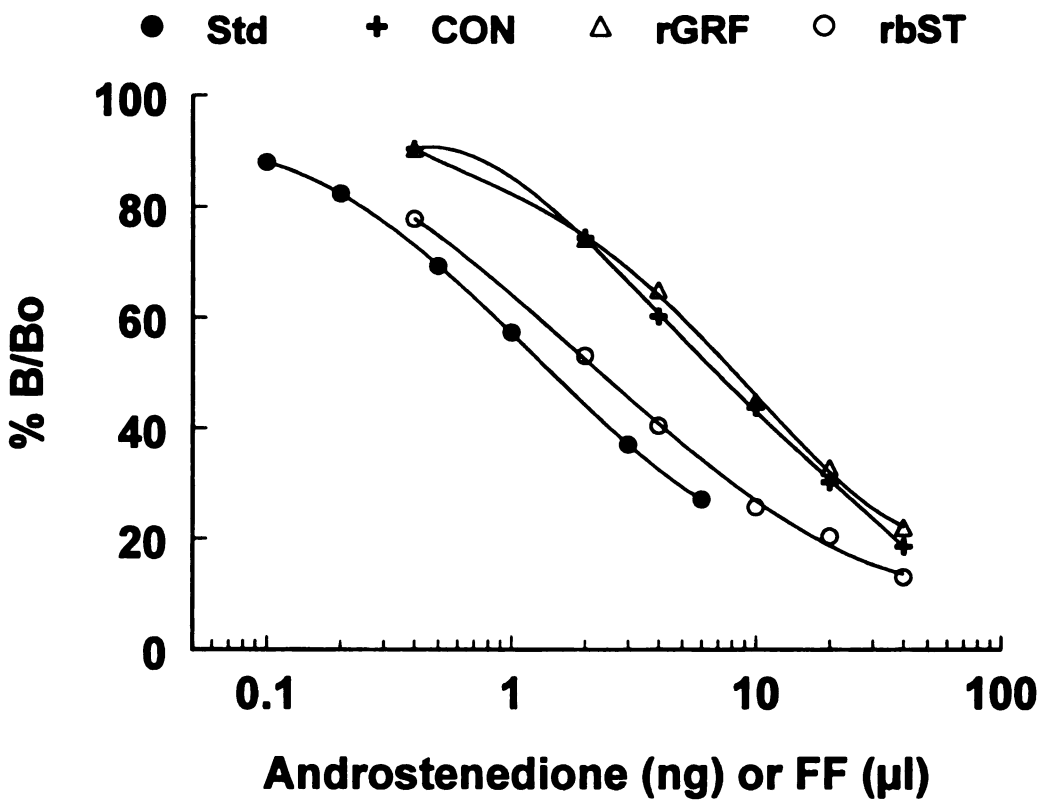
Dry matter, %	59
Crude protein, %	18
Neutral detergent fiber, %	31
Acid detergent fiber, %	19
Net energy lactation, Mcal/d	0.79

[1] Calculated values from NRC book values and forage report values (NRC, 1989)

APPENDIX B

Figure 15. Parallelism of ether-extracted follicular fluid with androstenedione. Concentration of androstenedione was determined by a solid-phase RIA according to Diagnostic Product Corporation's instructions. Different volumes of follicular fluid (0.5 to 40 µl/tube) were ether extracted and analyzed as described in *Materials and Methods*. Values for the androstenedione standard curve (Std) and follicular fluid dilutions are expressed as percent binding (% B/Bo). For the androstenedione standard curve, maximum binding (Bo) was 71%, ED_{50} was 1.44 ng/tube, and non-specific binding was 0.3%. Each point on the standard curves is the mean of duplicate samples. Follicular fluid from control (CON), recombinant bovine growth hormone-releasing factor (rGRF)-, and recombinant bovine somatotropin (rbST)-treated cows depressed binding parallel to the standard curve.

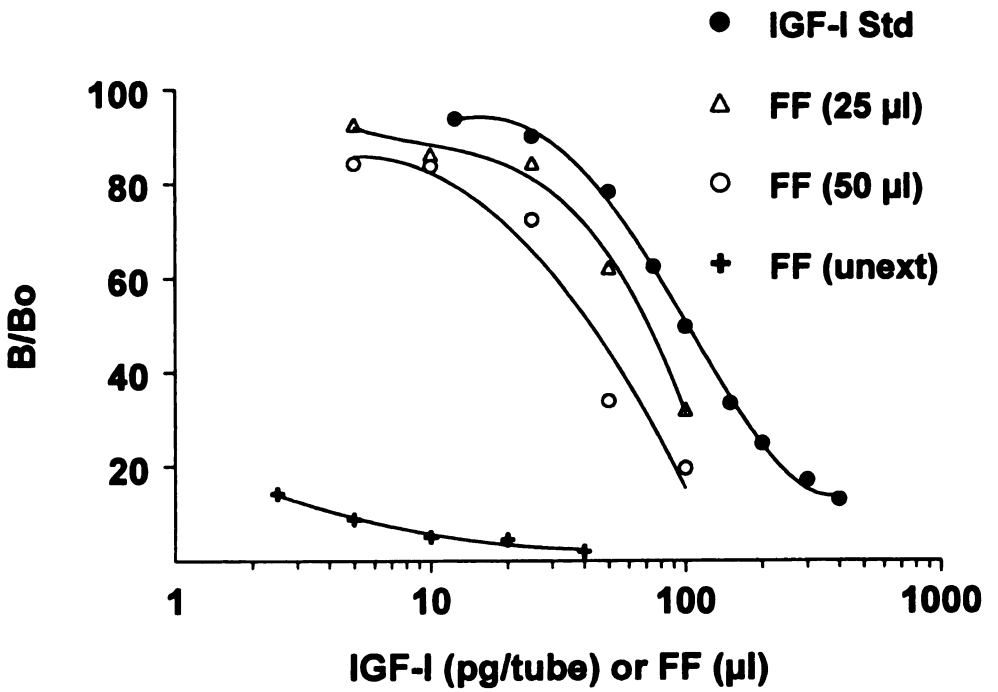
Figure 15.

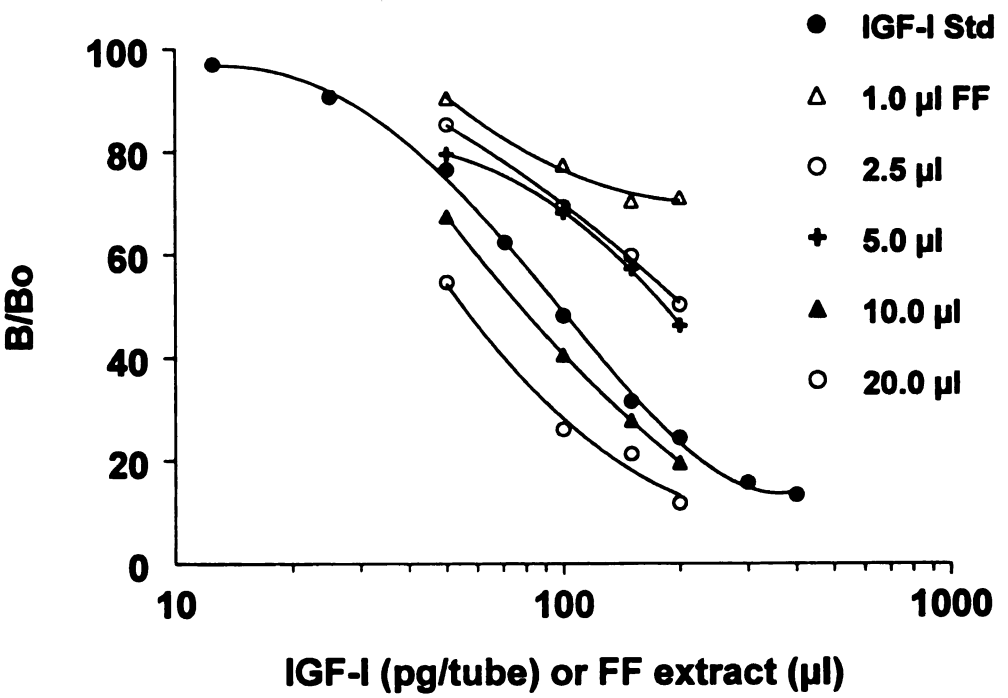

Std
CON
rGRF
rbST
100
80
60
40
20
0
% B/Bo
0.1
1
10
100
Androstenedione (ng) or FF (µl)

APPENDIX C

Figure 16. Parallelism of extracted and unextracted follicular fluid (FF) with insulin-like growth factor-I (IGF-I). Concentration of IGF-I was determined by RIA of FF as described in *Materials and Methods*. Values for the IGF-I standard curves (Std) and FF dilutions are expressed as percent binding (% B/Bo), and each point is the mean of three replicates. *Top panel*: 5 to 100 µl of FF was extracted with 12.5 µl of 2.4 M formic acid and 250 µl absolute ethanol, centrifuged at 600 xg for 30 min, and 100 µl of supernatant was mixed with 1 ml neutralizing buffer (0.11 M Na_2HPO_4, 0.154 M NaCl, 0.01 M EDTA, 0.015 M NaN_3, 0.5% Tween-20, pH 7.5; Bruce et al., 1991). Then, 25 (△) and 50 (○) µl of each different dose of FF extract, and 2.5 to 40 µl of unextracted FF (+, unext) were analyzed by RIA. For the IGF-I standard curve (•), maximum binding (Bo) was 31%, ED_{50} was 0.11 ng/tube and non-specific binding (NSB) was 8.6%. Results indicate that both 25 and 50 µl volumes of extracted FF depressed binding parallel to the standard curve. Unextracted FF suppressed binding to nearly 0. *Bottom panel*: To determine the minimal volume of FF required for RIA, the following assay was performed. One to 20 µl of FF was extracted and neutralized as indicated above. For each different amount of neutralized FF extract, 50, 100, 150 and 200 µl volumes (represented on the *x*-axis) were analyzed by RIA. For the IGF-I standard curve, maximum binding was 21%, ED_{50} was 0.12 ng/tube and NSB was 10.5%. Except for the 1 µl volume (△) of FF, all the different doses of the neutralized FF extract depressed binding parallel to the standard curve. To conserve sample volume, 100 µl volumes of the neutralized FF extract from 5 µl of follicular fluid were used to analyze IGF-I concentrations in samples.

Figure 16.

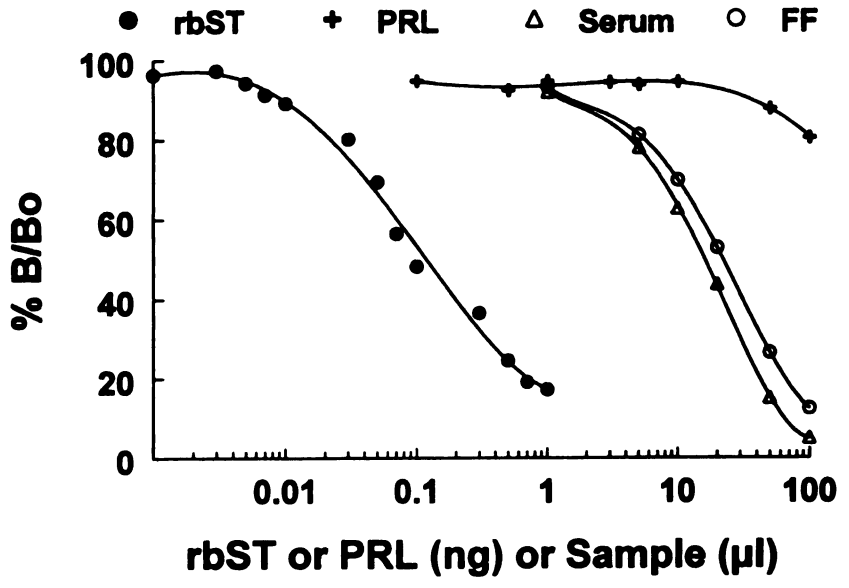

IGF-I Std
FF (25 µl)
FF (50 µl)
FF (unext)
100
80
60
40
20
B/Bo
1
10
100
1000
IGF-I (pg/tube) or FF (µl)

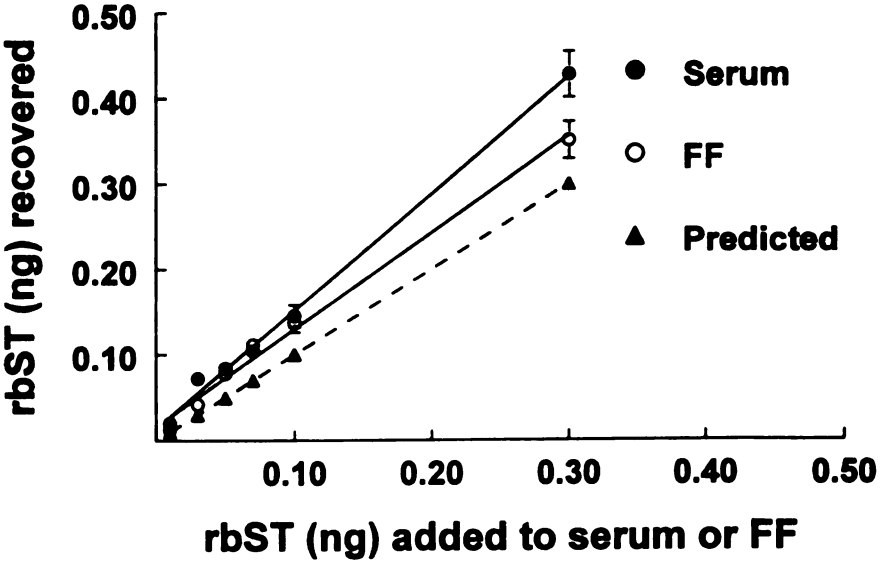

IGF-I Std
1.0 µl FF
2.5 µl
5.0 µl
10.0 µl
20.0 µl
100
80
60
40
20
0
B/Bo
10
100
1000
IGF-I (pg/tube) or FF extract (µl)

APPENDIX D

Figure 17. Validation of the growth hormone (GH) assay. Concentration of GH was determined in serum and follicular fluid (FF) as described in *Materials and Methods*. *Top panel*: Standard curves of recombinant bovine somatotropin (rbST) and bovine prolactin (PRL), and dilutions of serum and FF are expressed as percent binding (% B/Bo). One to 100 µl of serum or FF was analyzed by RIA. For the rbST standard curve, maximum binding (Bo) was 13%, ED_{50} was 0.11 ng/tube, and non-specific binding was 0.6%. Cross-reactivity with PRL (0.1 to 100 ng/tube) was < 0.01%. Serum and FF depressed binding parallel to the standard curve. Each point in the standard curves is the mean of duplicate samples. Based on these results, 5 µl volumes of serum or FF were used to determine concentration of GH by RIA. *Bottom panel*: rbST (0.01 to 0.3 ng) was added to 5 µl of serum or FF and then analyzed by RIA to measure hormone recovery. Recovery of rbST was 113 to 137%. The concentration of GH in serum and FF was 0.06 ± 0.02 and 0.03 ± 0.002 ng/5 µl, respectively. Percent recovery was calculated after subtracting endogenous GH from total GH concentration measured.

Figure 17.

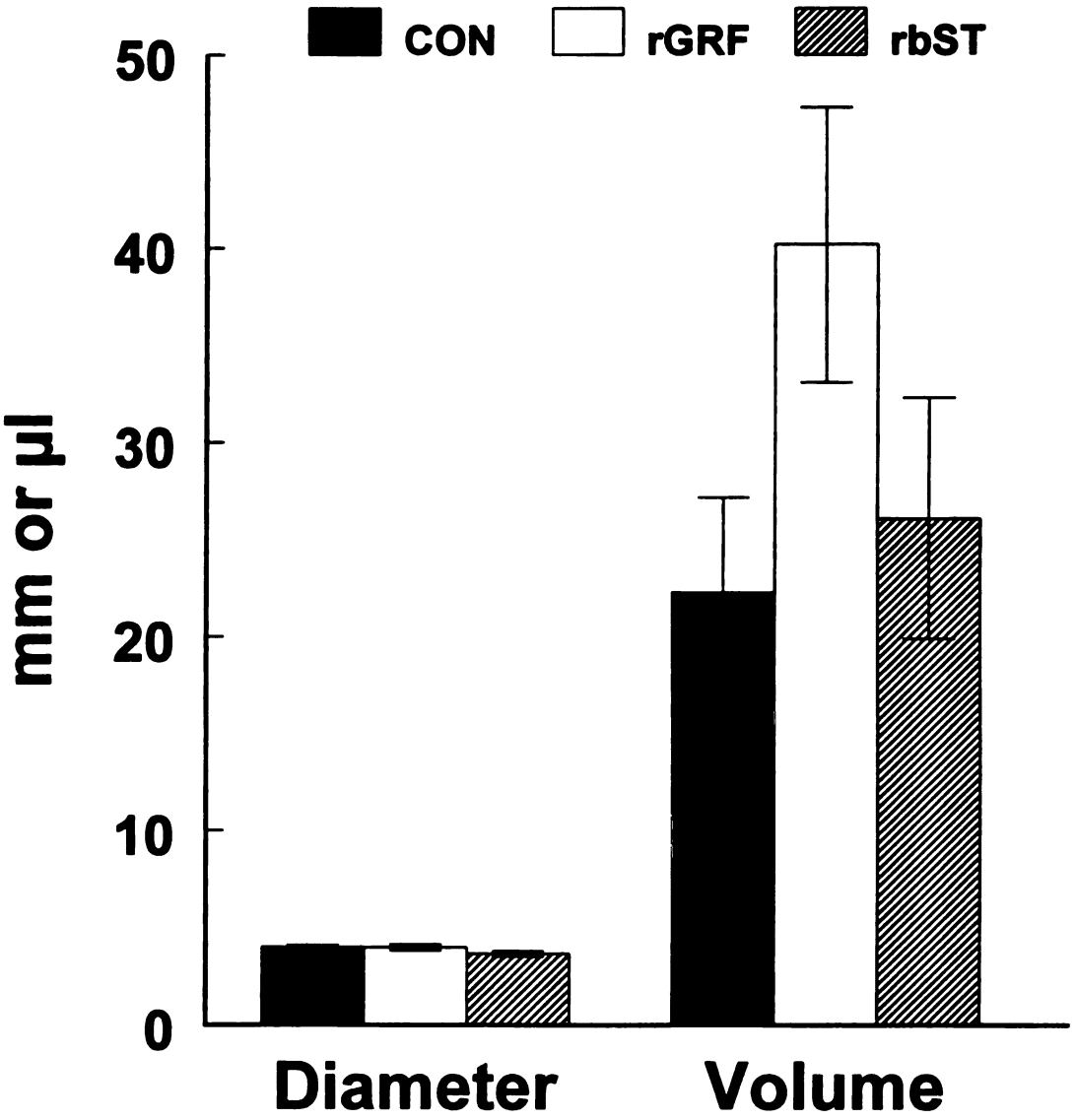

rbST
PRL
Serum
FF
100
80
60
40
20
0
% B/Bo
0.01
0.1
1
10
100
rbST or PRL (ng) or Sample (µl)


0.50
0.40
0.30
0.20
0.10
rbST (ng) recovered
Serum
FF
Predicted
0.10
0.20
0.30
0.40
0.50
rbST (ng) added to serum or FF

APPENDIX E

Figure 18. Effect of recombinant bovine growth hormone-releasing factor (rGRF) or recombinant bovine somatotropin (rbST) on the size (mm) and volume of follicular fluid (μl) from follicles ≤ 5 mm in diameter in Holstein cows at slaughter. Primiparous cows were infused with rGRF or rbST from 117 to 180 days postpartum. Sizes of five follicles ≤ 5 mm in diameter were measured with calipers, whereas volumes of follicular fluid were estimated using a calibrated micropipette. Bars represent mean (± SEM) of five follicles per treatment. There were no differences among treatments ($P > 0.10$)

Figure 18.



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