Original Paper

Cells Tissues Organs

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DOI: 10.1159/000481167

Accepted after revision: August 31, 2017
Published online: November 3, 2017

Changes in the Proliferation/Apoptosis Balance in the Bovine Ovary: A Key Early Event in Follicular Persistence

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Keywords
Proliferation · Apoptosis · Follicular persistence · Cow

Abstract
The objective of this work was to evaluate proliferation and apoptosis in the bovine ovary in a model of follicular persistence induced by low levels of progesterone to detect incipient changes during cystic ovarian disease development on the expected day of ovulation (day 0) and after 5, 10, and 15 days of follicular persistence. We analyzed cell proliferation by evaluating the expression of Ki-67 and apoptosis by evaluating caspase-3, BAX, and BCL2 expression. Proliferation was similar in the granulosa and theca cells of antral follicles in the P0 group (treated with progesterone up to the expected day of ovulation) and in the control group. A decrease in cell proliferation was detected after 5 days of persistence (P5) in relation to P0 ($p < 0.05$). Similar changes were found in the granulosa cells of the persistent follicles in relation to the control group ($p < 0.05$). Caspase-3 expression was similar in granulosa cells of antral follicles at early stages of persistence, with an increase after 15 days of persistence ($p < 0.05$). In the granulosa cells of group P10 (10 days of persistence), caspase-3 expression was reduced relative to that of antral follicles from the control group ($p < 0.05$). BCL2 expression was higher in granulosa cells of the persistent follicles of group P0 relative to the control follicles, with no changes in BAX expression, which was increased in persistent follicles of group P15 ($p < 0.05$). Similar results were observed in theca cells at initial stages of persistence. The results show that, initially, proliferation is maintained with low apoptosis and an increase in cell survival.

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Introduction

Follicular persistence is caused by the failure of ovulation and the consequent permanence of the follicular structure in the ovary, which alters the cyclicity of the female and causes infertility. This process is one of the main components of cystic ovarian disease (COD) and other diseases of ovarian origin [Hatler et al., 2008; Díaz et al.,

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Abbreviations used in this paper

AMH	anti-Müllerian hormone
BMP	bone morphogenetic protein
COD	cystic ovarian disease
FSH	follicle-stimulating hormone
IGF	insulin-like growth factor
LH	luteinizing hormone

2015; Ortega et al., 2015, 2016]. COD causes great economic losses in the dairy industry because of unsuccessful artificial inseminations, veterinary treatment, and a decrease in milk production related to the increase in the interval from calving to conception [Vanholder et al., 2006; Cattaneo et al., 2014].

Along folliculogenesis, the cells that compose the ovarian follicles normally proliferate and then differentiate. Finally, the follicle can take 1 of 2 pathways: ovulation, if the follicle is dominant in an ovulatory wave, or atresia, which occurs with most of the nondominant follicles [Robker and Richards, 1998; Adams et al., 2008]. Atresia involves the apoptosis of granulosa cells, oocytes, and eventually theca cells [Hsueh et al., 1994; Markström et al., 2002; D'haeseleer et al., 2006]. Successful follicle development depends on the presence of survival factors that promote follicle growth and also protect cells from apoptosis [Robker and Richards, 1998; Quirk et al., 2004]. These include factors produced within the ovary like 17-β estradiol, progesterone, insulin-like growth factor (IGF)-1, as well as gonadotropins [Quirk et al., 2004]. In the absence of survival factors, endogenous apoptotic pathways within the follicle become activated and lead to follicular atresia [Hsueh et al., 1994].

Two main systems are responsible for inducing apoptosis in the mammalian ovary: the FAS system and BCL2 (B-cell lymphoma 2) family members [Kim et al., 1999; Roughton et al., 1999]. Members of the BCL2 family, which are the main regulatory proteins acting in mitochondria, have been classified into those having an antiapoptotic function, like BCL2, and those having a proapoptotic function, such as BAX (Bcl2-associated X membrane protein) [Slot et al., 2006]. The antiapoptotic proteins block the activation of effector caspases, caspase-3, caspase-6, and caspase-7, which in turn transduce the apoptotic signals [Tilly, 1996]. Caspase-3 is functionally required for apoptosis [Das et al., 2008] and its activation is responsible for the cleavage of key substrates, such as DNA repair enzymes, and cytoskeletal and nuclear scaffold proteins [Scaffidi et al., 1998; Krammer, 1999; Slot et al., 2006]. As previously described, the failure in the process of ovulation can lead to follicular persistence, one of the main components of the pathogenesis of COD. Many studies have reported alterations in the processes of proliferation and apoptosis in ovarian follicular cysts in different species [Isobe and Yoshimura, 2007; Das et al., 2008; Salvetti et al., 2009, 2010; Sun et al., 2012; Ortega et al., 2015], and even in several experimental models developed in laboratory animals [Salvetti et al., 2009; Bas et al., 2011]. However, since these studies have been conducted in cystic follicles developed previously and with a long period since their formation in the ovary, it is not possible to discern whether the alterations found are due either to the disease itself (and thus part of the causes) or to their persistence for an extended period of time. For this reason, it is crucial to perform studies from the initial stages of development of follicular persistence associated with COD.

The aim of the present study was to evaluate proliferation and apoptosis in the bovine ovary in an experimental model of follicular persistence induced by low levels of progesterone to detect incipient changes during COD development that could contribute to its pathogenesis. We analyzed cell proliferation by evaluating the expression of Ki-67 by immunohistochemistry and apoptosis by evaluating caspase-3, BAX, and BCL2 expression on the expected day of ovulation (day 0), and after 5, 10, and 15 days of follicular persistence.

Materials and Methods

Induction of Follicular Persistence by Low Doses of Progesterone

All procedures were evaluated and approved by the Ethics and Security Committee of the Facultad de Ciencias Veterinarias of the Universidad Nacional del Litoral, Santa Fe, Argentina (protocol No. 131/12) and are consistent with the Guide for the Care and Use of Agricultural Animals in Research and Teaching (Federation of Animal Science Societies, 2010).

The model used here has been previously optimized [Díaz et al., 2015] and is summarized in Figure 1. This model was performed in nonlactating Holstein cows ($n = 25$) with regular estrous cycles. Ovarian activity was synchronized starting with the protocol G6G [Pursley et al., 1995; Bello et al., 2006] with modifications [Díaz et al., 2015]. Once synchronized, the cows were divided into 5 groups: the control group ($n = 5$), and groups P0 (treated with progesterone up to the expected day of ovulation; $n = 5$), P5 (5 days of follicular persistence since the expected day of ovulation; $n = 5$), P10 (10 days of follicular persistence since the expected day of ovulation; $n = 5$), and P15 (15 days of follicular persistence since the expected day of ovulation; $n = 5$). Control cows received no additional hormonal treatment. On day 16 after estrus synchronization, the cows of groups P0, P5, P10, and P15 were treated with an

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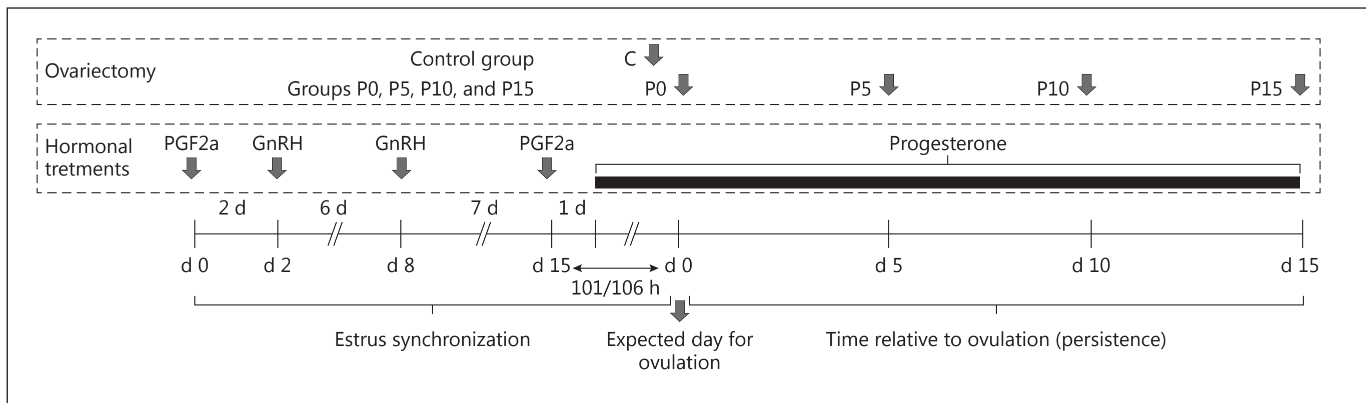

Fig. 1. Experimental design for the induction of follicular persistence by progesterone. Holstein cows were synchronized and received an intravaginal progesterone-releasing device to induce follicular persistence. Follicular dynamics were followed by daily ultrasound scanning as indicated. Ovariectomy was performed in proestrus (controls), and after 0 (expected time for ovulation), 5, 10, and 15 days (d) of follicular persistence.

Table 1. Antibodies, suppliers, and dilutions used for immunohistochemistry

Primary antibodies	Clone/source	Immunohistochemistry antigen retrieval	Dilution
Ki-67	Monoclonal; clone 7B11, Invitrogen	0.01 M citrate buffer (pH 6.0) in a microwave oven at 800 W	1:100
Caspase-3	Polyclonal; Ab-4051, Abcam	0.001 M EDTA buffer (pH 8.0) with 0.05% Tween 20 in a pressure cooker	1:100
BAX	Polyclonal; Ab32503 [E63], Abcam	0.01 M citrate buffer (pH 6.0) in a microwave oven at 800 W	1:100
BCL2	Polyclonal; Ab7973, Abcam	0.01 M citrate buffer (pH 6.0) in a microwave oven at 800 W	1:100

intravaginal progesterone device (750 mg of micronized progesterone; Pro-Ciclar P4-Zoovet®, Santa Fe, Argentina) to obtain the subluteal concentrations of progesterone (1–2 ng/mL), as described in Díaz et al. [2015]. The device was kept in the cows until the expected day of ovulation in group P0, for 5 days after the expected day of ovulation in group P5, and for 8 days in groups P10 and P15. In the latter 2 groups, a new intravaginal progesterone-releasing device was inserted 1 day before the removal of the first one to maintain a more consistent concentration of progesterone throughout the treatment period. In group P15, a third intravaginal progesterone-releasing device was inserted on day 11 of persistence, 1 day before the removal of the second one (Fig. 1) [for details see Díaz et al., 2015].

In control cows, bilateral ovariectomy was performed 2 days after completion of the synchronization protocol, whereas in groups P0, P5, P10, and P15, ovariectomy was performed on day 0 (the expected day of ovulation) and on days 5, 10, and 15 of follicular persistence, respectively [Díaz et al., 2015] (Fig. 1). Blood samples were obtained daily up to ovariectomy to test for progesterone, testosterone, and estradiol levels in a parallel study [Díaz et al., 2015].

Tissue Sampling and Follicle Classification

For histology and immunohistochemistry, the ovaries obtained by ovariectomy from control animals and animals with progesterone-induced follicular persistence were fixed in 4% buffered formaldehyde for 8–10 h at 25°C, washed in phosphate-buffered saline, and then dehydrated in an ascending series of ethanol, cleared in xylene, and embedded in paraffin. Sections that were 5 μm thick, obtained with a microtome with a high-precision motorized specimen feed (Leica RM2245), were mounted on slides previously treated with 2% (v/v) 3-aminopropyltriethoxysilane in acetone (Sigma-Aldrich, St. Louis, MO, USA) and primarily stained with hematoxylin-eosin for preliminary observation of the ovarian structures [Díaz et al., 2015]. For immunohistochemical analysis, follicles were classified into the following groups: primary, small preantral, large preantral, antral, and atretic follicles [Braw-Tal and Yossefi, 1997], and persistent follicles [Díaz et al., 2015].

Immunohistochemistry

The homology between the target peptide of each antibody and the corresponding bovine protein was tested using the Basic Local Alignment Search Tool (BLAST software; http://www.ncbi.nlm.nih.gov/BLAST) to determine the peptide locations and to confirm antigen specificity. Furthermore, the antibodies used were meticulously validated in bovine tissues in previous reports [Salvetti et al., 2010].

Detailed information of the suppliers and concentrations of the antibodies used is presented in Table 1. Each antibody was assayed in at least 5 sections of each ovary from each animal. A total of approximately 15 sections from each animal were taken for immunohistochemical quantification. A streptavidin-biotin immuno-

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peroxidase method was performed as previously described [Salvetti et al., 2010]. Briefly, after deparaffinization, antigen retrieval was performed (detailed in Table 1). Endogenous peroxidase activity was inhibited with 3% (v/v) H_2O_2 in methanol, and nonspecific binding was blocked with 10% (v/v) normal goat serum in phosphate-buffered saline. All sections were incubated with the primary antibodies for 18 h at 4°C and then for 30 min at room temperature with the biotinylated link (CytoScan™ HRP Detection System; Cell Marque, Rocklin, CA, USA). The antigens were visualized by the CytoScan™ HRP detection system, and 3,3-diaminobenzidine (DAB Liquid DAB-Plus substrate kit; Invitrogen, Camarillo, CA, USA) was used as the chromogen. Finally, the slides were washed in distilled water and counterstained with Mayer hematoxylin, dehydrated and mounted.

To verify the immunoreaction specificity, adjacent control sections were subjected to the same immunohistochemical method, replacing primary antibodies with rabbit and mouse nonimmune sera. The specificity of the secondary antibodies was tested by incubation with primary antibodies with negative reaction to bovine antigens: anti-human CD45 (clone: PD7/26; Dako, Carpinteria, CA, USA) and anti-human estrogen receptor alpha (polyclonal, Cell Marque). To exclude the possibility of nonsuppressed endogenous peroxidase activity, some sections were incubated with DAB (3,3-diaminobenzidine) alone. Serial sections of similarly processed tissue samples of control ovaries were used as positive controls in each assay to normalize the image analysis.

Image Analysis

Microscopic images were digitized with a color video camera Nikon DS-Fi2 mounted on a conventional light microscope Nikon Eclipse Ci-L *Ni* (Tokyo, Japan), with a plane apochromatic objective (×40 magnification), and analyzed with Image Pro-Plus 3.0.1 (Media Cybernetics, Silver Spring, MD, USA). The microscope was prepared for Koehler illumination, which 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 negative control slides contained a series of tissue sections in which the primary antibodies were replaced with rabbit and mouse nonimmune sera.

Microscopic fields covering the entire follicular wall area were digitized and stored in a 24-bit true color TIFF format. The resolution of the images was set to 2,560 × 1,920 pixels (5.07 effective megapixels).

The positive controls were used as interassay controls to maximize the levels of accuracy and robustness of the method [Ranefall et al., 1998]. The slides were scanned left to right from the top and all follicles of the selected categories were analyzed. The image analysis score was calculated separately in each follicular wall layer (granulosa and theca interna) from at least 50 images of the different categories of follicles from the ovaries of all groups.

Ki-67 staining was evaluated by counting positive cells/total cells for each layer to obtain an index of positive cells. The antigen expression of caspase-3, BAX, and BCL2 in tissue sections was expressed as a fraction of the stained area fraction (percent of immunopositive area), and was calculated as a percentage of the total area evaluated through the color segmentation analysis, which extracts objects by locating all objects of the specific color (brown stain). These values were verified and normalized with the controls carried across various runs using the same region (verified by image comparison) for calibration. Sections were analyzed with the observer blinded to the experimental group.

The methodological details of image analysis as a valid method for quantification have been described previously [Ortega et al., 2009; Wang et al., 2009; Salvetti et al., 2010]. The major strength of the imaging approach used in this study is visualization of in situ localization of proteins within the tissues and cells of interest. In the past decade, computerized image analysis systems have been developed to obtain an objective and accurate quantification of nuclear markers [Lejeune et al., 2008]. This approach has been successfully applied by other investigators to quantify immunostaining in different tissues and validated for diagnostic, prognostic, and therapeutic purposes [Shan et al., 1997; Wang et al., 1999, 2000, 2009; Zhu et al., 2000; Lejeune et al., 2008; Salvetti et al., 2010].

Statistics

The adequate number of images per follicle and the number of follicles per category were confirmed from a sample size calculation that evaluated the number of samples necessary to produce an estimate of the immunoreactivity that would fall within 0.4 units of the real value. SPSS 11.0 for Windows (SPSS Inc., Chicago, IL, USA) was used to perform the statistical tests. The distribution of data was tested for normality using the Kolmogorov-Smirnov test. Differences between 2 groups of data were detected by unpaired 2-tailed Student *t* test. The differences between more than 2 groups of data were assessed by 1-way ANOVA followed by Duncan multiple range tests. p values <0.05 were considered significant. Results are expressed as the mean ± SD.

Results

Representative images of granulosa and theca interna immunostaining for each marker are shown in Figure 2a and b.

Ki-67 Percentage of Positive Cells

In granulosa cells, comparison between the different persistence groups and the control group for each specific follicular category showed a lower Ki-67 percentage of positive cells of antral and atretic follicles from groups P5, P10, and P15 than in those from group P0 ($p < 0.05$), without differences compared with the control group (Fig. 3a). In addition, persistent follicles of group P0 showed a higher percentage of positive cells than those of groups P5 and P15 ($p < 0.05$) without differences compared with P10 (Fig. 3a). Comparison of the Ki-67 percentage of positive cells in antral and atretic follicles from the control group (as the reference structure) with persistent follicles showed a lower expression in persistent follicles of group P5 than in control structures (Fig. 2a, 3a).

In theca cells, the Ki-67 percentage of positive cells was lower in antral and atretic follicles from groups P5, P10,

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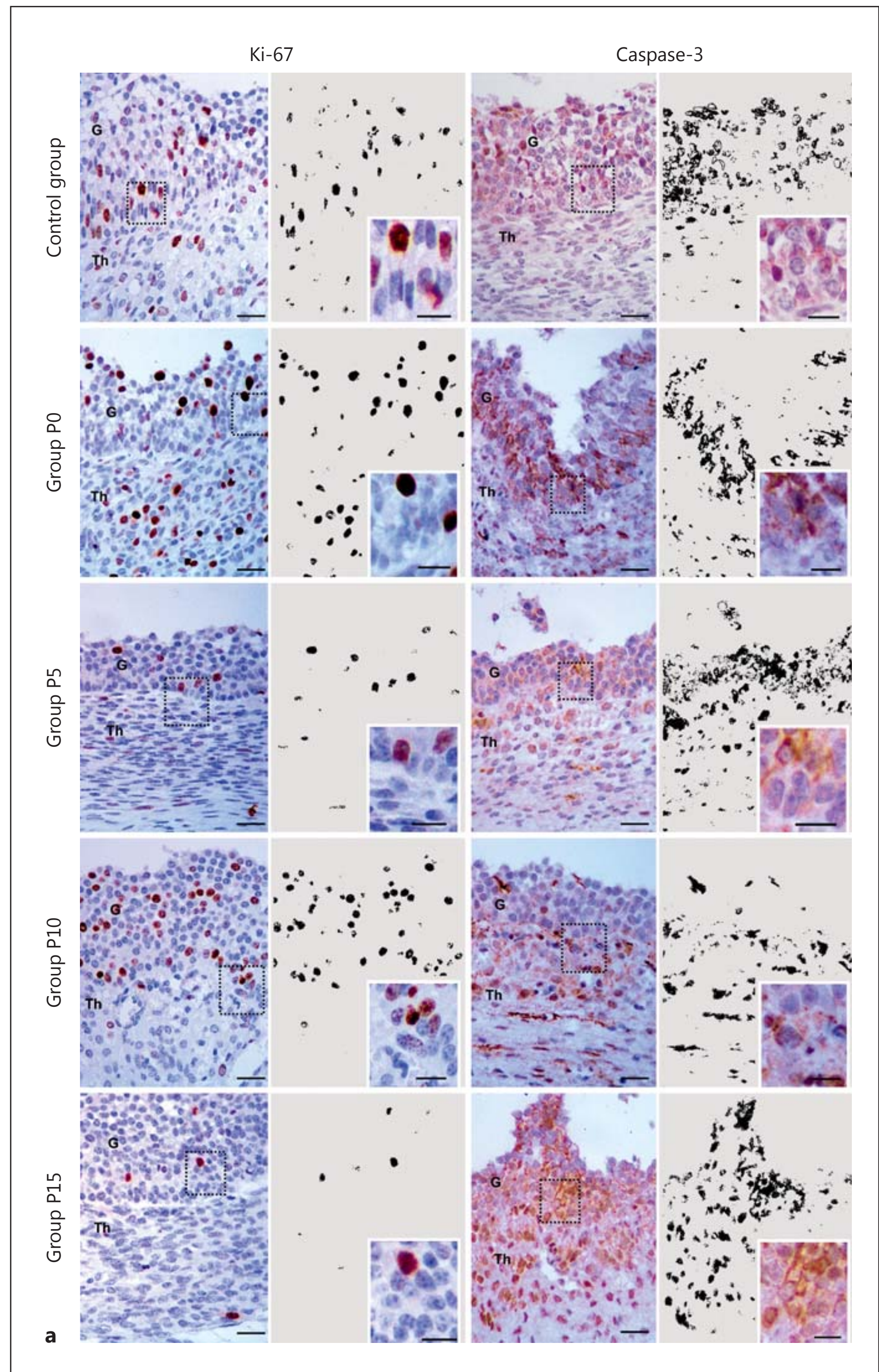

Fig. 2. Representative images of Ki-67 and caspase-3 (**a**), and BAX and BCL2 (**b**) immunostaining in the antral follicles (control group) and persistent follicles (groups P0, P5, P10, and P15). The immunopositive areas are indicated by brown staining. G, granulosa; Th, theca interna. Scale bar, 25 μm (left columns). The images were segmented by digital image analysis, showing the differential positive pattern in black in the right column for each marker. Additionally, an amplified area of the original image is illustrated in an inset in the right columns (scale bar, 12.5 μm).

(Figure continued on next page.)

and P15 than in those from group P0 ($p < 0.05$), without differences compared with the control group (Fig. 3b). In persistent follicles, group P0 showed a higher expression than the other persistence groups ($p < 0.05$). Comparison of the Ki-67 percentage of positive cells in theca cells of antral and atretic follicles from the control group (as the reference structure) with persistent follicles showed a higher expression in P0 persistent follicles than in control atretic follicles ($p < 0.05$), without differences compared with control antral follicles (Fig. 2a, 3b).

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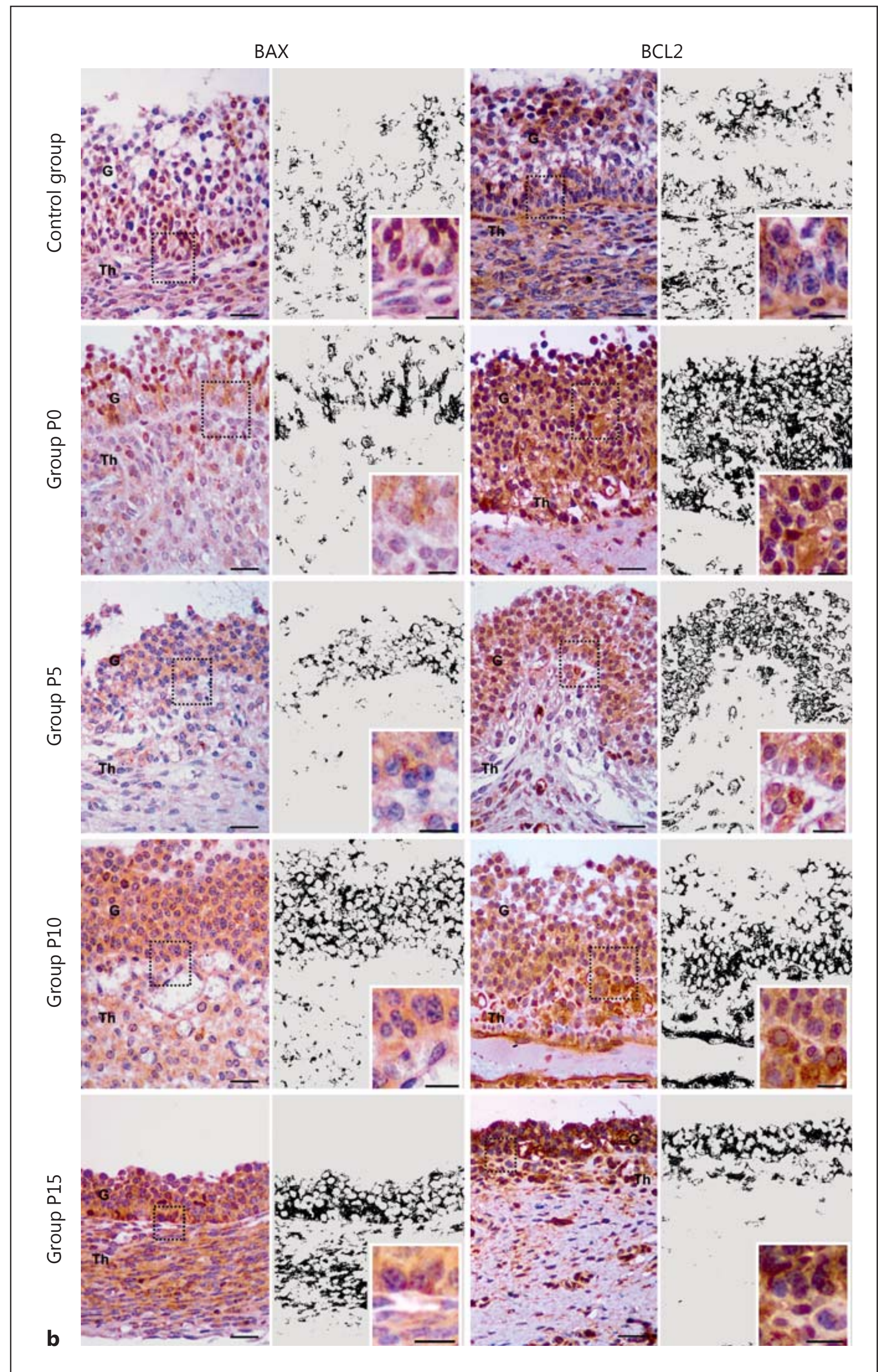

Caspase-3 Expression

In granulosa cells, caspase-3 expression was higher in the antral follicles of group P15 than in those of the control group and groups P0, P5, and P10 ($p < 0.05$; Fig. 3a), and higher in the persistent follicles of group P15 than in those of group P10 ($p < 0.05$). The comparison with reference structures (antral and atretic follicles of the control group) showed lower caspase-3 expression in the persistent follicles of group P10 than in the antral follicles of the control group ($p < 0.05$).

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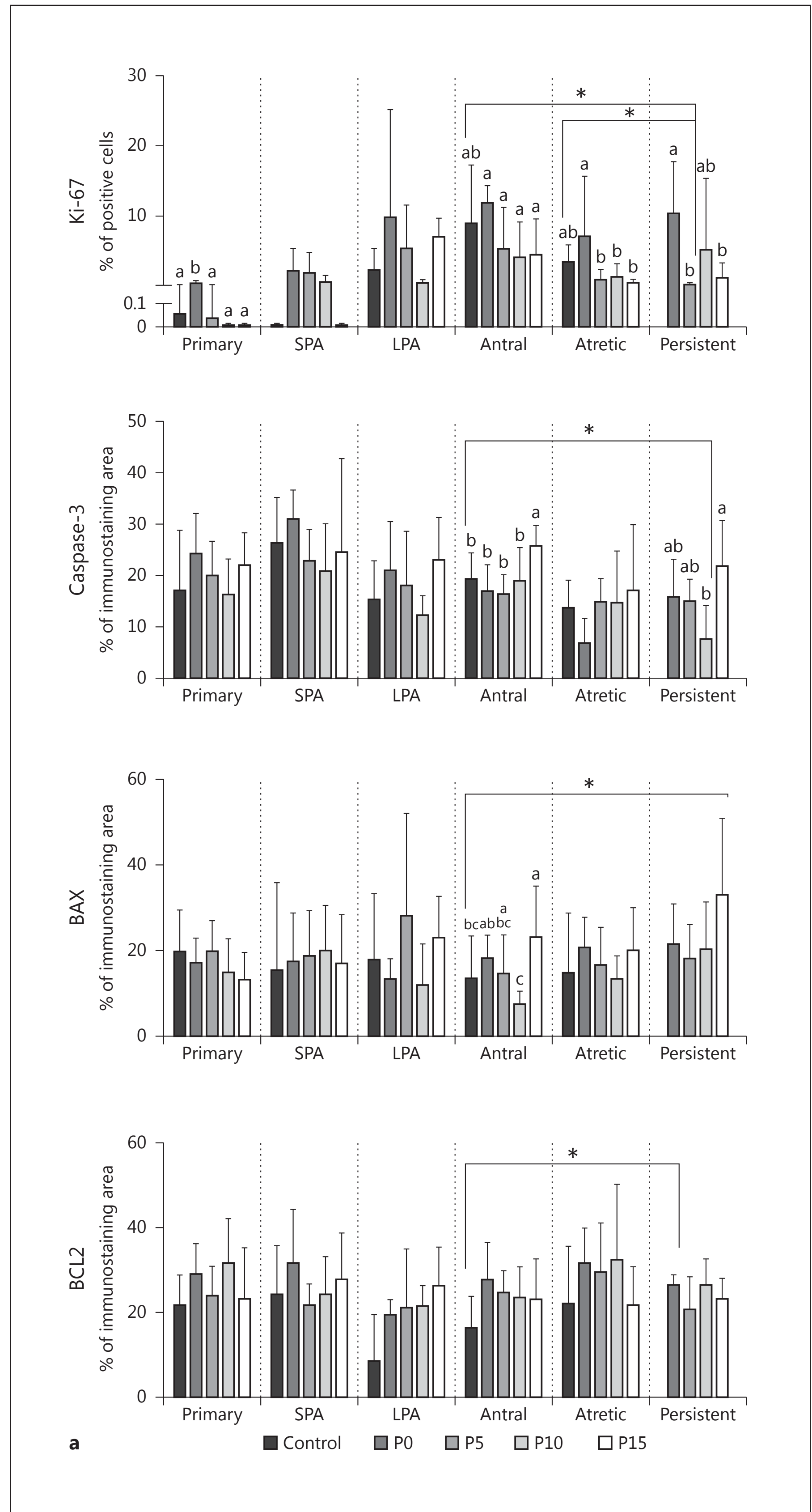

Fig. 3. Percentage of Ki-67-positive cells and relative protein expression (measured as the percentage of the immunopositive area) of BAX, BCL2, and caspase-3 in different follicular categories from the control group and progesterone-induced follicular persistence groups P0, P5, P10, and P15 in granulosa (**a**) and theca interna (**b**) cells of primary, small (SPA), and large preantral (LPA), antral, and atretic follicles from the control group and groups P0, P5, P10, and P15, and persistent follicles from groups P0, P5, P10, and P15. Values represent the mean ± SD. Bars with different letters within a category are significantly different ($p < 0.05$). Asterisks indicate differences between persistent follicles and antral or atretic follicles from the control group ($p < 0.05$).

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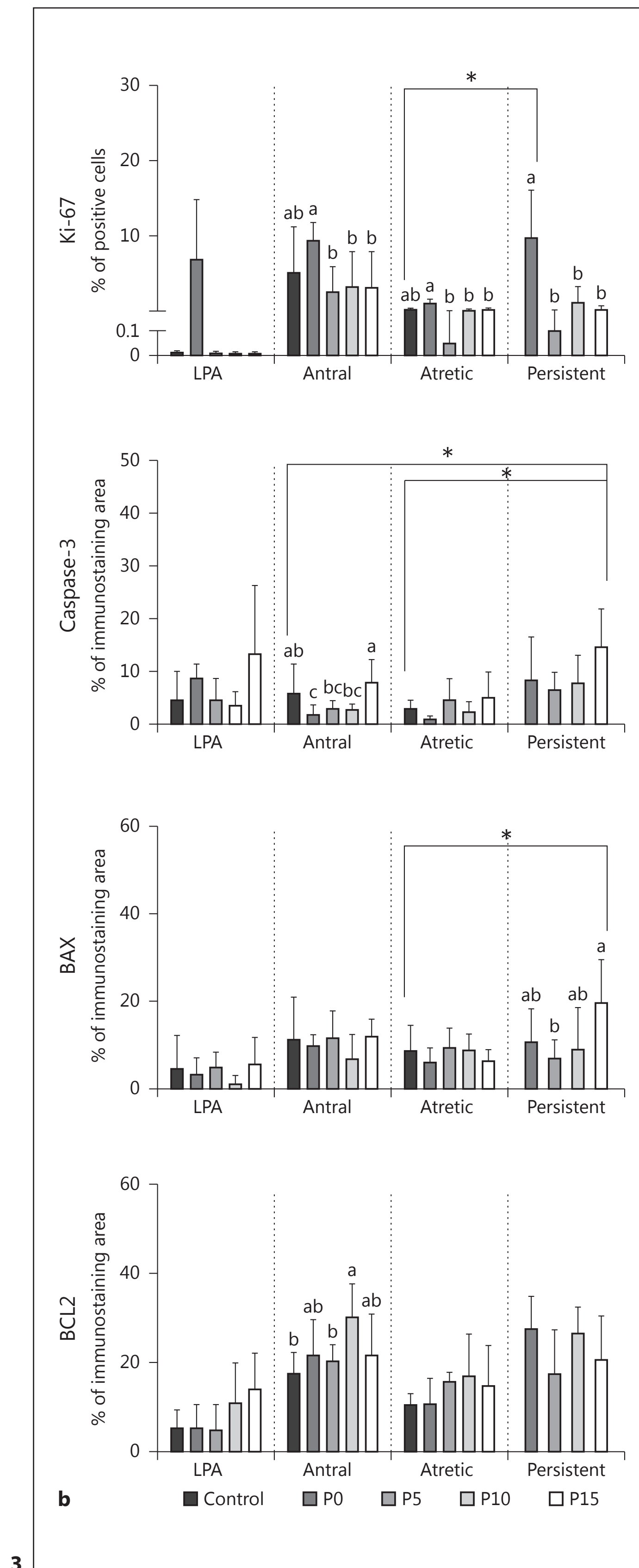

In theca cells, caspase-3 expression was also higher in the antral follicles of group P15 than in those of groups P0, P5, and P10 ($p < 0.05$). Also, caspase-3 expression was lower in the antral follicles of group P0 than in those of the control group ($p < 0.05$; Fig. 3b). Additionally, the comparison of caspase-3 expression with that in the reference structures of the control group (antral and atretic follicles) showed a higher expression in persistent follicles of group P15 than in antral and atretic follicles ($p < 0.05$; Fig. 2a, 3b).

BAX Expression

In granulosa cells, comparison between groups for each specific follicular category showed higher BAX expression of antral follicles from group P15 than those of the control group and group P10 ($p < 0.05$; Fig. 3a). Comparison between the antral follicles from the control group (as the reference structure) with persistent follicles showed higher BAX expression in the persistent follicles from group P15 ($p < 0.05$; Fig. 2b, 3a).

In theca cells, persistent follicles from group P15 showed a higher expression than those of group P5 ($p < 0.05$). Also, BAX expression was higher in the persistent follicles of group P15 than in the atretic follicles of the control group (as the reference structure; $p < 0.05$; Fig. 2b, 3b).

BCL2 Expression

In granulosa cells, BCL2 expression showed no differences between groups for each follicular category. However, BCL2 expression was higher in granulosa cells of persistent follicles of group P0 than in antral follicles of the control group (as reference structures; $p < 0.05$; Fig. 2b, 3a).

In theca cells, BCL2 expression was higher in the antral follicles of group P10 than in the control group and group P5 ($p < 0.05$). The comparison with the reference structure (antral follicles) of the control group showed a tendency towards a higher BCL2 expression in the persistent follicles of group P0 ($p = 0.067$; Fig. 2b, 3b).

Discussion

In the present study, no differences were found in the proliferation of granulosa and theca cells of the antral and atretic follicles of the progesterone-treated group on the expected day of ovulation (P0) relative to the control group. A decrease in cell proliferation was detected after 5 days of persistence (P5) relative to P0. In addition, a de-

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crease in cell proliferation was found in the granulosa cells of group P5 in relation to reference structures (antral and atretic follicles) from the control group. These data are complemented by the similar expression of caspase-3 found in granulosa cells of antral follicles in the early stages of persistence, which increased after 15 days of persistence. Also, in this cell layer, caspase-3 expression was lower in the persistent follicles of group P10 than in the antral follicles from the control group. In relation to BAX and BCL2, we found that in the initial stages of persistence (P0) there was an increase in BCL2 expression in granulosa cells of the persistent follicles relative to the control antral follicles, with no changes in BAX expression at this time. However, BAX expression was higher in the persistent follicles in more advanced stages of persistence (group P15) than in the reference structures of the control group.

Similar results were observed in the theca cell layer at initial stages of persistence. An increase was observed in the proliferation levels of group P0 in relation to the atretic follicles, without differences compared with the antral follicles of the control group. A low expression of caspase-3 was detected in this layer of antral follicles from group P0 relative to the control group. Moreover, BAX expression was higher in the persistent follicles of group P15 than in group P5 and higher in the persistent follicles of group P15 than in atretic control follicles. The antral follicles of group P10 showed a higher expression of BCL2 than those of the control group and group P5, without differences compared with the other groups.

Previous reports have shown that follicular cysts in cattle have lower levels of cell proliferation in both cellular populations (granulosa and theca), associated with an increase in survival of cells with low levels of expression of proapoptotic proteins and low levels of apoptosis measured by TUNEL [Isobe and Yoshimura, 2000a, b, 2007; Salvetti et al., 2010; Ortega et al., 2015]. Nevertheless, these studies have shown a difference in cysts formed a long time ago, where many factors probably influenced their maintenance. However, little is known about the cellular dynamics in the initial stages during the formation of structures that give rise to cysts. In the present study, we used an experimental model which has been previously characterized and has proven to be a useful tool for the study of the early stages of the formation of cysts in cattle [Díaz et al., 2015, 2016]. The results presented here show that, in the initial stages of the persistence process, the levels of proliferation remain similar to those of the control follicles. This is in agreement with the increase in the follicular size observed in the cysts already developed, both in spontaneous COD and in diverse animal models (ACTH-induced COD, progesterone-induced COD) [Amweg et al., 2013; Díaz et al., 2015]. The results in groups P10 and P15 agree with the results of previous reports, where low proliferation levels were found in developed cysts [Gümen and Wiltbank, 2002; Silvia et al., 2002; Amweg et al., 2013; Díaz et al., 2015]. In the same sense, from the beginning of the persistence, we observed that the antiapoptotic factors remained at levels similar to those of control follicles and that the proapoptotic factors increased in advanced stages of persistence, inclining the balance towards cell survival in the first stages of the disease.

Several studies have characterized the hormonal environment necessary for follicular cells to survive, proliferate, or differentiate. Estradiol and follicle-stimulating hormone (FSH) are required for follicular cells to divide rapidly and develop follicles, whereas luteinizing hormone (LH) is required for ovulation and differentiation in luteal cells [Robker and Richards, 1998]. In fact, in granulosa and even theca cells, LH is the main hormone that induces the expression of genes implicated in proliferation, differentiation, or atresia [Gilbert et al., 2011]. In the animal model used here, the hormonal environment is characterized by higher levels of serum 17-β estradiol until day 10 of persistence and an altered pattern of LH secretion without changes in FSH secretion. The LH pulse frequency on days 5 and 10 of follicular persistence in treated cows is similar to that in the follicular phase and significantly higher than that in the mid-luteal phase in controls [Díaz et al., 2015]. On the other hand, the pulse concentration and amplitude are lower in treated cows than in control cows [Díaz et al., 2015]. At a local level, 17-β estradiol in the follicular fluid is maintained at a concentration similar to that of the control dominant follicles, except for group P15 where we observed a decrease relative to the control group and the other persistence groups. Also, in this model, a low concentration of progesterone and high concentration of testosterone was detected as the follicular persistence progressed. A decrease in 17-β estradiol in follicular fluid at 15 days of persistence and a concomitant increase in testosterone concentration have been previously observed in animals with COD and would represent the loss of the integrity of the granulosa cell layer, which would interfere in its capacity of aromatization of androgens [Amweg et al., 2013; Díaz et al., 2015]. These data coincide with the increase in the expression of BAX and the decrease in cell proliferation (Ki-67-positive cells) in granulosa cells at advanced stages of persistence observed in the present study. On the

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other hand, at initial stages of persistence (group P0), we observed an increase in BCL2 expression without differences in Ki-67 expression in persistent follicles related to control antral follicles, which coincides with the high concentration of 17-β estradiol in this period [Díaz et al., 2015].

In the ovary, proliferation, differentiation, and apoptosis are also regulated by several growth factors, such as in IGF-1, vascular endothelial growth factor, and members of the transforming growth factor-β superfamily [Mazerbourg et al., 2003; Knight and Glister, 2006; Berisha et al., 2016]. In this regard, it has been previously reported that bone morphogenetic protein (BMP)-6 is increased in persistent follicles from early stages of persistence and that BMP4 has a higher expression in persistent follicles after 10 days of persistence [Díaz, 2016; Díaz et al., 2016]. BMPs inhibit FSH-induced progesterone synthesis and play a key role as luteinization inhibitors [Shimasaki et al., 1999, 2004; Otsuka et al., 2000, 2001, 2011; Erickson and Shimasaki, 2003; Inagaki et al., 2009; Otsuka, 2013] and BMP4 inhibits granulosa cell apoptosis [Shimizu et al., 2012]. Thus, the BMP4 increase observed in group P10 could be related to the decrease in caspase-3 expression in this group associated with the constant expression of BCL2 in follicular persistence. Furthermore, it has been reported that anti-Müllerian hormone (AMH), another member of the TGF-β superfamily, presents high levels in follicular fluid of persistent follicles after 10 days of persistence, and similar levels in groups P5 and P15 relative to the control group [Díaz, 2016]. In the ovary, AMH has inhibitory effects on granulosa cell proliferation [Kim et al., 1992; Seifer et al., 1993], aromatase activity, and LH receptor expression [Di Clemente et al., 1994]. The AMH concentration found in the animal model used could explain the poor proliferation observed in follicles and could contribute to the decrease in 17-β estradiol and higher concentration of testosterone present in the follicular fluid in group P15 [Díaz et al., 2015].

Conclusion

The results presented here coincide in part with those previously reported in spontaneous COD in cattle. In particular, the results referring to events after 10 days of persistence (groups P10 and P15) coincide fully with the concept that there is a decrease in cellular proliferation and an increase in the survival of the cells, which contribute to the persistence of these follicles, preventing their ovulation or regression. However, the study of initial events in the development of persistence indicates that, initially, the proliferation rate is maintained in the absence of ovulation, with low levels of apoptosis (indicated by the absence of differences in BAX and caspase-3 expression in the follicles of group P0 relative to the controls) and an increase in cell survival due to the increase in BCL2 levels. It is probable that the hormonal changes that occur later, both at the endocrine and paracrine level, are responsible for the alterations observed in the parameters of cell proliferation and differentiation of already developed cysts.

Acknowledgments

We are grateful to the staff of the Large Animal Health Hospital of the Facultad de Ciencias Veterinarias de la Universidad Nacional del Litoral, Santa Fe, Argentina, for animal care and help with the generation of the experimental animals and collection of samples. We also thank DVM Jose Bertoli and DVM Fabian Barberis for their assistance with animal care, the staff members of the Laboratorio de Biología Celular y Molecular Aplicada (ICIVET-Litoral UNL CONICET) for their technical support during processing of the slides, and Novartis Laboratories for the provision of 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.

Funding Sources

This study was supported by a grant from the Argentine National Agency for the Promotion of Science and Technology (ANPCyT) (PICT 2011-1274). M.M.L.V., P.U.D., B.E.M., F.R., H.H.O. and N.R.S. are Research Career Members; E.M.B. and A.F.S. are Fellows of the National Scientific Research Council (CONICET, Argentina); U.S.N. is a Fellow of ANPCyT.

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