Drug Development and Industrial Pharmacy, 25(3), 315–321 (1999)

RESEARCH PAPER

Gonadorelin-Induced Testosterone Release: A Biological Assay for Quality Assurance of Gonadorelin in Veterinary Medicine

D. J. Kesler,* R. N. Summers, C. A. Peterson, and T. L. Steckler

Department of Animal Sciences, University of Illinois, Urbana, IL 61801

ABSTRACT

Two experiments were conducted with bulls administered norgestomet and gonadorelin to determine if the gonadorelin-induced release of testosterone could be developed into a biological assay for quality assurance of gonadorelin. Implants containing norgestomet (0 to 36 mg) reduced the episodic release ($r = -.81$; $P < .05$*) and mean concentrations of testosterone (*$r = -.82$; $P < .05$*). Gonadorelin-induced testosterone release increased (*$r = .99$; $P < .05$*) with increasing dosage of gonadorelin (up to 5 μg) in norgestomet-implanted bulls (36 mg). Maximal testosterone was released (>sixfold increase) with 5 to 40 μg of gonadorelin. In summary, the gonadorelin-induced testosterone release in bulls administered a synthetic progestin is a sensitive (0.008 μg per kg body weight for 5 μg of gonadorelin) biological assay with a rapid turnaround time for the confirmation of gonadorelin potency. Based on a per-kg-body-weight basis, the norgestomet-treated bull is the most sensitive biological assay model.*

INTRODUCTION

The first therapeutic use for gonadorelin in veterinary medicine, treatment of ovarian follicular cysts in dairy cattle, was reported in 1975, three years before the Nobel Prize was awarded to the two scientists that chemically identified it (1,2). Gonadorelin is the hypothalamic-releasing factor responsible for the release of gonadotropins (luteinizing hormone [LH] and follicle-stimulating hormone [FSH]) from the anterior pituitary. The go-

* To whom correspondence should be addressed. 1207 West Gregory Drive. Telephone: (217) 333-2902. Fax: (217) 244-3169. E-mail: d-kesler@ux6.cso.uiuc.edu

nadorelin-induced LH release causes luteinization of ovarian follicular cysts and recovery in about 18 to 24 days (3–5). Gonadorelin has been marketed for more than two decades and is subjected to numerous quality control tests, including biological analysis, before and after manufacturing. Although numerous biological assays have been identified, all are time consuming and expensive. Although it has been well known that the gonadorelin-induced LH surge stimulates a significant release of testosterone (6–8), it has not been used as a bioassay because the pretreatment episodic variability of testosterone concentrations diminishes efficacy. The objective of these experiments was to determine if the gonadorelin-induced release of testosterone could be developed into a biological assay for quality assurance of gonadorelin after synthesis and/or manufacturing.

MATERIALS AND METHODS

Six beef bulls, 1 to 2 years of age and weighing 644.0 ± 9.3 kg, were included in two experiments. In the first experiment, on day 0, all bulls were bled hourly for 4 hr. After the last blood collection, bulls were implanted with norgestomet implants containing 6, 12, 18, 24, 30, or 36 mg. The implants were matrix-type silicone implants and were 0.36 cm in diameter and 1.2 cm long for each 6 mg of norgestomet. Two days after implantation, the bulls were bled hourly for 4 hr, and implants were then removed. Bulls were treated in four cycles, and each cycle was initiated every 7 days. Hourly bleeding on day 0 was only done in the first cycle. Bulls were assigned to treatment groups so that they were used only once for each dose. In the second experiment, the bulls were implanted with implants containing 36 g norgestomet (each 0.36 cm × 3.6 cm). Two days after implantation, the bulls were administered 0, 1, 5, 10, 20, or 40 μg of gonadorelin (Cystorelin; Merial, Inc., Athens, GA). Blood was collected 2 hr after gonadorelin treatment. As in the first experiment, bulls were treated in four cycles, and each cycle was initiated every 7 days. Bulls were assigned to treatment groups so that they were used only once for each dose.

Blood was collected via jugular venipuncture into syringes using 18 g needles that were 3.81 cm long. After collection, the blood was immediately placed in an ice-water bath and was held there until centrifugation, which was done within 6 hr after collection. Serum was harvested by centrifugation at 2000 × g for 15 min at 4°C. Serum samples were individually stored in 1-ml vials at −20°C until assayed. Testosterone concentrations were determined by a validated enzyme-linked immunosorbent assay (ELISA) (9). Data were analyzed by analysis of variance and linear regression (10).

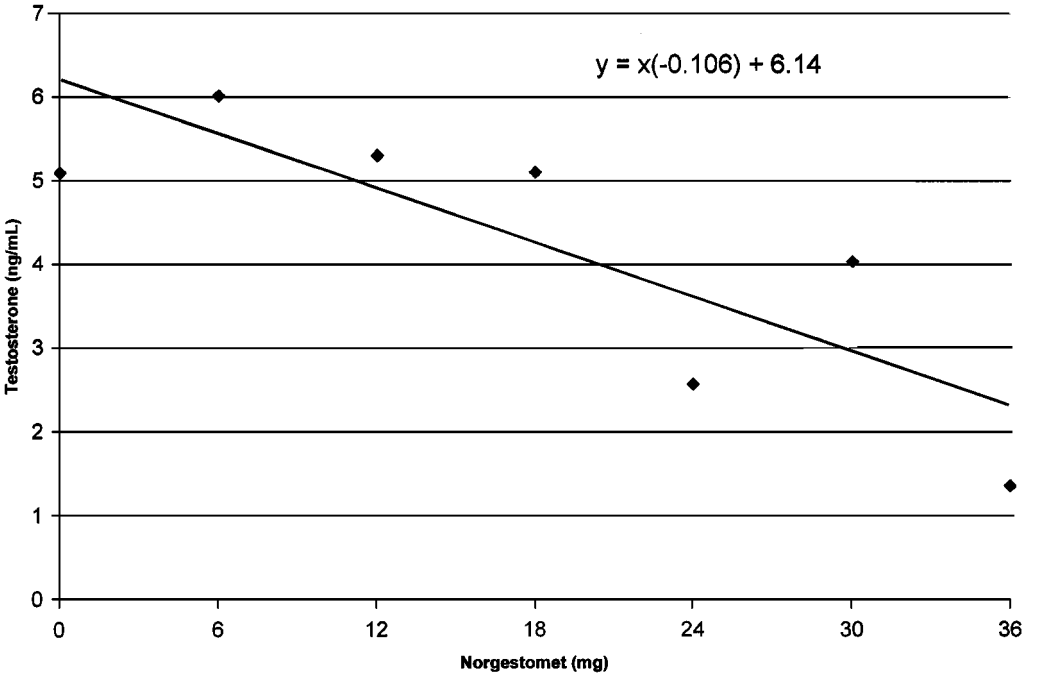

Figure 1. Mean testosterone concentrations (ng/ml) in bulls administered implants containing 0–36 mg of norgestomet. Mean testosterone concentrations were correlated ($r = -.82$) to dose of norgestomet administered.

RESULTS

Before implantation in experiment 1, testosterone concentrations averaged 5.09 ng/ml and were similar to concentrations previously reported for bulls of similar age (7,8,11,12). Because testosterone is released episodically, pretreatment concentrations were variable (standard deviation 3.21). Mean testosterone concentrations in bulls implanted with norgestomet implants decreased with increasing dose of norgestomet ($r = -.82$; $P < .05$) (Fig. 1). Variability in testosterone concentrations decreased with increasing dose of norgestomet ($r = -.81$; $P < .05$) and as mean testosterone concentrations decreased ($r = -.97$; $P < .01$). Figure 2 illustrates the episodic release of testosterone in bulls implanted with implants containing 6 or 36 mg of norgestomet. Although the magnitude of the testosterone concentrations was reduced, testosterone was still released episodically.

In experiment 2, testosterone concentrations in bulls administered implants containing 36 mg of norgestomet were similar to concentrations in experiment 1. Increasing the dose of gonadorelin to 5 μg increased testosterone release ($r = .99$; $P < .05$; Fig. 3). In this experiment, 1 μg of gonadorelin increased ($P < .05$) testosterone concentrations 2 hr later (compared to 0 μg), and 5 μg of gonadorelin induced a greater ($P < .05$) testosterone release than 0 or 1 μg of gonadorelin, but the release induced by 5 μg was similar ($P > .10$) to the releases induced by 10–40 μg. Based on these data, 5 μg (0.008 μg/kg body weight) to 40 μg of gonadorelin induced maximal testosterone releases. In norgestomet-treated bulls, 5 to 40 μg of gonadorelin increased testosterone by more than sixfold over basal concentrations.

DISCUSSION

Gonadorelin has been used with high consistency in effecting a cure for ovarian follicular cysts. All published studies that utilized 100 μg of the gonadorelin Cystorelin are included in Table 1, and they illustrate that efficacy of gonadorelin for the treatment of ovarian follicular cysts has not waned. The efficacy of gonadorelin since 1979 has been equal to or greater than the mean efficacy

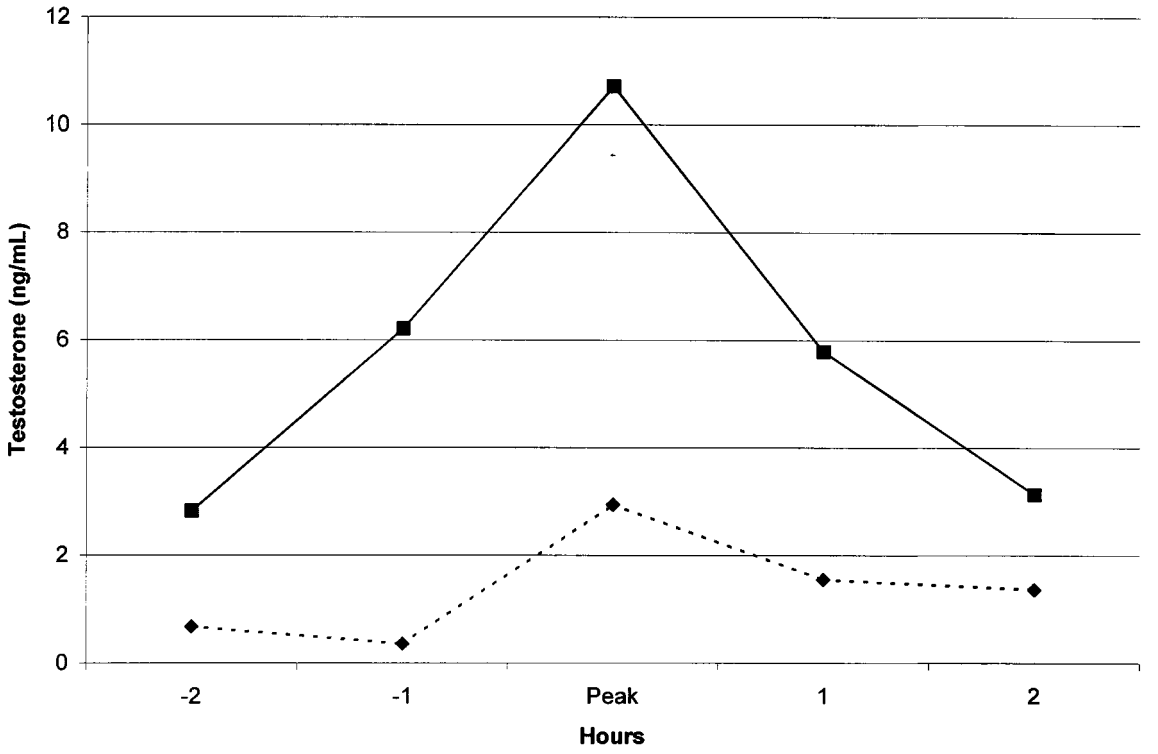

Figure 2. Mean testosterone concentrations (ng/ml) in bulls administered implants containing 6 mg (solid line) or 36 mg (broken line) of norgestomet. Testosterone concentrations are arranged around the peak (highest concentration during the 4-hr sampling period).

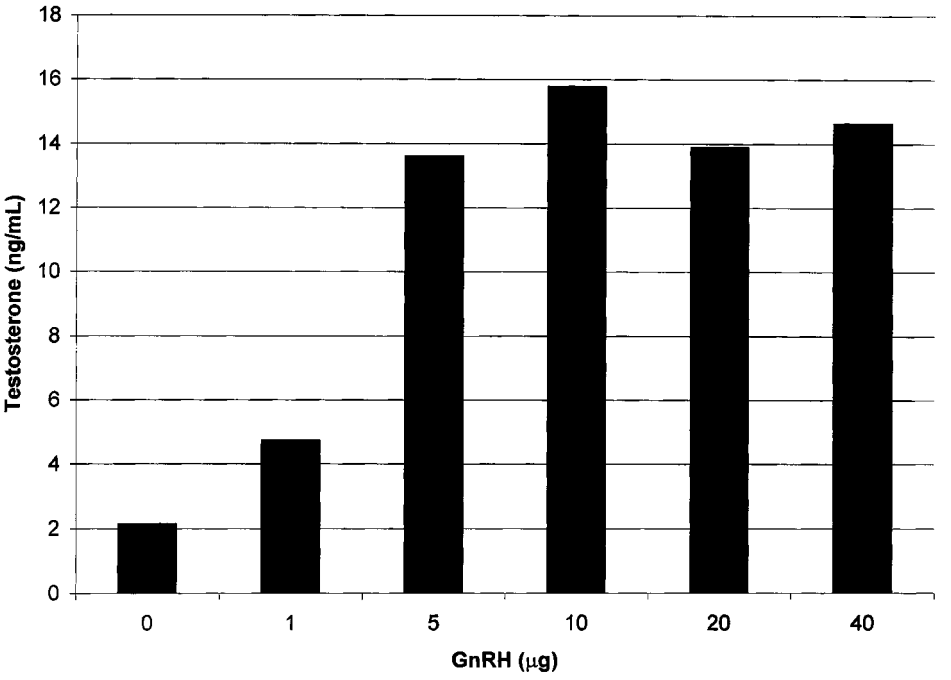

Figure 3. Mean testosterone concentrations (ng/ml) in bulls treated with norgestomet (36 mg) 2 hr after the administration of 0 to 40 μg gonadorelin.

(± 1 SD) for studies conducted before 1980. This degree of assurance must be maintained.

Numerous bioassays for gonadorelin exist as follows, but only the rabbit ovulation bioassay has been validated for quality assurance of gonadorelin.

- *Rabbit Ovulation*. This bioassay involves the administration of gonadorelin to female rabbits. The rabbits are euthanized and ovaries examined for evidence of ovulation 24 hr after administration via the marginal ear vein. The rabbit is an excellent biological model for the evaluation of gonadotropins and their releasing hormones (gonadorelin) because rabbits are induced ovulators (ovulation only occurs after copulation or gonadotropin stimulation). Studies in estrus have demonstrated that the ED_{100} (100% effective dose in inducing ovulation) is 0.5 μg (13), 1.0 μg (13,14), and 2.5 μg (15) (Table 2). The rabbit is a seasonal breeder and is suited for biological evaluation only when the rabbits are in estrus. Ovarian follicles capable of ovulating may not be present at certain times of the year, and LH or its releasing hormone (gonadorelin) will not induce ovulation. The variability observed by Humphrey et al. (15) may be due to seasonality and/or may be due to variability of the bioassay.
- *LH Release*. This involves the sampling of LH after gonadorelin treatment. Many species may be used for this bioassay; however, the species intended for veterinary product use is most appropriate. Since this is the mechanism of action for gonadorelin in effecting a cure in cows with ovarian cysts, it may appear to be a logical biological

Table 1

Response of Dairy Cows Treated with Gonadorelin (100 μg)

Reference No.	Cows with Positive Response/Cows Treated	Positive Response (%)
24	23/28	82
25	34/43	79
26	8/10	80
27	38/58	66
	14/20	70
	26/41	63
28	6/8	75
18	14/18	78
29	170/225	76
30	11/11	100
31	108/127	85
32	23/32	72
Combined	475/621	77

Table 2

Gonadorelin-Induced Ovulation in Rabbits

Dosage (μg)	Ref. 14	Ref. 15	Ref. 13
0	0%	0%	0%
0.1	0%	—[a]	33%
0.5	—[a]	—[a]	100%[b]
1.0	100%[c]	60%	100%
2.5	[a]	100%[d]	—[a]
5.0	—[a]	100%	—[a]
10.0	100%	100%	—[a]

[a] Dose was not included in the study.
[b] 0.176 μg/kg body weight.
[c] 0.352 μg/kg body weight.
[d] 0.880 μg/kg body weight.

evaluation (5). However, although LH is released in a dose-dependent manner in the cow, it is not highly sensitive (overlap exists between doses) (16,17). Furthermore, the quantity of LH released is not correlated to the efficacy of gonadorelin (18).

- *Progesterone Release*. This involves the sampling of progesterone after gonadorelin in heifers or cows with corpora lutea. Many species may also be used for this bioassay; however, again the species intended for veterinary product use is most appropriate. Although the gonadorelin-induced LH release stimulates a release of progesterone in the cow, the progesterone response is minimal (19). The minimal response does not allow sensitive biological evaluation for quality assurance purposes.
- *Bovine Ovulation*. This bioassay involves the administration of gonadorelin to heifers or cows 5 to 8 days after estrus, and ovulation is monitored via transrectal ultrasonography 2 days later. Ovulation response, however, is less than 100% and is reduced if administered on other days of the estrous cycle (19–21).
- *Hamster Ovulation*. This bioassay involves the administration of phenobarbital to golden hamsters exhibiting estrous cycles (15). The ovulation block induced by phenobarbital may be overcome by gonadorelin treatment. Although this bioassay is extremely sensitive, phenobarbital (a controlled substance) must be administered on the day of proestrus, followed by gonadorelin treatment 2 to 3 hr later. Ovulation is determined the next day on euthanasia of the hamsters.

Table 3

Gonadorelin-Induced Ovulation in Hamsters Administered Phenobarbital

Dosage (μg)	Ovulation Response (%)
0	0/20 (0%)
0.001	0/10 (0%)
0.005	6/10 (60%)
0.01[a]	6/6 (100%)
0.05	5/5 (100%)

From Ref. 15.
[a] 0.110 μg/kg body weight.

Results herein demonstrate that the gonadorelin-induced testosterone release may be used as a sensitive bioassay for gonadorelin. Pretreatment episodic variability of testosterone concentrations may be suppressed with norgestomet implants, as demonstrated with melengestrol acetate, another synthetic progestin (22). This is an important aspect because the variability reduces the efficacy of the assay. Based on a per-kg-body-weight basis, the gonadorelin-induced testosterone release was the most sensitive biological assay. The most sensitive laboratory animal bioassay was the hamster ovulation bioassay (0.110 μg per kg body weight; Table 3). Other advantages of this biological assay are that bulls are not affected by season, no barbituates are used, and bulls may be retained for production purposes after evaluation. Although the hamster ovulation bioassay is more sensitive on a per animal basis, it is a more time-consuming assay (2 days vs. less than 5 hr).

Furthermore, this is the first report demonstrating the high sensitivity of the bovine testis in releasing testosterone in response to gonadorelin (6–8,23). Without norgestomet treatment, most studies have used 40 μg or more of gonadorelin. Therefore, acute exogenous progestin therapy appears to reduce episodic release of testosterone, but does not abolish testosterone synthesis.

CONCLUSION

Gonadorelin-induced testosterone release is a sensitive and economical assay with rapid turnaround time for the determination of gonadorelin potency. This bioassay may be developed to ensure that non-biologically active material is not marketed, that consumers are receiving the dosage of gonadorelin that was established as efficacious for treating dairy cattle with ovarian follicular cysts, and that gonadorelin will continue to remain con-

sistent in effecting a cure in dairy cows with ovarian follicular cysts as it has for the past two decades.

REFERENCES

1. R. Guillemin, Peptides in the brain: the new endocrinology of the neuron, Science, 202, 390–402 (1978).
2. A. V. Schally, Aspects of hypothalamic regulation of the pituitary gland, Science, 202, 18–28 (1978).
3. D. J. Kesler, R. G. Elmore, E. M. Brown, and H. A. Garverick, Gonadotropin releasing hormone treatment of dairy cows with ovarian cysts. I. Gross ovarian morphology and endocrinology, Theriogenology, 16, 207–217 (1981).
4. D. J. Kesler and H. A. Garverick, Ovarian cysts in dairy cattle: a review, J. Anim. Sci., 55, 1147–1159 (1982).
5. D. J. Kesler, Therapeutic uses of gonadotropin-releasing hormone, Comp. Cont. Educ. Pract. Vet., 19(Suppl. 1), 1–9 (1997).
6. J. Zolman and E. M. Convey, GnRH: effect on serum FSH and androgens in bulls, J. Anim. Sci., 37, 334 [Abstr.] (1973).
7. K. Mongkonpunya, H. D. Hafs, E. M. Convey, H. A. Tucker, and W. D. Oxender, Serum luteinizing hormone, testosterone and androstenedione in pubertal and prepubertal bulls after gonadotropin releasing hormone, J. Anim. Sci., 40, 682–686 (1975).
8. T. D. Golter, J. J. Reeves, C. C. O'Mary, A. Arimura, and A. V. Schally, Serum LH levels in bulls treated with synthetic luteinizing hormone-releasing hormone/follicle stimulating hormone-releasing hormone (LH-RH/FSH-RH), J. Anim. Sci., 37, 123–127 (1973).
9. D. J. Kesler, H. Khazali, and R. J. Favero, Quantification of steroids via a polymer linked second antibody enzyme immunoassay system: methods of linking anti-rabbit IgG to polystyrene, in *Progress in Biomedical Polymers* (C. G. Gebelein and R. L. Dunn, eds.), Plenum, New York, pp. 157–170 (1990).
10. R. G. D. Steel and J. H. Torrie, *Principles and Procedures and Statistics*, McGraw-Hill, New York, 1960.
11. O. W. Smith, K. Mongkonpunya, H. D. Hafs, E. M. Convey, and W. D. Oxender, Blood serum testosterone after sexual preparation or ejaculation, or after injections of LH or prolactin in bulls, J. Anim. Sci., 37, 979–984 (1973).
12. N. E. Sitarz, R. E. Erb, T. G. Martin, and P. V. Malven, Effect of repeated sampling on concentrations of testosterone, LH and prolactin in blood of yearling Angus bulls, Theriogenology, 7, 151–158 (1977).
13. J. R. Reel, Luteinizing hormone-releasing hormone versus human chorionic gonadotropin: differential effect on the development of ovulatory refractoriness and antibodies, Fertil. Steril., 27, 59–64 (1976).
14. M. Amoss, R. Blackwell, and R. Guillemin, Stimulation of ovulation in the rabbit triggered by synthetic LRF, J. Clin. Endocrinol., 34, 434–436 (1972).
15. R. R. Humphrey, W. C. Dermody, H. O. Brink, F. G. Bousley, N. H. Schottin, R. Sakowski, J. W. Vaitkus, H. T. Veloso, and J. R. Reel, Induction of luteinizing hormone (LH) release and ovulation in rats, hamsters, and rabbits by synthetic luteinizing hormone-releasing factor (LRF), Endocrinology, 92, 1515–1526 (1973).
16. T. C. Cantley, H. A. Garverick, C. J. Bierschwal, C. E. Martin, and R. S. Youngquist, Hormonal responses of dairy cows with ovarian cysts to GnRH, J. Anim. Sci., 41, 1666–1673 (1975).
17. B. E. Seguin, E. M. Convey, and W. D. Oxender, Effect of gonadotropin-releasing hormone and human chorionic gonadotropin on cows with ovarian follicular cysts, Am. J. Vet. Res., 37, 153–157 (1976).
18. D. J. Kesler, H. A. Garverick, R. G. Elmore, R. S. Youngquist, and C. J. Bierschwal, Reproductive hormones associated with the ovarian cyst response to GnRH, Theriogenology, 12, 109–114 (1979).
19. B. E. Seguin, W. D. Oxender, and J. H. Britt, Effect of human chorionic gonadotropin and gonadotropin-releasing hormone on corpus luteum function and estrous cycle duration in dairy heifers, Am. J. Vet. Res., 38, 1153–1156 (1977).
20. J. R. Pursley, M. O. Mee, and M. C. Wiltbank, Synchronization of ovulation in dairy cows using $PGF_{2\alpha}$ and GnRH, Theriogenology, 44, 915–923 (1995).
21. J. L. M. Vasconcelos, R. W. Silcox, J. R. Pursley, and M. C. Wiltbank, Effect of day of estrous cycle on synchronization of ovulation using GnRH, J. Anim. Sci. 75(Suppl. 1), 89 (1997).
22. N. B. Haynes, T. E. Kiser, H. D. Hafs, and J. D. Marks, Prostaglandin $F_{2\alpha}$ overcomes blockade of episodic LH secretion with testosterone, melengestrol acetate or aspirin in bulls, Biol. Reprod., 17, 723–728 (1977).
23. D. J. Kesler and H. A. Garverick, Luteinizing hormone and testosterone concentrations in plasma of bull calves treated with gonadotropin releasing hormone, J. Dairy Sci., 60, 632–634 (1977).
24. C. J. Bierschwal, H. A. Garverick, C. E. Martin, R. S. Youngquist, T. C. Cantley, and M. D. Brown, Clinical response of dairy cows with ovarian cysts to GnRH, J. Anim. Sci., 41, 1660–1665 (1975).
25. R. G. Elmore, C. J. Bierschwal, R. S. Youngquist, T. C. Cantley, D. J. Kesler, and H. A. Garverick, Clinical response of dairy cows with ovarian cysts after treatment with 10,000 IU HCG or 100 mcg GnRH, Vet. Med./Sm. Anim. Clin., 70, 1346–1349 (1975).
26. H. A. Garverick, D. J. Kesler, T. C. Cantley, R. G. Elmore, R. S. Younquist, and C. J. Bierschwal, Hormone response of dairy cows with ovarian cysts after treatment with HCG or gonadotropin-releasing hormone, Theriogenology, 6, 413–425 (1976).

27. Abbott Laboratories, *Cystorelin*, Abbott Laboratories, Inc., North Chicago, IL, 1976.
28. D. J. Kesler, H. A. Garverick, A. B. Caudle, C. J. Bierschwal, R. G. Elmore, and R. S. Youngquist, Clinical and endocrine responses of dairy cows with ovarian cysts to GnRH and $PGF_{2\alpha}$, J. Anim. Sci., 46, 719–725 (1978).
29. H. L. Whitmore, J. P. Hurtgen, E. C. Mather, and B. E. Seguin, Clinical response of dairy cattle ovarian cysts to single or repeated treatments of gonadotropin-releasing hormone, J. Am. Vet. Med. Assoc., 174, 1113–1119 (1979).
30. L. A. Vasquez, H. L. Whitmore, B. O. Brodie, D. J. Kesler, and M. D. Brown, Comparison of GnRH treatment and manual rupture of ovarian cysts in dairy cattle, Proc. 10th Int. Cong. Anim. Reprod. AI, 476 (1984).
31. R. P. Dinsmore, M. E. White, C. L. Guard, D. J. Jasko, J. A. Perdrizet, P. M. Powers, and M. C. Smith, A randomized double blind clinical trial of two GnRH analogs for the treatment of cystic ovaries in diary cows, Cornell Vet., 77, 235–243 (1987).
32. R. P. Dinsmore, M. E. White, and P. B. English, An evaluation of simultaneous GnRH and cloprostenol treatment of dairy cattle with cystic ovaries, Can. Vet. J., 31, 280–284 (1990).