Original Paper

Cells Tissues Organs

Cells Tissues Organs
DOI: 10.1159/000442150

Accepted after revision: November 2, 2015
Published online: December 18, 2015

Role of Glucocorticoids in Cystic Ovarian Disease: Expression of Glucocorticoid Receptor in the Bovine Ovary

Ayelen N. Amweg[a, b] Fernanda M. Rodríguez[a] Emilia Huber[a] Belkis E. Marelli[a] Natalia R. Salvetti[a] Florencia Rey[a] Hugo H. Ortega[a]

[a]Laboratorio de Biología Celular y Molecular Aplicada, Instituto de Ciencias Veterinarias del Litoral (ICiVet-Litoral), Universidad Nacional del Litoral (UNL)/Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET), Esperanza, and [b]Fundación Bunge y Born, Buenos Aires, Argentina

Key Words
Bovine ovary · Cystic ovarian disease · Glucocorticoid receptor · Ovarian cysts

Abstract
The aim of this study was to characterize the expression of glucocorticoid receptor (GR) in the components of normal bovine ovary and in animals with cystic ovarian disease (COD). Changes in the protein and mRNA expression levels were determined in control cows and cows with COD by immunohistochemistry and real-time PCR. GR protein expression in granulosa cells was higher in cysts from animals with spontaneous COD and adrenocorticotropic hormone-induced COD than in tertiary follicles from control animals. In theca interna cells, GR expression was higher in cysts from animals with spontaneous COD than in tertiary follicles from control animals. The increase in GR expression observed in cystic follicles suggests a mechanism of action for cortisol and its receptor through the activation/inactivation of specific transcription factors. These factors could be related to the pathogenesis of COD in cattle.

Introduction

Cystic ovarian disease (COD) is a cause of temporary infertility and one of the most common reproductive disorders in dairy cows [Nelson et al., 2010]. Cysts are dynamic structures, described as single or multiple anovulatory follicles, located in one or both ovaries, which have a diameter >18 mm and persist for more than 6 days in the absence of luteal tissue without uterine tone and with disruption of normal estrous cycles [Silvia et al., 2002; Bartolomé et al., 2005]. It is known that one of the initial

Abbreviations used in this paper

ACTH	adrenocorticotropic hormone
CL	corpus luteum
COD	cystic ovarian disease
dNTP	deoxyribonucleotide triphosphate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GC	glucocorticoid
GR	glucocorticoid receptor
TNF	tumor necrosis factor

Dr. Hugo H. Ortega
Instituto de Ciencias Veterinarias del Litoral (ICiVet-Litoral), Universidad Nacional del Litoral (UNL)/Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET), R.P. Kreder 2805, Esperanza, Santa Fe 3080 (Argentina)
E-Mail hhortega@fcv.unl.edu.ar

causes that contribute to the pathogenesis of COD is a failure in the normal mechanism of ovulation, which leads to the absence or lack of coordination in the various endocrine components involved. Ovulation has been described as an inflammatory process where a series of events leads to proteolytic degradation of a specific point in the follicular wall to permit the release of the oocyte [Espey et al., 2004; Richards et al., 2008]. Glucocorticoids (GCs), which are stress-induced hormones, are synthesized under the control of the hypothalamic-pituitary-adrenal axis and have been described as anti-inflammatory agents that act to modulate the production of cytokines and prostaglandins in the different reproductive processes: ovulation, luteolysis, embryo implantation, fetal growth and placental development [Hillier and Tetsuka, 1998; Andersen, 2002; Myers et al., 2007; Tetsuka, 2007; Duong et al., 2012; Kuse et al., 2013]. Therefore, cortisol, an active GC released in response to stress, could inhibit ovulation due to its local action as an anti-inflammatory agent at the ovulatory site [Vanholder et al., 2006]. In this sense, it has been postulated that stress is one of the most important predisposing factors for COD and other reproductive diseases [Vanholder et al., 2006]. GCs exert their effects through binding to GC receptor (GR), a member of the nuclear steroid receptor superfamily [Nicolaides et al., 2010]. The GC-GR dimerizes, and then the complex is translocated to the nucleus and binds to specific DNA sequences, the GC response element in target genes, leading to the induction or repression of gene transcription [Lu and Cidlowski, 2006; Kino et al., 2011; Torres Uchoa et al., 2014]. Thus, GCs regulate mRNA expression of inflammatory molecules directly or indirectly through the synthesis of anti-inflammatory proteins, or, more importantly, by transrepression mechanisms [Adcock, 2000; Barnes, 2006; Lu and Cidlowski, 2006; Majewska et al., 2012].

In response to various stressors, adrenocorticotropic hormone (ACTH) stimulates the synthesis and secretion of GCs [Engelmann et al., 2004; Xing et al., 2010], which can affect reproduction directly through their actions on the hypothalamic-pituitary-ovarian axis, affecting the secretion of gonadotropin-releasing hormone in the hypothalamus, inhibiting gonadotropin secretion, especially of luteinizing hormone in the pituitary, and consequently altering follicular and luteal function [Charpenet et al., 1982; Rivier and Rivest, 1991; Tilbrook et al., 2000]. GCs act in the ovary and other tissues of the female reproductive system. Also, GCs directly regulate the action of gonadotropins and steroid biosynthesis in the ovary [Kawate et al., 1993; Andersen, 2002; Acosta et al., 2005; Sunak et al., 2007], where GR expression has also been demonstrated [Komiyama et al., 2008; Tetsuka et al., 2010; Park et al., 2012]. GCs also act as potent cytoprotective agents in the ovary, protecting against apoptosis and may play a role in minimizing the damage related to follicle rupture [Whirledge and Cidlowski, 2013]. On the other hand, cortisol modulates corpus luteum (CL) function by influencing progesterone secretion [Duong et al., 2012] and is responsible for the maintenance of the CL during early pregnancy in cattle [Duong et al., 2012; Majewska et al., 2012].

Although it has not been demonstrated that the ovary is able to produce GCs de novo, we have demonstrated that ACTH is able to stimulate the secretion of cortisol in the follicular wall in culture [Amweg et al., 2011], induce steroid hormone secretion and produce changes in 11β-hydroxysteroid dehydrogenase expression by the ovary [Amweg et al., 2013], indicating that ACTH may be involved in regulatory mechanisms related to ovarian function associated with ovulation, ovarian steroidogenesis, luteal function and COD pathogenesis [Tetsuka, 2007; Amweg et al., 2011, 2013].

The aim of this study was to characterize the expression of GR in the components of the bovine ovary and to establish changes in animals with COD. We examined the expression patterns of mRNA and protein of GR in ovaries from healthy cows and animals with spontaneous and ACTH-induced COD.

Materials and Methods

All the procedures with animals were approved by the Institutional Ethics and Security Committee (Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral, Santa Fe, Argentina) and are consistent with the Guide for the Care and Use of Agricultural Animals in Research and Teaching (Federation of Animal Science Societies 2010).

Induction of COD by ACTH Administration

Ten Holstein heifers (age, 18–24 months; body weight, 400–450 kg) were assigned to either the control (n = 5) or the ACTH-treated (n = 5) groups. Ovulation of all heifers was hormonally synchronized by the OvSynch protocol [Pursley et al., 1995]. The time of ovulation was monitored by daily transrectal ovarian ultrasonography and was designated as day 1 of the estrous cycle, because, as described by Pursley et al. [1995], ovulation occurs 24–32 h after the second injection of gonadotropin-releasing hormone.

The model of ACTH-induced ovarian follicular cysts used in the present study has been previously described in detail [Dobson et al., 2000; Ortega et al., 2008; Salvetti et al., 2010, 2012; Amweg et al., 2013]. Briefly, beginning on day 15 of a synchronized estrous cycle, 5 heifers received subcutaneous injections of 1 mg of a syn-

thetic polypeptide with ACTH activity (Synacthen Depot; Novartis, Basel, Switzerland) every 12 h for 7 days (treated group), whereas the other 5 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 (HS101V; Honda, Tokyo, Japan). The growth and regression of follicles >5 mm, CLs and follicular cysts were monitored. In control heifers, daily ovarian ultrasonography was performed through a complete estrous cycle (21–23 days), while in ACTH-treated heifers ultrasonography was performed from day 14 (day 1 = 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 ≥20 mm present for ≥10 days, without ovulation or CL formation [Dobson et al., 2000]. The first day of follicular cyst formation was the day on which a follicle attained ≥20 mm in diameter. The ovaries were removed 10 days later by flank laparotomy (approx. day 48).

In control heifers, ovariectomy was conducted when the dominant follicle reached a diameter >10 mm, in the absence of an active CL, to obtain preovulatory follicles (approx. day 18).

Blood samples were obtained daily throughout the entire experiment to test estradiol, progesterone, testosterone and cortisol concentrations, and these data have been published previously [Ortega et al., 2008; Amweg et al., 2013].

Spontaneous COD

Cases of spontaneous COD were diagnosed through routine reproductive controls in dairy herds, through the cooperation of veterinarians in the area of influence of the Faculty of Veterinary Science (UNL), Esperanza, Argentina. Ovarian alterations compatible with COD were verified by ultrasound (transrectal linear transducer, 5-MHz HS101V) taking into account the definition of COD described by Bartolomé et al. [2005]. Ten Argentinean Holstein cows that showed ≥1 follicular cysts of ≥20 mm in diameter persisting for at least 10 days in the absence of a CL were selected. The ovaries were removed by transvaginal ovariectomy [Marelli et al., 2014], and blood samples were collected just before surgery for 17β-estradiol, testosterone and progesterone determination. The follicular fluid obtained from the cystic follicles was stored at –20°C for 17β-estradiol, testosterone and progesterone determination. These data, which confirmed the physiological state of follicles, have been published previously [Amweg et al., 2013].

Additionally, ovaries from 60 cows were collected at a local abattoir, within 20 min of death, from mixed breeds of *Bos taurus* cows assessed visually as being nonpregnant and without macroscopic abnormalities in the reproductive system. Ovaries were collected from 45 normal cycling cows (ovaries showed macroscopically different follicular categories without active CL) and 15 animals with COD with cystic follicles diagnosed as follicle of ≥20 mm without active CL in both ovaries [Silvia et al., 2002]. The ovaries were washed, refrigerated and immediately transported to the laboratory. Each pair of ovaries was placed on ice, and the antral follicles (control and cystic) were removed using scissors and scalpel dissection. Before the ovaries were dissected, the follicular diameter was measured using calipers and the follicular fluid from each follicle was aspirated and stored separately at –20°C for 17β-estradiol, testosterone and progesterone determination. These data, which confirmed the physiological state of follicles, have been published previously [Marelli et al., 2014].

Tissue Sampling and Processing, and Classification of Follicles

Ovaries obtained by ovariectomy from spontaneous COD, ACTH-induced COD and estrus-synchronized control groups were immediately fixed in 4% buffered formaldehyde for 8 h at 4°C and then washed in PBS. For light microscopy, fixed tissues were dehydrated in an ascending series of ethanol, cleared in xylene and embedded in paraffin. Sections (5 μm thick) were mounted on slides previously treated with 3-aminopropyltriethoxysilane (Sigma-Aldrich, St. Louis, Mo., USA) and primarily stained with hematoxylin-eosin for preliminary observation of all ovarian structures [Ortega et al., 2008; Salvetti et al., 2010]. Primary, secondary and tertiary follicles from all groups, and cystic follicles from animals with spontaneous and ACTH-induced COD were analyzed [Dellmann and Priedkalns, 1993].

For RT-PCR and real-time PCR, samples from control animals and animals with COD obtained from the slaughterhouse were processed. Healthy tertiary follicles from normal cycling cows (controls) were classified into three categories according to their calculated follicle diameters, as described previously [Parrot and Skinner, 1998]: small (<5 mm, n = 15), medium (5–10 mm, n = 15) or large (>10 mm, n = 15). Follicles with an obviously atretic appearance (avascular theca and debris in the antrum) were discarded. Cystic follicles were diagnosed as a follicle of ≥20 mm without CL in both ovaries (n = 15) [Silvia et al., 2002]. Granulosa cells were obtained by centrifugation of follicular fluid, aspirated with a syringe from the different tertiary follicles and cysts, and immediately frozen at –80°C until total RNA extraction. Theca cells of tertiary follicles from control animals and cystic follicles from animals with spontaneous COD were washed with diethyl pyrocarbonate water (Genbiotech, Buenos Aires, Argentina) to remove granulosa cells and stored at –80°C until total RNA extraction.

Total RNA Extraction

Total RNA was isolated from granulosa and theca samples after treatment with TRIzol LS reagent (Invitrogen, Life Technologies Corporation, Carlsbad, Calif., USA) according to the manufacturer's instructions with slight modifications. Also, adrenal samples were processed as positive control. Briefly, 50–100 mg of tissue were homogenized with 750 μl of TRIzol LS reagent and incubated for 10 min at 25°C. Then, RNA was purified by vigorously homogenizing with chloroform and incubating for 15 min at 4°C. After centrifugation at 12,000 *g*, the aqueous phase was incubated overnight with an equal volume of isopropyl alcohol at –20°C and centrifuged at 12,000 *g* to obtain the pellet of mRNA that was then washed with 75% ethanol for 10 min at 4°C. Alcohol was replaced by diethyl pyrocarbonate water prewarmed at 60°C. The extracted RNA was DNase treated with deoxyribonuclease I (Invitrogen) to eliminate contaminating DNA and stored at –80°C pending further use.

PCR Primer Design

Bovine sequences for GR, cytochrome P_{450} aromatase (CYP-19a1) and cytochrome P_{450} 17-hydroxylase/17,20-lyase (CYP17a1) were obtained from GenBank (http://www.ncbi.nlm.nih.gov/Entrez/index.htm). Specific primers were designed using the PrimerSelect program in the Lasergene software (DNAStar, Madison, Wis., USA). The primers were purchased from Invitrogen, and the sequences are summarized in table 1. Oligonucleotide primers and amplification products were tested using the Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/BLAST) soft-

Table 1. Primer sequences, regions of the target genes and conditions used for quantitative RT-PCR

Name	Sequence (5′–3′)	Gene accession No.	Amplicon position	Amplicon size, bp	Annealing temp., °C	Cycles, n
GR						
Forward	TTCCTGCAACATTACCACAACTCA	NM_00120663	1,948–2,095	167	57	40
Reverse	TTTCACGGCTGCAATCACCT					
GAPDH						
Forward	CACCCTCAAGATTGTCAGCA	BC102589	492–594	103	52	35
Reverse	GGTCATAAGTCCCTCCACGA					
CYP17						
Forward	GGAGGCGACCATCAGAGAAGTGC	NM_174304	1,105–1,423	319	60.8	35
Reverse	CAGCCGGGACATGAAGAGGAAGAG					
CYP19						
Forward	TAAAACAAAGCGCCAATCTCTACG	BTCYP19	8–348	341	55.4	35
Reverse	GGAACCTGCAGTGGGAAATGA					

ware to confirm gene specificity and determine exon locations, assuring that they were not designed from any homologous regions coding for other genes. The primer sequences for CYP19a1 and CYP17a1 were designed and validated to confirm bovine granulosa and theca cell mRNA purity (no cross contamination) [Lagaly et al., 2008]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene [Shibaya et al., 2007].

Reverse Transcription

First-strand cDNA was synthesized from the RNA samples treated with DNase I (Invitrogen) using a master mix (MMLV), buffer, dithiothreitol, RNAout, MMLV reverse transcriptase, deoxyribonucleotide triphosphate (dNTP) and random primers (Invitrogen). The reverse transcription conditions were as described in a previous study [Amweg et al., 2011].

End-Point PCR

An optimized end-point PCR protocol was used to analyze the mRNA expression of CYP19a1 and CYP17a1. The reaction conditions were as described in a previous study [Amweg et al., 2011]. The primer sequences used are described in table 1. PCR was carried out in a final volume of 25 μl containing 10 ng cDNA (previously quantified by the Qubit method; Invitrogen), 1.5 mM $MgCl_2$, 0.5 μM forward primer, 0.5 μM reverse primer, 0.2 mM dNTP, 2.5 μl Taq buffer 10× and 2 U Taq polymerase (5 U/μl; all reagents were from Invitrogen). Amplification conditions included 35 cycles of denaturation at 94°C for 45 s, annealing at 55.4–60.8°C (table 1) for 30 s and extension at 72°C for 90 s. Finally, a final extension step at 72°C for 10 min was performed.

Real-Time PCR

An optimized real-time PCR protocol was used to analyze the mRNA expression of GR using SYBR Green I (Invitrogen) technology in a LightCycler Detection System [Pfaffl, 2001]. Transcript levels were measured by relative quantitative real-time PCR using a StepOne real-time PCR system (Applied Biosystems, Life Technologies Corporation, Foster City, Calif., USA). For GR, the following standardized protocol was used: initial denaturation at 98°C for 3 min followed by 40 cycles of denaturation at 98°C for 5 s, annealing at 57°C for 15 s, extension at 72°C for 20 s and fluorescence reading at 81°C for 10 s. A master mix of the reaction components was prepared using SYBR Green I. Each PCR was performed in a final volume of 20 μl containing 4 μl cDNA and 16 μl of a reaction mixture: forward primer (20 μM), reverse primer (20 μM), 0.2 mM of each dNTP, 1 μl SYBR Green I, 4 μl buffer 5× and 0.05 μl enzyme Phire (Hot Start II DNA Polymerase; Thermo Fisher Scientific Company, Espoo, Finland). For GAPDH, the following standardized protocol was used: initial denaturation at 95°C for 10 min followed by 35 cycles of denaturation at 95°C for 15 s, annealing at 52°C for 20 s, extension at 72°C for 30 s and fluorescence reading at 74°C for 10 s. Each PCR was performed in a final volume of 20 μl containing 4 μl cDNA and 16 μl of a reaction mixture: forward primer (20 μM), reverse primer (20 μM), 0.2 mM of each dNTP, 1 μl SYBR Green I, 2 μl buffer Taq 10×, 50 mM $MgCl_2$ and 0.32 μl Taq DNA polymerase (Invitrogen). All measurements for each sample were performed in duplicate. The efficiency of the PCR and relative quantities were determined from a six-point standard curve. Standard curves were constructed from 50% serial dilutions of a cDNA mixture of granulosa and theca cells, and PCR efficiency was calculated using the StepOne software v2.2. In standard curves, R2 equals 0.99 for GR and 0.98 for GAPDH. About 20 ng cDNA, previously quantified by the Qubit method (Invitrogen), were used. The primer sequences used are described in table 1. mRNA expression levels of genes were recorded as cycle threshold (C_T) values that corresponded to the number of cycles at which the fluorescence signal can be detected above a threshold value. C_T was calculated manually using StepOne v2.2. Negative DNA template controls were included in all the assays, and yielded no consistent amplification. Product purity was confirmed by dissociation curves, and random samples were subjected to agarose gel electrophoresis. Fold change was determined using the $2^{-\Delta\Delta C_T}$ method [Livak and Schmittgen, 2001].

The specificities of the PCR products were checked by direct sequencing to ensure amplification of the correct sequences using

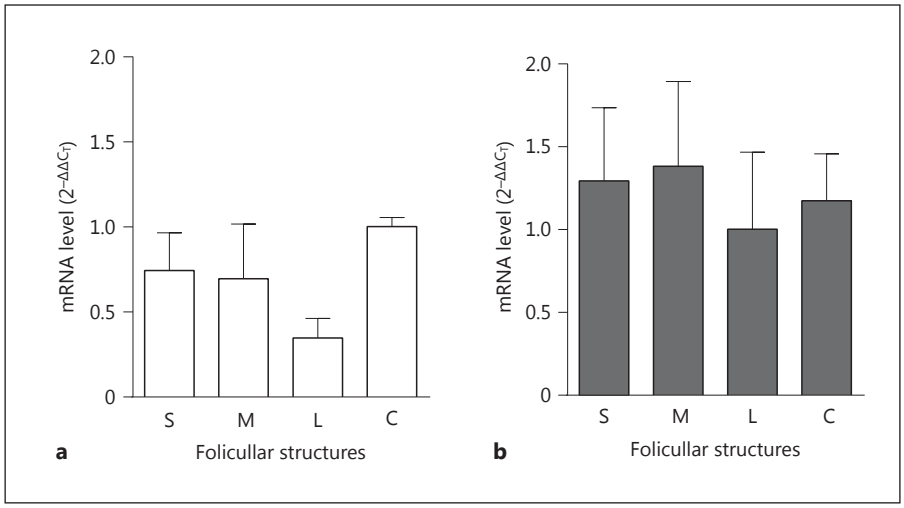


Fig. 1. Relative quantification of GR mRNA by real-time PCR in granulosa (**a**) and theca (**b**) cells of small (S), medium (M) and large (L) tertiary control follicles, and cystic follicles (C). The $2^{-\Delta\Delta C_T}$ values are represented as means ± SEM. $p > 0.05$.

the Macrogen sequencing service (Macrogen, Seoul, South Korea). The resulting sequences were verified using the MegAlign tool in the Lasergene software (DNAStar, Madison, Wis., USA).

Immunohistochemistry

Histological 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 GR protein expression in follicular walls. A streptavidin-biotin immunoperoxidase method was performed as previously described [Salvetti et al., 2004; Ortega et al., 2009; Amweg et al., 2013]. Briefly, after deparaffinization, microwave pretreatment (antigen retrieval) was performed by incubating the sections in 0.01 M citrate buffer (pH 6). Endogen peroxidase activity was inhibited with 3% (vol/vol) H_2O_2 in methanol, and nonspecific binding was blocked with 10% (vol/vol) normal goat serum. All sections were incubated with the GR primary antibody for 18 h at 4°C and then for 30 min at room temperature with polyvalent biotinylated link (CytoScan HRP Detection System; Cell Marque, Rocklin, Calif., USA). The antigens were visualized by HRP streptavidin label (CytoScan HRP Detection System; Cell Marque), and 3,3′-diaminobenzidine (liquid DAB-Plus substrate kit; Zymed, San Francisco, Calif., USA) was used as the chromogen. Finally, the slides were washed in distilled water, counterstained with Mayer's hematoxylin, dehydrated and mounted.

GR monoclonal primary antibody (ab2768; Abcam, Cambridge, Mass., USA) was used at a concentration of 1:25. GR antibody was measured in at least 5 sections of each ovary from each animal. The homology between the target peptide of the antibody and the corresponding bovine protein was tested with BLAST software to determine the peptide location and to confirm antigen specificity. Also, the specificity of the primary antibody was tested by Western blot as described previously [Ortega et al., 2009; Salvetti et al., 2010].

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, Tokyo, Japan), with an objective magnification of ×40, and analyzed with the Image Pro-Plus 3.0.1 system (Media Cybernetics, Silver Spring, Mass., USA). The positive controls were used as interassay controls to maximize the levels of accuracy and robustness of the method [Ranefall et al., 1998; Ortega et al., 2009, 2010].

Immunohistochemical preparations were evaluated according to those described by Salvetti et al. [2012]. In this case, using the color segmentation tool, the total area positively stained with DAB was measured [brown moderate-intense reaction product (to differentiate background color)] and expressed as the percentage of total area of nuclei (brown reaction product + blue hematoxylin). This procedure was performed in each follicular wall layer (granulosa and theca interna) from at least 50 images of the primary, secondary, tertiary and atretic follicles and follicular cysts.

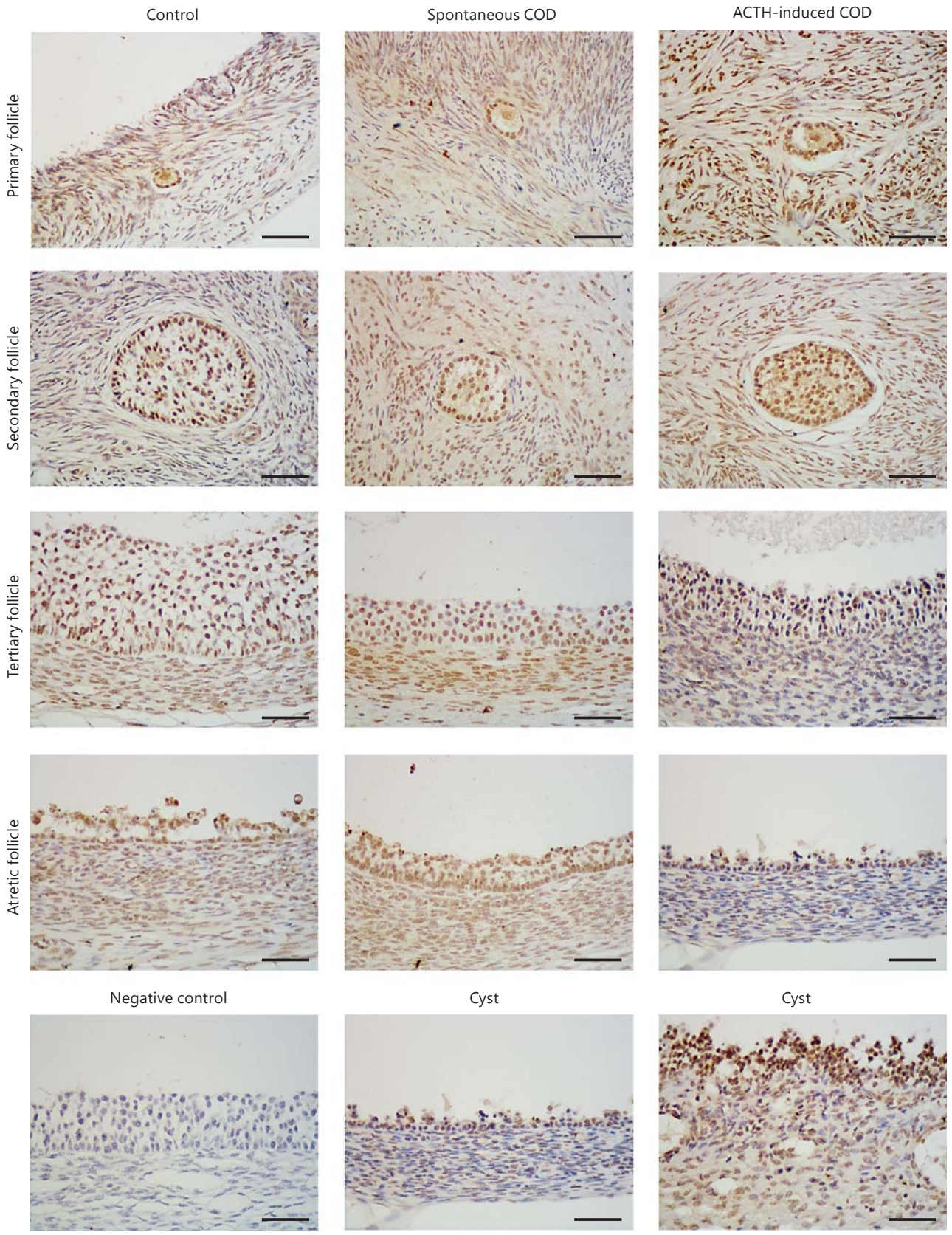
Statistical Analysis

The statistical software package SPSS 11.0 for Windows (SPSS Inc., Chicago, Ill., USA) was used to analyze the results of immunohistochemical analysis. The differences between two groups of data were analyzed using an unpaired two-tailed Student's t test. The differences between more than two groups of data were assessed by one-way ANOVA, followed by Duncan's multiple range tests. For all statistical analyses, $p < 0.05$ was considered significant. Results are expressed as means ± SEM.

Results

GR Gene Expression

We compared the expression of GR mRNA in real-time PCR products containing the same amount of GAPDH cDNA. All granulosa cell samples were positive for CYP19a1 mRNA and negative for CYP17a1 mRNA, whereas the theca cell samples were positive for CYP17a1 mRNA and negative for CYP19a1 mRNA expression. In addition, the negative controls performed using water instead of cDNA controls were negative to the reaction, and the positive control using adrenal tissue was positive.



2

(For legend see next page.)

The identity of the PCR products was confirmed by sequencing (100% homology with bovine sequences). GR mRNA was expressed in granulosa and theca cells in small, medium and large tertiary control follicles, and cystic follicles. No significant differences in GR gene expression were obtained in the different follicular categories analyzed in both cell populations ($p > 0.05$; fig. 1).

Immunolocalization and Expression of GR Protein

GR immunoexpression was observed in the nucleus of both granulosa and theca interna cells of all follicular categories analyzed (fig. 2) and an increased GR expression was observed in granulosa cells from atretic follicles ($p < 0.05$; fig. 3).

When we compared each structure between groups, GR immunoexpression in the granulosa cells was higher in the primary and tertiary follicles from the spontaneous COD group than in the control and ACTH-induced COD groups ($p < 0.05$). In atretic follicles, GR expression was higher in granulosa cells from animals with spontaneous COD ($p < 0.01$) and the control group ($p < 0.05$) than in the ACTH-induced COD group. No differences were observed in secondary follicles between groups. In theca interna cells, GR immunoexpression showed an increase in tertiary follicles from the spontaneous-COD group relative to the tertiary follicles from the control and ACTH-induced COD groups ($p < 0.05$; fig. 2, 4).

Also, GR protein expression in spontaneous or ACTH-induced cysts was compared with tertiary follicles from the control group. In granulosa cells, GR expression was higher in cysts from animals with spontaneous COD ($p < 0.01$) and ACTH-induced COD ($p < 0.05$) than in tertiary follicles from the control group. In theca interna cells, GR expression was higher in cysts from animals with spontaneous COD than in tertiary follicles from the control group ($p < 0.01$; fig. 2, 4).

Discussion

In the present study, we found no differences in GR mRNA expression in granulosa and theca cells of tertiary and cystic follicles, but did find differences in GR protein expression. In this sense, the differences in gene and protein expression found could be due to posttranscriptional and posttranslational GR modifications. GR is constituted by a DNA-binding domain flanked by an amino terminal (or N-terminal) variable domain and a carboxyl terminal (or C-terminal) relatively variable domain. The N-terminal domain contains the AF-1 (or hormone-independent) region, which has been associated with transcriptional activity and binding with coactivator proteins and transcription factors. Furthermore, the C-terminus contains the AF-2 region, which is responsible for hormone binding [Nicolaides et al., 2010]. GR contains nine exons: the first and last are subject to alternative splicing, giving rise to different variants or isoforms. GRα is the predominant isoform and the only one that can bind to GCs and, therefore, to perform functions of activation or repression [Lu and Cidlowski, 2006; Nicolaides et al., 2010]. Although GR is constitutively expressed, it shows distinct expression patterns in different cells and tissues due to differential regulation. Via posttranscriptional regulation, for example, microRNAs, single-stranded noncoding RNA molecules of about 21 nucleotides, can affect the expression levels of GR and its subsequent activity [Vandevyver et al., 2014]. Posttranslational modifications of GR, such as phosphorylation, acetylation, nitrosylation and oxidation, can affect GR ligand- and DNA-binding affinity, subcellular trafficking and cofactor recruitment, and hence its transcriptional activity [Vandevyver et al., 2014].

In the ovaries of control animals, evaluation of GR protein expression allowed observing high expression in the granulosa cells from atretic follicles in relation to the primary, secondary and tertiary follicles, and compared to the theca interna cells of all follicular categories. These results correlate with those described by Tetsuka et al. [2010], who found that GR mRNA expression did not change throughout the follicular growth in granulosa and theca cells, and increased in atretic follicles. Deprivation of key factors promoting survival, such as estradiol, insulin-like growth factor-1, follicle-stimulating hormone, interleukin-1β and interleukin-6 [Hsu and Hsueh, 1997; Mao et al., 2004; Maeda et al., 2007], or factors that stimulate cell death, such as tumor necrosis factor (TNF)-α, Fas and Fas ligand [Matsuda et al., 2006], are the main cause of apoptosis of granulosa cells. In this sense, it has been shown that GCs, through the interaction with GR, may attenuate the apoptosis induced by cyclic adenosine monophosphate, p53 and TNF-α, and by upregulation of Bcl-2 expression in granulosa cells, and therefore may play important roles in the process of tissue healing after ovulation, follicular rupture, and CL formation and

Fig. 2. Representative images of GR immunostaining in primary, secondary, tertiary and atretic follicles from all groups, follicular cysts from the spontaneous COD and ACTH-induced COD groups and a negative control. Scale bars = 25 μm.

maintenance during maternal recognition of pregnancy [Amsterdam et al., 2002; Sasson et al., 2002; Myers et al., 2007; Komiyama et al., 2008; Whirledge and Cidlowski, 2013]. This implication of GCs in the apoptotic process may be related to the increased GR expression that we found in granulosa cells from atretic follicles.

GR protein expression in granulosa cells was higher in tertiary follicles and cysts from animals with spontaneous COD and in cysts from animals with ACTH-induced COD than in tertiary follicles from control animals. GR immunoexpression was higher in theca interna of tertiary follicles and cysts from cows with spontaneous COD than in tertiary follicles from the control group. These results agree with those obtained in rats by Park et al. [2012], who showed that GR mRNA expression was higher in ovaries with cysts induced by ACTH or cold stress than in control animals, although they did not determine GR protein expression. We did not find this difference in mRNA expression level. These differences could be due to the different animal models used and methodological aspects since Park et al. [2012] analyzed whole ovaries for mRNA detection and we used specifically the isolated follicular cells of antral follicles.

The inflammatory injury caused by the rupture of the follicle at the time of ovulation can be diminished by the anti-inflammatory action of cortisol. In this sense, the increased concentration of cortisol found in follicular fluid from cysts [Amweg et al., 2013] and the increased expression of GR protein found in this work in the ovaries from animals with COD could suggest the existence of an increased anti-inflammatory reaction at the site of ovulation through the GC-GR complex, which could block ovulation with subsequent development and persistence of follicular cysts. On the other hand, ACTH increases endogenous cortisol secretion, thus inducing follicular cysts in cattle [Liptrap and McNally, 1976; Dobson et al., 2000; Salvetti et al., 2010]. In this sense, we have recently shown that 11β-hydroxysteroid dehydrogenase, an enzyme which converts cortisone to cortisol, is the predom-

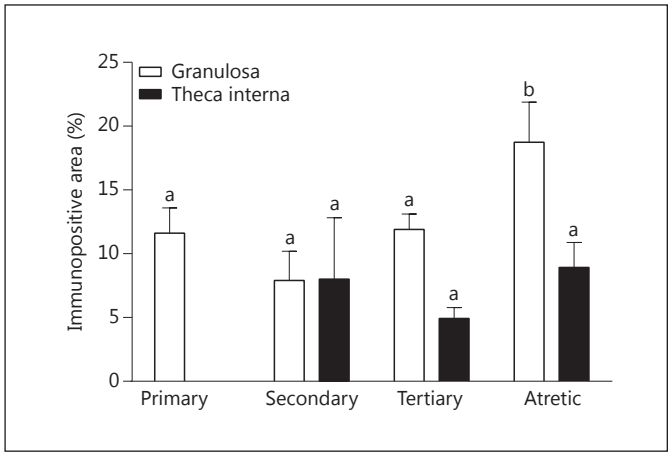


Fig. 3. Relative expression of GR in granulosa (white bars) and theca interna (black bars) of primary, secondary, tertiary and atretic follicles from the control group. Values represent means ± SEM. Bars with different letters are significantly different ($p < 0.05$).

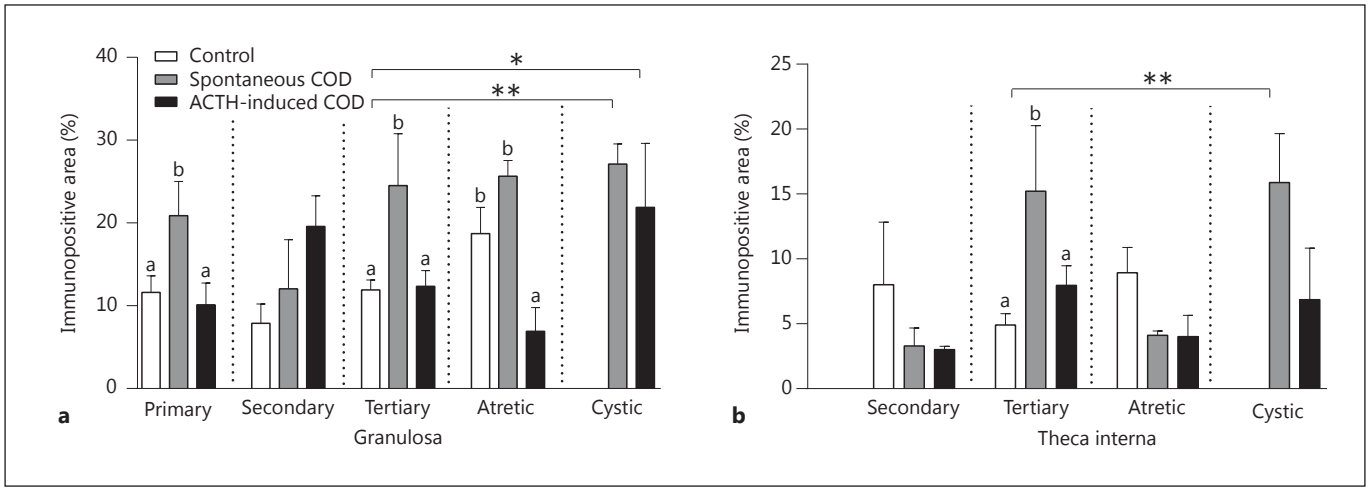


Fig. 4. Relative expression of GR in granulosa (**a**) and theca interna (**b**) of primary, secondary, tertiary and atretic follicles and follicular cysts from the control, spontaneous COD and ACTH-induced COD groups. Values represent means ± SEM. Bars with different letters within each follicular category are significantly 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 ** $p < 0.01$.

inant isoform in animals with COD [Amweg et al., 2013]. Altogether, previous and present results suggest a mechanism of action for cortisol and its receptor through the activation/inactivation of specific transcription factors. These factors could be related to the regulatory mechanisms involved in the process of ovulation and participate in the processes related to COD pathogenesis in cattle.

Acknowledgments

This study was supported by a grant from the Argentine National Agency for the Promotion of Science and Technology (ANPCyT; PICT 2010/2315 and 2011/1274). We are grateful to the staff of the Hospital de Salud Animal of the FCV-UNL for the animal care and help with the generation of experimental animals and collection of samples. We also thank the staff members of the Laboratorio de Biología Celular y Molecular Aplicada (FCV-UNL) for their technical support during the processing of slides and to Novartis Laboratories for providing the drugs.

Disclosure Statement

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

References

Acosta, T.J., M. Tetsuka, M. Matsui, T. Shimizu, B. Berisha, D. Schams, A. Miyamoto (2005) In vivo evidence that local cortisol production increases in the preovulatory follicle of the cow. J Reprod Dev *51:* 483–489.

Adcock, I.M. (2000) Molecular mechanisms of glucocorticosteroid actions. Pulm Pharmacol Ther *13:* 115–126.

Amsterdam, A., K. Tajima, R. Sasson (2002) Cell-specific regulation of apoptosis by glucocorticoids: implication to their anti-inflammatory action. Biochem Pharmacol *64:* 843–850.

Amweg, A.N., A.H. Paredes, N.R. Salvetti, H.E. Lara, H.H. Ortega (2011) Expression of melanocortin receptors mRNA, and direct effects of ACTH on steroid secretion in the bovine ovary. Theriogenology *75:* 628–637.

Amweg, A.N., N.R. Salvetti, M.L. Stangaferro, A.H. Paredes, H.E. Lara, F.M. Rodríguez, H.H. Ortega (2013) Ovarian localization of 11β-hydroxysteroid dehydrogenase (11βHSD): effects of ACTH stimulation and its relationship with bovine cystic ovarian disease. Domest Anim Endocrinol *45:* 126–140.

Andersen, C.Y. (2002) Possible new mechanism of cortisol action in female reproductive organs: physiological implications of the free hormone hypothesis. J Endocrinol *173:* 211–217.

Barnes, P.J. (2006) Corticosteroid effects on cell signalling. Eur Respir J *27:* 413–426.

Bartolomé, J.A., W.W. Thatcher, P. Melendez, C.A. Risco, L.F. Archbald (2005) Strategies for the diagnosis and treatment of ovarian cysts in dairy cattle. J Am Vet Med Assoc *227:* 1409–1414.

Charpenet, G., Y. Tache, M. Bernier, J.R. Ducharme, R. Collu (1982) Stress-induced testicular hyposensitivity to gonadotropin in rats. Role of the pituitary gland. Biol Reprod *27:* 616–623.

Dellmann, H.D., J. Priedkalns (1993) Histología Veterinaria, ed 2. Zaragoza, Acribia.

Dobson, H., A.Y. Ribadu, K.M. Noble, J.E. Tebble, W.R. Ward (2000) Ultrasonography and hormone profiles of adrenocorticotrophic hormone (ACTH)-induced persistent ovarian follicles (cysts) in cattle. J Reprod Infertil *120:* 405–410.

Duong, H.T., K.K. Piotrowska-Tomala, T.J. Acosta, M.M. Bah, E. Sinderewicz, M. Majewska, K. Jankowska, K. Okuda, D.J. Skarzynski (2012) Effects of cortisol on pregnancy rate and corpus luteum function in heifers: an in vivo study. J Reprod Dev *58:* 223–230.

Engelmann, M., R. Landgraf, C.T. Wotjak (2004) The hypothalamic-neurohypophysial system regulates the hypothalamic-pituitary-adrenal axis under stress: an old concept revisited. Front Neuroendocrinol *25:* 132–149.

Espey, L.L., A.S. Belilinger, J.A. Healy (2004) Ovulation: an inflammatory cascade of gene expression; in P.C.K. Leung, E.Y. Adashi (eds): The Ovary, ed 2. Vancouver, Elsevier, pp 145–165.

Hillier, S.G., M. Tetsuka (1998) An anti-inflammatory role for glucocorticoids in the ovaries? J Reprod Immunol *39:* 21–27.

Hsu, S.Y., A.J. Hsueh (1997) Hormonal regulation of apoptosis an ovarian perspective. Trends Endocrinol Metab *8:* 207–213.

Kawate, N., T. Inaba, J. Mori (1993) Effects of cortisol on the amounts of 17β-estradiol and progesterone secreted and the number of luteinizing hormone receptors in cultured bovine granulosa cells. Anim Reprod Sci *32:* 15–25.

Kino, T., E. Charmandari, G.P. Chrousos (2011) Glucocorticoid receptor: implications for rheumatic diseases. Clin Exp Rheumatol *29:* 32–41.

Komiyama, J., R. Nishimura, H.Y. Lee, R. Sakumoto, M. Tetsuka, T.J. Acosta, D.J. Skarzynski, K. Okuda (2008) Cortisol is a suppressor of apoptosis in bovine corpus luteum. Biol Reprod *78:* 888–895.

Kuse, M., H.Y. Lee, T.J. Acosta, T. Hojo, K. Okuda (2013) Expression of glucocorticoid receptor α and its regulation in the bovine endometrium: possible role in cyclic prostaglandin F2α production. J Reprod Dev *59:* 346–352.

Lagaly, D.V., P.Y. Aad, J.A. Grado-Ahuir, L.B. Hulsey, L.J. Spicer (2008) Role of adiponectin in regulating ovarian theca and granulosa cell function. Mol Cell Endocrinol *284:* 38–45.

Liptrap, R.M., P.J. McNally (1976) Steroid concentrations in cows with corticotropin-induced cystic ovarian follicles and the effect of prostaglandin F2alpha and indomethacin given by intrauterine injection. Am J Vet Res *37:* 369–375.

Livak, K.J., T.D. Schmittgen (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. Methods *25:* 402–408.

Lu, N.Z., J.A. Cidlowski (2006) Glucocorticoid receptor isoforms generate transcription specificity. Trends Cell Biol *16:* 301–307.

Maeda, A., N. Inoue, F. Matsuda-Minehata, Y. Goto, Y. Cheng, N. Manabe (2007) The role of interleukin-6 in the regulation of granulosa cell apoptosis during follicular atresia in pig ovaries. J Reprod Dev *53:* 481–490.

Majewska, M., H.Y. Lee, Y. Tasaki, T.J. Acosta, A.Z. Szostek, M. Siemieniuch, K. Okuda, D.J. Skarzynski (2012) Is cortisol a modulator of interferon tau action in the endometrium during early pregnancy in cattle? J Reprod Immunol *93:* 82–93.

Mao, J., M.F. Smith, E.B. Rucker, G.M. Wu, T.C. McCauley, T.C. Cantley, R.S. Prather, B.A. Didion, B.N. Day (2004) Effect of epidermal growth factor and insulin-like growth factor I on porcine preantral follicular growth, antrum formation, and stimulation of granulosal cell proliferation and suppression of apoptosis in vitro. J Anim Sci *82:* 1967–1975.

Marelli, B.E., P.U. Díaz, N.R. Salvetti, F. Rey, H.H. Ortega (2014) mRNA expression pattern of gonadotropin receptors in bovine follicular cysts. Reprod Biol *14:* 276–281.
Matsuda, F., N. Inoue, Y. Goto, N. Manabe (2006) The regulation of ovarian granulosa cell death by pro- and anti-apoptotic molecules. J Reprod Dev *52:* 695–705.
Myers, M., M.C. Lamont, S. van den Driesche, N. Mary, K.J. Thong, S.G. Hillier, W.C. Duncan (2007) Role of luteal glucocorticoid metabolism during maternal recognition of pregnancy in women. Endocrinology *148:* 5769–5779.
Nelson, S.T., A.D. Martin, O. Osteras (2010) Risk factors associated with cystic ovarian disease in Norwegian dairy cattle. Acta Vet Scand *52:* 60.
Nicolaides, N.C., Z. Galata, T. Kino, G.P. Chrousos, E. Charmandari (2010) The human glucocorticoid receptor: molecular basis of biologic function. Steroids *75:* 1–12.
Ortega, H.H., M.M. Palomar, J.C. Acosta, N.R. Salvetti, B.E. Dallard, J.A. Lorente, C.G. Barbeito, E.J. Gimeno (2008) Insulin-like growth factor I in ovarian follicles and follicular fluid of cows with spontaneous and induced cystic ovarian disease. Res Vet Sci *84:* 419–427.
Ortega, H.H., N.R. Salvetti, V. Padmanabhan (2009) Developmental programming: prenatal androgen excess disrupts ovarian steroid receptor balance. Reproduction *137:* 865–877.
Ortega, H.H., F. Rey, M.M. Velazquez, V. Padmanabhan (2010) Developmental programming: effect of prenatal steroid excess on intraovarian components of insulin signaling pathway and related proteins in sheep. Biol Reprod *82:* 1065–1075.
Park, E., J.F. Cockrem, K.H. Han, D.H. Kim, M.H. Jung, J.P. Chu (2012) Stress-induced activation of ovarian heat shock protein 90 in a rat model of polycystic ovary syndrome. J Obstet Gynaecol Res *38:* 396–407.
Parrott, J.A., M.K. Skinner (1998) Developmental and hormonal regulation of keratinocyte growth factor expression and action in the ovarian follicle. Endocrinology *139:* 228–235.
Pfaffl, M.W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res *29:* e45.
Pursley, J.R., M.O. Mee, M.C. Wiltbank (1995) Synchronization of ovulation in dairy cows using PGF2alpha and GnRH. Theriogenology *44:* 915–923.
Ranefall, P., K. Wester, A.C. Andersson, C. Busch, E. Bengtsson (1998) Automatic quantification of immunohistochemically stained cell nuclei based on standard reference cells. Anal Cell Pathol *17:* 111–123.
Richards, J.S., Z. Liu, M. Shimada (2008) Immune-like mechanisms in ovulation. Trends Endocrinol Metab *19:* 191–196.
Rivier, C., S. Rivest (1991) Effect of stress on the activity of the hypothalamic-pituitary-gonadal axis: peripheral and central mechanisms. Biol Reprod *45:* 523–532.
Salvetti, N.R., E.J. Gimeno, J.A. Lorente, H.H. Ortega (2004) Expression of cytoskeletal proteins in the follicular wall of induced ovarian cysts. Cells Tissues Organs *178:* 117–125.
Salvetti, N.R., M.L. Stangaferro, M.M. Palomar, N.S. Alfaro, F. Rey, E.J. Gimeno, H.H. Ortega (2010) Cell proliferation and survival mechanisms underlying the abnormal persistence of follicular cysts in bovines with cystic ovarian disease induced by ACTH. Anim Reprod Sci *122:* 98–110.
Salvetti, N.R., N.S. Alfaro, M.M. Velázquez, A.N. Amweg, V. Matiller, P.U. Díaz, H.H. Ortega (2012) Alteration in localization of steroid hormone receptors and coregulatory proteins in follicles from cows with induced ovarian follicular cysts. Reproduction *144:* 723–735.
Sasson, R., N. Winder, S. Kees, A. Amsterdam (2002) Induction of apoptosis in granulosa cells by TNF alpha and its attenuation by glucocorticoids involve modulation of Bcl-2. Biochem Biophys Res Commun *294:* 51–59.
Shibaya, M., A. Matsuda, T. Hojo, T.J. Acosta, K. Okuda (2007) Expressions of estrogen receptors in the bovine corpus luteum: cyclic changes and effects of prostaglandin F2alpha and cytokines. J Reprod Dev *53:* 1059–1068.
Silvia, W.J., T.B. Alter, A.M. Nugent, L.F. Laranja da Fonseca (2002) Ovarian follicular cysts in dairy cows: an abnormality in folliculogenesis. Domest Anim Endocrinol *23:* 167–177.
Sunak, N., D.F. Green, L.R. Abeydeera, L.M. Thurston, A.E. Michael (2007) Implication of cortisol and 11β-hydroxysteroid dehydrogenase enzymes in the development of porcine *(Sus scrofa domestica)* ovarian follicles and cysts. Reproduction *133:* 1149–1158.
Tetsuka, M. (2007) Actions of glucocorticoid and their regulatory mechanisms in the ovary. Anim Sci J *7:* 112–120.
Tetsuka, M., H. Nishimoto, A. Miyamoto, K. Okuda, S. Hamano (2010) Gene expression of 11β-HSD and glucocorticoid receptor in the bovine *(Bos taurus)* follicle during follicular maturation and atresia: the role of follicular stimulating hormone. J Reprod Dev *56:* 616–622.
Tilbrook, A.J., A.I. Turner, I.J. Clarke (2000) Effects of stress on reproduction in non-rodent mammals: the role of glucocorticoids and sex differences. Rev Reprod *5:* 105–113.
Torres Uchoa, E., G. Aguilera, J.P. Herman, J.L. Fiedler, T. Deak, M.B. de Sousa (2014) Novel aspects of glucocorticoid actions. J Neuroendocrinol *26:* 557–572.
Vandevyver, S., L. Dejager, C. Libert (2014) Comprehensive overview of the structure and regulation of the glucocorticoid receptor. Endocr Rev *35:* 671–693.
Vanholder, T., G. Opsomer, A. de Kruif (2006) Aetiology and pathogenesis of cystic ovarian follicles in dairy cattle: a review. Reprod Nutr Dev *46:* 105–119.
Whirledge, S., J.A. Cidlowski (2013) A role for glucocorticoids in stress-impaired reproduction: beyond the hypothalamus and pituitary. Endocrinology *154:* 4450–4468.
Xing, Y., C.R. Parker, M. Edwards, W.E. Rainey (2010) ACTH is a potent regulator of gene expression in human adrenal cells. J Mol Endocrinol *45:* 59–68.