Follicular cysts occur after a normal estradiol-induced GnRH/LH surge if the corpus hemorrhagicum is removed

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Abstract

The pathophysiology underlying follicular cysts appears to be lack of an estradiol (E_2)-induced GnRH/LH surge due to hypothalamic insensitivity to E_2 . In addition, progesterone (P_4) can cause animals with follicular cysts to resume normal cyclicity and normal hypothalamic responsiveness to E_2 . We postulated that follicular cysts may be a pathological manifestation of a physiological state that cows, and possibly other species, go through during the normal estrous cycle but the rise in P_4 following ovulation induces them back to normal hypothalamic responsiveness to E_2 . Based on this hypothesis, we expected that removal of the ovary containing the corpus hemorrhagicum would prevent the normal rise in P_4 following ovulation and induce development of follicular cysts. Cows ($n = 24$) on day 7 of the estrous cycle were treated with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and time of ovulation was detected by ovarian ultrasonography every 8 h. Immediately following detection of ovulation, cows were randomly but unequally assigned to have the ovary containing the corpus hemorrhagicum removed (TRT; $n = 16$) or the ovary opposite to the corpus hemorrhagicum removed (CON; $n = 8$). Cows were subsequently evaluated by daily ultrasound and blood sampling to determine follicular dynamics. Ovulation was detected at 93.7 ± 4.5 h after $PGF_{2\alpha}$ injection. All CON cows had a normal estrous cycle length (22.0 ± 0.6 days) after ovariectomy (OVX). Half of the TRT cows became anovular (TRT-ANO; $n = 8$) after OVX with large anovular follicles developing on the ovary (maximal size, 25.4 ± 1.4 mm; range, 20–32 mm). However, eight TRT cows ovulated (TRT-OV; $n = 8$) 7.3 $\pm$ 0.6 days after OVX. Control cows had a normal P_4 rise after ovulation. Removal of the newly formed corpus hemorrhagicum prevented the rise in circulating serum P_4 in TRT-ANO cows throughout the 25-day experimental period. The TRT-OV cows had a delayed increase in circulating P_4 but it was normal in relation to time of ovulation. Serum E_2 concentrations were similar among groups (TRT-OV, TRT-ANO and CON cows) until 7 days after OVX. Serum E_2 concentrations then decreased in TRT-OV and CON cows but remained elevated (>5 pg/ml) in TRT-ANO cows. Thus, the endogenous increase in circulating E_2 that induces the GnRH/LH surge and estrus is sufficient to induce cows into a physiological state that resembles follicular cysts if it is not followed by increased circulating P_4 .

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Introduction

Follicular cysts have been reported in many mammalian species, including cattle (Roberts 1971, Kesler & Garverick 1982), swine (Heinonen *et al.* 1998), dogs (Arbeiter 1993), rabbits (Lopez-Bejar *et al.* 1998), rats (Brawer *et al.* 1986), mares (McCue & Squires 2002), sheep (Christman *et al.* 2000), elephants (Brown *et al.* 1999) and women (Stein & Leventhal 1935). In cattle, this condition is characterized by large (generally ≥ 25 mm in diameter) anovular structures that persist on the ovary for at least 10 days in the absence of a corpus luteum (CL) (Roberts 1971). In earlier studies, this condition was detected with rectal palpation and was estimated to occur in 6–19% of dairy cows (Kesler & Garverick 1982, Garverick 1997). In recent

studies the incidence of anovulation (detected with either blood samples or ultrasonography) in dairy cattle ranged from 18 to 29% (Cartmill *et al.* 2001, Pursley *et al.* 2001, Gümen *et al.* 2003). Follicular cysts can cause economic loss in dairy operations (Youngquist 1986).

The pathophysiology of follicular cysts in any species is incompletely defined. Development of an appropriate model is essential for studies on the underlying physiology of follicular cysts. Several different methods have been used to induce animals into an anovular condition resembling follicular cysts including estradiol (E_2) treatment during diestrus (Wiltbank *et al.* 1961, Nadaraja & Hansel 1976), multiple treatments with high doses of progesterone (P_4) and E_2 (Erb *et al.* 1973, Cook *et al.* 1990, 1991,

Hamilton *et al.* 1995, Calder *et al.* 1999) and prolonged E_2 exposure (Dobson *et al.* 1996, Öztürk *et al.* 1998). Follicular cysts can also be induced with a single E_2 injection if E_2 is given at a time when the gonadotropin-releasing hormone (GnRH)/luteinizing hormone (LH) surge will occur in the absence of a potentially ovulatory follicle (Gümen & Wiltbank 2002, Gümen *et al.* 2002). Conversely, cows with either induced or naturally occurring follicular cysts return to cyclicity after treatment with P_4 (Calder *et al.* 1999, Gümen & Wiltbank 2002, 2005). Thus, the pathophysiology underlying follicular cysts appears to involve an unresponsiveness of the hypothalamus to increasing E_2 due to elevated E_2 inducing a GnRH/LH surge that is not followed by an increase in P_4 . Based on this model we postulated that follicular cysts may be a pathological manifestation of a physiological state that cows, and possibly other species, go through during the normal estrous cycle. However, the results providing support for this model are based on studies using treatments with high doses of exogenous E_2 . This study was designed to determine whether the normal E_2 rise leading to estrus, the GnRH/LH surge and ovulation can produce a similar E_2 -unresponsive condition if there was no subsequent exposure to luteal-phase P_4 concentrations. Specifically, we hypothesized that if the ovary containing the corpus hemorrhagicum was removed soon after ovulation (after natural E_2 -induced GnRH/LH surge but prior to increased circulating P_4), the cow would be left in a physiological condition similar to follicular cysts (i.e. lack of positive feedback by elevated E_2 , no GnRH/LH surge and development of large anovular follicles).

Materials and Methods

Animals and experimental design

The experiment was conducted at the University of Wisconsin-Madison Dairy Research Center. Lactating dairy cows ($n = 24$) were housed in individual stalls. Cows were fed twice daily with a high-energy lactating dairy cow ration fed as a total mixed ration following National Research Council (2001) recommendations. Cows at unknown stages of the estrous cycle were treated with 100 µg GnRH i.m. (Cystorelin; Merial Inc., Iselin, NJ, USA) to induce ovulation. All cows were treated with 25 mg prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) i.m. (Lutalyse; Pfizer Animal Health, Groton, CT, USA) 7 days after GnRH to regress functional CL and allow a new ovulation.

Transrectal ultrasound evaluation to monitor ovarian structures was performed with an ultrasound scanner (Aloka 500V; Corometrics Medical Systems Inc., Wallingford, CT, USA) equipped with a 7.5 MHz linear-array transducer. Measurements were made on a single frozen image of the apparent maximal area of each follicle, using the average diameter in two directions at right angles. Ovaries from cows were evaluated by ultrasonography daily from day 0 (GnRH injection) to day 9. In order

to detect ovulation, ovaries were evaluated by ultrasonography every 8 h, starting from day 9, until ovulation. Ovulation was determined by ultrasound scanning (disappearance of a large follicle followed by appearance of a corresponding CL). Follicular deviation was defined as occurring during the retrospectively identified ultrasound examination in which the beginning of the greatest difference in growth rates between the largest follicle and the second-largest follicle was identified. Co-dominant follicles were defined as two follicles of 10 mm in diameter that had not yet undergone diameter deviation in the first follicular wave after ovariectomy (OVX).

Unilateral OVX was performed using the colpotomy technique described by Drost *et al.* (1992). After ovulation was detected, each cow was placed in a chute. Following feces removal from the rectum, the vulva was washed with 10% povidone-iodine (Betadine antiseptic solution; The Purdue Frederick Co., Stamford, CT, USA). After collection of a blood sample from a tail vein, an i.v. injection of 5 mg acepromazine maleate (Phoenix Pharmaceutical Inc., St Joseph, MO, USA) and 10 mg xylazine (Xyla-ject; Phoenix Pharmaceutical Inc.) was given to tranquilize the cows. Tail hair was trimmed with clippers and epidural anesthesia was then administered using 4 ml of 2% lidocaine hydrochloride (Lidocaine Hydrochloride Injectable-2%; Phoenix Pharmaceutical Inc.). The vagina was scrubbed with 10% povidone-iodine and hemostatic forceps were introduced into the vagina in order to make the initial vaginal incision. The incision was enlarged by forceful introduction of individual fingers and ultimately, the entire hand directly into the peritoneal cavity. An ecraseur (Jorgensen Laboratories Inc., Loveland, CO, USA) was introduced and one ovary was removed. Cows were randomly but unequally assigned to two treatment groups. The ovary containing the newly formed corpus hemorrhagicum was removed in the treatment group (TRT; $n = 16$), whereas the control group (CON; $n = 8$) had the ovary that did not contain the corpus hemorrhagicum removed. The remaining ovary was evaluated by daily ultrasound and blood samples were taken every day for the next 25 days or until the next ovulation. All animal handling and care procedures were approved by the Research Animal Resources Center of University of Wisconsin-Madison.

Blood samples and hormone assays

Blood samples for P_4 , E_2 , cortisol and follicle-stimulating hormone (FSH) analyses were collected daily from day 0 to day 9, and then every 8 h until ovulation, from the coccygeal vein into Vacutainer tubes (Becton Dickinson Co., Franklin Lakes, NJ, USA). Blood samples also were collected daily after surgery until next ovulation as determined by ultrasonography or until 25 days after surgery. Blood was allowed to clot at 4°C for 24 h and centrifuged at 1500 g for 15 min. Serum was collected and stored at –20°C until further analysis.

Serum concentrations of FSH were determined using a RIA validated for use in cattle (Bolt & Rollins 1983, Bolt *et al.* 1990). The FSH assay incorporated bovine FSH (USDA-bFSH-I-2) for iodination and reference standards and NIDDK-anti-o-FSH-I-2 as the primary antiserum. Hormone sensitivity, calculated as two standard deviations below the mean c.p.m. at maximum binding, was 0.05 ng/ml (mean of two different assays). Interassay and intraassay coefficients of variation for FSH were 4.1 and 7.8% respectively using pooled plasma from cows near diestrus and estrus.

Serum concentrations of P₄ were determined by a solid-phase RIA kit containing antibody-coated tubes and ¹²⁵I-labelled progesterone (Coat-A-Count; Diagnostic Products Corporation, Los Angeles, CA, USA) as described previously (Gümen & Wiltbank 2005). Hormone sensitivity, calculated as two standard deviations below the mean c.p.m. at maximum binding, was 0.03 ng/ml (mean of six different assays). Interassay and intraassay coefficients of variation for P₄ were 5.8 and 6.5% respectively using a quality control sample with 2.0 ng/ml, and 5.5 and 6.1% respectively using a quality control sample with 10 ng/ml P₄.

Serum concentrations of E₂ were measured using modifications to a commercially available E₂ RIA kit (Third Generation Oestradiol Assay Kit; Diagnostics Systems Laboratories Inc., Webster, TX, USA) previously validated for use in cattle (Kulick *et al.* 1999). Hormone sensitivity, calculated as two standard deviations below the mean c.p.m. at maximum binding, was 0.19 pg/ml (mean of two different assays). A quality control sample was prepared from charcoal-stripped serum containing a known concentration of E₂. The quality control sample was evaluated multiple times in each assay and interassay and intraassay coefficients of variation for E₂ were 3.2 and 8.6% respectively.

Serum concentrations of cortisol were determined by a competitive ELISA that was previously validated in our laboratory (Reinemann *et al.* 2003). Hormone sensitivity, calculated as two standard deviations below the mean optical density (OD) of maximal binding wells, was 0.06 ng/ml (mean of five different assays). A quality control sample was prepared from charcoal-stripped serum containing a known concentration of cortisol. The quality control sample was evaluated multiple times in each assay and interassay and intraassay coefficients of variation for cortisol were 2.3 and 4.7% respectively.

Statistical analyses

Analyses of data (serum P₄, E₂, cortisol and FSH) were conducted using the Mixed Procedure of SAS with repeated measures in time (SAS 1999). The model was:

$$Y_{ijk} = \mu + \text{trt}_i + \text{cow}_j(\text{trt}_i) + \text{time}_k + \text{trt} * \text{time}_k + e_{ijk}$$

where: Y_{ijk} = dependent observations; μ = overall mean; trt_i = groups (i = anovular, ovular or control cows after

OVX (fixed effect)); $\text{cow}_j(\text{trt}_i)$ = cows (j = 1, 2, 3, 4, ..., 24 (random effect)); time_k = days (k = 0, 1, 2, 3, ..., 25 (fixed effect)); $\text{trt} * \text{time}_k$ = interaction of groups and time (fixed effect); e_{ijk} = residual (random effect). To account for correlated errors due to repeated measures on the same experimental unit, $\text{cow}_j(\text{trt}_i)$, the Autoregressive (1) covariance error structure was used (Littell *et al.* 1998). If the treatment was significant in the model, differences between treatments were determined using the PDIFF option (SAS 1999). Student's *t*-test was used to compare hours to ovulation after prostaglandin injection and time to deviation after OVX between treatment and control cows. Student's *t*-test was also used to compare follicle diameter at different stages of the experimental period (ovulatory follicle size, largest follicle after OVX). The data with a binomial distribution were analyzed by Fisher's Exact test. All data are presented as means $\pm$ S.E.M. or proportions. *P* values less than or equal to 0.05 were considered to be significant, and trends were discussed for *P* values between 0.05 and 0.1.

Results

Serum P₄ concentration was high in all cows at the time of PGF_{2 α} injection (3.3 ± 0.3 ng/ml). P₄ concentration decreased to basal level (0.05 ± 0.001 ng/ml) 3 days after PGF_{2 α} in all cows. Ovulation was detected in all cows at an average of 93.7 ± 4.5 h after PGF_{2 α} injection.

Serum P₄ concentrations were not different between control (CON; 3.3 ± 0.3 , 0.04 ± 0.01 ng/ml) and treatment (TRT; 3.3 ± 0.5 , 0.05 ± 0.01 ng/ml) groups at the time of PGF_{2 α} and OVX respectively. No difference was observed in average interval from PGF_{2 α} to ovulation between CON (94.1 ± 10.2 h) and TRT (93.5 ± 4.8 h) groups. The size of the dominant follicle that ovulated after PGF_{2 α} injection was not different between CON (15.4 ± 0.5 mm) and TRT (16.0 ± 0.4 mm) cows. A new follicular wave was detected at the time of OVX and diameter of follicles at this time was not different between CON (4.9 ± 0.3 mm) and TRT (4.8 ± 0.2 mm) cows. Days from OVX to deviation tended to be shorter ($P < 0.07$) in TRT (3.1 ± 0.2 days) than CON (3.9 ± 0.3 days) cows. The incidence of co-dominance was not different in CON (3/8; 37.5%) and TRT (8/16; 50%) cows. At the time of deviation, the diameters of the largest dominant follicle (F1; 10.7 ± 0.6 mm vs 10.3 ± 0.3 mm), the second-largest follicle (F2; in co-dominance; 9.3 ± 0.2 mm vs 9.0 ± 0.3 mm) and the subordinate follicle (SF; 7.6 ± 0.5 mm vs 7.9 ± 0.3 mm) were not different between CON and TRT cows respectively.

Individual follicular profiles for two cows in the CON group are shown in Fig. 1A and B). None of the CON cows became anovular following OVX. Of the CON cows, six cows had two follicular waves and the other two cows had three follicular waves during the estrous cycle. All CON cows had a normal length estrous cycle (22.0 ± 0.6 days) after OVX.

Figure 1 Growth patterns of dominant follicles and CL before and after OVX. Time of OVX is shown with a dotted vertical line (day 0). The ovary on the opposite side from the new ovulation (OV) was removed in control cows (A and B). The ovary containing the corpus hemorrhagicum was removed in treatment (TRT) cows and half of these cows ovulated (TRT-OV) after OVX (C and D). Time of removal of follicles and CL by OVX is shown with star symbols.

Half of the TRT cows (TRT-OV; $n = 8$) ovulated 7.3 ± 0.6 days after OVX. Individual follicular profiles for two cows in TRT-OV are shown in Fig. 1C and D. Estrous cycle length was 20.1 ± 1.4 days after ovulation in TRT-OV cows.

The other half of the TRT cows became anovular (TRT-ANO; $n = 8$) after OVX. These eight cows remained anovular 25 days after OVX. Individual follicular profiles for

four cows in TRT-ANO are shown in Fig. 2. Three cows had co-dominant follicles and five cows had a single dominant follicle during the first follicular wave after OVX. The maximal size of follicles reached 25.4 ± 1.4 mm (range, 20–32 mm) in TRT-ANO cows. Six of eight anovular cows had two follicular waves during the 25-day experimental period and two anovular cows had only one follicular wave during this time (Fig. 2).

Figure 2 Growth patterns of dominant follicles and CL before and after OVX. Time of OVX is shown with a dotted vertical line (day 0). The ovary containing the corpus hemorrhagicum was removed in treatment (TRT) cows and half of these cows became anovular (TRT-ANO) after OVX. Time of removal of follicles and CL with OVX is shown with star symbols.

The second follicle wave was detected 15.0 ± 2.4 days after OVX in the six anovular cows with two follicular waves.

Double ovulation rates were determined before OVX in all cows and after OVX in CON and TRT-OV cows (TRT-ANO did not ovulate post-OVX). Three of the eight CON cows (37.5%) had double ovulation before OVX (two with bilateral ovulations and one with unilateral ovulations). Two of the 16 TRT cows had double ovulation before OVX (two with unilateral ovulations). Five of eight cows in TRT-OV had co-dominant follicles and double ovulation after OVX, whereas the other three cows had a single dominant follicle and single ovulation. In contrast, after the normal estrous cycle, all of the CON cows had a single ovulation and only two of eight TRT-OV cows had double ovulation after the normal estrous cycle.

Serum P₄ concentrations decreased to basal levels after PGF_{2α} in all cows regardless of treatment (Fig. 3). Control cows had a normal P₄ rise after ovulation. Removal of the newly formed corpus hemorrhagicum prevented the rise in circulating P₄ in TRT-ANO cows throughout the 25-day experimental period. However, TRT-OV cows had a delayed increase in circulating P₄ but serum P₄ concentration was normal in relation to the time of ovulation (Fig. 3). Serum P₄ was greater ($P < 0.05$) in CON than TRT-ANO cows beginning from day 5 after OVX. Serum P₄ was similar between TRT-OV and TRT-ANO cows until day 9 after OVX and began increasing after day 9 in TRT-OV cows. Serum P₄ concentrations were greater ($P < 0.05$) from day 12 to day 20 in TRT-OV cows than in TRT-ANO cows. Also, P₄ concentrations were greater

($P < 0.05$) in CON than TRT-OV cows from day 5 to day 15 after OVX (Fig. 3).

Serum E₂ concentrations were similar among all groups (CON, TRT-OV and TRT-ANO cows) until 7 days after OVX (Fig. 4). Serum E₂ concentrations decreased in TRT-OV and CON cows between 5 and 8 days after OVX; whereas, serum E₂ remained elevated (> 5 pg/ml) during this time period in TRT-ANO cows. Serum E₂ concentrations were greater ($P < 0.001$) in TRT-ANO cows than in TRT-OV and CON cows from day 8 to day 12 after OVX.

Serum FSH concentrations were similar among groups from 3 days before until 9 days after OVX (Fig. 5). Control cows had greater ($P < 0.01$) FSH concentrations at day 10 after OVX than TRT-OV and TRT-ANO cows. Serum cortisol concentrations were not different among groups for any time before or after OVX (data not shown).

Discussion

The results of this study were consistent with our original hypothesis, namely that removal of the corpus hemorrhagicum would result in development of follicular cysts. Thus, the physiological increase in E₂ is sufficient to induce cows into an anovular condition that resembles follicular cysts if there is no subsequent increase in circulating P₄. Numerous previous studies have hormonally induced cows to have follicular cysts, using large doses of exogenous E₂. About 50% of cows became anovular following these treatments (Cook *et al.* 1990, 1991, Hamilton *et al.* 1995, Calder *et al.* 1999, Gümen & Wiltbank 2002). Cows with induced follicular cysts appear to be in a physiological state in which high circulating E₂ either produces a delayed

Figure 3 Serum P₄ concentrations (means $\pm$ S.E.M.) before and after OVX. Time of OVX is shown with a dashed vertical line (day 0). The ovary on the opposite side from the new ovulation was removed in control cows. The ovary containing the corpus hemorrhagicum was removed in treatment (TRT) cows. Half of these cows ovulated (TRT-OV) and the other half became anovular (TRT-ANO) after OVX. * $P < 0.05$ indicates differences between control vs TRT-OV and TRT-ANO cows. † $P < 0.05$ indicates difference in TRT-OV vs TRT-ANO cows.

Figure 4 Serum E₂ concentrations (means $\pm$ S.E.M.) before and after OVX. Time of OVX is shown with a dashed vertical line (day 0). The ovary on the opposite side from the new ovulation was removed in control cows. The ovary containing the corpus hemorrhagicum was removed in treatment (TRT) cows. Half of these cows ovulated (TRT-OV) and the other half became anovular (TRT-ANO) after OVX. * $P < 0.001$ indicates differences in TRT-ANO cows vs TRT-OV and control cows.

Figure 5 Serum FSH concentrations (means $\pm$ S.E.M.) before and after OVX. Time of OVX is shown with a dashed vertical line (day 0). The ovary on the opposite side from the new ovulation was removed in control cows. The ovary containing the corpus hemorrhagicum was removed in treatment (TRT) cows. Half of these cows ovulated (TRT-OV) and the other half became anovular (TRT-ANO) after OVX.

* $P < 0.01$ indicates difference in control vs TRT-OV and TRT-ANO cows.

GnRH/LH surge (Zaied *et al.* 1981) or does not cause a GnRH/LH surge (Dobson & Alam 1987, Refsal *et al.* 1987, Ribadu *et al.* 1999, Gümen & Wiltbank 2002, Gümen *et al.* 2002) due to altered hypothalamic responsiveness to E_2 . However, this is the first study, to our knowledge, that has shown that this lack of hypothalamic responsiveness to E_2 and follicular cysts can be induced by the normal increase in E_2 simply by preventing the post-ovulatory rise in circulating P_4 . Although not providing detailed mechanistic information, the results of this study provide key insights into the physiological events that underlie the development of follicular cysts.

It was not possible from our results to determine the reason that some cows did not enter this anovular condition even when the P_4 increase was eliminated (TRT-OV cows). There were no differences from 3 days before until 7 days after OVX in circulating P_4 , E_2 , FSH or cortisol concentrations between treated cows that became anovular versus treated cows that remained ovular. Hamilton *et al.* (1995) found that the circulating concentrations of E_2 and pulsatile LH were greater in anovula cows than in ovular cows. Similarly, we found greater E_2 concentrations in anovular cows than in control or treated cows that remained ovular from days 8–12 after OVX. Thus, at about 7 days after OVX the TRT-OV cows had an E_2 increase that induced a GnRH/LH surge and this produced a decrease in follicular E_2 production and subsequent ovulation. Conversely, the cows that became anovular (TRT-ANO) had follicles that continued to grow and E_2 concentrations remained elevated but there was no GnRH/LH surge or ovulation. As with our results, Yoshioka *et al.* (1998) found that circulating E_2 concentrations increased with greater follicular size in anovular cows. In each of our previous studies using injections of a

high dose of E_2 , we were also able to induce cows into an anovular condition (59–62% of cows became anovular) but we also failed to detect any hormonal differences that would explain why treated cows remained ovular or became anovular (Gümen & Wiltbank 2002, 2005).

It is possible that a continuous elevation in E_2 is a part of the mechanism that induced follicular cysts in those cows that developed this condition in our experiment. Continuous treatment with follicular-phase levels of E_2 blocked a subsequent E_2 -induced LH surge in ovariectomized ewes (Öztürk *et al.* 1998). Treatment with E_2 specifically downregulates hypothalamic estrogen receptor mRNA (Lauber *et al.* 1990, Simerly & Young 1991) in rats. E_2 appears to act on cells in the mediobasal hypothalamus (Blache *et al.* 1991, Caraty *et al.* 1998) by activating estrogen receptor- α (ER α) (Couse & Korach 1999). Several studies have shown that ER α knockout mice have very large anovular follicles (Couse *et al.* 1999, Scully *et al.* 1997, Schomberg *et al.* 1998). Thus, the E_2 increase near the time of estrus could not only induce a GnRH surge but also could downregulate expression of ER α (or some other aspect of E_2 signal transduction) in the lateral mediobasal hypothalamus producing hypothalamic unresponsiveness to E_2 . Our experimental results are consistent with this downregulation occurring not only during pharmacological treatments with E_2 , as shown in previous experiments, but also during the physiological rise in E_2 that produces the natural GnRH/LH surge. A continuing elevation in circulating E_2 from the subsequent follicular waves may prolong this downregulation of ER α and hypothalamic unresponsiveness and may be a critical component in development of persistent anovulation in animals with follicular cysts. However, the differences between TRT-ANO and TRT-OV cows do not appear to be solely explained by E_2 concentrations since there were no differences in circulating E_2 between these groups until 7 days after OVX when TRT-ANO follicles continue to grow and TRT-OV follicles ovulate.

It appears that a relatively simple model could explain our results in this study (Fig. 6). In normally cycling cows, an E_2 surge due to the final stages of preovulatory follicular growth could not only induce a GnRH surge but also could downregulate expression of ER α in the lateral mediobasal hypothalamus. The GnRH surge would be expected to cause an LH surge from the pituitary and ovulation of a dominant follicle. The subsequent increase in P_4 from the CL could reinstate responsiveness to E_2 at the hypothalamus by inducing ER α (Blache *et al.* 1991, Caraty & Skinner 1999). Removal of the CL after ovulation in the anovular cows prevents the rise in circulating P_4 , a new follicle wave grows and E_2 concentrations increase, so maintaining downregulation of hypothalamic ER α .

The mechanisms by which P_4 can reinstate normal cyclicity in cows with follicular cysts has been investigated in a number of studies (Johnson & Ulberg 1967, Nanda *et al.* 1991, Calder *et al.* 1999, Gümen & Wiltbank 2002, 2005). First, P_4 can decrease GnRH and LH pulses

Figure 6 Simplified model of possible underlying reproductive physiology explaining development of follicular cysts. In normally cycling cows, the E_2 increase associated with estrus could not only induce a GnRH surge but also could downregulate expression of ER α (or some other aspect of E_2 signal transduction) in the lateral mediobasal hypothalamus. Normally, the GnRH surge would cause an LH surge from the pituitary and ovulation of a dominant follicle. Progesterone secreted from the CL could reinitiate responsiveness to E_2 at the hypothalamus by inducing ER α (or some other aspect of E_2 signal transduction). Removal of the CL after ovulation prevented the rise in circulating progesterone, so a new follicular wave develops and the increasing circulating E_2 maintains ER α downregulation in the hypothalamus of anovular cows.

leading to a lack of trophic support for follicular cysts (Calder *et al.* 1999). This would lead to a decrease in E_2 production from the cyst, decreased circulating E_2 and potentially removal of the mechanisms that were down-regulating E_2 responsiveness in the hypothalamus. Indeed cystic cows have elevated LH secretion compared with non-cystic cows (Hamilton *et al.* 1995, Cook *et al.* 1991). Additional support for this idea is the finding that cystic cows that 'self-corrected' (similar to TRT-OV in this study) had mean and pulsatile LH levels that were intermediate between cows that remained cystic and normally cycling cows (Hamilton *et al.* 1995). There may also be direct effects of P_4 on the hypothalamus to induce E_2 responsiveness, possibly by increasing ER α receptor (Blache *et al.* 1991, Caraty & Skinner 1999). The results of our study extend these previous observations by indicating that similar P_4 -mediated mechanisms are needed in normally cycling cows to liberate them from a potentially cystic condition that occurs after estrus during each estrous cycle.

Thus, the endogenous increase in circulating E_2 that induces the GnRH/LH surge and estrus is sufficient to induce cows into a physiological state that resembles follicular cysts if it is not followed by increased P_4 . These cows exhibited the hallmarks of cystic cows: large anovular follicles and probably hypothalamic unresponsiveness to E_2 (elevated E_2 concentrations were not followed by ovulation probably due to lack of a GnRH/LH surge). Under natural conditions the rise in P_4 following ovulation would return the hypothalamus to a condition that would respond to elevated E_2 . It seems likely that the natural development of follicular cysts may also occur due to similar circumstances. This could involve either an inadequate GnRH surge, inadequate pituitary LH or lack of ovulatory capacity in the follicle that would prevent an ovulation after the E_2 rise that normally induces

estrus. Obviously, testing of this speculative model will require future research.

Other interesting observations on follicular development were also noted in this study. The TRT cows had earlier follicular deviation than CON cows. This could be due to lower circulating P_4 concentrations perhaps increasing follicular growth rate. In addition, the interval from emergence to deviation was longer in this study with lactating cows (80.0 ± 4.8 h) than in a previous study in heifers ($60.4\text{--}61.0$ h) (Kulick *et al.* 1999). Follicular size was also smaller at deviation (~ 8.5 mm) in previous studies with heifers (Ginther *et al.* 1996, Kulick *et al.* 1999) and lactating cows (9.8 mm, Sartori *et al.* 2004) than in the present study (10.3–10.7 mm). Unilateral OVX may have altered deviation due to removal of a large number of ovarian follicles and/or alteration in circulating hormonal concentrations. In addition, there was a high incidence of co-dominant follicles in the first follicular wave after OVX in both treatment (eight of 16; no CL and low P_4) and control (three of eight; with CL and increasing P_4). Similar to our findings, Mohan & Rajamahendran (1998) reported that unilateral OVX (performed on the day after ovulation) increased the number of ovulatory follicles (two follicles developed and ovulated in six of eight cycles). Also, Matsui *et al.* (2004) reported that absence of CL induced co-dominant follicles during the first follicular wave in six of eight cows. Thus, OVX appeared to alter a number of aspects of follicular development and selection through mechanisms related or unrelated to the decrease in circulating P_4 caused by removal of the corpus hemorrhagicum.

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