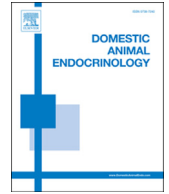




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## Ovarian localization of 11 $\beta$ -hydroxysteroid dehydrogenase (11 $\beta$ HSD): effects of ACTH stimulation and its relationship with bovine cystic ovarian disease

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### ABSTRACT

Cystic ovarian disease (COD) is an important cause of infertility in cattle, and ACTH has been involved in regulatory mechanisms related to ovarian function associated with ovulation, steroidogenesis, and luteal function. Here, we examined the localization of 11 $\beta$ -hydroxysteroid dehydrogenase type 1 (11 $\beta$ HSD1) and 11 $\beta$ HSD2 proteins in the ovary of healthy cows and animals with spontaneous and ACTH-induced COD and the in vitro response of the follicular wall exposed to ACTH. After stimulation by ACTH, we documented changes in 11 $\beta$ HSD expression and cortisol secretion by the follicular wall of large antral and follicular cysts. Follicular cysts showed a higher constitutive expression of both enzymes, whereas ACTH induced an increase in 11 $\beta$ HSD1 in tertiary follicles and follicular cysts and a decrease in 11 $\beta$ HSD2 in follicular cysts. Moderate expression of 11 $\beta$ HSD1 was observed by immunohistochemistry in granulosa of control animals, with an increase ( $P < 0.05$ ) from primary to secondary, tertiary, and atretic follicles. The level of immunostaining in theca interna was lower than that in granulosa. The expression of 11 $\beta$ HSD2 was lower in the granulosa of primary follicles than in that of secondary, tertiary, and atretic follicles and was lower in the theca interna than in the granulosa. In ACTH-induced and spontaneously occurring follicular cysts, differences from controls were observed only in the expression of 11 $\beta$ HSD1 in the granulosa, being higher ( $P < 0.05$ ) than in tertiary follicles. These findings indicate that follicular cysts may be exposed to high local concentrations of active glucocorticoids and indicate a local role for cortisol in COD pathogenesis and in regulatory mechanisms of ovarian function.

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### 1. Introduction

Stress-associated stimulation of the hypothalamic–pituitary–adrenal/ovarian axis has been reported to disrupt the reproductive function and has been associated with the pathogenesis of bovine cystic ovarian disease (COD) [1]. Poor follicle development, reduced ovulation rates, and

follicular cyst formation have been reported in association with ACTH treatment and increased concentrations of glucocorticoids [2–5]. Recently, we have confirmed the expression of ACTH receptors in bovine ovaries [6]. The fact that ACTH is able to induce steroid secretion from the ovary in vitro strongly indicates that ACTH could be involved in regulatory mechanisms related to ovarian function associated with ovulation, steroidogenesis, and luteal function [6].

The most widely accepted hypothesis to explain the formation of follicular cysts is that LH release from the

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hypothalamus-pituitary is altered [7–12]. In this sense, exogenous glucocorticoids prolong the estrous cycles of cattle [13,14], presumably by inhibiting LH secretion by the anterior pituitary [15,16] and by directly inhibiting the follicular function [17–19]. Exogenous ACTH treatment in cattle increases endogenous cortisol secretion, thus inducing follicular cysts [2,5,20]. It has been proposed that the action of glucocorticoids on ovarian function would be antagonistic to that on reproductive function [21], with a direct effect on granulosa cells down-regulating the production of steroids and prostaglandins and the expression of LH receptors [18,22,23]. Because ovulation is an inflammatory event characterized by increased synthesis of interleukins and prostaglandins [24–26], an increased generation of anti-inflammatory glucocorticoids during ovulation would help limit inflammatory process in the ovary [27–29]. However, extreme situations could affect the normal ovulatory mechanism, altering the estrous cycle.

Although the ovary would not be able to produce glucocorticoids *de novo* [30], ACTH is able to stimulate cortisol secretion from cultured bovine follicular walls [6]. Moreover, the access of active cortisol to target tissues is regulated by the relative expression of two 11 $\beta$ -hydroxysteroid dehydrogenase (11 $\beta$ HSD) enzymes: a bidirectional 11 $\beta$ HSD type 1 (11 $\beta$ HSD1), which mainly activates corticosterone to cortisol, and 11 $\beta$ HSD type 2 (11 $\beta$ HSD2), which inactivates cortisol to cortisone [31,32].

Both 11 $\beta$ HSD1 and 11 $\beta$ HSD2 mRNAs have been identified in the bovine ovary [32], and their expression and activity have been associated with follicular development and maturation [33,34]. In follicular fluid of bovine follicular cysts, changes have been observed in the profile of these enzymes, with a decrease in 11 $\beta$ HSD1 activity [35]. Although mRNA expression of the two 11 $\beta$ HSD isoforms has been detected in bovine ovaries, little is known about 11 $\beta$ HSD protein expression and localization. In the present study, we examined the *in vivo* expression of 11 $\beta$ HSD1 and 11 $\beta$ HSD2 proteins in the ovary of healthy cows and animals with spontaneous and ACTH-induced COD, and we studied the effect of *in vitro* stimulation of the follicular wall with ACTH on 11 $\beta$ HSD1 and 11 $\beta$ HSD2 protein expression, as well as changes in steroid hormones secretion.

## 2. Materials and methods

### 2.1. *In vivo* ACTH treatment

All the procedures were approved by the Institutional Ethics and Security Committee (Faculty of Veterinary Sciences – Universidad Nacional del Litoral, Argentina) and are consistent with [36]. Fifteen nulliparous Holstein heifers (age, 18–24 mo; body weight, 400–450 kg) were assigned to the control ( $n = 10$ ) or treated ( $n = 5$ ) groups.

Ovulation of all heifers was hormonally synchronized by the OvSynch protocol [37]. All heifers received 10  $\mu$ g of a gonadotropin-releasing hormone (GnRH) analog (buserelin acetate, Gonaxal; Biogénesis-Bagó, Garín, Argentina) on day –9 (see below), 150  $\mu$ g of a prostaglandin F2 $\alpha$  analog (D+Cloprostenol, Enzaprost D-C; Biogénesis-Bagó) on day –2, and 10  $\mu$ g of a GnRH analog on day 0. The time of the

ovulation was monitored by transrectal ultrasonography and was designated as day 1 of the estrous cycle (Fig. 1), because, as described by Pursley et al [37], ovulation occurs 24 to 32 h after the second injection of GnRH.

The model of ACTH-induced ovarian follicular cysts used in the present study has been previously described [5,20,38,39]. Briefly, beginning on day 15 of a synchronized estrous cycle, 5 heifers received subcutaneous injections of 1 mg of a synthetic polypeptide with ACTH activity (Synacthen Depot; Novartis, Basel, Switzerland) every 12 h for 7 d (treated group). The other 10 animals received saline solution (1 mL) (control group).

Ovarian ultrasonographic examinations were performed in all animals, using a real-time, B-mode scanner equipped with a 5-MHz, linear-array, transrectal transducer (Honda HS101V, Tokyo, Japan) [40]. The growth and regression of follicles >5 mm, corpora lutea, and follicular cysts were monitored. Daily ovarian ultrasonography was performed through a complete estrous cycle in untreated (control) heifers (21–23 d) and from day 14 (day 0 = day of ovulation) until ovariectomy on approximately day 48 in ACTH-treated heifers. Follicular cysts detected by ultrasonography were defined as any follicular structure with a diameter  $\geq 20$  mm present for  $\geq 10$  d, without ovulation or corpus luteum (CL) formation [5]. The first day of follicular cyst formation was the day a follicle attained  $\geq 20$  mm in diameter, and the ovaries were removed 10 d later by flank laparotomy (approximately day 48). In 5 heifers of the estrus-synchronized control group, ovariectomy was conducted when the dominant follicle reached a diameter >10 mm, in the absence of an active CL, to obtain preovulatory follicles (approximately day 18). The remaining 5 animals of the estrus-synchronized control group were subjected to daily blood sampling for hormonal determinations.

To clarify the effects of ACTH on steroid hormone concentrations, blood samples were taken from the coccygeal vein from 5 estrus-synchronized control and 5 ACTH-treated heifers every 24 h between day 15 and day 28 (Fig. 1). In addition, samples were collected just before ovariectomy in control and treated animals. Blood tubes were refrigerated after collection, and serum was separated within 2 h and stored at –20°C until hormone analysis.

### 2.2. Collection of spontaneous cysts

For immunohistochemistry, ovaries with spontaneous follicular cysts were collected from cows at local dairy farms. The diagnosis and confirmation was done with transrectal ultrasonography (5-MHz linear transducer, Honda HS101V). Ten Argentinean Holstein cows that showed  $\geq 1$  follicular cysts of  $\geq 20$  mm in diameter persisting for at least 10 d in the absence of a CL were selected. The ovaries were removed by transvaginal ovariectomy, and blood samples were collected just before surgery for hormone analysis.

For tissue culture, ovaries with large antral follicles (>10 mm;  $n = 12$ ) and with spontaneous follicular cysts ( $n = 12$ ) were collected at a local abattoir, within 20 min of death, from mixed breeds of Argentinean Holstein cows assessed visually as being nonpregnant without macroscopic

abnormalities in the reproductive system and immediately were transported to the laboratory.

### 2.3. Immunohistochemistry

Histologic ovarian sections from ACTH-treated cows, cows with spontaneous follicular cysts obtained from dairy herds, and estrus-synchronized controls were used for immunohistochemistry to locate and quantify the protein expression of 11 $\beta$ HSD1 and 11 $\beta$ HSD2 in follicular walls. During the dissection of the ovaries, the external follicular diameter was measured with calipers, and follicular fluid from each follicle was aspirated and stored separately at  $-20^{\circ}\text{C}$ . The health status of the follicles was confirmed by the hormone concentrations in the follicular fluid, considering normal follicles (healthy, nonatretic) those with high concentrations of estradiol ( $>150\text{ pg/mL}$ ), low progesterone ( $80\text{ ng/mL}$ ), and low testosterone-to-estrogen ratio. Ovaries were fixed in 4% buffered formaldehyde for 8 h at  $4^{\circ}\text{C}$ , dehydrated, and embedded in paraffin. Sections ( $5\text{ }\mu\text{m}$  thick) were mounted on slides previously treated with 3-aminopropyltriethoxysilane (Sigma-Aldrich, St. Louis, MO) and primarily stained with hematoxylin–eosin for a preliminary observation of all ovarian structures. For immunohistochemical studies, follicles were classified into the following groups: primary, secondary, tertiary (antral), and atretic follicles and follicular cysts [20,38,41,42].

The details, suppliers, and concentrations of the antibodies used are reported in Table 1. Each antibody was measured in at least 5 sections of each ovary from each animal. The homology between the target peptide of each antibody and the correspondent bovine protein was tested with the Basic Local Alignment Search Tool (BLAST software; <http://www.ncbi.nlm.nih.gov/BLAST>) to determine the peptide location and to confirm antigen specificity.

A streptavidin-biotin immunoperoxidase method was performed as previously described [43,44]. Briefly, after deparaffinization, microwave pretreatment (antigen retrieval) was performed by incubating the sections in 0.01M citrate buffer (pH 6). Endogen peroxidase activity was inhibited with 3% (vol/vol)  $\text{H}_2\text{O}_2$  in methanol, and nonspecific binding was blocked with 10% (vol/vol) normal goat serum. All sections were incubated with the primary antibodies for 18 h at  $4^{\circ}\text{C}$  and then for 30 min at room temperature with biotinylated secondary antibodies selected specifically for each of the 2 types of primary antibodies used. The antigens were visualized by streptavidin-peroxidase (BioGenex, San Ramon, CA), and 3,3-diaminobenzidine (Liquid DAB-Plus Substrate Kit; Zymed, San Francisco, CA) was used as the chromogen. Finally, the slides were washed in distilled water, counterstained with Mayer hematoxylin, dehydrated, and mounted.

To verify immunoreaction specificity, adjacent control sections were subjected to the same immunohistochemical method, replacing primary antibodies with nonimmune serum. To exclude the possibility of nonsuppressed endogenous peroxidase activity, some sections were incubated with diaminobenzidine alone.

Microscopic images were digitized with a CCD color video camera (Motic 2000; Motic China Group, Xiamen, China) mounted on a conventional light microscope (Olympus BH-2; Olympus Co, Tokyo, Japan), with an objective magnification of  $\times 40$ , and analyzed with the Image Pro-Plus 3.0.1 system (Media Cybernetics, Silver Spring, MD). The microscope was prepared for Koehler illumination. This was achieved by recording a reference image of an empty field for the correction of unequal illumination (shading correction) and calibrating the measurement system with a reference slide to determine background threshold values. The reference slides contained a series of tissue sections stained in the absence of a primary antibody. The positive controls were used as interassay controls to maximize the levels of accuracy and robustness of the method [44–46]. The methodologic details of image analysis as a valid method to quantify protein expression have been described previously [38,44,45–50]. The percentage of the immunopositive-stained area was calculated separately in each follicular wall layer (granulosa and theca) from at least 50 images of the primary, secondary, tertiary, and atretic follicles and follicular cysts.

### 2.4. In vitro ACTH treatment and Western blot analysis

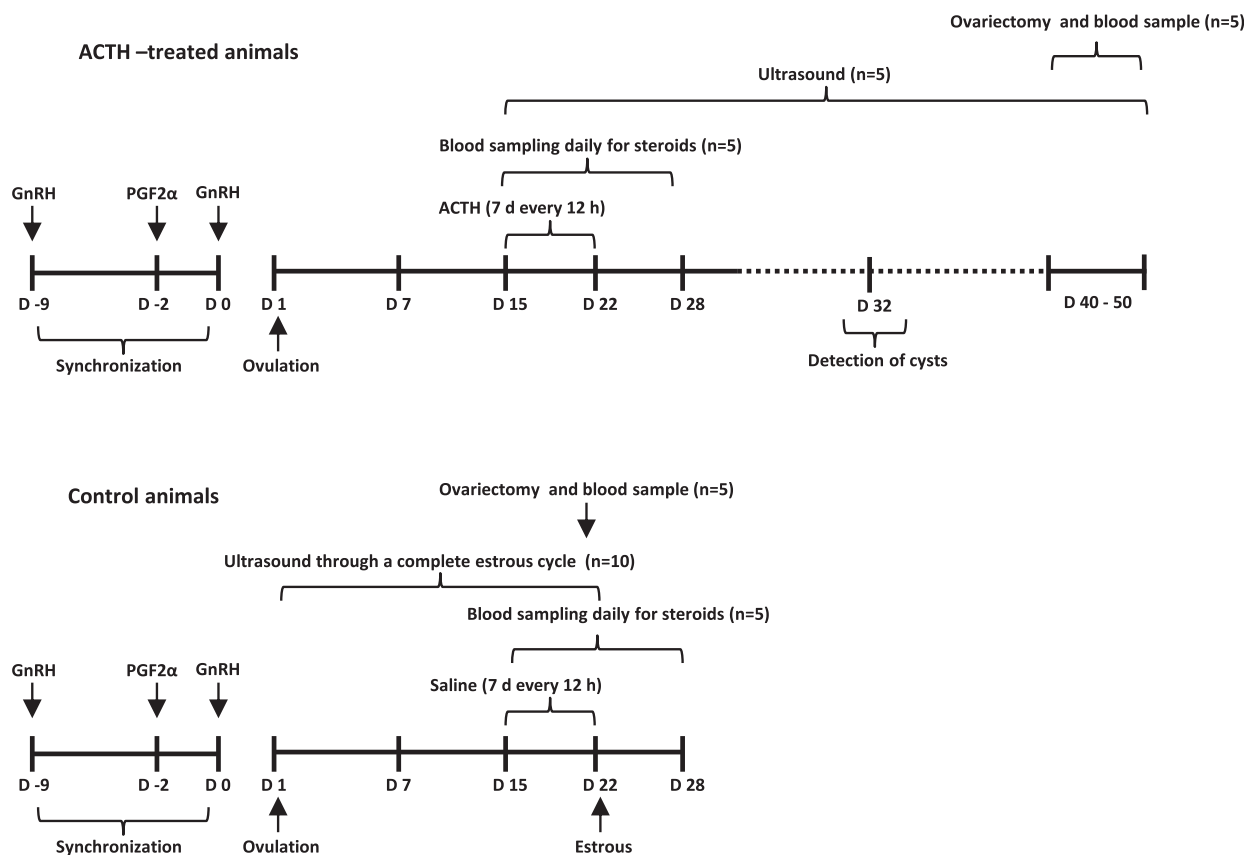
To analyze the effect of ACTH stimulation on the protein expression of 11 $\beta$ HSD1 and 11 $\beta$ HSD2 in vitro, follicular wall samples from cystic and large antral follicles collected at a local abattoir were used in the tissue culture assay (basal and ACTH-treated) and then processed for immunoblotting.

The protocol for in vitro ACTH treatment has been previously applied to bovine follicular walls to determine the effect of ACTH on the release of steroid hormones in vitro [6]. Briefly, in a laminar flow hood, ovaries were rinsed, follicular fluid was aspirated, and large antral follicles or follicular cysts were taken with a scalpel and placed in Krebs-Ringer bicarbonate buffer, pH 7.4. The culture medium had been previously equilibrated for 2 h in a humidified incubator (3154 Water-Jacketed Incubator; Forma Scientific, Asheville, NC) gassed with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$ .

**Table 1**  
Antibodies, suppliers, and dilutions used for IHC and WB analysis.

Antibodies	Clone/source	Dilution
Primary antibodies		
11 $\beta$ HSD1	Polyclonal, ab39364; Abcam (Cambridge, MA)	IHC: 1:100 WB: 1:500
11 $\beta$ HSD2	Polyclonal, ab37800; Abcam (Cambridge, MA)	IHC: 1:80 WB: 1:500
Secondary antibodies		
Biotinylated anti-rabbit IgG	Goat Polyclonal, 65-6140; Zymed (San Francisco, CA)	IHC: 1:100
Anti-rabbit IgG peroxidase	Goat Polyclonal (Amersham, UK)	WB: 1:1,000

Abbreviations: IHC, immunohistochemistry; WB, Western blot.



**Fig. 1.** Experimental design for the induction of follicular cysts. Holstein heifers were synchronized and treated with ACTH or saline, every 12 h, from days 15 to 22 of the estrus cycle. Follicular dynamics were followed by daily ultrasound scanning, and blood steroid hormones were measured as indicated. Ovariectomy was performed to obtain preovulatory follicles from control animals and cysts persisting for at least 10 d from treated animals. D, day; GnRH, gonadotropin-releasing hormone; PGF2 $\alpha$ , prostaglandin F2 $\alpha$ .

Each follicle was sectioned into 2 fragments (one for basal effects and one for ACTH treatment, approximately 50 mg each). The fragments were cultured on 24-well (1.5 mL medium/well) culture plates (Nunc plate; Applied Scientific, South San Francisco, CA), and incubated in Krebs-Ringer bicarbonate buffer, pH 7.4, for 20 min. After preculture, treated tissues were placed in medium with 100nM ACTH (Laboratorios ELEA, Buenos Aires, Argentina) for 3 h at 37°C. This dose has been described for a high cortisol secretion response in antral follicles in *in vitro* follicular wall cultures [6]. Control tissues were incubated in the same way without ACTH. Then, the culture medium was taken from each well and maintained at -20°C until hormone assays, and follicular wall samples were immediately frozen at -80°C until used for the determination of 11 $\beta$ HSDs protein expression by Western blot analysis. Each set of cultures, constituted by 6 follicular cysts and 6 large antral follicles (basal and ACTH-treated each), was performed twice, under identical conditions.

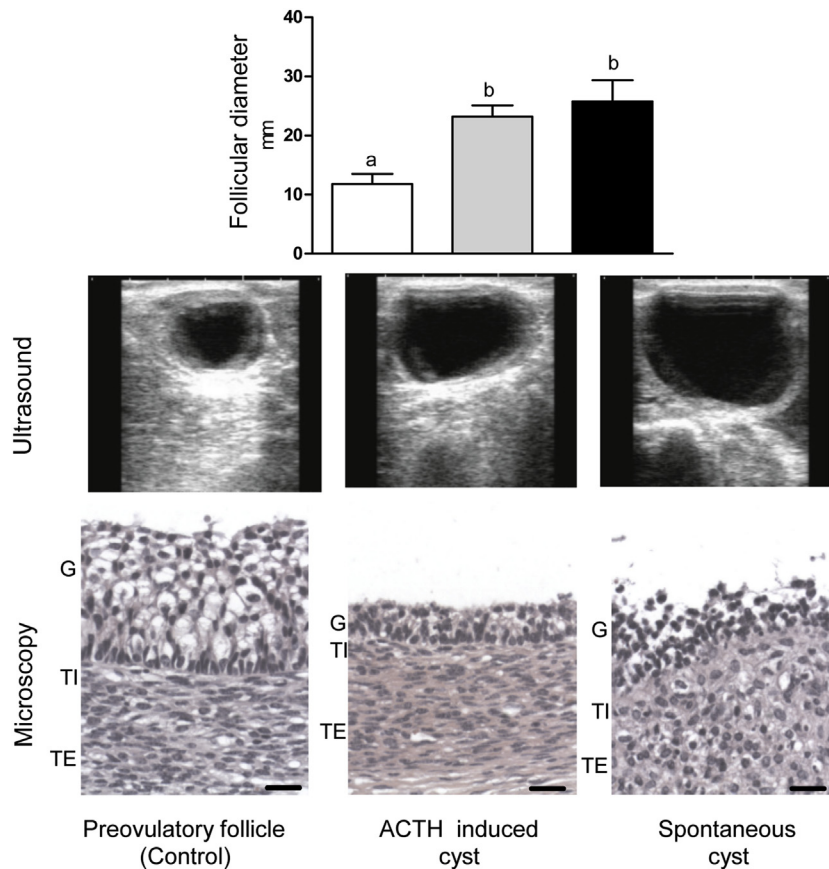
The tissue samples were homogenized in radioimmuno-precipitation assay lysis buffer and a protease inhibitor cocktail (Complete Mini Protease Inhibitor Cocktail Tablets; Roche, Mannheim, Germany), as previously described [15,39]. An aliquot of 40  $\mu$ g of proteins was

separated by SDS-PAGE (10% resolving gel). Proteins were transferred to nitrocellulose membranes (Amersham, Buckinghamshire, UK), blocked for 1 h in 2% wt/vol nonfat milk in Tris-buffered saline that contained 0.05% vol/vol Tween 20 (Sigma-Aldrich), and then incubated overnight at 4°C with specific primary antibodies (Table 1). After washing, membranes were treated for 1 h with the corresponding secondary peroxidase-conjugated antibody (Table 1). Immunopositive bands were visualized with a chemiluminescent detection kit (Amersham). To test specificity of the antibodies, bovine adrenal tissues were processed with the same protocol.

The exposed films were scanned at 1,200 dpi, and the protein expression levels of 11 $\beta$ HSD1 and 11 $\beta$ HSD2 were analyzed by densitometry with the Image Pro-Plus 3.0.1 system to obtain an integrated optical density value.

### 2.5. Hormone assays

The concentrations of estradiol, progesterone, testosterone, and cortisol in serum and follicular fluid from ACTH-treated cows, cows with spontaneous cysts, and controls, as well as in the incubation medium of tissue cultures, were measured by ELISA kits (Estradiol EIA,



**Fig. 2.** Morphologic characteristics of preovulatory follicles and follicular cysts at the time of ovariectomy. Mean  $\pm$  SEM follicular diameter, selected ultrasound pictures, and histologic features are shown. Bars with different letters are different ( $P < 0.05$ ). In microscopy image, scale bars = 20  $\mu$ m. G, granulosa; TE, theca externa; TI, theca interna.

DSL-10-4300; Progesterone EIA, DSL-10-3900; Testosterone EIA, DSL-10-4000; Cortisol DSL-1-200; Diagnostic Systems Laboratories, Webster, TX), according to the manufacturer's instructions. The assay sensitivity was 7 pg/mL for estradiol, 0.13 ng/mL for progesterone, 0.04 ng/mL for testosterone, and 0.10  $\mu$ g/dL for cortisol. For the incubation medium, results were normalized in relation to tissue weight and were expressed as hormone concentration per milligram of tissue [6].

## 2.6. Statistical analyses

A statistical software package (SPSS 11.0 for Windows; SPSS Inc, Chicago, IL) was used to perform all statistical tests. The serum concentrations of steroids in ACTH-treated and control animals during the experimental protocol were analyzed by multivariate ANOVA to determine the time and treatment effects and treatment-by-time interaction.

The immunohistochemical data and hormone concentrations in serum and follicular fluid from ACTH-treated cows, cows with spontaneous cysts, and controls were compared by ANOVA, followed by Duncan multiple range tests. The protein expression and hormone concentrations in the culture media between ACTH-treated and control

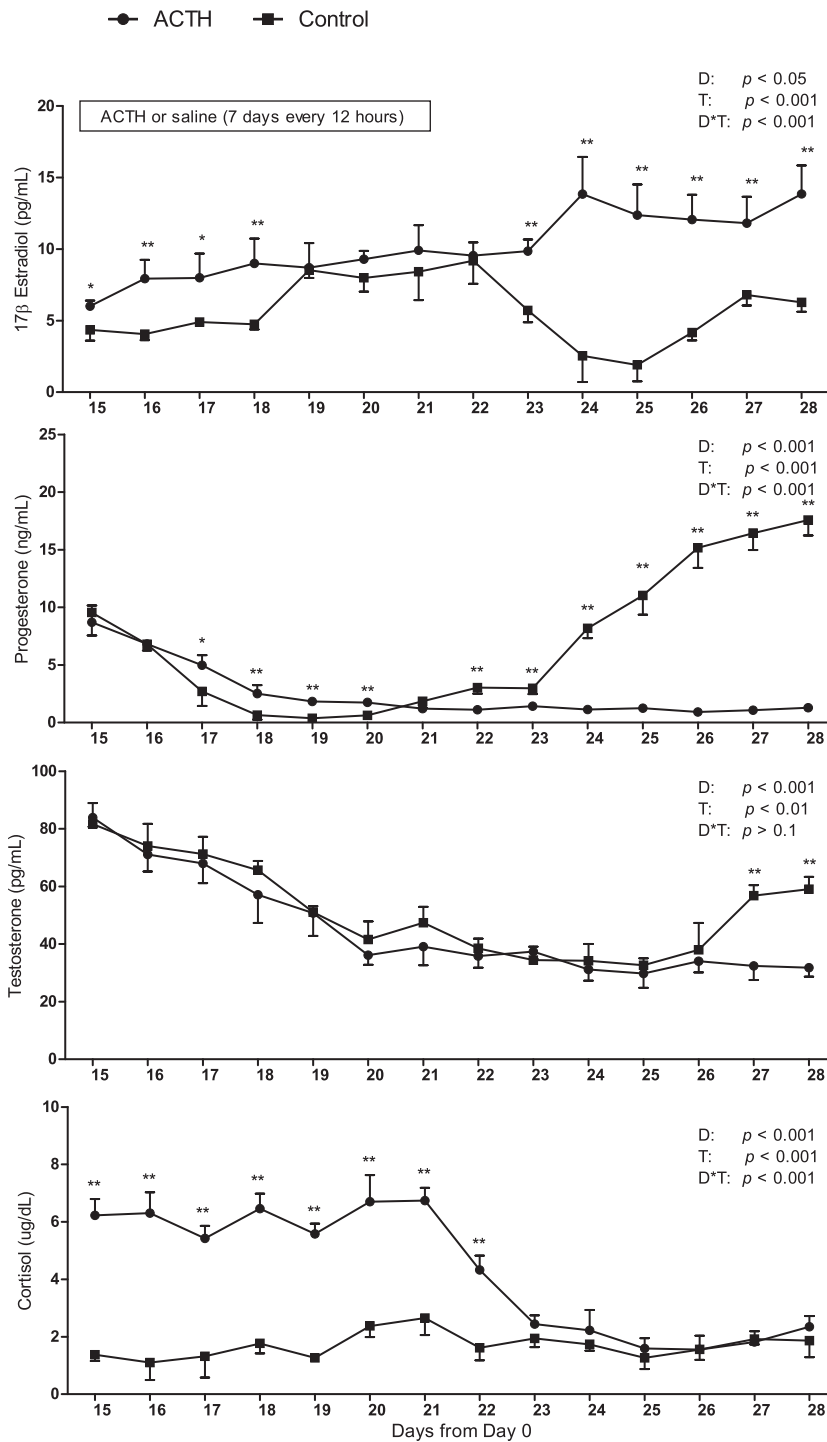
tissues were compared with a nonpaired 2-tailed Student *t* test. For all statistical analyses,  $P < 0.05$  was considered significant. Results are expressed as mean  $\pm$  SEM.

## 3. Results

### 3.1. Ultrasonography and follicular morphology

#### 3.1.1. ACTH-treated animals

The successful induction of COD with the ACTH treatment in heifers was confirmed by observing ovarian morphology and hormone concentrations. Ovarian follicular cysts were induced in all ACTH-treated animals. Detection of cysts began on day  $32.5 \pm 4$ , and the mean diameter of cysts was  $23.2 \pm 1.9$  mm, larger ( $P < 0.05$ ) than the mean diameter of ovulatory follicles, which was  $11.8 \pm 1.7$  mm at ovariectomy (approximately day 18). Ultrasonographically, the cysts appeared as uniformly non-echogenic ovarian structures with a wall thickness of  $\leq 3$  mm. Microscopically, healthy developing follicles, follicles showing different degrees of atresia, and 1 large cyst (in one ovary) with a complete and intact multilaminar granulosa layer were observed. Corpora lutea were absent in all cases (Fig. 2).



**Fig. 3.** Serum levels of estradiol, progesterone, testosterone, and cortisol in ACTH-treated and control animals during the experimental protocol. Results are mean  $\pm$  SEM. Data were analyzed according to the time after the ovulation (day 1) in the previous synchronized cycle. Values with superscripts indicate differences ( $*P < 0.05$  and  $**P < 0.01$ ) between ACTH-treated and controls in each time point ( $n = 5$  animals per group). D, day of experimental protocol effect; T, treatment effect; D\*T, day of experimental protocol-by-treatment interaction.

### 3.1.2. Control animals

Daily ultrasound monitoring in control untreated heifers during a complete estrous cycle showed normal

follicular dynamics. The preovulatory follicle was detected on day 15, and its mean diameter was  $11.8 \pm 1.7$  mm after ovariectomy (approximately day 18). Histologically, ovaries

exhibited follicles in various stages of development, including primary, secondary, and tertiary follicles, as well as atretic follicles and CL from previous cycles (Fig. 2).

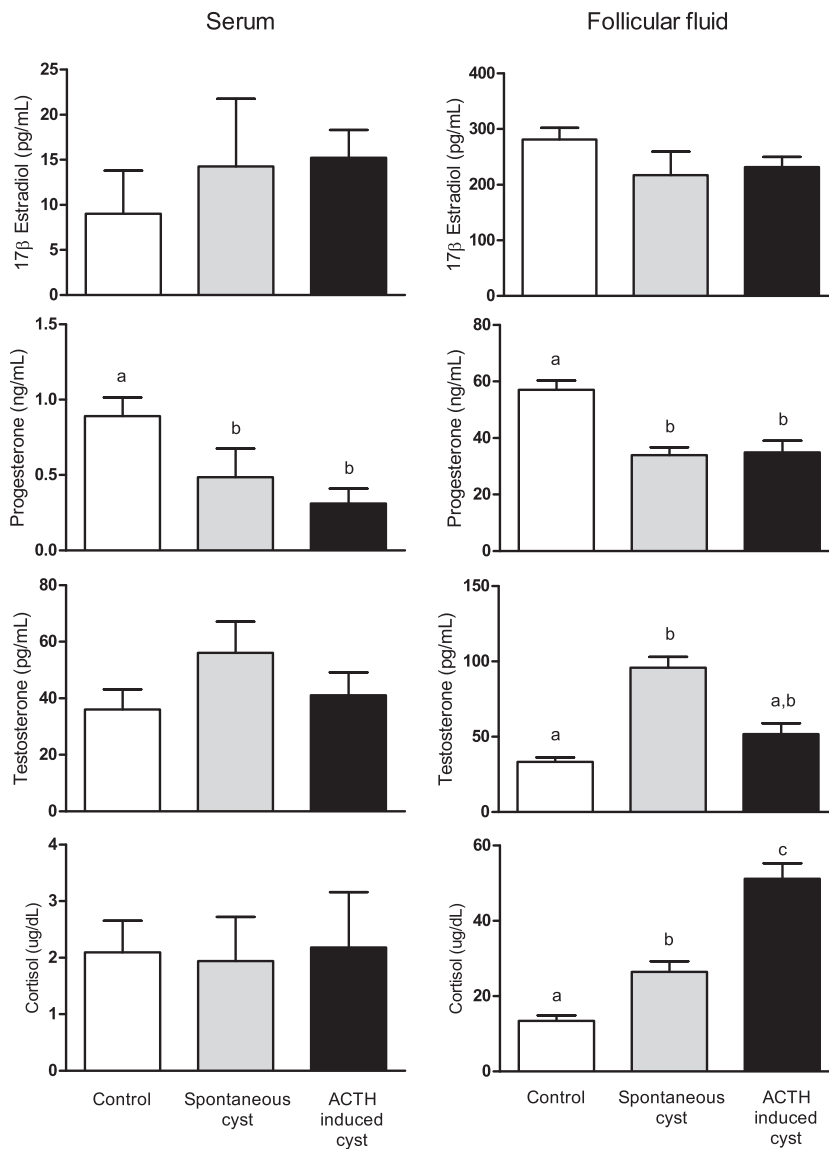
### 3.1.3. Animals with COD diagnosed at the dairy farm

Ultrasonographically, spontaneous follicular cysts diagnosed at the dairy farm were similar to ACTH-induced cysts, with a mean diameter of  $25.8 \pm 3.6$  mm, larger than ovulatory follicles in control animals ( $P < 0.05$ ), but not different from induced follicular cysts ( $P > 0.1$ ). Microscopically, healthy developing follicles, atretic follicles, and 1 or 2 large cyst(s) with a complete granulosa of variable thickness, with degeneration signs in some cases, were also observed. Corpora lutea were absent in all cases (Fig. 2).

### 3.2. Effects of ACTH on blood steroid hormone concentrations

Daily blood samples in estrus-synchronized heifers (controls) and ACTH-treated animals during the treatment and 1 wk after completion of treatment showed effects of the treatment and time ( $P < 0.05$ ) on concentrations of all hormones and a treatment-by-time interaction in estradiol, progesterone, and cortisol (Fig. 3).

Estradiol was higher ( $P < 0.05$ ) in blood of ACTH-treated animals during treatment (days 15–18). At the beginning of the subsequent estrous cycle (days 23–28), estradiol decreased ( $P < 0.05$ ) in control animals and continued to increase ( $P < 0.05$ ) in ACTH-treated animals. In ACTH-treated animals, progesterone was greater ( $P < 0.05$ ) at the end of the treatment (days 17–20) and then decreased



**Fig. 4.** Serum levels of estradiol, progesterone, testosterone, and cortisol in serum and follicular fluid at the time of ovariectomy. Bars represent the mean  $\pm$  SEM for each hormone in serum or follicular fluid. Bars with different letters are different ( $P < 0.05$ ) ( $n = 5$  animals for controls and ACTH-induced cysts and  $n = 10$  for spontaneous follicular cysts).

and increased ( $P < 0.05$ ) in estrus-synchronized controls from day 22 because of the formation of a new CL after ovulation. Testosterone showed a similar profile in both groups until day 27, when testosterone increased ( $P < 0.05$ ) in control animals. Cortisol was greater ( $P < 0.05$ ) during ACTH treatment (days 15–21) and then decreased to control values after day 23.

### 3.3. Steroid hormone concentrations in serum and follicular fluid at sampling time

The concentrations of estradiol, progesterone, testosterone, and cortisol in serum and follicular fluid of ACTH-treated animals, cows with spontaneous cysts obtained at the dairy herd, and estrus-synchronized heifers (controls) at ovariectomy are shown in Figure 4. Control animals had greater ( $P < 0.05$ ) progesterone concentration in serum, although always  $<1$  ng/mL. Estradiol and cortisol did not change ( $P > 0.05$ ), whereas testosterone tended to increase ( $P = 0.06$ ) in serum from cows with spontaneous cysts.

In follicular fluid, estradiol was similar in all groups but tended ( $P = 0.055$ ) to decrease in ACTH-induced follicular cysts in relation to preovulatory follicles. Progesterone was lower ( $P < 0.05$ ) in spontaneous and induced follicular cysts than in preovulatory follicles. Testosterone was greater ( $P < 0.05$ ) in spontaneous follicular cysts than in preovulatory follicles. Cortisol concentration increased ( $P < 0.05$ ) in animals with COD.

### 3.4. Immunolocalization of 11 $\beta$ HSDs proteins

To obtain information about the localization of 11 $\beta$ HSD proteins in different follicular structures, their expression was evaluated by immunohistochemistry and quantitative image analysis. In estrus-synchronized control animals, a moderate expression of 11 $\beta$ HSD1 was observed in granulosa, with an increase from primary to atretic follicles ( $P < 0.05$ ) (Figs. 5 and 6). The immunopositive area in theca interna was lower than that in granulosa ( $P < 0.05$ ), and no differences were found across the different follicle stages. The expression of 11 $\beta$ HSD2 was lower in the granulosa layer of primary follicles than in secondary, tertiary, and atretic follicles ( $P < 0.05$ ). The level of immunostaining of 11 $\beta$ HSD2 in theca interna was lower than in granulosa ( $P < 0.05$ ). In addition, secondary follicles showed a lower expression than tertiary and atretic follicles ( $P < 0.05$ ). The theca externa layer had a weak or absent immunostaining for 11 $\beta$ HSD1 and 11 $\beta$ HSD2, and the expression was not quantified because the values did not differ from the background.

In follicular cysts of ACTH-treated heifers and spontaneously happening at the dairy herd, differences were observed only in 11 $\beta$ HSD1 expression in granulosa relative to controls (Fig. 7). The immunopositive area of 11 $\beta$ HSD1 was higher in the granulosa of tertiary follicles and follicular cysts of animals with ACTH-induced COD than in tertiary follicles of estrus-synchronized controls ( $P < 0.05$ ) (Figs. 6 and 7). In animals with spontaneous cysts, the staining in follicular cysts was also higher than that in tertiary follicles of controls ( $P < 0.05$ ) (Figs. 6 and 7). The immunopositive area of 11 $\beta$ HSD1 decreased in atretic follicles from both animals with spontaneous cysts and animals with ACTH-

induced cysts, in relation to preovulatory follicles from control animals ( $P < 0.05$ ).

### 3.5. Effects of ACTH on 11 $\beta$ HSD1 and 11 $\beta$ HSD2 protein expression in the follicular wall

The analysis of the expression of 11 $\beta$ HSD1 and 11 $\beta$ HSD2 by Western immunoblot in the follicular wall of preovulatory and follicular cysts obtained at the abattoir is presented in Figure 8. The 11 $\beta$ HSD1 antibody recognized bands of 34 and 68 kDa, corresponding to monomeric and dimeric forms, and the dimeric form is the most functionally relevant. By contrast, the 11 $\beta$ HSD2 antibody recognized a band of 48 kDa. After 3 h of incubation, basal expression of both enzymes was found in all samples, with higher ( $P < 0.05$ ) expression in follicular cysts than in preovulatory follicles. These differences persisted in ACTH-stimulated tissues. After ACTH stimulation, an increase ( $P < 0.05$ ) was found in 11 $\beta$ HSD1 expression in large antral follicles and follicular cysts with a decreased 11 $\beta$ HSD2 expression (Fig. 8).

### 3.6. Effects of ACTH on secretion of steroids by the follicular wall

Steroid concentrations in the incubation medium of follicular wall cultures of preovulatory follicles and follicular

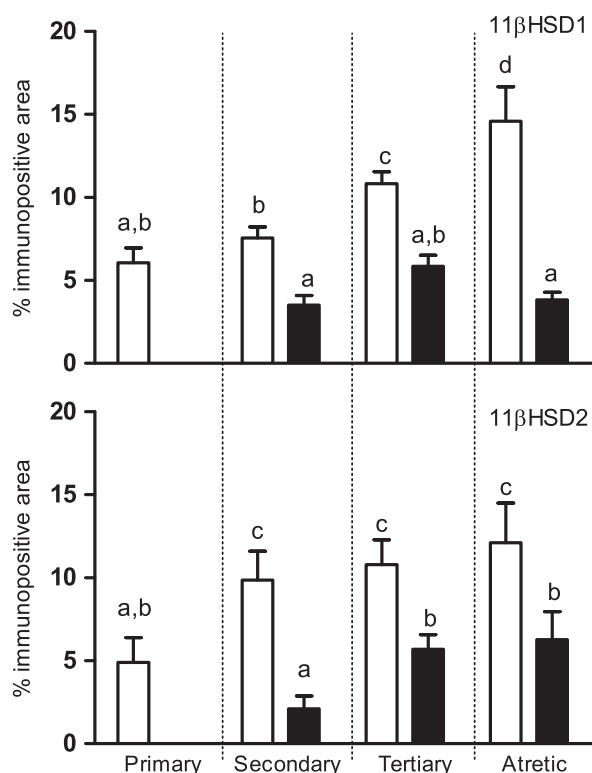
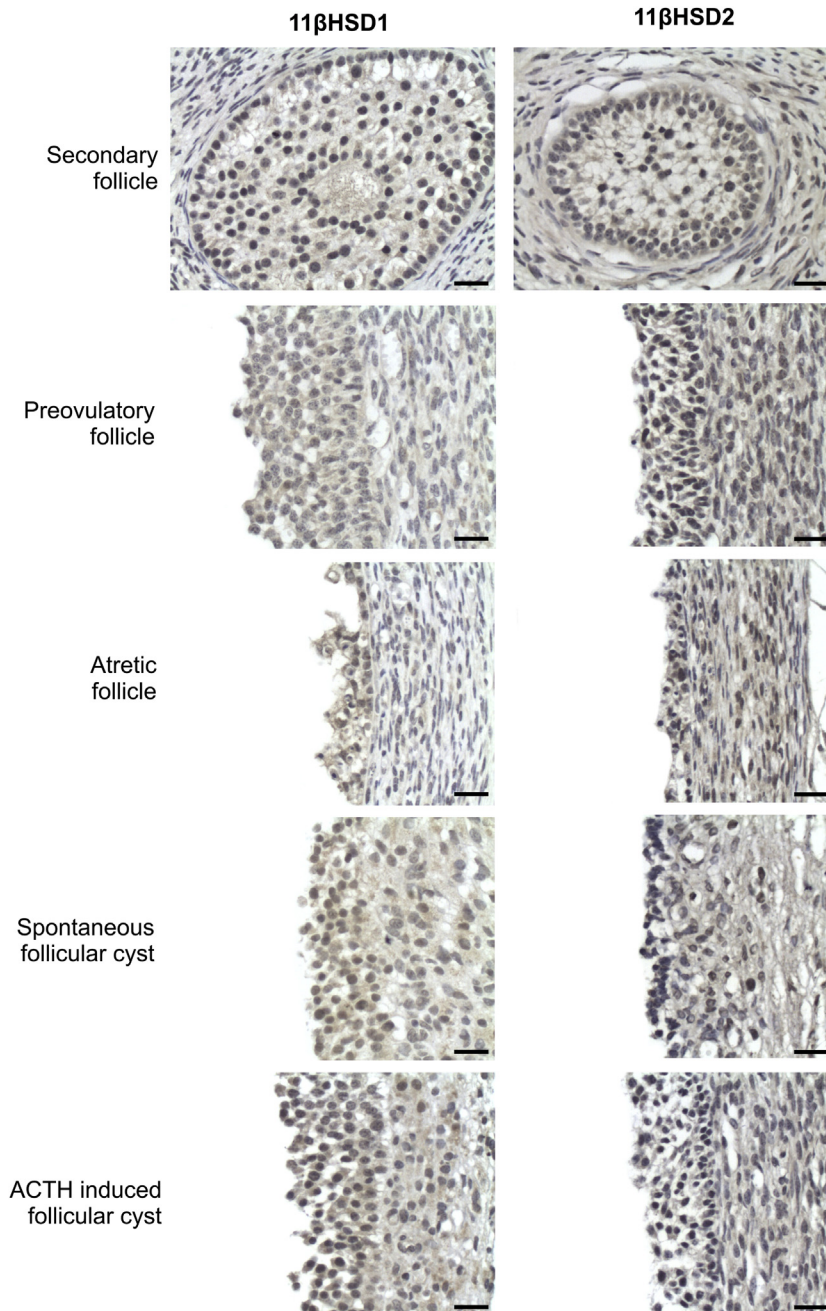


Fig. 5. Relative expression (measured as percentage of immunopositive area) of 11 $\beta$ HSD1 and 11 $\beta$ HSD2 in granulosa (white bars) and theca interna (black bars) of primary, secondary, tertiary, and atretic follicles from the estrus-synchronized control group. Values represent mean  $\pm$  SEM. Bars with different letters are different ( $P < 0.05$ ). 11 $\beta$ HSD1, 11 $\beta$ -hydroxysteroid dehydrogenase type 1; 11 $\beta$ HSD2, 11 $\beta$ -hydroxysteroid dehydrogenase type 2.





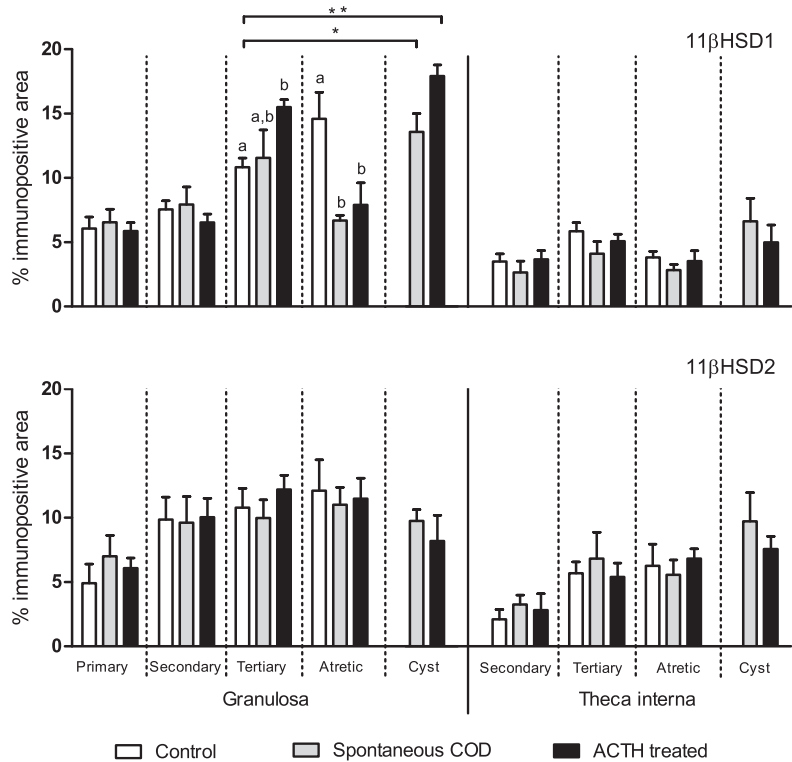
**Fig. 6.** Representative images of 11 $\beta$ HSD1 and 11 $\beta$ HSD2 immunostaining in secondary, preovulatory, and atretic follicles of the estrus-synchronized control group and follicular cysts of spontaneous and ACTH-induced COD groups. Scale bars = 20  $\mu$ m. COD, cystic ovarian disease; 11 $\beta$ HSD1, 11 $\beta$ -hydroxysteroid dehydrogenase type 1; 11 $\beta$ HSD2, 11 $\beta$ -hydroxysteroid dehydrogenase type 2.

cysts obtained at the abattoir are presented in Figure 9. After 3 h of incubation, basal concentrations of steroid secretion were detected in all samples, with higher ( $P < 0.05$ ) concentrations of estradiol and testosterone in follicular cysts than in large antral follicles. By contrast, progesterone showed a lower basal secretion in follicular cysts. These differences persisted in ACTH-stimulated tissues. After ACTH stimulation, testosterone and cortisol secretions increased

( $P < 0.05$ ) in large antral and follicular cysts. In addition, estradiol increased ( $P < 0.05$ ) in follicular cysts in response to ACTH.

#### 4. Discussion

Treatment with ACTH resulted in changes in serum steroid concentrations. Estradiol, progesterone, and cortisol



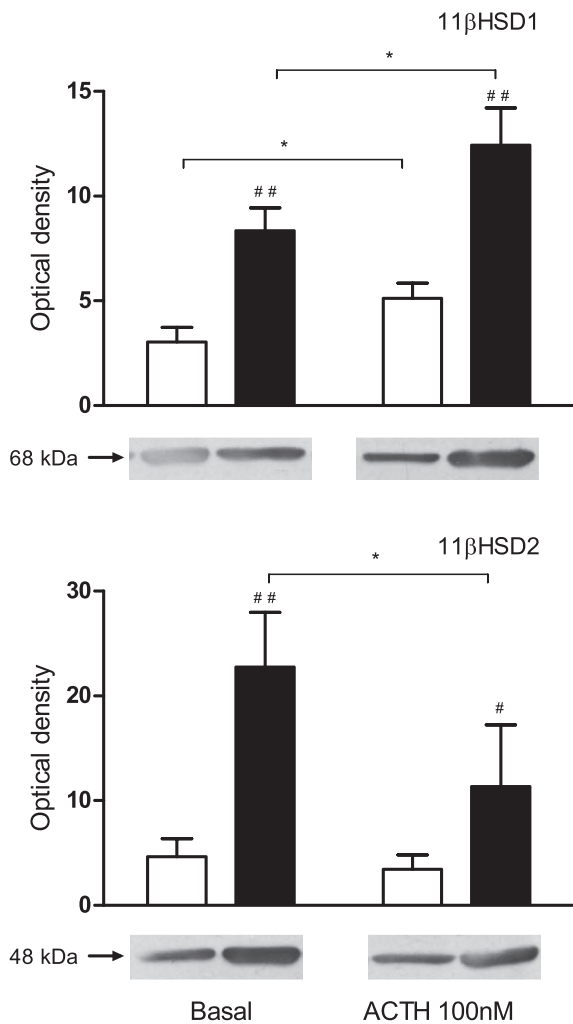
**Fig. 7.** Relative expression (measured as percentage of immunopositive area) of 11βHSD1 and 11βHSD2 in granulosa and theca interna of primary, secondary, tertiary, and atretic follicles and follicular cysts in estrus-synchronized controls, spontaneous COD, and ACTH-induced COD groups. Values represent mean ± SEM. Bars with different letters within each follicle category are different ( $P < 0.05$ ). Differences relative to the expression in follicular cysts in relation to control tertiary follicles are also indicated: \* $P < 0.05$  and \*\* $P < 0.01$ . COD, cystic ovarian disease; 11βHSD1, 11β-hydroxysteroid dehydrogenase type 1; 11βHSD2, 11β-hydroxysteroid dehydrogenase type 2.

concentrations increased during ACTH treatment, whereas testosterone showed no changes. After ACTH treatment, estradiol remained increased, whereas progesterone did not increase as in controls. This would reflect follicular persistence and lack of luteinization, respectively. This is consistent with results of Dobson et al [5], who described that estrogen remains high until approximately day 30 and proposed that estradiol, along with inhibin (but not ACTH, cortisol, or progesterone), exerts a strong negative feedback on FSH secretion [51]. However, these investigators did not analyze the concentration of testosterone.

Cortisol in follicular fluid was higher in cysts than in controls, confirming the existence of a mechanism to regulate its intrafollicular concentration. Cortisol concentration in follicular fluid was even greater in animals treated with ACTH, confirming our previous results that indicated a direct action of ACTH in the follicular wall [6]. In this sense, no alterations have been detected in adrenal function or in serum cortisol concentration between normal cycling cows and cows with ovarian follicular cysts [52], in agreement with our results. We found differences in serum cortisol only during treatment but not at the time of cyst formation. Thus, we found no evidence that cyclic cows and cows with COD differed in adrenal function. Because glucocorticoid is synthesized only in the adrenal cortex and is delivered systemically to the whole body, the presence of

an enzymatic pathway is crucial for glucocorticoids to modulate local concentration of active glucocorticoids [31,32].

It is well known that ACTH is acutely released from the pituitary gland in response to stressor stimulation and is the primary signal for adrenal cortisol biosynthesis. The stress-induced release of glucocorticoids is known to inhibit the immune [53], reproductive [4,54], and endocrine [55] systems. The expression of 11βHSD1 and 11βHSD2 in bovine ovarian tissues, together with the reported direct glucocorticoid action in cultured follicular cells [19,22,56], indicates that the bovine ovary is a glucocorticoid target organ that is also capable of modulating the local glucocorticoid environment [32]. In addition, the expression of melanocortin receptors (MCRs) in the follicular wall could indicate that the ovarian follicle is a direct target organ of ACTH, indicating a role for this hormone in modulating gonadal function through MCRs. Specifically, MC2R (the main signaling receptor for ACTH in the adrenal gland) mRNA and MC5R mRNA are expressed in the theca of bovine antral follicles [6]. Because the theca expresses CYP17A1, a key enzyme in the steroidogenic pathway that produces the precursors of cortisol [57], it was expected that cortisol secretion could be stimulated by ACTH, by the presence of its receptors in theca. In this sense, when we analyzed steroid hormone secretion in vitro, basal secretion



**Fig. 8.** Effects of ACTH on the expression of 11βHSD1 and 11βHSD2 analyzed by Western immunoblot analysis in the follicular wall of cultured preovulatory follicles (white bars) and follicular cysts (black bars). Values represent mean ± SEM. Differences in basal and stimulated secretion between preovulatory follicles and follicular cysts are indicated: \* $P < 0.05$ . Differences relative to basal secretion are also indicated: # $P < 0.05$  and ## $P < 0.01$ . 11βHSD1, 11β-hydroxysteroid dehydrogenase type 1; 11βHSD2, 11β-hydroxysteroid dehydrogenase type 2.

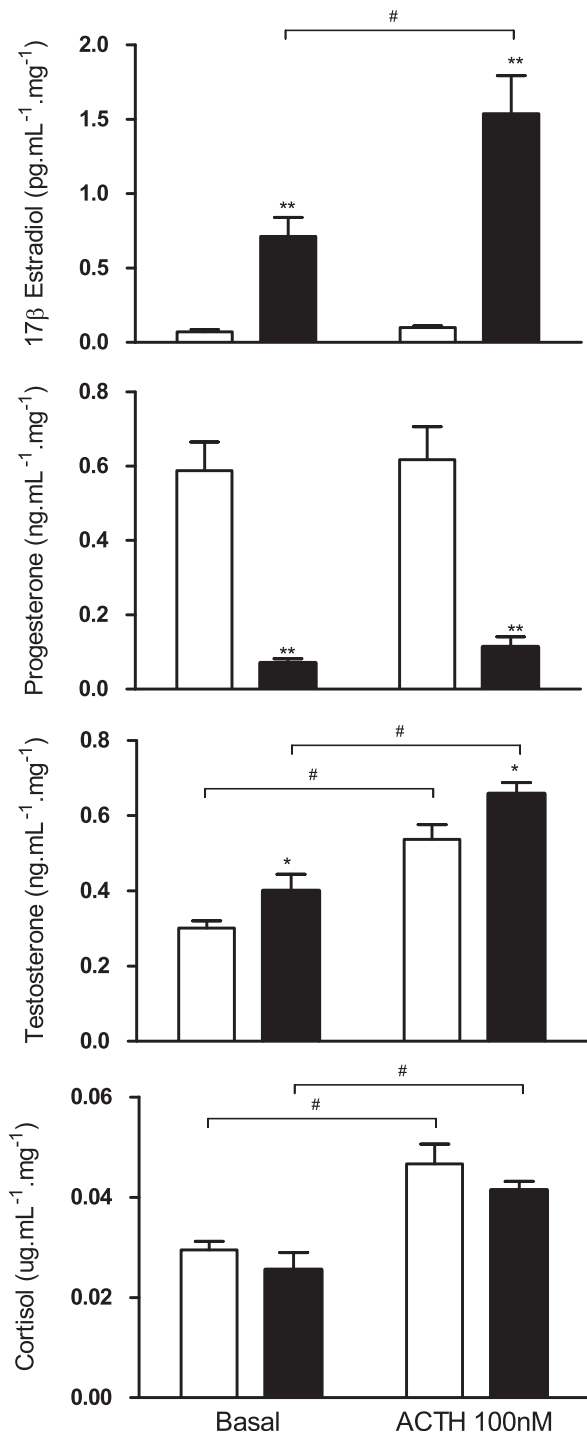
of estradiol and testosterone was lower in large antral follicles than in follicular cysts. In contrast, basal progesterone secretion was higher in preovulatory than in follicular cysts. However, ACTH stimulation has an effect on estradiol secretion by follicular cysts wall and also a stimulatory effect on testosterone and cortisol in the follicular wall of both preovulatory follicles and follicular cysts. These results are in agreement with our previous results in the bovine ovary [6].

Administration of ACTH to gilts during the luteal phase increases both serum and follicular cortisol concentrations [58] without disrupting gonadotropin secretion [59]. Acosta et al [21] reported a temporal increase in follicular cortisol concentration around the preovulatory LH surge in cattle. However, although concentrations of active

glucocorticoids were decreased during ovarian follicular development and maturation, glucocorticoids were increased during the ovulatory process triggered by an LH surge, suggesting a functional role for ACTH in the stimulation of ovarian cortisol secretion. In addition, the fact that the follicular wall of antral follicles had higher expression of MCRs could be related to the stimulatory effect of ACTH on estradiol and cortisol secretion. The expression of MC2R could also be associated with the stimulatory effect of ACTH on testosterone secretion in large antral follicles, perhaps associated with a decrease in P450 aromatase activity. If we take into account the differentiation of follicular walls, the increased ACTH secretion previously reported during stress in cattle could be responsible for the increase in testosterone that characterizes follicular cysts during COD in cattle [60].

In this study, we documented the immunolocalization of enzymes that catalyzed activation or inactivation of cortisol in the bovine ovary. In this sense, immunohistochemistry is currently the only technique that allows an integrative analysis of the cellular localization of proteins in nearby tissue compartments when it is not possible to separate cell populations to perform other more sensitive and specific techniques. We demonstrated that all follicular stages in the bovine ovary express 11βHSD1 and 11βHSD2 in a developmentally related way. In healthy ovaries, immunostaining of 11βHSD1 in granulosa increased as follicles matured and remained constant in theca cell layers. In addition, atretic follicles reached the highest expression of 11βHSD1 in granulosa. However, 11βHSD2 protein expression increased from secondary follicles onward in the granulosa and from tertiary follicles onward in the theca. Spontaneous and induced COD showed similar immunexpression patterns with differences only in the levels of 11βHSD1 in granulosa in relation to controls. Granulosa of follicular cysts evidenced higher levels of 11βHSD1 in relation to tertiary follicles of controls, and the expression in atretic follicles of COD ovaries was lower than that in healthy ovaries. The pattern of expression evidenced by immunohistochemistry is partially related to that previously described by other investigators.

With the use of real-time PCR, Tetsuka et al [33] showed that gene expression of 11βHSD1 mRNA in healthy follicles increased as follicles matured, both in granulosa and theca. In addition, the expression of 11βHSD2 mRNA was low and unchanged during follicular maturation, whereas in atretic follicles a drastic increase in the expression of 11βHSD2 was observed by these investigators. [33] In another study, using PCR and Western blot analysis, Thurston et al [34] documented changes in 11βHSD protein expression and cofactor-dependent enzyme activities across the bovine ovarian cycle in granulosa. Trends in 11βHSD activities that depend on nicotinamide adenine dinucleotide phosphate and the oxidized form of nicotinamide adenine dinucleotide paralleled changes in the expression of the 11βHSD1 and 11βHSD2 proteins, which in turn reflected the expression of mRNA encoding these enzyme proteins. These investigators suggested that the balance of 11βHSD enzyme activities may be set, to a large extent, at the level of enzyme translation, if not at the level of enzyme transcription. This could explain the differences observed



**Fig. 9.** Effects of ACTH on the secretion of estradiol, progesterone, testosterone, and cortisol by the follicular wall of large antral follicles (white bar) and follicular cysts (black bar). Bars represent the mean  $\pm$  SEM. Differences in basal and stimulated secretion between preovulatory follicles and follicular cysts are indicated: \* $P < 0.05$  and \*\* $P < 0.01$ . Differences relative to basal secretion are also indicated: # $P < 0.05$  ( $n = 6$  replicates).

between gene expression analyzed by PCR and protein localization studied by immunohistochemistry. In this study, we described changes in 11 $\beta$ HSD expression and

cortisol release in the follicular wall of large antral follicles and follicular cysts after the stimulation by ACTH. Follicular cysts showed a higher expression of both enzymes at basal conditions, whereas ACTH induced an increase in 11 $\beta$ HSD1 in both follicular categories and a decrease in 11 $\beta$ HSD2 in follicular cysts. This response is similar to that induced by the preovulatory gonadotropin surge that induces the expression of 11 $\beta$ HSD1 and decreases the expression of 11 $\beta$ HSD2 in follicles [61,62]. This results in a rapid increase in follicular cortisol just before follicular rupture [63]. The physiological importance of this event is not clear but may either enhance oocyte maturation [64,65] or prevent excess inflammation caused by ovulation [21,27]. Although this mechanism may temporarily increase the local concentration of glucocorticoids, forming an integral part of the regulatory mechanism in ovarian physiology, this could be part of a process leading to the formation of follicular cysts.

It has been established that glucocorticoids modulate ovarian function in cattle. Taking into account that the circulating concentration of cortisol is largely unchanged throughout the bovine estrous cycle [66–68], this enzymatic system may play an important role in regulating ovarian glucocorticoid action. However, the regulatory mechanisms have not been fully elucidated. In humans, the relative expression of 11 $\beta$ HSD1 and 11 $\beta$ HSD2 has been shown to determine the concentration of cortisol in follicles [62]. In addition, the levels of enzyme cofactors also influence the activities of 11 $\beta$ HSDs, and it has been found that follicular fluid of large antral follicles and cysts contain ovarian modulators of 11 $\beta$ HSD activity that depended on nicotinamide adenine dinucleotide phosphate. Moreover, because of a decreased content of 11 $\beta$ HSD stimuli accompanied by increased content of 11 $\beta$ HSD inhibitors, the follicular fluid of cysts exerts a net inhibitory effect on 11 $\beta$ HSD2 activity [35], with a decreased inactivation of cortisol in follicular cysts [69]. By contrast, because progesterone is a strong intrafollicular inhibitor of 11 $\beta$ HSD1 activity [70–75], ovulation failure and consequent lack of luteinization and low progesterone concentration, characteristic of COD, could exacerbate 11 $\beta$ HSD1 activity and cortisol production.

As has been previously determined by many investigators cysts undergo 1 of 3 fates: cysts may remain dominant for a prolonged period without other follicular growth, cysts may lose dominance and be replaced by a cyst from a new follicular wave (cyst turnover), or cysts may lose dominance and a new dominant follicle may develop and ovulate [76,77]. Delay of follicle regression after ovulation is a possible cause of cysts because a preovulatory follicle that cannot be ovulated will not grow further if it regresses immediately; therefore, no follicular cysts will be formed. In terms of the implications of these findings in the intra-ovarian COD pathogenesis, this study has shown that COD ovaries are exposed to higher concentration of cortisol and have the ability to further increase these concentrations in response to specific stimuli. Previous works have shown alterations in the apoptosis rate in follicles from cows with spontaneous and induced follicular cysts, and these findings support the notion that follicular persistence is an important component of COD pathogenesis [20,78]. In addition, in bovine and rat follicular cysts, we have previously described changes in cell differentiation, mostly

related to overexpression of cytoskeletal components [43,49]. In this context, the ability of glucocorticoids to inhibit granulosa apoptosis [79] may also be important in limiting atresia in persistent antral follicles (and may even prevent apoptotic degeneration of follicular cysts) [69,80,81]. In addition, glucocorticoids have been shown to stimulate granulosa differentiation [18] and so may participate in the differentiation of granulosa described in follicular cysts.

Stress-associated stimulation of the hypothalamic–pituitary–adrenal/ovary axis has been reported to disrupt reproductive function and has been associated with bovine COD pathogenesis [1]. Poor follicle development, reduced ovulation rates, and follicular cyst formation have been reported in association with ACTH treatment and increased concentrations of glucocorticoids [2–5]. Recently, we have confirmed the expression of ACTH receptors in bovine ovaries [6], and the fact that ACTH is able to induce steroid secretion and changes in 11 $\beta$ HSD expression from the ovary strongly indicates that ACTH could be involved in regulatory mechanisms related to ovarian function associated with ovulation, steroidogenesis, and COD pathogenesis. In conclusion, these findings indicate that ovarian follicles may be exposed to high local concentrations of active glucocorticoids and may indicate a local role for cortisol in COD pathogenesis and in regulatory mechanisms of ovarian function.

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