INFLUENCE OF EXOGENOUS GONADOTROPIN-RELEASING HORMONE ON OVARIAN FUNCTION IN BEEF COWS AFTER SHORT AND LONG TERM NUTRITIONALLY INDUCED ANOVULATION

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This thesis is dedicated to the memory of my grandfather Miguel Angel Prado who represented an example of a man that always found a good justification for better education.

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NOMENCLATURE

CL	corpus luteum
d	day
DNA	deoxyribonucleic acid
FSH	follicle stimulating hormone
g	gram
GH	growth hormone
GnRH	gonadotropin-releasing hormone
h	hour
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
kDa	kilodalton
kg	kilogram
LH	luteinizing hormone
μg	microgram
min	minute
mm	millimeter
mL	milliliter
mRNA	messenger ribonucleic acid
ng	nanogram
NEFA	nonesterified fatty acid
RIA	radioimmunoassay
wk	week

CHAPTER I

INTRODUCTION

Cattle are economically important due to their ability to convert low quality roughage into high quality protein and other nutrients that are important in human nutrition. The beef industry has increased in size and is of paramount importance to the economic activity of the world. Cattle have been a source of food since they were domesticated and beef consumption in most societies is an indicator of the standard of living.

Efficiency of beef production is markedly affected by reproductive performance. Acceptable reproductive performance is a function of early return to estrous cycles after calving and conception in most cows after mating. This performance can be influenced by management, disease, and genetics. Reduced fertility in beef cows is difficult to study because a majority of beef cows are maintained under range conditions. Animals are grazed on pasture and the quantity and quality of forage is often limited. This nutritional restriction is critical to cows that are bred by natural service affecting both percentages of mating and fertilization. However, markedly improved performance can be achieved when careful consideration is paid to adequate management of herd nutrition. Nutrition is one of the major factors that controls reproductive efficiency.

It is often difficult to apply results from nutritional studies in research herds to commercial herds. However, because nutrition is so economically important for a successful cow-calf operation, experimental models to study the effect of undernutrition on reproduction have been developed. Nutrition and reproduction are bi-directional, in that reproductive status of the cow alters nutrient requirements, and the amount of nutrients assimilated by the animal will alter reproductive functions. A beef cow should always be either pregnant, when the fetus has a high priority for nutrients, or lactating, when nutrients are repartitioned to support lactation (Bauman and Currie, 1980). Several aspects of reproduction are influenced by nutrition. For example, malnutrition of beef cows during the last trimester of gestation can be detrimental to fetal growth resulting in increased calf mortality and reduced subsequent reproductive performance (Dunn and Kaltenbach, 1980). Undernutrition delays puberty in heifers and increases the interval from calving to conception (Wettemann, 1980) and ultimately causes reduced reproductive performance in cattle (Johnson et al., 1987). Nutritional restriction of cows or heifers results in loss of body weight and body condition, which results in cessation of normal estrous cycles (Richards et al., 1989a; Bossis et al., 1999). Rasby et al. (1991) found that restriction of feed intake affected the release of gonadotropin releasing hormone (GnRH) from the hypothalamus, which in time reduces gonadotropin secretion and likely cause cessation of normal estrous cycles.

Previous studies have evaluated the effects of GnRH on ovarian function in nutritionally induced anovulatory cows at 3 to 4 weeks after luteal regression but not in cows that were anovulatory for extended intervals. Therefore, an experiment was designed to determine the effects of pulsatile treatment with GnRH on reproductive

function of nutritionally induced anovulatory cows after cessation of luteal activity for 4 or 18 weeks. Follicular growth, concentrations of estradiol and progesterone in plasma and in follicular fluid, and concentrations of androstenedione, IGF-I, and IGF-I binding proteins in follicular fluid were determined after short and long periods of anovulation.

CHAPTER II

REVIEW OF LITERATURE

Puberty

The estrous cycle in domestic animals can be defined as the restricted period of sexual receptivity or estrus in the female and the correlated changes in the reproductive tract and behavior from one estrus to the next.

The first estrous cycle is defined as puberty. At this time, heifers exhibit standing estrus, ovulate, and a corpus luteum (CL) of normal duration is formed (Moran et al., 1989). Age at first estrus is variable, and heifers may have an anovulatory estrus about three months before the first normal cycle. The incidence of anovulatory estrus ranges from 13% (Nelsen et al., 1985) to 60% (Allrich, 1994). Abnormally long or short estrous cycles and early ovulation without estrus occurred in 45% of dairy heifers during the first two estrous cycles (Del Vecchio et al., 1990). This indicates that there may be anomalies in the hormonal events of the ovary and uterus necessary for normal estrous cycles during the onset of puberty.

Estrous cycle

Sexual activity continues for many years once it commences. Number of primordial follicles in cattle remains the same until about the fourth year (mean 133,000), and decreases to near zero by year 15 or 20 (Erickson, 1966). However, fertility increases from the first pubertal estrus to the third (Byerley et al., 1987), and there is a greater lifetime productivity in beef cattle that calve for the first time at two years of age compared with those that calve at three years or older (Donaldson, 1968).

Estrous cycles in cattle average 21 days in length, with a range of 14 to 28 days (Plasse et al., 1970). Ovarian morphology and behavioral changes allow the division of the estrous cycle into four phases: estrus, metestrus, diestrus, and proestrus.

Estrus is usually designated as day 0 and is the day when concentrations of progesterone in plasma are minimal, and the animal is under the influence of follicular estrogen (Hansel and Convey, 1983). Duration of estrus is 12.1 hours. However, Bos indicus cows have a shorter less intensive estrus than Bos taurus cows (Plasse et al., 1970). Bos indicus cows also have a shorter estrus to ovulation interval, smaller CL, and concentrations of progesterone in plasma are less during the luteal phase then in Bos taurus cows (Randel, 1976; Irvin et al., 1978; Adeyemo and Heath, 1980). Estrous behavior is more frequently observed during the night. Hurnik et al. (1975) monitored estrous activity in dairy cows in a free stall barn and observed that 70% of mounting occurs between 7 PM and 7 AM. Evaluation of estrus in dairy cows by Nebel et al. (1992), using an externally mounted pressured-sensitive electronic device (radiotelemeters), revealed that 48% of the mounts occur between midnight and 0800, 27% between 0900 and 1600, and 25% between 1700 and 2400. Nebel et al. (1992) also

showed that duration of estrus (range .4 to 37.8 h) averaged 12.1 h in lactating dairy cows. Estrus was shorter in winter (14.4 h) than summer (18.4 h) when estrous behavior of beef cattle was detected by an electronic heat detector device (The Heat-Watch system; M.L Looper personal communication). Metestrus (day 1-3), is the period between ovulation and formation of the functional CL. Ovulation is spontaneous and occurs about 32 h after the luteinizing hormone (LH) surge in dairy heifers (Swanson and Hafs, 1971) and 27.6 h after the onset of estrus in dairy cows (Walker et al., 1996). In beef cattle, the interval from onset of estrus to ovulation is 32 h (Looper et al., 1998). Diestrus (day 4-18) is the longest phase of the estrous cycle and is the period when the CL is fully functional and progesterone production is maximal. Proestrus (day 19 to behavioral estrus), is characterized by transition from progesterone dominance to a period of estrogen dominance and the onset of estrus (Swanson et al., 1972; Wettemann et al., 197

Regulation of estrous cycles

After the first estrus at puberty, cyclical changes in morphology, function, and behavior occur in response to endocrine stimuli of the reproductive organs. The hypothalamus produces gonadotropin releasing hormone (GnRH), a decapectide, (Matsuo et al., 1971) which is transported from the median eminence of the hypothalamus to the anterior pituitary gland by the hypophyseal portal system (Green and Harris, 1947) and stimulates release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Walters and Schallenberger, 1984). The secretion of FSH and LH is controlled by tonic and surge centers. The tonic center is responsible for the continuous basal secretion of gonadotropins, which stimulate the germinal and endocrine components of the ovary. The

surge center controls the massive short-lived secretion of LH and FSH, responsible for ovulation (Lamming et al., 1979). Bovine ovarian follicles produce estradiol and inhibin as they become dominant, which control growth of subordinate follicles (Padmanabhan et al., 1984; Fortune, 1994). Lussier et al. (1987) reported that continuous growth and atresia of follicles occurs during bovine estrous cycles and the follicle destined to ovulate requires at least two cycles to grow through the antral phase (0.1 mm) to preovulatory size (8.6 mm).

The CL is formed after ovulation. The bovine CL consists of small and large luteal cells that produce maximal concentrations of progesterone under the influence of LH (Hansel and Dowd, 1986). The uterine endometrium produces prostaglandin F2α (PGF2α) which causes luteal regression during late diestrus. During luteolysis, luteal cells release oxytocin in response to PGF2α stimulation from the uterus, which causes increased production of PGF2α by the endometrium (Pate and Townson, 1994). Episodic release of prostaglandin from the uterus at 6 hour intervals, for 36 hours, induces luteal regression in ruminants, which causes a decline in plasma concentrations of progesterone (Kindahl et al., 1976; Pate and Townson, 1994).

Folliculogenesis

A major function of the ovary is folliculogenesis and production of fertilizable ova. During follicular growth, a Graafian or ovulatory follicle forms from a pool of primordial follicles through a series of subcellular and molecular transformations that occur in the oocyte, granulosa and theca cells (Spicer and Echternkamp, 1986; Hirshfield, 1991).

Control of follicular development is not completely defined, and the process by which some follicles are selected to growth and others become atresic has not been elucidated (Wandji et al., 1996). Folliculogenesis in cattle starts during fetal life between day 90 to 130 of gestation (Henricson and Rajakoski, 1959). At puberty, follicles acquire a number of properties that allow them and their oocytes to grow and mature, and those that fail to mature, regress (Campbell et al., 1995).

Efforts to estimate the duration of follicular growth started 28 years ago when Pedersen, (1970) developed a method to estimate the parameters of cell proliferation at each state of growth. Waves of follicular growth in cattle were first described by Rajakoski, (1960), and three stages in the process of folliculogenesis were described as recruitment, selection and dominance in women and nonhuman primates by Hodgen, (1982).

Follicular dynamics.

Rajakoski (1960) suggested that follicular growth throughout the bovine estrous cycle occurs in waves. Bane and Rajakoski, (1961) demonstrated two waves of growth, with the first wave beginning on day 3 to 4 and the second one on day 12 to 14. Others have observed three (Sirois and Fortune, 1988; Savio et al., 1990), or four follicular waves in bovine estrous cycles, with the occurrence of three waves the most common (Ginther et al., 1989a; Fortune, 1994). The numbers of waves appears to be associated with cycle length. Estrous cycles of three waves have a longer luteal phase (19.2 vs 16.5 d), and longer intraovulatory interval (22.8 vs 20.4 d) than two-waves cycles (Ginther et al., 1989a). Since 1984, bovine follicular waves have been determined by ultrasonography.

The combination of ultrasonography with concentrations of hormones in plasma allows a better understanding of the mechanisms involved in follicular dynamics.

Follicular wave emergence is initiated by an increase in serum FSH (Adams et al., 1992). A secondary increase in FSH follows the preovulatory surge, which initiates the first wave. Detectable increases in FSH precede the initiation of all other waves by 1 or 2 days (Adams et al., 1992). There is a functional relationship between increased concentrations of FSH in plasma and the emergence of follicular waves (Adams et al., 1994). One or two days after a wave is detected, dominant follicles are significantly larger than other follicles and the remainder of the follicles (subordinates) undergo atresia within a few days (Ginther et al., 1989b). Antral follicles become dominant and control follicular dynamics. This may be due to a size advantage over the subordinates at early stages of development (Ginther et al., 1996). Increased concentration of mRNA for LH receptors in granulosa cells may be important in the process of selection of follicles (Xu et al., 1995).

During the growing phase, dominant follicles cause regression of subordinate follicles and delay the emergency of the next follicular wave. A new follicular wave does not appear while a dominant follicle is in its growing phase or early static phase (Ginther, 1989a,c). Subordinate follicles are suppressed whether they are on the same or opposite ovary of the dominant follicle, which suggested a systemic rather than local effect (Ginther et al., 1989d). Suppression of plasma FSH concentrations and reduction in sensitivity to FSH is apparently the way dominant follicles inhibit subordinates (Driancourt, 1991). Secretion of FSH from the anterior pituitary is inhibited by estradiol and inhibin which are produced by granulosa cells. Estradiol also has a role in controlling secretion of LH

(Kesner et al., 1981; Clarke et al., 1986; Findlay and Clarke, 1987). In addition, dominant follicles may secrete hormonal factors or proteins (e.g., follicle-regulatory protein) that regulate growth and differentiation of subordinate follicles (Ireland, 1987). Glencross et al. (1994) found increase in the number of large follicles that developed during both the preovulatory and postovulatory period in cows immunized against inhibin. Law et al. (1992) indicated that dominant follicle seems to secrete factors other than inhibin that can suppress follicular growth, because administration of inhibin-free follicular fluid to heifers decreased follicular growth.

Steroidogenesis

Steroidogenesis in ovarian granulosa and theca cells is induced by gonadotropin stimulation of cAMP, which results in increased concentrations of estrogen in plasma and follicular fluid of antral follicles. Concentration of estrogen in follicular fluid of the selected follicle is important in the process of dominance. (Fortune, 1994). In the early phases of follicular development, FSH has an important role in recruitment of follicles (Ginther et al., 1996). In the late stages of folliculogenesis, LH has a major role. When plasma progesterone decreases due to the demise of the CL, basal concentrations of LH and LH pulse frequency increase (Rahe et al., 1980; Spicer et al., 1981). This change in LH secretion coincides with a parallel pulsing of estradiol secreted into the circulation (Baird, 1984; Walters and Schllenberger, 1984). Follicular growth is stimulated by FSH. However, maximal synthesis of estradiol occurs with a combination of FSH and LH (Hansel and Convey, 1983). Luteinizing hormone may increase the production of

androgen by theca cells and increase the aromatization capacity of granulosa cells (Fortune, 1994).

A coordinated stimulation of granulosa cells by both LH and FSH is necessary for follicular maturation (Hansel and Convey, 1983; Campbell et al., 1995). Luteinizing hormone binds to theca cells and stimulates production of androgens that diffuse through the basement membrane into the granulosa cell. FSH bind increases aromatase activity in granulosa cells which converts androgens to estradiol. Increasing concentrations of FSH and estradiol, up-regulates receptors for LH in granulosa cells in preovulatory follicles. Up-regulation of receptors increases LH binding by granulosa cells and stimulates follicular maturation and ovulation. The process by which LH and FSH regulate granulosa and theca cells is called the two-cell, two-gonadotropin model (Hansel and Convey, 1983; Fortune, 1994).

As concentrations of progesterone in plasma decrease, basal LH and frequency of LH pulses increase (Rahe et al., 1980; Spicer et al., 1981). The increase in LH causes secretion of estradiol that stimulates the surge center for LH release and results in ovulation of the mature follicle (Hansel and Convey, 1983). Ovulation of the Graafian follicle is the final phase of follicular development. Ovulation occurs after the preovulatory surge of LH, which produces biochemical, morphological and endocrinological changes in thecal and granulosa cells (Priedkalns et al., 1968; Jablonka-Shariff et al., 1993; Zheng et al., 1994). Mechanisms associated with inflammatory reactions have been likened to the process of ovulation (Espey, 1994). Following ovulation the remaining follicular cells (theca interna and granulosa) undergo a transformation known as luteinization. A highly vascular CL is formed that secretes

increased amounts of progesterone, which maintains a favorable uterine environment for pregnancy (Donaldson and Hansel, 1965; Fields and Fields, 1996).

During luteinization, theca cells differentiate into small luteal cells and granulosa cells differentiate into large luteal cells. Both types of cells secrete progesterone in cattle and concentrations of progesterone are maximal by day 8 to 10 of the cycle, which is coincident with the maximum weight of the CL (Donaldson and Hansel, 1965; O'Shea, 1989; Niswender et al., 1994). Plasma progesterone and diameter of the CL are correlated in dairy cows (Rajamahendran and Taylor, 1990; Ribadu et al., 1994; Wiltbank et al., 1995). Of the total luteal cell population in cattle, 80% are large luteal cells and 20% are small luteal cell. Most of the progesterone produced by the CL (80 %) comes from large luteal cells during the midluteal phase of the estrous cycle (Niswender et al., 1985,1994). Small luteal cells are six time more responsive to LH for progesterone production in vitro than large luteal cells. This indicates that small cells have most of the LH receptors (Urseley and Leymarie, 1979; Hansel et al., 1991; Niswender et al., 1994). However, large luteal cells have most of the receptors for prostaglandin (PGF2α and PGE2), and also produce oxytocin and neurophysin (Wiltbank et al., 1995; Niswender et al., 1994).

Factors that stimulate and inhibit follicular development

Follicular development is regulated by stimulatory and inhibitory factors that influence granulosa and theca cells. Many of these factors may act in cooperation with gonadotropins to modulate folliculogenesis and steroidogenesis.

Growth hormone stimulates the synthesis and secretion of somatomedins by the liver. Somatomedins, also known as the insulin-like growth factors (IGF-I - IGF-II), have lipolytic effects on adipose tissue and increase the uptake of glucose and amino acids by skeletal muscle, similar to actions of insulin. Cells synthesize IGF which binds to receptors, increasing the rate of proliferation of the cells (autocrine stimulation) or near neighbor cells (paracrine stimulation). IGF-I is present in many tissues in the body, including granulosa, theca, stroma, and luteal cells of the ovary (Hammond et al., 1985; Murphy et al., 1987; Einspanier et al., 1990). Granulosa cell proliferation is stimulated by IGF-I which results in stimulating follicular growth (Spicer et al., 1993; Gong et al., 1993). Concentrations of IGF-I are increased in follicular fluid during terminal phase of follicular growth (Hammond et al., 1985), and have a stimulatory effect on DNA synthesis and differentiation of ovine and porcine granulosa cells in vitro (Baranao and Hammond, 1984; Monniaux and Pisselet, 1992).

Number of IGF-I receptors is greater in granulosa than thecal cells (Stewart et al., 1996). Concentration of IGF-I in follicular fluid is not correlated with follicular diameter in cattle (Stanko et al., 1994) because dominant and first subordinate follicles have similar concentration of IGF-I (Stewart et al., 1996). In sheep and pigs, IGF-I stimulates granulosa cell differentiation, proliferation and DNA synthesis in vitro. However, in sheep, IGF-I stimulates granulosa cell proliferation in small follicles (1-3 mm), but not in large (> 5 mm) follicles (Monniaux and Pisselet, 1992).

The stimulatory effect of IGF-I on follicular estradiol production is inconsistent in cattle (Spicer et al., 1988; Echternkamp et al., 1990). Concentrations of IGF-I and estradiol in follicular fluid during the estrous cycle are associated with increase

steroidogenesis (Spicer and Enright, 1991). However, IGF-I can inhibit FSH-induced estradiol production by granulosa cells of small follicles in vitro, and slightly increases estradiol from large follicles (Spicer et al., 1993). In pigs, IGF-I stimulates estradiol production by granulosa cells in vitro (Howard and Ford, 1994), and a synchronous increase in IGF-I and estradiol occurs in preovulatory follicles (Hammond et al., 1988).

Progesterone production is stimulated by IGF-I in granulosa, theca, and luteal cells of sheep (Monniaux and Pisselet, 1992), cattle (Spicer et al., 1993), and pigs (Caubo et al., 1989; Samaras et al., 1993). In sheep, IGF-I stimulates secretion of progesterone by granulosa cells from large follicles but not from small follicles. Monniaux and Pisselet (1992) demonstrated that, IGF-I enhance both basal and FSH-induce progesterone secretion by ovine granulosa cells from large follicles (> 5 mm), but it was decreased in small follicles (1-3 mm). IGF-I increases granulosa cell numbers and progesterone production by large and small bovine follicles in vitro (Spicer et al., 1993). Concentrations of progesterone are positively correlated with concentrations of follicular wall IGF-I mRNA in porcine follicular fluid (Samaras et al., 1993).

Insulin-like growth factor binding proteins (IGFBPs) are in body fluids and regulate the action of growth factors by modulating their bioavailability in the follicle (Stewart et al., 1996). Six IGFBPs have been identified in plasma of horses (Prosser and McLaren., 1992) and pigs (McCusker et al., 1989), and IGFBP-2, -3, -4, and -5 are in follicular fluid of sheep and cattle (Echternkamp et al., 1994a; Besnard et al., 1996; Steward et al., 1996).

The function of IGFBPs in the ovary is to regulate the biological activity of IGF-I, which regulates mitogenesis and steroidogenesis. IGFBP-3 inhibited the IGF-I-induced

increase in bovine theca cell numbers in vitro by 76%, and theca cell progesterone and androstenedione production were reduced by 52 and 89%, respectively. IGFBP-2 inhibited IGF-I induced androstenedione production by 18-30%. However, in the presence of LH and (or) IGF-I, IGFBP-2 did not influence progesterone production or theca cell proliferation (Spicer et al., 1997). During normal estrous cycles in gilts, IGFBP-2 mRNA expression is negatively correlated with follicular fluid progesterone (Samara et al., 1993), and IGFBP-3 is inhibitory to various functions of porcine granulosa cells (Samaras and Hammond, 1995). Concentrations of estradiol in bovine and ovine follicles are negatively correlated with follicular fluid IGFBP-2 (Echternkamp et al., 1994a; Spicer et al., 1995). Concentrations of IGFBP-2 are greater in atretic follicles than actively follicles in sheep, cows, and pigs (Monget et al., 1993; Echternkamp et al., 1994a; Guthrie et al., 1995; Stewart et al., 1996). Concentrations of IGFBP-3 are unchanged while IGFBP-2 decrease progressively in growing antral follicles in sheep and cows (Monget et al., 1993; Echternkamp et al., 1994a; Stewart et al., 1996).

Factors affecting normal estrous cycles

Follicular development is minimal in cows shortly after parturition, and corpora lutea have regressed and are not functional. This suppressed ovarian function, and a lack of behavioral estrus or anestrus may be due to insufficient gonadotropin secretion.

Control of the pituitary-ovarian axis by the brain is through the release of GnRH by the hypothalamus. Because GnRH induces a marked release of LH in most species, it was first called luteinizing hormone-releasing hormone (LH-RH; Matsuo et al., 1971).

Later, Schally, (1978) reported that LH-RH also induced release of follicle-stimulating hormone (FSH) in many species. Now it is generally agreed that GnRH is the gonadotropin-releasing hormone in mammals. Synthesis of GnRH in some species is restricted to a group of neurons located in specific areas of the hypothalamus, such as the arcuate nucleus in monkeys (Knobil, 1980). However in most mammals, GnRH is controlled by two separated areas. The ventromedial and arcuate nuclei comprise the tonic center which releases small pulses of GnRH over days or weeks, whereas the anterior hypothalamic area and the preoptic and suprachiasmatic nuclei comprise the preovulatory center or surge center which stimulates the LH surge that causes ovulation.

Release of GnRH in pulses, at different intervals, is important for reproductive function in most species. Use of push-pull perfusion in the stalk median eminence of rhesus monkeys, revealed that GnRH neurons have endogenous pulse generator mechanisms. In vivo studies revealed that pulsatile secretion of GnRH is modulated by input from neuropeptide Y (NPY), norepinephrine (NE), and gamma-aminobutyric acid (GABA) neurons. These neurons may relay the action of steroid hormones on LH secretion (Terasawa, 1994).

Hypothalamic cells in vitro also release pulses of GnRH spontaneously. In diestrus rats, hypothalamic cells release GnRH pulses spontaneously at 48 min intervals (Dluzen and Ramirez, 1986), while in other species like sheep (Clarke et al., 1987) and horses (Alexander and Irvine, 1987; Irvine and Alexander, 1988) discharges of GnRH that induce gonadotropin pulses occur at intervals of less than 30 min.

It is essential that beef cows initiate normal estrous cycles within 60 to 70 days after calving to maintain a 12-month calving interval. Factors such as suckling (Clapp,

1937; Short et al., 1972), nutrition and (or) body energy reserves (Wiltbank et al., 1962; Dunn and Kaltenbach, 1980) have major effects on the duration of postpartum anestrus. However, other factor such as, age (Erickson et al., 1976; Bellows et al., 1982), breed (Reynolds et al., 1979), milk yield (Oxenreider and Wagner, 1971), postpartum disorders (Stevenson and Call, 1988; Bosu and Peter, 1987; Peter and Bosu, 1988), the presence of a male (Zalesky et al., 1984; Alberio et al., 1987), and season (Hansen and Hauser, 1983; Hansen, 1985), could also interact and affect postpartum anestrus and reproductive performance of cows. Many of this factors are presumed to act via GnRH secretion that controls gonadotropin secretion.

Factors Affecting Estrus

Fertility is markedly reduced after the age of 14 years in beef cattle (Erickson et al., 1976). However, producers rarely maintain cows for more than 8-10 years. Exposing postpartum beef cows to bulls decreases the period of anestrus (Zalesky et al., 1984; Alberio et al., 1987). High milk yield in dairy cows prolongs postpartum anestrus by affecting ovarian activity (Oxenreider and Wagner, 1971). Interval to the first postpartum estrus is shorter in Bos taurus cows than in Bos indicus cows (Reynolds et al., 1979).

Season may influence anestrus in cattle in northern latitudes. This could be related to the effect of day length on secretion of gonadotropins (Critser et al., 1987). Others studies have indicated that fall-calving primiparous cows may have longer intervals to first ovulation and estrus unless 16 to 18 h of light supplementation is provide per day. The duration of postpartum anestrus is often shorter for cows calving in the summer or autumn than for cows calving in the winter or spring. However, seasonal differences also could be

modified by factors such as nutrition, rainfall, breed, and suckling. Evidence that cows are seasonal breeders is inconclusive (Hansen and Hauser, 1983; Hansen, 1985).

An event that occurs during the early postpartum period is the involution of the previously gravid uterus (Kiracofe, 1980). Involution, first estrus and first ovulation could be delayed in cows with uterine infections (Stevenson and Call, 1988). Prostaglandin $F_{2\alpha}$ may play a role for the early elimination of infection and may be effective in improving the postpartum performance of cows with uterine infections (Bekana et al., 1996; Nakao et al., 1997)Postpartum uterine infections may induce anestrus by delaying initiation of folliculogenesis (Peter and Bosu, 1988). Production of endotoxins by microorganisms in the uterus may induce a hormonal imbalance that contributes to anestrus. Endotoxins may trigger prostaglandin ($PGF_2\alpha$) release that stimulates release of cortisol and suppression of the preovulatory release of LH. This could induce the development of follicular cysts and anestrus in some cows (Bosu and Peter, 1987).

Suckling

Suckling prolongs the period of anestrus in beef cows. Anestrus occurs most frequently in suckling beef cows and in cows with poor or thin body condition. Radford et al. (1978) found that crossbred beef cows that nursed their calves did not return to estrus until at least 98 days after parturition. However, crossbred beef cows that did not suckle their calves returned to estrus by 10 to 33 days after parturition. The duration of anestrus was shorter if calves were removed at birth or when suckling was limited (Oxenreider, 1968). Although part of the suckling effect in postpartum anestrus has been attributed to the negative energy balance and weight loss, the neural stimulus that occurs with

frequently suckling blocks secretion of LH. Decreasing the suckling stimulus by early weaning (Walters et al., 1982) or calf separation of calves from cows for a 48 h may stimulated to onset of estrous cycles. Treatment with progesterone may hasten reestablishment of estrous cycles in postpartum anestrous cows (Kiser et al., 1980). The interval to the ovarian activity after early weaning can be influenced by BCS and is correlated with the LH pulse frequency before weaning (Bishop et al., 1994).

The effect of suckling on reproduction in beef cows is mediated through inhibition of gonadotropin release from the anterior pituitary and a decrease in pulsatile LH release. (Carruthers and Hafs, 1980; Lamming et al., 1981; Williams et al., 1983). After parturition, responsiveness of the anterior pituitary to stimulation with GnRH is reduced for about 20 days in suckling beef cows and only 9 days in lactating dairy cows. Concentrations of LH in plasma increase gradually from nearly nondetectable concentrations at calving until day 10 postpartum in dairy cows (Kesler et al., 1977; Moss et al., 1985). The reason for this refractory period may be due to the progesterone-induce negative feedback during pregnancy (Lamming et al., 1979). Another hypothesis is that opioid peptides secreted in response to suckling may inhibit secretion of LH. Whisnant et al. (1986) found that infusion of an opioid antagonist, such as naloxone or naltrexone, increased concentrations of LH in suckled cows. This supports the hypothesis that suckling induces anestrus is due to a block at the hypothalamic level. Decreased pulsatile LH release results in inadequate stimulation of follicular growth and reduced theca cell production of androgens, which are precursors of estradiol produced by granulosa cells (Williams et al., 1982). However, pituitary content of LH and responsiveness to GnRH are not decreased in suckling cows (Moss et al., 1985; Williams et al., 1982). Pulses of

GnRH every one or two hours increased pulses of LH, and induced estrous cycles in suckling cows (Bishop and Wettemann, 1993; Vizcarra et al., 1997).

It has been accepted that suckling prolongs anestrus in beef cows. However, suckling per se may not be essential to prolong anestrus. When cows were maintained with calves, but calves were not allowed to nurse or suckle by using nose plates or muzzles, the cows had an equal duration of anestrus as those that were allow to be suckled by their calves (Williams, 1990). Furthermore, an intact udder is not essential for the suckling inhibition. Attempted suckling induces secretion of prolactin, cortisol, and oxytocin and reduces concentrations and pulses of LH in mastectomized and udder-intact cows. In either case, when calves were present and attempted to suckle mastectomized cows, or suckled from udder-intact cows, the effect was similar and anestrus was prolonged (Stevenson et al., 1994). Williams et al. (1993) subjected a group of cows to complete mammary denervation and found that blood concentrations of LH were identical to those of intact neural pathway cows during the suckling period. These results indicated that there is not a direct neural stimulation from the mammary gland that inhibits gonadotropin release in cows. Factors such as visual and olfactory sensory imputes to the dam (individual or combination of all of them) when she encounters the offspring will induce the anestrus effect. Postpartum anestrus is induced only by a cow's own calf. When a natural calf is replaced with an alien calf that is allowed to nurse, LH surges occur with a restoration of ovarian activity (Silveira et al., 1993; Griffith and Williams, 1996).

Nutrition and body condition

Adequate nutrition is essential to maintain ovarian activity and normal estrous cycles (Randel, 1990), Nutritional requirements vary with the reproductive status of the animal. Grasses, legumes, and other plant sources contain considerable quantities of cellulose, hemicellulose, and starch. Rumen microbes breakdown complex carbohydrates in forages to produce volatile fatty acids. These volatile fatty acids then provide energy to the animal (Bergman et al., 1965, 1974).

A practical way to evaluate nutritional status of the animal is by measurament of body condition score (BCS). Body condition is directly proportional to weight loss or gain and is also highly correlated with the amount of fat present in the body (Wagner et al., 1988). Body condition scoring systems have been developed that use visual and tactile appraisal of muscle and adipose tissue. The most commonly used system for beef cattle in the United States is the nine point scale, with BCS 1 being extremely thin and 9 being extremely obese (Richards et al., 1986; Wagner et al., 1988). The nine point scale is based on visual observation and palpation of vertebral processes, ribs, and pin bones. Body condition score of an animal can be used as a predictor of boneless carcass composition, fat and body energy reserves in heifers (Yelich et al., 1996) and cows (Wagner et al., 1988).

Body condition requirements for optimal reproductive performance are affected by season of calving. Fall calving cows, due to the availability of feed, usually have better body condition at calving than spring calving cows in Oklahoma. However, body condition changes from calving to breeding could be more critical for fall calving cows than spring calving cows because cows tend to lose weight during the fall due to

decreased quality of forage (Wettemann, 1994). Cows in good body condition pre- and post calving have early resumption of ovarian activity and a short postpartum anestrous period (Dunn and Kaltenbach, 1980; Richards et al., 1986). Similarly, primiparous beef heifers that receive increased dietary energy intake during late gestation have shorter postpartum anestrous intervals (Echternkamp et al., 1982; Spitzer et al., 1991; Vizcarra et al., 1998). Wright et al. (1992a) confirmed that body condition at calving was a better indicator of the duration of the postpartum anestrous period than postpartum feed intake. Time between calving to conception and pregnancy rate are influenced by body condition at calving. Cows with a BCS of 4 or less exhibited estrus 12 d later and were open 14 d longer than cows calving with a BCS 5 or greater (Richards et al., 1986). Pregnancy rate is greater for cows calving with BCS of 6 and 7 (87.0 and 90.7%) than cows calving with BCS of 4 and 5 (64.9 and 71.4%; DeRouen et al., 1994). Selk et al. (1988) described a similar relationship between pregnancy rate and precalving body condition score for cows with condition scores of 3 through to 7.

Metabolic signal and gonadotropin secretion and steroidogenesis

Leptin

Leptin, a hormone product of the obese gene, is expressed by adipocytes and may be involved in regulation of food intake and metabolism (Zhang et al., 1994). Leptin serves as a metabolic signal to indicate that fat stores are adequate to meet the necessary caloric demands for reproduction in normal animals, obese mice (ob), diabetic mice (db), and fatty (fa) Zucker rat (Coleman and Hummel, 1969). The genetic mutation of the

obese (ob) gene mouse results in a protein that is expressed in white adipose tissue, and serves as a satiety factor (Zhang et al., 1994; Campfield et al., 1995). Concentrations of leptin in plasma are decreased when animals are subjected to metabolic stress such as food restriction, metabolic wasting diseases or severe exercise. However, it is still unclear if increased concentrations of leptin in plasma will activate the reproductive axis or if it only serves as a permissive signal to maintain reproductive functions (Barash et al., 1996).

Leptin may be a metabolic signal to the reproductive system via direct action on the ovary to influence on sex steroid production. Physiological concentrations of leptin attenuated insulin-induced steroidogenesis of granulosa and theca cells without affecting proliferation of the cells (Spicer and Francisco, 1997, 1998). The inhibitory effect of leptin on steroidogenesis in vitro is due to binding to its own receptors and not through inhibiting the binding of insulin to its receptors (Spicer and Francisco, 1997, 1998).

Metabolic signals are recognized by the brain and serve as indices of metabolic state or nutritional status. These signals may regulate reproduction by controlling gonadotropin secretion and steroidogenesis. However, further investigation is needed to determine if leptin is one of these factors in the bovine.

Undernutrition in beef cows

Reduced nutrient intake may alter endocrine function, follicular growth and normal estrous cycles in beef cows. Cows on restricted diets lose body condition and plasma concentrations of glucose, insulin, and IGF-I are decreased while concentrations of NEFA and growth hormone are increased compared with cows maintaining BCS.

Alteration in these hormones and metabolites in the animal may signal anestrus if restriction of nutrition is for a prolonged period of time (Bossis et al., 1999).

Glucose and NEFA

Ruminants only absorb small amounts of dietary glucose. After fermentation in the rumen, a considerable amount of propionic acid passes across the rumen wall into blood. Propionic acid is then transported to the liver and converted into glucose. Gluconeogenesis is critical in ruminant metabolism (Dunn et al., 1967; Bergman, 1973).

Murahashi et al. (1996) suggested that the area postrema, at the lower brain stem, could be an important glucosensor that controls or modulates LH secretion in rats. Sen et al. (1979) demonstrated in the rat, that glucose is important for GnRH stimulation and LH release. Furthermore, reduced energy intake that causes nutritionally induced anestrus in cows, results in reduced concentrations of glucose in plasma (Vizcarra et al., 1998). This reduction in glucose concentration may decrease insulin secretion and increase NEFA by mobilization of fat in adipocytes (Richards et al., 1989b; Bossis et al., 1999). Increased concentration of NEFAs in plasma is an indicator of the negative energy balance of the animal and fatty acid release from adipocytes (Bines and Hart, 1982).

Gonadotropins

A profound suppression of LH secretion occurs in adult male rhesus monkeys after one day of fasting. This suppression was rapidly reverse when animals were fed (Cameron and Nosbisch, 1991). One day of fasting of monkeys, decreased plasma concentrations of insulin and reproductive hormones, and the hormones increased after refeeding (Helmreich

and Cameron, 1992). These results agree with the findings of Schreihofer et al. (1993) which indicate that metabolic signals lead to the resumption of pulsatile LH secretion after feed restricted monkeys are fed. Changes in insulin secretion during fasting and refeeding of male monkeys did not affect serum concentrations of LH (Williams et al., 1996). However, they found a positive correlation between feeding induced changes in LH and T_3 secretion, suggesting that T_3 could have a role in mediating nutrition- induce changes in the central neural control of the reproductive axis.

The mechanism by which nutritional deprivation regulates estrous cycles in cows may involve a metabolic signal (s) that modulates LH secretion (Richards et al., 1989b). Decreased secretion of GnRH from the hypothalamus with consequent decreases in LH release from the hypophysis, occurs with inadequate nutritional intake in heifers and cows (Procknor et al., 1986; Rasby et al., 1991). Insulin could be a signal that stimulates GnRH release (Arias et al., 1992). Decreased energy intake in pre- and postpartum cows decreases pulse frequency of LH in serum (Perry et al., 1991). Body condition of postpartum cows influences LH pulse frequency independent of energy status. Poor BCS of cows reduces the frequency of GnRH pulses from the hypothalamus. This can result in reduced pulsatile secretion of LH without an effect of BCS directly on pituitary function. Body condition score and reduced nutritional intake could alter the sensitivity of the hypothalamus to estradiol producing a negative feedback that declines with time after calving (Imakawa et al., 1986; Wright et al., 1990). Rasby et al. (1990) found that estradiol and estrone are greater in cows with thin BCS than in moderate cows at 260 d of gestation. Frequency of LH pulses are decreased in nutritionally induced anestrous cows (Richards et al., 1989a; Bossis et al., 1999). Luteinizing hormone pulse frequency is

greater at 3 weeks postpartum in cows that calved in moderate BCS compared with cows that calved in thin or poor BCS (Wright et al., 1992b).

Concentration and frequency of FSH pulses in plasma are not affected by body condition or dietary energy before or after parturition in cows or sheep (Wright et al., 1987; Huffman et al., 1987). Postpartum beef cows fed low quality roughage diets after calving had reduced concentrations of LH with moderate concentration of FSH compared with well fed cows (Jolly et al., 1991). Vizcarra et al. (1997) found that infusion of 1 pulse / 4 h of GnRH to nutritionally induced anovulatory cows, reduced pituitary content of FSH but not LH. This indicates that less frequent pulses of GnRH are required to stimulate FSH secretion than LH during nutritionally induced anestrus in cows. Rhodes et al. (1996) and Bossis et al. (1999) observed that concentrations of LH and estradiol in plasma were decreased, but mean concentrations of FSH in serum were increase during nutritionally induced anestrus in beef heifers.

IGF-I

Rinderknacht and Humbel, (1978) purified and sequenced insulin and insulin-like growth factor-I and -II (IGF-I; IGF-II). Many functions of the IGFs have been identified in many organs and tissues (LeRoith et al., 1993; Spicer and Echternkamp, 1995). Concentration of IGF-I in plasma can be used as an indicator of short-term changes in nutritional status in humans patients (Donahue and Phillips, 1989; Thissen et al., 1994). Concentration of IGF-I in plasma are also regulated by nutritional state in beef cattle (Breier et al., 1988; Richards et al., 1991; Spicer et al., 1992; Bossis et al., 1999). During negative energy balance in dairy cows, concentrations of luteal progesterone and IGF-I are

decreased in serum (Spicer et al., 1990). In high producing dairy cows, a severe negative energy balance can increase the days from calving to first ovulation (Butler and Smith, 1989). When beef cows were fed restricted diets, IGF-I in plasma decreases as cows became anestrus (Richards et al., 1989c; Bossis et al., 1996). Body energy reserves influence IGF-I in serum and the interval to the onset of ovarian activity in postpartum anovulatory beef cows after early weaning (Bishop et al., 1994). These changes in serum IGF-I may directly impact ovarian function because IGF-I stimulates ovarian cell proliferation and steroidogenesis. Specific receptors for insulin and insulin mRNA have been found in granulosa cells of pigs (Baranao and Hammond, 1984), cattle (Spicer et al., 1993), rats (Davoren et al., 1986), and humans (El-Roeiy et al., 1993).

Follicular dynamics

Number of follicles, as well as size, growth, and regression of dominant follicles are affected by nutrient intake before and after parturition. Perry et al. (1991) indicated that the number of small follicles (5.0 to 7.9 mm) was not affected by energy intake before and after calving, but numbers of medium (8.0 to 9.9 mm) and large sized follicles (≥10 mm) were greatly reduced in cows fed low energy diets. Undernutrition may inhibit the growth of large follicles, but it has little effect on steroidogenesis in small or medium follicles (Prado et al., 1990; Ryan et al., 1994). When feed intake was restricted to beef heifers, ovulatory and nonovulatory follicles were smaller than in heifers maintaining BCS (Bossis et al., 1999). Concentrations of IGF-I, NEFA, glucose and insulin were reduced during the two cycles before heifers became anovulatory, and concentrations of NEFA and GH were increased. Concentration of IGF-I were less during the anovulatory cycle

compared with the previous cycle. These findings suggest that IGF-I and (or) NEFA may influence GnRH secretion from the hypothalamus, resulting in decreased secretion of LH and consequently less production of estradiol by dominant follicles which failed to ovulate (Bossis et al., 1999).

GnRH and undernutrition

Postpartum anestrus can be reduced in cows by treatment with GnRH. Roberge et al. (1992) found that continuous infusion of low doses of the LHRH agonist D-Trp^{6}-LHRH (microcapsules of 25 mg that release 15μg/d for 30 days) after day 5 postpartum, reduced the interval to first estrus by 12 days (43 day for treatment vs 55 days for control) in suckled beef cattle. When dairy cows, that had been anestrus for over 32 days, were treated with GnRH (two treatments of 50 μg daily, at one hour apart for 6 weeks), concentration of progesterone in plasma was greater compared with controls (Hussein et al, 1992).

Nutritional restriction that resulted in a 25% loss of initial body weight, induced anovulation in beef cows. Bishop and Wettemann, (1993) and Vizcarra et al. (1997) considered a cow to be nutritionally anestrus (anovulatory) when concentrations of progesterone in plasma were less than 1 ng/ml for three consecutive weeks. Treatment of nutritionally induced anovulatory cows with 2 μg (one pulse) of GnRH at hourly intervals for 13 days, results in luteal activity (Bishop and Wettemann, 1993; Vizcarra et al., 1997)

Summary

Undernutrition and consequent loss of body condition are the major causes of decreased pituitary function and absence of ovarian luteal activity in beef cattle. Reduced pulsatile secretion of LH restricts growth of follicles. Treatment of nutritionally induced anovulatory beef cows with GnRH or analogues, restores secretion of LH and ovarian activity. However, the influence of an extended duration (greater than 4 wk) such as 18 wk of anovulation on responsiveness of the pituitary and ovaries to pulsatile GnRH has not been evaluated.

CHAPTER III

Influence of exogenous gonadotropin-releasing hormone on ovarian function in beef cows after short and long term nutritionally induced anovulation

Abstract

The effect of pulsatile infusion of GnRH on follicular function was evaluated in nutritionally induced anovulatory beef cows. After 4 wk (n = 12) or 18 wk (n = 12) of anovulation, cows were randomly assigned within anovulatory group to either 2 μg GnRH or saline (control) (iv) every hour for 5 d. Cows were exanguinated, and uteri and ovaries were removed. Follicles were classified as large (≥ 5 mm) or small (< 5.0 mm) and fluid from follicles was collected. Growth rate of the largest follicle, as determined by transrectal ultrasonography, was greater (P< .002) for GnRH (1.1 ± .1 mm/d) vs control cows (0.7 ± .2 mm/d), and maximum size of the largest follicle during treatment was greater (P< .005) for GnRH (11.3 ± .9 mm) vs control cows (7.4 ± .5 mm). At slaughter, size of the largest follicle was greater (P< .08) in long anovulatory cows (10.8 ± .6 mm) than in short anovulatory cows (8.8 ± 1.0 mm), however, size of the second largest follicle was greater (P< .03) for short anovulatory cows (7.0 ± .6 mm) than for long anovulatory cows (5.4 ± .3 mm). Number of small follicles in the ovaries was affected by duration of

anovulation x treatment (P< .06). Short anestrous cows treated with GnRH had more small follicles (31.1 ± 4.0 follicles) than short term saline treated cows (23.3 ± 2.9 follicles), or long anestrous cows treated with GnRH (17.3 ± 5.0 follicles) or saline (24.3 ± 2.0 follicles). Number of large follicles was greater (P< .08) in the right ovary (1.2 ± .3 follicles) than in the left ovary (0.5 ± .2 follicles). More cows (83.3%: P< .002) treated with GnRH for 5 d had concentrations of estradiol, greater than 1 pg/mL, in plasma than control cows (25%). However, concentrations of progesterone in plasma were not influenced by treatment of cows with GnRH. Cows that were anovulatory for 18 wk tended (P< .11) to have greater concentrations of estradiol in large follicles (47.7 ± 20.9 ng/mL) than small follicles (3.7 ± 1.0 ng/mL) or in large (10.7 ± 5.0 ng/mL) and small (15.1 ± 9.0 ng/mL) follicles of cows that were anovulatory for short term. Treatment with GnRH tended (P< .12) to increase concentrations of estradiol in follicles (24.4 ± 9.0 ng/mL) compared with control cows (9.8 ± 4.5 ng/mL). Concentrations of androstenedione in follicular fluid were greater (P< .09) in GnRH treated cows (59.3 ± 21.1 ng/mL) compared with control cows (25.8 ± 6.7 ng/mL). Large follicles had greater (P< .0001) concentrations of progesterone (195.8 ± 50.6 ng/mL) than small follicles (54.6 ± 6.3 ng/mL). There was a duration of anovulation x follicular size effect on follicular fluid IGF-1 (P< .02). Cows that were anovulatory for 4 wk had greater IGF-I concentrations in large follicles (14.1 ± 1.8 ng/mL) compared with small follicles (10.2 ± 1.4 ng/mL), or large (8.7 ± .9 ng/mL) and small (8.3 ± .8 ng/mL) follicles of cows that were anovulatory for 18 wk. Amount of 20 kDa IGF binding proteins (IGFBP) was greater (P< .10) in 4 wk (5.8 ± .8 units) than 18 wk (4.0 ± .7 units) anovulatory cows. Amount of IGFBP-2 (34 kDa IGFBP) and the 22 kDa IGFBP were greater (P< .07) in

control cows (16.8 ± 2.2 and 6.3 ± .9 units; respectively) than GnRH-treated cows (11.2 ± 1.4 and 4.2 ± .7 units; respectively). Cows treated with GnRH had greater IGFBP-3 (40-44 kDa IGFBP; P< .09) in short anovulatory (31.2 ± 3.5 units) than long anovulatory cows (15.3 ± 2.8 units). These results indicated that pulsatile infusion of GnRH in anovulatory cows increases growth rate of largest follicles, concentrations of estradiol in plasma, concentrations of estradiol and androstenedione in follicular fluid, and decreases the amount of IGFBP-2 (34 kDa IGFBP) and the 22 kDa IGFBP in follicular fluid. The duration of anovulation, and its interaction with GnRH treatment, influences size of largest and second largest follicles, number of small follicles, and the amount of the 20 kDa IGFBP and IGFBP-3 (40-44 kDa IGFBP) in follicular fluid. Estradiol and IGF-I in follicular fluid were affected by duration of anovulation and its interaction with follicular size. Progesterone in follicular fluid is only affected by follicular size. We conclude that when cows are given pulsatile treatment with GnRH, the increased growth rate of follicles and concentrations of hormones in follicular fluid, as well as the decreased amounts of IGFBP are similar when cows are anovulatory for 4 or 18 weeks. However, the duration of anovulation and interactions with GnRH treatment and follicular size affect size and number of follicles, as well as amounts of IGFBP and hormones in follicular fluid.

Key words: Anovulation, Beef Cows, Estradiol, GnRH, IGF, Ovarian Function.

Introduction

One of the principal causes of reduced reproductive performance in beef cattle is an extended anestrous period after calving (Bellows et al., 1979). Less than adequate nutrient intake is a major cause of an extended postpartum anovulatory interval (Randel,

1990; Bishop et al.,1994). Undernutrition results in loss of body weight, body condition and cessation of estrous cycles (Richards et al., 1989a; Bossis et al., 1999). An extended anestrous interval (Richards et al., 1986) reduces the opportunity for a cow to have a calf every year.

Adequate LH secretion by the hypophysis is essential for stimulation, selection and development of large follicles (Xu et al., 1995; Bodensteiner et al., 1996). Treatment with GnRH may induce ovulation in anestrous beef cows (Britt et al., 1974; Kesler et al., 1981; Troxel et al., 1980). Infusion of GnRH (2 μg) at a frequency of one pulse every hour stimulated LH secretion and resumption of luteal activity in cows that were nutritionally anestrus for 3 to 4 weeks (Bishop and Wettemann, 1993; Vizcarra et al., 1997).

Ovarian concentration of insulin-like growth factor-I may have a role in the process of follicular dominance (Stewart et al., 1996), and the number of IGF-I receptors increases as follicular size increases (Spicer et al., 1994). Cows that are anovulatory due to nutritional deficiencies have decreased plasma IGF-I and LH concentrations (Richards et al., 1995; Bossis et al., 1999) which could decrease concentrations of aromatizable androstenedione and inhibit theca cell differentiation. In addition, changes in insulin-like growth factor binding proteins (IGFBPs) in follicular fluid may be involved in the dominance of follicles by regulating IGF-I bioactivity (Spicer and Echterkamp, 1995; Stewart et al., 1996). Progesterone and androstenedione production in vitro by bovine theca cells is inhibited by IGFBP-3 (40-44 kDa IGFBP; Spicer et al., 1997). Infusion of GnRH at 3 weeks after cows became anovulatory due to nutritional restriction did not affect IGFBPs. However, amounts of IGFBP-2 (23 kDa IGFBP), the 29-32 kDa IGFBP

and the 22 kDa IGFBP were affected by follicular size (Hamilton et al., 1999). Roberts et al. (1997) indicated that plasma concentration of IGFBP-2 were greater and IGFBP-3 concentrations were less in cows the remained anestrus compared with postpartum cows the resumed estrous cycle after they were given greater energy intake. These results suggested that circulating concentration of IGF-I, IGFBP-2 and IGFBP-3 may be indicators of the capacity of energy-restricted cows to resume estrous cycle after realimentation.

The objectives of this study were to determine the effect of the duration of nutritionally induced anovulation (4 weeks or 18 weeks) and pulsatile treatment with GnRH on: 1) follicular growth 2) concentrations of estradiol and progesterone in plasma and follicular fluid, and 3) concentrations of androstenedione, IGF-I, and IGFBPs in follicular fluid.

Material and Methods

Animals and Treatments. Twenty-four nonlactating, Hereford x Angus cows (2 to 8 yr of age) exhibiting normal estrous cycles were maintained in a drylot and fed a restricted diet to lose 1% of their initial body weight per week. Cows consumed 2.72 kg of prairie hay and 35 g of mineral mix per day. When ambient temperature was below 0° C, an additional 1.4 kg of hay per day was provided. Body weight and body condition scores (BCS; 1 = emaciated and 9 = obese; Wagner et al., 1988) were determined every 14 d. Blood samples (10 mL) were collected every 7 d via venipuncture into tubes containing EDTA. Blood samples were immediately placed on ice and centrifuged (3000 x g for 20 min) within 4 h after collection. Plasma was decanted and stored at -20°C until

progesterone was quantified. Cows were determined to be anovulatory when concentrations of progesterone in plasma were less than 1 ng/mL for three consecutive weeks.

At 4 wk (3.7 ± .1 wk) or 18 wk (18.1 ± 1.2 wk) after the onset of anovulation, cows were confined in individual stalls with controlled environmental conditions (21± 4°C, 50± 10% relative humidity). Cows were fed a diet of prairie hay (5.5 kg) and mineral mix (35 g) every day at 0900. We have previously determined that this would maintain the anovulatory condition (Richards et al., 1989a). A polyvinyl jugular cannula (i.d. 1.68 mm, o.d. 2.39 mm, Bolab, Lake Havasu City, AZ) was placed in each jugular vein of cows 2 d before initiation of treatment. One cannula was used for GnRH infusion and the other for collection of blood.

Within each group (4 wk or 18 wk), cows were randomly assigned to receive either 2 μg GnRH or saline (control) iv every hour for 5 d. Treatments began at 0800 h on d 0 and continued until 0600 h on d 5. Heparin (1 USP/mL) and penicillin (50 units/mL) were added to sterile GnRH in saline or saline to prevent clotting and bacterial contamination of cannulas during infusion. Pulsatile infusions were administered via a Harvard Infusion pump (Model 931, Harvard Infusion/Withdrawal Pump, South Natick MA) controlled by an automatic timer (Model CD-4, ChronTrol, Lindburg Ent, San Diego, CA). The pump-timer unit was calibrated to deliver the pulse of saline or GnRH (1.8 mL) in a 5 min interval. Blood samples (10 mL) were collected daily from d 1 through d 5 via cannulas, and plasma was obtained and stored at -20°C.

Ovarian follicular growth was assessed daily by rectal ultrasonography using an Aloka 500V ultrasound scanner with a 7.5 MHz transducer (Corometrics Medical

Systems, Inc, Wallingford, CT). Follicles were categorized as small (< 5.0 mm) and large (≥ 5.0 mm). Ultrasound examinations were recorded to facilitate measurement of follicular growth. Follicles that were growing at least 0.5 mm/d for three days were considered to be dominant follicles (Bossis et al., 1996).

After 5 days of treatment, cows were exsanguinated, within 2 h after the cessation of GnRH treatment. Uteri and ovaries were removed, placed on ice and transported to the laboratory. Ovaries were separated from uteri, trimmed, and follicles were counted and diameters were measured with calipers. Follicular fluid from small follicles (< 5.0 mm; pooled within ovary) and individual large follicles (≥ 5 mm) was collected with 1 mL tuberculin syringes (Becton Dickinson & Co., Frankling Lakes, NJ) and placed into 12 x 75 mm culture tubes. Tubes were capped and placed on ice until all follicles were aspirated. Samples were stored at -20°C until estradiol, progesterone, androstenedione, and IGF-I were quantified by radioimmunoassays and IGFBPs by one dimensional SDS-PAGE.

Radioimmunoassays. Concentrations of each hormone in plasma or follicular fluid, and IGFBPs in follicular fluid were determined in one assay for each constituent. A solid-phase RIA (Coat-A-Count progesterone kit, Diagnostic Products Corp., Los Angeles, CA) was used to determine concentrations of progesterone in plasma (Vizcarra et al., 1997). The intraassay coefficient of variation was 3 %. Estradiol concentrations in plasma were analyzed by RIA using a modification of the Serono Estradiol MAIA assay kit (Biodata SpA, Montecelio, Italy) as described by Vizcarra et al. (1997). The intraassay coefficient of variation was 10 %. Progesterone and estradiol in follicular fluid were

quantified by RIA (Spicer and Enright, 1991), with intraassay coefficients of variation of 7.5% and 12.4% for progesterone and estradiol, respectively. Concentrations of IGF-I in follicular fluid were determined by RIA after acid-ethanol extraction (Echternkamp et al., 1990), with an intraassay coefficient of variation of 10.4%. Concentrations of androstenedione in follicular fluid were determined by solid-phase RIA (ICN Biomedicals, Costa Mesa, CA), as describe by Stewart et al. (1996), with an intraassay coefficient of variation of 11.3%.

IGFBPs in follicular fluids were quantified by one dimensional SDS-PAGE, (Echternkamp et al., 1994b). Buffer (21 μl) and 4 μl of sample were heat denatured and then separated on a 12% polyacrylamide gel via electrophoresis. Proteins in each gel were electrophoretically transferred to nitrocellulose after separation, and ligand-blotted for 17 h with ^{125}I-IGF-I. Gels were washed and exposure to X-ray film at -70°C for 24 h. Intensity of bands on autoradiographs was determined using scanning densitometry (Bio-Rad Molecular Imager and Imaging Densitometer, Bio-Rad Laboratories; Hercules, CA).

Statistical Analyses. Follicular growth, maximum follicular size, and number of cows that had concentrations of estradiol greater than 1 pg/mL and progesterone greater than 1 ng/mL in plasma were analyzed by analyses of variance. Number of follicles was analyzed by split-plot analyses of variance with duration of anovulation (D), treatment (T), and DxT in the main plot, and ovarian side and the interactions with the main effect in the split-plot. Hormone concentrations in follicular fluid and IGFBPs were analyzed by split-plot analyses of variance with duration of anovulation (D), treatment (T), and DxT in the main plot, and follicular size and the interactions with the main effects in the split-plot. If

there were significant interactions between the main effects, a Duncan test (Steel and Torrie, 1980) was used to compare means. Because of heterogeneous variance (Steel and Torrie, 1980), concentrations of estradiol, progesterone, androstenedione, and the 20 kDa IGFBP were transformed to natural Log (Y + 1) prior to analyses.

Results

Anovulation. Consistent with previous experiments (Bishop and Wettemann, 1993; Vizcarra et al., 1997) cows became anovulatory when loss in weight resulted in a BCS of 3.45 ± .03 and a body weight of 350.8 ± 8.5 kg.

Follicular Growth. Rate of growth of the largest follicles during treatment with GnRH was greater (1.1 ± .1 mm/d; P< .002) compared with follicular growth in control cows (0.7 ± .2 mm/d; Fig 1a). Three GnRH treated cows (two 4 wk anovulatory cows and one 18 wk anovulatori cow) ovulated the last day of the treatment before slaughter. Data from these cows were included in follicular growth, but follicular fluid was not analysed because the large follicles ovulated. Rate of growth of the largest follicles was not affected (P> .10) by duration of anovulation or the interaction between duration of anovulation and GnRH treatment.

Follicular Size. Maximum size of the largest follicle was greater (P< .005) for GnRH treated cows (11.3 ± .9 mm) than for control cows (7.4 ± .5 mm; Fig 1b). Maximum size of follicles was not affected (P> .10) by the interaction between duration of anovulation and GnRH treatment. Duration of anovulation influenced the size of the

largest follicle (P< .08), and second largest follicle (P< .03) at slaughter. Size of the largest follicle was greater in cows that were anovulatory for 18 wk (10.8 ± .6 mm) than in cows the were anovulatory for 4 wk (8.8 ± 1.0 mm). However the second largest follicle was greater in cows that were anovulatory for 4 wk (7.0 ± .6 mm) compared with cows that were anovulatory for 18 wk (5.4 ± .3 mm; Fig 2).

Number of Follicles. Number of small (<5.0 mm) follicles in the ovaries was affected by duration of anovulation x treatment (P< .06). Treatment of cows that were anovulatory for 4 wk with GnRH, increased the number of small follicles (31.1 ± 4.0 follicles) compared with 4 wk control cows (23.3 ± 2.9 follicles), and 18 wk treated and control cows (17.3 ± 5.0 follicles; 24.3 ± 2.0 follicles; respectively). However, control cows that were anovulatory for 18 wk had more small follicles (24.3 ± 2.0 follicles) compared with 18 wk GnRH treated cows (17.3 ± 5.0 follicles; Fig 3). Number of large follicles was greater (P< .08) in the right ovary (1.2 ± .3 follicles) than the left ovary (0.5 ± .2 follicles; Fig 4).

Hormones in Plasma and Follicular Fluid. Treatment with GnRH increased (P< .002) the percentage of cows with greater than 1 pg/mL of estradiol. Eighty-three percent of cows treated with GnRH had greater than 1 pg/mL of estradiol on at least one day of treatment, whereas only 25% of the control cows had greater than 1 pg/mL of estradiol at any sampling time (Fig 5). Concentrations of progesterone in plasma during the 5 d of treatment were not influenced by treatment and were less than 1 ng/mL in 83% of the

treated cows and 92% of control cows. Duration of anovulation did not influence concentrations of estradiol or progesterone in plasma during treatment.

There was an effect of duration of anovulation x follicular size on concentration of estradiol in follicular fluid. Concentration of estradiol tended (P< .11) to be greater in large follicles (47.7 ± 20.9 ng/mL) of 18 wk anovulatory cows than in small follicles (3.7 ± 1.0 ng/mL) of 18 wk anovulatory cows and in large (10.7 ± 5.0 ng/mL) and small follicles (15.1 ± 9 ng/mL) of 4 wk anovulatory cows (Fig 6). GnRH-treated cows tended (P< .12) to have greater concentrations of estradiol in follicular fluid (24.4 ± 9.0 ng/mL) than in control cows (9.8 ± 4.5 ng/mL; Fig 7).

Duration of anovulation and size of follicles did not influence concentrations of androstenedione in follicular fluid. Concentrations of androstenedione were greater (P< .09) in follicular fluid of GnRH-treated cows (59.3 ± 21.1 ng/mL) compared with control cows (25.8 ± 6.7 ng/mL; Fig 7). Duration of anovulation and GnRH treatment did not influence concentration of progesterone in follicular fluid. However, concentrations of progesterone in follicular fluid were greater (P< .0001) in large follicles (195.8 ± 50.6 ng/mL) than in small follicles (54.6 ± 6.3 ng/mL; Fig 8).

There was a duration of anovulation x follicular size effect on IGF-I in follicular fluid (P< .02; Fig 9). Cows that were anovulatory for 4 wk had greater IGF-I concentrations in large follicles (14.1 ± 1.8 ng/mL) compared with small follicles (10.2 ± 1.4 ng/mL) in cows that were anovulatory for 4 wk and large (8.7 ± .9 ng/mL) and small (8.3 ± .8 ng/mL) follicles of 18 wk anovulatory cows. In cows that were anovulatory for 18 wk, concentrations of IGF-I were similar in large and small follicles. Treatment with GnRH did not influence concentration of IGF-I in follicular fluid.

IGF binding protein in follicular fluid. IGF binding proteins are reported as arbitrary densitometric units as identified by ligand blotting. Amount of 20 kDa IGFBP activity was greater (P< .10) in 4 wk anovulatory cows (5.8 ± .8) compared with 18 wk anovulatory cows (4.0 ± .7; Fig 10). Treatment with GnRH influenced the amount of IGFBP-2 (34 kDa IGFBP) and the 22 kDa IGFBP (P<.07), but duration of anovulation did not influence the amounts of these binding proteins. Control cows had more IGFBP-2 (16.8 ± 2.2) and 22 kDa IGFBP (6.3 ± .9) compared with GnRH treated cows (11.2 ± 1.4 and 4.2 ± .7; respectively; Fig 11). Duration of anovulation x treatment affected the amount of IGFBP-3 (40-44 kDa IGFBP; P< .09; Fig 12). Short term anovulatory cows that were treated with GnRH had greater IGFBP-3 (31.2 ± 3.5) than long term anovulatory cows (15.3 ± 2.8). However, amount of IGFBP-3 in short and long term anovulatory control cows (24 ± 2.6; 21.2 ± 2.8; respectively) treated with saline were not different from either short or long term anovulatory cows treated with GnRH. Follicular size did not influence the amount of IGFBPs, and the IGFBP with a molecular weight of 29-32 kDa was not affected by treatment with GnRH, duration of anovulation, or size of follicles.

Discussion

The number of follicles in postpartum cows increases with time after parturition (Wagner and Hansel, 1969; Spicer et al., 1986cd; Perry et al., 1991). The largest follicle averaged 10.8 mm in diameter by day 7 postpartum in suckled beef cows (Spicer et al., 1986b). Follicles greater than 10 mm in diameter are present by 5 wk postpartum before

the first estrus (Wiltbank et al., 1964). Nonovulatory follicles can be similar in size to those during normal estrous cycle (Staigmiller et al., 1982; Braden et al., 1986). Poor body condition and undernutrition probably do not influence follicular growth and development of large follicles (Prado et al., 1990) and follicular waves occur in nutritionally anovulatory cows (Bossis et al., 1996). Decrease nutritional intake, which causes major decreases in body weight, will decrease the size of dominant follicles, but not the number of follicles when cows maintaining body condition of 4 are compared with obese or optimal BCS cows (Ryan et al., 1994). Treatment with a low dose of GnRH induced ovulation in postpartum anestrous ewes (Wright et al., 1983, 1984), postpartum anestrous sows (Armstrong and Britt, 1985), and anestrous mares (Johnson, 1987; Turner and Irvine, 1991; Mumford et al., 1994a,b). Treatment of adequately fed postpartum anestrous cows with GnRH, induced gonadotropin secretion (Kesler et al., 1980; Riley et al., 1981; Spicer et al., 1986a; Jagger et al., 1987). Suckling cows maintained on low nutritional intake and treated with GnRH between 5 and 9 wk postpartum, had an increase in follicular activity (Prado et al., 1990). These results indicated that neither time nor body condition altered the pattern of follicular growth or the incidence of follicular atresia (Prado et al., 1990).

Duration of anovulation did not affect the rate of growth of the largest follicles in our study. However, treatment with GnRH significantly increased the daily growth rate of large follicles. Cows treated with saline, had follicular growth similar to that observed by Bossis, (1997) in nutritional anestrous cows, but the rate of growth was increased when pulses of GnRH were given. Follicular waves occur in nutritionally induced anovulatory cow but the largest follicles fail to mature and ovulate (Rhodes et al., 1995; Bossis et al.,

1999). It is known that infusion of nutritionally induced anovulatory beef cows with GnRH every hour induced luteal activity within 12 days in 75% of the animals (Bishop and Wettemann, 1993; Vizcarra et al., 1997). Dominant follicles in nutritionally anovulatory cows had a growth rate of .9 mm/d (Bossis, 1997). However, to our knowledge, this is the first report that quantify the dynamics of follicular growth in short and long term nutritionally induced anestrous cows treated with GnRH.

Duration of anovulation influenced the size of follicles. The largest follicle was bigger in 18 wk than 4 wk anovulatory cows, but size of the second largest follicle was greater in 4 wk than 18 wk anovulatory cows. Follicular sizes for long and short nutritionally induced anovulatory cows have not been previously reported. However, evaluation of follicles from normal anestrous dairy cows during 35 days after parturition revealed that the number of small antral follicles decreased significantly from day 15 to 25 postpartum while the percentage of large nonatretic antral follicles increased (Dufour and Roy, 1985). Diameter of follicles increase with time after parturition in anestrous suckling beef cows (Spicer et al., 1986cd). Reestablishing feed intake to nutritionally induced anovulatory beef cows resulted in a linear increase in persistence, growth, and size of dominant follicles (Rhodes et al., 1995; Bossis, 1997)

Number of follicles is not affected by GnRH (500 ng of LHRH every 2 h for 96 h) treatment of postpartum anestrous cows (Spicer et al., 1986a). However, number of follicles could be affected by body condition. More small follicles were observed at 5 wk postpartum in good body condition anestrous beef cows compared with low body condition cows treated with GnRH (Prado et al., 1990). The number of small and medium size follicles are decreased in postpartum anestrous dairy cows during negative energy

balance (Lucy et al., 1991). This indicated that there is a physiological mechanism responsible for ovarian follicular recruitment and that reduced energy intake or body condition may increase persistency of small subordinates follicles. Our results revealed that an interaction between time of anovulation x treatment with GnRH on follicular size. The number of small follicles was greatest in those cows that were anovulatory for a short period and were treated with GnRH. However, these results disagree with Hamilton et al. (1999) who found that treatment with GnRH at the rate of one pulse/h for 12 d to short term nutritionally anovulatory cows did not affect the number of follicles.

Rajakoski (1960) found that the incidence of the largest normal follicle was greater in right ovaries of cattle (66.7%) and that the left ovary is less functional. Decreased follicular and luteal activity in the left ovary may occur because it is positioned near the rumen and blood flow is restricted. We found that the number of large, but not small follicles was greater in the right ovary than in the left ovary regardless of time of anestrus or treatment. This indicates that differences in blood flow to the ovary due to the location in relation to the rumen may affect the amount of gonadotropin supplied to the ovary and this alters growth of large follicles.

Concentration of estradiol in blood decrease drastically after parturition (Echternkamp and Hansel, 1973; Humphrey et al., 1983) and does not increase until the first postpartum estrus (Humphrey et al., 1983). Concentration of estradiol in plasma also increases about three days before ovulation in realimented nutritionally induced anovulatory heifers (Bossis, 1997). Concentrations of estradiol in plasma were increased in dairy cows treated with GnRH compared to controls regardless of the duration of anestrus (Britt et al., 1974). Vizcarra et al. (1997) indicated that infusion of one pulse of

GnRH every 4 h for 12 d did not alter plasma estradiol in cows that were anestrus for at least 3 wk. However, when cows were given pulsatile infusion of GnRH once every hour for 12 d, concentrations of estradiol in plasma were increased compared with controls. Our results confirm that pulsatile infusion of GnRH stimulates estradiol synthesis in follicles.

Treatment with GnRH and duration of anovulation did not influence concentrations of progesterone in plasma. However, the duration of treatment was not sufficient to allow ovulation and CL formation. Concentration of progesterone are minimal in plasma of anestrous cows until the first ovulation (Wettemann, 1980; Humphrey et al., 1983).

Follicular size could influence concentrations of estradiol in follicular fluid. Increased follicular size and consequently increased numbers of granulosa cells will increase conversion of androstenedione to estradiol (McNatty et al., 1984a). Concentrations of estradiol are less in medium size atretic follicles compared with normal follicles (Spicer et al., 1987), and only large non-atretic follicles produce significant amounts of estradiol (Ireland and Roche, 1982). Large follicles ($\geq$ 8 mm) produce and contain greater concentrations of estradiol than small follicles ($<$ 8 mm) in postpartum anovulatory cows as well as in normal cycling cows (Staigmiller et al., 1982; Spicer et al., 1986b; Spicer and Enright, 1991). Bodensteiner et al. (1996) indicated that during normal estrous cycles, an increase in estradiol production by dominant follicles occurs before there is an increased in the number of gonadotropin receptors. However, differences in concentrations of follicular fluid estradiol, but not number of gonadotropin receptors in

granulosa cells were associated with the early growth of dominant and subordinates follicles during the normal estrous cycle (Stewart et al., 1996).

Nutritional intake could affect estradiol concentrations in follicular fluid. Prepuberal heifers that were adequately fed had greater concentration of estradiol in dominant follicles compared with feed restricted heifers (Bergfeld et al., 1994). Prado et al. (1990) found more than one large estrogenic follicle per cow at 5 weeks than at 9 weeks postpartum cows. In the present study, duration of anovulation and follicular size tended to affect estradiol concentration in follicular fluid. Concentration of estradiol in large follicles of 18 wk anovulatory cows were greater than in short anovulatory cows. McNatty et al. (1984a) indicated that aromatase activity increased in granulosa cells of healthy follicles, but not in atretic follicles. In our study, all follicles are atretic based on estradiol progesterone ratio (Ireland and Roche, 1982,1983). It is likely that short term anovulatory cows may have large atretic follicles with granulosa cells that are immature and have decreased aromatase activity when compared to long term anovulatory cows. Furthermore, ability to convert testosterone to estradiol may be increased in large follicles compared with small follicles during long periods of anovulation, but not after a short duration of anovulation.

Concentrations of estradiol in follicular fluid are increased when postpartum anovulatory cows are treated low doses of GnRH (Spicer et al., 1986b). Treatment with GnRH affected concentrations of estradiol in follicular fluid regardless of follicular size or duration of anovulation. Hamilton et al. (1999) found that when short term (3 wk) anovulatory cows were treated with GnRH 1 pulse every four hours, concentrations of estradiol in follicular fluid were increased in small and large follicles, but not when cows

were treated 1 pulse/h. Wright et al. (1990) found that mean LH concentrations, pulse frequency, and pulse amplitudes increased with time postpartum and increases in LH were associated with increased rate of estrogenic secretion by the follicle. However, greater LH secretion was not associated with increased numbers of large follicles when cows were treated with 2.5 μg of GnRH at 5 and 9 weeks postpartum (Wright et al., 1990). Vizcarra et al. (1995,1997) found that pulsatile treatment of cows that were anovulatory for 3 wk with 2.0 μg of GnRH, did not influence concentration of FSH in serum and concluded that FSH was not a rate-limiting factor in nutritionally induced anestrous cows. Based on these results, we suggest that different mechanisms may control follicular function and stimulate ovarian granulosa cells to produce estradiol when there is an adequate secretion of gonadotropins in nutritionally anovulatory cows. Treatment with pulses of GnRH once every hour may stimulate follicular estradiol synthesis regardless of the duration of anovulation or size of the follicles.

Androgens are precursors for follicular estrogen production (Hillier, 1981) and theca cells are the major source of follicular androstenedione (McNatty et al., 1984b). This indicates that androgen synthesis by theca cells was not a factor that limited production of estradiol by follicles in this study. Concentrations of androstenedione were greater in anovulatory cows treated with GnRH regardless of the duration of anestrus or follicular size. Hamilton et al. (1999) found that treatment of nutritionally induced anovulatory cows with GnRH did not increased concentrations of androstenedione in follicles. This increase in androstenedione in GnRH treated cows may account for the tendency for increased concentrations of estradiol in anovulatory cows. Echternkamp et

al. (1994a) and Spicer et al. (1986b) found that follicular concentration of androstenedione were not affected by follicular size or GnRH treatment.

Concentration of progesterone in follicular fluid normally increase from day 2 to 10 of the bovine estrous cycle as follicular size increases during the first follicular wave (Bodensteiner et al., 1996; Stewart et al., 1996). Short, (1962) and Ireland et al. (1979) found that follicular fluid contains large quantities of progesterone. This ability of bovine follicles to produce progesterone appears to increase near the time of ovulation (Ireland and Roche 1982,1983; Fortune and Hansel 1985), and both granulosa and theca cells are able to produce large amounts of progesterone (McNatty et al., 1984a; Spicer et al., 1993; Spicer and Francisco, 1998). On the other hand, treatment of postpartum beef cows with 500 ng of GnRH for 4 days decreased concentrations of progesterone in follicular fluid of large follicles, but did not affect progesterone concentrations in small or medium size follicles (Spicer et al., 1986b). Our results demonstrated that large follicles have a significant increase in progesterone compared with small follicles, regardless of treatment or duration of anovulation. This is in contrast with results of Hamilton et al. (1999) in which follicular size did not influence progesterone concentration in follicular fluid.

Duration of anovulation influenced the effect of follicular size on the concentrations of IGF-I in follicular fluid. It has been suggested that IGF-I could be a mediator of nutritional effects on reproduction (Spicer et al., 1990) because there is decreased secretion of IGF-I in cows subjected to underfeeding or short term fasting (Elsasser et al., 1989; Richards et al., 1989c; Spicer et al., 1992; Bossis et al., 1999). Ryan et al. (1994) found that serum and follicular fluid concentrations of IGF-I increased with increasing body condition. Insulin-like growth factor-I increases in follicular fluid as

follicular diameter increases during the normal estrous cycle in cattle (Spicer and Enright, 1991; Stewart et al., 1996). A gradual increase in IGF-I is associated with a gradual increase in the size of dominant follicles during refeeding nutritionally induced anovulatory heifers (Bossis, 1997). In swine, IGF-I stimulates estradiol production by granulosa cells and there is a positive correlation between follicular wall IGF-I mRNA and follicular fluid estradiol (Samaras et al., 1993; Howard and Ford, 1994). However, concentrations of IGF-I in follicular fluid do not consistently regulate estradiol in follicular fluid in cattle and they may be either positively or negatively correlated. Increases in follicular fluid concentrations of IGF-I did not influence follicular fluid concentrations of estradiol, and increases in follicular fluid concentrations of estradiol did not alter concentrations of follicular fluid IGF-I (Spicer et al., 1988, 1991; Echternkamp et al., 1990). Richards et al. (1991) indicated that production of estradiol by the ovaries of cyclic cows, but not in feed restricted cows, would increase secretion of IGF-I by the liver. This suggested that communication between the ovary and the liver is interrupted when energy intake of cows is restricted.

Concentrations of IGF-I were significantly increased in large versus small follicles of short anovulatory cows. In contrast, Hamilton et al. (1999) found similar concentration of IGF-I in small and large follicles in cows 3 wk after the onset of anovulation. We suggest that different mechanisms may increase concentrations of IGF-I during growth of dominant follicles, or that IGF-I concentrations may influence growth of the dominant follicles. However, regardless of the mechanism controlling follicular growth, IGF-I does not appear to be involved in the ability of follicles to produce estradiol. Production of progesterone by growing follicles could be related to increased IGF-I in large follicles.

Insulin-like growth factors in biological fluids are usually bound to a family of homologous proteins called insulin-like growth factor-binding proteins (IGFBPs). Binding proteins regulate growth factor action by prolonging the half-life of IGFs and by transporting them into the extracellular matrix and to the cell surface (Rechler, 1993). The function of IGFBPs in the ovary is to regulate the local endocrine actions of IGFs in an autocrine and (or) paracrine fashion (Clemmons and Underwood, 1991). Pigs, sheep, and cattle have decreased amounts of IGFBPs in follicular fluid as follicles develop and produce estrogens, and the amount of IGFBPs increase as follicles becoming atretic and estrogen inactive (Spicer and Echternkamp, 1995). Most IGFBPs are associated with these changes but IGFBP-3 is not. Concentrations of IGFBP-2, -5, and -4 decrease and IGFBP-3 remains unchanged in the growing preovulatory follicle of sheep and cattle (Monget et al., 1993; Echternkamp et al., 1994a). However in sheep, concentrations of IGFBP-2, -5, and -4 increase and IGFBP-3 decreases during follicular atresia (Monget et al., 1993). Although increases or decreases in the amounts of IGFBPs seem to correlate with follicular size, this was not the case in the present study. Thissen et al. (1994) reported that human diet restriction increases the clearance and degradation of IGF-I in serum due to changes in amounts of circulating IGFBPs. These results agree with our study in which duration of anovulation, treatment with GnRH and their interaction, were important factors that modulated amount of IGFBPs in follicular fluid.

Stanko et al. (1994) identified a 31 to 32-kDa IGFBP in the bovine follicular fluid as IGFBP-5. None of the factors evaluated, such as duration of anovulation, treatment with GnRH, follicular size or they interactions affected the amounts of 29- to 32-kDa IGFBPs. The 20-kDa IGFBP (likely IGFBP-4) was increased in cows that were

anovulatory for 4 wk compared with cows that were anovulatory for 18 wk. However, estradiol was low in 4 wk and greater in 18 wk anovulatory cows. These results suggest that when cows have been anestrous for short period, follicles are atretic, estradiol is minimal and the amounts of 20-kDa IGFBP is greater. However, in long anestrous cows, time has allowed follicles to develop and grow, estradiol is increased and the amount of the 20 kDa-IGFBP is decreased.

IGFBP-2 and other low molecular weight binding proteins, such as the IGFBP-4 and -5, may inhibit estradiol synthesis by cultured rat and human granulosa cells, by inhibiting the effect of FSH on estradiol production (Mason et al., 1992; Liu et al., 1993). These binding proteins are present in pig and human ovaries (Hammond et al., 1991). Stewart et al. (1996), Spicer et al. (1995), and Echternkamp et al. (1994a) found that IGFBP-2 in follicular fluid of the cows and ewes, is negatively correlated with concentration of estradiol in follicular fluid. These authors also found that amounts of IGFBP-3 does not change while IGFBP-2 and other low molecular weight binding proteins are decreased in preovulatory and dominant follicles of adlibitum fed animals. Besnard et al. (1996) suggested that specific proteinases in follicular fluid may increase in activity and degrade IGFBP-2 while other proteinases decrease in activity and degrade less IGFBP-3 during growing and terminal development of large antral follicles in the sheep.

Activity of IGFBP-2 and the 22 kDa-IGFBP were significantly increased in control cows compared with GnRH treated cows regardless of the duration of anovulation and follicular size. However, IGFBP-3 was greater in short anovulatory cows treated with GnRH than in control cows regardless of follicular size. These results are in disagreement with those from Hamilton et al. (1999) who found that 13-day GnRH treatment did not

affect IGFBP-3 in cows that were anovulatory for 3 wk. We observed that IGFBP-2 and the 22 kDa-IGFBP were significantly decreased in cows that were treated with GnRH. Besnard et al. (1996) observed an increase in degradation of IGFBP-2 and 22 kDa-IGFBP, but not of the IGFBP-3 during terminal growth of ovine antral follicles. This indicates that GnRH treatment may activate a mechanism or specific intrafollicular proteinases that decrease concentration of IGFBP-2 and 22 kDa-IGFBP in follicular fluid and their influence on follicular steroidogenesis. Alternatively, follicular production of various IGFBPs may be differentially regulated by GnRH treatment. The reduction in these binding proteins may allow FSH-induced estradiol production by granulosa cells.

Implications

Restriction of nutritional intake induces anovulation in beef cattle and affects reproductive efficiency by causing cessation of estrous cycles. Ovarian activity is reinitiated after the cows are realimentated. Pulsatile treatment with GnRH for five days stimulates follicular growth and ovarian steroidogenesis in nutritionally induced anovulatory cows regardless of the duration (4 wk or 18 wk) that the animal has been anovulatory. This indicated that duration of anovulation is not a factor that limits the reestablishing of ovarian activity when anovulatory cows are treated with GnRH or realimented.

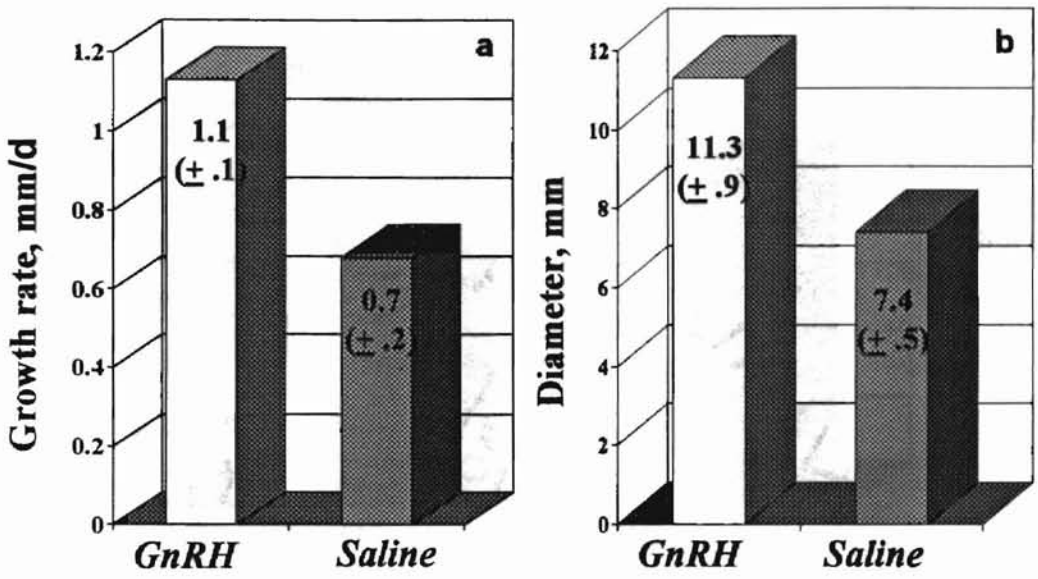

Figure 1. Influence of GnRH treatment on (a) follicular growth (P< .002) and (b) size (P < .005) of the largest follicle

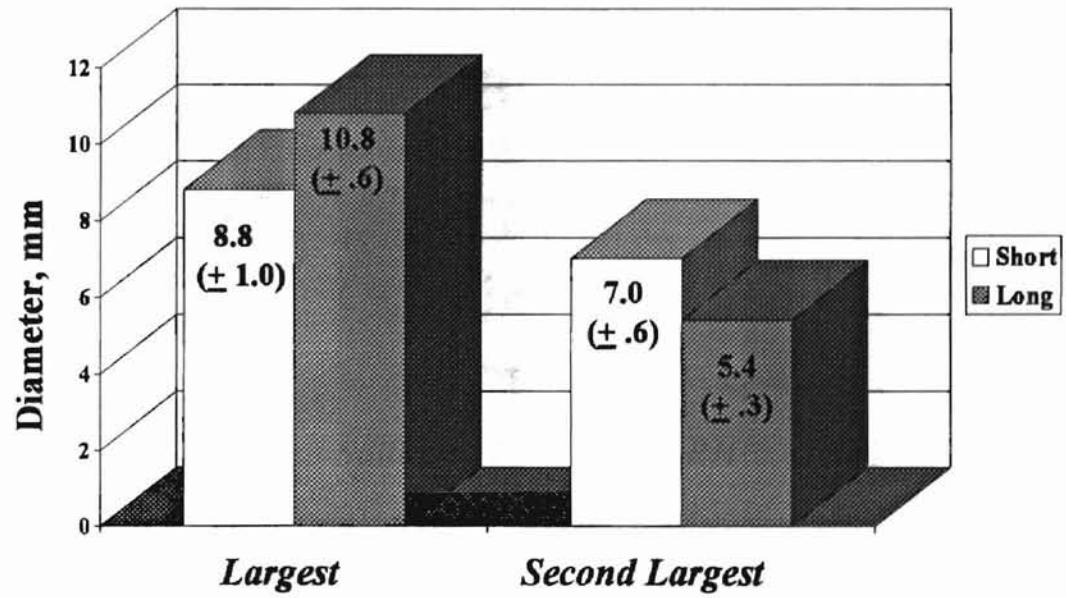

Figure 2. Influence of duration of anovulation on the size of the largest (P< .08) and second largest (P< .03) ovarian follicles at slaughter

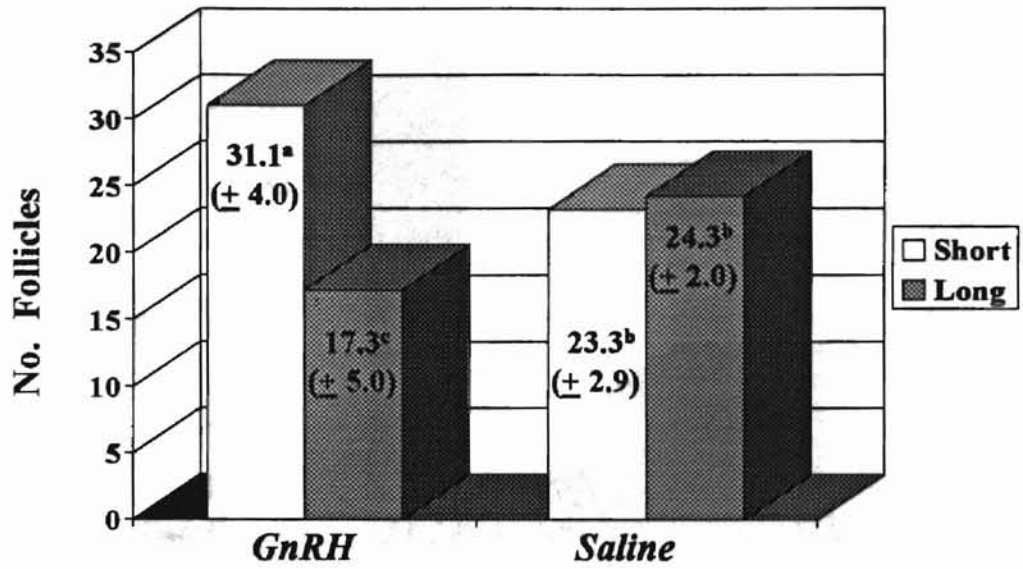

Figure 3. Influence of duration of anovulation and treatment with GnRH on the number of small (< 4.9) ovarian follicles (P< .06). [a,b,c] Means without a common letter differ (P< .05)

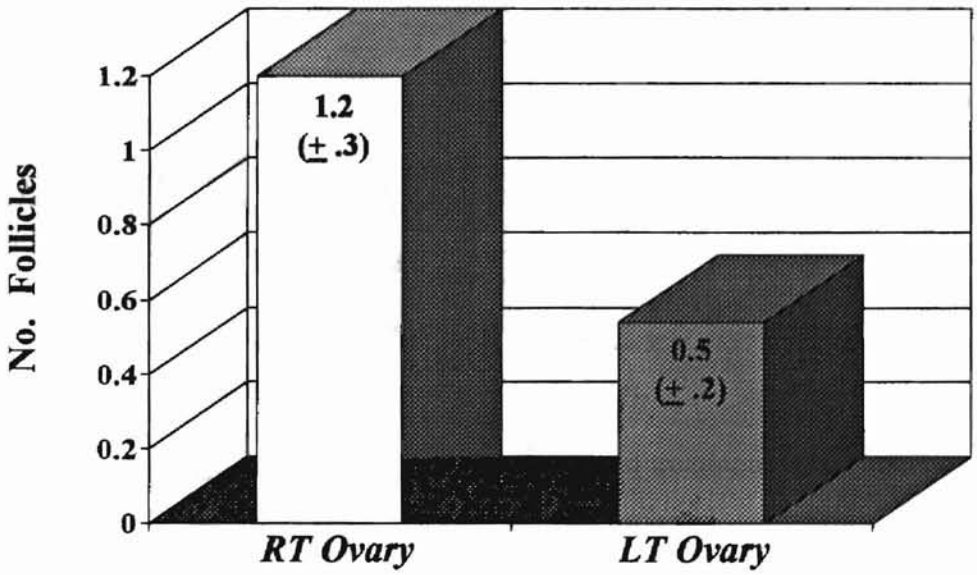

Figure 4. Influence of ovarian side (Right, RT; Left, LT) on the number of large (≥ 5.0) ovarian follicles (P< .08)

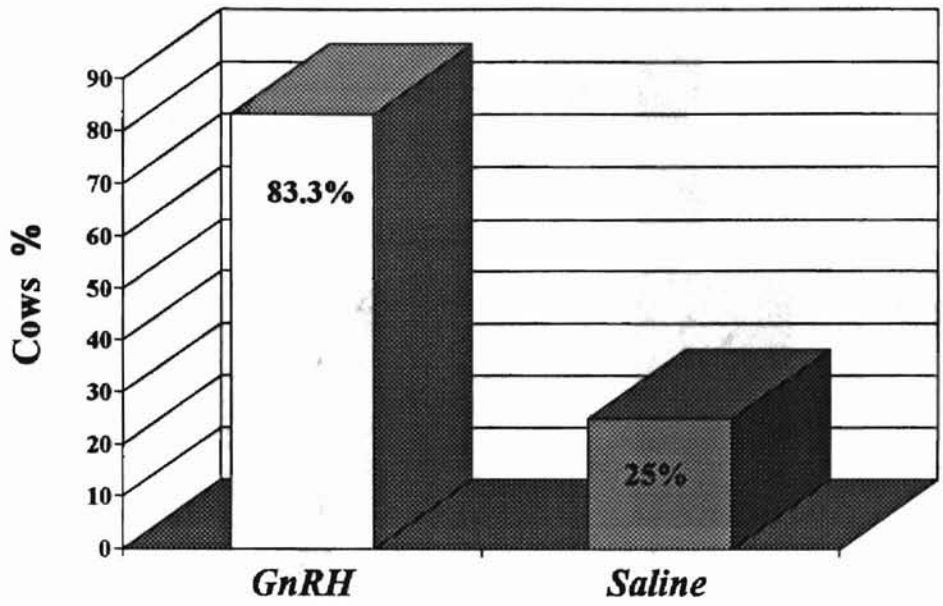

Figure 5. Influence of GnRH treatment on percentage of cows with plasma estradiol > than 1 pg/mL ($p < .002$)

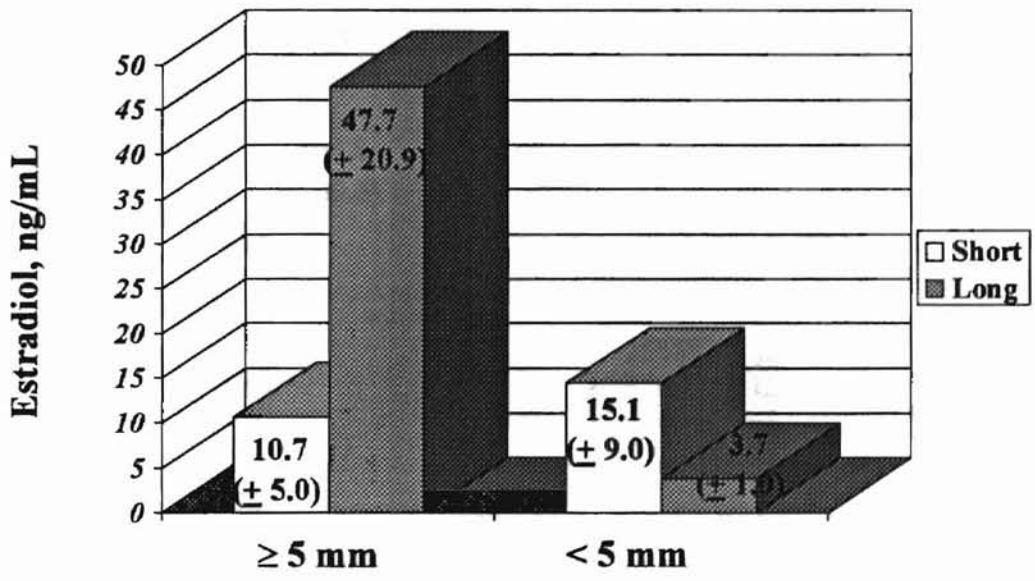

Figure 6. Influence of duration of anovulation and follicular size on concentration of estradiol in follicular fluid ($P < .11$)

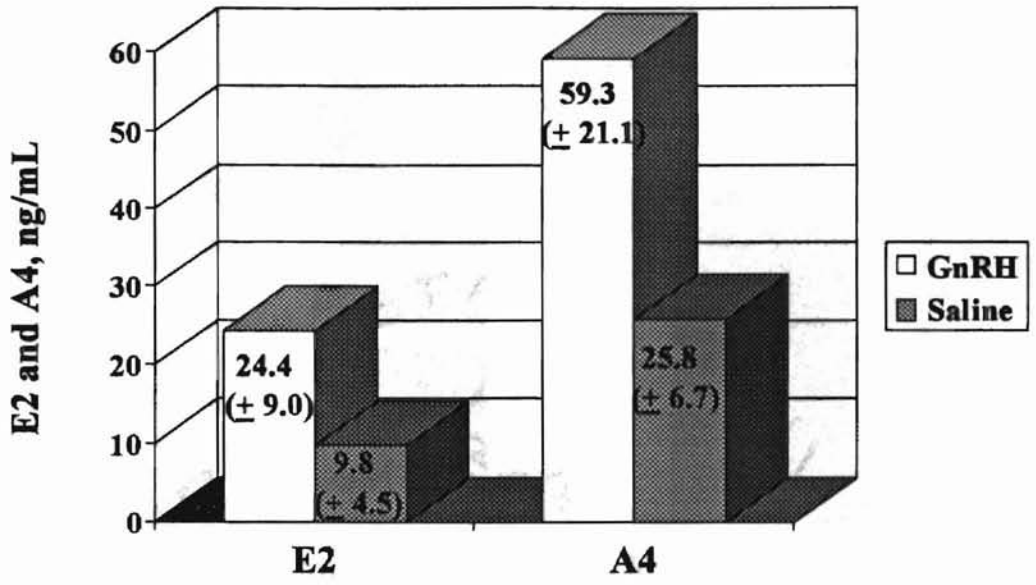

Figure 7. Influence of treatment with GnRH on estradiol (E2; P < .12) and androstenedione in follicular fluid (A4; P < .09)

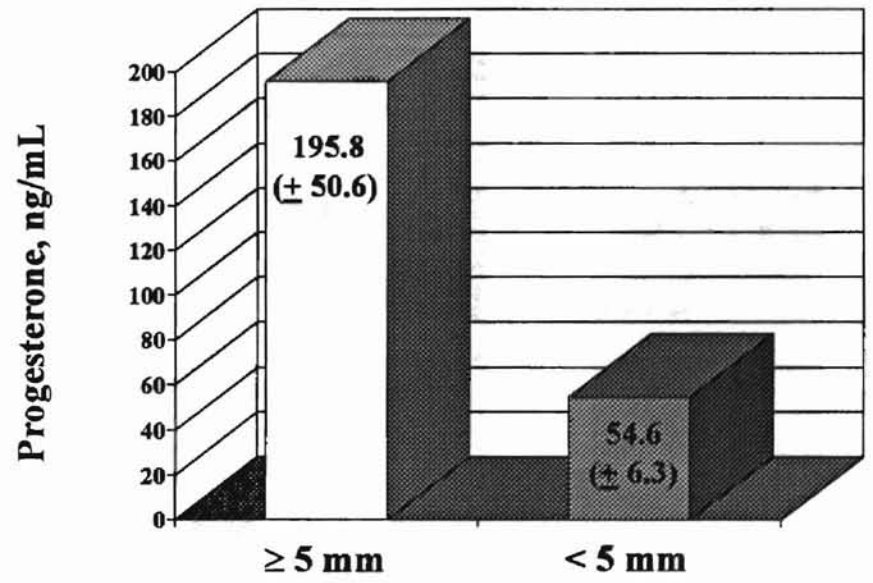

Figure 8. Influence of follicular size on concentrations of progesterone (P < .0001) in follicular fluid

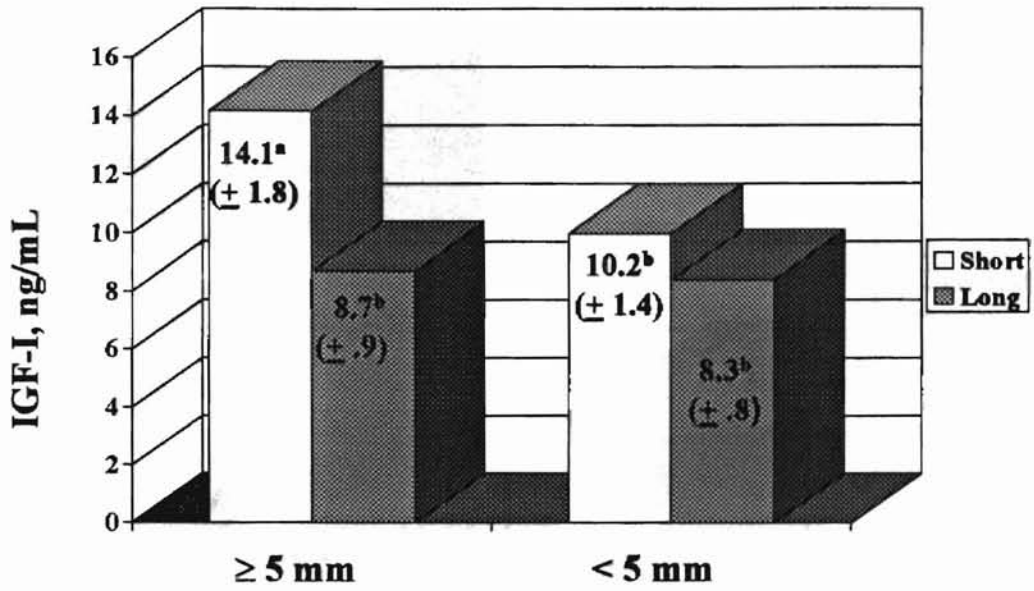

Figure 9. Influence of duration of anovulation and follicular size on concentration of IGF-1 in follicular fluid (P < .02)

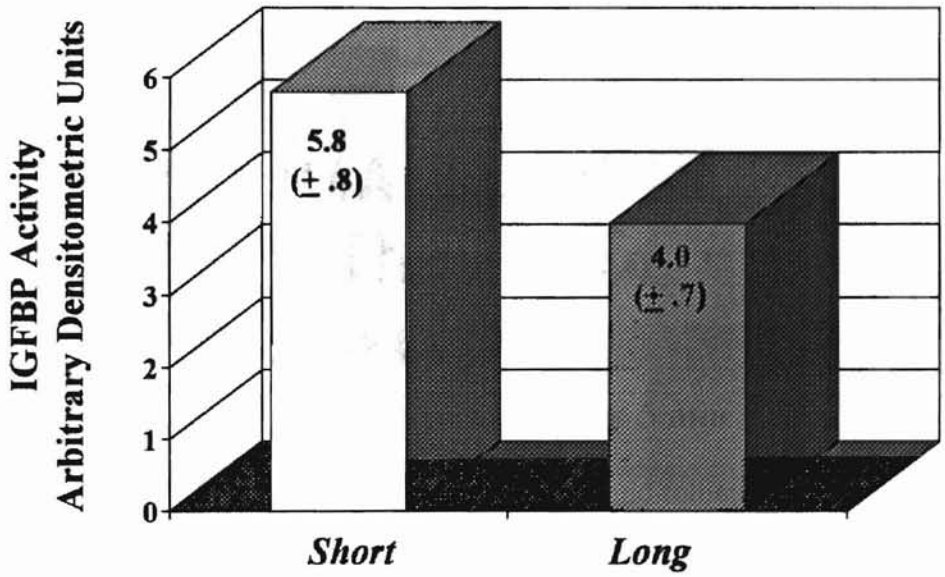

Figure 10. Influence of duration of anovulation on the 20 kDa IGFBP (P< .10) in follicular fluid

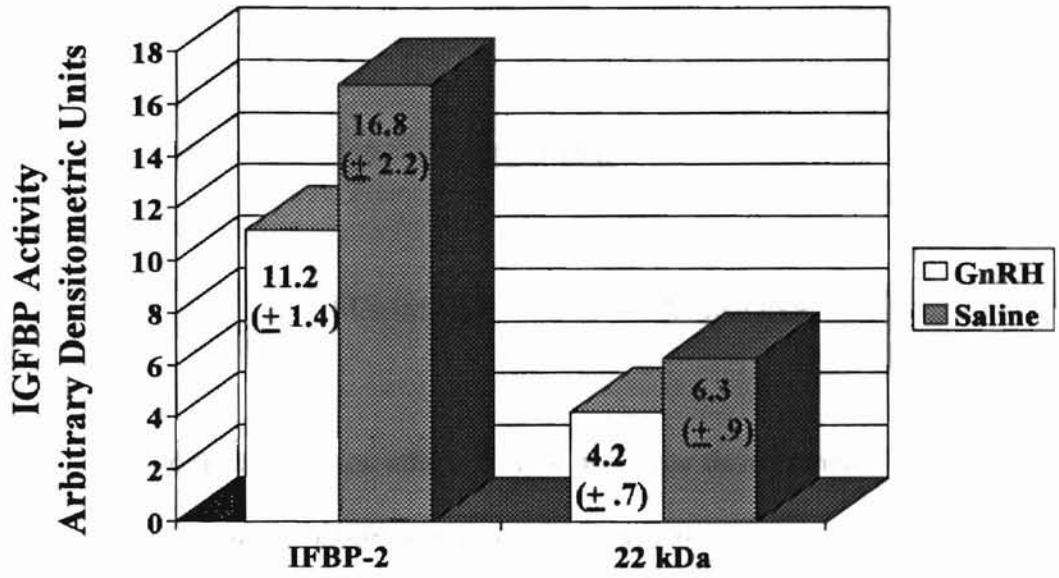

Figure 11. Influence of treatment with GnRH on IGFBP-2 and the 22 kDa IGFBP in follicular fluid (P< .07)

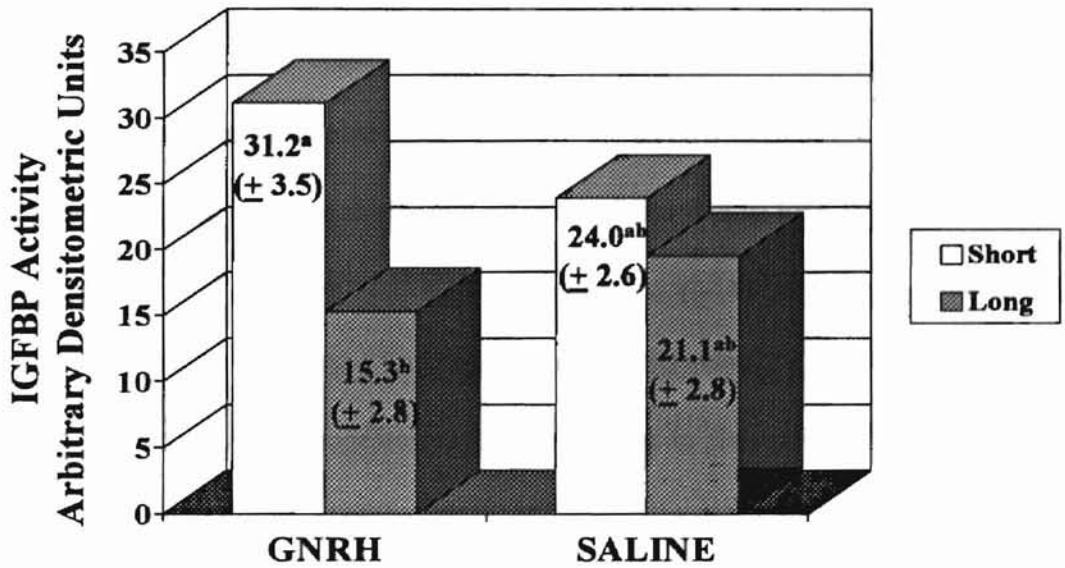

Figure 12. Influence of duration of anovulation and treatment on IGFBP-3 in follicular fluid (P< .09). [a,b,c] Means without a common letter differ (P< .05)

CHAPTER IV

SUMMARY AND CONCLUSIONS

Nutritional deprivation affects ovarian follicular development in beef cattle and this effect is mediated by decreased secretion of LH from the pituitary. The effect of GnRH treatment on ovarian function of nutritionally restricted cows, after 4 wk or 18 wk of anovulation has not been evaluated. The objectives of this study were to determine the effect of pulsatile treatment with GnRH on follicular growth and concentrations of hormones in plasma and follicular fluid of nutritionally induced anovulatory cows at 4 weeks or 18 weeks after cessation of luteal activity.

Twenty-four nonlactating, Hereford x Angus cows exhibiting normal estrous cycles were maintained in a drylot and fed a restricted diet to lose 1% of their initial body weight per week. Cows were fed a diet of prairie hay and mineral daily to maintain the anovulatory condition. Blood samples were collected every 7 d. Cows were determined to be anovulatory when progesterone concentrations in plasma were less than 1 ng/mL for three consecutive weeks.

A polyvinyl jugular was placed in each jugular vein of cows 2 d before initiation of treatment. One cannula was used for GnRH infusion and the other for collection of blood. Within each duration group (4 wk or 18 wk), cows were randomly assigned to receive

either 2 μg GnRH or saline (control) iv every hour for 5 d. Pulsatile infusions were administered via Harvard Infusion pumps. The pump-timer unit was calibrated to deliver the pulse of saline or GnRH (1.8 mL) in a 5 min interval. Blood samples (10 mL) were collected daily from d 1 through d 5 via cannulas. Ovarian follicular growth was assessed daily with rectal ultrasonography using an Aloka 500V ultrasound scanner with a 7.5 MHz transducer. Follicles were categorized as small (< 5.0 mm) and large (≥ 5.0 mm). Largest follicles that were growing at least 0.5 mm/d for three days were considered to be dominant follicles.

After 5 days of treatment, cows were exsanguinated within 2 h after the cessation of GnRH treatment. Uteri and ovaries were removed and transported to the laboratory. Ovaries were separated from uteri, follicles were counted and the diameter was measured with calipers. Follicular fluid from small follicles (<5.0 mm; pooled within ovary) and individual large follicles (≥ 5 mm) was collected. Samples were stored at -20°C until estradiol, progesterone, androstenedione, and IGF-I were quantified by radioimmunoassays and IGFBPs by one dimensional SDS-PAGE.

Cows became anovulatory when loss in weight resulted in a BCS of 3.45 ± .03 and a body weight of 350.8 ± 8.5 kg. Rate of growth of the largest follicle was greater when cows were treated with GnRH, but was not affected by duration of anovulation or the interaction between duration of anovulation and GnRH treatment. Maximum size of the largest follicle was greater for GnRH treated cows. At slaughter, size of the largest follicle was greater in cows that were anovulatory for 18 wk. However, the second largest follicle was greater in cows that were anovulatory for 4 wk versus 18 wk. Treatment of cows with GnRH that were anovulatory for 4 wk, increased the number of

small follicles compared with control cows and treated and control cows that were anovulatory for 18 wk. However, control cows that were anovulatory for 18 wk had increased numbers of small follicles compared with GnRH treated cows. Number of large follicles was greater in the right ovary than the left ovary.

Duration of anovulation did not influence concentrations of estradiol or progesterone in plasma during treatment. However, treatment with GnRH increased the percentage of cows with greater than 1 pg/mL of estradiol in plasma. Eighty-three percent of cows treated with GnRH had greater than 1 pg/mL of estradiol at one or more sampling times. Concentrations of progesterone in plasma during the 5 d of treatment were less than 1 ng/mL for treated and control cows.

Concentrations of steroids in follicular fluid were influenced by duration of anovulation x follicular size. Concentrations of estradiol tended to be greater in large than small follicles of 18 wk anovulatory cows, and GnRH-treated cows tended to have greater concentrations of estradiol in follicles after the long duration of anovulation. Duration of anovulation and size of follicles did not influence concentrations of androstenedione in follicular fluid. Concentrations of androstenedione were greater in follicular fluid of GnRH-treated than control cows. This indicates that androgen synthesis by theca cells was not a factor the limited the production of estradiol when animals were treated with GnRH. Duration of anovulation and GnRH treatment did not influence concentration of progesterone in follicular fluid. However, concentrations of progesterone in follicular fluid were greater in large follicles.

There was a duration of anovulation x follicular size effect on IGF-I conentrations in follicular fluid. Cows that were anovulatory for 4 wk had greater IGF-I concentrations

in large follicles compared with small follicles and large and small follicles of 18 wk anovulatory cows. However, in cows that were anovulatory for 18 wk, concentrations of IGF-I were similar in large and small follicles. Treatment with GnRH did not influence concentrations of IGF-I in follicular fluid. The amount of the 20 kDa IGFBP was greater in 4 wk anovulatory cows than 18 wk anovulatory cows. Treatment with GnRH influenced the amount IGFBP-2 (34 kDa IGFBP) and the 22 kDa IGFBP, but duration of anovulation did not influence the amounts of these binding proteins. Control cows had more IGFBP-2 and the 22 kDa IGFBP than GnRH treated cows. Duration of anovulation x treatment affected the amount of IGFBP-3. Cows that were anovulatory for 4 wk and were treated with GnRH had greater IGFBP-3 than cows that were anovulatory for 18 wk. This suggests that specific intrafollicular proteinases degrade the amounts of the IGFBP-2 and the 22 kDa-IGFBP, but not IGFBP-3. Follicular size did not influence the amount of IGFBPs, and the 29-32 kDa (likely IGFBP-5) was not affected by any of the factors evaluated.

Growth rate of follicles and concentration of hormones in follicular fluid, as well as decreased amount of low molecular weight IGFBPs, can be altered by treatment of nutritionally induced anovulatory cows with GnRH regardless of the duration of anovulation. However, follicular size, number of follicles, IGFBPs and hormones in follicular fluid were only affected by duration of anovulation when it interacted with GnRH treatment or follicular size. These results indicate the importance of GnRH as a major factor that regulates ovarian function in nutritionally induced anovulatory beef cows.

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VITA

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