A bovine model for examining the effects of an aromatase inhibitor on ovarian function in women

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Objective: To test the hypothesis that treatment with an aromatase inhibitor (letrozole) will terminate dominant ovarian follicle growth and result in the emergence of a new follicular wave, regardless of the stage of follicular development at the time of treatment.

Design: Prospective study.

Setting: Academic research center.

Animal(s): Postpubertal beef heifers.

Intervention(s): Randomized trial involving treatment with letrozole on days 1–3, 3–5, 5–7 (day 0 = pretreatment ovulation) or no treatment.

Main Outcome Measure(s): Follicular development, corpus luteum (CL) development, hormone profiles, and plasma aromatase inhibitor concentration.

Result(s): Multiple doses of letrozole lengthened the period of follicular dominance, delayed emergence of the next follicular wave, and resulted in a larger CL regardless of the stage of the follicular wave in which treatments were initiated. No effects on circulating FSH concentrations were detected, but the stimulatory effects on the dominant follicle and CL were associated with increased plasma concentrations of LH in letrozole-treated animals. Plasma P concentrations were numerically higher throughout the luteal phase in letrozole-treated versus control heifers, but differences were not significant.

Conclusion(s): The results provide rationale for the hypothesis that the mechanism of action responsible for the stimulatory effect of aromatase inhibitors on ovarian function involves an elevation in circulating concentrations of LH rather than FSH. (Fertil Steril® 2011;96:434–8. ©2011 by American Society for Reproductive Medicine.)

Key Words: Aromatase, aromatase inhibitor, bovine model, corpus luteum, dominant follicle, estrogens, follicular dynamics, letrozole, ovary

Letrozole is a nonsteroidal inhibitor that inactivates the aromatase enzyme responsible for the synthesis of estrogens by reversibly binding to the heme group of this member of the cytochrome P450 enzymes (1). Aromatase inhibitors have been used as an adjuvant or first-line treatment for hormone-dependent breast cancer in postmenopausal women (2, 3). The effects of estrogen deprivation on reproductive function in premenopausal women have not been critically examined, but aromatase inhibitors have been used in assisted reproduction based on the notion of removing the negative feedback effects of E_2 on gonadotropin secretion. Theoretically, reduction of E_2 in the circulation will result in a surge in circulating FSH that will cause new follicular development and possibly the selection of more than one ovulatory follicle (4–6). Letrozole has been used in a single- or a 5-day regimen for ovarian stimulation (5, 7, 8) and in higher or increasing doses to induce ovarian superstimulation in women (9, 10).

Ethical and practical constraints limit observational and interventional studies in humans, thus a bovine model has been validated to investigate human ovarian function (11–16). Follicular wave recruitment was detected during both the follicular and luteal phases of the menstrual cycle and was associated with the same endocrine mechanisms as those described in cows and mares (11, 13–15, 17). The bovine model was chosen because of accessibility and suitability for frequent examination and sampling and because it has been useful for understanding several features of human reproduction including follicular dynamics (11, 13–15), menopause (18), and the effects of reproductive senescence on oocyte competence (19, 20).

The objective of the present study was to test the hypothesis that an extended period of aromatase inhibitor exposure will terminate dominant follicle growth and result in the emergence of a new follicular wave, regardless of the stage of follicular development at the time of treatment.

MATERIALS AND METHODS
Animals and Treatments

Hereford-cross heifers (nulliparous cattle) were chosen from a herd of 14- to 20-month-old animals, weighing between 233 and 404 kg and maintained at University of Saskatchewan Goodale Research Farm, based on an initial ultrasound examination (7.5 MHz linear-array transducer, Aloka SSD-900) to confirm the presence of a corpus luteum (CL), that is, postpubertal (21). To facilitate data collection, ovarian synchrony among heifers was induced by

transvaginal ultrasound-guided follicular ablation of the two largest ovarian follicles (22, 23). Three days after the expected emergence of the dominant follicle (i.e., 4 days after follicular ablation), heifers were given 500 μg of cloprostenol (PGF, Estrumate, Schering-Plough Animal Health) IM to induce regression of the CL and synchronize ovulation (24).

The experiment was performed in two replicates (n = 20–27 heifers per replicate), and each heifer was used only once. In replicate 1, heifers were assigned randomly at the time of ovulation (day 0) to the following groups and given a 3-day regimen of letrozole on days 1–3 (n = 5), days 3–5 (n = 5), days 5–7 (n = 5), or no treatment (control group, n = 5). In replicate 2, heifers were similarly assigned to groups and given letrozole from days 1 to 3, (n = 5), days 3 to 5 (n = 4), or days 5 to 7 (n = 4), but untreated control heifers were arranged in three subgroups to serve as contemporaneous controls for each aromatase inhibitor group (i.e., control days 1–3, n = 4; control days 3–5, n = 4; and control days 5–7, n = 5). For practical purposes, the total dose of letrozole (250 μg/kg) was calculated on the basis of an average weight of 350 kg for all heifers. Hence, a total dose of 87.5 mg was given to each heifer, divided into three daily doses (29.2 mg per day) and administered IV. Letrozole was prepared in 95% ethanol to a final concentration of 5 mg/mL, resulting in an injection volume of $\approx$6 mL/day.

Animal procedures were performed in accordance with the Canadian Council on Animal Care and were approved by the University of Saskatchewan Protocol Review Committee.

Ovarian Ultrasound

Ultrasound examinations were recorded on a sketch sheet in which each ovary and its structures (CL and follicles $\geq$4 mm in diameter) were represented by size and relative location (25, 26).

Ovulation was defined as the disappearance of a follicle $\geq$8 mm between consecutive daily examinations and was confirmed by the subsequent development of a CL (12). The dominant follicle of a wave was defined as the one that reached the largest diameter, and a subordinate follicle was defined as one that appeared to originate from the same follicular pool as the dominant follicle (25). Follicular wave emergence was defined retrospectively as the day when the follicle that was destined to become dominant was first identified at a diameter of 4 or 5 mm (27, 28). If the dominant follicle was not identified until it reached 6 or 7 mm, the previous day was considered the day of follicular wave emergence (29). The manifestation of selection of the dominant follicle of a wave was defined as the time of divergence in the growth profiles of the dominant versus subordinate follicles of the wave (27, 30). The day of onset of follicular and luteal regression was defined as the first day of an apparent progressive decrease in follicular and luteal diameters, respectively (27).

Collection of Blood Samples

Blood samples were collected daily from pretreatment ovulation to posttreatment ovulation by jugular or coccygeal venipuncture into 10 mL heparinized vacuum tubes (Vacutainer tubes; Becton Dickinson Vacutainer Systems). Additional samples were collected from aromatase inhibitor–treated heifers every 12 hours from the beginning of treatment to the next wave emergence. Blood samples were centrifuged at 1500 $\times$ g for 20 minutes, and plasma was separated and stored in plastic tubes at $-20°$C.

Hormone Assays

Please refer to the Supplemental Material for details on hormone assays.

Plasma Letrozole Concentration

Plasma concentrations of letrozole were determined from samples collected every 12 hours from pretreatment to 1 day after cessation of treatment using high-performance liquid chromatography tandem mass spectrometry (LC/MS/MS; Supplemental Material).

Statistical Analyses

Serial data were compared among groups by analysis of variance for repeated measures using the Mixed Procedures of SAS (Statistical Analysis System Institute Inc.) to determine the effects of treatment, time, and treatment-by-time interaction. Initial inspection of LH data revealed an apparent difference between morning (when letrozole treatment was applied) and afternoon values (12 hours after letrozole treatment), and therefore LH data were examined by analysis of variance for repeated measures to determine the main effects of treatment (during and after treatment), stage of follicular development (days 1–3, days 3–5, and days 5–7), time of the day (A.M. vs. P.M.), and day of treatment (first, second, and third dose). Since P.M. samples were not obtained from control animals, the control group was not included in the analysis. Single-point measurements were analyzed by analysis of variance. If no differences were detected among aromatase inhibitor–treated groups, data were combined and reanalyzed as a single aromatase inhibitor treatment group for comparison with nontreated controls. If significant main effects or interactions ($P \leq .05$) were detected, Tukey's post hoc test was used for multiple comparisons.

Due to individual variability in circulating hormone concentrations, and because our objective was to determine the effect of treatment within individuals, LH and FSH concentrations were transformed to a percentage of the mean concentration of the two first data points collected (days 0 and 1, day 0 = treatment) for each individual heifer before statistical analysis. For the same reasons, E_2 concentrations were transformed to a percentage of the mean concentration of two pretreatment data points (i.e., for each individual heifer) before statistical analysis. Plasma E_2 concentrations for each treatment group were compared independently with the respective control subgroup for each treatment period (data from replicate 2).

RESULTS
Circulating Concentrations of Letrozole

Plasma letrozole concentrations in all three treatment groups were elevated by 12 hours after initiation of treatment and reached a maximum at 60 hours, followed by a decline to approximately half peak values by 108 hours (time effect, $P < .001$). Heifers treated from days 1 to 3 had greater circulating letrozole concentrations than those treated from days 3 to 5, while those treated from days 5 to 7 were intermediate (overall means, 68.6 $\pm$ 4.79, 43.9 $\pm$ 4.75, and 55.6 $\pm$ 4.75 ng/mL, respectively; $P < .01$).

Ovarian Follicles and E_2

The diameter profiles of the extant dominant follicle (i.e., the dominant follicle present at the time of treatment) were similar among aromatase inhibitor treatment groups; hence, data were combined for comparison with controls. Compared with controls, the dominant follicle grew to a greater diameter ($P < .01$) and the interwave interval was longer ($P < .05$) in letrozole-treated heifers (Fig. 1; Table 1). However, the interovulatory interval did not differ between groups. The diameter profile of the largest subordinate follicle in heifers treated with letrozole from days 1 to 3 was larger than that of control heifers ($P < .05$; Fig. 2). The diameter profile of the largest subordinate follicle in heifers treated with letrozole from days 3 to 5 was not different from that of the control group (Fig. 2). As expected, the largest subordinate follicle had already regressed in heifers treated from day 5 to 7, and therefore no data were available for analysis.

No differences were detected in plasma E_2 concentration between letrozole-treated and control heifers (no main effects of treatment or time no an interaction).

CL and Plasma P

No differences in CL diameter or P concentration were detected among letrozole-treated groups; hence, data for the three treatment groups were combined for comparison with untreated controls. The day-to-day CL diameter profile of heifers treated with letrozole was larger than that of controls ($P < .004$; Supplemental Fig. 1). Differences in plasma P concentrations were not significant, despite that

Effects (mean ± SEM) of a 3-day regimen of letrozole given at three different stages of the follicular wave in heifers on the intervals between new wave emergence and ovulation.

	Letrozole treatment			
	Days 1–3 (n = 10)	Days 3–5 (n = 9)	Days 5–7 (n = 9)	Control (n = 17)
Interwave interval, days	8.9 ± 0.35[a]	10.0 ± 0.37[a,b]	10.7 ± 0.37[b]	7.6 ± 0.27[c]
Interovulatory interval, days	20.9 ± 0.46[a]	20.1 ± 0.49[a]	21.2 ± 0.49[a]	20.5 ± 0.35[a]
Dominant follicle diameter at treatment, mm*	6.8 ± 0.36[a]	10.1 ± 0.45[b,c]	12.1 ± 0.43[d]	6.5 ± 0.27 (day 1),[a] 9.4 ± 0.32 (day 3),[b] 11.2 ± 0.30 (day 5)[c,d]

[a,b,c,d] Within rows, values with no common superscript are different ($P < .05$).

* Compared with respective controls at the same stage of follicular development.

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values were numerically higher throughout the sampling period in the letrozole-treated groups than in the control group (Supplemental Fig. 1).

Gonadotropins

No differences were detected in plasma FSH concentration between letrozole-treated heifers compared with their respective controls, nor were there any treatment-by-time interactions (Supplemental Fig. 2). No differences in plasma LH concentrations were detected among groups using daily samples ($P = .78$). The overall model revealed no significant effect or interaction involving follicular stage; hence, the effect of follicular stage was removed from the model before further analysis. Interactions between day (first, second, and third) and time (A.M. vs. P.M.) and between treatment period (during vs. after treatment) and time (A.M. vs. P.M.) were significant. Concentrations of LH were significantly greater in the P.M. samples (12 hours after letrozole treatment) collected during the period of treatment (188% ± 28.5%) than in the A.M. samples (24 hours after letrozole treatment) collected during the period of treatment (94% ± 14.9%) or in A.M. and P.M. samples collected after cessation of treatment (79% ± 14.6% and 79% ± 27.9%, respectively; Fig. 3).

DISCUSSION

We hypothesized that a 3-day treatment regimen of aromatase inhibitor would, as a consequence of decreasing E_2 production, induce a surge in circulating FSH and hasten the emergence of a new follicular wave in cattle, regardless of the stage of development of the dominant follicle at the time of treatment. However, results of the present study do not support this hypothesis. On the contrary, letrozole treatment caused the extant dominant follicle to grow larger and prolonged its period of dominance, extending the interval to emergence of the next follicular wave regardless of whether treatment was initiated before, during, or after selection of the dominant follicle. In women, treatment with letrozole from days 3 to 7 after the beginning of menses was reported to cause emergence of a new wave of follicular development shortly after the initiation of treatment (5). The mechanism responsible for this effect was hypothesized to involve removal of the negative feedback effect of E_2 on pituitary FSH secretion, resulting in an endogenous surge in plasma FSH which, in turn, causes recruitment of a new cohort of growing follicles (4–6). However, the present study challenges this proposed mechanism of action.

The results of the present study are consistent with those previously reported in which treatment with single intravenous dose of

letrozole on day 3 postovulation in heifers did not induce follicular atresia or hasten emergence of a new follicular wave (35). Surprisingly, letrozole treatment did not induce an elevation in circulating FSH concentrations, despite the fact that circulating concentrations of E_2 were affected. Rather, letrozole treatment increased mean plasma LH concentrations and resulted in a prolonged period of dominance of the extant dominant follicle and delayed emergence of the next follicular wave (35).

Continued growth of the dominant follicle and CL in aromatase inhibitor–treated heifers in the present study was attributed to the effect of increased circulating concentrations of LH (36). A biphasic pattern of LH secretion was observed in the three aromatase inhibitor–treated groups as a result of an elevation in LH concentration by 12 hours after treatment followed by a decline to baseline within 24 hours. That the evening increase in circulating LH concentration observed in the present study was not a result of natural diurnal variation (37–39) was verified by the observation that the effect was seen only during the period of letrozole treatment and not after.
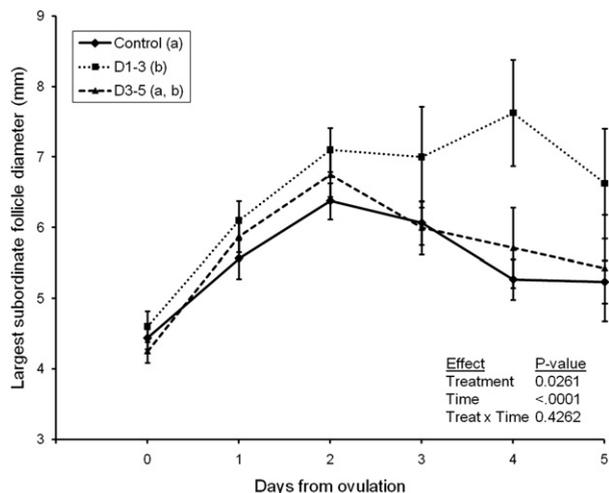
Diameter profile (mean ± SEM) of the dominant follicle in heifers treated with letrozole on days 1–3, 3–5, or 5–7 (day 0 = ovulation; treatment groups combined, n = 28) compared with untreated controls (n = 17).
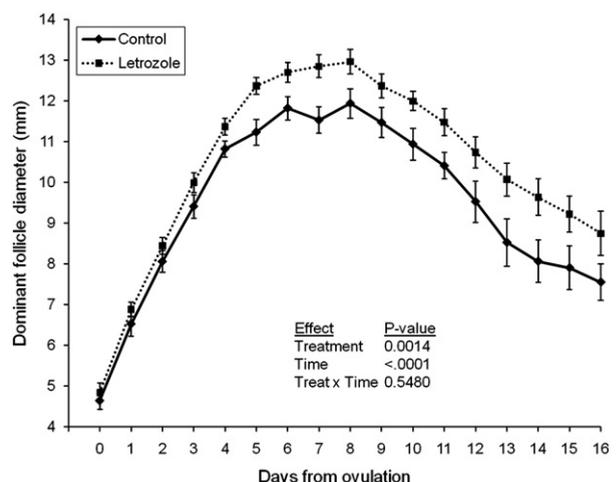
Yapura. Effects of AI on ovarian dynamics in cattle. Fertil Steril 2011.

Diameter profile (mean ± SEM) of the largest subordinate follicle in heifers treated with letrozole on days 1–3 (n = 10) or days 3–5 (n = 9), compared with untreated controls (n = 17). Heifers treated with letrozole from days 5 to 7 were not included in the analysis since the largest subordinate follicle was no longer detectable at initiation of treatment on day 5. [a,b,c]Overall means for treatment groups with no common superscript were different (P < .05).

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Effect of letrozole on plasma LH concentrations (expressed as a percent of the mean of days 0 and 1 postovulation; mean ± SEM) in heifers treated in the morning for 3 consecutive days (aromatase inhibitor groups combined, n = 28) during the 3 days of treatment and during the 3 days after treatment.

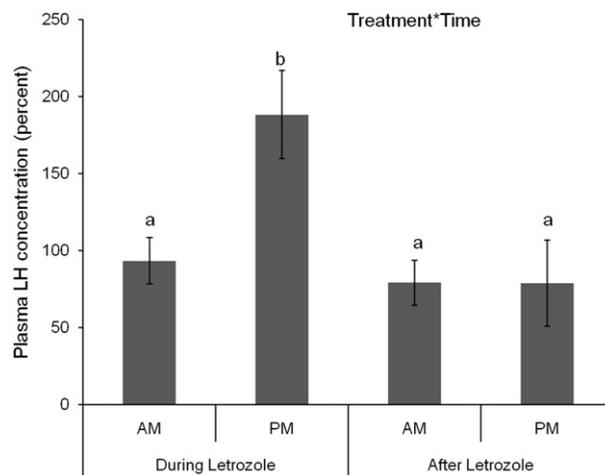Yapura. Effects of AI on ovarian dynamics in cattle. Fertil Steril 2011.

The largest subordinate follicle in heifers treated with letrozole from days 1 to 3 after ovulation grew larger and for a longer period of time compared with controls. This observation resembled an FSH-dependent superstimulatory effect (27, 40); however, it was not associated with an increase in circulating concentrations of FSH. Elevated LH may have been responsible for the continued growth of subordinate follicles during treatment on days 1–3. As the switch from FSH to LH responsiveness is not a all-or-nothing event, it is plausible that the growing subordinate follicles become responsive to LH before selection is complete (e.g., day 2 of the follicular wave) (30, 41).

Higher circulating letrozole concentrations in heifers treated from days 1 to 3 compared with those treated from days 3 to 5 may be attributed to a greater proportion of binding to aromatase in the latter. Follicular E_2 production, and hence aromatase activity, is low during the first few days after follicular wave emergence but increases to maximum levels between days 3 and 5 (42).

The bovine model used in the present study served as a tool to better understand the effect of aromatase inhibitors on ovarian function in women. Results using the bovine model are consistent with observations reported in a prospective study conducted in premenopausal women (43) in which letrozole treatment on days 5–7 after menses was associated with elevated LH concentrations and mild ovarian superstimulation. Results are also consistent with those of a preliminary report in which treatment of premenopausal women with a single dose of letrozole at different stages of follicle development failed to recruit a new follicular wave (44). Results of the present study, taken together with those involving treatment of premenopausal women, provide a rationale for the hypothesis that the mechanism of action responsible for the stimulatory effect of aromatase inhibitors on ovarian function involves an elevation in circulating concentrations of LH rather than FSH. A test of this hypothesis and exploration of treatment possibilities using aromatase inhibitors deserves further attention, including verification of dose-related effects.

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SUPPLEMENTAL MATERIAL

Hormone Assays

Plasma LH concentrations were determined in duplicate using a double-antibody radioimmunoassay (NIDDK-bLH4) (31, 32). The minimum and maximum values along the standard curve were 0.06 and 8 ng/mL, respectively. The intra- and interassay coefficients of variation were 7.9% and 2.5%, respectively, for low reference samples (mean, 0.85 ng/mL) and 8.6% and 9.5%, respectively, for high reference samples (mean, 2.5 ng/mL).

Plasma FSH concentrations were determined in duplicate using a double-antibody radioimmunoassay using NIDDK-anti-oFSH-1 primary antibody and expressed as United States Department of Agriculture bovine FSH-Il units (31, 32). The minimum and maximum values along the standard curve were 0.12 and 16 ng/mL, respectively. The intra- and interassay coefficients of variation were 11.1% and 11.2%, respectively, for low reference samples (mean, 1.9 ng/mL) and 5.2% and 4.1%, respectively, for high reference samples (mean, 4.0 ng/mL).

Plasma E_2 concentrations were determined in duplicate by enzyme-linked immunosorbent assay (Cayman Chemical Company). The minimum and maximum values along the standard curve were 6.6 and 4,000 pg/well, respectively. The intra- and interassay coefficients of variation for reference samples (100 pg/mL) assayed in duplicate were 11.7% and 12.7%, respectively. A concentration procedure using diethyl ether extraction was performed before the assay in all samples to increase estrogen concentration to measurable levels (33). A ^{3}H-labeled steroid was added to each plasma sample before extraction as an internal recovery standard (34).

Plasma P concentrations were determined in duplicate using a commercial solid-phase kit (Coat-A-Count; Diagnostic Products Corporation). The range of the standard curve was 0.1–40.0 ng/mL. The intra- and interassay coefficients of variation for samples assayed in duplicates were 10.1% and 15%, respectively, for low reference samples (mean, 1.8 ng/mL) and 5.5% and 7.7%, respectively, for high reference samples (mean, 17.5 ng/mL).

Plasma Letrozole Concentration

Plasma concentrations of letrozole were determined using high-performance liquid chromatography tandem mass spectrometry (LC/MS/MS). Letrozole was extracted from 250 μL of plasma with 250 μL of 0.1 M ammonium acetate followed by the addition of 5 mL of methyl t-butyl ether and vortexed for 15 seconds. The organic layer was removed and transferred to a fresh 15-mL plastic tube and dried by gentle nitrogen gas flow. The dried extract was reconstituted in 1 mL of 100% ethanol, sonicated for 5 minutes, and transferred to a labeled vial for further analysis. Separation was accomplished by HPLC (Agilent 1200) fitted with an analytical column (50 × 2.1 mm, 3 μm particle size; Thermo Scientific Betasil C18) operated at 35°C. Gradient conditions were used at a flow rate of 250 μL/minute, starting at 85% A (0.1% acetic acid) and 15% B (0.1% acetic acid in acetonitrile). Initial conditions were held for 2 minutes and then ramped to 100% B at 6 minutes, held until 9 minutes, decreased to 0% B at 11 minutes, returned to initial conditions at 13 minutes, and held constant until 15 minutes. Mass spectra were collected using a tandem mass spectrometer (Applied Bioscience SCIEX 3000) fitted with an electrospray ionization source that was operated in the negative ionization mode. Chromatograms were recorded using multiple reaction monitoring mode, where at least two transitions per analyte were monitored. The following instrument parameters were used: desolvation temperature 450°C, desolvation (curtain) gas 6.0 arbitrary units (AU), nebulizer gas flow 4 AU, ion spray voltage 4,500 V, collision gas 12 AU, collision energy 46 AU, declustering potential 30 AU, and a dwell time of 100 ms. Quantification using these transitions was performed using Analyst 1.4.1 software provided by SCIEX (Applied Bioscience). The minimum and maximum values along the standard curve were 0.25 and 500 μg/mL, respectively. The limit of quantification used in this method was 250 ng/mL, and the mean recovery was 70%.
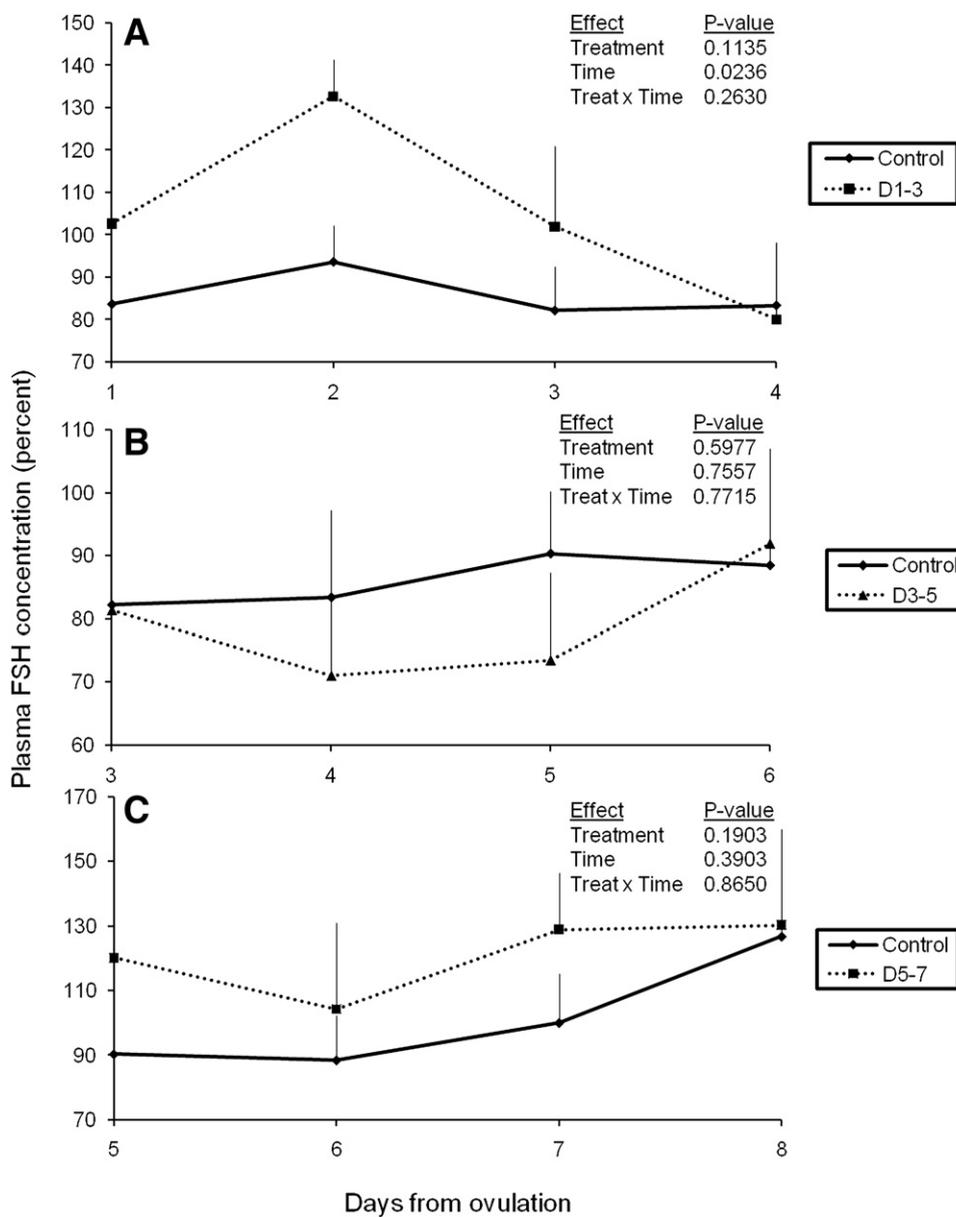
(**A**) Diameter profile of the corpus luteum and (**B**) plasma progesterone concentration (mean $\pm$ SEM) in heifers treated with letrozole (85 μg/kg/day for 3 days; data from treatment periods of Days 1 to 3, 3 to 5, and 5 to 7 combined; n = 18), compared to untreated controls (n = 17).
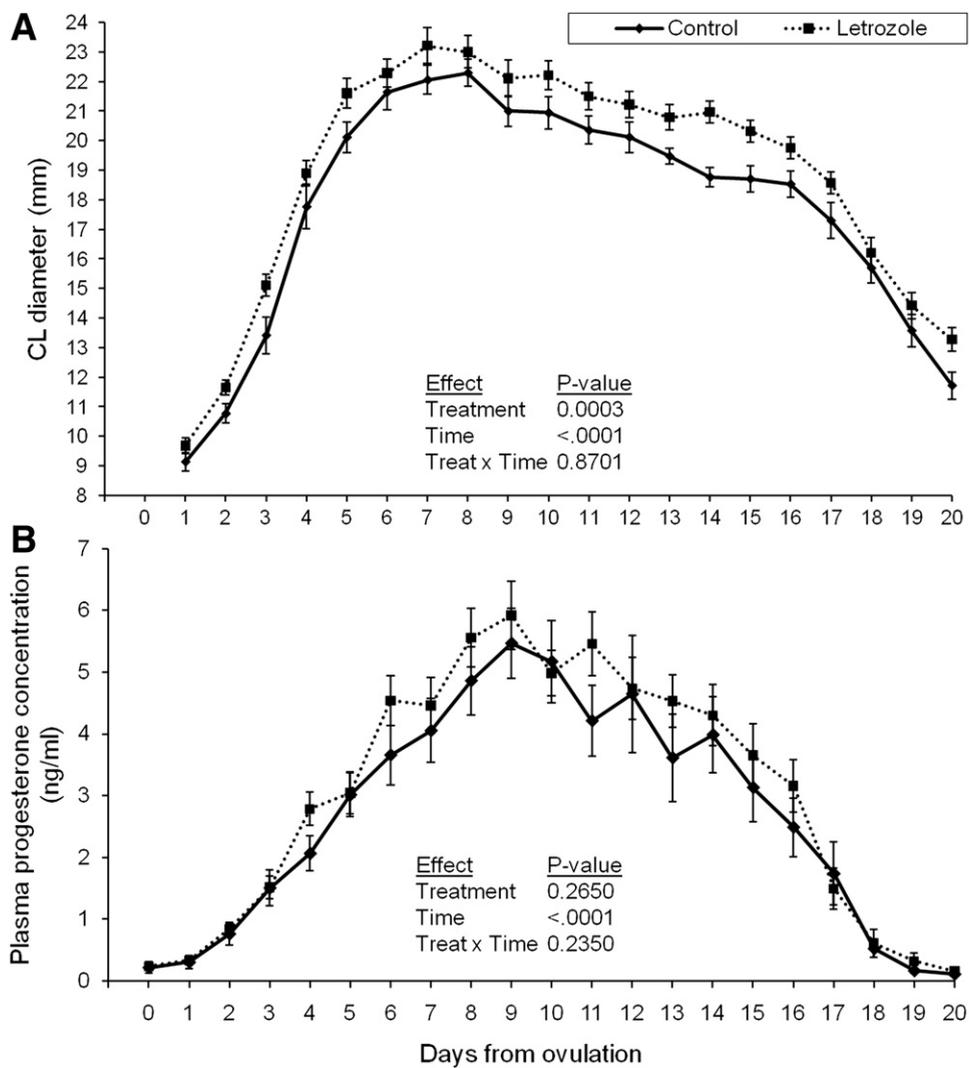
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Plasma FSH concentration, expressed as a percent of the mean of Days 0 and 1 (mean ± SEM), in heifers treated with letrozole on Days 1 to 3 (n = 10), Days 3 to 5 (n = 9), or Days 5 to 7 (n = 9; Day 0 = ovulation), compared to untreated controls (n = 17).

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