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Characterization of persistent follicles induced by prolonged treatment with progesterone in dairy cows: An experimental model for the study of ovarian follicular cysts

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ABSTRACT

Cystic ovarian disease (COD) is a major factor contributing to poor reproductive efficiency of lactating dairy cows. The objective of the present study was to analyze the endocrine profile, growth dynamics, and histologic characteristics of persistent ovarian follicles-cysts developing in response to long-term administration of intermediate levels of progesterone. To this end, after synchronization of cows, a low dose of progesterone was administered for 5, 10, and 15 days after the expected day of ovulation in treated cows (groups P5, P10, and P15, respectively), using an intravaginal progesterone-releasing device. A significant increase in diameter was detected on Day 11 of progesterone treatment and thereafter ($P < 0.05$), and at Day 15 of persistence, the diameter of the persistent follicle reached a mean of 23 ± 0.6 mm. Microscopically, the persistent follicles had a complete granulosa, an intensely vascularized theca interna, and a collagenous theca externa layer. Temporal changes in the serum concentrations of estradiol, progesterone, and FSH were detected (effects of time, $P < 0.01$). Progesterone treatment completely inhibited the LH preovulatory surge in treated cows and affected the basal concentration of LH. The pulse frequency remained high at 5 and 10 days of persistence and declined ($P < 0.05$) after 15 days of persistence. The LH pulse concentration and pulse amplitude had a significant reduction ($P < 0.05$) during follicular persistence. Changes in the serum levels of estradiol, progesterone, 17-hydroxyprogesterone, and testosterone in serum and follicular fluid were also observed. In serum, estradiol increased gradually from proestrus to Day 10 of follicular persistence ($P < 0.05$), progesterone showed an increase ($P < 0.05$) at Day 5 of follicular persistence, 17-hydroxyprogesterone showed a significant decrease at 5 days of follicular persistence in relation to proestrus, and testosterone showed a significant increase ($P < 0.05$) from proestrus and Day 5 of persistence through Day 15 of follicular persistence. Correlation between serum and follicular fluid steroid concentrations was significant for testosterone ($P < 0.0001$) and not significant for estradiol and progesterone. These findings indicate that ovarian cysts in COD are similar in many ways to the persistent follicles induced by progesterone, with an analogous hormonal and morphologic context, thus

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confirming a local role of subluteal levels of progesterone in COD pathogenesis and in the regulatory mechanisms of the ovarian function.

1. Introduction

Cystic ovarian disease (COD) is a major factor contributing to poor reproductive efficiency of lactating dairy cows [1]. However, the pathogenesis of COD has not been clearly established. Cysts develop from preovulatory follicles that fail to ovulate, persist, and then interfere with normal ovarian function [2]. The most widely accepted hypothesis is that COD is the result of a "hormonal imbalance" within the hypothalamic–pituitary–gonadal axis [2]. Silvia et al. [3] have proposed that the primary defect resides in the hypothalamus, which fails to release a surge of GnRH in response to follicular estradiol. This failure leads to a lack of stimulus for the anterior pituitary gland to secrete the preovulatory surge of LH. This hypothalamic insensitivity to estradiol may be due to the intermediate concentrations of progesterone commonly found in cows with COD [4–9].

The study of the processes that lead to ovulatory failure and persistence of the dominant follicle is the key to understand the pathogenesis of COD. The major difficulty in investigating cysts is that their formation can only be retrospectively recognized after the follicle has undergone extensive pathologic changes. Therefore, prediction of the time of cystic structure formation through follicular development in experimental models is a formidable opportunity to understand their pathogenesis. In this sense, numerous experimental models have been developed to induce the formation of follicular cysts [10–23]. However, the possible role of intermediate levels of progesterone in promoting the formation of ovarian follicular cysts has been investigated only in short-term models without reaching the time of follicular persistence necessary to define ovarian structures as follicular cysts. Thus, the objective of the present study was to analyze the endocrine profile, growth dynamics, and histologic characteristics of persistent ovarian follicles and cysts developing in response to long-term administration of intermediate levels of progesterone.

2. Materials and methods

2.1. Animals

All the procedures were approved by the Institutional Ethics and Security Committee of the Faculty of Veterinary Sciences of the Universidad Nacional del Litoral, Argentina (protocol no. 131-12) and are consistent with the "Guide for the Care and Use of Agricultural Animals in Research and Teaching, Third Edition" (Federation of Animal Science Societies, 2010). This study was performed in nonlactating Holstein cows (N = 25) with regular estrous cycles, all of which had calved at least once. Cows were obtained at the end of lactation from local commercial farms and housed outside in an open lot, except during blood collection or ultrasound examinations (when they were moved to a stanchion barn). The cows were fed a diet based on alfalfa pasture, oat, or rye grass grazing supplemented with corn silage, alfalfa silage, corn grain, soybean expeller, and hay, following the recommendations of the Nutrient Requirements of Dairy Cattle (2001).

2.2. Experimental model

2.2.1. Synchronization

Ovarian activity was synchronized starting with the procedure commonly referred to as G6G [24] with some modifications (see Fig. 1). Holstein cows with one or more corpora lutea (CL) identified by transrectal ovarian ultrasonography were enrolled to start the experiment. The synchronization protocol consisted of two doses of $PGF_{2\alpha}$ (150-μg D-Cloprostenol, Enzaprost D-C; Biogénesis-Bagó, Argentina) administered 12 hours apart on Day 0 to induce luteolysis, followed by a dose of GnRH (20-μg buserelin acetate, Gonaxal; Biogénesis-Bagó) 2 days later to stimulate ovulation of the preovulatory follicles present. Six days after the first GnRH dose, the cows started Ovsynch with an injection of GnRH. Seven days later, cows received two doses of $PGF_{2\alpha}$, 12 hours apart, to ensure luteolysis (completion of the modified synchronization protocol).

2.2.2. Groups and treatments

After synchronization, the cows were divided into five groups: C1 (control 1; n = 5), C2 (control 2; n = 5), P5 (5 days of follicular persistence; n = 5), P10 (10 days of follicular persistence; n = 5), and P15 (15 days of follicular persistence; n = 5).

Control cows (groups C1 and C2) received no additional hormonal treatment. Cows from group C2 were used to determine the time of ovulation, defined as Day 0 through a sequence of ovarian ultrasonography and blood samples (described in the following). On average, ovulation occurred around 4 days after administration of the first $PGF_{2\alpha}$ dose (range, 101–106 hours).

Treated cows (groups P5, P10, and P15) were administered a low dose of progesterone until 5, 10, and 15 days after the expected day of ovulation (Day 0). Progesterone was administered using an intravaginal progesterone-releasing device (750 mg of micronized progesterone; Pro-Ciclar P4-Zoovet) inserted 1 day after the first $PGF_{2\alpha}$ injection of Ovsynch. This device was kept in the cows for 5 days after the expected day of ovulation in group P5 and for 8 days in groups P10 and P15. In the latter two groups, a new intravaginal progesterone-releasing device was introduced 1 day before the removal of the first one, to maintain a more consistent concentration of progesterone throughout the treatment period. In group P15, a third intravaginal progesterone-releasing device was introduced on Day 11 of persistence, again 1 day before removal of the second one.

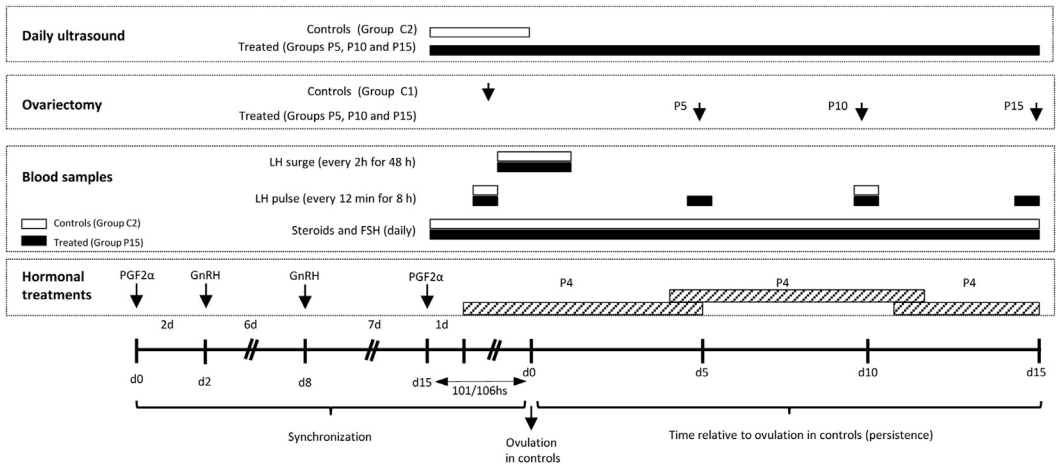

Fig. 1. Experimental design for the induction of follicular persistence. Holstein cows were synchronized and received an intravaginal progesterone-releasing device to induce follicular persistence. Follicular dynamics were followed by daily ultrasound scanning, and levels of blood steroid and gonadotrophic hormones were measured as indicated. Ovariectomy was performed in proestrus (controls) and after 5, 10, and 15 days of follicular persistence.

2.2.3. Ovariectomy

Bilateral ovariectomy was performed in cows from group C1, 2 days after completion of the synchronization protocol (48 hours after the first $PGF_{2\alpha}$ injection of Ovsynch). In treated groups, ovariectomy was performed at Days 5, 10, and 15 of follicular persistence in groups P5, P10, and P15, respectively (Fig. 1).

2.3. Blood sampling

Control cows of group C2 were used to determine the average time of ovulation and to determine hormone profiles. Group P15 was used to determine the percentage of ovulation failure and to monitor progesterone, estradiol, and FSH concentrations, LH pulse frequency, and LH surge (Fig. 1).

For estradiol, progesterone, and FSH determination, blood samples were collected *via* coccygeal venipuncture daily from the day of the last $PGF_{2\alpha}$ injection until the end of the protocol (Day 15 of persistence) in treated (group P15) and control cows (group C2). For LH pulse frequency, blood samples were collected through an indwelling jugular cannula every 12 minutes for 8 hours, starting 48 hours after the first $PGF_{2\alpha}$ injection for the follicular phase (groups C2 and P15), on Days 5, 10, and 15 of follicular persistence (group P15) and on Day 10 after ovulation, during midluteal phase (group C2). For LH surge, blood samples were collected every 2 hours for 48 hours, from 56 hours to 104 hours after $PGF_{2\alpha}$ injection in groups C2 and P15.

At the time of ovariectomy, blood samples were taken from groups C1, P5, P10, and P15 to determine the levels of estradiol, progesterone, 17-hydroxyprogesterone, and testosterone. All blood samples were kept at room temperature (22 °C–25 °C) for 30 minutes, placed at 4 °C to 8 °C for 1 hour, and finally centrifuged at 1500 × *g* for 20 minutes in a refrigerated centrifuge at 5 °C. Serum was separated and stored at −20 °C until hormone analysis.

2.4. Ovarian ultrasonography

Ovarian ultrasonographic examinations were performed on all animals using a real-time B-mode scanner equipped with a 5-MHz linear-array transrectal transducer (Honda HS101V, Japan). The effectiveness of the synchronization protocol was monitored by ultrasonography on Days 0, 2, 8, and 15. Daily ovarian ultrasonography was performed either from the day of the last $PGF_{2\alpha}$ injection until ovulation in control cows (group C2) or on the day of ovariectomy in the treatment groups (P5, P10, and P15; Fig. 1). The size, growth, and regression of all follicles greater than 5 mm, CL, and persistent follicles were monitored.

2.5. Collection and preparation of tissues

For histologic examination, ovaries from control (group C1, proestrus) and treated cows (groups P5, P10, and P15) were obtained by transvaginal ovariectomy [25]. After follicular aspiration, follicular fluid was refrigerated and immediately transported to the laboratory for processing. Ovaries, as well as follicular structures of interest, were measured, photographed, and immediately fixed in 4% buffered formaldehyde for 8 hours at 25 °C after obtaining samples of follicular wall of persistent (P5, P10, or P15) or preovulatory follicles (C1) for molecular biology analysis. Then, the ovaries were washed in PBS, sectioned and reduced for an appropriate histologic processing, dehydrated in an ascending series of ethanol, cleared in xylene, and finally embedded in paraffin.

2.6. Histologic observation

Five-micrometer-thick sections were mounted on slides previously treated with 3-aminopropyltriethoxysilane (Sigma–Aldrich, St. Louis, MO, USA) and stained with hematoxylin–eosin for the observation of all ovarian structures [26].

Digital image analysis of histologic slides was performed using Image Pro-Plus 3.0.1 (Media Cybernetics, Silver Spring, MA, USA). Images were obtained using a Nikon DS-Fi2 (Tokyo, Japan) digital camera attached to a Nikon Eclipse Ni optical light microscope. The thickness of the granulosa and theca layers of the dominant (group C1) and persistent follicles (groups P5, P10, and P15) was determined using specific tools in the accompanying Nikon software.

2.7. Hormone assays

Follicle-stimulating hormone and LH serum levels were determined by RIA using kits provided by NIDDK (USA), as previously described and validated [27,28]. Intra-assay and interassay coefficients of variation for LH and FSH were less than 8% and 12%, respectively. Minimum detectable concentrations were 0.16 and 1.18 ng/mL of serum for LH and FSH, respectively.

The concentrations of estradiol, progesterone, and testosterone in serum and follicular fluid were measured by electrochemiluminescence immunoassay kits (Roche Diagnostics GmbH, Germany) in a cobas e411 system (Roche Diagnostics), according to the manufacturer's instructions. The assay sensitivity was 5 pg/mL for estradiol, 0.03 ng/mL for progesterone, and 0.02 ng/mL for testosterone. The concentrations of 17-hydroxyprogesterone in serum and follicular fluid were measured by a commercially available competition RIA without extraction (Immunotech, Marseille, France) with a sensitivity of 0.05 ng/mL.

To validate the electrochemiluminescence immunoassay and RIA of steroids, pooled follicular fluid and serum of ovariectomized cows were made steroid free by charcoal treatment [29]. Serial dilutions of estradiol, testosterone, progesterone, and 17-hydroxyprogesterone (Sigma–Aldrich) were prepared by using steroid-free follicular fluid or serum as diluents. Recovery of steroid standards added to individual samples before the assay was between 82% and 96% for high-concentration samples and 79% and 93% for low-concentration samples. The intra-assay and interassay coefficients of variation were less than 7% and less than 6%, respectively.

2.8. Statistical analyses

The pulse frequency and amplitude of LH pulses were characterized in a manner similar to that used by D'Occhio et al. [30]. Basal concentration was calculated from values not associated with either the ascending or descending portions of a pulse. A sample was defined as a peak (start of a pulse) if (1) the value was at least 40% greater than the preceding sample and (2) it was followed by at least two successive values that were declining or represented basal levels. Pulse amplitude was determined by subtracting the preceding basal value from the highest value associated with the pulse (pulse concentration) [31].

The frequency and amplitude of LH pulses, steroid hormone concentrations in serum and follicular fluid at ovariectomy, and the morphometric data were compared by ANOVA, followed by Duncan's multiple comparisons test.

Repeated-measures ANOVA using the general linear model procedure was used to compare the serum concentrations of estradiol, progesterone, and FSH in treated and control animals during the experimental protocol to determine the time and treatment effects and treatment-by-time interaction.

Pearson's coefficient was used to analyze a possible correlation between the serum and follicular fluid concentrations of the different steroids at ovariectomy.

All statistical tests were performed using a statistical software package (SPSS 11.0 for Windows; SPSS Inc., Chicago, IL, USA). A $P < 0.05$ value was considered significant. The results are expressed as the mean ± standard error of the mean.

3. Results

A single dominant follicle was detected in all animals at the time of the last $PGF_{2\alpha}$ injection. The mean diameter of the preovulatory follicle in control animals at the last ultrasonographic examination before ovulation was 17.6 ± 0.3 mm. This follicle ovulated in each animal 4 days after the last $PGF_{2\alpha}$ injection. The diameter of the largest follicle in treated animals, measured 24 hours before the time of ovulation in the controls, was 15.6 ± 1.8 mm, without difference between groups.

Successful induction of follicular persistence was confirmed by observing the failure of the dominant follicle to ovulate and increase in diameter throughout the 15 days of progesterone treatment. The largest follicle increased gradually throughout the experimental period. A significant increase in diameter was detected on Day 11 of progesterone treatment and thereafter ($P < 0.05$). By Day 15 of persistence (15 days after ovulation in the controls), the diameter of the persistent follicle reached a mean of 23 ± 0.6 mm (Fig. 2A). Ultrasonographically, the persistent follicles appeared as uniformly nonechogenic ovarian structures with a wall thickness of 3 mm or less (Fig. 2B).

Ovaries from control animals presented healthy follicles in all stages of development and atresia. Corpora lutea were absent or in regression in all cases (Fig. 3A). Ovaries from treated cows (groups P5, P10, and P15) presented all stages of follicle growth and development, including primordial, small preantral, large preantral, and antral follicles, follicles showing different degrees of atresia, and a single large persistent follicle. An active CL was absent in all cases.

All persistent follicles had a complete granulosa, an intensely vascularized theca interna, and a collagenous theca externa layer. Microscopically, as follicle persistence progresses, the granulosa cell layer showed higher proportion of apoptotic cells and a loss of the cells nearest the antrum, which are frequently separated and within the antral cavity. A high proportion of granulosa cells of the basal zone maintained the integrity and health. Furthermore, the follicular wall folds included granulosa and theca cells. In some areas of these follicles, the basement membrane was little evident. No signs of luteinization were observed in the granulosa or theca cell layers in any of the persistent follicles of any group (Fig. 3A).

The average thickness of the granulosa and the two theca layers declined with time and by Day 15 of

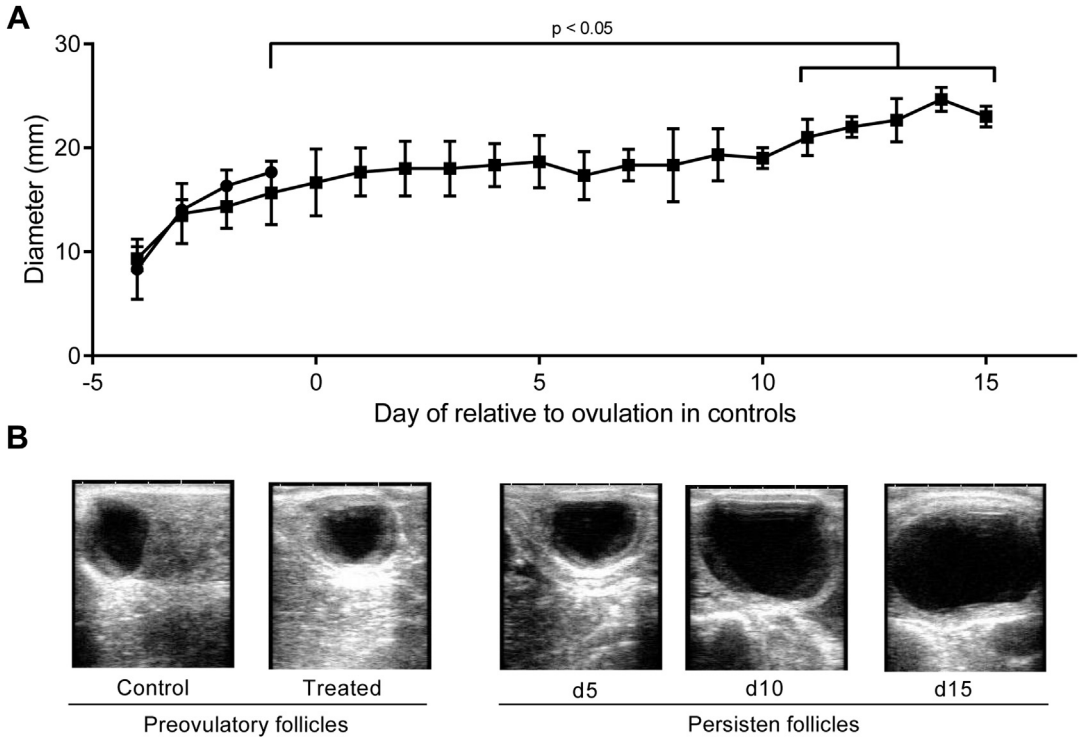

Fig. 2. (A) Maximal diameter of dominant and persistent follicles in controls (n = 5; ●) and treated (n = 5; ■) cows, respectively. Mean (±standard error of the mean) of follicular internal diameter and differences with preovulatory follicle in controls are shown. (B) Selected ultrasound pictures of preovulatory follicles from control and treated cows and follicles with 5, 10, and 15 days of follicular persistence from treated cows.

persistence; the granulosa and theca layers were significantly thinner than in the preovulatory follicles ($P < 0.05$; Fig. 3B).

Temporal changes in the concentrations of estradiol and progesterone were observed in both control and progesterone-treated animals from the day of the last $PGF_{2\alpha}$ injection until Day 15 of follicular persistence (group P15) (effects of time, $P < 0.05$ and $P < 0.001$, respectively; Fig. 4). An effect of the treatment on the concentrations of estradiol and progesterone was also observed ($P < 0.05$ and $P < 0.001$, respectively). Significant treatment-by-time interactions were observed for both steroid hormones ($P < 0.001$; Fig. 4). The concentrations of estradiol were greater in progesterone-treated animals than in controls from Day 2 until the end of the experiment. After ovulation, estradiol concentration in control animals decreased, whereas that in treated animals continued to increase significantly until Day 10 of persistence and then remained at low values, although at values significantly higher than those of controls (Fig. 4). In treated animals, progesterone values remained approximately at 2 ng/mL from Day 0 to the end of experiment, showing small increases when intravaginal releasing devices were changed for new ones. In controls, the concentration of progesterone increased significantly from Day 5 because of the formation of a new CL after ovulation (Fig. 4).

Follicle-stimulating hormone concentrations did not change over time and did not differ between treatment groups. Follicle-stimulating hormone concentrations in the control group showed an increase around the time of growth of each new follicular wave (Days 6 and 13; Fig. 4).

The basal concentration of LH, pulse frequency, pulse concentration, and pulse amplitude of control and treated cows at different times are depicted in Table 1. Progesterone treatment completely inhibited the LH preovulatory surge in treated cows. In control animals, the surge was observed from 70 to 80 hours after the $PGF_{2\alpha}$ injection.

The basal concentration of LH was lower ($P < 0.05$) in the follicular phase of controls (0.195 ± 0.004 ng/mL) than in the follicular phase of treated animals (0.242 ± 0.008 ng/mL). In treated cows, the basal concentration remained lower at 5, 10, and 15 days of persistence (0.139 ± 0.007, 0.167 ± 0.006, and 0.157 ± 0.008 ng/mL, respectively), similar to that of the midluteal phase in controls (0.137 ± 0.009 ng/mL).

The pulse frequency of LH was relatively high and similar ($P > 0.05$) in control (7 ± 0.5 pulses/8 hours) and treated groups (6.4 ± 0.2 pulses/8 hours) during the follicular phase. In controls, the LH pulse frequency was reduced ($P < 0.05$) in the midluteal phase (3.2 ± 0.21 pulses/8 hours). In treated cows, the pulse frequency remained high at 5 and 10 days of persistence (8 ± 0.4 and 6.2 ± 0.2 pulses/8 hours, respectively) and the pulse frequency declined ($P < 0.05$) after 15 days of persistence (2.2 ± 0.02 pulses/8 hours).

The LH pulse concentration was higher in the follicular phase of control (0.763 ± 0.019 ng/mL) and treated cows (0.705 ± 0.029 ng/mL), with a significant reduction

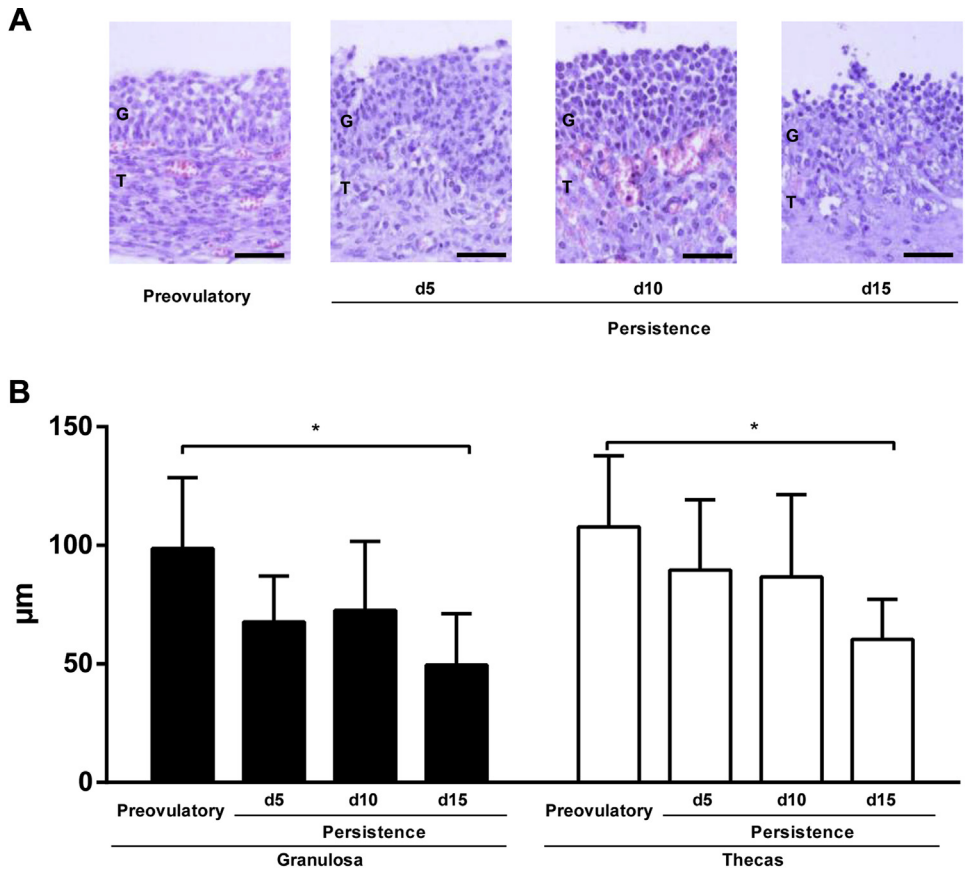

Fig. 3. Representative microscopy images (A) and histomorphometric analysis (B) of the follicular wall of preovulatory follicles from control cows (n = 5) and follicles with 5, 10, and 15 days of follicular persistence from treated cows (n = 5 in each day). A decrease in the thickness of granulosa (G) and theca (T) cells was observed at 15 days of persistence in relation to preovulatory follicles (*P < 0.05). Values represent mean ± standard error of the mean. Bars = 50 µm.

(P < 0.05) during follicular persistence in treated cows and midluteal phase in controls. Also, the mean LH pulse amplitude was higher (P < 0.05) in the follicular phase of control (0.562 ± 0.029 ng/mL) and treated cows (0.478 ± 0.033 ng/mL) than in midluteal phase from controls (0.352 ± 0.006 ng/mL) and in 5 (0.332 ± 0.023 ng/mL), 10 (0.388 ± 0.025 ng/mL), and 15 (0.399 ± 0.007 ng/mL) days of persistence from treated groups.

The levels of estradiol, progesterone, 17-hydroxyprogesterone, and testosterone in serum and follicular fluid of progesterone-treated cows and controls at the time of ovariectomy are shown in Figure 5. In serum, estradiol increased gradually from proestrus to Day 10 of follicular persistence and then reduced to values similar to those at proestrus (P < 0.05). Cows with 5 days of follicular persistence had a significantly higher concentration of progesterone in serum (P < 0.05) compared to the other experimental groups. 17-Hydroxyprogesterone showed a significant decrease at 5 days of follicular persistence in relation to proestrus and Day 10 of persistence (P < 0.05), whereas at Day 15 of persistence, a significant increase was observed (P < 0.05). Testosterone levels showed a significant increase (P < 0.05) from proestrus and Day 5 of persistence through Day 15 of follicular persistence.

In follicular fluid, estradiol concentrations were relatively higher in samples collected during the follicular phase and after 5 or 10 days of persistence and lower in those from 15-day persistent follicles (P < 0.05). Progesterone concentrations were higher in follicular fluid from preovulatory follicles (P < 0.05) than in from persistent follicles. 17-Hydroxyprogesterone concentrations increased progressively from preovulatory follicles throughout the persistence period (P < 0.05). Testosterone concentrations were relatively low in preovulatory follicles and remained low until 15 days of persistence when it increased significantly (P < 0.05).

Correlation between serum and follicular fluid steroid concentrations was significant for testosterone ($r = 0.782$, P < 0.0001) and not significant for estradiol or progesterone. For 17-hydroxyprogesterone, a relevant tendency was observed ($r = 0.399$, P = 0.081) for a correlation between serum and follicular fluid concentration.

4. Discussion

The main difficulty in studying COD in cattle is that cyst formation can only be recognized retrospectively after the follicle has undergone extensive morphologic and physiological changes [32]. Most previous studies on the

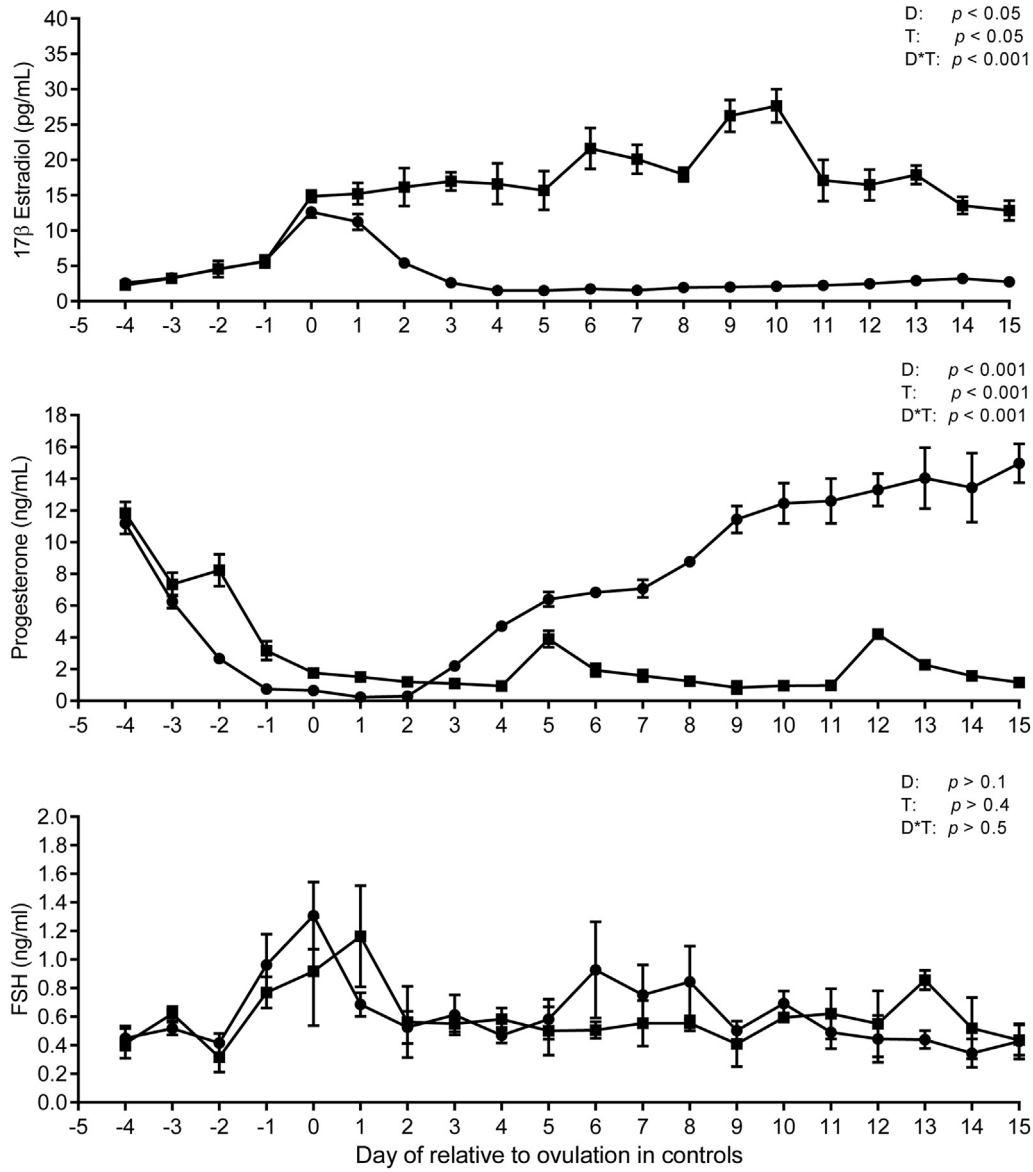

Fig. 4. Serum concentrations of estradiol, progesterone, and FSH in control (n = 5; ●) and treated (n = 5; ■) cows. Results are mean ± standard error of the mean. Data were analyzed according to the time of ovulation in controls (Day 0). D: effect of the day of the experimental protocol; T: treatment effect; D*T: day of experimental protocol-by-treatment interaction.

histologic and endocrine characteristics of bovine cysts have relied on slaughterhouse material, where the reproductive history and persistence of cysts are not known. In the present study, a cyst-like condition was induced by chronic treatment with low doses of progesterone. We characterized the endocrine environment and follicular dynamics before and after the development of ovarian cysts induced in lactating cows. The length of the progesterone treatment (15 days) was designed to allow demonstration of the temporal changes in follicular development that occur during cystogenesis on the basis of some of the most common definitions for follicular cysts.

Although similar models have been characterized, no data about intrafollicular levels of steroids and histologic characteristics through the persistence period of non-ovulatory follicles had been published previously. As in numerous previous experiments, subluteal concentrations of progesterone were effective in suppressing the occurrence of spontaneously occurring preovulatory surges of LH and ovulation in treated lactating cows [9,33–42].

The LH pulse frequency on Days 5 and 10 of follicular persistence in treated cows was similar to that in the follicular phase and significantly higher than that in the midluteal phase in controls, confirming that changes in the frequency of LH pulses occur rapidly after exposure to subluteal concentrations of progesterone [5,38,39,43–45]. The continuity of a high frequency beyond the follicular phase is consistent with the fact that the formation of new cysts

Table 1
Mean (±standard error of the mean) LH basal concentration, pulse frequency, pulse concentration, and pulse amplitude during the follicular and midluteal phases in control (n = 5) and 5, 10, and 15 days of persistence (n = 5 in each day) in progesterone-treated animals.

Sampling time	Basal concentration (ng/mL)	Pulse frequency (pulses/8 h)	Pulse concentration (ng/mL)	Pulse amplitude (ng/mL)
Control animals				
Follicular phase	0.195 ± 0.004^{b}	$7.0 \pm 0.52^{b,c}$	0.763 ± 0.019^{c}	0.562 ± 0.019^{c}
Midluteal phase	0.137 ± 0.009^{a}	3.2 ± 0.21^{a}	0.468 ± 0.015^{a}	0.352 ± 0.006^{a}
Progesterone-treated animals				
Follicular phase	0.242 ± 0.008^{c}	6.4 ± 0.24^{b}	0.705 ± 0.029^{b}	0.478 ± 0.033^{b}
5 Days of persistence	0.139 ± 0.007^{a}	8.0 ± 0.44^{c}	0.477 ± 0.026^{a}	0.332 ± 0.023^{a}
10 Days of persistence	$0.167 \pm 0.006^{a,b}$	6.2 ± 0.20^{b}	0.573 ± 0.022^{a}	0.388 ± 0.025^{a}
15 Days of persistence	0.157 ± 0.008^{a}	2.2 ± 0.02^{a}	0.522 ± 0.025^{a}	0.399 ± 0.007^{a}

Different letters are significantly different ($P < 0.05$).

is associated with changes in LH secretion [2,46]. Also, taking into account that the pulse concentrations during follicular persistence in treated animals was higher than those in the luteal phase, our results support the hypothesis that hypersecretion of LH may play a role in cyst persistence. Although the pulse frequency was similar during follicular phase in both control and treated animals, the pulse concentration and amplitude were higher in controls than in treated cows. These data suggest that ovulation failure could be related to a reduced LH pulse concentration and amplitude rather than changes in the frequency. Furthermore, the pulse frequency increased by 5 days of persistence compared to follicular phase in treated animals, but ovulation did not occur. In fact, the pulse concentration and amplitude by 5 days of persistence were still lower than in control cows, suggesting again that the last two parameters are essential to induce ovulation. In terms of the implications of these findings in COD pathogenesis, this study confirms that cystic ovaries may be formed when the anterior pituitary gland fails to release a surge of LH in response to the preovulatory rise in estradiol.

On the other hand, FSH concentration was not affected by progesterone, and changes were observed only related to the normal follicular dynamics during the estrous cycle in controls. The observed results are consistent with a feedback mechanism functioning properly and suggest that estradiol (not progesterone) is the main regulator of FSH secretion, with preovulatory elevations preceding the emergence of a new follicular wave. This indicates that changes in the secretory pattern and concentrations of FSH may not be a factor in the etiology of follicular persistence and ovarian cysts.

The concentrations of estradiol in serum of control and treated cows were similar until shortly after ovulation, when the prolonged follicular growth in treated animals produced higher levels of estradiol, which reached its highest concentration on Day 10 of follicular persistence. This increase in estradiol concentration has been associated with an increased frequency of LH pulses, as we observed on Day 10 of follicular persistence, and appears to be a representative of those in the early stage of development of prolonged dominance [39,45]. Also, the serum concentrations of estradiol suggest that estradiol production from persistent follicles decreased by Day 11 of persistence, in agreement with the histologic changes observed in the granulosa of persistent follicles on Day 15. These data highlight the convenience of our model to study the initial histologic and hormonal changes during cyst formation, given that most other studies have been conducted with slaughterhouse samples or spontaneous cysts, where the follicular persistence of cysts was not known.

This is the first article that described the morphologic and histologic changes throughout the induction of persistent follicles in an experimental model. During the experimental induction, persistent follicles increased their size until Day 15 of persistence, showing granulosa and theca layers similar to those of preovulatory follicles on Days 5 and 10. In contrast, at 15 days of persistence, both layers showed a reduced thickness. Spontaneous follicular cysts, in which the persistence time is not known, show a variety of features, such as partial or total loss of the granulosa cell layer, luteinization of theca cells, hyalinization of the theca cell layer, or complete absence of differentiated cells (granulosa or theca cells), leaving only the cystic cavity surrounded by stromal tissue. This has been described and classified in different ways by several authors who have also attempted to correlate the time of persistence of cysts with morphologic and functional characteristics [47–51]. In this model, we observed that the follicles that may potentially become cysts remained similar to healthy follicles and gradually lost granulosa cells but never showed a complete loss of granulosa or the luteinization of the theca layer, at least until Day 15, maintaining an active steroidogenesis.

The concentrations of estradiol in follicular fluid were constant between proestrus and Day 10 of persistence, decreasing significantly at Day 15 of persistence. The increase in serum concentrations could reflect estradiol production by healthy granulosa cells until Day 10 of persistence with an increase in serum concentrations. In this sense, it has been proposed that the large diameter of persistent follicles is coupled with an increase in the mass of steroidogenic tissue and that prolonged dominant follicles have steroidogenic capacities similar to those of large antral follicles [52]. Also, the higher LH pulse frequency may promote follicular androgen production, leading to increased synthesis of estradiol [53], and has been associated with follicular cyst pathogenesis [4].

Progesterone showed an increased intrafollicular concentration in preovulatory follicles but remained low in persistent follicles. This increase has been described as a key event in the ovulatory mechanism. Although the mechanism of these hormones has not been fully elucidated, progesterone appears to stimulate the

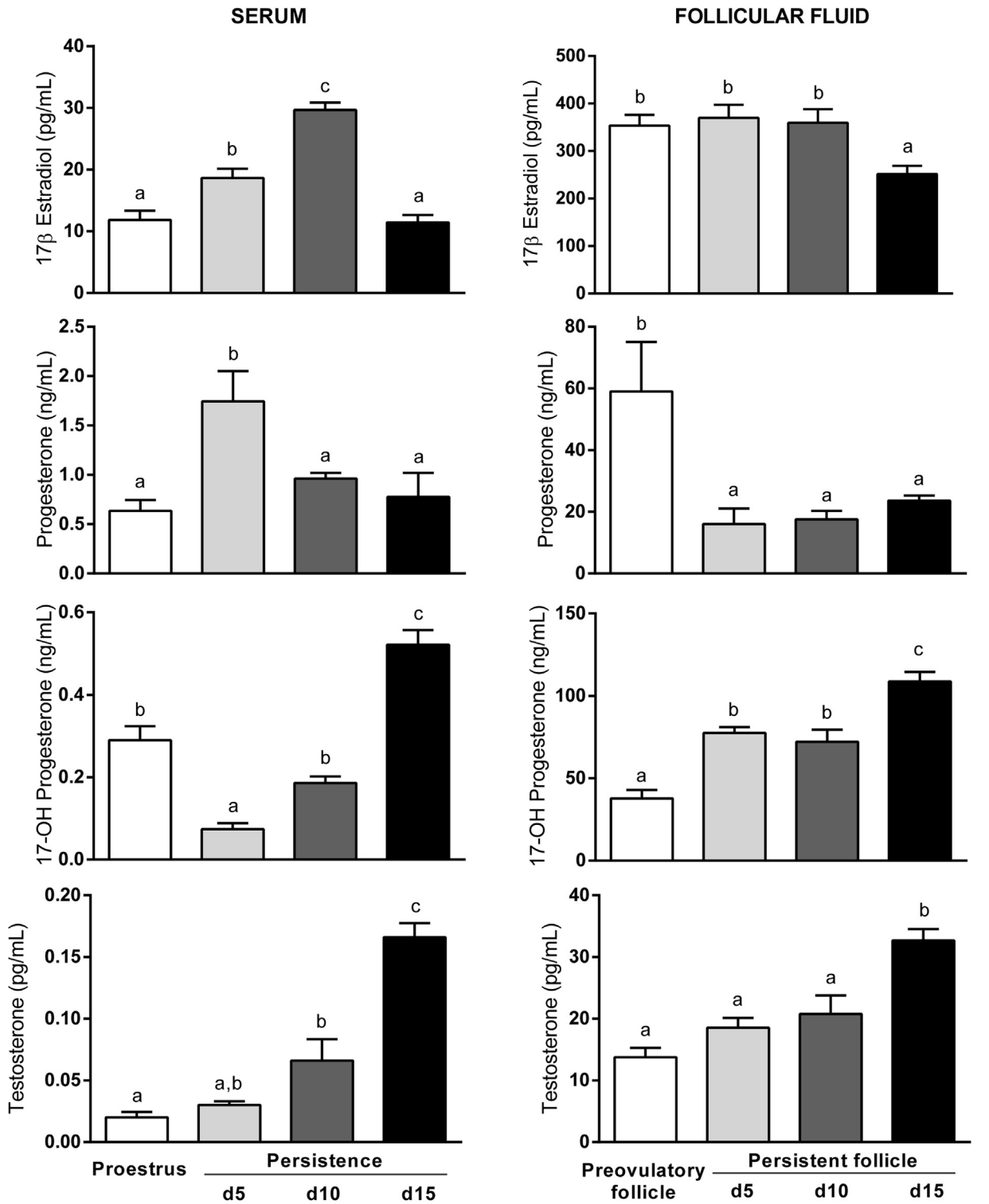

Fig. 5. Concentration of estradiol, progesterone, 17-hydroxyprogesterone, and testosterone in serum and follicular fluid at the time of ovariectomy (n = 5 in each point). Bars represent the mean ± standard error of the mean for each hormone in serum or follicular fluid. Bars with different letters are significantly different ($P < 0.05$).

transformation from prostaglandin E2 to PGF2α and regulate the metalloproteinase induction [54].

Testosterone showed a gradual increase from the preovulatory stage to Day 15 of follicular persistence, with a significant correlation between serum and intrafollicular concentrations. These results suggest that persistent follicles were able to maintain capacity for androgen synthesis for an extended period, but that at Day 15 of persistence might have started to decrease their capacity for estrogen synthesis (aromatization), as previously described in cysts [23]. Therefore, this deficient aromatization by changes in the granulosa cells could be responsible for the increase in testosterone that characterizes cystic follicles during COD in cattle [23,55].

This study is the first to document the relation between serum and intrafollicular concentrations of 17-hydroxyprogesterone. Serum concentrations of 17-hydroxyprogesterone increased gradually in animals with persistent follicles, with the higher concentrations at Day 15 of persistence. This increase was accompanied by a correlated increase in follicular fluid concentrations, showing that the follicles are still metabolically active, which is related to the histologic structure evidenced. It has been described that estrone and estradiol are the main steroids produced from acetate-14C in the mature follicle [56], whereas 4-androstenedione, dehydroepiandrosterone, and 17-hydroxyprogesterone are the main products in the atretic follicle under identical experimental conditions [57]. Until today, 17-hydroxyprogesterone has not been studied in cattle in relation to reproduction. In contrast, it has been observed that women with polycystic ovarian syndrome exhibit 17-hydroxyprogesterone hyperresponsiveness to LH and hCG tests [58]. The precise mechanisms responsible for this steroidogenic response in women with polycystic ovarian syndrome have not been established, although an intrinsic abnormality within the ovarian theca cells has been proposed and associated with ovarian androgen overproduction [59]. In this sense, the increase observed in serum and follicular fluid 17-hydroxyprogesterone was in parallel with the increase in testosterone, and both hormones showed similar concentration patterns.

Previous works have also shown significant alterations in the apoptosis rate in follicles from cows with spontaneous and induced ovarian cysts, and these findings support the notion that follicular persistence is an important component of COD pathogenesis [60,61]. Delayed follicle regression after ovulation failure is an alternative cause of cyst formation because a preovulatory follicle that cannot be ovulated will not grow further if it regresses immediately; therefore, no cystic follicles will be formed. In addition, we have previously described significant changes in steroid receptors, including the progesterone receptor (PGR) [62–64]. In this context, progesterone has been shown to stimulate granulosa cell differentiation [65,66]. Also, animals with COD usually present alterations in PGR expression, which, when added to the subluteal hormone levels, could induce changes in the mechanisms of proliferation/apoptosis of follicular cells, contributing to follicular persistence [60].

In conclusion, these findings indicate that ovarian cysts are similar in many ways to persistent follicles induced by long-term administration of progesterone. Persistent follicles induced by this model appear to adequately mimic cystic follicles that arise spontaneously, providing a new tool to study their early stages. The endocrine profile, growth dynamics, and histologic characteristics of persistent ovarian follicles are analogous to those of spontaneous cysts and confirm a local role of subluteal levels of progesterone in COD pathogenesis and in regulatory mechanisms of the ovarian function.

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Competing Interests

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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