ENDOCRINE STATUS OF COWS WITH OVARIAN CYSTIC DEGENERATION AND THERAPEUTIC EFFICACY OF GONADOTROPIN RELEASING HORMONE AND HUMAN CHORIONIC GONADOTROPIN IN AFFECTED COWS

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ABSTRACT

ENDOCRINE STATUS OF COWS WITH OVARIAN CYSTIC DEGENERATION AND THERAPEUTIC EFFICACY OF GONADOTROPIN RELEASING HORMONE AND HUMAN CHORIONIC GONADOTROPIN IN AFFECTED COWS

By

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Ovarian cystic degeneration is a major endocrine cause of dairy cattle infertility recognized clinically by veterinarians. It is characterized by persistence of a follicular structure (cyst) which is usually larger than a normal mature follicle, the absence of a corpus luteum, and cessation of normal estrous cycles. For the past 3 decades the most common treatment for the condition has been administration of products with luteinizing hormone (LH) activity such as pituitary LH extract (PLH) or human chorionic gonadotropin (HCG) to cause luteinization of the cyst. Recent synthesis of a hypothalamic factor controlling pituitary LH release (gonadotropin releasing hormone, GnRH) has provided an alternate method of causing elevated serum LH, in this case from an endogenous source, in cows.

The major objectives of my research were 1) to determine the endocrine status of cows with ovarian cysts before and after treatment and 2) to test the therapeutic efficacy of a practical method of GnRH administration (single intramuscular [IM] injection) in cows with this condition.

In Trial 1, cows with ovarian follicular cysts were treated with 0 (saline solution), 25, 50, 100, 150, or 250 μg of GnRH or 10,000 units of HCG by IM injection, 5 cows per treatment. Blood samples were collected before treatment (0 hour); at 0.25, 0.5, 0.75, 1, 2, 3, and 4 hours (after saline and GnRH only); and at 1, 7, 11, 15, and 20 days after treatment. Serum concentrations of estradiol, estrone, LH, and progesterone were determined by specific radioimmunoassays.

Prior to treatment, the cows with ovarian follicular cysts had very low ($\bar{X}$ = 0.4 ng/ml) serum concentrations of progesterone indicating very little luteal tissue was present. Serum LH values were slightly higher (2.7 ng/ml) than those normally found during the luteal phase of the bovine estrous cycle (≈ 1.0 ng/ml). And serum estradiol concentrations (3.9 pg/ml) in these cows with ovarian follicular cysts were approximately equal to those found in cows during proestrus.

Serum LH was not altered in cows given saline solution, but increased in a dose related manner in cows given any of the doses of GnRH. LH peaked about 2 hours after GnRH administration, and was declining by 4 hours. Serum progesterone concentrations increased by more than 2.0 ng/ml by day 11 in 18 of 20 cows treated with 50, 100, 150, or 250 μg of GnRH. Progesterone responses in these cows were greater ($P<0.05$) than in cows given saline solution or a 25-μg of GnRH. Mean progesterone response to the 4 large doses of GnRH was similar in magnitude and duration to serum progesterone changes during the luteal phase of the bovine estrous cycle. Serum

progesterone values were similar in cows treated with HCG and those given GnRH (50 to 250 µg).

In 19 cows irrespective of treatment (0-250 µg GnRH) in which serum progesterone increased by more than 2.0 ng/ml by day 11 after treatment, serum estradiol, estrone, and LH concentrations decreased ($P<0.01$) over the same interval. In contrast, serum estradiol, estrone, and LH remained at pretreatment concentrations in 9 cows in which progesterone did not change over the 11-day period.

Estrus was observed within 30 days of treatment in 11 of 20 cows given 50, 100, 150, or 250 µg of GnRH and probably occurred in 5 additional cows (no signs) according to serum progesterone changes. Thus, 16 of 20 cows seemed to come into estrus within 30 days of treatment. Too few cows were bred after these treatments to make meaningful observations on fertility.

In Trial 2, cooperating veterinarians detected and treated ovarian cysts in 106 cows. Forty-nine were treated with LH preparations (usually 5,000-10,000 units of HCG) and 57 with GnRH (100 µg). At the time of treatment cows averaged 5.9 years of age, were about 110 days postpartum, were in their fourth lactation, and were more commonly showing signs of nymphomania (abnormally frequent signs of estrus) than anestrus. A blood sample was taken before treatment and serum progesterone quantitated. In 66% of cows progesterone was low (<1.0 ng/ml) indicating the presence of follicular cysts. Serum progesterone concentrations were intermediate (1.0 to 3.0 ng/ml) in 16% and high (>3.0 ng/ml) in 18% of the cows, indicating significant luteal tissue activity. Thirty-eight of 57 (66.7%) cows treated with 100 µg GnRH and 27 of 49 (55.2%) cows treated with LH preparations

were observed in estrus within 30 days of treatment. The average interval to first estrus after GnRH and LH treatment was 19.1 and 19.8 days, respectively. First service conception rates of cows inseminated within 30 days of treatment were 59 and 55% after GnRH and LH, respectively. The differences between treatments were not significant ($P>0.20$), nor were responses significantly affected by the amount of serum progesterone present at the time of treatment.

In summary, 1) cows with ovarian follicular cysts had low serum progesterone concentrations and slightly elevated serum LH and estradiol concentrations as compared to normal luteal phase values; 2) single IM injections of GnRH consistently caused a rapid elevation in serum LH in affected cows; and 3) when doses of 50 to 250 µg of GnRH were given the LH released was highly effective in initiating luteal tissue formation. GnRH (100 µg) was at least equally as effective as available LH preparations in causing cows with ovarian cysts to have normal estrous periods within 30 days of treatment and fertility at first estrous periods appeared to be normal. The average interval to estrus after treatment with GnRH or LH preparations was about 3 weeks. These studies indicate that GnRH (100 µg), as a single IM injection, represents an efficacious therapeutic alternative to presently available treatments for cows with ovarian cystic degeneration.

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By

Bradley Edson Seguin[II]

A DISSERTATION

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Department of Dairy Science

1975

To my late friend, teacher and colleague in the area of theriogenology, Dr. E. J. Carroll

BIOGRAPHICAL SKETCH

Bradley Edson Seguin[II] was born on December 22, 1944, in Black River Falls, Wisconsin. He attended public schools in Alma Center, Wisconsin, and graduated from high school in June 1963. Following three semesters in the civil engineering curriculum at the University of Wisconsin, he decided to follow his interest in animal agriculture and completed the preveterinary medicine requirements at Wisconsin State University - River Falls in June 1966. He was accepted into the University of Minnesota's College of Veterinary Medicine and received the Bachelor of Science degree in June 1968 and the Doctor of Veterinary Medicine degree in June 1970.

Pursuing his interest in theriogenology, he served a one-year internship at the Animal Reproduction Laboratory at Colorado State University, Fort Collins. He was granted a Post-DVM Fellowship in September 1971 at Michigan State University in the Department of Large Animal Surgery and Medicine and received the Master of Science degree in December 1972. His thesis was entitled "Progesterone and LH Secretion and Estrous Cycle Alteration Following Endometrial Irritation in Cows."

He then entered a Ph.D. program in the area of theriogenology in the Department of Dairy Science at Michigan State University under the directorship of Dr. W. D. Oxender and with the support of a National Institutes of Health Post-DVM Fellowship. He received the

Doctor of Philosophy degree in December 1975 and accepted a position as assistant professor in the Theriogenology Section, Department of Clinical Sciences, College of Veterinary Medicine, University of Minnesota at St. Paul, Minnesota.

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To my advisor, colleague and friend, Dr. W. D. Oxender, I express my thanks and appreciation for his efforts on my behalf during this graduate program.

The participation and consent of the other members of my guidance committee, Drs. R. W. Erickson, N. E. Robinson, S. D. Sleight, and D. L. Whitenack, are also gratefully acknowledged.

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To my wife, Patricia, I express my gratitude for her patience and support throughout my graduate studies and consider the completion of this program as much her accomplishment as mine.

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INTRODUCTION

Ovarian cysts are a major endocrine form of dairy cattle infertility that can be diagnosed by veterinarians. McKay and Thomson (1959) reported 12 to 14% of cows with breeding problems had cystic ovaries. Zemjanis (1970) reported the incidence of ovarian cysts was 5.7% in approximately 21,000 postpartum examinations of dairy cows. The condition costs dairymen dollars by lengthening the calving interval, decreasing the number of calves produced by individual cows, increasing culling due to infertility thereby decreasing culling based on production or conformation, and increasing managerial and veterinary expenses.

Ovarian cysts, ovarian cystic degeneration or dysfunction, cystic ovaries and "cystic cows" are general terms used to describe the condition, with follicular and luteal cysts being specific forms of the problem. Briefly the condition is characterized by persistence of an ovarian follicular structure(s) which is usually larger than the normal Graafian follicle (>2.5 cm diameter) in the absence of active luteal tissue. Abnormal estrous behavior, varying from lack of estrus (anestrus) to abnormally frequent estrus (nymphomania) to masculinization (virilism), is associated with the condition.

Methods of treatment used to date have been designed to initiate normal luteal function in affected cows, hoping that subsequent estrous periods will be normal. For the last 30 years one of the

most common treatments has been administration of human chorionic gonadotropin (HCG), which has luteinizing hormone (LH) activity, to cause luteal tissue formation. Recently gonadotropin releasing hormone (GnRH), which stimulates LH release from the anterior pituitary, has provided a new means of administering LH therapy to cows with cystic ovaries. Preliminary evidence by Kittok, Britt and Convey (1973) indicated that this compound initiated normal estrous cycles in cows with ovarian cystic dysfunction.

The purpose of my research was to determine the endocrine (estradiol, estrone, LH, and progesterone) status of cows with ovarian cysts prior to treatment and to test the ability of GnRH when administered as a single intramuscular (IM) injection to cause endocrine and reproductive patterns indicative of recovery from the cystic condition. In addition, I compared this response to that after HCG treatment.

LITERATURE REVIEW

In the subsequent literature review, ovarian cystic dysfunction in cattle will be discussed in terms of diagnostic characteristics, etiology and methods of therapy. First, a brief physiologic and endocrine review of the normal bovine estrous cycle is presented to provide a basis for discussion of the cystic condition.

Normal Bovine Estrous Cycle

The bovine estrous cycle averages about 21 days in length, with the normal range being 18 to 24 days (Asdell, 1964). The main stages of the estrous cycle are estrus, the period of sexual receptivity, which lasts about 12 to 18 hours, and diestrus, the period of luteal function, lasting about 13 days. Metestrus is the period of luteal development between estrus and diestrus lasting 3 to 4 days. And proestrus is the 3- to 4-day preparation period before estrus involving luteal regression and follicular growth (Zemjanis, 1970; Roberts, 1971). Days of the estrous cycle are numbered from the day of estrus, which will be day 0 in this discussion.

Endocrine Pattern in the Bovine Estrous Cycle

Chenault *et al.* (1975) have recently described the progesterone, estradiol, and LH interrelationships from late diestrus through estrus and ovulation in the cow (Figure 1). Although others, as discussed by Chenault *et al.* (1975), had previously reported on various aspects

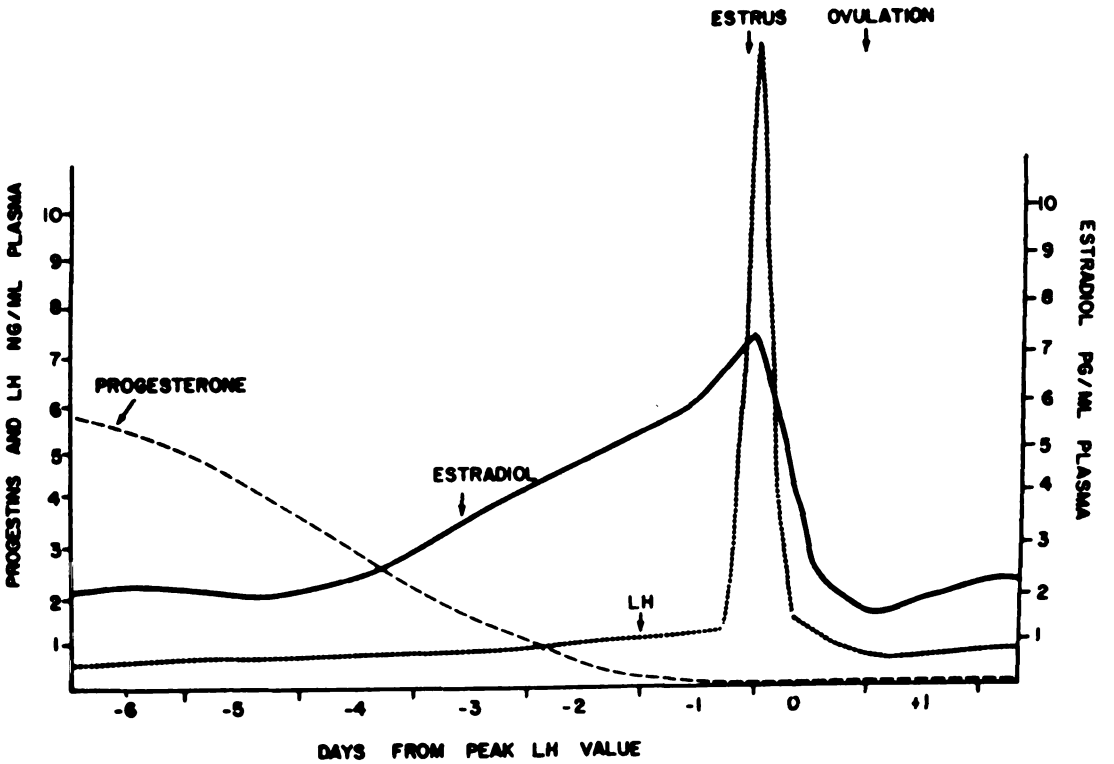

Figure 1. Pooled within animal least square regressions for plasma progestins, estradiol, and LH approaching ovulation in the bovine. From Chenault *et al.* (1975).

of this period, this is the most comprehensive endocrine study available of the proestrous and estrous periods in cattle. Progesterone declines from high serum concentrations in late diestrus, 5.7 ng/ml on day 6 prior to estrus, to very low concentrations at estrus, 0.07 ng/ml (Chenault *et al.*, 1975). When data from individual cows are studied, the decline in progesterone is very abrupt, more than a 50% decline within a 12- to 24-hour period (Stabenfelt, Ewing and McDonald, 1969). The luteolytic factor which controls corpus luteum (CL) regression is of uterine origin as discussed in reviews of the utero-ovarian relationship in cattle by Ginther (1968a,b) and Anderson, Bland, and Melampy (1969). Although the luteolytic factor in cattle has not been positively identified, evidence is suggestive that it may be prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Goding *et al.*, 1972). With conception this uterine luteolytic factor is apparently inactivated or overridden so the CL and pregnancy are maintained.

With the decrease in serum progesterone at the end of diestrus, a period of follicular growth occurs (Rajakowski, 1960) which is classically thought to be controlled by follicle stimulating hormone (FSH) from the anterior pituitary. Pituitary concentrations of FSH decrease in the cow during this time (Hackett and Hafs, 1969), and assuming that this decrease indicates pituitary release and increased blood FSH concentrations, this report supports the role of FSH in follicle growth. However, recent direct measurement of peripheral serum FSH by specific radioimmunoassay (Akbar *et al.*, 1974) did not indicate an increase in FSH during proestrus but did show elevated FSH near estrus coincident with the LH surge. A conclusive statement

on the control of follicle growth in cattle must await further clarification.

Serum estradiol concentrations gradually increase through proestrus and early estrus as the Graafian follicle develops and, at or near the outset of estrus, LH increases to 10 to 20 ng/ml as compared to the 1 to 2 ng/ml found during the rest of the estrous cycle (Chenault *et al.*, 1975). Serum LH is elevated for about 8 hours, and this is termed the preovulatory LH surge. As previously stated, it was at this time that Akbar *et al* (1974) detected elevated FSH. Data from Chenault *et al.* (1975) and others support the hypothesis that progesterone must decline to low levels before estrogen begins to rise, that estrogen must be elevated for several hours before LH is released, and that the preovulatory surge of LH is probably initiated by elevated estradiol. The latter point is supported by observations of Hobson and Hansel (1972) that in ovariectomized heifers estradiol benzoate injections caused elevations of LH about 40 hours after injection similar in magnitude and duration to the preovulatory LH surge occurring at estrus. LH did not increase after estradiol injection if progesterone was administered simultaneously or in intact heifers during diestrus.

Estrus lasts about 12 to 18 hours in cattle, although duration and intensity of behavioral signs of estrus vary greatly as discussed by Asdell (1964). Ovulation occurs spontaneously about 10 hours after the end of estrus (Asdell, 1964) or 20 to 30 hours after the beginning of estrus (Swanson and Hafs, 1971). With ovulation, blood vessels cross the basal membrane of the follicle and the steroidogenic activity of the granulosa cells changes from estrogen to progesterone

production with development of the CL of the subsequent diestrum (Rajakowski, 1960).

Types of Ovarian Cysts

Since Gurlt first described the ovarian cystic condition of cattle in 1831 (see Garm, 1949), some variation has existed regarding the classification of ovarian cysts in cattle, but recent descriptions of follicular cysts, luteal cysts, and CL with cystic centers (cystic CL) by Morrow *et al.* (1966), McEntee (1970), Zemjanis (1970) and Roberts (1971) show a consolidation in terminology. Follicular and luteal cysts are considered pathologic, while the cystic CL is considered a nonpathologic variant of the normal CL (Figure 2). Cystic CL's form after ovulation when for unknown reasons a fluid-filled cavity forms within the developing luteal tissue creating the cystic structure. An ovulatory papilla or crown marking the ovulation site is detectable by palpation and is used clinically to differentiate the cystic CL from follicular and luteal cysts (Figure 2). Morrow *et al.* (1966) concluded that cystic CL's did not adversely affect either estrous cycle length or the maintenance of pregnancy, and thus stated that treatment of cows with cystic CL's was not indicated.

Follicular and luteal cysts develop from Graafian follicles which initially appear to develop; but estrus may or may not be exhibited in the usual manner, ovulation does not occur, and the follicle usually continues to grow to a size greater than that at which ovulation normally occurs (>2.0 to 2.5 cm diameter--Morrow *et al.*, 1966). Follicular cysts have a thin wall and are fluctuant on palpation, whereas luteal cysts have a definite layer of luteal tissue

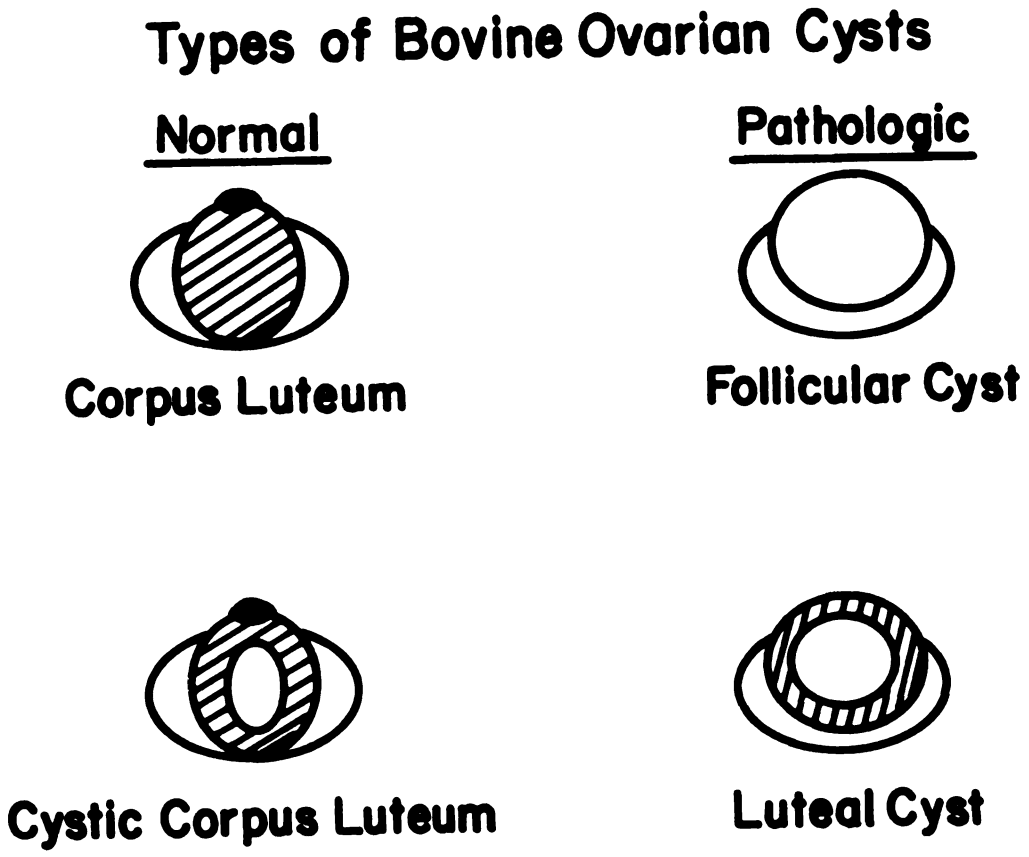

Figure 2. Types of bovine ovarian cysts. Cross-hatched areas represent luteal tissue and the blackened areas represent ovulation papillae.

in the cyst wall and are more resilient on palpation (Figure 2). Luteal cysts may appear more frequently as single structures, while follicular cysts are frequently multiple (McEntee, 1970; Zemjanis, 1970; Roberts, 1971). These authors also cite a more frequent occurrence of anestrus in cows with luteal cysts and more nymphomania with follicular cysts. They conclude that both types share a common etiology and that luteal cysts are probably a less severe form of the same defect which causes follicular cysts. Clinically, follicular and luteal cysts are rarely differentiated and are usually treated similarly (Zemjanis, 1970). Morrow *et al.* (1966) reported that normal CL's, cystic CL's and cystic follicles (follicular and luteal cysts combined) occurred during 62.5%, 25.2% and 12.3%, respectively, of 357 postpartum cycles, i.e., through 60 days postpartum.

It has frequently been assumed that cows with cystic ovaries, especially those with raised tailheads, relaxed sacrosciatic ligaments, edematous vulvae, vaginal mucous secretion, and frequent estrual activity, are being influenced by excessive ovarian estrogen production (Short, 1962; McEntee, 1970; Roberts, 1970). Short (1962) quantitated the steroids (progesterone, 17 α-hydroxyprogesterone, androstenedione, estrone, and estradiol-17β) present in mature follicles in cows at estrus and in ovarian cysts. Estradiol-17β was the major steroid present in normal follicles and was significantly greater in normal follicles than in cysts. The steroid profile of cyst fluid varied greatly both between and within affected animals. In some "cystic cows" progesterone was the most plentiful steroid while estradiol-17β was the most plentiful in others. Short (1962) was not able to make any correlation between the behavioral characteristics

of "cystic cows" and the amount or type of steroids present in the cyst fluid. He concluded that the cyst was not the primary defect in cystic ovarian disease but was a degenerating structure as indicated by atresia and degeneration of the granulosa and theca interna cells of the cyst wall (also described by Yamauchi and Inui, 1954). In the normal follicle, follicular growth and steroidogenesis occur simultaneously; but in the cyst, follicular growth continues while steroidogenesis decreases so that dilution of steroids in the cyst fluid occurs (Short, 1962).

A condition in women has been described which has at least some superficial characteristics similar to those described for cows with ovarian cystic degeneration. Polycystic ovarian disease, also known as the Stein-Leventhal syndrome, in general involves cessation of estrous cycles (amenorrhea), some masculinization and defeminization of secondary sex characteristics, hirsutism, and enlarged, sclerosed, polycystic ovaries (Goldzieher, 1973). The most common method of treatment is surgical resection of a wedge of ovarian tissue which appears to be quite effective although the reasons for its success are not clear.

Etiology of Ovarian Cysts

Two etiologic hypotheses were proposed a half century ago to explain the occurrence of ovarian cysts in cattle. Albrechtsen (1916) believed that ovarian cysts resulted from uterine infections either by the action of bacterial toxins from the infected uterus on the ovaries or by the ascension of bacteria from the uterus to the ovaries. His hypothesis was based on clinical observations that

endometritis was nearly always present in cows with ovarian cysts, that cysts disappeared if the endometritis was cured, and that ovarian cysts could be prevented by prophylactic measures preventing the development of puerperal infections.

Meanwhile Hess, in 1921 (see Garm, 1949), maintained that ovarian dysfunction was primary in cases of ovarian cysts and considered any accompanying endometritis to be noninfectious in nature and secondary to the ovarian changes. In support of Hess's viewpoint, Garm (1949) found histologic evidence of endometritis in only 4 of 62 cows with ovarian cysts and thus discounted any cause and effect relationship between uterine infection and ovarian cysts.

As previously discussed, Short (1962) concluded that the ovary was not the site of the primary defect in cows with ovarian cysts. Rather, he cited Garm's (1949) observation of pituitary hypertrophy in cows with cystic ovaries as evidence that the defect may be located at the pituitary level. It was not possible, however, to determine if the pituitary hypertrophy observed by Garm (1949) in cows with ovarian cysts was the cause of or the result of the cystic condition.

Morrow *et al.* (1966), in a study of postpartum ovarian activity and uterine involution in dairy cows, observed a significantly higher incidence of cystic follicles in cows which had postpartum complications such as milk fever, twins, retained placenta, metritis, etc., than those with no complications, 23.4% vs. 8.3%, respectively. They suggested that LH release patterns from the pituitary may have been altered in these abnormal cows.

Casida, McShan and Meyer (1944) cited the 1927 reports by Ascheim and Zondek and by Smith and Engle showing the physiological

relationship between the anterior pituitary gland and the ovaries as the stimuli for speculation that gonadotropins might be utilized in the treatment of ovarian cysts in cattle. Casida *et al.* (1944) reported that large numbers of cows with cystic ovaries were successfully treated with an unfractionated sheep pituitary extract, presumably rich in LH. This curative effect of LH was cited by Roberts (1971) as evidence that the cystic condition develops as a result of depressed preovulatory LH surge. Other evidence to support this

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degrees of LH deficiency. Direct measurement of LH at estrus in cows developing ovarian cysts has not been reported. When the sampling frequency necessary to determine peak serum LH concentrations at estrus and the incidence of the cystic condition are considered, the resources required to obtain direct proof of this hypothesis may be prohibitive.

What causes a deficiency in the preovulatory LH surge at estrus in cows developing ovarian cysts? The data previously cited upon which the LH deficiency hypothesis is based indicate that there is no LH deficiency in the pituitary glands of these cows but only an abnormality regarding LH release. Convey (1973) reviewed the evidence which indicates that the hypothalamus controls pituitary hormone release via releasing and inhibiting hormones (factors). An LH releasing factor (LHRF), also named gonadotropin releasing hormone (GnRH), has been isolated, described and synthesized (see GnRH discussion, page 24). Control of hypothalamic releasing factors is apparently at least partially due to inputs from higher central nervous system (CNS) centers and may reflect both external and internal stimuli. Relatively little is presently known about the effect of the CNS and hypothalamus on pituitary gonadotropic function in cattle, particularly in cows developing ovarian cysts. Many predisposing factors have been cited as being positively correlated with the incidence of ovarian cysts in cattle, and some of these could be exerting an influence through the CNS and hypothalamus.

Predisposing Factors for the Ovarian Cystic Condition

Roberts (1971) reported the incidence of ovarian cysts from 1948 to 1968 in New York State dairy cattle to have increased at a

rate in excess of 100% per 5-year period in an area where cow numbers were declining. In a time when dairy cattle management has been changing rapidly, it is indeed difficult to pinpoint the cause(s) of the greater incidence. Predisposing factors frequently associated with the cystic condition include heredity, level of milk production, lead feeding--especially heavy concentrate feeding, season and age, complications associated with parturition and the early postpartum period, and ingestion or injection of exogenous estrogens. Much of the supportive evidence for these factors is circumstantial and confounded, and is discussed here only to demonstrate the complexities involved in establishing the pathogenesis of the condition.

Heredity

While the probable genetic transmission of ovarian cystic dysfunction has been proclaimed by clinicians for many years, few researchers have investigated the genetic influence on the incidence of ovarian cysts in dairy cattle. Casida and Chapman (1951) studied the lifetime reproductive records of 144 dams and 245 daughters in a large purebred Holstein herd. Of the dams, 43 had ovarian cysts and 26.8% of their 82 daughters were affected. The remaining 101 dams had no history of ovarian cysts and only 9.2% of their 163 daughters developed ovarian cysts. The heritability estimate for ovarian cysts was 0.43 in this herd.

The bull's role in transmission of ovarian cysts was described by Bane (1964) from Sweden, where bulls used in artificial insemination (AI) were culled if their daughters had a greater than average incidence of ovarian cysts. Under this policy the incidence of

ovarian cysts in the national dairy herd decreased from 10.8% at its inception in 1954 to 5.1% in 1964. Roberts (1971) cited the possible concentration of the genetic tendency toward ovarian cysts via the widespread adoption of AI over the last 30 years in the U.S. as a possible cause for the increasing incidence of this condition he observed. To my knowledge, bulls used in AI in the U.S. are not evaluated as to the incidence of ovarian cysts in their daughters; nor are bulls from cow families with a high incidence of cystic ovaries excluded from AI studs.

Level of Milk Production

Garm (1949), among others, reported that cows with ovarian cysts produced more milk than noncystic herdmates. Johnson, Legates and Ulberg (1966) also found that cows with follicular cysts had higher 90- and 305-day milk production, adjusted to mature equivalent and for days open, during the lactation in which they were cystic than their noncystic herdmates. However, no significant differences in milk production were detected in lactations prior to those in which cows became cystic and those of their contemporary herdmates. Part of the difference in 305-day milk production between cows with and without cysts was due to the negative effect of pregnancy on milk production in noncystic cows. But 90-day milk production, which was also greater in cystic than noncystic cows, would not be influenced by pregnancy. These data indicate that cystic cows are not higher producers before they become cystic, but that circumstances associated with the cystic condition apparently cause increased production. These authors suggested that an altered endocrine status in cystic cows may cause increased milk production.

Feeding Practices

More liberal concentrate (grain) feeding and the practice of lead feeding to maximize milk production per cow are frequently incriminated among management factors which may increase the incidence of ovarian cysts. However, Morrow, Tyrrell and Trimberger (1969) detected only nonsignificant differences in the incidence of cystic follicles between cows liberally fed concentrate feeds and those fed a conventional ration. In a similar study Whitmore, Tyler and Casida (1974) examined the incidence of ovarian cysts in cows fed high levels of nutrients (150% of requirements) as compared to controls (100% of requirements). Again no difference in the occurrence of cysts was detected. Thus, the available data do not substantiate the contention that intensive feeding of concentrates or total nutrients increases the incidence of ovarian cysts.

Season and Age

Among others, Roberts (1955) and Morrow *et al.* (1966), working in New York State, reported that nearly half their cases of cystic ovaries occurred during December, January, and February. It was not determined in these studies which factors associated with winter, such as short photoperiod, low temperature, reduced exercise, stored feed, management emphasis, caused the increase in cystic ovaries.

Ovarian cysts are rarely found in virgin heifers (Roberts, 1955), the incidence of the condition increasing as cows approach maturity. With no adjustment made for the age distribution within the dairy cow population, Garm (1949) found the cystic condition to be most common in cows milking in their second to fifth lactations. The recurrence

rate of cystic ovaries in subsequent lactations is higher than the incidence in previously unaffected cows of similar ages (Roberts, 1971). Again the reason for an age or parity effect on the incidence of cystic ovaries has not been resolved.

Periparturient Disease

While the hypothesis of Albrechtsen (1916) that uterine infection caused ovarian cysts has been largely discounted by Garm (1949) and others, the predisposition for cystic ovaries reported by Morrow *et al.* (1966) in cows which had complications at or shortly after parturition remains unexplained. Uterine infection may alter hypothalamic-pituitary-gonadal interactions such that ovulation is hindered resulting in cystic ovaries (Morrow *et al.*, 1966). Indeed, the uterine influence on ovarian function has been demonstrated in several ways, including experimentally induced endometrial irritation causing altered estrous cycle lengths in cows (Seguin, Morrow and Louis, 1974).

As Morrow *et al.* (1966) pointed out, the incidence of cysts is greatest during the early postpartum period (first 60 days). Nearly 50% of these early-occurring cysts spontaneously regressed by 60 days postpartum without treatment and in many clinical situations these early-occurring cysts may not have been detected.

Exogenous Estrogens

Wiltbank, Ingalls and Rowland (1961) reported that 7 of 10 heifers given 5 mg of estradiol valerate on day 15 or 16 of the estrous cycle became cystic, whereas none of 20 heifers treated on day 2 or 3 or day 8 or 9 developed cysts. They hypothesized that the exogenous estrogen may have altered the preovulatory LH surge

such that insufficient LH to cause ovulation was released at the outset of estrus. Others have reported clinical impressions that exogenous estrogens used to treat reproductive problems in cows increased the incidence of cystic ovaries. While the report by Wiltbank *et al.* (1961) indicates potential problems involved with estrogens used at a specific stage of the estrous cycle, estrogens have definite clinical applications; for example, uterine evacuation in cases of pyometra (Roberts, 1971), for which their efficacy overcomes the possibility that ovarian cysts might result from estrogen therapy. Brodie *et al.* (1970) examined the relationship between the clinical use of diethylstilbestrol (DES) in cases of retained fetal membranes, metritis or pyometra and anestrus and the incidence of ovarian cysts in dairy cows. The incidence of ovarian cysts in 323 cows given DES was not different than that in 447 herdmates not given DES (7.7% and 7.3%, respectively), indicating that the risk of induction of cystic ovaries by estrogen therapy should not deter its clinical use by veterinarians.

Erb *et al.* (1973) reported a high percentage of cows developed cystic follicles following a 7-day period of twice daily injections of progesterone and estradiol-17β. Treatment regimes of this type have recently been used to initiate lactation in dairy cows (Smith and Schanbacher, 1973).

Adler and Trainin (1960) reported an apparent hyperestrogenic syndrome in dairy cattle, the likely source of the estrogen being alfalfa hay. Symptoms observed included ovarian cysts, swollen, hyperemic vulvae, enlarged uteri, estrus in pregnant cows, abnormal cycle lengths, and low conception rates. Bickoff *et al.* (1961),

among others, have described estrogenic activity in forage legumes such as alfalfa and several clovers frequently used as feedstuffs for cattle. Therefore, in herds suddenly developing a high incidence of ovarian cysts, an investigation of the herd's nutritional program may be indicated.

Methods of Therapy

Roberts (1971) stated that "rational treatment (of cows with ovarian cysts) should have as its aim the development of a functional normal corpus luteum" and that the most common treatment methods are "rupture of the cysts, administration of luteinizing hormone or preventing the continuous release of luteinizing hormone from the pituitary gland by administration of progestational compounds." An extensive summary of the literature regarding these forms of therapy for the cystic condition of cattle has been compiled by Roberts (1971) and only selected reports sufficient to describe the relative status of each method are provided here.

Manual Rupture of Cysts

Albrechtsen (1916) reported that manual rupture of cysts when combined with irrigation of the uterus with an antiseptic was a successful therapeutic approach to the problem of ovarian cysts. As mentioned previously, Albrechtsen felt that formation of ovarian cysts resulted from a uterine infectious process. Of 107 cows with cysts treated by manual rupture plus intrauterine infusion, 92 recovered, 54 became pregnant, and 2 "had to be castrated." However, only 36 recovered after a single treatment. Another group of 67 cows

with cysts were treated by manual rupture only and 46 "had to be castrated."

Roberts (1971), summarizing literature reports and personal observations, reported a 37% recovery rate among 188 cows with cysts treated by manual rupture. Duration of the cystic condition prior to treatment by manual rupture (and probably other methods also) significantly influences the recovery rate (Schjerven, 1965). This duration effect may be partially explained by spontaneous recoveries inflating the recovery rate in cows with recently formed cysts as compared to those more chronically affected. Morrow *et al.* (1966) reported that 48% of cows which developed ovarian cysts within 60 days of parturition recovered without treatment.

Complications, such as hemorrhage and adhesions, may occur following manual rupture of cysts, but the incidence of these complications has not been reported.

LH Therapy

Casida *et al.* (1944) were the first to successfully use an unfractionated sheep pituitary extract to treat a significant number of cows with follicular and luteal cysts, and they reported that it was not necessary to manually rupture cysts in addition to treatment with the pituitary extract. Since that report LH, either as pituitary extract (PLH) or as human chorionic gonadotropin (HCG), has been used by veterinarians to treat cows with ovarian cysts. Yamauchi (1955) observed 3 types of responses in cows treated for ovarian cysts with HCG: 1) the cyst may rupture and a CL develop; 2) the cyst may luteinize without rupturing; or 3) the cyst may remain unchanged

after treatment. The latter response occurred in those cows in which the granulosa and theca interna layers of the cyst wall had degenerated prior to treatment, also indicating that duration of the cystic condition prior to treatment influences the recovery rate.

Of 352 cows with ovarian cysts treated intravenously (IV) with PLH or HCG (Roberts, 1955), equal percentages (85%) resumed normal cyclic activity and 71 and 76%, respectively, conceived. No advantage was observed from using manual rupture of cysts in addition to injection of PLH or HCG. It might be pointed out that no time limit was established for recovery in this study, so that only 26% of the conceptions occurred within 30 days of treatment with over 100 days passing before the 71 and 76% of treated cows had conceived. Bierschwal (1966) also reported on the results of HCG administration (5000 units IM) to cows with ovarian cysts. Two-thirds of 145 cows treated had a CL within 1 to 2 weeks after treatment and nearly 60% of those treated conceived after one treatment. The interval to first estrus after successful treatment was about 3 weeks in these studies, similar to the normal interestrous interval in cattle. Morrow *et al.* (1969) recommended that at least 30 days pass before HCG treatment is repeated unless signs of nymphomania persist following treatment.

Roberts (1955) reported that dosages of 5,000 units of HCG IV or 10,000 units IM were slightly superior compared to 2,500 units IV for ovarian cyst treatment. No advantage was detected in administering HCG directly into follicular cysts (Roberts, 1957). Reduced responses and development of antibodies to repeated gonadotropin injections have been reported by Willett, Buckner and McShan (1954) and therefore

it is frequently recommended that, should retreatment be necessary, a product of different origin (i.e., pituitary vs. chorionic) be used for the second treatment (Roberts, 1971). Since spontaneous recovery from ovarian cysts is common during the early postpartum period, it has sometimes been recommended that hormonal therapy be delayed until the cow is about 60 days postpartum and that manual rupture of cysts might be used during the early postpartum period (Morrow *et al.*, 1969; Roberts, 1971).

Progesterone Therapy

Based on the report by Ulberg, Christain and Casida (1951) that daily progesterone injections would prevent estrus and ovulation in cattle, Beck and Ellis (1960) treated 100 cows which had ovarian cysts and which were showing signs of nymphomania with a single injection of a long acting form of progesterone (1 mg per pound of body weight). In most cases nymphomaniac behavior ceased in 36 to 72 hours and 67 cows conceived. Metritis was an undesirable complication arising from the use of progesterone, probably due to infection arising from preexisting bacterial contaminants. Others (Black *et al.*, 1953; Rowson, Lamming and Frye, 1953) have reported that the bovine uterus is more susceptible to infection during the progestational than the estrogenic phase of the cycle. Johnson and Ulberg (1967) used daily subcutaneous injections of 50 or 100 mg of progesterone in oil per day for 14 days to treat cows that had ovarian follicular cysts. Regardless of dosage used, 62% of the 40 cows in each group began normal estrous cycles within a few days of the last injection and about 50% of those treated conceived at an average of

45 days after treatment. Of 30 untreated control cows (with cystic ovaries), only 4 began normal cycles and conceived. These investigators suggested that progesterone blocks release of LH from the anterior pituitary and promotes its storage there so that when progesterone therapy is stopped and estrus occurs, the pituitary contains adequate LH to cause ovulation. Use of orally active progestins has been investigated as an alternative to the injected progesterone, but results to date are not adequate to compare with the LH methods of therapy (Roberts, 1971).

Present Status of Therapy for Ovarian Cysts in Dairy Cattle

The LH products, HCG and PLH, are presently generally accepted as the best treatment available for follicular and luteal cysts of cattle. In a survey of 46 Michigan veterinarians (unpublished data, 1971), 77% preferred HCG to treat ovarian cysts of cattle, 9% preferred pituitary gonadotropins (PLH), 9% used progesterone and 5% primarily used manual rupture of the cyst. The preferred therapy may have changed somewhat in the last 2 years as the supply of HCG has decreased resulting in a 2- to 5-fold increase in its cost.

Clapp (1934) observed a high incidence of twins from cows which had been cystic, 37.5% in 24 cows, compared to the incidence in non-cystic herdmates, 3.1%, and therefore recommended that cows not be bred for 1 or 2 estrous periods after recovery. In contrast, Roberts (1955 and 1971) recommends breeding cows at the first estrus after treatment with LH. He observed no increase in twinning or delayed conception due to this practice in nearly 300 cases treated. He reasoned that since pregnancy prevents the recurrence of cysts, to

minimize chances of recurrence and to keep the calving interval in affected cows as short as possible, it is better to start breeding at the first estrus after treatment.

Gonadotropin Releasing Hormone (GnRH)

Basic neuroendocrine relationships involving the hypothalamus-pituitary-target organ complex have recently been reviewed by Convey (1973). In addition, the chemistry, physiology and possible applications of GnRH to animal agriculture were also summarized. GnRH has also been referred to as LH-releasing hormone or factor (LHRH or LHRF) or LH-releasing hormone/FSH-releasing hormone (LHRH/FSHRH). Several reviews are available describing the proposed existence, physiological function and search for structure of GnRH (see summary by Convey, 1973). Amoss *et al.* (1971) and Schally *et al.* (1971) independently reported isolation of ovine and porcine GnRH, respectively. The decapeptide structure of GnRH (Figure 3) was subsequently proposed by Matsuo *et al.* (1971b). Synthesis of GnRH by Matsuo *et al.* (1971a) followed and the synthesized form of GnRH was shown to have activity in laboratory animals similar to that of the purified ovine and porcine extract by Burgus *et al.* (1972) and Schally *et al.* (1972), respectively. Zolman *et al.* (1973) demonstrated that purified porcine hypothalamic extract and synthetic GnRH had similar LH releasing activities on bovine pituitary tissue *in vitro*.

With synthesis of GnRH possible applications to farm animals have been widely studied, as reviewed by Convey (1973). To summarize, GnRH causes pituitary release of LH in cattle, swine and sheep in a dose related manner, with FSH release by GnRH also reported in sheep and cattle.

Gn-RH

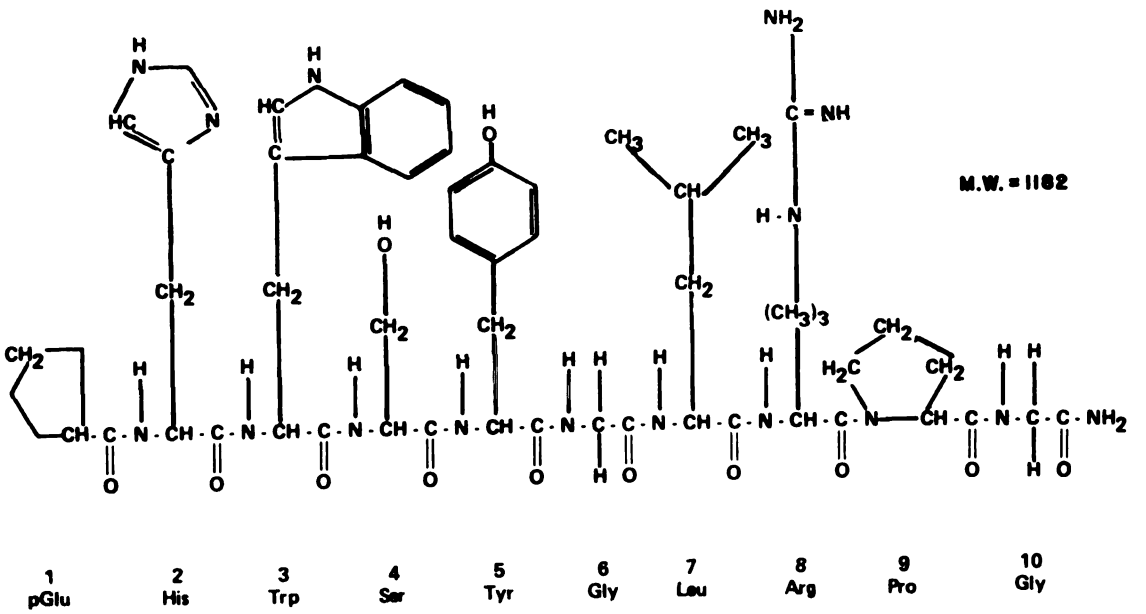

Figure 3. Amino acid sequence of gonadotropin releasing hormone (GnRH).

Kittok, Britt and Convey (1973) tested the ability of GnRH to induce pituitary release of LH, initiate formation of luteal tissue and restore estrous cycle activity in cows with ovarian follicular cysts. They gave 3-100 μg doses of GnRH IV at 2-hour intervals to 5 cows with follicular cysts and found LH elevated approximately 20-fold within 2 hours of the first injection. Luteal tissue developed in these cows as indicated by a rise in serum progesterone from 0.9 ng/ml at treatment to 3.6 ng/ml on day 11 after treatment. Estrus was observed 20 to 24 days after treatment in all 5 cows. They concluded that "repeated doses of GnRH produced an LH response similar to the preovulatory surge in the bovine and initiated estrous cycles in cows with ovarian follicular cysts."

The objectives of my dissertation research involved investigation of the endocrine status of cows with ovarian cystic degeneration and a more extensive and practical evaluation of the therapeutic effect of GnRH in cows with ovarian cysts. Specific objectives were:

1) determination of the endocrine (estrogens, LH, and progesterone) status of cows with ovarian follicular cysts;

2) quantitation of the LH response to single intramuscular (IM) injections of GnRH in these cows;

3) comparison of the luteotropic effect of GnRH and HCG in cows with cysts;

4) determination of the endocrine status of cows treated clinically by veterinary practitioners for the cystic condition; and

5) evaluation of the therapeutic efficacy of GnRH and HCG used clinically to treat cows with ovarian cysts.

MATERIALS AND METHODS

Two trials were conducted to determine the endocrine status of cows with ovarian cysts and to evaluate the potential of GnRH as a treatment for cows with this condition. In Trial 1, cows with ovarian follicular cysts were treated by single IM injection with a placebo (negative control), various doses of GnRH, or a standard dose of HCG (positive control). Parameters measured included the endocrine status of these cows prior to treatment, in response to treatment, and after recovery.

A larger field trial (Trial 2) was designed to determine the endocrine status of cows detected by veterinary practitioners as having cystic ovaries and to compare the therapeutic effectiveness of GnRH and HCG in "on the farm" situations. Primary endpoints here were the occurrence of estrus and fertility rates within 30 days of treatment.

Experimental Design

Trial 1: Endocrine Status (Estradiol, Estrone, LH and Progesterone) of Cows with Ovarian Follicular Cysts Before and After Treatment with Saline, GnRH, or HCG

Ovarian follicular cysts in this experiment were defined as follicular structures at least 2.5 cm in diameter which persisted for at least 10 days in the absence of a corpus luteum (CL). This diagnosis was based on 2 examinations by palpation *per rectum* 10 to 14 days

apart and on low serum progesterone (<2.0 ng/ml) at the time of treatment. These criteria were designed to exclude cows with luteal cysts, cystic CL's, and noncystic CL's (Figure 2) from treatment. Records and observations by owners were used to verify the absence of irregularity of estrous behavior (anestrus and nymphomania, respectively) in these cows prior to treatment.

Thirty-five dairy cows (5 cows per group) meeting these criteria were injected IM with either 0 (saline control), 25, 50, 100, 150, or 250 µg of GnRH[a] or 10,000 units of HCG[b] in saline (groups 1 through 7, respectively). The total volume injected in each case was 10 ml. Coccygeal arterial or venous blood (10 ml) was collected just before treatment (0 hour) and 1, 7, 11, 15, and 20 days after treatment. To determine acute changes in serum LH after GnRH, blood was also collected at 0.25, 0.5, 0.75, 1, 2, 3, and 4 hours after treatment from cows in groups 1 through 6. Blood serum was stored at -15°C until assayed.

Serum estradiol, estrone, LH, and progesterone concentrations were determined in selected samples by specific radioimmunoassays as described by Britt, Kittok and Harrison (1974) and Hafs *et al.* (1974), Britt *et al.* (1974), Convey *et al.* (in press), and Louis, Hafs and Seguin (1973), respectively. Specific procedural details for each of these assays are included in the appendices of this dissertation.

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[a]GnRH was supplied by Dr. M. D. Brown, Abbott Laboratories, North Chicago, IL 60064.

[b]Follutein, E. R. Squibb and Sons, Inc., Princeton, NJ 08540.

Serum LH response was quantitated for each cow given GnRH or saline by calculating the area under the LH response curve from 0 through 4 hours after treatment.

Serum progesterone concentrations were used as indicators of luteal tissue function throughout this study. Increasing serum progesterone concentrations were interpreted as indicative of formation of luteal tissue (luteinization). Serum progesterone concentrations on day 11 after treatment were analyzed by 1-way analysis of variance and Dunnett's t-test was used to compare progesterone after each treatment with that in saline-treated controls.

The effect of luteinization of ovarian cysts on serum concentrations of estradiol, estrone, and LH was investigated in groups 1 through 6 by comparing these hormones on day 11 after treatment with their concentrations prior to treatment (day 0) on a within-cow basis. Endocrine responses were grouped by cow irrespective of GnRH treatment group into those in which progesterone increased by at least 1.0 ng/ml by day 11 after treatment and those in which it decreased, did not change, or increased but by less than 1.0 ng/ml by day 11. Hormone concentrations for individual cows prior to and 11 days after injection were compared using a t-test for paired observations. Endocrine data from cows exhibiting signs of estrus by or near day 11 after treatment were excluded from these analyses since endocrine changes which occur near estrus would confound the effect of luteinization.

Cows were located in several privately-owned and 2 Michigan State University dairy herds. Where possible (university herds), ovaries of cows were reexamined by palpation for possible changes on days 7 and

15 after treatment. Interval to first estrus, within 30 days of treatment, and first service conception rates during that interval were summarized. At least 30 days were allowed after administration of treatments before retreatment was considered.

Trial 2: Reproductive Performance of Cows with Ovarian Cysts after Treatment with GnRH or LH Preparations

In a subsequent field trial, 11 veterinarians whose practices primarily involved dairy cattle were asked to treat cows which had ovarian cysts with 100 µg GnRH or the LH preparation (HCG or PLH) of their choice. The criteria for ovarian cysts were left to the discretion of each practitioner so that the cows treated would be representative of the type usually treated. Twelve cows were treated by each cooperating practitioner, 6 per treatment.

A blood sample was collected at the time of treatment from each cow and serum was frozen until assayed for progesterone (Louis *et al.*, 1973). The purpose of the progesterone determination was to compare the types of cysts being treated by practitioners with those treated in Trial 1. Although the serum progesterone status of cows with various types of cysts--follicular, luteal, cystic CL's--has not been described, cows were ranked into those with low (<1.0 ng/ml), intermediate (1.0 to 3.0 ng/ml) and high (>3.0 ng/ml) serum progesterone at the time of treatment.

Reproductive performance after treatment, i.e., estrus within 30 days of treatment and first service conception rates during that interval, was summarized by treatment. Pregnancy was determined by palpation *per rectum* at 50 to 90 days after breeding (Zemjanis, 1970). Historical data such as age, lactation number and days postpartum at

the time of treatment were summarized. Treatment response by type of cyst (low, intermediate, and high progesterone) was also examined.

RESULTS AND DISCUSSION

Trial 1

Pretreatment Historical Data

The median postpartum interval to treatment was 117 days, $\bar{X}$ = 152.2 days, with a range of 42 to 380 days for the 35 cows. Eight cows in groups 1 through 6 were 8 to 13 months postpartum at the time of treatment with GnRH and had been treated, usually with HCG, for the cystic condition sometime (>30 days) prior to receiving GnRH. The postpartum interval to treatment did not affect the endocrine response to treatment in these cows and therefore data from these 8 cows were combined with data from non-pretreated cows for statistical analysis. Based on ovarian palpation *per rectum*, the estimated diameter of the cysts averaged 3.8 cm at the time of treatment and in all 35 cases the cysts were classified by palpation as being of the follicular type. Pretreatment behavioral characteristics which were equally divided between cows showing nymphomania and those with anestrus did not affect responses to treatment. These pretreatment characteristics of cows with ovarian follicular cysts are similar to those previously described by Zemjanis (1970) and Roberts (1971).

Endocrine Status Prior to Treatment

Blood serum progesterone at the time of treatment (Table 1) averaged 0.4 ± 0.1 ng/ml (± standard error of the mean, SEM). Although the majority of cows treated had low serum concentrations of progesterone (<1.0 ng/ml), 4 of the 35 cows had between 1.0 and 2.0 ng/ml of serum progesterone at the time of treatment, indicating the

Table 1. Serum progesterone, LH, and estrogens in cows with ovarian follicular cysts and in normal cows during the luteal and estrual phases of the estrous cycle

Subjects		Progesterone (ng/ml)	LH	Estradiol (pg/ml)	Estrone (pg/ml)
Cows with ovarian follicular cysts (n=35)	Mean	0.4	2.7	3.9	23.0
	SEM	(0.1)	(0.2)	(0.5)	(2.0)
	Range	(0.1-2.0)	(0.2-7.2)	(1.1-7.1)	(8.8-33.7)
Normal cows[a]					
Luteal phase		≃6.0	≃1.0	2.0	N.D.
Estrus		<0.5	≥10.0	7.0	N.D.

[a]Chenault *et al.* (1975).

N.D. = not determined

SEM = standard error of mean

presence of some luteal tissue and these cows may therefore have had luteal cysts. By palpation, however, the cysts in these cows were classified as the follicular type. Since serum LH and progesterone responses to GnRH did not differ between these cows and those with less than 1.0 ng/ml of serum progesterone, the data were combined.

Thus, serum progesterone concentrations at the time of treatment in the cows in this study were similar to progesterone values normally found in cows without functional luteal tissue, such as near estrus (Table 1) or shortly after parturition (Smith *et al.*, 1973). Peripheral serum progesterone concentrations have not been correlated with the various types of bovine ovarian cystic dysfunction, but low serum progesterone concentrations as observed in the present investigation probably indicate the presence of follicular-type cysts lacking significant luteal tissue in the majority of these cows.

Serum LH concentrations averaged (± SEM) 2.7 ± 0.2 ng/ml prior to treatment in cows with ovarian follicular cysts (Table 1). This concentration is low compared to the magnitude of the preovulatory LH surge in cattle but is slightly higher than the usual LH concentrations (≈1.0 ng/ml) during the remainder of the estrous cycle (Figure 1 and Table 1, Chenault *et al.* [1975]). Cows with ovarian follicular cysts may have slightly increased serum LH due to lack of steroid (progesterone) feedback on the pituitary. This is similar to the effect of castration on serum LH in cows (Hobson and Hassel, 1972; Beck *et al.*, in press).

Serum estradiol and estrone averaged (± SEM) 3.9 ± 0.5 and 23.0 ± 2.0 pg/ml, respectively, prior to treatment (Table 1). This level of estradiol is similar to values reported for cattle during proestrus from our laboratory (Stellflug *et al.*, 1975) and by Chenault *et al.* (1975). Serum estrone concentrations prior to treatment were approximately double those reported from our laboratory for dairy cows during the early postpartum period by Britt *et al.* (1974). High urinary excretion

rates of estradiol and estrone have been reported for cows with ovarian cysts by Lunaas, Refsdal and Garm (1974). Contradictory results have been reported regarding serum estradiol and estrone in cows with this condition. In one study Kittok *et al.* (1973) reported estradiol and estrone concentrations were similar in cows with ovarian follicular cysts and cows in the luteal phase of the cycle. But in a second study Kittok *et al.* (1974) found that serum estradiol was greater in cows which developed ovarian follicular cysts than in non-cystic herdmates on days 2 to 7 after estrus. It should be pointed out that due to the very small (pg/ml) amounts of estrogen present in the peripheral serum of cows direct comparison of absolute values between different studies is probably not valid. Rather relative changes following treatment (luteinization) or between normal and abnormal cows are preferable.

The serum concentrations of estradiol and estrone in cows with ovarian follicular cysts reported here do not appear to substantiate the commonly held belief that cows with ovarian follicular cysts are influenced by excessively high levels of estrogen. Ovarian cysts may, however, produce excessive amounts of estrogen shortly after their formation but decrease in steroidogenic activity as the theca interna and granulosa cell layers degenerate with time (Yamauchi and Inui, 1954; Short, 1962). I was unable to determine when the cystic structures had formed in the cows in this study and therefore could not examine this effect.

LH Response

Average serum LH responses through 4 hours after injection are shown in Figure 4 for each dose of GnRH tested. Serum LH was low

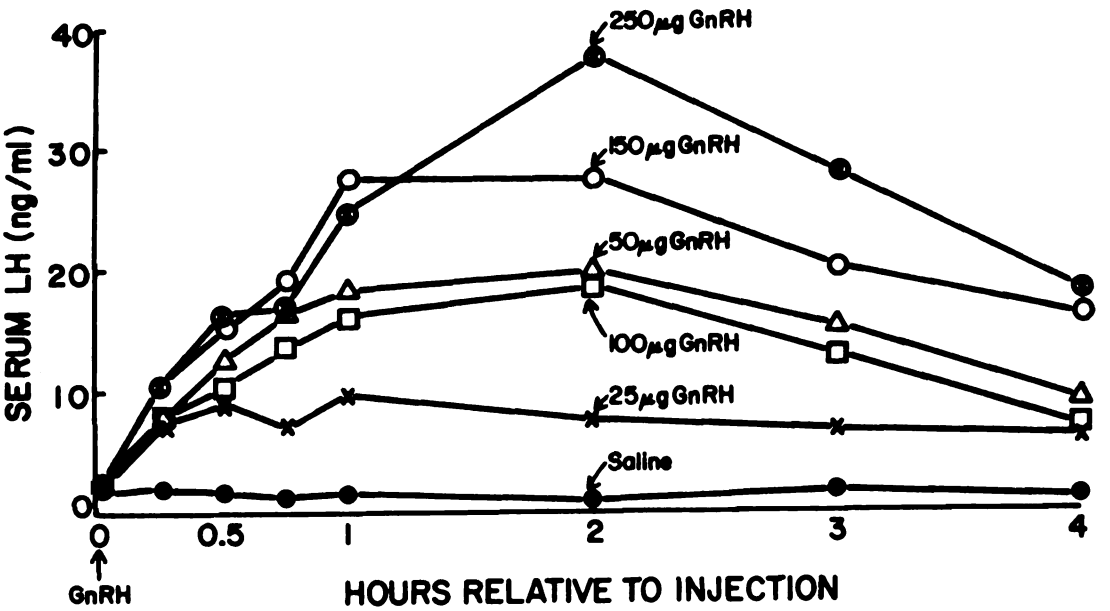

Figure 4. Average serum luteinizing hormone concentrations in cows with ovarian follicular cysts after intramuscular injection of 0 (saline), 25, 50, 100, 150, or 250 μg of gonadotropin releasing hormone (GnRH), 5 cows per treatment (n=30).

initially ($\bar{X} \pm$ SEM = 2.7 $\pm$ 0.2 ng/ml) in all groups (1 through 6) and did not increase following injection of saline. Serum LH increased rapidly for all groups given GnRH (Figure 4) and, based on regression analysis of area under individual response curves, LH responses increased ($P<0.01$) linearly with dose of GnRH. In all 25 cows given GnRH, serum LH increased within 4 hours, and the modal time to peak LH was 2 hours. Serum LH concentrations declined from peak responses by 4 hours after GnRH and did not differ from pretreatment values by 24 hours.

Magnitude of LH responses observed following 50, 100, 150, and 250 µg GnRH was similar to that reported after comparable doses of GnRH were given (IV--Kittok *et al.*, 1973; or IM--Cantley *et al.*, 1974) to cows with ovarian cysts or given (IM) to bulls (Mongkonpunya *et al.*, 1974) or normal heifers (Kaltenbach *et al.*, 1974). The increase in serum LH after GnRH was similar in magnitude and duration to the normal surge of LH occurring near the beginning of estrus in cattle (Swanson and Hafs, 1971; Chenault *et al.*, 1975). It is apparent from these data that IM administration of GnRH offers an effective method of causing elevated serum LH in cows with ovarian follicular cysts.

The etiology of the ovarian cystic condition may involve a failure of basophilic delta cells of the anterior pituitary to release the preovulatory surge of LH, resulting in lack of ovulation (Jubb and McEntee, 1955). One possible explanation for this failure of LH release is an inability of these cells to recognize and/or respond to the hypothalamic LH releasing factor (GnRH). However, these results, and those of Kittok *et al.* (1973), show no impairment

in LH release following administration of GnRH to cows with ovarian cysts, thereby suggesting that a deficiency in synthesis or release of hypothalamic releasing hormone may precipitate the ovulation failure.

Serum Progesterone after GnRH and HCG

Serum progesterone was low ($\bar{X} \pm$ SEM = 0.4 $\pm$ 0.1 ng/ml) at the time of treatment. Since the serum progesterone response was not different ($P>0.25$) among groups given 50, 100, 150, or 250 µg GnRH, progesterone data from these cows were averaged (Figure 5). By day 11 after treatment, 18 of 20 cows given 50, 100, 150, or 250 µg GnRH had increases in serum progesterone of more than 2.0 ng/ml over pre-treatment concentrations. Serum progesterone on day 11 after treatment (Figure 5) was greater ($P<0.05$) in cows which were given 50, 100, 150, or 250 µg GnRH than those given saline of 25 µg GnRH. The slight increases in average concentrations of serum progesterone in cows given saline or 25 µg GnRH (Figure 5) reflect increasing progesterone in 1 and 2 cows, respectively. When doses of 50 to 250 µg of GnRH were given to cows with follicular cysts the LH released was effective in initiating formation of functional luteal tissue as indicated by increased serum progesterone after treatment. These serum progesterone changes were similar in magnitude and duration to those described for normal bovine estrous cycles by Wettemann *et al.* (1972) and Chenault *et al.* (1975).

Progesterone also increased after injection of HCG and on days 7 and 11 was not different from the progesterone concentrations at these times after the 4 higher doses of GnRH (Figure 5). Serum progesterone concentration appeared to decline sooner after HCG treatment

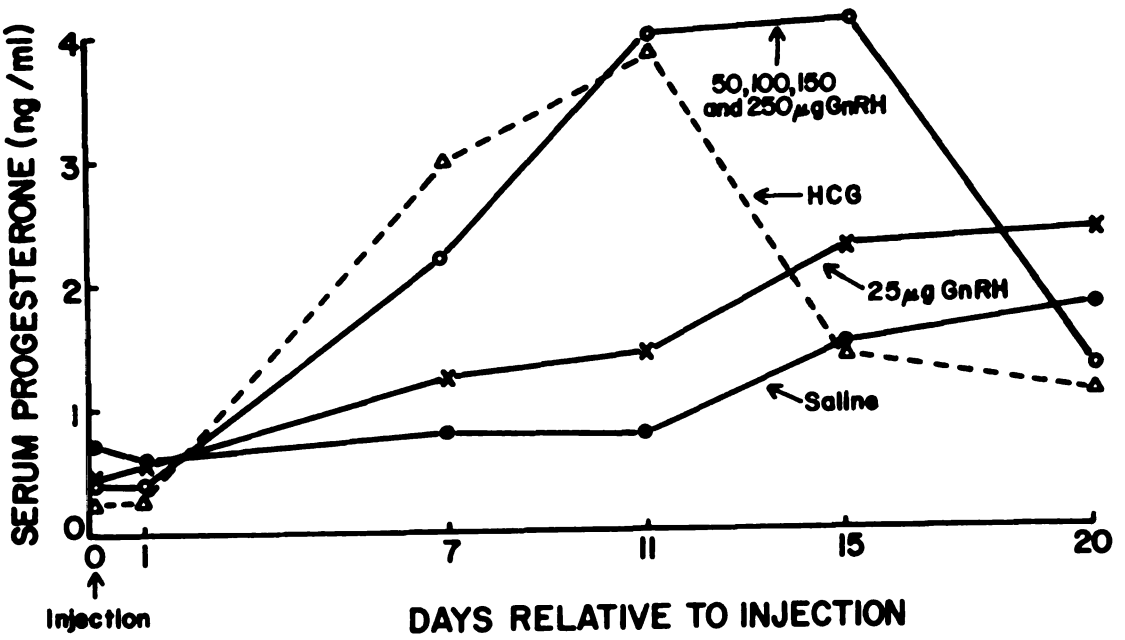

Figure 5. Average serum progesterone concentrations in cows with ovarian follicular cysts after intramuscular injection of 0 (saline), 25, 50, 100, 150, or 250 µg of gonadotropin releasing hormone (GnRH) or 10,000 units of human chorionic gonadotropin (HCG), 5 cows per treatment (n=35).

than after GnRH (groups 3 through 6), but this difference was not statistically significant (P>0.10). The increase in progesterone by day 7 and 11 after HCG in cows with follicular cysts is consistent with previous reports by Erb *et al.* (1973) and Kittok, Britt and Edgerton (1974) and indicates that HCG also stimulates luteinization. To my knowledge, duration of luteal function after HCG as indicated by serum progesterone has not been reported although an interval of 21 to 24 days has been cited as the likely interval to estrus after HCG treatment of cows with ovarian cysts by Bierschwal (1966) and Roberts (1971). Although the luteal phase appeared shorter after HCG treatment than after GnRH, more cows need to be studied before such a conclusion would be valid.

Ovarian Characteristics after Treatment

Twelve of the 18 cows in which progesterone increased more than 2.0 ng/ml following treatment with 50, 100, 150, or 250 µg GnRH were palpated on days 7 and 15 after treatment. In 7 of the 12 cows ovulation had occurred after GnRH treatment as indicated by the presence of a CL, usually on the ovary opposite the one containing the cyst(s). In the remaining 5 cows CL's were not detected and changes in the character of the cyst(s) were not evident on palpation even though luteal tissue had apparently developed since serum progesterone had increased substantially. Serum progesterone response did not differ (P>0.25) between these 2 groups through 20 days after treatment. In contrast to the GnRH response, CL's were not detected in any of the cows treated with HCG, although serum progesterone had increased by at least 2.0 ng/ml in all 5 cows treated.

Apparently luteal tissue developed after GnRH treatment either by luteinization without ovulation of cystic or non-cystic follicles or by ovulation and CL development of non-cystic follicles. Since theca interna and granulosa cell layers of the cyst wall degenerate with time after onset of the cystic condition (Yamauchi and Inui, 1954), duration of the cystic condition and stage of development of non-cystic follicles at the time of treatment probably determine which occurred.

Effect of Luteinization on Serum Estradiol, Estrone, and LH

Serum progesterone increased ($P<0.01$) by at least 2.0 ng/ml by day 11 post-treatment in 19 of 30 cows given GnRH or saline (Table 2). In contrast, serum estradiol, estrone, and LH concentrations were decreased ($P<0.01$) in these 19 cows by day 11 relative to comparable values at day 0. In 9 cows, progesterone was not increased ($P>0.25$) at day 11 relative to day 0 and serum estradiol, estrone, and LH were also unchanged ($P>0.25$). The remaining 2 cows were observed in estrus on days 10 and 12 after treatment and were excluded from these analyses.

Both estrogen and progesterone are involved in negative feedback control of LH release in cows (Beck *et al.*, 1974). The increased serum LH in cows with ovarian follicular cysts prior to luteinization is consistent with the report of Beck *et al.* (1974) since progesterone concentrations were low prior to treatment in these cows. When pretreatment LH was compared to LH on day 11 after treatment in those cows in which luteinization occurred, a consistent decrease in LH ($P<0.005$) was observed after treatment demonstrating the negative feedback control of progesterone and estrogen on LH release (Table 2). The ability of

Table 2. Effect of luteinization (increased serum progesterone) of bovine ovarian follicular cysts on serum estradiol, estrone, and LH

Group	No. of cows[b]	Day relative to treatment	Progesterone (ng/ml)	Estradiol (pg/ml)	Estrone (pg/ml)	LH (ng/ml)
Progesterone increased[a]	19	0	0.5±0.1[c]	4.1±0.5	23.7±1.9	2.6±0.2
		+11	4.4±0.4*	1.2±0.1*	16.4±1.8*	1.7±0.1*
Progesterone unchanged	9	0	0.5±0.1	3.6±0.5	21.8±3.0	2.9±0.4
		+11	0.6±0.1	3.2±0.3	17.6±2.1	3.1±0.7

[a]Serum progesterone increased by more than 1.0 ng/ml from day 0 to day 11 after injection.

[b]Two cows were observed in estrus at 10 and 12 days after treatment and were not included in these analyses. Only cows from groups 1 through 6 considered.

[c]Mean ± standard error of the mean.

*Interval difference significant at $P<0.005$.

increasing serum progesterone to decrease serum LH, presumably by decreasing pituitary release of LH and increasing storage of LH in the anterior pituitary, was the basis for progesterone treatment of cows with ovarian cysts reported by Beck and Ellis (1960) and Johnson and Ulberg (1967).

A significant decrease in serum concentrations of both estradiol and estrone also occurred by day 11 after treatment if serum progesterone increased more than 2.0 ng/ml over the same interval (Table 2). Therefore, near normal luteal phase estradiol-progesterone ratios were established in these cows. Edqvist *et al.* (1974) also reported a decline in serum estradiol as serum progesterone increased in cows with ovarian cysts after treatment with luteinizing hormone-releasing hormone (LH-RH). This decrease in estrogens may reflect the conversion of cells of the granulosa and theca interna layers of the follicle wall from estrogen to progesterone secreting activity.

Reproductive Performance after Treatment

I allowed 30 days for the occurrence of estrus after treatment and recorded only first service conception rates on breedings during that period. In many cases this is the usual interval between scheduled reproductive examinations by veterinarians and would be the interval allowed before retreatment is considered. I recognize that this interval is arbitrary but, for the reasons stated, it would appear to have practical merit.

Sixteen of the 30 cows given GnRH or saline were observed in estrus within the 30-day time limit and no dose effect was evident ($P>0.50$). The average interval to estrus for those cows given GnRH was 21.8 days with a range from 10 to 28 days. Three cows treated

with saline were observed in estrus within 30 days of injection. Five cows which responded to GnRH with luteinization had decreases in progesterone consistent with a change from the luteal to follicular phase of the cycle between 15 and 20 days after GnRH but were not observed in estrus. Therefore, estrus probably occurred in 16 of 20 cows given 50, 100, 150, or 250 µg GnRH. Ten of the 16 cows observed in estrus were inseminated and 6 of the 10 were diagnosed pregnant by palpation *per rectum* 50 to 90 days after insemination. Estrus was observed within 30 days in 3 of 5 cows treated with HCG and serum progesterone indicated that estrus had probably been missed in an additional cow.

The apparent occurrence of estrus within 30 days of treatment in 80% of the cows treated with GnRH (50 to 250 µg) compares favorably with reports on the efficacy of other gonadotropic preparations used in treating this condition. Casida *et al.* (1944) reported that within 31 days of treatment with an unfractionated sheep pituitary extract, 79% of 96 cows with ovarian cysts had normal CL's (by palpation *per rectum*) and 50% had been observed in estrus. After treatment with 5,000 units of HCG, Bierschwal (1966) reported 67% of 145 cows developed a CL within 1 to 2 weeks after treatment.

These data indicate that serum concentrations of progesterone are very low, LH concentrations are slightly elevated from usual baseline values, and estrogens appear similar to normal proestrual values in cows which have developed ovarian follicular cysts. These results also indicate that single IM injections of GnRH in whole body doses of 50 to 250 µg are capable of consistently causing elevated serum LH

which induced formation of luteal tissue in a high proportion of cows with ovarian follicular cysts.

Trial 2

Pretreatment Historical Data

One hundred six cows were treated for ovarian cysts on the field study, 49 with LH preparations (primarily HCG) and 57 with GnRH. Age distributions of the cows treated and of the United States dairy cattle population from 1964 through 1968 (Norman *et al.*, 1974) are presented in Figure 6-A and 6-B, respectively. These distributions differ significantly ($P<0.01$) by Chi-square analysis indicating an age effect on the incidence of the condition. In Figure 6-C the relative incidence of ovarian cysts on an age corrected basis is presented. This adjustment makes it increasingly evident that the incidence of ovarian cysts is affected by age, the distribution differing significantly from an equal distribution across all ages ($P<0.01$, Chi-square analysis). If the incidence was not influenced by age, the relative frequency in Figure 6-C should be 1.0 for all ages. These data indicate that the incidence of cysts was lower in younger cows (<5 years) than in mature cows ($\geq$5 years). Similar observations have been reported by Garm (1949), Roberts (1955) and others.

Average age in years, lactation number, days postpartum, and type of estrual behavior (anestrus or nymphomania) of the cows treated on the field trial are presented in Table 3. No significant differences ($P>0.25$) were detected in these parameters between treatment groups. Average days postpartum at treatment, 111.1 days, was about 40 days

Figure 6-A. Age distribution of cows with ovarian cysts (n = 106).

Figure 6-B. Age distribution of cows completing DHI and DHIR records in U.S. - 1964 through 1968 (n = 3,200,348).

Figure 6-C. Adjusted frequency of the incidence of ovarian cysts in dairy cows. Calculated by dividing A by B for each age category.

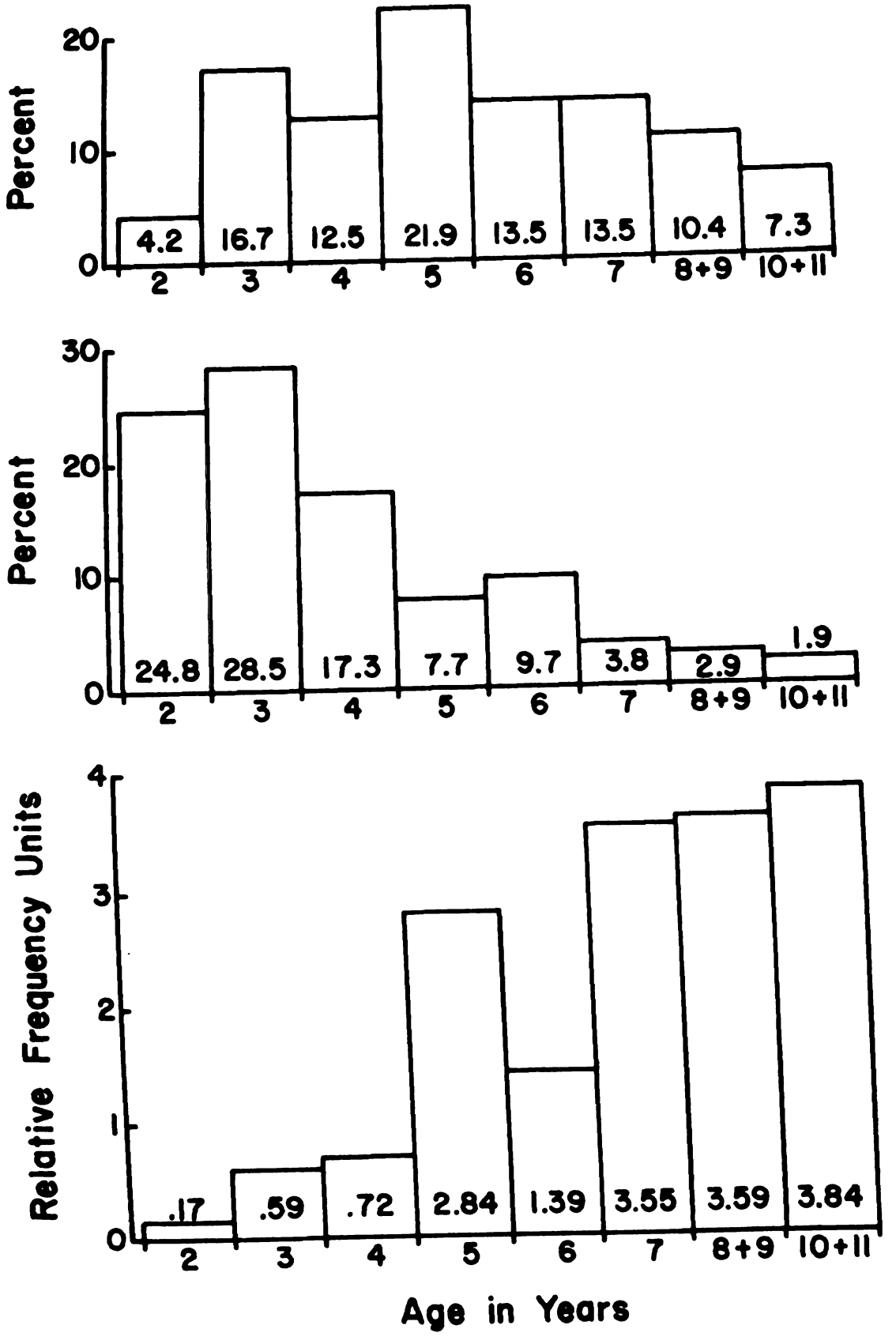
Percent
20
10
0
4.2
16.7
12.5
21.9
13.5
13.5
10.4
7.3
2
3
4
5
6
7
8+9
10+11
Percent
30
20
10
0
24.8
28.5
17.3
7.7
9.7
3.8
2.9
1.9
2
3
4
5
6
7
8+9
10+11
Relative Frequency Units
4
3
2
1
0
.17
.59
.72
2.84
1.39
3.55
3.59
3.84
2
3
4
5
6
7
8+9
10+11
Age in Years

Figure 6

Table 3. Pretreatment historical data for cows with ovarian cysts (n = 106)

Treatment	Age (yr)	Lactation	Days postpartum at treatment	Behavior	
				Anestrus	Nymphomania
LH	5.6	3.7	108.7	32.8%	67.4%
GnRH	6.2	4.1	113.5	36.2%	63.2%

shorter for cows treated in the field trial than those treated in Trial 1. There were, however, 8 previously treated cystic cows in Trial 1 that inflated that figure. The average from the field trial is probably a more realistic indication of the time at which commercial dairy cows are treated for ovarian cysts.

The greater proportion of cows showing signs of nymphomania rather than being anestrus probably indicates that the majority of cows were selected for examination by the dairyman because of abnormal estrous behavior rather than being detected on routine postpartum or pregnancy examinations. Roberts (1955) reported nearly 75% of cows treated clinically for cysts had symptoms of nymphomania (frequent estrus) whereas Bierschwal (1966), Morrow *et al.* (1966) and Morrow *et al.* (1969) reported 60 to 80% of cows detected as having ovarian cysts on periodic scheduled examinations were anestrus. Thus, where herd health programs including regular examinations of postpartum cows are employed, a high percentage of anestrous cystic cows should be expected, whereas a higher percentage of nymphomania in cystic cows will probably be reported when cows are only examined upon complaint by dairymen.

Serum Progesterone at Treatment

The distribution of serum progesterone concentrations in the 106 cows diagnosed as having ovarian cysts is presented in Table 4. The distribution did not differ between treatment groups ($P>0.25$). Progesterone was low (<1.0 ng/ml) in 66.0% of the cows treated, intermediate (1.0 to 3.0 ng/ml) in 16.0%, and high (>3.0 ng/ml) in 18.0%. Cows with intermediate and high serum progesterone apparently had luteal cysts, although cows with normal CL's at various stages of development would also have similar progesterone concentrations (Chenault *et al.*, 1975; Figure 1). The distribution of serum progesterone in cows clinically designated cystic has not been reported, nor has the endocrine status of cows with different types of ovarian cysts--follicular, luteal, and cystic CL's--been reported. Therefore, it is not possible to determine from these data whether cows with serum progesterone in excess of 1.0 ng/ml had luteal cysts or normal cystic and non-cystic CL's. The majority of cows treated (66.0%) had low serum progesterone concentrations indicating the presence of follicular type cysts lacking significant luteal tissue similar to those cows treated in Trial 1.

Table 4. Distribution of serum progesterone (ng/ml) in cows with ovarian cysts

Treatment	Serum Progesterone (ng/ml)			Total
	1.0	1.0 to 3.0	3.0	
LH	34(69.4%)	8(16.3%)	7(14.3%)	49
GnRH	36(63.2%)	9(15.8%)	12(21.1%)	57
Combined	70(66.0%)	17(16.0%)	19(18.0%)	106

Reproductive Performance after Treatment

As indicated in Table 5, 38 (66.7%) of 57 cows treated with 100 µg GnRH and 27 (55.2%) of 49 cows treated with LH preparations, usually HCG, were observed in estrus within 30 days of treatment. The average interval to first estrus after GnRH and LH treatment was 19.1 and 19.8 days, respectively. First service conception rates of cows inseminated within 30 days of treatment were 59.4 and 55.0% for GnRH and LH preparations, respectively. None of these 3 parameters varied significantly between treatments (P>0.20), nor were responses significantly affected by serum progesterone concentration at the time of treatment.

Table 5. Reproductive performance of cows with ovarian cysts after treatment with LH preparations or GnRH

Treatment	No. of cows	Estrus by 30 days	Days to first estrus	Fertility rate (no. preg./no. bred)
LH	49	27(55.2%)	19.8	11/20 (55.0%)
GnRH	57	38(66.7%)	19.1	19/32 (59.4%)

The proportion of cows observed in estrus within 30 days of GnRH treatment, 66.7%, is similar (P>0.10) to results in Trial 1 where 55% were observed in heat after treatment with 50, 100, 150, or 250 µg GnRH. A recovery rate of 66.7% within 30 days of GnRH treatment compares favorably with reports on the efficacy of other products used to treat ovarian cysts in cattle. For example, Casida *et al.* (1944) observed estrus within 31 days of treatment in 50% of 96 cows treated for ovarian cysts with an unfractionated sheep pituitary extract

which some (Roberts, 1971) have interpreted as "a highly specific and curative effect." As reviewed earlier (page 21), higher rates of recovery have frequently been reported when time limits for recovery have not been imposed.

The average interval to estrus of 19.1 days after GnRH is similar to the 21.8 day average in Trial 1. The distribution of the occurrence of estrus after treatment of cystic cows with LH preparations and GnRH is presented in Figure 7. In both cases the majority of estrous activity occurred about 3 weeks (18 to 24 days) after treatment. Although luteal function was not monitored after treatment in Trial 2, the distribution probably indicates that luteal function developing in response to LH or GnRH treatment was preventing the occurrence of estrus until after luteal tissue regression occurred about day 15 after treatment. These results also support the hypothesis that these treatments establish luteal function which is then subject to the usual endogenous luteolytic influences occurring during normal estrous cycles. And they also support the observation that the first estrus after treatment in cows with ovarian cysts occurs near the normal interestrual interval (18 to 24 days) after treatment (Roberts, 1971).

First service conception rates in these cows following treatment for ovarian cysts (55 to 60%) are similar to the 50% conception rates reported recently for New York dairy cows by Spalding, Everett and Foote (1975). Thus, results of this trial indicate that if normal estrous cycle activity is initiated in cows with ovarian cysts, fertility is normal.

Figure 7. Distribution of estrus in cows with ovarian cysts after treatment with LH preparations or gonadotropin releasing hormone (GnRH).

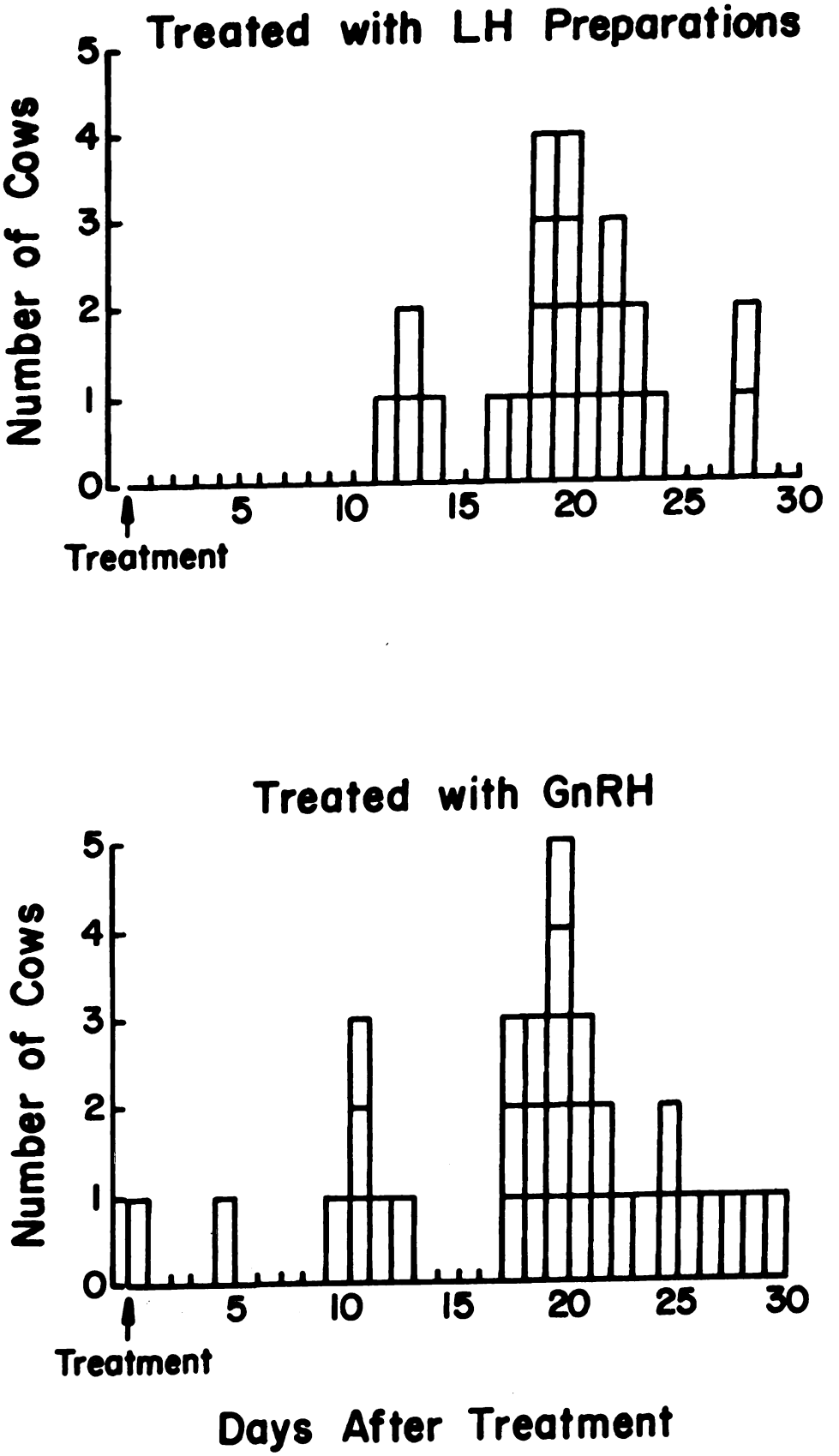
Treated with LH Preparations
Number of Cows
0
1
2
3
4
5
5
10
15
20
25
30
Treatment
Treated with GnRH
Number of Cows
0
1
2
3
4
5
5
10
15
20
25
30
Treatment
Days After Treatment

Figure 7

In summary, results of this trial indicate that a single IM injection of 100 µg of GnRH is at least equally effective as presently used LH preparations for the treatment of cows with ovarian cysts, both the follicular and luteal type cysts. Cows with ovarian cysts responding to GnRH treatment usually return to estrus about 3 weeks after treatment and fertility at that first estrus appears normal.

GENERAL DISCUSSION

While my studies were in progress at least 2 other groups reported results on the efficacy of treating cows with ovarian cysts with GnRH (or LH/FSH-releasing hormone). Grunert, Muller-Schlosser and Ahlers (1973) reported that of 15 cows treated with 1.0 or 1.5 mg of synthetic LH/FSH-releasing hormone, 13 developed a palpable CL by day 7 after treatment. Eleven cows were kept for breeding after treatment with 6 observed in estrus by 42 days. Six of 7 cows bred after treatment conceived.

Researchers at the University of Missouri have reported LH responses and reproductive performance after administration of GnRH, also from Abbott Laboratories, to cows with cystic ovaries. They (Cantley *et al.*, 1974) reported serum LH responses similar to those which I observed. A second report by this group (Bierschwal *et al.*, 1974) described the reproductive performance of cows with cystic ovarian follicles treated with GnRH. Responses to treatment were termed positive if normal estrous cycles or conception occurred after treatment and negative if character of the cyst did not change within 2 to 4 weeks of treatment. Positive responses were observed in nearly 75% of cows given GnRH (50, 100, or 250 µg) but in only 21% of cows given a placebo. The mean interval to estrus in those responding positively to GnRH was 22.5 days.

My data plus those of Grunert *et al.* (1973), Bierschwal *et al.* (1974), and Cantley *et al.* (1974) on the efficacy of single IM injections of GnRH to cows with ovarian cysts indicate that this more practical method of administration can be substituted for the initial method (3-100 μg doses of GnRH given IV at 2-hour intervals) described by Kittok *et al.* (1973).

While my data describe to some extent the endocrine status of cows with ovarian follicular cysts, much remains unknown about the etiopathogenesis of the condition. The endocrine events leading to and during development of ovarian cysts in cattle remain unresolved. The hypothesis of an insufficient preovulatory LH surge to cause ovulation (Jubb and McEntee, 1955) remains an hypothesis. The primary site of malfunction and its cause are also unknown, and a factual explanation of the abnormal sexual behavior described in affected cows, ranging from anestrus to nymphomania to virilism, is not available. In many ways these unanswered questions are also applicable to the polycystic ovarian disease in women (Goldzieher, 1973). Although these conditions may or may not prove to have similar etiopathogeneses, it seems likely that similar investigational procedures might be utilized in both species for answering such questions.

Why should GnRH rather than HCG or PLH be used to treat cows with ovarian cysts? The antigenicity of GnRH is probably less than that of HCG although the degree to which this will enhance the effectiveness of GnRH over HCG in the treatment of cows with ovarian cysts is probably small. Differences in the characteristics of GnRH and HCG, such as molecular weight (1182 versus 30,000), blood half life (7 minutes versus 24 hours), and number of sialic residues (zero versus

12) (Niswender, Nett and Akbar, 1974) indicate the likelihood of reduced antigenicity of GnRH relative to HCG. GnRH may be the drug of choice for treating cows with ovarian cysts which have failed to respond to LH preparations such as HCG.

Another major reason for the use of GnRH over HCG will hopefully prove to be the relative cost of the 2 products. For reasons not clear to me, the demand for HCG has exceeded the supply in the past 18 months to the point that veterinarians have had difficulty obtaining the product and, if available, the cost has been prohibitive to its use in many situations. Thus, the veterinary profession would be receptive to an alternative product that was effective and economical. Since GnRH is synthetically produced whereas HCG is extracted from the urine of pregnant women, the supply of GnRH should be more elastic than that of HCG and its price will hopefully be economically compatible with its application to animal agricuture. The Federal Food and Drug Administration (FDA) has yet to rule on the use of GnRH in food-producing animals and therefore its market price is not yet determined.

My data indicate that another compound which is in the experimental stages of development may have application to treatment of ovarian cysts in cows. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) has a luteolytic (causes regression of luteal tissue) effect in cattle which are in the luteal phase of the estrous cycle with estrus and ovulation occurring about 72 and 96 hours, respectively, after its administration (Louis *et al.*, 1973). The luteolytic property of $PGF_{2\alpha}$ should be tested in cows with ovarian cysts, especially those with significant serum progesterone where a luteolytic effect would be most likely.

Nearly 40% of the cows on my field trial had over 1.0 ng/ml of serum progesterone at the time of treatment. To my knowledge the luteolytic effect of $PGF_{2\alpha}$ in dairy cows with ovarian cysts has not been tested since at the present time FDA's investigational permits only allow the use of $PGF_{2\alpha}$ in beef cows and non-lactating dairy heifers. The advantage of $PGF_{2\alpha}$ would be that in cows in which luteolysis occurred following treatment, estrus would occur 3 to 4 days after treatment rather than about 21 days as is the case with GnRH or HCG. Thus, the more rapid response after $PGF_{2\alpha}$ would be economically and psychologically advantageous.

SUMMARY AND CONCLUSIONS

The purpose of these studies was to evaluate gonadotropin releasing hormone (GnRH) as a therapeutic agent for ovarian cysts of cattle. Trials were designed to examine the endocrine status of cows with ovarian cysts, to determine the endocrine response (LH and progesterone) in these cows to GnRH and to human chorionic gonadotropin (HCG), and to compare reproductive performance (occurrence of estrus within 30 days of treatment and fertility rates during that interval) of cows with cysts after GnRH and HCG treatment.

Prior to treatment serum progesterone concentrations were very low (0.4 ng/ml), estradiol was about equivalent with proestrual values (4.0 pg/ml), and LH was approximately 2-fold higher than normal baseline values (2.7 ng/ml) in cows with ovarian follicular cysts.

In the first experiment cows with ovarian follicular cysts were treated with single IM injections of saline, GnRH, or HCG. Serum LH was not altered after saline, but all doses of GnRH significantly increased serum LH in a dose related manner. Peak LH responses occurred about 2 hours after GnRH and by 4 hours LH was declining from peak concentrations. Serum progesterone concentrations increased more than 2.0 ng/ml by day 11 after treatment in 18 of 20 cows treated with 50 to 250 μg of GnRH and were greater than serum progesterone on day 11 after saline or 25 μg of GnRH. Mean progesterone response in cows given 50 to 250 μg of GnRH was similar in magnitude and duration

to serum progesterone during the luteal phase of the bovine estrous cycle. Cows given 10,000 units of HCG had increases in serum progesterone similar to those observed in cows given the high doses of GnRH.

In those cows in which serum progesterone increased significantly after treatment, serum estradiol, estrone, and LH concentrations decreased. Serum concentrations of these hormones did not change in those cows in which serum progesterone did not rise after treatment.

Secondly, a field trial was conducted in which cooperating veterinarians treated cows having ovarian cysts with GnRH (100 µg) or commonly used LH preparations (usually HCG). A total of 106 dairy cows were treated on this trial. These cows were, on the average, 5.9 years old, in their fourth lactation, about 110 days postpartum, and more commonly showing signs of nymphomania rather than being anestrus at the time of treatment. About two-thirds (66.0%) of them had follicular-type cysts, serum progesterone being less than 1.0 ng/ml. The remaining third (34.0%) had over 1.0 ng/ml of serum progesterone indicating the presence of a significant amount of functional luteal tissue. Thirty-eight of 57 (66.7%) cows treated with 100 µg of GnRH and 27 of 49 (55.2%) cows treated with LH preparations were observed in estrus within 30 days of treatment. The average interval to first estrus after GnRH and LH treatment was 19.1 and 19.8 days, respectively. First service conception rates of cows inseminated within 30 days of treatment were 59 and 55% after GnRH and LH, respectively. None of these responses varied significantly between treatments.

In conclusion, these data indicate that cows with ovarian follicular cysts have very low serum concentrations of progesterone,

slightly increased serum LH concentrations, and serum estradiol values in the range normally expected in cows approaching estrus. It was also determined that single IM injections of GnRH are capable of consistently causing elevated serum LH with subsequent formation of luteal tissue in cows with ovarian follicular cysts. A high percentage of cows treated with GnRH had normal estrous periods within 30 days of treatment with normal rates of fertility, responses which were at least equivalent to those observed after treatment with presently used LH preparations, principally HCG.

APPENDICES

APPENDIX A

EXTRACTION AND RADIOIMMUNOASSAY OF ESTROGEN (ESTRADIOL OR ESTRONE)

Michigan State University

Duplicate aliquots of serum or plasma (0.1 ml if estrogen is high as in late pregnancy or 0.5 ml if estrogen is low as during the estrous cycle) are placed in disposable culture tubes (16 x 125 mm). To account for procedural losses, 3,000 dpm of ^{3}H-1,2,6,7-estradiol or estrone (New England Nuclear; 100 c/mM; repurified by column chromatography) is added to a third aliquot from a representative number (10 to 20 within each assay) of unknowns. Samples of serum with ^{3}H-1,2,6,7-estradiol or estrone are vortexed for 10 sec and endogenous and labeled hormones are allowed to equilibrate for 30 min. Serum is extracted by vortexing with 5.0 ml of distilled benzene or a 1:5 ratio of serum to benzene for 60 sec. The samples are then equilibrated for 30 min while the serum or plasma settles out from the benzene phase. The benzene is then aspirated off with disposable pasteur pipettes into disposable culture tubes (12 x 75 mm) and those for procedural losses are aspirated off into scintillation vials. Radioactivity of these extracts is averaged to determine a single correction factor to account for procedural losses of estrogen in all serum samples.

Standard estrogen (Sigma Chemical Co.) is pipetted from a stock solution of 100 pg/ml and at least 3 sets (0.0, 0.5, 1.0, 2.0, 4.0, 7.0, 10.0, 20.0, 50.0, and 100.0 pg) are included in each assay. Standard estrogen and serum extracts are dried under nitrogen. Tube walls are rinsed once with minimal amounts of distilled benzene and dried under nitrogen.

0.1% Knox Gelatin in 0.01 M phosphate buffered saline is used to dilute the antibody[1] to 1:20,000 for estradiol and 1:30,000 for estrone. Antibody (0.2 ml) is added to each tube, vortexed for 10 sec and allowed to incubate at room temperature for 30 min. Two hundred microliters of 0.1% gelatin (Knox Gelatin, Inc., Johnstown,

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[1]Anti-estrogen, generously supplied by Dr. G. D. Niswender, Department of Physiology, Colorado State University, Fort Collins. The rabbit antiserum #825-E_2 was prepared against 6-oxime-estradiol conjugated to bovine serum albumin. The sheep antiserum #84-E_1 was prepared against 11-oxime-estrone conjugated to bovine serum albumin.

N.Y.) in 0.01 M phosphate buffered saline, containing about 18,000 dpm of ^{3}H-1,2,6,7-estradiol or estrone (New England Nuclear; 96 c/mM), is added to each tube. The contents of the tubes are mixed for 5 sec and incubated for 3 hr at 5°C.

To separate bound and free estrogen, 0.5 ml of 1.0% dextran T70 (Pharmacia, Uppsala, Sweden) and 0.50% carbon decolorizing neutral norit (Fisher Scientific Co.) in glass distilled water is added to each tube. Contents are mixed, incubated in an ice bath for 10 min and then centrifuged at 2,500 g for 10 min at 5°C. A 0.5-ml aliquot of the supernatant fluid is diluted with a liquid scintillation fluid[2] for quantification of radioactivity in a liquid scintillation spectrometer (Nuclear Chicago Corp., Mark 1).

For comparison among assays, standard sera with high and low estrogen, and extracts from blank extraction tubes, are assayed with each set of unknown serum samples.

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[2] 3a70B preblend scintillation cocktail, Research Products, International Corp., Elk Grove Village, IL.

APPENDIX B

RADIOIMMUNOASSAY FOR BOVINE LUTEINIZING HORMONE (LH)

Michigan State University

Dilution duplicates of serum, usually 100 µl and 200 µl, diluted to 500 µl with PBS-0.1% Knox Gelatin, are incubated at 4°C with first antibody (guinea pig antibovine LH) for 24 hr. Then ^{125}I-LH is added and incubation continued for another 24 hr. The second antibody (sheep anti-guinea pig gamma globulin) is then added. After an additional 72 hr, 3 ml of PBS is added and the samples are centrifuged at 2,500 g for 30 min. The supernatant fluid is decanted and ^{125}I in the precipitate is counted in a gamma counter.

Laboratory Procedure

I. Incubation with first antibody

Each unknown is assayed in dilution duplicate. One hundred and 200 µl of each unknown are added to separate disposable glass culture tubes (12 x 75 mm) with a micromedic automatic pipette. A total volume of 500 µl is obtained in each tube by adding PBS-0.1% Knox. Twelve tubes containing 0.062, 0.126, 0.176, 0.250, 0.352, 0.500, 0.704, 1.000, 1.408, 2.000, 4.000, or 8.000 ng of standard LH (NIH-LH-B8) in 500 µl of PBS-0.1% Knox are placed at each end of the assay. Two hundred microliters of LH antibody (first antibody) is added at a dilution of 1:600,000 to each of the culture tubes and the tubes are incubated at 4°C for 24 hr. Each tube is vortexed gently after each addition and covered during incubation to retard evaporation.

II. ^{125}I-LH

Methods for radioiodination of purified bovine LH (LER-1072-2) were essentially those of Niswender *et al.* (1969), except that ^{125}I was used and the column of Bio-Gel P-60 was coated with EWA. The stock solution of ^{125}I-LH is diluted with PBS-0.2% Knox so that 100 µl contain about 20,000 CPM. One hundred microliters of ^{125}I-LH solution is then added to each tube. Incubation is continued at 4°C for 24 hr.

III. Second antibody

Sheep anti-guinea pig gamma globulin (SAGPGG), referred to as second antibody, diluted to a titer which will optimally precipitate the gamma globulin, is used to form an antigen-antibody-antibody complex large enough to be precipitated by centrifugation. Two hundred microliters of SAGPGG is added to each tube and incubation is continued for 72 hr.

IV. Precipitation

Following final incubation, 3 ml of cold PBS is added to each tube to dilute the unbound ^{125}I-LH. Centrifugation at 2,500 g for 30 min in a refrigerated centrifuge is used to precipitate the bound ^{125}I-LH. The supernatant fluid is decanted and the tubes are allowed to drain for 30 min. Any fluid adherent to the neck and lip of the tube is removed with absorbent tissue. The bound ^{125}I-LH of the precipitate is then quantified in an automatic gamma counter. Samples are counted for 10 min or to a total of 4,000 counts, whichever accumulates first.

V. Calculations

The counting time for each sample is punched automatically on paper tape by a Teletypewriter. The standard curve is calculated by multiple regression analysis on a CDC 6500 computer. These regression coefficients are entered manually into an Olivetti computer, the counting time for each unknown is entered into the computer from the punched tape, and LH concentrations in the unknowns are computed by multiple regression analysis.

Control tubes are included in each assay to determine background radioactivity (tube containing 1:400 control guinea pig serum in place of first antibody), total counts added (tube containing only ^{125}I-LH) and counts in the precipitate (tube containing no unknown or standard). Values for the duplicate standards are averaged and plotted as the percent of ^{125}I-LH precipitated at each dose of LH standard.

APPENDIX C

EXTRACTION AND RADIOIMMUNOASSAY OF PROGESTERONE

Michigan State University

Duplicate aliquots of serum or plasma (0.1 ml) are placed in disposable culture tubes (15 x 80 nm). To account for procedural losses, 3,000 dpm of ^{3}H-1,2-progesterone (New England Nuclear; 34 c/mM; repurified by column chromatography) is added to a third aliquot from a representative number (10 to 20 within each assay) of unknowns. Samples of serum with ^{3}H-1,2-progesterone are vortexed for 10 sec and endogenous and labeled hormones are allowed to equilibrate for 30 min. Serum is extracted by vortexing with 2.0 ml of nanograde benzene:hexane (1:2) for 30 sec. Tubes are then stored at -20°C for at least 1 hr to freeze the aqueous phase. With precautions taken to avoid thawing the aqueous phase, extracts destined for radioimmunoassay are decanted into disposable culture tubes (12 x 75 mm) and those for procedural losses are decanted into scintillation vials. Radioactivity of these extracts is averaged to determine a single correction factor to account for procedural losses of progesterone in all serum samples.

Standard progesterone (Sigma Chemical Co.) is pipetted from a stock solution of 10 ng/ml and at least 2 sets (0.0, 0.025, 0.05, 0.1, 0.25, 0.50, 0.75, and 1.0 ng) are included in each assay. Standard progesterone and serum extracts are dried under nitrogen. Tube walls are rinsed once with minimal amounts of analytical grade diethyl ether and dried under nitrogen.

Normal rabbit sera, diluted 1:400 with 0.05 M EDTA (or 0.1% Knox Gelatin) in 0.01 M phosphate buffered saline, is used to dilute the antibody[1] to 1:4,500. Antibody (0.2 ml) is added to each tube, vortexed for 10 sec, and allowed to incubate at room temperature for 30 min. Two hundred microliters of 0.1% gelatin (Knox Gelatin, Inc., Johnstown, N.Y.) in 0.01 M phosphate buffered saline, containing 45,000 dpm of ^{3}H-1,2,6,7-progesterone (New England Nuclear; 96 c/mM),

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[1]Anti-progesterone, generously supplied by Dr. G. D. Niswender, Department of Physiology, Colorado State University, Fort Collins. The rabbit antiserum (#869) was prepared against 6β-succinyl progesterone conjugated to bovine serum albumin.

is added to each tube. The contents of the tubes are mixed for 5 sec and incubated for 12-18 hr at 5°C.

To separate bound and free progesterone, 1.0 ml of 0.5% dextran T70 (Pharmacia, Uppsala, Sweden) and 0.25% carbon decolorizing neutral norit (Fisher Scientific Co.) in glass distilled water is added to each tube. Contents are mixed, incubated in an ice bath for 10 min, and then centrifuged at 2,500 g for 10 min at 5°C. A 0.5-ml aliquot of the supernatant fluid is diluted with a liquid scintillation fluid (Bray, 1960) for quantification of radioactivity in a liquid scintillation spectrometer (Nuclear Chicago Corp., Mark 1).

For comparison among assays, standard sera with high and low progesterone, and extracts from blank extraction tubes, are assayed with each set of unknown serum samples.

The results from 16 bovine serum samples as determined by assay of benzene:hexane serum extracts were compared with competitive protein binding assays on the same samples and with radioimmunoassay of progesterone isolated from the same samples by Sephadex LH-20 column chromatography. Radioimmunoassay estimations of benzene:hexane extracts of serum (2.9 $\pm$ 0.7 ng/ml) did not differ significantly from those by radioimmunoassay of progesterone eluted from Sephadex LH-20 column (2.7 $\pm$ 0.8 ng/ml, r = 0.94) or from those by competitive protein binding assay of progesterone (3.9 $\pm$ 1.4 ng/ml, r = 0.78). The specificity of the antibody was described by Niswender (1972).

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