IMMUNIZATION AGAINST GONADOTROPIN RELEASING HORMONE ALTERS PUBERTY, SECRETION OF LUTEINIZING HORMONE AND OVARIAN ACTIVITY IN BEEF HEIFERS

By

CATHERINE MARIE O'CONNELL

Bachelor of Science in Agriculture

University of Tennessee at Martin

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Thesis Approved:

Robert P. Wettemann
Thesis Advisor

Rodney D. Geisert

Keith S. Lusby

Norman N. Durham
Dean of the Graduate College

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CHAPTER I

INTRODUCTION

Gonadotropin releasing hormone (GnRH) is necessary to establish and maintain normal estrous cycles in females. After synthesis by hypothalamic neurons, GnRH is released into the hypophyseal portal system and transported to the anterior pituitary where it stimulates the synthesis and release of luteinizing hormone (LH) and follicle stimulating hormone (FSH). Physical separation of the pituitary from hypothalamic control by pituitary stalk transection and hypothalamic lesions, have been used in rodents, primates and domestic animals to study the physiology and function of the pituitary gland in the absence of GnRH. Hypothalamo-pituitary disconnection causes a reduction in gonadotropins and other pituitary hormones and abolishes reproductive function (Clarke et al., 1983).

Selective inhibition of endogenous GnRH by active immunization has been achieved in many species including rats (Arimura et al., 1973), sheep (Lincoln and Fraser, 1979; Adams and Adams, 1986), horses (Garza et al., 1986), pigs (Falvo et al., 1986; Esbenshade and Britt, 1987), and cattle (Robertson et al., 1981; Johnson et al., 1988). Males typically experience testicular atrophy and reduced

spermatogenesis (Schanbacher, 1984) while, immunization of females against GnRH prevents ovulation and estrous cycles (Jeffcoate and Keeling, 1984). Immunization against GnRH suppresses fertility by reducing gonadotropin and gonadal steroid concentrations without disrupting other pituitary hormones, that are essential for normal endocrine function (Adams and Adams, 1986).

Reproduction is the primary factor which limits production efficiency in beef cattle (Koch and Algeo, 1983). Further evaluation of the physiological function of GnRH on gonadotropin secretion, follicular development, induction of the preovulatory LH surge and gonadal steroid feedback mechanisms will help to elucidate the role of GnRH during puberty, estrous cycles and anestrous in beef cattle.

Immunization against GnRH has potential for practical application in the livestock industry. Heifers which are pregnant upon entering the feedlot are an economic liability. Increased costs and possible mortality may occur if small heifers are aborted or allowed to calve. Cycling heifers in the feedlot are less efficient due to increased physical activity concurrent with estrus. The ability to induce sterility in heifers could be used to prevent estrous behavior and reduce the number of pregnant heifers entering feedlots.

The objectives of this study were: to determine the effects of active immunization of heifers against GnRH on puberty and secretion of LH, to evaluate the functional

ability of the pituitary after immunization and to determine the reversibility of the suppressive effects of immunization against GnRH.

CHAPTER II

REVIEW OF LITERATURE

Puberty in Heifers

Puberty in heifers is defined as the age at first expressed estrus with ovulation (Bearden and Fuquay, 1984). Kinder et al. (1987) further define puberty to include behavioral estrus accompanied by the development of a corpus luteum that is maintained for a period characteristic of a particular species. Puberty in heifers is dependent upon a variety of both genetic and environmental factors and is associated with a change from a state of ovarian inactivity to one in which regular ovulations occur. This maturational process involves an increase in the secretion of the pituitary gonadotropins, LH and FSH, to amounts sufficient to stimulate follicular growth, oocyte maturation and ovulation.

Puberty normally occurs about 11-15 months after birth in beef heifers (Jainudeen and Hafez, 1987), although it can occur anytime between 6 (Glencross, 1984) and 24 (Robinson, 1977) mo of age. Age at puberty is an important factor that influences optimum reproductive performance in a cow herd. Early onset of sexual maturity provides an economic advantage by increasing an animals lifetime reproductive

rate. Heifers bred to calve as 2 yr olds wean more calves during their lifetime than heifers which calve at 3 yr of age (Short and Bellows, 1971). Furthermore, heifers which calve early in the season continue to calve early in subsequent years and wean heavier calves (Short and Bellows, 1971; Lesmeister et al., 1973).

Factors influencing puberty in heifers

Breed. Age at puberty of heifers is influenced by breed (Wiltbank et al., 1966; Gregory et al., 1978; Stewart et al., 1980; Ferrell, 1982). Bos indicus cattle generally reach puberty later than Bos taurus breeds (Laster et al,. 1976). Breeds differ in the mean age and weight at which they reach puberty. Heterosis affects age at puberty in addition to those effects exerted through growth rate in heifers (Wiltbank et al., 1966). Similarly, Laster et al. (1972) indicated that heterosis and maternal effects influence age at puberty, but not weight at puberty. Breeds selected for greater milk production attain puberty at an earlier age and lighter weight than those selected solely for beef production (Laster et al., 1979). This may result from direct maternal effects expressed through greater preweaning gain by calves of breeds that produce more milk (Arije and Wiltbank, 1971).

Weight and body condition. Heifers reach puberty at a certain weight rather than age when reared on different planes of nutrition (Crichton et al., 1959; Wiltbank et al.,

1966). The timing of puberty is more closely related to body weight as opposed to chronological age. It has been proposed that puberty is initiated after a critical body weight is achieved. Frisch and Revelle (1970) suggest that puberty in humans occurs at a common body weight rather than a specific age. The influence of body weight on puberty in cattle is conflicting. Although puberty was initiated earlier when a greater plane of nutrition was maintained, it was also evident that increased feed consumption was associated with heavier weights at puberty in heifers (Short and Bellows, 1971).

It has also been proposed that puberty occurs after an alteration in metabolic rate caused by changes in body fatness. This theory is based on findings in which the ratio of body water to body weight and body fat at first estrus is similar between rats fed high and low energy diets (Frishe et al., 1977). Work by Siebert and Field (1975) indicates that the onset of estrous cyclicity in heifers is closely related to content of body fat. This study utilized body weight and total estimated body water at puberty to predict a body fat content of 18.8 to 21.8 kg at puberty. Results of Brooks et al. (1985) are inconclusive, however, concerning both the critical weight and body composition hypotheses for puberty in heifers.

Growth rates before and after weaning are important factors influencing the onset of pubertal estrous cycles. Greater preweaning growth rates and heavier weaning weights

are associated with early puberty in heifers (Arije and Wiltbank, 1971). Similarly, reduced rates of postweaning gain inhibited the onset of puberty in heifers from early maturing breeds (Ferrell, 1982). In contrast, Rhodes et al. (1978) suggest that increased growth rate and increased fattness in beef heifers does not necessarily result in an earlier onset of puberty. Heifers were fatter and grew faster when fed a protein encapsulated fat which bypassed the rumen, however, age at puberty was increased.

Nutrition. Nutrient intake influences the time of initiation of reproductive cabability. Adequate nutrient intake allows the necessary prepuberal increase in LH secretion to occur (Schams et al., 1981; Day et al., 1984). Variation in feed intake affect the age at which heifers reach puberty (Joubert, 1954; Wiltbank et al., 1969). Heifers maintained on a lower plane of nutrition reached puberty at an older age (Day et al., 1986). Day et al. (1984) restricted dietary energy intake to delay puberty and demonstrated that LH secretion remained responsive to the negative feedback effects of estradiol. Dietary restriction of energy may act by extending the period of suppressed LH secretion due to estradiol negative feedback, since the negative effects of estradiol on LH secretion lessen upon initiation of a high energy diet (Day et al., 1984). Similarly, an increase in nutritional energy intake stimulated ovulation in heifers maintained in a prepuberal condition (Gonzalez-Padilla et al., 1975a).

Feed intake is related to the initiation of estrous cyclicity, however, the qualitative aspects of the diet may also be important. Volatile fatty acids (VFA) supply 50-85% of the metabolizable energy available to ruminants (Owens and Goetsch, 1988). The total concentration of VFA's in rumen fluid is dependent upon the composition of the diet. Moseley et al. (1978) indicated that the addition of dietary monensin to the diet may reduce the age at puberty in heifers. Monensin is a biologically active compound which acts by increasing the ruminal production of the VFA, propionate (Raun et al., 1976). McCartor et al. (1979) further investigated the effects of monensin in heifers and found that the age at puberty was reduced when ruminal propionate production was increased either by the addition of monensin to the diet or by an increase in the percentage of concentrate in the diet. Bushmich et al. (1980) reported a greater ovarian response in prepuberal heifers fed monensin and challenged with FSH, which included an increase in ovarian weight, more corpora lutea and greater follicular growth. Furthermore, increased propionate in the rumen resulted in greater LH release from the pituitary in response to exogenous GnRH administration in heifers fed monensin (Randel and Rhodes, 1980) or administered abomasal infusions of propionate (Rutter et al., 1983). Similarly, when a large portion of energy and protein in the diet was rumenally bypassed, thus, decreasing the quantity of fermentable carbohydrates in the rumen, heifers were older

and fatter at puberty (Rhodes et al., 1978). These results indicate that propionate is involved in the control of endocrine secretions associated with the onset of estrous cycles. However, it has not been established whether propionate directly affects reproductive function or if the mechanism is related to an alteration in metabolizable energy levels.

Season and photoperiod. Reproductive activity in mature cattle is not restricted to a particular time of the year. However, a correlation exists between date of birth and age at puberty in heifers (Menge et al., 1960; Schillo et al., 1982a; Little et al., 1981). Spring-born heifers reached puberty at an earlier age than those born in the fall (Menge et al., 1960). In addition, a winter environment prolonged the onset of puberty (Grass et al., 1982). These results indicate the possibility that prepuberal heifers may be responsive to photoperiod effects. Part of the seasonal effect on puberty may be attributed to photoperiod since supplemental lighting has been shown to hasten the onset of estrous cycles in heifers (Peters et al., 1978; Hansen et al., 1982; Hansen et al., 1983). The mechanism through which season exerts its stimulatory effects may involve changes in growth rate, ovarian sensitivity, or secretion of gonadotropins. Peters et al. (1978) indicate that supplemental lighting during the winter increases average daily gain. Furthermore, growth rate and feed efficiency were increased in heifers exposed to 16 h

light/d which resulted in reduced age at puberty (Petitclerc, 1983). In contrast, earlier age at puberty for heifers exposed to spring-fall conditions, while in environmental chambers (Schillo et al., 1983), or supplemental lighting (Hansen et al., 1983) was not related to an increase in body weight. Kamwanja et al. (1980) concluded that the influence of season was mediated by mechanisms independent of growth or feed intake. Hansen et al (1983) indicated that early puberty associated with supplemental light exposure was accompanied with an increase in ovarian volume. Exposure of prepuberal heifers to 18 h of light/d during the winter resulted in a greater estrogen induced LH release after ovariectomy (Hansen et al., 1982). These results suggest that photoperiod may exert its effects by stimulating the rate of maturation of the estradiol positive feedback system. Season and photoperiod are closely related and appear to influence the timing of sexual maturity in heifers so that they calve in the spring or summer (Kinder et al., 1987).

Social environment. In many species, age at first ovulation is influenced by the social environment (Vandenburgh, 1967; Eastham et al., 1984). The presence of a male hastens the onset of puberty and the onset of the breeding season in gilts (Brooks et al., 1970; Kirkwood et al., 1980) and mice (Bronson et al., 1975). Studies regarding the influence of social environment on first estrus in heifers are not conclusive. Neither short term

(Bernardinelli et al., 1978) nor long term (Roberson et al., 1987) exposure of prepuberal heifers to mature bulls altered the age at the onset of puberty. In contrast, bull exposure successfully increased the percentage of heifers reaching puberty prior to the breeding season (Pennel et al., 1986). Social interaction of prepubertal heifers with estrous heifers also failed to decrease the age or weight at first estrus (Roberson et al., 1983). However, Izard and Vandenbergh (1982) found that application of bull urine into the nose and mouth of heifers increased the percentage of heifers reaching puberty. This response is thought to be mediated by a pheromone present in bull urine. An androgen priming pheromone present in the urine of adult male mice accelerates puberty in female mice (Colby and Vandenbergh, 1974). Conflicting results on the influence of social environment on puberty in heifers suggest that the length of exposure and form of stimulation may be important in determining the physiological response of heifers to the social environment.

Endocrine regulation of puberty in heifers

Puberty is achieved through a gradual maturational process which begins before birth and continues through the prepuberal period. The onset of estrous cyclicity is regulated by endocrine factors, specifically hypothalamic hormones, pituitary gonadotropins and ovarian steroids. Ovarian function is controlled by the hypothalamus and

pituitary gland. Sexual maturation is influenced by the synthesis and secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary in response to synthesis and secretion of GnRH by the hypothalamus.

Gonadotropin secretion. LH is secreted in a pulsatile manner and concentrations of LH in blood fluctuate greatly from minute to minute. Blood serum concentrations of LH in heifers increased from birth to 3 mo of age, declined from 3 to 6 mo and then gradually increased until puberty (Schams et al., 1981). Mean concentration of LH in serum increased prior to first estrus (Swanson et al., 1972; Day et al., 1984). Gonzalez-Padilla et al. (1975a) failed to observe an increase in concentrations of LH in serum at 2 mo prior to the first ovulation. Concentrations of LH decreased as puberty approached and then fluctuated considerably preceding puberty. Basal concentrations of LH, however, were greater during the prepuberal period than after the onset of estrous cycles.

Schams et al. (1981) found increased frequency and decreased amplitude of LH pulses from 1 mo of age until puberty in heifers. Similarly, Kinder et al. (1987) observed increased LH pulse frequency prior to puberty. Frequency of LH pulses was a better predictor of age at puberty than was amplitude or mean LH, indicating that pulse frequency probably plays a major role in the initiation of cycles. Increasing mean concentration of LH was attributed

to an increase in LH pulse frequency near puberty (Dodson et al., 1988). In contrast, McLeod et al. (1984) found no increases in LH pulse frequency with increasing age.

Pituitary content of FSH (Desjardins and Hafs, 1968) and concentration of FSH in serum (Gonzalez-Padilla et al., 1975a; Dodson et al., 1988) were greatest shortly after birth, then declined and remained relatively constant until puberty. Static concentrations of FSH near puberty (Dodson et al., 1988) suggest that FSH plays a permissive role in the onset of puberty. In addition, FSH in prepuberal heifers did not differ significantly from concentrations during the adult estrous cycle (Akbar et al., 1974).

Desjardins and Hafs (1968) observed greater content of LH and FSH in the pituitary in prepuberal verses postpuberal heifers. Furthermore, the onset of puberty was associated with a reduction in concentrations of LH in the pituitary, which may reflect increased serum concentrations that have been observed in other studies (Swanson et al., 1972; Schams et al., 1981; Day et al., 1984).

Exogenous GnRH administration resulted in an increase in LH and FSH secretion from the pituitary of prepuberal heifers (Schams et al., 1981; McLeod et al., 1985). Moreover, responses to GnRH injections were greater as age increased (Schams et al.,1981). Frequent administration of GnRH (2 ug/2h) to 5 mo old heifers elicited a preovulatory surge of gonadotropins (McLeod et al., 1985). This suggests that the anterior pituitary of the sexually immature heifer

has the ability to synthesize and store LH long before the onset of pubertal cycles. Secretion of FSH in response to GnRH treatment is extremely variable between animals (McLeod et al., 1984). In summary, gonadotropins are available for secretion by the pituitary of the prepubertal heifer and are released in response to exogenous stimulation long before puberty occurs.

Ovarian steroids. Estradiol has both positive and negative feedback effects on LH secretion (Karsch, 1987). In sexually mature bovine females, the preovulatory surge of LH is preceded by an increase in estradiol in plasma. During the follicular phase of the estrous cycle, increasing estradiol concentrations (Wettemann et al., 1972; Echternkamp and Hansel, 1973) probably act at the hypothalamo-pituitary axis to stimulate the preovulatory surge of LH. The opposite effect is apparent in the prepuberal heifer in which case estradiol is inhibitory to secretion of LH (Gonzalez-Padilla et al., 1975b). The inhibitory effects of estradiol on LH secretion begin early in life and continue throughout the prepuberal period. Furthermore, an increase in secretion of LH is detectable after ovariectomy of prepuberal heifers (Day et al., 1984; Anderson et al., 1985). It has been proposed that ovarian estradiol acts at the hypothalamus to suppress pulses of GnRH (Kinder et al., 1987). This results in inadequate amounts of LH secreted to produce follicular growth and ovulation.

The initiation of puberty occurs as a result of increased gonadotropin secretion. The "gonadostat hypothesis" (Dodson et al., 1988) explains the pubertal increase in LH secretion. This theory suggests that reduced concentrations of LH are maintained due to the sensitivity of the hypothalamo-pituitary axis to inhibitory feedback of estradiol (Kinder et al., 1987). The first ovulation occurs when the sensitivity of the hypothalamo-pituitary axis to estradiol declines. As a result, secretion of LH increases and stimulates follicular growth, thereby enhancing estradiol secretion and inducing the preovulatory surge of gonadotropins (Kinder et al., 1987). Supporting the gonadostat hypothesis, Schillo et al. (1982b) found that estradiol administration to prepuberal heifers suppressed secretion of LH. Furthermore, LH suppression after estradiol lasted longer in younger heifers than in older prepubertal heifers. Exogenous estradiol administration inhibited the increase in secretion of LH that normally occurs after ovariectomy in sexually immature heifers (Staigmiller et al., 1979; Day et al., 1984). The inhibitory effects of estradiol on suppression of LH decreased in ovariectomized heifers, concurrently with first ovulation in intact heifers of the same age (Day et al., 1984). The negative feedback actions of estradiol on pulsatile secretion of LH in sheep have been studied in detail. Prior to the first ovulation in ewes, estradiol suppresses LH secretion by inhibiting pulse frequency.

Gradually the potency of estradiol negative feedback declines and becomes ineffective in suppressing pulse frequency (Foster et al., 1985).

The involvement of opioid neuropeptides in the process of sexual maturation in the female has been suggested. A decrease in opioid inhibition of LH secretion is associated with puberty in the rat (Bhanot and Wilkinson, 1983). Wolfe et al. (1989) indicated that opioid neuropeptides and estradiol appear to interact in regulating the secretion of LH associated with puberty in heifers. Opioids may control the responsiveness of the hypothalamus to estradiol. A decrease in the concentration of estradiol receptors in the anterior and medial basal hypothalamus and the anterior pituitary is associated with the time of puberty in heifers (Kinder et al., 1987). Opioids may act by altering the concentrations of estradiol receptors in the hypothalamus. A decline in estradiol receptor concentration may result in reduced negative feedback effects of estradiol on LH secretion during maturation.

Temporary increases in concentrations of progesterone, in blood have been demonstrated prior to first estrus in heifers (Gonzalez-Padilla et al., 1975a; Schams et al., 1981; Glencross 1984). The source of this progesterone is probably of ovarian origin, due to the presence of compact luteal tissue located on the ovary (Berardinelli et al., 1979). Corah et al. (1974) and Humphrey et al. (1976) reported a similar phenomenon in postpartum beef cows.

Concentrations of progesterone increased and remained elevated for 2 to 7 days prior to the resumption of estrous cycles. Ovariectomy revealed that elevated progesterone prior to first estrus in cows was from ovarian luteal tissue formed after ovulation without estrus (Castenson et al., 1976). Ovulation does not appear to be necessary for these temporary increases in progesterone, although it may occur (Berardinelli et al., 1979). Short lived luteal structures, similar to those in heifers, are not required for sexual maturation of ewes (Keisler et al., 1983). However, progesterone priming of ewes resulted in follicles with greater estradiol secretion and granulosa cells with increased capacity to bind LH compared with follicles of non-primed ewes (Hunter et al., 1987). Progesterone increases the sensitivity of granulosa cells to LH which may increase the response to the ovulatory LH surge. Gonzalez-Padilla et al. (1975a) suggested that progesterone may be acting to establish a phasic pattern of LH release. Short periods of elevated progesterone prior to puberty in heifers appear to function to synchronize the ovulatory and estrus systems, consequently the LH surge is associated with standing estrus and ovulation, followed by a normal luteal phase (Moran et al., 1989). Although progesterone appears to be a significant factor in the establishment of estrous cyclicity, the mechanism is not fully understood.

Function of GnRH in Reproduction

The structure of GnRH was revealed through isolation and characterization of porcine hypothalamic extracts (Matsuo et al., 1971). The releasing hormone is a decapeptide [(pyro)Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2]. Moreover, this structure appears to be common to all species studied. GnRH is synthesized by neurons in the hypothalamus and is released in a pulsatile manner into the hypophyseal portal vessels and stimulates anterior pituitary. Binding specifically to the pituitary gonadotroph cells (Naor and Childs, 1986), GnRH results in the synthesis and secretion of LH and FSH. Physical separation of the hypothalamus from the pituitary (Clarke et al., 1983) or active immunization against GnRH (McNeilly et al., 1986) causes reduced secretion of gonadotropins and an impairment of reproductive function.

The mechanisms controlling the secretion of GnRH are not fully understood. Release of GnRH from storage sites in the hypothalamus of ewes is pulsatile (Clarke and Cummins, 1982) and regulated by the hypothalamic GnRH pulse generator. Pulsatile secretion of LH in many species, further supports that GnRH release is pulsatile. Pulses of GnRH in hypothalamic portal blood correspond with LH in jugular blood of sheep (Clarke and Cummins, 1982), indicating that LH secretion occurs as a direct result of pulsatile GnRH stimulation.

Stage of the estrous cycle directly influences the secretion of LH in the cyclic cow (Rahe et al., 1980) and ovarian steriods may act to modify the pattern of LH secretion. During the estrogen dominated follicular phase of the estrous cycle, LH pulses were more frequent than in the progesterone dominated luteal phase (Schallenberger et al., 1984). Ovarian steroids may influence the secretion of gonadotropins by directly affecting the release of hypothalamic GnRH, or by altering the sensitivity of the pituitary to GnRH stimulation.

Existence of a separate releasing hormone for FSH has been suggested due to variations in secretion of FSH and LH. Reevaluation of bovine hypothalamic extracts found no evidence to support this proposal (Schally et al., 1976), however, the subject remains controversial. Knobil (1980) found that changes in the frequency of GnRH stimulation affected the circulating concentrations of the gonadotropins and the ratios of LH to FSH. Alterations in the frequency of GnRH stimulation have been demonstrated to influence gonadotropin subunit mRNA expression in vivo (Dalkin et al., 1989). Since GnRH pulse frequency changes throughout the estrous cycle, differences in the synthesis and release of LH and FSH may be related to differences in gene expression, thus supporting the existence of a single releasing hormone for both gonadotropins.

The number of receptors for GnRH vary under different physiologic conditions (Yenn, 1986). Clayton et al. (1982)

concluded that GnRH regulates its own receptor number at the pituitary and consequently, the synthesis and secretion of gonadotropins. Thus, it appears that the number of GnRH receptors reflects changes in the secretion of GnRH. Continuous GnRH stimulation results in reduced synthesis and secretion of gonadotropins due to down regulation of GnRH receptors (Knobil, 1980). In contrast, pulsatile administration of GnRH increased the concentration of GnRH receptors in seasonally anestrous ewes (Khalid et al., 1987). Furthermore, Amann et al. (1986) found increased numbers of GnRH receptors in the pituitary of bull calves after the onset of pulsatile LH release.

Endogenous opioid peptides (EOP) have been implicated in the control of LH secretion in many species. Evidence indicates that EOP inhibit the secretion of LH (Leshin et al., 1988). Treatment with an opioid receptor antagonist (naloxone) resulted in increased LH secretion in postpartum cows (Whisnant et al., 1986). In contrast, naloxone administration failed to alter LH secretion in cycling cows but was successful in cycling heifers and postpartum anestrous cows (Mahmoud et al., 1989). Hence, it was suggested that EOP involvement during the estrous cycle of the cow was possibly related to age, parity, or stage of the estrous cycle. Short et al. (1987) found that opioids controlled LH secretion during the follicular phase but not during the luteal phase of the estrous cycle in heifers. Administration of naloxone did not alter serum LH in luteal

phase cows (Schoenemann et al., 1990). Similarly, naloxone administration did not result in increased LH secretion in postpartum beef cows pretreated with progesterone to simulate the luteal phase (Cross et al., 1987). However, in gilts (Barb et al., 1986) and ewes (Malven et al., 1984), naloxone stimulated an increase in LH only during the luteal phase. Thus, it may be possible that progesterone during the luteal phase of the bovine cycle directly inhibits LH by a mechanism independent of EOP. The mechanism by which EOP exert their effect on LH is thought to be through the regulation of GnRH secretion from the hypothalamic neurons (Kalra and Kalra, 1983). Administration of the opioid, morphine, suppresses the electrophysiological activity of the GnRH pulse generator (Kesner et al., 1986).

Immunization against GnRH

Following the availability of synthetic GnRH, attempts were made to develop radioimmunoassays to measure endogenous hormone concentrations. Production of antibodies to GnRH was often accompanied by gonadal atrophy (Arimura et al., 1973). Since then, neutralization of GnRH has been successfully accomplished by active or passive immunization in several species including the rat (Fraser et al., 1974), ewe (Clarke et al., 1978), gilt (Esbenshade et al., 1985), mare (Garza et al., 1986) and heifer (Wettemann and Castree 1988). This technique is an effective way to evaluate the physiological role of GnRH in reproduction and has provided

an alternative method of fertility control in domestic species.

GnRH antibody production

Carriers. Due to its low molecular weight (1183), the endogenous GnRH peptide is not very immunogenic. To enhance immunogenicity the releasing hormone must be conjugated to a larger molecule and emulsified with an adjuvant (Jeffcoate et al., 1976). Conjugation of GnRH to a protein results in the most effective and reliable production of antisera (Fraser, 1980). The choice of carriers is dependent upon the species being immunized, to prevent the production of antibodies which could be detrimental to the animal. Several protein carriers have been successfully utilized including tetanus toxoid (Ladd et al., 1989; Upadhyay et al., 1989), keyhole limpet haemocyanin (Adams and Adams, 1986), human serum albumin (Wettemann and Castree, 1988), and ovalbumin (Goubau et al., 1989a). However, bovine serum albumin remains the most commonly used carrier protein (Clarke et al., 1978; Fraser et al., 1982; Esbenshade and Britt, 1985; Garza et al., 1986). Work by Goubau et al. (1989a) concluded that antibody response against GnRH is affected by the carrier used.

Methods of conjugation. Various methods have been utilized to couple GnRH to the desired carrier protein. The simplist and most extensively used method of conjugation involves the use of carbodiimide (Fraser, 1980).

Carbodiimides act by joining free amino or carboxyl groups on a peptide to respective groups on the carrier molecules (Fraser, 1980). Neither of these groups however, exists on the GnRH molecule, therefore the reaction must involve the hydroxyl groups on serine or tyrosine (Jeffcoate et al., 1976). Diazotization is a less frequently used technique in which two carbon rings are incorporated between GnRH and the protein carrier (Fraser et al., 1974) resulting in the formation of a chemical bridge. Furthermore, the glutaraldehyde condensation reaction has been used to polymerize GnRH with the chosen carrier (Jeffcoate and Keeling, 1984). Regardless of the method used, it is difficult to predict the resultant antibody specificity and the amount of GnRH incorporation to the carrier molecule.

Adjuvants. An adjuvant is a compound which enhances the normal immune response by slowing the release of antigen into the body (Tizard, 1984). Immunization procedures routinely utilize an adjuvant in conjuction with the GnRH-protein conjugate to further stimulate the immune response. Freund's complete adjuvant (FCA) is the best and most consistent stimulator of the immune response against a GnRH conjugate (Johnson et al., 1988; Goubau et al., 1989b). Use of FCA involves incorporating the antigen into a water in oil emulsion. Freund's complete adjuvant is composed of an emulsifier, mineral oil and Mycobacterium tuberculosis (Tizard, 1984). This extremely potent adjuvant has some disadvantages which include a local inflammatory response

resulting in the formation of a granuloma at the site of the injection (Goubau et al., 1989b). In addition, FCA interferes with the tuberculin test (Robertson et al., 1984) due to the presence of mycobacterial cell wall fractions. An additional adjuvant, Bortella pertussis has been given in combination with FCA (Fraser, 1975; Schanbacher, 1982) however, it does not necessarily increase the antibody response from that of FCA alone (Fraser, 1980). There is growing concern regarding the adverse effects which accompany the use of FCA, especially when considering the potential use of a commercial vaccine for domestic species. Presently, alternative adjuvants are being examined. Factors which need to be considered when selecting an adjuvant are its ability to consistently produce a sufficient, long lasting immune response with minimal side effects. Goubau et al. (1989b) compared various adjuvants and found FCA to elicit the greatest immune response in cattle. However, Alhydrogel showed some potential as an alternative adjuvant with no observed side effects. Furthermore, Silversides (1988) reported some success with with 2 additional adjuvants (Havlogen and dimethyldiocta-decylammonium bromide).

Booster immunizations are frequently given to increase GnRH antibody titer. The number of booster injections given varies between species and individuals because of differences in the immune response. When FCA is used with primary immunization, Freund's incomplete adjuvant (FIA) has

traditionally been used for booster immunizations, but FCA may be used as well. Freund's incomplete adjuvant is not as potent as FCA and does not cause adverse side effects.

GnRH protein conjugates have been administered by intradermal, subcutaneous, intramuscular and lymph node routes (Jeffcoate and Keeling, 1984). However, multisite intradermal and/or subcutaneous injections are used most frequently.

Specificity. The possibility exists that a single conjugate will generate antisera of varying specificity in different animals. Antisera formed as a result of unconjugated GnRH are generally not highly specific (Jeffcoate et al., 1976) and therfore not as useful as conjugated preparations. A conjugation procedure that gives consistently high specific antisera to GnRH has not been developed (Schanbacher, 1984).

Copeland et al. (1979) classified GnRH antisera into 3 groups according to their specificity. Highly specific antisera are termed conformational in that they are not directed against a portion of the hormone sequence but rather the overall configuration of the hormone. Sequential antisera, however, cross react with certain fragments of GnRH. The third group recognizes several fragments of GnRH but with no regard to sequence, suggesting that they are composed of several subpopulations of antibodies. Antisera which recognize several portions of the GnRH sequence have the potential of detecting that same sequence in a similar

molecule and as a result may cross react with other hormones.

Evaluation of antisera specificity is possible due to the availability of many GnRH analogs and synthetic fragments. Specificity has been evaluated by determining the ability of GnRH and GnRH analogs to compete with labeled GnRH for binding sites (Adams and Adams, 1986). In addition, measurement of other anterior pituitary hormones in actively immunized animals allows an additional assessment of specificity achieved. Complete antibody specificity is not necessary for immunization against GnRH in vivo (Fraser, 1980). However, it is important that resultant antisera do not inhibit other endogenous hormones.

Immunization against GnRH in vivo

Immunization techniques can be used to neutralize the effects of GnRH in vivo, thereby, allowing an assessment of the physiological role of the releasing hormone. Long term inhibition of the releasing hormone can be accomplished through active immunization and short term through the passive transfer of antibodies.

The inhibitory effects of GnRH antisera are proposed to act at the hypophyseal portal vessels by preventing GnRH from reaching the pituitary gonadotrophs (Lincoln and Fraser, 1979; Schanbacher, 1984). Clayton et al. (1982) demonstrated the necessity of GnRH for the maintenance of its own receptors at the pituitary. Gilts immunized against

GnRH had decreased GnRH receptors within the pituitary (Esbenshade and Britt, 1985). This work further supports the idea that antibodies bind at the hypophyseal portal system and prevent the releasing hormone from reaching the pituitary.

Passive immunization. Temporary neutralization of endogenous GnRH can be accomplished through passive immunization. Administration of antibodies provides specific, short term suppression of GnRH and is an ideal method to study the physiological role of the releasing hormone under a variety of conditions.

The most noticable effect of passively transferring antibodies occurs in females when ovulation is prevented. Infusion of antisera after maximal concentrations of estradiol during the estrous cycle inhibits ovulation by suppressing the preovulatory surges of LH and FSH in rats and ewes (Arimura et al., 1974; Fraser and McNeilly, 1982). These studies clearly show the necessity of GnRH for the LH surge and subsequent ovulation.

Pulsatile secretion of LH ceased immediately following administration of GnRH antisera in rams (Lincoln and Fraser, 1979) and during the late follicular phase of the cycle in ewes (Clarke and Cummins, 1982). Exogenous GnRH was unsuccessful in stimulating LH release from the pituitary the day following treatment, demonstrating the ability of antisera to rapidly neutralize exogenous hormone as well (Lincoln and Fraser, 1979). Frequent blood collection

enabled Fraser and McNeilly (1983) to demonstrate the immediate inhibitory effects of GnRH antisera on pulsatile LH release. They found that, although pulsatile secretion of LH was blocked, basal concentrations of gonadotropins were maintained. Estradiol was suppressed concurrent with inhibition of pulsatile LH, suggesting that the episodic secretion of LH is necessary to increase follicular estradiol secretion (McNeilly et al., 1984).

Passive immunization against GnRH drastically alters LH secretion. Unlike LH, FSH concentrations were relatively unaffected immediately following injection of specific antibodies against GnRH (Clarke and Cummings, 1982; Lincoln and Fraser, 1979; McNeilly et al., 1984). Failure of FSH to respond in these studies may be due to the removal of estradiol negative feedback effects on FSH due to immunization (Fraser and McNeilly, 1982). In contrast, following infusion of antisera against GnRH, no immediate response in FSH in serum was apparent, however, FSH concentrations were greater than controls within 24 hours of treatment (McNeilly et al., 1984). Studies have found a decline in concentrations of FSH following passive immunization against GnRH. However, the reduction in FSH occurred slower and to a lesser extent when compared to LH (McCormack et al., 1977; Gledhill et al., 1982). Complete inhibition of GnRH through the administration of antibodies is unlikely, suggesting that a small amount of GnRH may still be active, thus, preventing total suppression of

gonadotropin secretion. Minimal concentrations of GnRH in serum may enhance the release of FSH in immunized rams (Fraser, 1980). Several days were necessary to alter the secretion of FSH in rams administered pulsatile GnRH and when treatment was discontinued it took several days for FSH to decline (Lincoln, 1979). These results further demonstrate the difference in LH and FSH release following GnRH stimulation.

It is evident that LH and FSH are not controlled identically by GnRH due to the variation in responses to antisera against GnRH. Secretion of FSH appears unresponsive to short term removal or variation in GnRH release (Lincoln and Fraser, 1979; Fraser and McNeilly, 1983).

Normal ovulatory cycles after passive transfer of antibodies against GnRH have been reported to return anywhere from 1 to 6 wk (Fraser and McNeilly, 1982) after antisera administration. This corresponds to the reinitiation of normal circulating gonadotropin concentrations in ovariectomized animals given GnRH antiserum (Gledhill et al., 1982).

Passive transfer of antibodies has advantages over long term inhibition of GnRH in that the effects are immediate and use of predetermined antisera will limit some of the variation in response between different animals. Some species, however, may not respond as well as another to the same antisera. Passive immunization also allows

manipulation of the suppressive effects following transfer of antibodies. Administration of an immunologically crossreactive, biologically inactive, fragment of GnRH can be utilized to saturate the antibodies when desired (Fraser, 1975). There is a risk involved with introducing a large volume of foreign protein into an animal, making continued inhibition through repeated GnRH antisera injection impractical.

Active immunization. Active immunization against GnRH suppresses estrous cycles and prevents ovulation (Kerdelhue et al., 1976; Fraser and Baker, 1978). Selective inhibition of GnRH, through the production of antibodies, has been achieved in many species and is associated with a reduction in serum gonadotropin concentrations and gonadal steroids. Suppression of reproductive function using this technique may take 2-3 months to reach its maximum inhibitory capacity (Fraser and McNeilly, 1982), but, may be influenced by the immunization schedule. Active immunization of monkeys successfully inhibited ovulatory cycles, however, 2 to 3 normal cycles occurred after the primary immunization, demonstrating the slow increase in antibody production (Fraser, 1983). During the period when antibody titers against GnRH develop, a gradual decrease in the synthesis and release of gonadotropins occurs resulting in reduced follicular growth and consequently, decreased ovarian steriod secretion (Fraser and McNelly, 1982). The degree of GnRH neutralization can be assessed through the measurement

of GnRH antibody titers. Anti-GnRH titers are usually determined by the ability of serum dilutions to bind radiolabeled GnRH and are expressed as a percentage of radioactivity bound at a particular serum dilution or as a dilution which binds a predetermined quantity of labeled GnRH (Jeffcoate and Keeling, 1984).

Female. Leukocytic vaginal smears confirmed the disruption of estrous cycles in rats actively immunized against GnRH (Fraser, 1975) while basal progesterone concentrations verified the cessation of estrous cycles in gilts (Esbenshade and Britt, 1985) and heifers (Johnson et al., 1988; Wettemann and Castree, 1988) following active immunization. Immunization against GnRH in mares produced a condition similar to seasonal anestrus (Garza et al., 1986). Ovaries of GnRH immunized rats weighed significantly less than controls and contained no evidence of recently active luteal structures (Fraser, 1975), however, follicles were present at various stages of development. Similarly, active immunization of ewes against GnRH resulted in ovarian follicular growth, but an absence of corpora lutea (Clarke et al., 1978). Ovarian weight, number of follicles (>10mm) and number of corpora lutea were reduced in GnRH immunized mares (Garza et al., 1986) and heifers (Johnson et al., 1988) compared to control animals. Antibody titers are correlated with physiological changes that occur after immunization against GnRH. Ewes with greater GnRH titers had smaller ovaries and pale, small uteri (Jeffcoate et al.,

1978). Larger follicles, and uterine weights similar to nonimmunized controls were found in rats with low vs high titers against GnRH (Fraser and Baker, 1978). Thus, it appears that gonadotropin secretion is maintained when minimal titers are present, but concentrations are inadequate to produce an LH surge.

A rapid decline in concentrations of LH occurred following initial immunization of gilts against GnRH, however, FSH was unchanged until a booster was administered, then FSH was reduced to nondetectable amounts (Esbenshade and Britt, 1985). Adams and Adams (1986) found basal LH and FSH secretion and reduced pituitary stores of gonadotropins in ewes immunized against GnRH. In contrast, Clarke et al. (1978) failed to see a decrease in FSH in ewes immunized against GnRH. In the mare, FSH decreased but was still detectable after immunization against GnRH (Garza et al., 1986). Secretion of FSH appears to be less susceptible than LH to the effects of GnRH neutralization by immunization providing a possible explanation for maintained follicular development. Furthermore, an alteration in secretion of FSH varies among species in response to active immunization against GnRH.

Immunoneutralization of GnRH prevented the positive feedback responses of the gonadotropins from occurring following exogenous estradiol administration in ewes (Adams and Adams, 1986) and monkeys (Fraser, 1983). This may be due to a lack of releasable gonadotropins since pituitary

stores of gonadotropins in ewes immunized against GnRH are reduced (Adams and Adams, 1986). The positive feedback response to estrogen could be mediated at the hypothalamus through stimulation of GnRH secretion. If this is true, antibodies produced against GnRH may bind to the GnRH released from the hypothalamus and prevent the release of gonadotropins

Ovariectomized females are the ideal model to study the effects of immunization against gonadotropin secretion, due to elimination of steroid negative feedback effects on LH and FSH. Ovariectomized rats experienced decreased gonadotropins in serum and reduced pituitary content of LH and FSH following immunization against GnRH (Fraser, 1975). Immunoneutalization of GnRH prevented the normally occurring post ovariectomy increase in gonadotropins in ewes (Jeffcoate et al., 1978), gilts (Esbenshade and Britt, 1985) and mares (Garza et al., 1986). Thus, the normally occurring increase in gonadotropin secretion in post-ovariectomized animals is likely a result of increased GnRH secretion, since neutralization of GnRH by antibodies prevented the increase in gonadotropins.

Prolactin is frequently measured to evaluate antibody specificity and the functional ability of the pituitary gland after immunization against GnRH. Prolactin concentrations were unaltered following immunoneutralization of GnRH in rats (Fraser, 1975), gilts (Esbenshade and Britt, 1985) and ewes (Adams and Adams, 1986). An alteration in

prolactin release would be expected following separation of the pituitary from hypothalamic stimulation by physical methods. However, this is not the case when considering active immunization against GnRH due to the establishment of a selective barrier between the hypothalamus and pituitary gonadotrophs. In contrast, concentrations of prolactin increased in the serum of ewes immunized against GnRH, but decreased after ovariectomy to concentrations similar to those for controls (Clarke et al., 1978). Reasons for the increase in concentration of prolactin are unknown, but the authors suggest that changes in prolactin might be due to reduced concentrations of progesterone and normal estrogen concentrations occurring after cessation of estrous cycles.

Male. Selective immunoneutralization of GnRH in males results in a reduction in serum gonadotropins and testicular atrophy in several species (Fraser et al., 1974; Schanbacher, 1982; Robertson et al., 1984; Chase et al., 1988). Presence of anti-GnRH titers is frequently correlated with a reduction in concentrations of serum LH, FSH and testosterone, as well as, reduced testicular and accessory sex organ weights. Titers against GnRH in serum are not correlated with concentration of testosterone in serum or testis size, and a wide range of titers against GnRH (5-55%) inhibited normal testicular function (Chase et al., 1988). Evaluation of testicular histology in rats immunized against GnRH revealed a reduction in seminiferous tubule diameter, shrunken Sertoli cells, atrophied Leydig

cells and an absence of elongated spermatids in the lumen (Ladd et al., 1989). Production of antibodies against GnRH delayed normal devolopment of the reproductive system in ram lambs and bull calves (Jeffcoate et al., 1982). Active immunization against GnRH results in reproductive function similar to that observed in hypophysectomized animals.

Inhibition of gonadotropin secretion, testicular growth, testosterone secretion and sperm production confirmed the effectiveness of GnRH neutralization in rams (Schanbacher, 1982; Chase et al., 1988). In addition, a GnRH challenge was unsuccessful in eliciting LH or testosterone release (Schanbacher, 1982).

Immunization against GnRH in cattle has produced variable responses regarding the degree of inhibition achieved and the duration of response (Robertson et al., 1979; Jeffcoate et al., 1982; Robertson et al., 1984). In addition to reduced serum testosterone and decreased testicular volume in young bulls immunized against GnRH, behavior became docile and was comparable to that of steers (Robertson et al. 1981; Robertson et al., 1984). Reduction in spermatogenesis was confirmed by semen collection and related to decreased secretion of testosterone (Robertson et al., 1984). Furthermore, immunization of bulls resulted in increased growth rate and greater production of lean meat than for steers (Robertson et al., 1984).

Reversibility of Active Immunization In - Vivo

Successful immunoneutralization of GnRH produces a state of sexual infertility in both males and females in many species. As discussed previously, females undergo a loss of estrous cyclicity while males experience testicular atrophy, accompanied by impaired spermatogenesis. Administration of frequent booster immunizations against GnRH could impair reproductive activity for an indefinite period of time. Selective inhibition of GnRH would be more practical as a research tool, and to the livestock producer, if reversal of the inhibitory effects could be controlled. Possible means of reversing the suppressive effects of active immunization against GnRH include the natural decline in antibody titers and artifical reversal through administration of GnRH analogs.

Reinitiation of estrous cycles in ewes and testicular regeneration in rams was reported between 1 and 2 yr after GnRH immunization and was related to a gradual decline in GnRH antibody titers (Keeling and Crighton, 1984). Complete reversal of the suppressive effects of active immunization against GnRH has been demonstrated through the production of offspring in previously immunized cows, following a natural decline in anti-GnRH titers (O'Connell and Wettemann, in press). Similarly, actively immunized ewes conceived and carried pregnancies to term after titers were allowed to decline (Keeling and Crighton, 1984). Lambs born to GnRH

immunized ewes experienced normal growth and sexual development indicating a lack of detrimental effects associated with GnRH antibodies obtained in colostrum (Keeling and Crighton, 1984). A tendency for abnormally low concentrations of serum progesterone was observed following the first ovulatory cycle after the resumption of menstrual cycles in GnRH immunized monkeys (Fraser, 1983). However, subsequent ovulations resulted in normal luteal development. Upadhyay et al. (1989) found evidence of cellular resorption and degradation of the androgen dependent epididymides of GnRH immunized male rats, indicating that reversibility of GnRH immunoneutralization may be a function of the level of inhibition achieved. Exogenous testosterone administration to immunized male rats restored libido without restoring spermatogenesis, suggesting that supplemental testosterone may help prevent irreversible damage due to prolonged androgen deprivation. Variations in the immune response between individual animals results in differing levels of reproductive inhibition which make it difficult to determine the time of fertility reinitiation. A majority of animals actively immunized against GnRH will resume normal reproductive activity when titers are allowed to decline in the absence of booster immunizations. Further work is necessary to determine the reproductive status of animals previously immunized against GnRH.

Single injections of GnRH analogs, which did not bind to antibodies against GnRH generated in immunized animals,

stimulated LH and FSH responses in females actively immunized against GnRH. However, ovulation did not occur (Clarke et al., 1978; Esbenshade and Britt, 1985; Traywick and Esbenshade, 1988). The timing of gonadotropin release in response to administration of an analog in gilts immunized against GnRH was similar to that in nonimmunized controls but the magnitude of response was reduced (Esbenshade and Britt, 1985). Clayton et al. (1982) demonstrated the neccessity of endogenous GnRH secretion for maintainance of pituitary GnRH receptors and consequently, gonadotropin secretion. Thus, long-term absence of GnRH stimulation of the pituitary gonadotrophs could result in the reduction in LH and FSH secretion in animals immunized against GnRH and treated with an analog to GnRH. Attempts to initiate gonadotropin secretion in ovariectomized ewes actively immunized against GnRH, with repeated GnRH agonist treatment (100 ng/hr) increased secretion of LH within 2 days (Adams and Adams, 1986). Serum concentrations of LH were similar to those in controls after 6 d of treatment with a GnRH agonist. Pituitary gonadotrophs may be capable of responding to stimulation with normal gonadotroph function after extended deprival of GnRH stimulation.

Efforts to induce sustained follicular growth and ovulation in animals actively immunized against GnRH have been unsuccessful. Neither a single dose of PMSG nor increasing doses at regular intervals for 50 d stimulated follicular growth in gilts immunized against GnRH

(Esbenshade, 1987). Exogenous gonadotropin administration for 9 d at 6 h intervals also failed (Esbenshade, 1987). Pulsatile administration of a GnRH agonist for 72 or 144 h (100 ng/2h) was unsuccessful in inducing follicular growth or ovulation in gilts actively immunized against GnRH (Traywick and Esbenshade, 1988). However, testosterone implants successfully restored spermatogenesis in GnRH immunized rats (Awoniyi et al., 1989). Pulsatile delivery of a GnRH agonist (400 ng every hour) for 10 days to immunized ram lambs increased concentrations of serum LH and testosterone, however, testosterone was only partially restored and testis weight was not increased by agonist treatment (Sabeur and Adams, 1989). We interpret results utilizing exogenous hormone administration to suggest that either the duration of treatment or intensity of stimulation was insufficient or that artificial reversal was not possible with the methods employed. Additional work is necessary to establish a method to overcome the suppressive effects of active immunization against GnRH.

CHAPTER III

IMMUNIZATION AGAINST GONADOTROPIN RELEASING HORMONE ALTERS PUBERTY, SECRETION OF LUTEINIZING HORMONE AND OVARIAN ACTIVITY IN BEEF HEIFERS

Abstract

Twelve Angus x Hereford heifers at 11 mo of age and 317 ± 6 kg BW were utilized to evaluate the effects of active immunization against GnRH on reproduction. Prepuberal heifers (n=6) received a primary immunization (wk 0) against GnRH conjugated to human serum albumin (GnRH-HSA). The conjugate was emulsified in Freund's complete adjuvant and injected into mammary tissue. Booster immunizations, emulsified in Freund's incomplete adjuvant, were given at 6, 38 and 66 wk. Blood was obtained weekly from immunized and nonimmunized control (n=6) heifers for determination of concentrations of progesterone and LH and antibody titers to GnRH. Antibodies against GnRH were produced in all treated heifers. Puberty occurred at 5.3 and 25.8 wk ($P<.002$) after initial treatment in control and immunized heifers, respectively. Mean concentrations of LH in serum during the 4 mo after immunization were not different between GnRH immunized heifers and controls (2.89 vs 2.90 ng/ml,

respectively). After pubertal ovarian cycles were established, heifers were treated intravenously with GnRH and an analog to GnRH (GnRH-A), [(des-Gly^{10}(D-Ala^{6})-LHRH)]. Immunized heifers failed to respond to GnRH administration, whereas, LH in serum increased after GnRH treatment of control heifers. Infusion of the GnRH-A, [(des-Gly^{10}(D-Ala^{6})-LHRH)], increased concentrations of LH in serum over time but tended ($P<.10$) to differ in immunized and control heifers . At 42 and 70 wk, immunized heifers received pulsatile infusions of GnRH-A or saline for 2 min every 2 h, for 14 d. Pulsatile infusion of GnRH-A resulted in increased ($P<.07$) concentrations of LH in serum and increased ($P<.08$) pulse amplitude compared to saline treatment, but had no effect on the frequency of LH pulses. The interval from the booster immunization to the onset of luteal activity was not influenced by GnRH-A infusion. We conclude that active immunization against GnRH inhibits reproductive activity in heifers. Pulsatile infusion of an analog to GnRH for 14 d did not reverse the suppressive effects of active immunization on reproductive function.
(Key Words: GnRH, Heifer, Immunization, LH, Puberty)

Introduction

The hypothalamus synthesizes GnRH which is essential for the establishment and maintenance of normal reproductive function. GnRH is released episodically from the hypothalamus into the hypophyseal portal vessels. Upon

arrival at the anterior pituitary, GnRH binds specifically to the pituitary gonadotrophs and stimulates the synthesis and secretion of LH and FSH. Gonadotropins govern ovarian activity through stimulation of follicular growth and ovulation. Removal of the influence of GnRH on the pituitary has been accomplished by physical separation of the hypothalamus from the pituitary gland (Clarke et al., 1983). This results in a reduction in serum gonadotropins and inhibition of reproductive activity.

Neutralization of endogenous GnRH through the production of specific antibodies has been achieved in rats (Arimura et al., 1973), ewes (Adams and Adams, 1986), gilts (Esbenshade and Britt, 1985), mares (Garza et al., 1986) and heifers (Johnson et al., 1988). This procedure selectively inhibits the releasing hormone without disrupting other pituitary hormones (Adams and Adams, 1986). Active immunization against GnRH is usually characterized by a suppression of reproductive function due to a reduction in gonadotropin and gonadal steroid concentrations. In males, this is manifested by testicular atrophy accompanied by impaired spermatogenesis. Inhibition of GnRH by immunization in females is most evident by the cessation of estrous cyclicity (Fraser, 1975; Esbenshade and Britt, 1985). Ovarian weights are reduced and the number of follicles and corpora lutea are decreased in GnRH immunized females (Garza et al., 1986; Esbenshade, 1987; Johnson et al., 1988).

Administration of GnRH analogs, which do not cross-react with GnRH antibodies in immunized animals, cause release of gonadotropins (Clarke et al., 1978; Esbenshade and Britt, 1985). Infusion of a GnRH analog every hour increased secretion of LH within 48 h, with LH returning to pretreatment concentrations 6 d after treatment initiation (Adams and Adams, 1986). However, neither frequent exogenous gonadotropin administration (Esbenshade, 1987) nor pulsatile treatment with a GnRH analog (Traywick and Esbenshade, 1988) has been effective in inducing follicular growth or ovulation in gilts actively immunized against GnRH.

Selective inhibition of endogenous GnRH through the production of antibodies can be used to evaluate the role of the releasing hormone in reproductive function. Immunization against GnRH can be used to study regulation of gonadotropin synthesis and secretion, maintenance of pituitary GnRH receptors, and feedback mechanisms involving steroid hormones. Utilization of this technique may also prove beneficial in the feedlot industry. A significant population of cattle that enter the feedlot are heifers. Sexually mature heifers have reduced feed efficiency, which is partially attributed to increased physical activity during estrus. Furthermore, many heifers entering the feedlot are pregnant, resulting in increased weight loss at slaughter. Additional expenses and possible mortality may occur if small heifers are aborted or if parturition occurs

while in the feedlot. Extended suppression of reproductive activity through active immunization against GnRH may prevent estrus and ovulation and reduce the number of pregnant heifers entering feedlots.

The objectives of this study were (1) to determine the effects of active immunization of heifers against GnRH, on the initiation of pubertal estrous cycles and secretion of LH, (2) to evaluate the functional status of the pituitary after immunization against GnRH, by administration of GnRH and a GnRH-A, and (3) to determine the effects of pulsatile infusion of a GnRH-A, on secretion of LH and resumption of luteal activity after immunization against GnRH.

Materials and Methods

Twelve prepuberal Angus x Hereford heifers at approximately 11 mo of age and 317 ± 6 kg BW were used. Heifers were maintained in four, 4 x 4.5 M slotted floor pens, with 3 heifers per pen. Animals were exposed to ambient temperature and light, and fed in accordance with NRC requirements, with water available ad libitum.

Heifers were randomly allocated to two treatments. Six prepuberal heifers were actively immunized (wk 0) against GnRH conjugated to human serum albumin (HSA). The remaining heifers were untreated and served as controls (n=6). Treated heifers received a booster immunization 45 d following the primary injection. Blood (50 ml) was obtained

weekly for 22 mo by jugular venipuncture. Blood (40 ml) was added to 50 ml tubes containing 32 mg oxalic acid and immediately placed on ice. Samples were centrifuged within 1 h of collection (20 min, 4000 x g), plasma was decanted and frozen at -20° C until progesterone was quantified. The remaining blood (10 ml) was allowed to clot for 24 h at 4° C. Samples were centrifuged (20 min, 3000 x g) and serum was decanted and stored at -20° C until concentrations of LH and antibody titers against GnRH were determined. BW (non-shrunk) were recorded biweekly until puberty was initiated.

Following the initiation of estrous cycles in all previously immunized heifers, animals were given a booster immunization against GnRH (wk 38). Cessation of ovarian luteal activity was verified by concentrations of progesterone in plasma. Immunized (n=5) and control (n=5) heifers were fitted with polyvinyl jugular cannulae[1] to enable frequent blood sampling (wk 42). Prior to cannulation, estrus was synchronized in control heifers with two intramuscular injections of prostaglandin F_2 [2] (25 mg), 11 d apart. Heifers were confined in individual stalls with stanchions in a temperature (21 ± 4° C) and light (14 h/d) controlled environment. On the following three days, blood serum (10 ml) was collected at 10 min intervals for 4 h (1300-1700). On the first day, heifers received no treatment, on d 2, all heifers were infused with 5 μg of

[1]Bolab Inc., BB 317-v10, i.d. 1.57 mm, o.d. 2.08mm, Lake Hayasu City, AZ.
[2]Lutalyse, The Upjohn Co., Kalamazoo, MI.

GnRH[3] and on d 3, heifers were infused with 3.5 μg of a GnRH analog, [des-Gly[10],(D-Ala[6])-LHRH][3]. The hormones were administered (i.v.) 1 h after sampling was initiated. Cannulae were flushed with 3 ml of 2.9% sodium citrate after each blood sample to prevent clotting. A single 30 ml plasma sample was obtained each day, at the start of sampling, to assess luteal activity.

In an attempt to reinitiate ovarian cyclicity, immunized heifers were administered pulsatile infusions of the GnRH-A. Five immunized heifers were fitted with two jugular cannulae to facilitate simultaneous GnRH-A infusion and blood collection. Three heifers were infused with 2 μg of GnRH-A every 2 h for 14 d (336 h) and two control heifers were infused with saline. The GnRH-A solution (.5 μg/ml) was prepared in sterile saline with the addition of heparin[4] (1 USP unit/ml) and penicillin[5] (50 units/ml) to prevent clotting and bacterial contamination of infusion cannulae. Pulsatile infusions were achieved using a variable speed Harvard[6] infusion/withdrawal pump connected to an automatic digital timer[7]. Pumps were calibrated to deliver 4 ml of analog (.5 μg/ml) or saline in 2 min at 2 h intervals commencing on d 1. Blood serum was obtained every 10 min from 0700 to 1100 on d 0, 1, 2, 4, 6, 8, 10, 12 and 14 of

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[3]Sigma Chemical Co., St. Louis, MO.
[4]Elkins-Sinn Inc., Cherry Hill, NJ.
[5]E.R. Squibb and Sons, Inc., Princeton, NJ.
[6]Harvard infusion/withdrawal Pump, Model 931.
[7]Graylab Timer, Model 900, Dimco-Gray Co., Centerville, OH.

GnRH-A or saline treatment. An additional plasma sample was obtained daily throughout the treatment period and during the wk following treatment, and progesterone was quantified to evaluate luteal activity. Plasma was collected weekly, by venipuncture, from the time of treatment with GnRH-A until the resumption of estrous cycles. Heifers were given a third booster immunization against GnRH on wk 66. Following the booster immunization, the above protocol was repeated (wk 70). Animals which were previously treated with saline were pulsed with GnRH-A and those pulsed with GnRH-A, received saline. This resulted in a total of 5 heifers per treatment.

Heifers were actively immunized against GnRH that was conjugated to HSA (GnRH-HSA) by the carbodiimide reaction (Fraser et al., 1974). In a 12 x 75 glass culture tube, GnRH (4.87 mg) and HSA[8] (5.3 mg) were combined. To this mixture was added 15 mg of 1-ethyl-3(3-Dimethylamino)propyl-carbodiimide hydrochloride (JBL Scientific Inc., 16.6 mg) and ^{125}I-GnRH diluted in H_2O (27,000 CPM). Tracer quantities of ^{125}I-GnRH were included to determine the percentage conjugation of GnRH to HSA. Contents of the reaction tube were incubated for 20 h at room temperature (27° C). Following incubation, contents were transferred to dialysis tubing[9] and dialyzed against distilled H_2O, twice, for 24 h at 4° C.

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[8]Sigma Chemical Co., St. Louis, MO.
[9]Spectra, Por 3, MW cutoff 3,500, Baxter Scientific Products, McGraw Park, IL.

Prior to the primary immunization, GnRH-HSA conjugate (3 ml) was dissolved in saline (8 ml) and emulsified in Freund's complete adjuvant[10] (15 ml). Subcutaneous and intradermal injections were given at six sites in the posterior portion of the mammary gland of each treated heifer (.052 mg/heifer). Booster immunizations used the same procedure as primary immunization, except Freund's incomplete adjuvant[10] was used. Boosters were given on wk 6, 38 and 66.

Antibody titers against GnRH were determined by the ability of serum from immunized heifers to bind radiolabeled GnRH, similar to procedures described by Esbenshade and Britt (1985). Serum was diluted 1:10, 1:100, and 1:1000 in ethylenediaminetetraacetate(EDTA)-phosphate buffered saline (PBS), (pH 7.0). Two hundred microliters of diluted serum were added to 12 x 75 culture tubes in duplicate. Radioiodinated GnRH (15,000 CPM) in 100 μl of PBS plus .01 % gelatin (pH 7.0) was added and incubated for 24 h at 4° C. Following incubation, antibody bound ^{125}I-GnRH was separated from labeled GnRH by the addition of 1.5 ml of ethanol (4°C) followed by centrifugation (2,800 x g for 15 min). Supernatant was decanted and the precipitate was counted for 4 min in a gamma spectrometer. Antibody titers were expressed as the percentage of ^{125}I-GnRH bound by a serum dilution. Percentage bound was determined by dividing the quantity of radioactivity (CPM) bound in the precipitate by

[10]Sigma Chemical Co., St. Louis, MO.

the total radioactivity added to each dilution. Nonspecific binding was determined in each assay by using serum from nonimmunized control heifers.

GnRH was iodinated using the chloramine-T procedure. Three micrograms of GnRH suspended in 20 μl H_2O and 25 μl phosphate buffer (.5 M) was combined with .75 mCi ^{125}I diluted in 7.5 μl H_2O. Ten microliters of chloramine-T (2 mg/ml in H_2O) were added to the mixture and allowed to react for 45 s. The reaction was stopped with the addition of 10 μl of sodium metabisulfite (10 mg/ml in H_2O). Free ^{125}I and ^{125}I-GnRH were separated by column chromotography. The column was prepared using a 10 cc disposable glass pipette packed with LH-20 swelled in .05 M phosphate buffer (pH 7.1). A plastic tube and metal clamp was attached to the bottom of the column to control flow. Prior to use, the column was washed with .05 M phosphate buffer (pH 7.1). Contents of the reaction vial were transferred to the column in 250 μl phosphate buffer (.05 M, pH 7.1) and eluted with phosphate buffer containing .1 % gelatin. Two milliliter fractions were obtained in tubes containing 1 ml of PBS with .1 % gelatin (pH 7.0) using a fraction collector. The labeled GnRH was eluted from the column after the free ^{125}I.

Progesterone concentrations in daily and weekly samples were quantified by a single antibody RIA (Lusby et al., 1981). Onset of puberty and luteal activity (LA) were determined by concentrations of progesterone in plasma greater than or equal to 1 ng/ml for two consecutive weeks.

Cessation of luteal activity after booster immunizations against GnRH was affirmed by concentrations of progesterone in plasma less than 1 ng/ml for a minimum of 3 consecutive wk.

Concentrations of LH in serum were quantified in weekly blood samples obtained after the primary immunization (wk 0-30) and in all serum collected during intensive sampling periods. LH was quantified by a double antibody RIA, similar to that described by Hallford et al. (1979). Duplicate serum aliquots of 20 to 250 μl were diluted in PBS with .1 % gelatin in 12 x 75 culture tubes for a total volume of 500 μl. Tubes were maintained at 4° C throughout the procedure. Bovine LH (NIH-LH-B9) was the standard and was diluted in PBS containing .1 % gelatin to produce varying concentrations (0, .1, .2, .4, .8, 1.6, 3.2, 6.4, 12.8 ng per tube) which were included in triplicate in each assay. First antibody to LH (OSU BLH 4-1) was diluted 1:160,000 in PBS/EDTA and 200 μl was added to all tubes and gently vortexed. After incubation for 24 h at 4° C, 100 μl ^{125}I-LH (10,000 CPM) was added, vortexed and incubated for an additional 24 h at 4° C. Radiolabeled LH was prepared by the chloramine-T method and an anion exchange column was used to separate ^{125}I-LH from free ^{125}I. The anion exchange column consisted of a 3 cc plastic syringe and disposable stopcock packed with glass wool (3 mm). Anion exchange resin[11] was swelled in .05 M phosphate buffer (pH 7.5) and

[11]Anion Exchange Resin, Bio-Rad Lab., Richmond, CA.

layered into syringe. The column was rinsed sequentially with 2 ml .5 M phosphate buffer, 2 ml bovine serum albumin (5 % in .05 M phosphate buffer) and 3 ml of phosphate buffer. Contents of the reaction vial were layered on the column and after the addition of 2 ml of phosphate buffer (.05M), the effluent was collected in .5 ml .01 M phosphate buffer with .1 % gelatin added (pH 7.0). To separate bound from free ^{125}I-LH a second antibody (OSU #0833) was used (1:40 dilution of ovine anti-rabbit serum in PBS/EDTA). After the addition of 200 µl of second antibody, tubes were mixed and incubated for 72 h at 4° C. Separation of bound from free ^{125}I-LH was achieved by adding 1.5 ml of cold PBS to tubes and centrifuging (30 min, 3000 x g). Supernatant was decanted, tubes were inverted to dry and radioactivity was quantified (Packard Multi-Prias).

Concentrations of LH in weekly blood samples from all heifers were quantified in one assay. Concentrations of LH in blood serum collected from all heifers during treatment with GnRH or GnRH-A were determined in a single assay. All samples for an individual heifer during the infusion period were included in a single assay.

Effects of treatment on age and weight at the onset of puberty were analyzed by analyses of variance using the General Linear Models Procedure of SAS (SAS, 1985). Concentrations of LH in samples collected weekly after immunization were analyzed by analyses of variance.

Regression analysis was used to analyze LH responses to treatment with GnRH and GnRH-A, and response curves were tested for homogeneity of regression. Characteristics of serum LH, including mean concentration, frequency of LH pulses and amplitude of LH pulses, were determined in repeated samples for each individual heifer on each day of frequent sampling and analyzed by split-plot analyses of variance. The model included treatment, heifer within treatment, day and treatment by day interaction. Concentration of LH was the average of all samples (n=25) in a 4 h sampling period from an individual heifer. Each LH pulse was characterized using a modification of parameters identified by Goodman and Karsch (1980). An LH pulse was defined as an increase in LH greater than one standard deviation above the mean for that day, followed by a minimum of 2 values of lesser concentration. Pulse amplitude was calculated by subtracting the minimal value of LH occurring 30 min prior to a pulse from the greatest value during a pulse. Amplitude for a heifer on a given day was the average amplitude of all pulses in a 4 h sampling period.

Results and Discussion

Antibody production against GnRH, occurred after immunization of all treated heifers (Table 1). Two of 6 heifers had an increase in antibody titer during the 6 wk after the primary immunization. A rapid increase in titers occurred in the 2 wk after the booster immunization on wk 6

(Figure 1). Prior to the booster immunization, titers in immunized heifers were not different from controls with the exception of 2 heifers (Table 2). Antibody titers increased in all heifers within 4 d after the booster immunization and continued to increase during the next 7 d. Nonspecific binding of ^{125}I-GnRH to diluted serum was < 2 % in nonimmunized control heifers throughout the experiment. Granulomas developed at the injection site in treated heifers. This undesirable side effect has been attributed to the use of Freund's complete adjuvant at the time of initial immunization.

Puberty occurred (onset of LA) at 5.3 wk and 25.8 ($P<.002$) wk after the primary immunization in control and treated heifers, respectively (Figure 2). BW at puberty was greater ($P<.05$) in treated vs control heifers (Table 3). In a similar study, the onset of LA in heifers was delayed 11 wk in response to immunization against GnRH (Wettemann and Castree, 1988). The reason for the greater delay in puberty in response to immunization in the present study is unclear. Possible causes include variations in the immune response of individual animals, different antisera specificity and/or varying degrees of GnRH conjugation to the carrier protein. A wide range in titers against GnRH existed in heifers at puberty. There was also much variation in time of puberty after the primary immunization (Table 4). Week at the onset of LA and corresponding antibody titers in immunized heifers ranged from 7-35 wk and

9-67% binding of ^{125}I-GnRH in serum, respectively. Antibody titers against GnRH at wk 10 and titers at puberty were not related (P>.10) to the week of onset of LA. For example, heifers 531 and 257 had the lowest titers at wk 10 and at the onset of LA (Table 4), however, LA was detected 14 wk after initial immunization of heifer 531, as compared to, 35 wk for heifer 257. Chase et al. (1988) found that a wide range of antibody titers against GnRH (5-55 %) induced inhibitory characteristics in actively immunized rams. In contrast, Wettemann and Castree (1988) indicated a direct relationship between titer and length of delay in puberty following immunization against GnRH in heifers.

Production of antibodies to GnRH before pubertal cycles were initiated prevented ovulation and subsequent luteal development. In the present study, it is possible that titers against GnRH were not established rapidly enough to prevent ovulation from occurring at wk 7 in heifer 580, the youngest heifer at puberty. However, normal cycles continued despite subsequent elevated anti-GnRH titers (Figure 3). Keeling and Crighton (1984) found a large range of differences between individual ewes in regard to antibody titers against GnRH and the interval from immunization to the resumption of reproductive activity. Much of the variation was attributed to differences in genetic factors between individual ewes. Reproductive activity in marmoset monkeys was inhibited for similar lengths of time in animals

with high and low titers against GnRH (Hodges and Hearn, 1979).

There are few reports on the production of GnRH antisera in the bovine. Antibody titers against GnRH in young bulls have been minimal (Schanbacher, 1984) and extremely variable (Robertson, 1979; Robertson et al., 1984). Concentrations of progesterone were suppressed and follicular development was absent in heifers immunized against GnRH that developed antibody titers greater than 20% at a serum dilution of 1:1000 (Johnson et al., 1988).

Control heifers exhibited normal estrous cycles throughout the study, after the attainment of puberty. Figure 4 depicts the concentrations of progesterone in a typical control heifer. Treated heifers which attained sexual maturity at wk 7 and 14, continued to exhibit normal estrous cycles, similar to controls. Immunized heifers that attained puberty later, had a tendency to have short-lived increases in progesterone at infrequent intervals prior to the first estrus (Figure 5). Temporary increases in progesterone have been demonstrated prior to puberty in heifers (Berardinelli et al., 1979; Schams et al., 1981; Glencross, 1984). Berardinelli et al. (1979) found compact luteal tissue in the ovaries of heifers displaying short term increases in plasma progesterone before a normal ovulation. Ovulation sometimes occurs, but is not required for transient increases in progesterone before puberty

(Berardinelli et al., 1980). Reproductive maturity was attained in all heifers within 9 mo of initial treatment.

Concentrations of LH in serum of immunized and control heifers is illustrated in figure 6. Mean concentrations of LH in the 4 mo following immunization were not influenced (P>.10) by treatment and averaged 2.89 ng/ml in heifers immunized against GnRH and 2.90 ng/ml in controls. These results disagree with studies in sheep and pigs in which immunization against GnRH was associated with reduced serum concentrations of LH (Clarke et al, 1978; Jeffcoate et al., 1978; Esbenshade and Britt, 1985). Secretory patterns of LH in immunized mares were comparable to seasonally anestrous mares (Garza et al., 1986). Serum concentrations of LH were not different between nonimmunized ewes and ewes actively immunized against LH (Roberts and Reeves, 1989). Suppression of estrous cycles and uterine weights similar to controls were observed in rats immunized against GnRH that had minimal titers against GnRH, suggesting that gonadotropin secretion was maintained but was probably insufficient to produce an LH surge and ovulation (Fraser and Baker, 1978). It is not clear as to why concentrations of LH in weekly samples were not reduced in the immunized heifers in the present study. Release of LH in the cyclic cow is directly influenced by stage of the estrous cycle (Rahe et al., 1980). Negative feedback effects of progesterone on LH secretion in normally cycling control heifers may contribute to the lack of a treatment effect on

concentrations of LH when comparing prepuberal immunized heifers and cyclic control heifers. Minimal development of antibody titers in heifers, compared to other species, may have prevented a reduction in serum LH. Although secretion of LH failed to decline following immunization, ovarian progesterone secretion was suppressed, indicating that ovulation was prevented. Basal gonadotropin secretion was maintained in ovariectomized ewes after immunization against GnRH (Jeffcoate et al., 1978). Hypothalamic GnRH may have fully saturated antibodies produced against GnRH, thereby allowing some GnRH to escape and reach the pituitary gonadotrophs, resulting in synthesis and secretion of LH.

The effects of immunization against GnRH, on secretion of FSH in heifers, has not been documented. Furthermore, results in other species are inconsistent. If concentrations of FSH in the present study, were not reduced by immunization against GnRH, ovarian estradiol synthesis and secretion may have continued. Thus, estradiol may have increased the sensitivity of the pituitary to minimal GnRH stimulation, resulting in maintained concentrations of LH in immunized heifers. However, the quantity of LH was insufficient to produce an LH surge.

One immunized heifer died at wk 37. Therefore, the number of animals in the immunized group was reduced from 6 to 5. Antibody titers against GnRH were induced in the 5 remaining heifers following booster immunizations at wk 38 and 66. Concentrations of progesterone in plasma were less

than 1 ng/ml for a minimum of 3 wk, confirming the cessation of estrous cycles following booster treatment. Concentrations of LH in serum obtained at 10 min intervals for 4 h on wk 42 (Figure 7) did not differ (P>.10) between immunized and control heifers (2.38 vs 2.42 ng/ml, respectively). Estrous cycles of control heifers were synchronized with prostaglandins so that control heifers were between d 1 and 3 of the estrous cycle. This was based on concentrations of progesterone in plasma. Thus, the negative feedback effects of progesterone on LH were minimal or absent in these cycling heifers. Frequency of LH pulses and amplitude of the pulses in the heifers on the two treatments were also similar (P>.10), averaging 2.6 vs 2.4 pulses/4 h and 2.25 vs 1.68 ng for control and treated heifers, respectively.

The response to exogenous GnRH stimulation is depicted in Figure 8. Analysis of time trends on secretion of LH indicated differences (P<.01) in LH response following GnRH infusion in immunized and control heifers (Table 5). A fifth order poylnomial regression equation was used to describe the overall LH response to GnRH. GnRH stimulation resulted in an increase in serum concentrations of LH in controls within 10 min of treatment and maximal concentrations of LH (8.89 ± .56 ng/ml) occurred 20 min after treatment. Immunized heifers failed to respond to GnRH, indicating that antibodies produced in response to immunization successfully neutralized exogenous GnRH.

Concentrations of LH were nondetectable in immunized gilts after injection of 100 μg of GnRH (Esbenshade and Britt, 1985). An inverse relationship existed between LH response to GnRH administration and antibody titer in actively immunized ewes (Jeffcoate et al., 1978).

Administration of a GnRH analog (GnRH-A; des-gly^{10}(D-ala^{6})-LHRH), increased concentrations of LH in the serum of immunized and control heifers. Concentrations of LH in response to GnRH-A treatment tended to differ ($P<.10$) for immunized and control heifers and the overall fit was best described by a third order polynomial regression equation (Figure 9, Table 6). The LH response between treatments tended ($P<.10$) to lack homogeneity in that immunized heifers responded to analog stimulation with a greater initial release of LH over the first hour after infusion. However, concentrations of LH in control heifers increased above immunized heifers during the second and third hour following treatment. Mean concentrations of LH in the present study were maximal at 130 min after analog infusion in both control and treated heifers (30.3 vs 25.5 ng/ml, respectively). Esbenshade and Britt (1985) found that LH release in immunized gilts after treatment with the GnRH agonist, D-(Ala^{6},des-Gly-NH$_2$^{10}) ethylamide, was similar to that for controls, however, the magnitude of response was reduced (Esbenshade and Britt, 1985). Immunized mares did not respond to GnRH analog with an increase in LH release, however, FSH response was comparable to that for controls

(Garza et al., 1986). The response of heifers to GnRH-A are consistent with studies stimulating secretion of LH in immunized animals after injection of a non-cross reactive GnRH analog (Clarke et al., 1978; Esbenshade and Britt, 1985). Administration of the GnRH analog to control heifers resulted in a greater response in secretion of LH than when heifers were treated with GnRH. Moreover, the maximal concentration of LH was achieved later (20 vs 130 min post infusion) and was of greater duration. Increased biological activity of the analog is probably due to a reduction in enzymatic degradation, thus, increasing the half-life over that of naturally occurring GnRH (Yen, 1986). These studies provide evidence that the pituitary gonadotrophs remain capable of responding to stimulation despite the presence of antibodies against GnRH.

Endogenous GnRH is neccessary for maintenance of pituitary GnRH receptors and for the synthesis of LH and FSH by the pituitary gonadotrophs (Clayton et al., 1982). It is probable that endogenous GnRH was not completely neutralized by circulating antibodies in this study, thereby sustaining LH synthesis by the gonadotrophic cells and maintaining adequate pituitary GnRH receptors. In addition, the period of endogenous GnRH deprival after immunization may not have been long enough to reduce concentrations of LH in the pituitary of immunized heifers. However, it appears that the gonadotropin surge occurring prior to ovulation was inhibited since luteal development was prevented.

The final objective was to determine if estrous cycles could be reinitiated in immunized heifers through pulsatile administration of a GnRH-A. Antibody titers were present in all immunized heifers during the 2 wk when GnRH-A was infused and ranged between 44 and 58% binding at a 1:1000 serum dilution (Table 7). All heifers were acyclic prior to treatment. Concentrations of LH in serum, frequency of LH pulses and amplitude of LH pulses were similar in immunized heifers before (d 0) initiation of GnRH-A or saline pulse infusions (Table 8). Infusion of saline every 2 h did not influence concentrations of LH, which averaged 2.12 ± .61 ng/ml during the 14 d infusion period (Figure 10). Concentration of LH in serum was influenced ($P<.07$) by GnRH-A treatment every 2 h. Concentrations of LH in the serum of all heifers were increased immediately following the initiation of treatment on d 1. Concentrations of LH increased from 2.34 ± .35 ng/ml on d 0 (prior to treatment) to 4.79 ± .35 ng/ml on d 14 (Figure 10). A reduction in LH secretion occurred on d 2 of GnRH-A treatment, and may represent a depletion of pituitary gonadotropin reserves after GnRH-A stimulation. Then on d 4 through d 14 of GnRH-A treatment, concentrations of LH remained greater than 4 ng/ml, peaking on d 12 (4.84 ± .35 ng/ml). Nett et al. (1987) indicated that following an LH surge in cows, the anterior pituitary has the ability to restore concentrations of LH within one day.

Frequency of LH pulses was not altered (P>.10) by treatment with GnRH-A and averaged 2.14 ± .2 pulses/4 h throughout the treatment period (Figure 11). Pulse amplitude was greater (P<.08) in heifers infused with GnRH-A (2.52 ng) than in heifers that received saline (0.94 ng) (Figure 12).

Concentrations of progesterone in daily plasma samples of heifers were not altered by infusion of GnRH-A and remained < 1 ng/ml during the 14 d infusion regime, in all but one heifer. Figure 13 illustrates the concentrations of progesterone in heifer 531, while receiving pulses of GnRH-A every 2 h. Concentrations of progesterone in plasma increased to > 1 ng/ml on d 7 of treatment and remained increased for 6 d. This temporary increase in progesterone may be the result of a luteinized follicle that was caused by GnRH-A treatment.

Treatment with GnRH-A every 2 h for 14 d resulted in increased concentrations of LH and increased amplitude of LH pulses, but had no effect on LH pulse frequency. Rahe et al. (1980) suggested that ovarian activity in cows is directly related to LH pulse frequency. The amount of analog, frequency of administration or duration of treatment in this study may have been insufficient to establish the necessary LH pulse frequency and thus, influence the resumption of estrous cycles. Similar attempts to stimulate follicular growth and ovulation, in gilts immunized against GnRH, with repeated gonadotropin (Esbenshade, 1987) or

pulsatile GnRH analog (Esbenshade and Britt, 1985) administration have been unsuccessful. A preovulatory LH surge can be induced in prepubertal heifers with pulsatile administration of GnRH, however, estrous cycles are not initiated earlier (Skaggs et al., 1986).

The onset of LA following the booster immunizations against GnRH was not influenced by pulsatile infusion of GnRH-A or saline. Resumption of LA occurred at an average of 18 ± 3 wk in 4 out of 5 heifers following the second booster immunization. One heifer had a persistent corpus luteum prior to treatment with GnRH-A, and was therefore omitted from the analyses. Reinitiation of estrous cycles occurred 18.8 ± 5 wk after the final (third) booster immunization in 4 out of 5 heifers. The remaining heifer had not reinitiated LA as of 35 wk after booster administration.

In summary, the production of antibodies against GnRH in heifers induced temporary sterility for varying periods of time. Ovarian activity in heifers immunized against GnRH, was suppressed for 4 to 5 mo after administration of a booster immunization. Contrary to work in other species, serum concentrations of LH were not significantly reduced by immunization against GnRH. Active immunization, however, prevented ovulation and subsequent luteal development. Exogenous GnRH failed to stimulate LH release in immunized heifers. In contrast, administration of an analog to GnRH stimulated secretion of LH, with concentrations similar to

those in nonimmunized heifers. Pulsatile treatment with an analog to GnRH increased concentrations of LH and increased LH pulse amplitude, however, pulse frequency was not influenced. The duration of anovulation induced by immunization against GnRH was not influenced by pulsatile treatment with an analog to GnRH.

TABLE 1. DEVELOPMENT OF ANTIBODY TITERS IN INDIVIDUAL HEIFERS IMMUNIZED AGAINST GNRH[a] AT WEEK 0

Week	Heifer					
	580	581	552	531	257	930
0	2.0	2.0	2.0	.9	1.0	1.0
1	2.0	2.0	4.0	1.1	1.6	1.3
2	2.2	1.8	1.1	1.0	1.2	.9
4	2.9	2.5	2.6	1.4	1.7	18.1
6	2.0	2.0	7.0	1.4	1.3	28.4
7	21.0	4.2	16.2	4.6	7.0	36.5
8	62.0	19.0	40.0	6.6	4.5	47.5
10	50.7	13.6	36.3	8.7	6.0	64.2

[a]Titers expressed as % ^{125}I-GnRH bound to serum at 1:100 dilution.

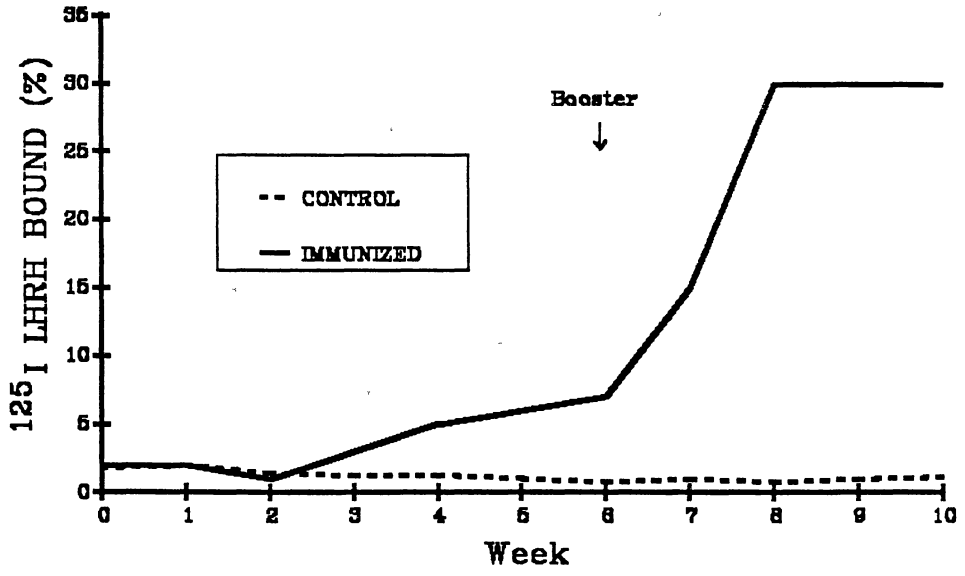

Figure 1. Antibody titer development (% ^{125}I-GnRH bound at 1:100 serum dilution) in control heifers and heifers immunized against GnRH at wk 0.

TABLE 2. ANTISERA TITERS IN HEIFERS BEFORE AND AFTER BOOSTER IMMUNIZATION AGAINST GNRH AT WEEK 6

Heifer	Trt	^{125}I-GnRH bound (%)[a] Day[b] 0	+4	+11
531	I[c]	1.4	4.6	6.6
257	I	1.3	7.0	4.5
581	I	2.0	4.2	19.0
580	I	2.0	21.0	62.0
552	I	7.0	16.2	40.0
930	I	28.4	36.5	47.5
---	C[d]	<2	<2	<2

[a]Percent ^{125}I-GnRH bound at 1:100 serum dilution.

[b]Day 0=immediately prior to booster immunization.

[c]Immunized.

[d]Controls (n=6).

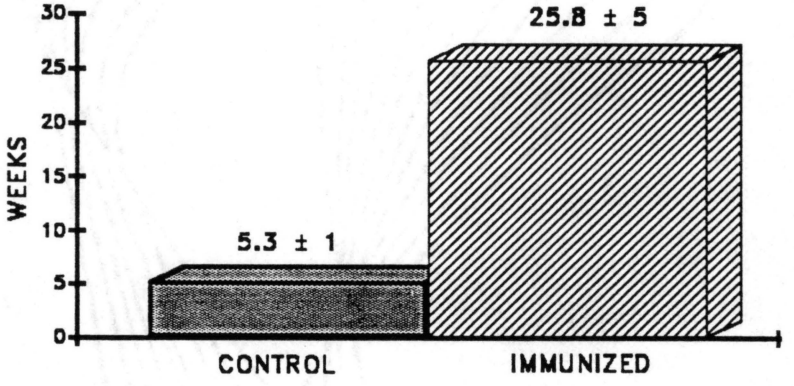

Figure 2. Least squares means for the onset of luteal activity in control and immunized heifers.

TABLE 3. LEAST SQUARES MEANS FOR INITIAL WEIGHT AND WEIGHT AT PUBERTY OF IMMUNIZED AND NONIMMUNIZED CONTROL HEIFERS

Weight (kg)	N	Control	Immunized	SE
Initial	6	319	314	6.7
Puberty	6	354[a]	407[b]	11.2

[a,b] Means within rows with different superscripts differ ($P<.05$).

TABLE 4. ONSET OF LUTEAL ACTIVITY (LA) AND ANTIBODY TITERS IN CONTROL AND IMMUNIZED HEIFERS

Heifer	Trt	Onset of LA (wk)	^{125}I-GnRH bound (%)[a]	
			Wk 10	Wk of LA
580	I[b]	7	51	21
531	I	14	9	9
581	I	32	14	27
552	I	33	36	54
930	I	34	64	67
257	I	35	6	19
---	C[c]	5 ± 1	<2	<2

[a]Percent ^{125}I-GnRH bound at 1:100 serum dilution.

[b]Immunized.

[c]Controls (n=6).

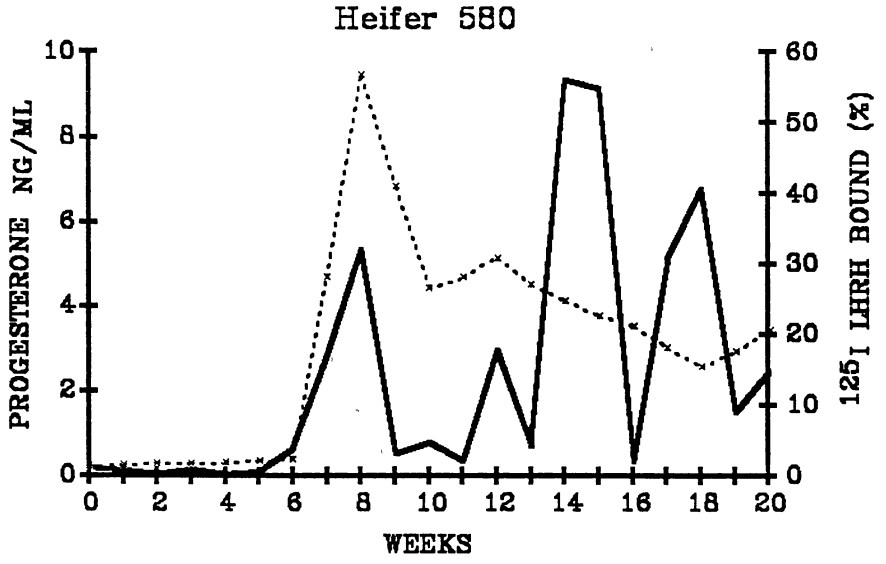

Figure 3. Concentrations of progesterone in plasma and antibody titers against GnRH in an immunized heifer with luteal activity 7 wk after the primary immunization.

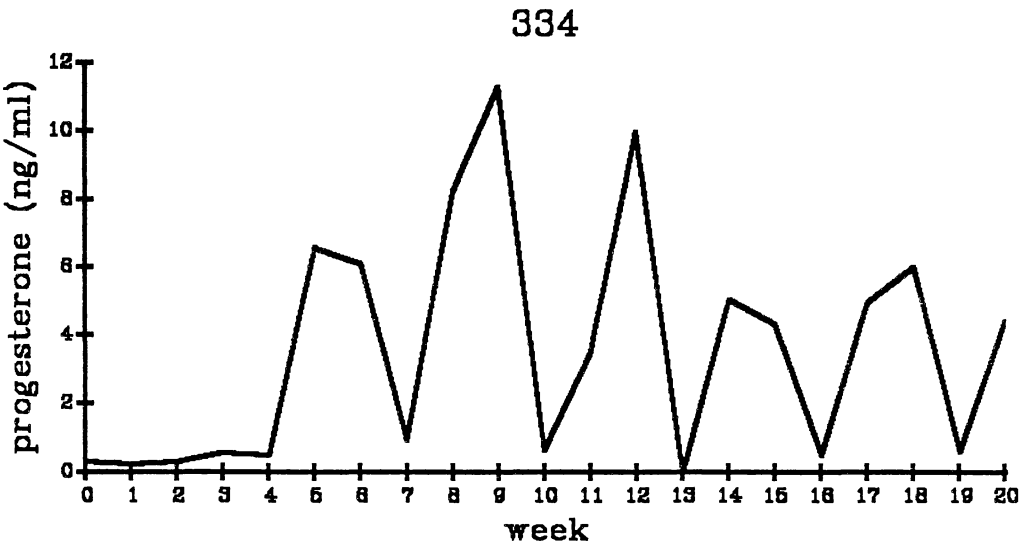

Figure 4. Concentrations of progesterone in plasma of a control heifer.

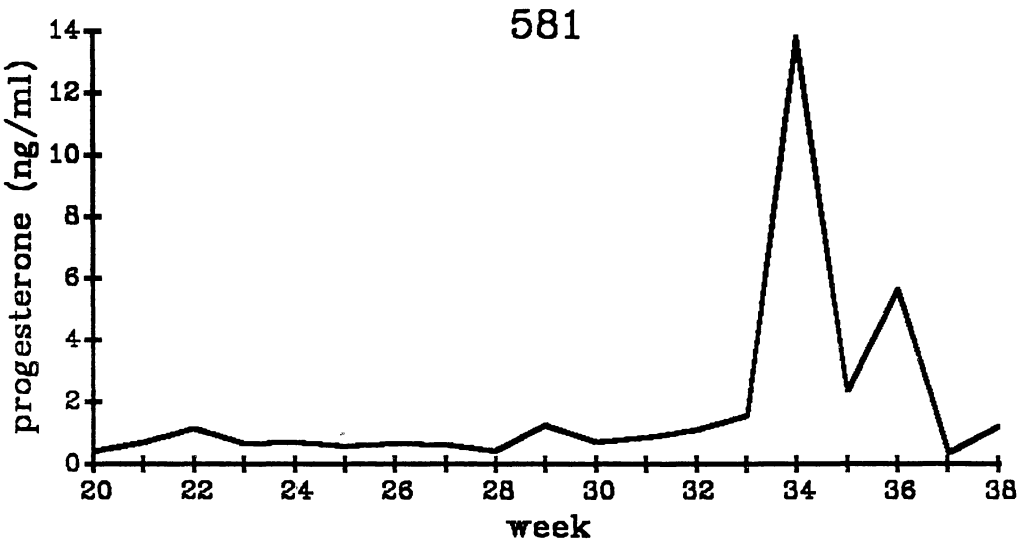

Figure 5. Concentrations of progesterone in plasma of an immunized heifer with temporary increases in progesterone at wk 22 and 29 prior to the onset of luteal activity.

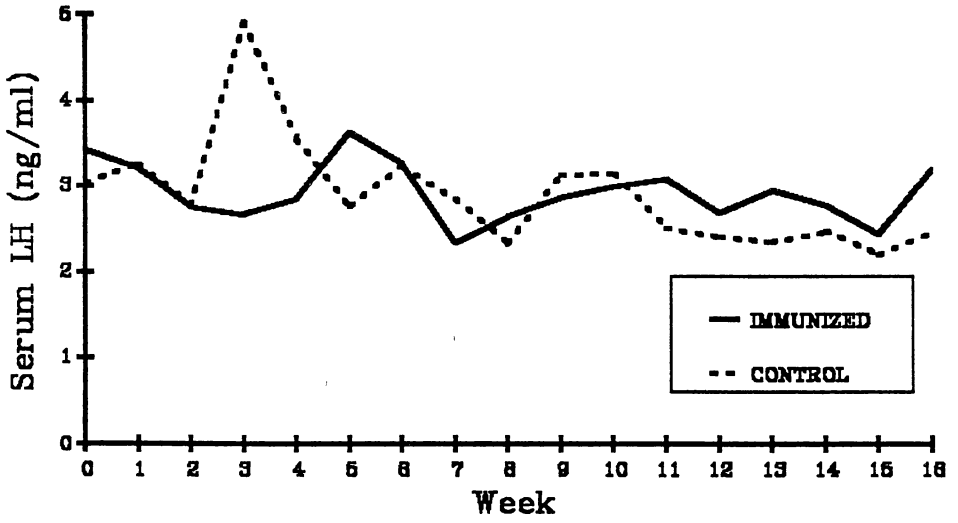

Figure 6. Least squares means (SEM ± .47) for concentrations of LH in serum of immunized and control heifers after primary (wk 0) and booster (wk 6) immunization.

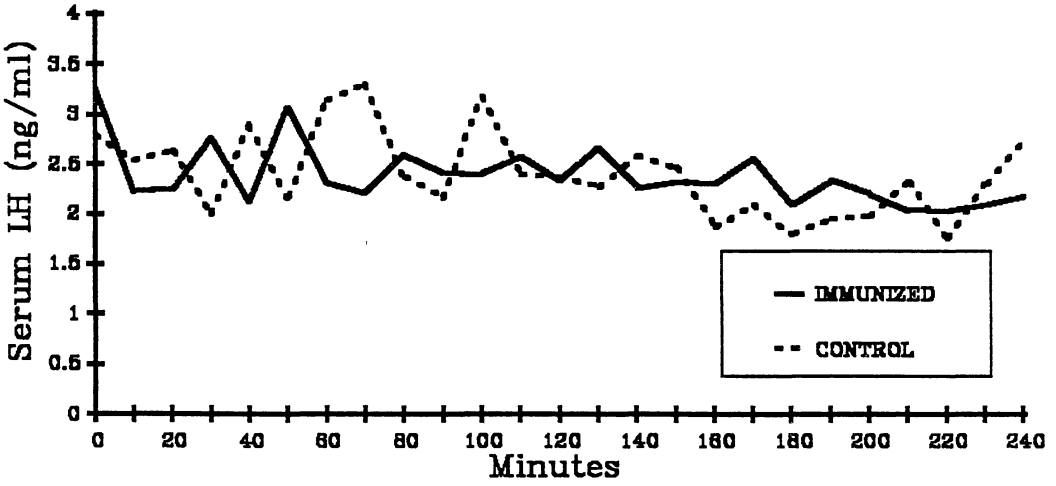

Figure 7. Least squares means (SEM ± .32) for concentrations of LH in serum in immunized and control heifers at wk 42.

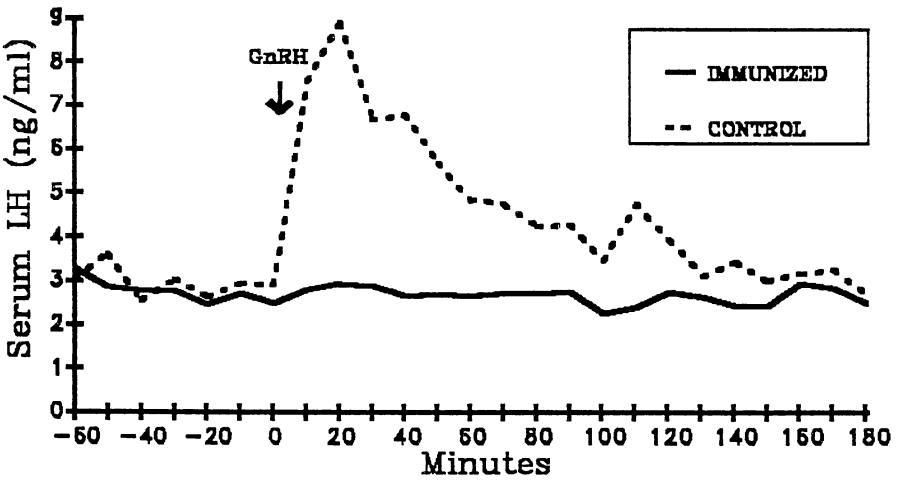

Figure 8. Least squares means (SEM ± .35) for concentrations of LH in immunized and control heifers after treatment with GnRH.

TABLE 5. ANALYSIS OF VARIANCE USED TO TEST FOR HOMOGENEITY OF REGRESSION COEFFIENTS FOR POLYNOMIAL RESPONSE CURVES FOR CONCENTRATIONS OF LH IN IMMUNIZED AND CONTROL HEIFERS AFTER TREATMENT WITH GNRH

Error	D.F.	S.S.	M.S.	F
Immunized	111	225.34		
Control	109	20.00		
Total	220	245.34	1.12	
Immunized, Control	225	346.09		
Difference	5	100.75	20.15	17.99*

*(P<.01).

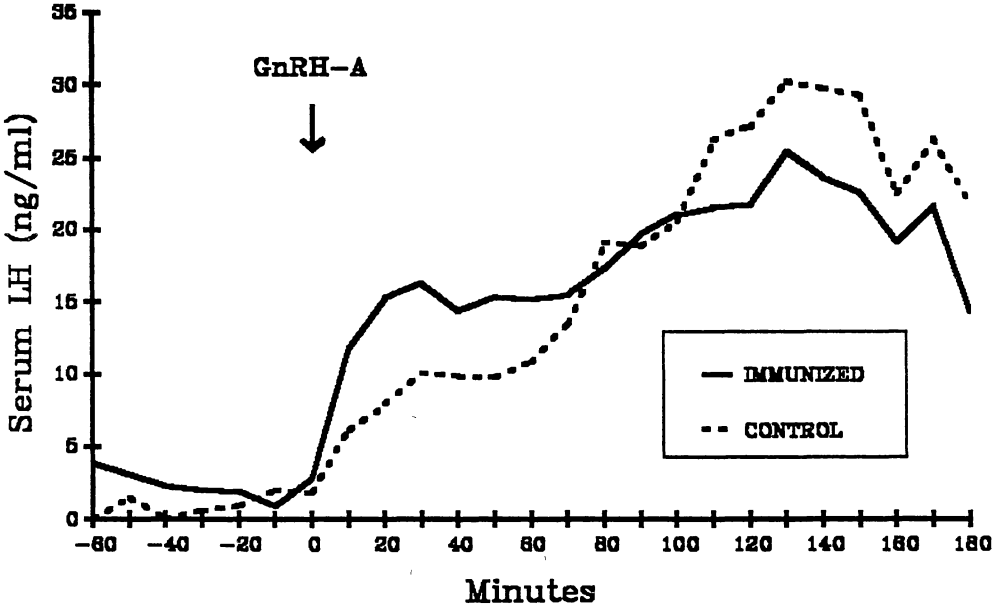

Figure 9. Least squares means (SEM ± 2.5) for concentrations of LH in immunized and control heifers after treatment with an analog to GnRH.

TABLE 6. ANALYSIS OF VARIANCE USED TO TEST FOR HOMOGENEITY OF REGRESSION COEFFIENTS FOR POLYNOMIAL RESPONSE CURVES FOR CONCENTRATIONS OF LH IN IMMUNIZED AND CONTROL HEIFERS AFTER TREATMENT WITH AN ANALOG TO GNRH

Error	D.F.	S.S.	M.S.	F
Immunized	100	1530.35		
Control	102	5071.47		
Total	202	6601.82	32.68	
Immunized, Control	205	7296.72		
Difference	3	694.90	231.63	7.09*

*(P<.10).

TABLE 7. ANTIBODY TITERS[a] DURING INFUSION OF GNRH ANALOG (A) OR SALINE (S) IN HEIFERS ACTIVELY IMMUNIZED AGAINST GNRH

Heifer	Trt	Period	Day of infusion		
			0	8	14
257	S	1	61	55	42
552	S	1	60	55	44
531	A	1	62	59	54
581	A	1	54	57	53
257	A	2	62	54	44
552	A	2	62	56	57
531	S	2	50	44	39
581	S	2	53	47	40
580	A	2	55	51	40

[a]Percent ^{125}I-GnRH bound at a 1:1000 serum dilution.

TABLE 8. LEAST SQUARES MEANS (± SEM) FOR CONCENTRATIONS OF SERUM LH, PULSE FREQUENCY AND PULSE AMPLITUDE IN IMMUNIZED HEIFERS PRIOR TO PULSATILE INFUSION OF GNRH-A OR SALINE

Treatment	LH (ng/ml)	Pulse	
		Frequency[a]	Amplitude (ng/ml)
Saline	2.23 ± .34	2.50 ± .57	1.51 ± .28
GnRH-A	2.39 ± .31	2.00 ± .51	1.05 ± .25

[a]Number of pulses occurring in 4 h.

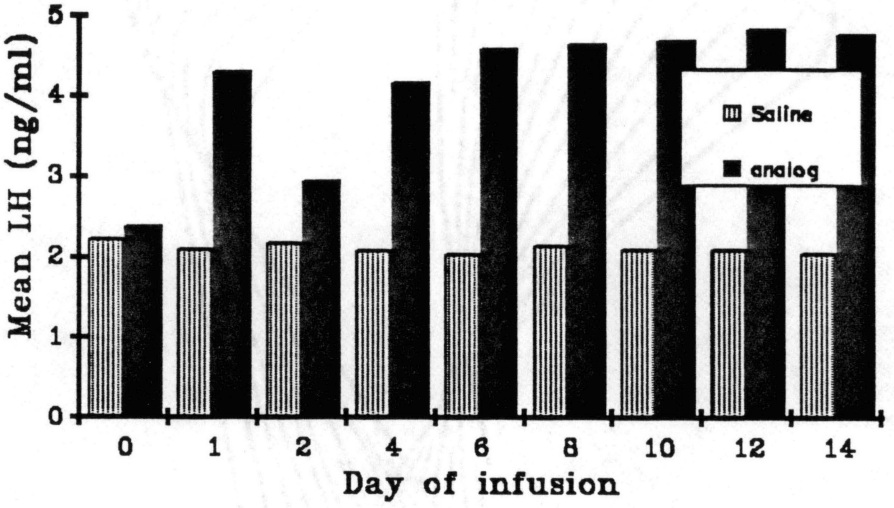

Figure 10. Least squares means (SEM ± .32) for concentrations of LH in serum of heifers immunized against GnRH and infused with saline or an analog to GnRH.

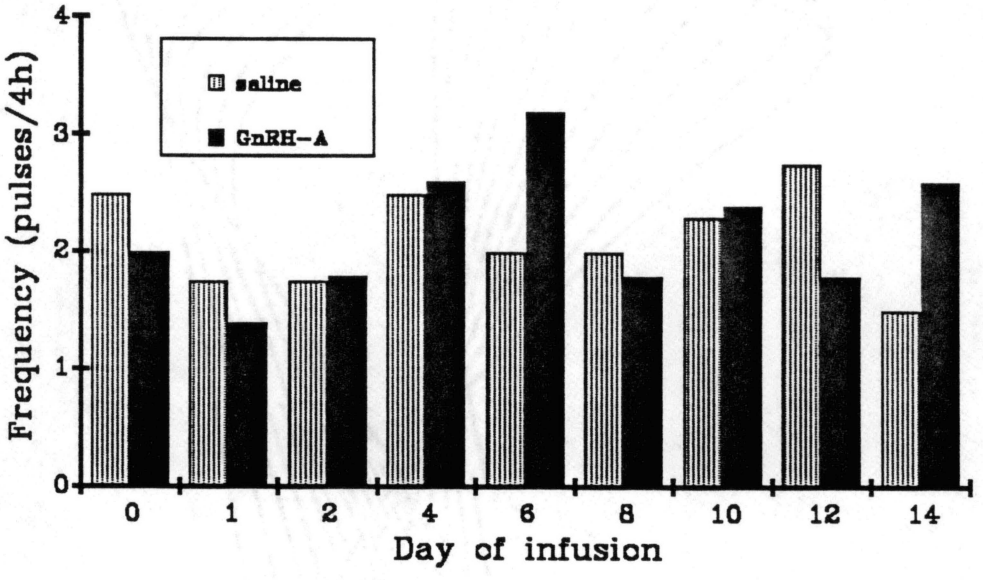

Figure 11. Least squares means (SEM ± .53) for frequency of LH pulses in heifers immunized against GnRH and infused with saline or an analog to GnRH.

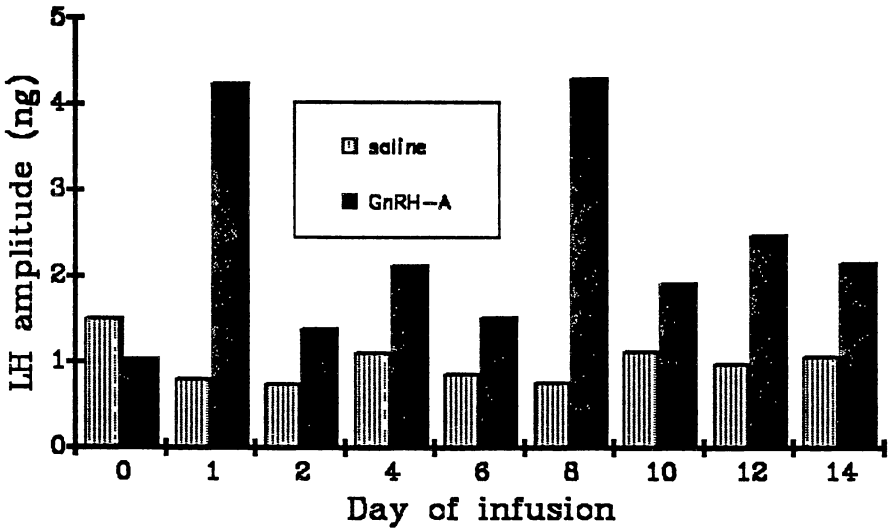

Figure 12. Least squares means for amplitude of LH pulses in heifers immunized against GnRH and infused with saline or an analog to GnRH.

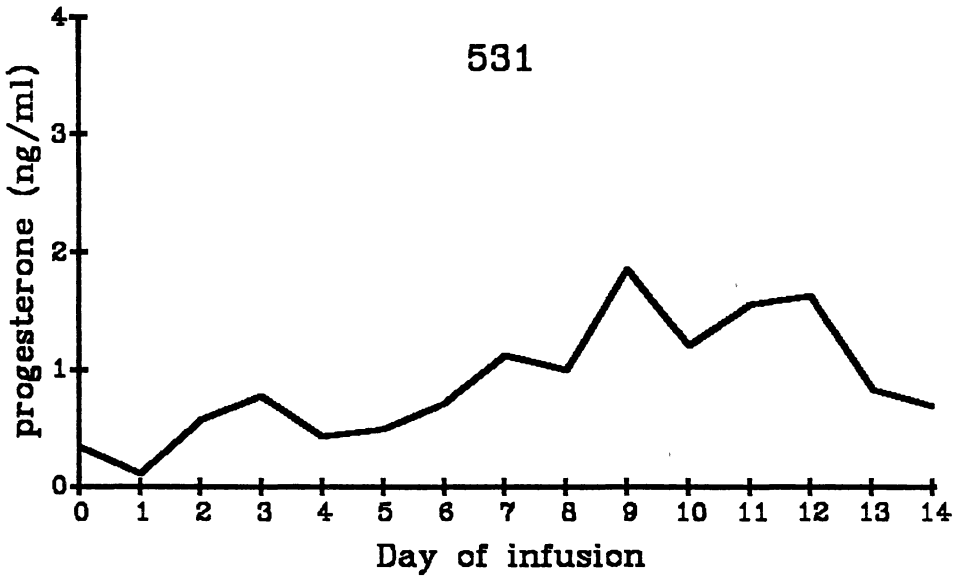

Figure 13. Concentrations of progesterone in an immunized heifer during infusion with an analog to GnRH.

Implications

Active immunization against GnRH can be used to suppress fertility in heifers through the production of specific antibodies. This technique could be useful to study reproductive function in the absence of endogenous GnRH. Application of immunization against GnRH in the livestock industry has the potential to reduce the number of pregnant heifers entering feedlots. Use of this technique may also aid in optimizing the production efficiency of sexually mature feedlot heifers by limiting physical activity associated with estrus.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Gonadotropin releasing hormone has a primary role in the series of hormonal events which culminate in ovulation and subsequent luteal development in females. Physical disruption of the hypothalamic-pituitary axis has been accomplished by transection of the infundibular stalk and removal of the medial basal hypothalamus. These procedures lead to a reduction in gonadotropin secretion and cessation of reproductive function. However, the secretion of other anterior pituitary hormones are also disrupted, which alters normal endocrine function.

Six heifers were actively immunized against GnRH to selectively neutralize endogenous GnRH. The decapeptide, GnRH, was conjugated to human serum albumin (GnRH-HSA), emulsified in Freund's adjuvant and administered at several sites in the mammary gland (week 0). Booster immunizations were given at 6, 38 and 66 weeks after the primary treatment. Blood serum and plasma were collected weekly for 22 mo via venipuncture. Heifers were weighed biweekly until puberty was achieved.

Following the establishment of pubertal estrous cycles, heifers were given a booster immunization against GnRH (wk

38). After estrous cycles were abolished, cannulae were inserted into the jugular veins of immunized (n=5) and control (n=5) heifers. During the next 2 days, heifers were treated with a single dose of GnRH and the GnRH analog, des-Gly^{10}(D-Ala^{6})-LHRH. After treatment, immunized heifers received an additional cannula in the contralateral vein to facilitate simultaneous blood collection and episodic infusion of the analog of GnRH. Immunized heifers were given pulses of the analog to GnRH or saline for 2 min, every 2 h, for 2 wk. Frequent blood samples were collected every 10 min over a period of 4 h during treatment with GnRH or the analog and on alternating days during analog infusion.

Concentrations of progesterone in weekly plasma samples, and in samples obtained daily during frequent sampling, were quantified by radioimmunoassay (RIA). Antibody titers against GnRH were confirmed by the ability of serum to bind radiolabeled GnRH. Concentrations of luteinizing hormone were determined by RIA in weekly samples and in serum obtained during intensive sampling periods.

Antibodies against GnRH were generated in all heifers immunized against GnRH. The onset of luteal activity, associated with puberty initiation, was delayed 5 months in treated heifers. Concentrations of LH in serum were greater ($P<.01$) in immunized heifers compared to control heifers over the 7 mo following the initial immunization. Other studies have indicated a reduction in LH secretion after

immunoneutralization of GnRH. We propose that since concentrations of serum LH were not reduced in treated heifers, GnRH immunization suppressed reproductive activity by inhibiting the preovulatory surge of LH.

Immunized heifers that were treated with GnRH, did not respond with an increase in secretion of LH, however, concentrations of LH in serum increased in immunized heifers following treatment with a GnRH analog. Our results indicate that the pituitary retained its ability to function despite active immunization against GnRH.

Pulsatile GnRH analog delivery over an extended period increased concentrations of LH and LH pulse amplitude but LH pulse frequency was not influenced. The interval from booster immunization against GnRH to the resumption of ovarian luteal activity was not reduced by episodic treatment with the GnRH analog. Previous studies have also failed to induce ovulation and luteal development with gonadotropins or analogs to GnRH in animals immunized against GnRH .

The results of this study indicate that active immunization against GnRH influenced ovarian function in heifers. Initiation of pubertal cycles in heifers was delayed and estrous cycles were abolished following booster immunizations. The lack of a reduction in concentrations of LH is unclear, but suggests that GnRH secretion was not completely inhibited by the antibodies that were produced against GnRH.

The effects of active immunization against GnRH on the secretion of FSH in heifers is unknown. Results reported in other species are inconsistent. Determination of pituitary content of LH and the effects of exogenous estrogen administration on induction of the LH surge in immunized heifers, should be investigated. Information about secretion of FSH and ovarian follicular characteristics following immunization against GnRH may be helpful in elucidating the mechanism whereby GnRH neutralization suppresses reproductive function in heifers.

This technique to immunize animals against GnRH could be utilized to temporarily sterilize livestock. Permanent inhibition of fertility could be achieved with regular booster immunizations. Immunization against GnRH would eliminate problems associated with surgical sterilization such as hemorrhaging and infection. Therefore, aseptic sterilization can be accomplished without sacrificing animal productivity. In conclusion, with the increasing awareness and attention to the humane treatment of animals, immunological sterilization is an alternative form of fertility control and may obtain greater approval from animal rights activists than conventional methods of surgical castration.

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VITA

Catherine Marie O'Connell

Candidate for the Degree of

Master of Science

Thesis: IMMUNIZATION AGAINST GONADOTROPIN RELEASING HORMONE ALTERS PUBERTY, SECRETION OF LUTEINIZING HORMONE AND OVARIAN ACTIVITY IN BEEF HEIFERS

Major Field: Animal Science

Biographical:

Personal Data: Born in Monterey, California; January 27, 1965, the daughter of Michael J. and Barbara R. O'Connell.

Education: Graduated from Memphis Catholic High School, Memphis, Tennessee, June 1983; received Bachelor of Science in Agriculture from the University of Tennessee at Martin, May 1987; completed the requirements for the Master of Science Degree in Animal Science at Oklahoma State University, May 1990.

Professional Experience: Graduate research and teaching assistant at Oklahoma State University 1987-1990.

Professional Organizations: Alpha Zeta Honorary Agricultural Fraternity, American Society of Animal Science, Animal Science Graduate Student Association.