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Luteinizing Hormone an Progesterone Respnse to GnRH Administration at Insemination in Repeat-Breeder Holstein Cows

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LUTEINIZING HORMONE AND PROGESTERONE RESPONSE

TO GnRH ADMINISTRATION AT INSEMINATION

IN REPEAT-BREEDER HOLSTEIN COWS

by

Robert Joseph Callan

A thesis submitted in partial fulfillment
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Robert J. Callan

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Luteinizing Hormone and Progesterone Response

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in Repeat-Breeder Holstein Cows

by

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Utah State University, 1988

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Several studies suggest that the administration of GnRH near the time of insemination improves pregnancy rates in cattle. It has also been reported that there is greater improvement in repeat-breeder animals than at first service. The mechanism for this observation has not been established. Twenty-eight lactating Holstein cows that returned to estrus after one or more inseminations from the USU Caine Dairy were used in the study. Animals were randomly divided into two treatment groups, intramuscular administration of 100 ug GnRH or saline control at the time of insemination. Blood samples were collected at 0, 1, 1.5, 2, 2.5, 3 and 4 hours post-insemination for LH determination and on days 0 through 7, 10, 16 and 22 for progesterone determination. Pregnancy status was determined by rectal palpation 40 to 47 days post insemination.

Serum LH concentrations reached peak concentrations (9.33 $\pm$ 5.5 ng/ml) by one hour following GnRH administration. This was significantly different from saline controls (p<0.005). There was no

relationship between LH peak and subsequent progesterone levels and pregnancy status.

Serum progesterone levels increased as expected from day 0 to day 16 in all animals. Animals treated with GnRH that became pregnant tended to have the highest progesterone levels beginning from day 4. Animals treated with GnRH that were non-pregnant at 40 to 47 days tended to have the lowest progesterone levels from days 4 through 10 but were high on day 16. Pregnant animals had higher progesterone levels than non-pregnant animals from days 4 to 16. These differences approached significance $(0.25 > p < 0.10)$. These results support the contention that GnRH administration affects progesterone levels but do not conclusively establish increased early progesterone levels as the mechanism for improved pregnancy rates. Other hormonal and functional factors may be involved.

(54 pages)

INTRODUCTION

Hypothalamo-Hypophyseal
Anatomy and Physiology

The endocrine system is made up of organs and hormones connected
by neurons, blood and lymph vessels which influence the physiologic
function of all cell types. The hypothalamus responds to external
stimuli and is partially under direct neuronal modulation. The
unique anatomic relationship of the hypothalamus with the pituitary
results in integration of the nervous system with the endocrine
system (1,2).

The hypothalamus is a portion of the brain which lies below the
thalamus. It forms the ventral and lateral walls of the third
ventricle. It has been divided into multiple areas called nuclei.
The hypothalamus provides control over the autonomic nervous system
as well as providing neuroendocrine functions. Neurons originating
in various nuclei produce specific hormones. These hormones are
packaged in secretory granules and are transported down the axons to
the terminal where they are stored (1,2).

The pituitary lies below the hypothalamus and consists of two
portions which are embryologically and histologically distinct. The
neurohypophysis develops from neural tissue and forms the
infundibulum and pars nervosa of the pituitary. It contains axons
and nerve terminals that originate in the hypothalamus. Both
oxytocin and vasopressin, produced in the hypothalamus, are stored
and released from the neurohypophysis. The adenohypophysis develops
from an outpocketing of oral ectoderm termed Rathke's pouch. It is

divided into three portions, the pars distalis or anterior pituitary, the pars intermedia and the pars tuberalis (1,2).

Several of the hormones released by the hypothalamus act specifically on the adenohypophysis. The neuron terminals that release these neurohormones reside in the median eminence of the tuber cinereum. Upon stimulation of the neuron cell body in the hypothalamus by natural pacemakers or external stimuli, secretory granules fuse with the axonal end plate membrane, releasing their contents at the median eminence. The neurohormones are picked up by the capillaries in the median eminence and are delivered to the adenohypophysis via the hypothalamo-hypophyseal portal system. The neurohormones finally diffuse from the pituitary capillary bed to act on specific chromophil cells in the adenohypophysis (1,2).

Early experiments in which the pituitary stalk was sectioned demonstrated the intimate functional relationship of the hypothalamus with the anterior pituitary (3). Hypothalamic extracts were observed to stimulate the release of other hormones from the adenohypophysis. A purified extract was later demonstrated to specifically increase the concentration of luteinizing hormone (LH) in plasma (4). A specific chemical substance was later isolated and its chemical structure determined (5). At this time it was referred to as luteinizing hormone releasing hormone (LHRH). Since this substance has also been shown to stimulate the release of follicle stimulating hormone (FSH) from the pars distalis, it is now termed gonadotropin releasing hormone (GnRH).

GnRH: Molecular and Cell Biology

GnRH is a decapeptide hormone produced by specific neurons located in the hypothalamus. It acts on cells in the pars distalis to stimulate release of LH and FSH. Several synthetic analogs, both agonists and antagonists, have been developed. In general, substitution of a D-amino acid at position six confers metabolic stability and thus increases half life of the peptide. Substitution of ethylamide for Gly^{10} increases activity by enhancing receptor binding affinity (1,6,7).

GnRH is released from the hypothalamus in pulses. There are two major hypothalamic regions where the cell bodies of GnRH neurons are located. The arcuate region lies caudally over the arcuate nucleus and is responsible for the tonic pulsatile release of GnRH. This region controls basal LH levels. The preoptic region lies over the optic chiasm and is responsible for the acute GnRH release which results in the preovulatory LH surge (1,6).

Pulsatile release of GnRH appears to be related to electrical activity in the hypothalamus (6). The neurons that secrete GnRH are partially under neuronal control (8). Norepinephrine appears to stimulate the release of GnRH. It is postulated there are noradrenergic neurons that originate in the brain stem that synapse with the GnRH neurons in the hypothalamic nuclei (6). The actions of norepinephrine can be blocked by alpha-adrenergic blockers. Dopamine appears to inhibit GnRH release. There is also evidence for a tuberoinfundibular dopaminergic tract which terminates near GnRH neuron terminals. Serotonergic, histaminergic and epinephrinergic

neurons are also present within the hypothalamus and may function to provide neural control over GnRH release (6,8).

GnRH release is also influenced by feedback from several hormones including estrogen, progesterone, LH and a factor called inhibin (1,6,7). Progesterone inhibits GnRH release. Estrogen inhibits the tonic center of the hypothalamus but higher levels stimulate the acute centers (6). Inhibin is believed to be released from the ovary and may result in negative feedback at the hypothalamus (1,7). LH causes negative feedback at the hypothalamus, inhibiting GnRH release. LH is delivered back to the hypothalamus both by a direct short loop circulatory pathway and the general peripheral circulation (1,7).

Mechanism of LH Release

GnRH is transported to the pituitary through the hypothalamo-hypophyseal portal system. After diffusion out of the capillary beds in the pars distalis, GnRH binds to specific membrane receptors on basophil cells. Changes in GnRH receptor numbers are related to the estrous cycle, lactation, castration and aging (7,9). After binding, the receptor-ligand complex undergoes patching and capping on the cell membrane surface, followed by internalization. However, these events are not necessary for LH release (7). Current information suggests that receptor-receptor interactions following binding result in microaggregation of receptor complexes. Microaggregation of receptor complexes is believed to stimulate the opening of calcium ion channels. Calcium acts as a second messenger, binding to calmodulin and activating previously inactive enzymes resulting in

the release of stored LH (7). The findings that theophylline augments LH release, by preventing the inactivation of cyclic AMP, and that dibutyryl cyclic AMP can also stimulate LH release suggest that adenyl cyclase is also involved in the release mechanism (6).

Following initial stimulation by GnRH, there is a refractory period where additional GnRH will not initiate additional LH release (7,10). This phenomenon is not calcium dependent and is not due to LH depletion but is associated with receptor binding (7). The refractory period lasts at least 15 minutes (10).

Stimulation by GnRH can result in both sensitization and desensitization of pituitary cells to additional GnRH pulses. A period of increased sensitivity to GnRH that lasts up to 3 hours from the initial GnRH stimulus has been observed (11). This phenomenon occurs during estrus and probably plays a role in the preovulatory LH surge. This has been referred to as the self priming effect of GnRH (6). Following this period, the pituitary becomes less sensitive to additional GnRH pulses (11,12). The period of desensitization lasts up to 24 hours but depends on the stage of the estrous cycle and other hormonal influences. This desensitization does not appear to be due to LH depletion (7,12).

Control of LH Release at the Pituitary

Every LH pulse from the anterior pituitary is preceded by a GnRH pulse. However, not all GnRH pulses elicit an LH response (13). This may be due either to desensitization of pituitary gonadotropes or to changes in threshold levels. There are many hormonal factors that affect the response of the pituitary to GnRH. Low levels of

estrogen suppress LH release (6). However, as estrogen increases, as in the follicular phase of the estrous cycle, the pituitary becomes sensitized and LH response increases (6,7,14). Progesterone decreases the LH response to GnRH (6,7,14,15). Progesterone also inhibits the sensitizing effect of estrogen (7,14).

Following ovariectomy, there is a relatively constant frequency of LH pulses within a species (6). This is due to the removal of the influence of gonadal steroids (1,6). The basal activity has been termed the ultradian rhythm of LH. LH pulse frequency and amplitude varies with different physiological states. As an example, pulses are of low frequency, high amplitude during the luteal phase and high frequency, low amplitude during the follicular phase of the bovine estrous cycle (16).

As mentioned previously, GnRH stimulation of the pituitary results in a refractory period followed by sensitization and/or desensitization. The duration of the refractory period (approximately 15 minutes) is shorter than the natural pulse frequency. During the follicular phase, GnRH demonstrates a self priming effect and pituitary response to GnRH increases approximately 50 times (6).

Direct Ovarian Effects of GnRH

It has been demonstrated that GnRH has direct inhibitory actions on both the ovary and the testes in the human and rat (7,17,18,19,20). GnRH inhibits both FSH stimulation of steroidogenesis in granulosa cells and the FSH mediated induction of LH and prolactin receptor formation on granulosa cells (17).

Hypothalamic GnRH does not reach high enough peripheral concentrations to directly affect the ovary. However there is evidence for a GnRH-like peptide that is produced in the ovary. This substance may perform a local regulatory role (18). Direct ovarian affects however, may not occur in the bovine since GnRH receptors have not been detected in luteal or follicular tissues of the bovine ovary (21).

Physiology of the Bovine Estrous Cycle

The estrous cycle is controlled by the complex interactions of several hormones with several endocrine organs. The hypothalamus and GnRH secretion play a key role in the events leading to estrus and ovulation. While there are still many unanswered questions involving hormonal events and regulation of the estrous cycle, a relatively complete and orderly mechanism has been established. To discuss the estrous cycle, one can divide the hormonal events into four stages; the luteal phase, the pre-surge phase, the preovulatory LH surge, and the post-surge phase. These hormonal events can also be related to behavioral and functional stages.

The luteal phase is characterized by the presence of a functional corpus luteum on one or both ovaries. The predominant steroid hormone is progesterone, produced by the corpus luteum (1). The corpus luteum develops from both thecal and granulosa cells of the ovulatory follicle. It is composed of two distinct cell types. Small luteal cells are of thecal origin and possess a large number of LH receptors but few prostaglandin F_2-alpha and PGE_2 receptors. Large luteal cells are predominantly derived from granulosa cells.

They contain few LH receptors and a large number of PGF_2-alpha and PGE_2 receptors (16). As the luteal phase progresses, small cells can be converted to large cells. The corpus luteum reaches mature size by approximately day 7. While large cells contain fewer LH receptors, they are responsible for the majority of progesterone secretion. Even though LH is luteotropic, it appears that mid-cycle progesterone levels are determined more by the large cell population and LH receptor numbers rather than LH levels (16).

Gonadotropin secretion is influenced by the steroid hormones. During the luteal phase, LH pulses are of low frequency (1 pulse/ 3-4 hours) and high amplitude (4 ng/ml) due to the influence of progesterone (16,22). Follicular growth occurs in waves during the luteal phase. There are generally two waves of folliculogenesis that end in atresia prior to the third and final ovulatory wave during the bovine estrous cycle (16,23). Progesterone concentrations in peripheral blood reach peak levels of 5 to 6 ng/ml between days 10 to 16 of the estrous cycle (22,24).

Factors influencing the development and regression of the corpus luteum are not clearly understood. While LH is the major luteotropic hormone, research suggests LH receptor numbers on the corpus luteum rather than LH levels govern corpora lutea activity (16,22). Prostaglandin F_2-alpha produced by the uterus is considered the major luteolytic hormone. The corpus luteum is not responsive to PGF_2-alpha during the first 5 days of the cycle. This may be related to the higher number of small cells with few prostaglandin receptors during the early luteal phase. Normal luteolysis in cycling cows

occurs near day 16 of the estrous cycle. The PGF_2-alpha is apparently produced by the endometrium and delivered to the ovary and corpus luteum via a local circulatory pathway of uterine veins and lymphatics with the ovarian artery (1,16,22).

Oxytocin is involved in corpora lutea regression but the mechanism is not clearly understood. Oxytocin administration during the first five days of the luteal phase will prevent corpora lutea maturation and induce estrus and ovulation independent of PGF_2-alpha (16,22). When given later in the luteal phase, oxytocin appears to induce luteal regression by stimulation of uterine release of PGF_2-alpha (22,24). Oxytocin is present within both the ovine and bovine corpus luteum but the significance of this source is not known (22,25).

Prostacyclin (PGI_2) and PGE may also be involved in corpora lutea function and regression. Recent evidence suggests that PGI_2 is luteotropic and functions to increase corpora lutea maturation and progesterone production in the early luteal stages. Both the endometrium and luteal tissue have been shown to synthesize PGI_2. Prostaglandin E_2 appears to have an antiluteolytic role in the ewe. It has been shown to prevent the decreased secretion of progesterone by luteal tissue following PGF_2-alpha administration. Prostaglandin E_2 is secreted by the uterus and levels are greater in pregnant ewes than cycling ewes on days 13 to 17 post estrus (16,22).

Following luteolysis, peripheral progesterone levels drop to basal levels of less than 1 ng/ml within 2 to 4 days (24). The removal of the negative feedback of progesterone on GnRH and LH

secretion results in a moderate increase in basal LH levels (22). This is the beginning of the pre-surge phase. Rising LH levels stimulate increased steroidogenesis by the developing tertiary follicles. Increased LH pulse frequency (>1 pulse/min) and decreased pulse amplitude (2 ng/ml) are observed at this time (16,22).

In the bovine, thecal cells have LH but not FSH receptors and respond to the increased LH levels by increasing androgen production, primarily androstenedione (16,23). Thecal cells lack the ability to aromatize androgens to estradiol. The androgens are secreted and enter the granulosa cells where they are converted to estradiol. While granulosa cells have both LH and FSH receptors, this process does not appear to be influenced by LH or FSH (23). Rising estradiol levels have a positive feedback on estradiol production by promoting androgen syntheses by thecal cells (23).

The increase in estradiol levels is the signal that initiates the preovulatory surge. Several hormonal events act in synchrony to induce the LH surge. Elevated estradiol levels stimulate the acute center of the hypothalamus for increased GnRH release. Estradiol also increases LH release from the pituitary in response to GnRH pulses. The self priming effect of GnRH on the pituitary is increased by elevated estradiol. Finally, progesterone levels must be low, reducing the negative feedback on GnRH and LH release (22,26).

The preovulatory LH peak occurs 2 to 4 hours after the onset of estrus in the bovine (26,27). Plasma LH levels reach a peak of 30 to 35 ng/ml (26). The duration of the LH peak is 10 to 12 hours and

ovulation follows the LH peak by approximately 20 to 25 hours (26,27).

The most significant hormonal event following the preovulatory surge is the dramatic drop in estradiol levels both in the follicular fluid and in the blood (22,23,27). It appears the LH surge initiates a decrease in estradiol synthesis by the mature follicle. The LH surge is also the signal initiating the events of ovulation. The current proposed mechanism of ovulation involves an inflammatory type response in the ovulatory follicle(s) initiated by the preovulatory LH surge (16). Following ovulation, luteinization and development of the corpus luteum begins and the cycle returns to the luteal phase. Normal cycle length varies from 21 to 22 days in the bovine (1).

GnRH: Clinical Applications in
the Bovine

The development and availability of synthetic GnRH has resulted in increased usage of the drug in the dairy industry for a variety of reproductive disorders. Perhaps the earliest report of clinical use of GnRH in the bovine was for the initiation of ovulation in heifers following progesterone synchronization (28). Reports of GnRH administration for the treatment of cystic ovarian degeneration, initiation of early postpartum ovulation and improved fertility soon followed (29,30,31).

Cystic ovarian disease is a relatively common reproductive abnormality in dairy cattle. These are defined as one or more anovulatory follicle(s) generally greater than 2.5 cm in diameter that persist for more than ten days. The incidence has been reported

to be 6 to 19 percent, however, many cystic ovaries resolve spontaneously and go undiagnosed (32). The direct cause of cystic ovaries has not been established although three theories are increased estrogen levels, decreased preovulatory LH release and decreased LH receptors on follicular cells (1). Many underlying causes have been established including hereditary predisposition, nutritional deficits and intrauterine infections (1,32,33). Recent work investigating the relationship of intrauterine infections with cystic ovaries suggests a hormonal mechanism where bacterial endotoxins cause increased uterine prostaglandin release resulting in elevated ACTH and cortisol levels which inhibit the preovulatory LH surge (33).

The administration of GnRH has become one of the standard treatments for cystic ovarian disease in dairy cattle. Reported recovery rates range from 62 to 97 percent (32,34). Following GnRH administration there is a characteristic increase in serum LH concentration. Peak LH levels are attained by two hours and vary from 18 to 40 ng/ml relative to GnRH dosages of 100 to 250 ug administered intramuscularly (34). Serum progesterone subsequently increases to more then 2 ng/ml by day 11 after GnRH treatment (34). Recovery rates of 62 to 97 percent have been reported. This is based on return to estrus within 30 days of GnRH treatment (32,33,34).

Reproductive performance is influenced during the postpartum period (parturition to first breeding). It has been observed that the number of estrous periods prior to breeding is related to conception rates (35). It was demonstrated very early that GnRH

administration 14 days postpartum induces LH release and ovulation in the bovine. These cows were observed to have normal estrous cycles through day 65 postpartum. However, while ovulatory cycles began earlier in GnRH treated cows, the interval to first observed behavioral estrus was not different from controls (30).

Numerous studies involving administration of GnRH in the early postpartum period have been performed. A recent review discusses many of the findings (36). This review reports that the pituitary is not responsive to exogenous GnRH until 7 to 10 days postpartum. This is apparently due to insufficient estradiol levels since LH release is related to pretreatment estradiol concentrations and previous injection of estradiol results in pituitary response to GnRH as early as 2 days postpartum (36).

Several benefits resulting from GnRH administration 12 to 14 days postpartum have been reported. There is an early initiation of ovulation and subsequent estrous cycles (30,36,37). The estrous cycle lengths are of normal duration up to the time of first breeding. The incidence of cystic ovaries is also reduced. Treated animals generally show improvement in reproductive performance over controls providing breeding begins early postpartum (ie. average days to first breeding ≤ 80 days) (36). When breeding occurs later postpartum, no difference is observed. Improved reproductive performance is reported with early postpartum GnRH administration in cows with periparturient disease, especially retained placenta or metritis (36,37). However, it is also reported that early postpartum treatment with GnRH increases the incidence of pyometra (38).

Another report noted that the use of GnRH resulted in less involuntary culling for reproductive failure (36).

The use of GnRH at the time of breeding to improve fertility has been widely investigated and has produced variable results (39-48). A summary of several studies where GnRH is administered at the time of breeding is presented in table 1.

Overall, there seems to be the potential for an increase in pregnancy rate up to 25 percent. In almost all cases, a numerical increase in pregnancy rate is observed. In several of the reports, this increase in pregnancy rate has been statistically significant (40,41,42,44,45,46). There is some indication that there may be a better response to GnRH in repeat-breeder ($\geq$ 3 services) cattle than in first or second service cattle (46).

Reproductive Failure in Cattle

For many years, investigators have been trying to identify the various causes of reproductive failure. A recent study has attempted to estimate the economic impact of repeated inseminations in dairy cattle (49). Under the conditions examined in 22 Michigan dairy herds in 1985, a loss of approximately $385 was associated with the repeat breeder syndrome in a given lactation per cow. Reported incidence of repeat-breeder syndrome has ranged from 1 to 24 percent (49,50,51).

An animal with repeat breeder syndrome has been defined as a cow or heifer that is not pregnant after at least three inseminations at regular intervals and has shown no clinical signs of genital disease (49,50). This is distinguished from repeat-breeders which are

Table 1: Summary of results from several reports on the effect of
GnRH administration at breeding on pregnancy rate.

| | PERCENT PREGNANT | | | |
EXPERIMENTAL GROUP	CONTROL	GnRH	DIFFERENCE	REFERENCE
FIRST SERVICE BREEDING INTERVAL				
<61 days postpartum	34.5	41.7	7.2	39
40 to 60 days postpartum	49.5	58.7*	9.2	40
>60 days postpartum	50.6	59.7	9.1	39
3 to 6 weeks postpartum	41.7	86.7*	45.0	41
4 to 6 weeks postpartum	35.7	83.7*	48.0	42
7 to 9 weeks postpartum	60.0	75.0	15.0	41
FIRST SERVICE COWS	54.1	58.8	4.7	43
	50	57 *	7.0	44
	46	47	1.0	45
	31.9	48.4	16.5	46
FIRST SERVICE HEIFERS	68.8	64.4	-4.4	46
SECOND SERVICE COWS	46	55	9.0	45
	56.6	58.1	1.5	43
THIRD OR GREATER SERVICE COWS	51	66*	15.0	45
	48	73*	25.0	46
	53	44	-11.0	43
FOURTH SERVICE COWS	37.7	47.0	10.0	47

*statistically different from controls (p<0.05)

animals that are not pregnant after one or more inseminations with or without signs of genital disease (51).

There are many possible causes of reproductive failure in the bovine. These causes may be placed in two categories depending on whether they result in failure of fertilization or embryonic death. Some of these causes may fall into both categories. A considerable amount of work has been done to identify the relative incidence of either fertilization failure or embryonic death (51-64). It is commonly agreed that fertilization rates in cattle can approach 85 to 95 percent or more (57-62). However, there is considerable variation in the reported frequency of fertilization failure in repeat-breeder cattle. Several reports indicate fertilization failure approaches 30 to 40 percent in repeat-breeder cattle and is significantly greater than in normal cattle (54,57,63). Other reports show no difference between repeat-breeder and normal cattle (52,53,55,62).

There is standard agreement that embryonic death is responsible for the majority of reproductive failure (51-57,62,64). Up to 40 percent of fertilized ova suffer embryonic death in normal dairy cattle (52,53,56). It has been estimated that 75 to 80 percent of embryonic and fetal death occurs before day 18 after breeding (61,65). The remainder occurs near implantation (10-15%) and between implantation and full term (5-8%). It has since been demonstrated that the critical period for early embryonic death is between days 6 and 7 after breeding (51,54,55,57). It also seems apparent that repeat-breeders have a higher incidence of early embryonic death than do normal cattle (55,57). Embryos collected 7 days post-breeding

showed only 28 percent normal embryos in repeat-breeder heifers while virgin heifers had 74 percent normal embryos (62).

As indicated previously, several causes of reproductive failure can result in either fertilization failure or embryonic death. Failure of ovulation at estrus has been reported in dairy cows (1,66). Obviously, this would result in failure of fertilization. It has been reported that this defect occurs in up to 8.8 percent of the estrous cycles (66).

There are very few nutritional experiments associated with fertility. One study showed that beef heifers subjected to short-term undernutrition had a lower percentage of normal fertilized ova and lower plasma progesterone levels than controls (60). A recent report of investigation in a dairy herd with decreased fertility noted that increased degradable protein in the diet can raise blood urea nitrogen levels and cause decreased pregnancy rates in dairy cattle (67).

It has been observed that climate can affect fertility. Environmental heat stress can result in decreased pregnancy rates in dairy cattle. Excessive environmental temperature can increase body temperature in cattle and cause a significant increase in early embryonic death (68,69,70).

Technique and timing of artificial insemination are critical in attaining optimum fertilization rates and embryo survival. Proper semen handling and insemination techniques have been described. The timing of insemination with ovulation is also related to both fertilization failure and early embryonic death (71,72,73).

Artificial insemination in the uterine body should occur between 18 and 7 hours before ovulation for optimum fertilization rates. For optimum embryo survival, fertilization should occur within six hours after ovulation. Early embryonic death increases when animals are inseminated later than six hours after ovulation (73).

Abnormal uterine environment has been associated with embryonic death. One study has shown increased concentrations of potassium, zinc, phosphorous and calcium in uterine flushes of animals with abnormal embryos at 7 days post breeding (57). It is not clear whether this is related to the cause of embryonic death or a result of embryonic death. Further, it does not appear that abnormal uterine environment is a significant factor in the repeat-breeder syndrome after 7 days post breeding. It has been observed that repeat-breeders are suitable embryo recipients at day 7 and have pregnancy rates comparable to normal cattle (64).

Several reports have described conditions of hormonal asynchrony both before and after breeding (51,57,74-77). Differences in estradiol patterns between normal and repeat-breeder cattle and between animals with normal and abnormal embryos have been reported (51,57). Other studies have shown abnormal variations in progesterone levels in some infertile cows (76,77). Preovulatory LH peak heights and basal LH levels after insemination did not differ between repeat-breeders and normal cows, nor are they related to pregnancy status (51,78). However, the interval from the beginning of estrus to the LH peak has been reported to be shorter in normal cattle (3.3 hr) than in repeat-breeders (20.0 hr) (51).

It is recognized that progesterone is necessary for the maintenance of pregnancy. Luteolysis of the corpus luteum will consistently result in termination of pregnancy up to 150 days gestation in the bovine (79). The critical stage for the recognition of pregnancy is between days 15 to 17 of the estrous cycle in the bovine. At this time, non-pregnant animals will undergo spontaneous luteolysis and enter the period of estrus. Considerable work has compared progesterone levels between pregnant and non-pregnant animals. Several studies show no significant difference up to day 16 of the estrous cycle (74,77,78). Other studies have demonstrated a difference in early progesterone levels between pregnant and non-pregnant cattle and between animals with normal and abnormal embryos (51,80). It has also been reported that repeat-breeder cattle have lower progesterone levels than normal cattle as early as 6 days post breeding (51).

Information has led to attempts of progesterone supplementation or stimulation to increase pregnancy rates. A recent review has addressed this topic (81). Several studies have demonstrated a trend toward increased pregnancy rates (10 to 60%) in cows supplemented with progesterone. However, control animals in these studies tended to have low pregnancy rates (16 to 42%) Most of these results were not statistically significant. Treatment with GnRH or human chorionic gonadotropin (HCG) at estrus as well as mid-cycle HCG administration have been reported to stimulate progesterone levels but there was no increase in pregnancy rates observed in these studies (51,81).

Several general concepts can be obtained from this review of reproductive failure. First, early embryonic death and not fertilization failure is the predominant cause of reproductive losses in normal cattle. It also appears that early embryonic death is more significant in repeat-breeders as well. The critical period when the majority of embryonic death is first apparent is 6 to 7 days after breeding. However, this may be due to factors preceding this period. Early embryonic death may be a result of several interrelated factors including abnormal time of ovulation, nutrition, climate, insemination conditions, and hormonal abnormalities. While increased progesterone levels seem to be associated with pregnancy, it is not clear whether this is a cause or effect relationship.

Purpose of Study

As discussed earlier, GnRH administration at insemination has been used in the dairy industry to improve conception rates. Several researchers have investigated the effect of GnRH on pregnancy rates in dairy cattle when administered at the time of artificial insemination (39-48). Results vary from a slight decrease in pregnancy rates to 25 percent increase in pregnancy rates. Several studies report increased pregnancy rates that are statistically significant (40,41,42,44,45,46). A greater benefit has been reported in repeat-breeder animals than in normal or first service animals (46,47).

The wide variation in results may be attributed to several factors including, experimental design, dosage, route of administration, experimental population, and reproductive management

factors. Reproductive failure in cattle is a result of many different conditions (50,57). A portion of the variability in reported results may be attributed to different distributions of factors responsible for reproductive failure in different experimental populations.

The mechanism by which GnRH administration at insemination may increase pregnancy rates in cattle has not yet been identified. GnRH may have a general affect on reproductive failure or it may have a specific affect on individual causes of reproductive failure. Several theories for the action of GnRH have been proposed including the affects of GnRH on corpora lutea development, progesterone levels, timing of ovulation with insemination, uterine environment, fertilization and embryonic survival.

A recent article discusses the economic return associated with the use of GnRH to improve fertility in dairy cows (82). This report suggested the use of GnRH at second and/or third service is profitable in most dairy herd conditions. Potential profit increases in herds with low heat detection efficiency and low conception rates. An understanding of the mechanism by which GnRH administration at breeding may increase pregnancy rates could help to identify animals which are more likely to respond favorably to GnRH. The purpose of this study was to examine the LH and progesterone response to GnRH administration at insemination in repeat-breeder cows. This information may lead to an understanding of the possible mechanism by which GnRH administration at insemination may improve pregnancy rates.

MATERIALS AND METHODS

Twenty eight lactating Registered Holstein dairy cows were used
in the study. The animals were maintained at the Caine Dairy
Teaching and Research Center, Utah State University. The Caine Dairy
milks approximately 250 cows two times per day. Cows are divided
into pens based on production level and research project.
Approximately 100 cows were available for use on this project,
however many of these were already pregnant. Approximately 60 cows
were maintained indoors in tie stalls and the remainder were outdoors
in freestall pens. Animals were fed twice daily following milking
with a balanced total mix ration (Appendix B). Long stem alfalfa hay
was also provided twice daily prior to milking.

Selected animals had normal postpartum histories with normal
estrous cycle lengths. Postpartum reproductive exams were performed
by USU veterinarians at 30 to 40 days after parturition. All animals
were previously bred at least once but returned to estrus. Estrus
detection was performed by the herdsman following milking and during
his daily routine. Milkers and other dairy personnel also observed
animals for signs of estrus during their daily work. Estrous signs
observed and recorded included standing, riding, presence of vaginal
mucous and red K-mar[R]. Artificial insemination is recommended
between ten to twelve hours after observed heat at the USU dairy but
subject to the herdsman's discretion. Artificial insemination in
this group of animals ranged from 5.5 to 16 hours after first

observed estrus. Individual cow data was collected at the time of insemination. This included the number of times bred, number of lactations, average daily milk production, number of days since parturition, body condition score, time of first observed signs of estrus, estrous signs observed and time of insemination.

Immediately following artificial insemination (time 0), a 10 ml blood sample was obtained from the coccygeal vein. Animals were then randomly treated with either 100 ug GnRH (2 ml Cystorelin[R], 50 ug/ml, Ceva Laboratories, Inc.) or 2 ml sterile saline intramuscularly. Additional blood samples were collected at 1, 1.5, 2, 2.5, 3 and 4 hours post insemination for LH determination and on days 1 through 7, 10, 16 and 22 for progesterone determination. In addition, three animals were selected and blood samples were collected every two hours during estrus up to the time of insemination. These samples were analyzed for serum LH concentration.

Blood samples were centrifuged within 30 minutes to minimize the effect of progesterone degradation by red blood cells (83). Serum was collected and stored at -4° C in plastic vials (Securivial, Diagnostic Products, Inc.). Animals were observed for subsequent signs of estrus following insemination until confirmed pregnant. Pregnancy was determined by rectal palpation at 40 to 47 days post insemination. Pregnancy was reconfirmed by rectal palpation at 60 to 70 days post insemination.

Hormone analysis were performed at the Reproduction Physiology Lab, Utah State University. Serum LH concentrations were determined on 0, 1, 1.5, 2, 2.5, 3 and 4 hour samples. A double antibody

radioimmunoassay procedure was used to quantitate LH concentration (Appendix C). Anti-ovine-LH #15 and CSU-240 were used as the first antibody. Goat anti-rabbit antibody was used for the second antibody (Diagnostic Products Corporation, catalog #5N6). Serum progesterone concentrations were quantitated on blood samples from days 0 through 7, 10, 16 and 22. The Coat-A-Count^R Progesterone RIA (Diagnostic Products Corporation) was used to determine serum progesterone concentrations (Appendix D).

Individual cow parameters were analyzed using the chi square test for discrete variables for both treatment and condition (pregnancy status). LH and progesterone levels were analyzed with respect to cow parameters using one way analysis of variance. Comparison of LH and progesterone levels over time with respect to treatment and condition were performed by using analyses of variance for split-plot in time. Progesterone levels for individual treatment and condition combinations on given days were compared using the least significant difference (LSD) test.

RESULTS

Of the twenty eight cows used in the study, four were found not to be in estrus at breeding based on high progesterone levels at day 0. One of these animals subsequently had low progesterone levels on days 7 and 10 and was presumed not to have been in estrus at the time of insemination. The other three were confirmed pregnant to an earlier service based on persistently high progesterone levels and estimation of the number of days pregnant at the time of pregnancy exam. Only the remaining 24 animals were used in the subsequent analysis, resulting in 12 GnRH treated and 12 saline control animals.

Both GnRH and saline treated groups were observed to have a 50 percent pregnancy rate following pregnancy exam by rectal palpation. This resulted in 6 animals pregnant and 6 animals non-pregnant in each treatment group. Significant differences in pregnancy rates were not expected in this study due to the small sample size.

Discrete cow parameters are summarized in table 2 by both treatment and condition of pregnancy. There were no statistically significant differences in these parameters for either the treatment or condition groups. Variation in LH and progesterone levels were not related to any of the individual cow parameters measured. There were no significant correlations between LH levels and progesterone levels.

Of the three animals bled during estrus, one demonstrated an endogenous preovulatory LH peak of 32.3 ng/ml 3 hours after the

Table 2. Averages and ranges of individual cow parameters by treatment group
(GnRH at insemination or saline controls) and pregnancy condition in
experimental cows from the USU Caine Dairy.

| | GnRH | | SALINE | | PREGNANT | | NON-PREGNANT | |
PARAMETER*	AVERAGE	RANGE	AVERAGE	RANGE	AVERAGE	RANGE	AVERAGE	RANGE
No. of Animals	12		12		12		12	
No. BREEDINGS	3.4	2-5	3.9	2-8	3.9	2-8	3.4	2-7
No. LACTATIONS	1.9	1-4	2.4	1-6	2.2	1-5	2.1	1-6
AVE. DAILY MILK PRODUCTION (kg)	31.4	15-43	32.7	19-46	33.7	25-42	30.6	15-46
DAYS SINCE PARTURITION	180	116-373	194	106-364	200	106-373	174	109-343
BODY CONDITION SCORE	3.2	3-4	3.1	2.5-4	3.1	3-4	3.2	2.5-4
ESTRUS TO BREEDING (hrs)	11.2	5.5-16	9.8	6-14.5	11.3	7.8-15.5	9.7	5.5-16

*At the time of insemination and treatment.

animal was first observed in estrus. The LH level dropped to 6.1 ng/ml by the time of insemination 7 hours after estrus was first observed. The other two animals bled during estrus maintained low LH levels (<1 ng/ml) for all samples taken during estrus.

Luteinizing hormone concentration increased and peaked by one hour after GnRH administration (table 3, figure 1). Peak levels averaged 9.33 ± 5.5 ng/ml at one hour. There was no statistically significant difference in peak LH response between pregnant versus non-pregnant cows after receiving GnRH (figure 2). Average peak LH levels for GnRH pregnant and GnRH non-pregnant animals were 9.67 ± 5.92 and 9.00 ± 5.58 ng/ml respectively. There was no difference in LH response over time between GnRH pregnant and GnRH non-pregnant animals. LH response over time was significantly different with respect to treatment (p<0.005). While GnRH treated animals showed an LH surge, LH concentration in saline control animals continued to fall to basal levels with time (figure 1).

Average progesterone levels are summarized in table 4 by treatment and condition. There was a consistent trend for pregnant animals (GnRH and saline animals combined) to have higher serum progesterone concentrations than non-pregnant animals starting by day 4 (figure 3). The difference approached statistical significance (0.25>p>0.10). Serum progesterone concentrations over time were significantly higher in pregnant animals than in non-pregnant animals when carried through day 22 (p<.001).

GnRH treated animals that were diagnosed pregnant tended to have higher progesterone levels than any other treatment and condition

Table 3. Average serum luteinizing hormone concentration (ave. ± sem, ng/ml) in cows from the USU Caine Dairy following intramuscular administration of 100 ug GnRH or saline at insemination (0 hour).

	GnRH			SALINE		
HOUR	PREGNANT	NON-PREGNANT	COMBINED	PREGNANT	NON-PREGNANT	COMBINED
(n)	(6)	(6)	(12)	(6)	(6)	(12)
0	1.15 ± 0.86	1.42 ± 2.34	1.29 ± 1.69	0.29 ± 0.33	6.65 ± 14.2	3.47 ± 10.2
1.0	7.88 ± 6.90	9.00 ± 5.58	8.44 ± 6.01	0.59 ± 0.48	3.53 ± 7.94	2.06 ± 5.58
1.5	5.16 ± 4.93	5.65 ± 4.98	5.40 ± 4.73	0.28 ± 0.25	2.38 ± 4.97	1.33 ± 3.53
2.0	4.37 ± 3.30	3.85 ± 3.45	4.11 ± 3.23	0.23 ± 0.18	2.49 ± 5.26	1.36 ± 3.74
2.5	4.97 ± 4.44	2.08 ± 2.14	3.53 ± 3.65	0.36 ± 0.34	1.73 ± 2.89	1.05 ± 2.09
3.0	1.75 ± 2.13	1.68 ± 2.16	1.71 ± 2.05	0.36 ± 0.40	0.89 ± 1.87	0.63 ± 1.32
4.0	0.90 ± 1.06	0.69 ± 0.70	0.80 ± 0.86	0.40 ± 0.49	0.57 ± 0.90	0.48 ± 0.69
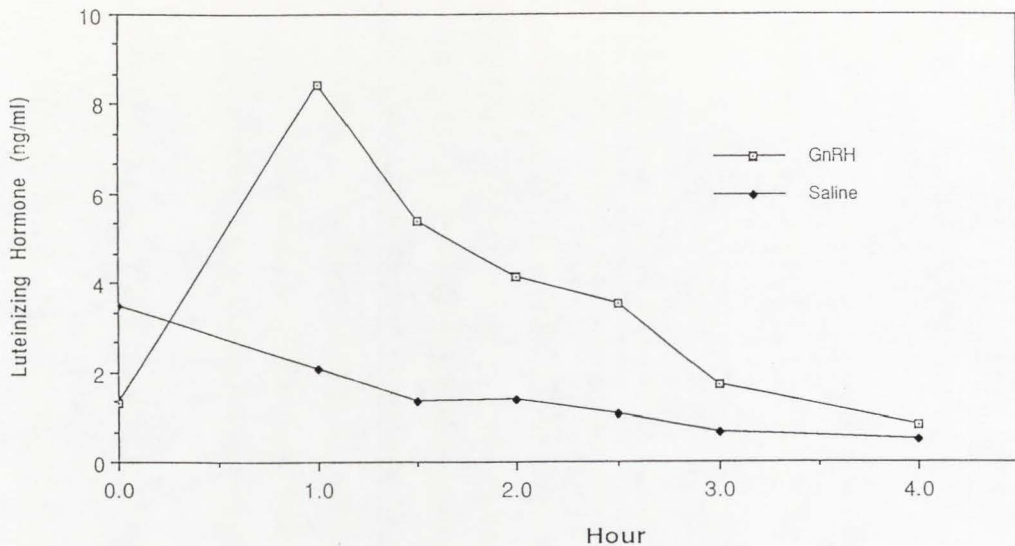
Figure 1. Comparison of serum luteinizing hormone concentration (ng/ml) in cows from the USU Caine Dairy following administration of 100 ug GnRH or saline at insemination (0 hour). LH levels over 4 hour period differ between GnRH and saline animals (p<0.005).
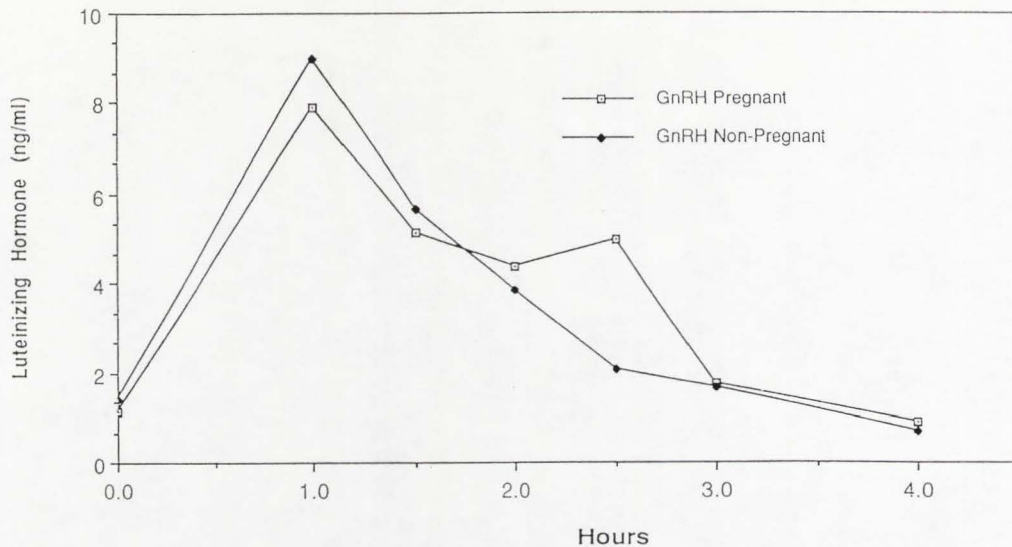
Figure 2. Comparison of serum luteinizing hormone concentration (ng/ml) in cows from the USU Caine Dairy that were subsequently diagnosed pregnant or non-pregnant following administration of 100 ug GnRH at the time of insemination (0 hour). LH levels over 4 hour period are not different between GnRH pregnant and GnRH non-pregnant animals.

Table 4. Average serum progesterone concentrations (ave $\pm$ sem, ng/ml) in cows from the USU Caine Dairy following intramuscular administration of 100 ug GnRH or saline at insemination (day 0).

| DAY | | PREGNANT | | | NON-PREGNANT | |
| | GnRH | SALINE | COMBINED | GnRH | SALINE | COMBINED |
(n)	(6)	(6)	(12)	(6)	(6)	(12)
0	0.05 $\pm$ 0.03	0.06 $\pm$ 0.04	0.06 $\pm$ 0.03	0.07 $\pm$ 0.03	0.10 $\pm$ 0.09	0.08 $\pm$ 0.07
1	0.06 $\pm$ 0.04	0.04 $\pm$ 0.02	0.05 $\pm$ 0.03	0.06 $\pm$ 0.04	0.10 $\pm$ 0.10	0.08 $\pm$ 0.07
2	0.12 $\pm$ 0.09	0.08 $\pm$ 0.03	0.10 $\pm$ 0.07	0.05 $\pm$ 0.04	0.10 $\pm$ 0.08	0.07 $\pm$ 0.06
3	0.32 $\pm$ 0.10	0.21 $\pm$ 0.09	0.26 $\pm$ 0.11	0.15 $\pm$ 0.10	0.29 $\pm$ 0.22	0.22 $\pm$ 0.18
4	0.62 $\pm$ 0.31	0.47 $\pm$ 0.12	0.55 $\pm$ 0.24	0.39 $\pm$ 0.25	0.40 $\pm$ 0.28	0.40 $\pm$ 0.25
5	0.94 $\pm$ 0.27	0.86 $\pm$ 0.19	0.90 $\pm$ 0.22	0.75 $\pm$ 0.67	0.75 $\pm$ 0.42	0.75 $\pm$ 0.54
6	1.52 $\pm$ 0.51	1.20 $\pm$ 0.45	1.36 $\pm$ 0.49	1.18 $\pm$ 0.97	1.34 $\pm$ 0.50	1.26 $\pm$ 0.74
7	1.94 $\pm$ 0.62	1.80 $\pm$ 0.83	1.87 $\pm$ 0.70	1.49 $\pm$ 0.99	2.03 $\pm$ 0.68	1.76 $\pm$ 0.86
10	3.14 $\pm$ 1.00	3.08 $\pm$ 1.57	3.11 $\pm$ 1.26	2.34 $\pm$ 1.55	2.97 $\pm$ 0.35	2.66 $\pm$ 1.12
16	4.15 $\pm$ 1.19	3.71 $\pm$ 1.48	3.93 $\pm$ 1.30	4.21 $\pm$ 2.31	3.26 $\pm$ 1.66	3.73 $\pm$ 1.98
22	4.32 $\pm$ 1.13	3.60 $\pm$ 1.37	3.96 $\pm$ 1 26	0.13 $\pm$ 0.13	0.15 $\pm$ 0.12	0.14 $\pm$ 0.12
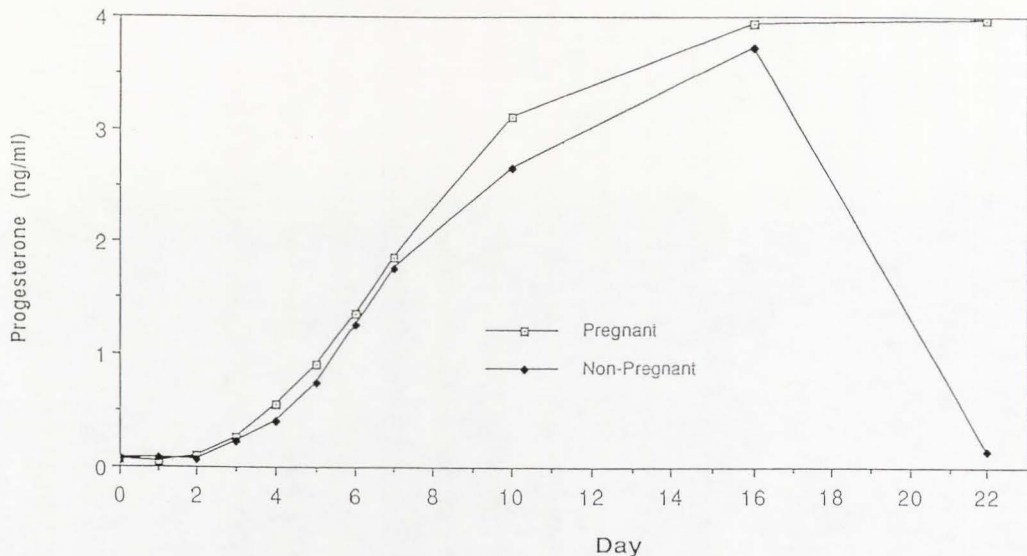
Figure 3. Comparison of serum progesterone concentrations in pregnant and non-pregnant cows from the USU Caine Dairy that received either 100 ug GnRH or saline intramuscularly at the time of insemination (day 0). Pregnancy was diagnosed by rectal palpation at 40 to 47 days post insemination. The difference in progesterone levels between pregnant and non-pregnant animals through day 16 approached significance $(0.25 > p > 0.10)$.

combination (figure 4). GnRH treated animals diagnosed non-pregnant tended to have the lowest progesterone concentrations through day 10, but were high on day 16. These trends were first evident by day 4 $(0.25 > p > 0.10)$.

Comparisons of progesterone concentration for each treatment and condition combination on individual days showed no significant differences at $p < 0.05$ ($LSD_{0.05} = 0.95$ ng/ml). However, the difference between both pregnant and non-pregnant GnRH treated animals (4.15 and 4.21 ng/ml respectively) and non-pregnant saline controls (3.26 ng/ml) approached significance ($p < 0.10$) on day 16. The difference between GnRH pregnant (3.14 ng/ml) and GnRH non-pregnant (2.34 ng/ml) animals also approached significance ($p < 0.10$) on day 10 ($LSD_{0.10} = 0.78$ ng/ml)

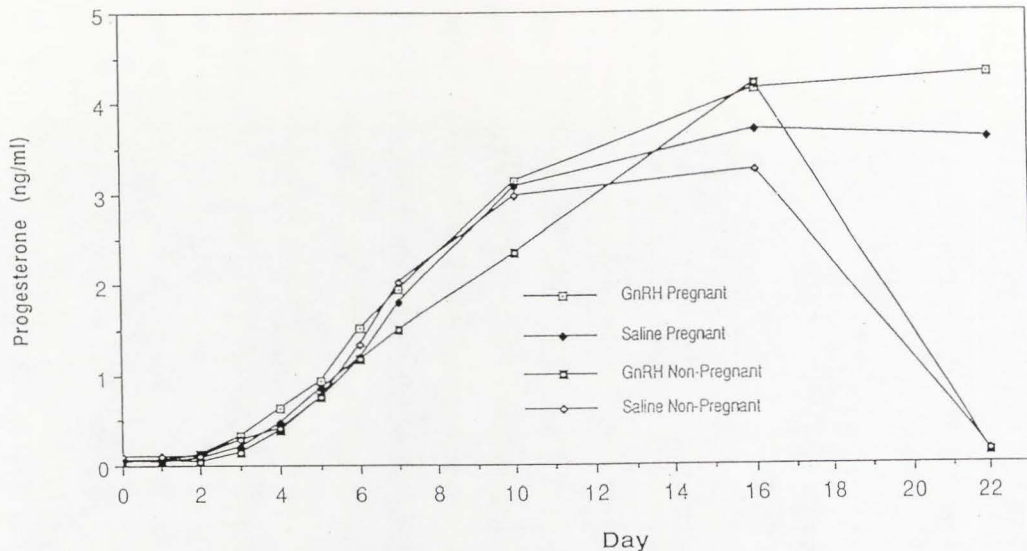Figure 4. Comparison of serum progesterone concentrations for all treatment and condition combinations. Cows from the USU Caine Dairy were treated with either 100 ug GnRH or saline intramuscularly at the time of insemination (day 0). Cows were diagnosed as pregnant or non-pregnant by rectal palpation at 40 to 47 days post-insemination. Treatment by condition interaction approached significance (0.25>p>0.10) on days 4 through 16.

DISCUSSION

The endogenous preovulatory LH surge normally occurs between two to four hours following the onset of estrus in the bovine (26,27,84). This timing was observed in one of the animals bled during estrus . However, the endogenous preovulatory LH surge was not observed in the other two animals although they had low progesterone levels at insemination. This suggests there was difficulty detecting the beginning of estrus in these animals under the management system used.

This study demonstrated the pituitary is still responsive to GnRH at the time of insemination, 10 to 12 hours after the onset of estrus. This is in agreement with other reports (84,85). Peak LH levels of approximately 9 ng/ml in response to intramuscular injection of 100 ug GnRH were observed. This is similar to the response previously reported in dairy cows treated with GnRH at first postpartum breeding (85), and in cows and heifers treated with GnRH 72 hours following synchronization with prostaglandin F_2-alpha (84). However, these peak levels are considerably less than the 60 to 80 ng/ml peaks reported following intravenous administration of 100 ug GnRH on days 2 or 10 of the estrous cycle (86).

The duration of the stimulated LH peak in other reports coincides with our observation of approximately four hours. We did not observe the variability in LH response and its association with subsequent pregnancy status that has been reported previously (85).

Although LH levels prior to insemination and treatment were only obtained in three animals, we did not observe any apparent association with the spontaneous preovulatory LH surge. GnRH was apparently administered after the endogenous LH surge in all animals. Three different LH responses in relation to the endogenous preovulatory LH surge following GnRH administration 72 hours after estrus synchronization with prostaglandin have been described (84).

Stimulation of progesterone levels following GnRH administration at insemination is one proposed theory to account for the improved pregnancy rates associated with this treatment. Progesterone is necessary for the maintenance of pregnancy. The association of progesterone levels with pregnancy has been reported (51,74,77,78,80,81). Several reports indicate pregnant animals show higher progesterone concentrations as early as four days post insemination (51,80,81). However, several reports indicate no difference between pregnant and non-pregnant cows prior to luteal regression (74,77,78). Further, it has not been determined if increased progesterone in pregnant cattle is a cause or effect relationship.

This study demonstrated a consistent trend of slightly higher progesterone levels in pregnant animals through day 16 (figure 3). This difference approached statistical significance (0.25>p>0.10). Some researchers have reported statistically higher progesterone levels in pregnant animals also using analysis of variance split-plot in time (84,85). It is unclear in these reports whether the analysis was terminated on day 16 or continued beyond normal luteal

regression. Progesterone levels in this study were significantly different when carried through day 22 (p<0.001). However, this is due to the drop in progesterone in non-pregnant animals following luteal regression and not due to earlier progesterone levels.

Progesterone supplementation has been used to demonstrate a causal relationship of progesterone with pregnancy. Progesterone supplementation to increase pregnancy rates in cattle has shown variable results. Increased pregnancy rates of 10 percent and greater have been reported following progesterone supplementation (81). The effect of GnRH administration at insemination on subsequent progesterone levels is inconclusive. This study demonstrated higher progesterone levels in animals treated with GnRH that were diagnosed pregnant (figure 4). This difference was first evident by day 4. However, the increase was not statistically significant. Animals treated with GnRH that were diagnosed non-pregnant tended to have the lowest progesterone levels through day 10. These findings are consistent with a similar report using GnRH in first service cows (85).

Another study has reported that both pregnant and non-pregnant animals previously treated with GnRH near insemination had lower progesterone levels than saline controls (84). This difference may in part be due to the different experimental design where animals synchronized with prostaglandin were treated 72 hours later and inseminated at 80 hours. Our study utilized animals in natural estrus and inseminated approximately 10 to 12 hours following first observed signs of estrus.

The consistent observation of decreased progesterone levels in non-pregnant cows following GnRH administration at breeding has not been explained. Intravenous injection of 100 ug GnRH on days 2 and 10 of the estrous cycle has been shown to stimulate an LH response and result in lower subsequent progesterone levels than saline controls (86). This same study reports decreased LH receptor numbers in luteal tissue following GnRH administration on day 2 of the estrous cycle. It is suggested that inappropriate LH surges may cause down regulation of LH receptors in luteal tissue and result in decreased progesterone synthesis by the corpus luteum (84,86).

The results from this study and others (84,85) do not conclusively demonstrate whether or not GnRH administration near insemination improves pregnancy rates by stimulating progesterone levels. The results from this study and the study by Lee, et. al. (85), suggest there may be a specific time during ovulation when GnRH may increase luteinization and subsequent progesterone levels. Elevated progesterone levels may then be responsible for the improved pregnancy rates. GnRH administration slightly earlier or later may impair corpora lutea maturation and/or function, possibly through down regulation of LH receptors. This time factor may account for some of the variability in reported increases in pregnancy rates attributed to GnRH administration at insemination. It could also have applications for recommendations in proper administration of GnRH near insemination.

Other theories regarding the mechanism by which GnRH may improve pregnancy rates have been suggested. Proper timing of insemination

with ovulation is critical for optimum fertilization and embryo survival (71,72,73). It is possible that the secondary LH surge following GnRH administration may help synchronize ovulation with insemination. To date, it is not known whether or not GnRH administration at insemination has an effect on the timing of ovulation.

The transition from estrogen active to estrogen inactive follicles may be affected by GnRH administration. The preovulatory LH surge is considered to be the trigger which decreases estrogen synthesis by the maturing follicle (16,22,23,27). The fall in estradiol level following the LH surge is associated with changes in oviductal and uterine motility (27). The secondary LH surge initiated by GnRH administration may affect the fall in estradiol near ovulation and thus may alter gamete transport and fertilization. This could be a beneficial affect in animals where the endogenous LH surge is not appropriately synchronized with follicle maturation thereby increasing fertilization rates in some animals. Measurements of estradiol levels immediately following GnRH administration at insemination have not yet been performed. Daily monitoring of estradiol levels following GnRH administration near insemination has shown higher estradiol levels in GnRH treated animals during the first week than in saline controls (84).

The limited information available describing the effect of GnRH administration at insemination indicates that there is a definite hormonal response. GnRH consistently initiates the release of luteinizing hormone. It would seem that the action of GnRH is

mediated through this LH surge. There is strong evidence that subsequent progesterone levels are altered. However, in some cases they appear to be decreased while in others they appear to be increased in comparison to controls. This phenomenon has not been completely explained but may be related to LH receptor numbers on the developing corpus luteum. Whether the observation of improved pregnancy rates is due to altered progesterone levels, synchronization of ovulation with insemination, gamete transport, or other hormonal or functional alterations is not yet established. Further studies investigating ovulatory events, fertilization rates, embryo survival, and hormonal responses may identify one or more mechanisms for increased pregnancy rates following GnRH administration at insemination in cattle.

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APPENDIXES

Appendix A
Data Collection Form

GNRH PROJECT

Cow ID#:   Date of Insemination:
Treatment: Inseminator:
Lactation #:   Breeding #:
Production: Estrous Signs Observed:
Days in Milk:
Condition Score:
Date and Time Estrus First Observed:
Uterine Tone at Insemination:
Dates of Previous Heats or Breedings:

Blood Sample	Date/Time	LH	Progesterone
0 hr.			
1 hr.			
1.5 hr.			
2 hr.			
2.5 hr.			
3 hr.			
4 hr.			
1 day			
2 day			
3 day			
4 day			
5 day			
6 day			
7 day			
10 day			
16 day			
22 day			

Pregnancy Exam - Date:
            Findings:

Previously in Study?   Date: Treatment:

Additional Comments:

Appendix B
USU Dairy Herd Ration

GRAIN MIX   PERCENT AS FED

    Rolled Barley   38.2
    Molasses   2.3
    Beet Pulp   19.8
    Distillers Grain   12.2
    Whole Cottonseed   24.4
    Premix   3.1

TOTAL MIXED RATION   PERCENT AS FED

	High	Middle	Low
Alfalfa	14.0	14.8	14.4
Alfalfa Haylage - 1st	21.9	37.0	48.2
Alfalfa Haylage - 4th	6.1	----	----
Corn Silage	12.1	21.0	20.5
Earlage	6.1	----	----
Grain Mix	39.8	27.2	16.9

Appendix C
Luteinizing Hormone Assay Procedure

1. Pipet 100 ul PBS-gel buffer into all 12 x 75 mm polypropylene tubes except non-specific binding (NSB) and zero-binding (B_0) tubes. Vortex tubes to coat walls with buffer.

2. Add 100 ul Standard to all appropriately labeled standard curve tubes. Vortex.

3. Add 100 ul unknown serum sample (run in duplicate) to labeled tubes. Vortex.

4. Add 200 ul NRS (1:25 Normal Rabbit Serum) to NSB tubes. Vortex.

5. Add 200 ul 1st Ab in NRS (1:20,000 Anti-ovine LH #15 Antibody in 1:25 NRS) to B_0, standard curve and unknown sample tubes. Vortex.

6. Add 100 ul tracer (Diagnostic products Corp., ovine LH tracer or own LH tracer at 40,000 cpm/min.) to each tube. Vortex.

7. Cover tubes with parafilm and foil and place in refrigerator (4°C) for 4 days.

8. After 4 days remove tubes from refrigerator and add 1 ml cold (4°C) 2nd Ab (DPC Goat Anti-rabbit Gamma Globulin with PEG). Vortex and let sit at room temperature 30 minutes.

9. Centrifuge tubes at 1000 x g, room temperature, for 30 minutes.

10. Carefully pour off supernatant. Allow to stand inverted on absorbent paper for 1 - 3 minutes. Count the pellet in the gamma counter for 1 minute per sample.

Appendix D
Progesterone Assay Procedure (a)

1. PLAIN TUBES: Label four plain (uncoated) 12 x 75 mm
   polypropylene tubes T (total counts) and NSB (nonspecific
   binding) in duplicate.

   COATED TUBES: Label fourteen Progesterone Antibody-Coated tubes
   A (maximum binding) and B through G in duplicate. Label
   additional antibody-coated tubes, also in duplicate, for
   controls and patient samples.

2. Pipet 100 ul of the zero calibrator A into the NSB and A tubes,
   and 100 ul of each of the calibrators B through G into
   correspondingly labeled tubes. Pipet 100 ul of each control and
   patient sample into the tubes prepared.

3. Add 1.0 ml of Buffered [^{125}I] Progesterone to every tube.
   Vortex.

4. Incubate for 3 hours at room temperature.

5. Decant thoroughly.

6. Count for 1 minute in a gamma counter.

(a) Coat-A-Count^R Progesterone Assay Kit,
     Diagnostic Products Corporation
     Los Angeles, CA 90045

Appendix E
Analysis Of Variance Tables
(split-plot in time)

LH levels 0 to 4 hours post-treatment

Source	DF	MS	F-value	P-value
Treatment	1	190.5072	14.0177	0.005>p
Condition	1	41.5015	3.0537	0.10>p>0.05
T X C	1	66.2264	4.8730	0.05>p>0.025
Error A	20	13.5905		
Hour	6	54.5581	2.4672	0.05>p>0.025
Error B	30	22.1131		
T X H	6	45.3096	2.4227	0.05>p>0.025
C X H	6	11.2552	0.6019	p>0.5
T X C X H	6	6.3447	0.3393	p>0.9
Error C	90	18.7009		
Total	167	21.6845		

Progesterone levels 0 to 16 days post-treatment

Source	DF	MS	F-value	P-value
Treatment	1	0.07141	0.1483	p>0.5
Condition	1	0.82134	1.7060	0.25>p>0.10
T X C	1	0.60200	1.2500	0.50>p>0.25
Error A	20	0.48150		
Day	9	41.36332	94.68	0.0005>p
Error B	45	0.43687		
T X D	9	0.40320	0.6374	p>0.75
C X D	9	0.11598	0.1833	p>0.95
T X C X D	9	0.18792	0.2956	p>0.95
Error C	135	0.63259		
Total	239	2.07033		

Progesterone levels 4 to 16 days post-treatment

SOURCE	DF	MS	F-value	P-value
Treatment	1	0.11041	0.2036	p>0.5
Condition	1	1.10208	2.0325	0.25>p>0.10
T X C	1	1.30208	2.4014	0.25>p>0.10
Error A	20	0.54222		
Day	4	21.29979	31.8878	0.0005>p
Error B	20	0.66796		
T X D	4	0.17168	0.3270	p>0.75
T X C X D	4	0.12106	0.2306	p>0.9
Error C	60	0.52503		
Total	119	1.21932		